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Wound Healing and Skin Physiology

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I. History of Wound Healing

Theory and Practice of Wound Healing in the Past

I. Müller

Treatment of Wounds in the Age of Homer

The problem of wound healing is presumably as old as mankind itself. As virtually no other discipline, the development of traumatic surgery has depended upon outside factors, particularly those of warfare technology and material. The infinite military conflicts throughout human evolution have presented abundant opportunities for testing the most varied of techniques. A survey of the history of wound healing can be found in the classical works by Gurlt (1898) and von Brunn (1928) and in those by Bardeleben (1878), Bibbings (1986), Brunner (1916), Caldwell (1990), Churchill (1964; 1965), Cope (1958), Crissey and Parish (1984), Elliott (1964), Forrest (1982), Gribel (1936), Küster (1915), Leibecke-Englisch (1979), Majno (1975), Marchand (1901), Moerl (1968), Schadewaldt (1975), Schoenbauer (1948), Stelzner (1969), Teubner (1973), Wangensteen and Sarah (1972), Whipple (1963), and Wolzendorff (1879).

In his epic on the Trojan war Homer described a multitude of injuries inflicted by arrows, lances, and swords, ranging from fractures, dislocations, and damage to the scull cap and hip joint to perforation of the diaphragm. Frölich (1879) counted a total of 147 injuries in the *Iliad*: 106 spear wounds with deaths in 80%, 17 wounds from sword cuts with fatal consequences, 12 injuries from arrows with deaths in 42%, and 12 from catapults of which 66% were lethal. (See also Albarracín Teulón 1971; Friedrich 1956; Körner 1929; Majno 1975, pp. 141–147; Schoenbauer 1948.) Because of the frequency of such injuries in Homeric times the physician's job then was principally that of a surgeon (Kudlien 1967, p. 16). His activities concentrated on the removal of projectiles, cleaning wounds with lukewarm water, and covering them with analgesic and hemostatic powder or ointment (*Iliad*, IV, 218; XI, 830). Occasionally a drink containing wine was given to soothe the pain (*Iliad*, XI, 639; XIV, 5), and in desperate cases warriors resorted to magical practices and sought to arrest bleeding by exorcism (*epodé*)

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(*Odyssey*, XIX, 457) – a method of treatment that was widespread until late in the Middle Ages, when it was practiced as wound blessing (Stuart and Walla 1987; see the discussion of Guy de Chauliac, below). Apparently, wound dressing was not common as it is mentioned by Homer in only two passages (*Iliad*, XIII, 599, from plaited sheep's wool; *Odyssey*, XIX, 457, without referring to the bandage material).

Wound Sutures in Egyptian Surgery

In ancient Egypt, however, injuries were not only dressed but in special cases even closed with surgical sutures, as we know from the oldest preserved wound book of papyrus Edwin Smith. This textbook was written as early as 1580 BC and comprises 48 types of surgery of simple and complicated injuries to the head, thorax, and spinal column. In the treatment of wounds a suture is mentioned here for the first time in medical literature (Westendorf 1966, p. 49). As an additional measure it recommends a dressing with fresh meat, which was a standard feature of wound treatment in ancient Egypt and may have derived from magical practices in the form of a sympathetic remedy (see Buchheim 1958). Linen strips such as those used by embalmers served as bandaging material proper, and the bandaging technique, which reached a high level in ancient Egypt, probably benefited from experiences with mummification.

Wound Healing in the Hippocratic Works

The collection of writings of Greek physicians, the Hippocratic works, contains no reference to wound sutures or suture material, and their surgical instrumentarium seems to have been extremely modest (Whipple 1963, p. 26; Gurlt 1897, vol. 1, pp. 246–293). Important in the later Hippocratics' theory of wound healing was the distinction between the healing of wounds with and without pus. According to their doctrine, simple gashes were to be treated with dry products such as alum and copper salts to prevent the formation of pus; for contusions, on the other hand, the formation of pus was expressly desired, because "The contused and crushed soft tissue should putrefy, become pus and dissolve, and new flesh particles spring up from it" (Hippocrates 1839–1861, vol. 6 (Peri Helkon), p. 402).

Wounds were normally cleaned with wine and sealed with resin or pitch plaster, and pure linen served as bandaging (vol. 6, pp. 401–403). The fact that an entire, detailed chapter was devoted to bandaging techniques indicates that the emphasis in wound treatment was placed on such knowledge (vol. 3, pp. 262–337). Extensive instructions for cleanliness served the prophylaxis of wound infection; that adverse incidents nevertheless occurred is indicated by the demand of surgeons that they be experienced not only in

the practice of wound healing but also in the diagnosis and therapy of such complications as phlegmonous inflammations, erysipelas, gangrene, tetanus and septicemia (Brunner 1916, p. 8; for examples see Gurlt 1898, vol. 1, pp. 251–293; see also statements relating to the qualification of a surgeon in Hippocrates 1839–1961, vol. 6, p. 150).

Wound Healing Techniques of the Romans

A substantial innovation in wound healing technique, that of vascular ligation, was introduced by the Romans. The first report of its use is by the Roman encyclopedist Cornelius Celsus (25 BC–50 AC), who recommended injuries of the great blood vessels to be treated with double ligature and subsequent separation to achieve a contraction of vessels (Celsus 1915, book V, chap. 26, 21–23; for the history of vessel ligature and wound suture see Kraft 1970; Mackenzie 1973; Teubner 1973). According to Celsus, this technique permitted among other things the extirpation of testicular tumors (book VII, chap. 19). When vessel ligation failed, the use of branding irons was also recommended to stop bleeding, but the use of caustics for scabbing was rejected.

Sutures with a soft stitch (*acia*) were already in use for the rapid closure of wounds, and gaping wounds were closed by a *fibula* after the wound had been cleaned of blood clots (regarding the confusion caused by this term, which was used to denote a stitched suture and not a clamp, see Benedum 1970). Subsequently the lesion was to be covered and kept moist by a sponge soaked with vinegar, wine, or water.

Galen's Contribution to Wound Surgery

Whereas Celsus used only strong thread for suturing, Galen, the Greek physician practicing in Rome (second century AC) recommended the use of silk and thin gut string, or *chordae*, as suturing material, which was easily obtained in the ancient world because of its use in music instruments. Galen stressed particularly that the suturing material should not decompose too rapidly, i.e., that it be *aseptos*, as rapid dissolution of the ligature may cause new hemorrhages (Galen 1822–1833, vol. 10, p. 942). Much later, in the nineteenth century, the English surgeon John Lister rediscovered these properties of the animal material. Galen considered these to be of special benefit, and he therefore recommended carbolized catgut string as suturing material because it could be treated antiseptically and was also resorbing (Lister 1881; see Gibson 1990).

As physician to the gladiators Galen had experience of his own in the practical treatment of wounds (on Galen's contribution to surgery see Mani 1991; Toledo-Pereyra 1973). According to his concept, the technique for the

arresting of bleeding consisted not only of local measures but also general ones (see Mani 1991). Local arrest of bleeding was therefore always combined with diverting the humors from the wound area to other body areas, either by derivation, revulsion, astringent products, or the application of cold (1823–1831, vol. 10, book V, chap. 3, pp. 313ff). The local treatment proper was by compression with the finger (vol. 10, p. 317; see Majon 1975, p. 404), ligation of the bleeding vessel (vol. 10, p. 317), bandage, or torsion of the bleeding vessel by punching a hook through the artery wall and cautious turning (vol. 10, pp. 318ff).

Galen recommended initial washing of the wound with wine after arresting of the bleeding and its closure with a suture after removal of the foreign body. Although Galen had frequently observed that the wound healed without pus formation, he later advocated the application of ointments and pharmaceuticals to compensate for the loss in substance and to promote pus formation and secondary wound healing. For such purposes he preferred a mixture of aloe and incense, which was given a honeylike consistency by adding egg white and mixed with fine rabbit hairs (vol. 10, p. 320).

Theory of Pusless and Pus-Forming Healing

Until the modern age no agreement could be reached among surgeons as to whether pusless healing or pus formation was desirable in the treatment of wounds. The majority of surgeons followed the ancient doctrine of Galen for healing per secundam intentionem. With similar persistence they advocated the doctrine of the adverse effect of air on wound healing.

Serious criticism of this concept was first expressed by surgeons during the Middle Ages in Salerno and Bologna in the twelfth century (Keil 1983). However, surgeons advocating pusless wound healing remained a minority even there. One of these was Teodorico dei Borgognoni (1205–1298), from the Bolognese school of surgeons. He gave clear instructions as to how this pusless wound healing could be achieved (Campbell and Colton 1955–1960, book II, chap. 1):

> In the first place the sides of the wound should be debrided, or abraded (*abradantur*), and then the wound should be completely cleansed of fuzz, hair and anything else, and let it be wiped dry with lint moistened with warm wine; thus the sides of the wound may be united as well as possible in accordance with their original state; and let it be bandaged in such a way that the re-approximation of the wound edges cannot be disturbed at all.

Teodorico also expressly contradicted the traditional concept of *pus laudabile* as an essential feature of the healing process (Campbell and Colton 1955–1960, book II, chap. 27):

> But it is not necessary, as Roger and Roland [surgeons of the Salerno school; see Keil 1983] have written, as many of their disciples teach, and as all modern surgeons profess, that pus should be generated in wounds. No error can be greater than this. Such a practice is indeed to hinder Nature, to prolong the disease and to prevent the conglutination and consolidation of the wound.

Criticism of the traditional approach to wound healing was also expressed by the royal court surgeon under the French Kings Philipp IV and Louis X, Henry of Mondeville (*c.*1260–*c.*1320), who – upset by the bad habits of the surgeons – gives the following vivid account of everyday wound healing in the Middle Ages (Pagel 1890, vol. 40, pp. 736ff; translation I.M.):

> Older surgeons treated wounds as follows: If the wound was small, they first stuck their little finger into it and violently pulled it open, then using their middle and finally first fingers to widen it further. It was then enlarged even more by stuffing cloth into it that had been made cold and sticky with some moist substance such as egg white. After this they applied tampons soaked in the same substance.

Instead of this Henry of Modeville recommended not interfering with the healing process and leaving the wound alone after the edges of the wound had been closed with a suture; cloth soaked in wine was not to be placed in the wound but only outside of it. He maintained that rapid closure of the wound edges was necessary to avoid irritation of pus formation by the air (Pagel 1891, vol. 41, pp. 138ff). His explanation shows the force of tradition upon him despite his reformist ideas.

These early attempts by a small group of outsiders to introduce some form of antiseptic or aseptic process into wound healing were not allowed to develop at the time, however, Guy de Chauliac (died 1368), the personal physician to three popes, dominated the practice of surgery for centuries with his classical *Chirurgia Magna* (1363), which took a strictly Galenist position. Among the more than 100 medical authorities quoted, Galen is cited 890 times, Avicenna 661 times, and Hippocrates only 120 times. Guy de Chauliac (1585, p. 131) mocked the concept of Teodorico and Mondeville based on healing without pus and administering hot, wine-containing drinks as a diet. With unshaken confidence in the ancient authorities, he recommended instead to treat wounds that show a loss in substance by pus forming after they had been covered with the usual powders, plasters, rose honey ("apostles' salve"), and Egyptian ointment. For deep wounds he counseled insertion of *turundae sive ellychnia*, strips of cloth made from old linen, or metallic drain tubes "*ex canna aeris, vel argenti perforata*" to drain the pus (1585, p. 130; on the history of wound drainage see Moss 1981).

Pharmacotherapy as Part of Wound Treatment

Closely associated with wound treatment was the practice of pharmacotherapy, particularly the application of ointments and plasters, which often accounted for two-thirds of surgical text books (Guy de Chauliac 1585, pp. 400–408; see also Goltz 1978). To this was added the administering of medicinal drinks and purgatives. Following the advice of Galen, the medieval surgeon had to supplement the local findings with a diagnosis based on the diseased person's complexion and numerous other features of importance in terms of humoral pathology. In the regimen that he then established for the patient's treatment the slightly purgative medicinal draught and blood

letting played an important part (Bock 1919; Raubach 1898). Without a wound draught, it was thought that healing of the wound is impossible, for only the draught would cleanse the wound, keep it closed, and induce healing (Guy de Chauliac 1585, p. 131). Henry of Mondeville also considered this remedy to promote the healing process (Paget 1891, vol. 41, pp. 172ff) whereas Guy de Chauliac expressly rejected such measures and dissociated himself from such superstitious practices (1585, p. 127: "De incantationibus et iniurationibus vocatis Nicodemi . . . non curo"). Still, the otherwise very critical physician and opponent of tradition, Paracelsus (1493–1541), vehemently defended wound draughts because "they contain a power which intrinsically aids nature in healing every wound and injury (1536, pp. 22ff).

A principal objective of pharmacotherapy was the relief of pain, for which preparations of opium and nightshade plants (mandragora, black henbane) were used. A popular method of administration was with so-called somniferous sponges (*spongia somnifera* or *spongia soporifera*). These were prepared from fresh marine sponges soaked in the juice of plants with narcotic effect and dried in the sun. The sponge was moistened with hot water and inserted into the patient's nostrils to put him quickly to sleep (Goltz 1976, p. 200; see Sudhoff 1914–1918, vol. 2, pp. 482–487; Guy de Chauliac 1585, p. 273). The advice on ways in which to reanimate the patient which often accompanied such remedies suggests that the administration of these difficult-to-dose somniferous sponges was not free of risks.

Healing of Injuries from Firearms

The invention of gunpowder and firearms in the fourteenth century led to new controversy. (A precise date for the first wounds from firearms cannot be determined; Gurlt 1898, vol. 3, p. 513; for a summary of such data see Gurlt, vol. 1, pp. 686ff.) With the first report on such injuries in 1460 by the German surgeon Heinrich von Pfohlsprundt (or pfalzpaint, Pfolspeunt; see Haeser and Middeldorpf 1868) the question was raised as to whether bullet wounds are to be considered as poisoned and therefore requiring special treatment. It was especially the Strasbourg surgeon Hieronymus Brunschwig (1450–1512) who established the doctrine that gunpowder poisons the wound. [His *Wundartzny* (1497) was still being frequently printed as late as the sixteenth century.] The most effective remedy for such wounds was considered to be the branding iron. One of the main advocates of the branding iron method had been the Arabian physician Albucasis (Abul-Quasim, 936–1013). The entire first book of his surgery is concerned with cauterization, which is described *a capite ad calcem* (Abulcasis 1973; see Goltz 1978). This commonly generated a burn of the third or fourth degree; subsequently the burned spot was covered with soaked, greased cotton until the scab came off. Brunschwig also recommended removing the "poison" by

inserting a hair rope into the path of the projectile and pulling it back and forth; to promote pus formation a piece of bacon was to be placed into it. Another method of treatment was to pour hot oil into the wound after extracting the projectile, a measure described in detail by the Strasbourg surgeon Hans von Gersdorff in 1517, although he personally rejected the hypothesis of poisoning. Gersdorff, who was renowned for his numerous amputations, also recommended inserting a "chisel" into the path of the projectile to prevent the hole from closing. The French surgeon and personal physician of several kings, Ambroise Paré (1510–1590), described, for lack of boiling oil, his use of a mixture of rose oil, egg yolk, and terpentine (1585, pp. 419–422). Treating the wounded on a battlefield in 1536, he reported better healing results than in similar cases before and thereafter ceased using the branding iron and boiling oil. He referred to these practices as a "chose très horrible et cruelle (p. 491)" and regretted that he himself had operated on patients in the past using the branding iron, for among six patients who had an arm or lower leg amputated only two could be expected to escape with their lives. His determined opposition to these painful methods contributed greatly to their abolition.

Paré also improved the technique for arresting the loss of blood which was becoming a substantial problem particularly in view of the increasing rate of amputations. In the place of the branding iron or of mere compression, he gripped the vessel with pliers, pulled it out, and ligated it with a double string (pp. 489–491). With this technique of vessel ligature and artery clamp Paré in several cases even ligated the carotid artery. Following the amputation Paré dressed the stump by applying a powder of alumina, dust meal and spruce resin, covering the wound with charpie, and placing on it a resin plaster consisting of egg white, Armenian clay, dragon's blood, gypsum, sealing earth, aloe, mastic, and burnt gallnuts.

Numerous complications such as the dreaded secondary hemorrhages and wound infections due to contaminated suture material prevented Paré's procedure from earning wider recognition. Many surgeons, including the famous German physician Fabry von Hilden (1560–1624), considered ligature during the amputation to be too time consuming. Although Fabry had designed ligature pliers of his own (1965, pp. 134ff), he still preferred the branding iron (1615, chap. 19; 1965, pp. 131ff).

Weapon Salve

Paracelsus (1536) and his followers considered every manipulation of the wound to be detrimental and relied solely upon the healing power of nature, rejecting all instrumental interventions. Paracelsus also rejected the suturing of wounds as an out-dated method and one that is opposed to nature. He also imputed selfish economic interests when he reproached surgeons (1536, p. 13; translation, I.M.):

> I oppose suturing not only because of its coarseness but also because of the pain that it causes. Moreover, it is with suturing as it is with inspection of the urine: for every suture, a farthing, for every inspection, a penny.

Such surgical nihilism no doubt encouraged the resort to supposed miracle cures, such as sympathetic ointments and powders. One of these was weapon salve, which consisted of bear fat, earthworms, mummia, pork brain, bloodstone, and moss growing on the crane shells of hanged persons. It was maintained that merely smearing this concoction on the weapon that had injured the patient would bring about the cure of his wound, even without the wounded person being present. [On the weapon salve, which dates from methods practiced by Paracelsus, see Van Helmont (1648) p. 1008. A detailed description of the Paracelsian concept of "magnetic curing" of wounds is presented in Goclenius (1609). A survey of further relevant writings is given by Gurlt (1898) vol. III, p. 153.] The often reported success of this cure was presumably due to the simple neglect of the wound proper. While the weapon salve was being applied, instead of charpie being tamped to the wound or drains introduced into it, the wound was bandaged only with a linen cloth and thus largely protected from contaminations (see Churchill 1964).

Theory of the Detrimental Effect of Air on Wound Healing

By making the world of hitherto unseen organisms visible the invention of microscope technology gave the doctrine maintaining a detrimental effect of air new impetus. Many believed the fantastic patterns now presented to the human eye to provide proof of the invisible *miasmata*, the air-suspended *contagia animata* that had been postulated by the ancients as responsible for wound putrefaction, and that were said to be exhaled from the diseased or to circulate in the air (e.g., Onomatologica Medico-Chirurgica Completa 1775). The method of covering and bandaging a wound with several layers of dressing to protect it from the dangerous air appeared as reasonable as the attempt to cure putrefaction in the wound by applying so-called "antiseptic" remedies.

However, it was the physical properties of the air itself that were believed by many to have a detrimental effect on wound healing because air caused contraction of the vessels and prevented the formation of new tissue. This was the position taken by the leading surgeon of the eighteenth century, Lorenz Heister (1683–1758), professor in Altdorf and Helmstedt, Germany (1719, pp. 48ff). According to Heister, air could be kept away in three ways: by quick bandaging, by tamping the wound with balm and charpie, and by covering the charpie with a wound plaster, applying a compress, and wrapping a bandage around it. Thus, occlusive dressing was again given priority in the practice of wound dressing.

The term "antiseptic" was coined at this time, during the feverish search for the putrefactive substances that were believed to be everywhere. Only a century later was it given its modern definition by Lister. The term was first used in 1750 by the English military and naval physician John Pringle (1707–1782). In his search for putrefaction inhibitors Pringle carried out an astonishing series of experiments, testing inorganic chemicals such as sea salt, ammonium chloride, saltpeter, borax, and alaun for suspected antiseptic properties. He dissolved the substances in water and used them to soak putrefying meat, judging their respective antiseptic effectiveness by the pungency of their smell. The standard that he used was sea salt, and accordingly he calculated a NaCl coefficient (Pringle 1749, 1753; see Wangensteen and Wangensteen 1967).

Similar aims, although with different methods, were pursued somewhat later by the English physician William Alexander (died 1783). He fed infusoria with substances recommended as antiseptics, such as mercurous chloride (calomel) and cinchona bark, and in each case determined the lethal dose of the substance under the microscope; this offered him a yardstick for the evaluation of antiseptic properties (Alexander 1768; see Stelzner 1969).

The dogma of the detrimental effect of air was given new impetus when Scheele and Priestley discovered oxygen in 1774 and thus drew attention to a new, possibly detrimental factor as a component of air. This discovery not only seemed to justify wound curing under complete exclusion of air but also stimulated the invention of numerous bizarre devices for pumping air from above the wounds, supplying the wound with carbon dioxide, or spraying it with alternately warm and ice-cold baths (Brunner 1916, p. 50; De Moulin 1988, pp. 291ff).

The Horrors of "Hospital Gangrene"

Polluted air was held to be the cause of the hospital gangrene that became an increasing problem in the overcrowded hospitals after the middle of the eighteenth century. [On the history of hospital infection see Churchill (1965); Popp (1991); Schadewaldt (1989); Schmitt (1968); Wangensteen and Wangensteen (1967).] The threat continued to spread throughout the century with the introduction of anaesthesia because the new possibilities for pain killing led to a considerable increase in the number of surgical activities, while also promoting the mass transmission of wound infection in the hospitals in which the operations were performed. Fear of the pathogenic character of air lead to covering and bandaging the wounds with dry or impregnated charpie in several layers after the defect was filled. This contributed to the massive spread of hospital gangrene because the dressing material was made from used and sometimes unwashed linen. Theodor Billroth (1829–1894), one of the most prominent surgeons of the nineteenth

century reported often having to reject compresses because they were still covered with crusts of pus from previous applications.

Moreover, there was no understanding of the need to wear clean clothing during surgery. Surgery was considered a dirty job, and the surgeon often wore an operating gown stained with blood and pus because the degree of such ostensible contamination was seen as a measure of the surgeon's frequency of operations and for the presumed success of these (Schadewaldt 1986). It is therefore not surprising that 60%–80% of those operated on as late as the middle of the nineteenth century fell victim to hospital gangrene.

However, not all surgeons accepted the hypothesis of the detrimental effect of air. The English surgeon John Hunter (1729–1793) in 1794 had published a wealth of observations about healing per primam intentionem, inflammation, pus formation, new formation of vessels, and granulation. The German surgeon Philipp Franz von Walther (1781–1849) was resolutely opposed to polypragmatism and supported a return to open wound treatment; he mocked as a *horror vacui* (1826, p. 210) the practice by contemporary surgeons of immediately tamping every wound opening and pouring something into it. He advised his colleagues: "In treating wounds, abscesses, cysts, and fistulas, ignore two-thirds of all the traditional rules prescribing how to perform an operation" (p. 180).

However, the acceptance of progressive attitudes failed to find general support because of the continuing predominance of the miasma doctrine. This doctrine later delayed even the recognition of the revolutionary findings of the Vienna gynecologist Ignaz Philipp Semmelweis (1818–1865), whose statistical investigations showed the high death rate in his hospital to be due not to infectious substances in the air but to the contaminating hand of the physician, transmitting "organic poison." Semmelweis' insights into the causes of puerperal fever and wound diseases was so contradictory to the concepts of conventional infection theory in the middle of the nineteenth century that they received proper attention only after his death.

"Patient Scattering" to Reduce Wound Diseases

Nikolai Iwanowitsch Pirogoff (1810–1881), the leading army surgeon in Russia, was finally able to radically change the treatment of wounds and achieve a reduction in dreaded wound infections. According to his findings, more than half of all the surgically treated casualites in the 1853–1855 Crimean War had ended in the death of the injured (Pirogoff 1864, p. 14). He demonstrated that dangerous wound infections occurred almost exclusively in the overcrowded hospitals, and that surgically treated patients living at home in the country, even those living in the most primitive of conditions, were generally spared such "hospital diseases." (A very similar observation had been made by the English surgeon P. Clare in 1779, how-

ever, without drawing the consequences for wound treatment: "It has been generally remarked, that the cure of compound fractures does not succeed so well in London hospitals as in the country, where the air is more pure and conducive to health".) Pirogoff initiated the practice of scattering recently operated patients to the dwellings on his own estate. Here Pirogoff reported "the most fortunate results" of his surgical practice (pp. 8ff).

Pirogoff also further improved hospital practice by abolishing the sponges which had usually been used to clean the wounds of numerous hospital patients successively; he considered these to be the "principal carrier of the infectious substance." The practice was replaced by rinsing of the wound with a water jet from a kettle. Pirogoff also considered probing the wound with the naked finger to be a "surgical capital crime" not worthy of the nineteenth century, and this was prohibited. In addition, he demanded that infected patients, including their bedding and clothing, be separated from each other in smaller isolation rooms, and that used dressing material be disposed of immediately and instruments be thoroughly cleaned.

Antisepsis and Asepsis as a Precondition of Modern Wound Treatment

A radical change was brought about by the introduction of antiseptic wound treatment by the English surgeon Joseph Lister (1827–1912) in 1867. Prompted by the work of Pasteur, Lister developed an antiseptic bandage which provided secure sealing against germs presumed to be in the air while avoiding continued contact with the carbolic acid used as antiseptic. As a protection against the irritating effect of carbolic acid, Lister used a piece of impermeable cloth as first cover after the wound had been treated with 5% carbolic acid. On this was placed an eightfold layer of antiseptic gauze, extending far beyond the edges of the wound; this was then normally covered with a layer of antiseptic dry cotton wool and secured with a gauze bandage (Küster 1916, p. 27).

A subject of vehement dispute in these germicidal procedures was the spraying apparatus which Lister introduced into antiseptic treatment in 1871. This served to shroud the wound area, the surgeon's hands, and the instruments in a mist of finely diluted carbolic acid, and the process was therefore accompanied by undesired side effects, including occasional poisoning of both surgeon and patient. A particularly annoying feature was the atomizer: "The carbolic acid spray attached itself so fast in the hair and beards of the personnel that its smell betrayed them even at a great distance" (Küster 1916, p. 40). Lister continued to improve his method further, and despite his incorrect premise of contaminated air being the cause of infection, his carefully devised treatment system produced brilliant practical results.

Elucidation of the true cause of wound infection diseases resulted finally from the investigations carried out by Robert Koch (1843–1910). These laid the foundation upon which modern aseptics and wound treatment have developed. In a path-breaking work in 1878 Koch presented the results of experiments demonstating that most of the bacteria contaminating the operation field derived not from the air but from the hands of personnel and from bandages.

Koch's bacteriological advance allowed the carbolic spray to be dispensed with and also led to modifications and further development in antiseptic procedure. The then traditional chemical disinfection was replaced by physical disinfection, which remains today the foundation of modern asepsis. Its development was largely the work of the Berlin clinician Ernst von Bergmann (1836–1907) and his colleague Curt Schimmelbusch (1860–1895), who observed in 1891 that only simple cleaning by mechanical methods and heat can guarantee the elimination of all dangerous germs. He presented precise instructions for the sterilization of bandaging material in hot steam and that of metallic instruments in soda solution as well as information on the disinfection of medical brushes and hands. As an additional germicial prophylaxis, William Stewart Halsted (1852–1922) introduced the use of rubber gloves in the United States in 1891. [As early as 1843 Sir Thomas Watson (1792–1882) had recommended the use of rubber gloves for germicidal prophylaxis.]

Less well known but also important were the bacteriological investigations of the Leipzig surgeon Paul Leopold Friedrich (1864–1916) that contributed to aseptic wound healing. Impressed by the research of Koch, Friedrich developed the practice of wound incubation as early as in 1889–1899. First in animal experiments and later in humans he demonstrated that wounds infected with earth germs do not develop infection but undergo primary healing provided the wound is excised within 6 h. The observation that germs remain latent for up to 7–8 h led him to develop the method of "operative sterilization," which brought a substantial improvement in wound healing methods. This innovation, however, at first received little attention, as the authority of von Bergmann prevented Friedrich's results from finding general acceptance (Magnus 1933, p. 19). Only during World War I was his operating method rediscovered and practiced on a wide scale. In the meantime it too has been replaced by further improvements in the methods of modern wound healing.

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Fig. 1. A portrait of Cesare Magati from the work of D.A. Sancassani *Dilucidazioni Fisico-mediche*, Roma, 1733

A Brief Outline of the Life and Works of Cesare Magati

A biography of Cesare Magati was first published by his nephew Prospero and is well described in the works of Putti [10] and Busacchi [2].

Magati was born in Scandiano, near Reggio Emilia, in 1577. He studied medicine at the University of Bologna, from where he graduated in 1597. He then moved to Rome, where he spent a period of further education in the hospitals. This stage was very important for the young physician, as he

observed that the surgeons' methods for treating wounds were quite different from those that he had learned in his classes at the University.

The next important step in the life of Magati was the call, to Ferrara, by the Marquis Enzo Bentivoglio; at this university he was lecturer of surgery from 1612 to 1618; he became also Chief Surgeon in the St. Anna Hospital. During this period, in 1616, he published his masterpiece *De rara medicatione vulnerum seu de vulneribus raro tractandis* (Fig. 2), in which he collected the results of his experiences and proposed a method to treat wounds.

In 1618 Magati entered the Order of Capuchin Friars, with the name of Frà Liberato, but he did not stop practicing medicine. Suffering from urinary stones, he died in Bologna, after a lithotomy operation, in 1647.

De rara medicatione vulnerum [4], printed in folio, is divided into two parts: the first volume is devoted to wound treatment in general and contains the principles of his doctrine; the second is devoted to injuries of different regions and organs.

The book had three editions (1616, 1676, 1733), the last two posthumous. The second and third editions contain, as an appendix, the *Apologia seu tractatus quo rara vulnerum curatio defenditur contra Sennertum*, signed by a brother of Magati, Giovanni Battista, in which the theses that the distinguished physician Daniel Sennert (1572–1637) proposed to challenge Magati's thoughts are countered.

In the following survey, the roman numerals between brackets indicate the volume and chapter of *De rara medicatione vulnerum* from which the quotations are derived.

De rara medicatione: The Fundamentals of a New Method

The essence of the doctrine of Magati is very simple: the healing of wounds is better and faster when the surgeon leaves the lesion in absolute rest. But how did Magati convince himself of this procedure, which was so different from the current practices of his age? We have already emphasized that during the stage in Rome he observed "that the surgeons of the city followed a new method to treat wounds" founded on a major respect for the damaged tissues, but they were not able to justify their behavior rationally and asserted empirically that "all was based on experience" [5]. For Magati experience had an important place in forming his ideas, but he was also able to link the observation with an attempt at rational explanation. The history of the case that led him to go a new way is included in volume I, chapter XXXIV: "I dressed a sore on the leg of a girl once or twice daily" writes Magati, but there was no improvement in the lesion. So "tired of the lengthy treatment I initiated to dress it only every other day, later medicating the ulcer every three days, in a short time it was healed. I experienced the same method in other patients, with positive results."

CAESARIS MAGATI
SCANDIANENSIS
IN ALMO FERRARIENSI
Gymnasio publici Medicinæ Professoris.

DE RARA MEDICATIONE VULNERVM,
seu De Vulneribus rarò tractandis.

LIBRI DVO.

IN QVIBVS NOVA TRADITVR METHODVS,
qua fœliciſſimè, ac citius quàm alio quouis modo ſanantur Vulnera.

Quæcunque præterea ad veram, & perfectam eorum curationem attinent, diligenter excutiuntur: permultaq. explicantur Galeni, & Hippocratis loca eò ſpectantia.

HAEC AVTEM DVPLICI QVAESTIONE.

I. Vtrum melius ſit vulnera quotidiè ſoluere, ac procurare, an pluribus interiectis diebus.
II. Vtrum turundarum, & pencillorum vſus in Curatione Vulnerum ſit neceſſarius.

NOVVM ARGVMENTVM EST, A NVLLO HACTENVS attentatum, ſed pulcherrimum, & Vulnera tractantibus maximè fructuoſum.

AD ILLVSTRISSIMOS VIROS

D. ALEXANDRVM FLASCVM
Equitem Habitus Calatrauæ, ac Sapientum Iudicem.

SAPIENTESQVE MAGISTRATVS

D. MARCHIONEM GALEATIVM GVALENGVM.
D. MARCHIONEM ALOYSIVM BEVILAQVIVM.
Ferrarienſis Gymnaſij Moderatores.

Cum triplici Indice Capitum, Quæſtionum, & Rerum omnium memorabilium.
Superiorum permiſſu, & Priuilegio.

VENETIIS, M DC XVI.
Apud Ambroſium, & Bartholomæum Del', Fratres.

Fig. 2. The frontispiece of the first edition of *De rara medicatione vulnerum*, Venezia, 1616 (Library of Istituti Ortopedici Rizzoli, Donazione Putti)

From these observations he concluded that repeated handling of the wounds hindered tissue repair, while "leaving in absolute rest the injured part, nature had time to carry out the work necessary for healing; with a daily dressing the work was hindered because nature was distracted from its effort. . . . By keeping the wounds covered and quiescent, secretion is minimal, the danger of bleeding is removed, and there is no pain" (I, XL).

However, Magati also emphasized that even drugs without well characterized properties were effective in wound healing (I, XXXVII). Nature became the main protagonist in the field of wound treatment; until then the scenery had been overlooked by the physician with his tools and liniments. Magati is explicit: "it is nature and not the physician with the medicaments that heals the wounds, because it is nature that drains the pus, regenerates the flesh, repairs bone fractures, agglutinates and releases secretions" (I, XL). In this concept, he reveals his agreement with the hippocratic belief: the *vis medicatrix naturae* carries on a primary task that the physician can only help. "The perfection of medicine consists therefore in relieving nature as much as one can" (I, XL).

The practical advantages of the new method are threefold: indeed it is "less hard for the physician, less troublesome for the patient and less expensive" (I, XL). Naturally there are also some situations in which it is necessary to uncover and dress the wound: these are listed in the chapter XXXVIII and include bleeding, gangrene and decay, the presence of foreign bodies, inflammation, obstruction of drainages, and excessive collection of purulent matter.

Between the topics of main interest in Magati's work, we can find his opinions on the influence of the contaminated environment on the injuries. In a prebacteriological era he raised the question about "what therefore happens to a wound exposed to polluted, contaminated or poisonous air" (I, LXVII). The answer is clear with regard to the consequences: "Abundant putrefaction and decaying often occur because of . . . depraved air composition and its pollution". With respect to the cause of those events two centuries later, Joseph Lister (1827–1912) [3] stated positively: "Since it has been demonstrated by the experiences of Pasteur that the harmful properties of air result . . . from certain tiny organisms in suspension, the idea occurred to me that it may be possible to avoid putrefaction of wounds". Regardless of the source of contamination, Magati applied an occlusive bandage: "The physician will easily prepare a dressing with small cloths folded in two, three, four or more . . . to cover abundantly also the surrounding healthy tissues" (I, XXXV). Medication defended the lesion against environmental contamination and allowed the maintenance of the "natural warmth," that is, the main process by means of which nature could fulfil the work of repair.

The principles outlined by *De rara medicatione vulnerum* excited a great deal of debate: they agreed with the doctrines of the renowned physician Lodovico Settala (1552–1633) but were challenged mostly by Daniel Sennert, and this opposition aroused a heated controversy [6].

The basis of the scientific method of Magati is clearly expressed in a passage of the third chapter: "When a doctrine opposes an observation, and there is a contrast between them, it is advisable to follow the observation." In a period of transition, in which doctrinal tradition and the excitement of new experimental methods existed together in medicine, this statement

assumes a double significance: Methodologically, it expresses the ability to criticize any dogmatic assertion on the basis of direct experience; practically, there is the suggestion that all practitioners build their skill not only with a theoretical education, but also by elaboration of their own observations.

As stated by Baglivi (1668–1707) [1] "*Ratio et observatio*" are the two "*praecipui medicinae cardines*" on which the medical act is founded. In this perspective Magati can actually be placed in the new scientific dimension that characterized the seventeenth century.

Concluding, we can refer to the judgement of Antoine Portal [9], who, regarding Magati, wrote, "He was the first that ensured simplification of surgery. His thoughts and principles about the treatment of wounds are worthy of a profound philosoper and an acute observer of nature."

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II. Morphology and Physiology

Fetal Wound Repair

I.K. Cohen and J.H. Haynes

Introduction

The process of wound repair has phylogenetically evolved as an essential survival process after injury. While some lower species retain the ability to regenerate, humans require rapid healing lest they succumb to infection, predators or starvation during evolution. Thus it is likely that wound healing in the form of rapid collagenous scar deposition evolved. The original demonstration that the mammalian fetus retains the ability to heal in a scarless fashion [1–3], resembling regeneration, reinforced the importance of restoration of original structure and function. These studies have suggested important therapeutic strategies in modulating pathologic adult wound healing. For example, can we modulate adult repair to prevent the formation of keloids and hypertrophic scarring? Will the study of the noncontracting fetal skin wound teach us how to control burn scar contracture or common bile duct stenoses? In an attempt to address these and other questions, fetal wound repair has been a major focus of research in our laboratory for the past 5 years. It is hoped that by delineating the mechanisms whereby the fetus carries out optimal wound repair, resembling regeneration, pathologic adult wound healing problems may be ameliorated.

Model

In vivo studies have been carried out in the pregnant New Zealand white rabbit. Does are operated on day 23 of gestation and the fetuses then returned in utero after manipulation. The fetuses are harvested at selected time points until term (31 days) and analysis carried out. This has been a cost-effective model because surgery can be performed on six fetuses

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per doe. The animals tolerate anesthesia well and fetal viability after manipulation exceeds 90% [4].

Characteristics of the Fetal Wound

Initial characterizations of fetal wounds were made in our laboratory when Krummel et al. [5] studied skin wounds in the fetal rabbit. Linear, incisional wounds healed without gross evidence of scarring, while wounds left open demonstrated no evidence of contraction, but rather appeared to increase in size. To further investigate these findings, a polyvinyl alcohol (PVA) sponge wound implant developed at the MCV Wound Healing Center was used. The PVA implant consists of a perforated silicone carrier tube into which the sponge is placed [6]. The device is then placed in the wound to sample the wound environment as healing progresses. We have used this device in numerous human and animal studies. It provides a sensitive indication of matrix metabolism and reflects well the cellular events occurring in the wound. PVA implants removed from linear sutured wounds were assayed for collagen using highly sensitive high pressure liquid chromatography (HPLC) capable of measuring hydroxyproline in the picomole range. No collagen could be detected at assay [7]. However, we reasoned that there *must* be collagen synthesis in order for healing to occur and could only hypothize that collagen could not be detected due to rapid turnover. This hypothesis has been strengthened further by simultaneous findings that scars could not be detected in 5-day-old primarily closed fetal incisions. This phenomenon was observed both on routine histology and electron microscopy. Histologically, the epithelium appeared completely normal with restoration of the basement membrane. The dermis contained a normal appearing matrix without any evidence of collagen disorganization, which would suggest scar deposition [8].

To further investigate the role of collagen in fetal wound repair, we measured collagen synthesis in adult and fetal fibroblasts under room air and hypoxic conditions. In both environments, fetal fibroblasts produced collagen at a rate greater than adult fibroblasts [7]. Thus it was apparent that a lack of collagen deposition at the wound site was not due to an intrinsic defect of the fetal fibroblast to synthesize collagen. This strengthened our supposition that there was probably a significant collagen turnover in fetal wound healing.

Subsequently, collagen synthesis was measured in these primarily closed fetal wounds and in adult rabbit wounds. In the adult, the absolute rate of collagen sythesis is increased, but noncollagen protein was not increased [7]. In contrast, in the fetus the absolute rate of both collagen and noncollagen protein synthesis are elevated significantly after wounding. Therefore, we hypothesized that the increased fetal noncollagen protein synthesis is

related to the rapid metabolic activity of the developing fetus and that a portion of this protein may be the collagenase required for the rapid turnover that occurs in the fetal environment.

Collagenase activity in the fetus has not been measured directly in our rabbit model. However, mRNA for collagenase has been detected in maternal and adult rabbit wounds. Preliminary studies indicate that there is significantly greater collagenase mRNA in fetal wounds suggesting that fetal collagenase activity is greater than that of adult wounds.

Although collagen turnover appears to be very rapid in the fetus, the breaking strength of healed fetal incisional wounds develops at a rate dramatically greater than that in adults. Therefore there appears to be a rapid remodeling and removal of the newly synthesized fetal collagen to account for the fact that collagen is not detected in abundance as seen in adult repair.

We are currently focusing our efforts in this area on understanding the role of collagenase in this remodeling process. Moreover, data accumulated thus far indicate that matrix *organization*, rather than the quantity of collagen synthesis and deposition, is the major factor in the development of wound tensile strength.

Hyaluronic Acid in Fetal Wound Repair

The finding of a lack of collagen deposition in the PVA implants from fetal wounds led us to identify the extracellular matrix which had been deposited instead of collagen. Using alcian blue stains, we demonstrated the matrix to be rich in glycosaminoglycans. Treatment of specimens with hyaluronidase revealed the presence of hyaluronic acid (HA) [5]. Electrophoresis later confirmed the presence of almost 100% hyaluronic acid [9].

These findings linked the fetal repair system to previous literature suggesting that HA controlled the differentiation and movement of cells early in embryonic development. When HA content in fetal wounds was compared to that of normal adult rabbit skin or wounds, the content of fetal wounds was always significantly higher. To test the hypothesis of a regulatory role of HA in fetal wound repair, we placed PVA sponges containing hyaluronidase at the fetal wound site [10]. This treatment lowered the amount of HA accumulating in the sponges. Concurrently, an inflammatory response was seen as well as the deposition of collagen. Therefore, as predicted, removal of HA allows the transition from the fetal wound healing to one which is more adult-like. It is not known if this occurs by a direct mechanism of HA on the cell walls, by the binding of receptors or perhaps related to activation or inhibition of various cytokines. Further work is underway that addresses the role of specific HA fragments in regulating fetal wound repair.

Role of Growth Factors in Fetal Wound Repair

With the recent accumulation of large volumes of data on the role of growth factors in adult wound healing, it seemed logical to test the responsiveness of the fetal system to well known adult wound healing mediators. Using the PVA implant, both platelet derived growth factor (PDGF) and transforming growth factor-β (TGF-B) were placed at the fetal wound site for various time periods and concentrations [11, 12]. Both growth factors induced an inflammatory and fibrotic response as sampled by the implant. Additionally, the PDGF treated implants demonstrated neovascularity at higher doses. These studies again demonstrated the capacity of the fetus to change from a regenerative type of repair of a more adult, fibrotic state.

Fetal Wound Contraction

One of the earliest observations in fetal wound repair was the finding that open fetal rabbit wounds do not contract in utero [13]. This work was confirmed first morphologically, then histologically with the finding that fetal rabbit wound edges did not contain myofibroblasts, the cellular element presumed responsible for contraction, nor was there inflammation or collagen deposition characteristic of adult wound contraction [8]. It should be noted, however, that myofibroblasts are associated with contracting fetal sheep wounds, reflecting interspecies variations [14].

To further address the issue of noncontraction, amniotic fluid (AF) which bathes the open fetal wound has been postulated as an inhibitor of wound contraction. When open fetal wounds are covered by a silicone patch, they decrease in size with time. Histology of the wound edge, however, demonstrates a mesenchymal cell proliferation suggesting a regenerative process. To address the effect of AF on open fetal wound closure, HA levels were measured in wounds both exposed and excluded from AF contact. We found those wounds not exposed to AF (and subsequently closing) contained a higher HA content than those exposed [15].

In summary, wound healing is entering a new age due to the availability of molecular biological techniques that will allow us to discover the mechanisms which precisely control wound healing and are responsible for its excesses and deficiencies. The application of these techniques to elucidate the sequence of events responsible for the seemingly optimal fetal system of repair are present goals. Unlocking these secrets will allow clinicians to deal with difficult wound healing problems such as anastomotic structures and burn wound contractures.

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Histological Examination of Skin Grafts

A. Fisseler-Eckhoff and K.-M. Müller

Introduction

Wound Healing of the Skin

Surface epithelial tissue of the skin belongs to the *Wechselgewebe* (regenerating tissue) due to the rapid proliferation of the cells. Under physiological conditions the continuous supply of cells results from the mitotic cells of the stratum germinativum (Cottier 1980). In traumatic injury of the skin, there is an interruption of tissue continuity with destruction of all epidermal cell layers and of concomitant mesenchymal and vascular tissue; thus a restitutio ad integrum due to proliferation of the germinative cell layer of the epithelial surface of the skin is not possible. Complex reparative and regenerative changes of wound healing, proceeding in three phases, are necessary: In the first phase the wound is covered with blood and a fibrin clot, which is the first barrier against infection. Tissue at the border of the damage becomes necrotic with migration and demarcation of necrosis by granulocytes. The second phase of wound healing is characterized by proliferation of angioblasts, fibroblasts, histiocytes, lymphocytes and plasma cells resulting in granulation tissue. Proliferation of connective tissue leads to skin scarring in a third phase.

Wound Healing in Burns

Healing of skin wounds primarily depends on the kind, extent and localization of skin damage (Lindner and Huber 1973, 1983; Dinges et al. 1979, 1980). In extended skin damage due to thermal injury, destruction of all epithelial cells makes physiological wound healing impossible. Additionally, problems of secondary microbial wound contamination with possible

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complications such as bacteremia and sepsis determine the clinical development. Therefore definite closure of the damaged skin areas in burns ensures the survival of the patients.

Fibrin Sealing – Historical Aspects

In the beginning of the twentieth century scientific work on wound healing in burns was mainly concentrated on finding a tissue glue that fulfilled the desire for nontraumatic tissue synthesis. The central role of fibrin in blood coagulation (Beneke and Schmidt 1971; Müller and Berkel 1983) and its importance as a protective and healing substance led to the use of powdered fibrin preparations from horse and bull in animal experiments in 1909. Grey (1915) and Harvey (1916) used, respectively, fibrin tampons and thin fibrin plates to stop bleeding of parenchymal organs. Genuine interest in an effective fibrin sealing system was prompted by the discovery of clotting factor XIII by Laki and Lorand in 1948 and its isolation by Loewy et al. (1961). The era of fibrin sealing was interrupted by the discovery of the glue-like quality of synthetic acrylate monomers by Coover in 1959 (see Lick et al. 1966). In 1972 Matras et al. took up the idea of fibrin sealing again. Preliminary results on the sealing of nerve anastomoses in animal experiments by use of fibrin glue were reported. Since then the fibrin sealing system has been the object of numerous clinical and experimental examinations (Braun et al. 1975; Staindl 1977; Spängler et al. 1973; Spängler 1976; Matras 1980). In spite of the frequent use of fibrin glue in surgery the importance and effect of fibrin sealing for wound healing, mainly in the operative care of extended degree burns, is still controversial. In wound healing in patients with different degrees of burns, good adaption and atraumatic fixation is particularly necessary (Braun et al. 1975). Till now no quantitative and qualitative morphological examinations concerning the question of wound healing in burns with sealed or nonsealed transplants have been available in humans.

Material and Methods

Investigated Collective

In cooperation with the Department of Plastic Surgery and Burns of the Berufsgenossenschaftlichen Kliniken Bergmannsheil, Bochum (University Clinic), 23 sealed and 13 nonsealed bioptically obtained biopsies of 21 patients (13 male and eight female) were investigated histologically. The biopsies were obtained at different time intervals after surgery. The percentage of skin burned was between 7.5% and 60%. All patients had second

degree burns, i.e., partial necrosis of the epidermis without destruction of the skin adnexes, or third degree burns, i.e., necrosis of epidermis and corium with destruction of skin adnexes. Microbial contamination of the burned skin with *Staphylococcus aureus*, *Streptococcus epidermidis* and *S. faecalis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans* and other germs could be observed. After surgical excision of superficial destroyed skin areas treated by a tangential or deep excision and hemostasis, skin grafts were taken from other body regions of patients as autotransplants. In one patient the wound was covered with the skin of a pig, in two patients skin grafts of their parents were used. These, respectively, hetero- and homotransplants were replaced by autologous transplants in a later operation. In seven patients necrosis extended up to the deep layer of the corium, therefore grafts were directly placed on the subcutis. In cases of sealed transplants a small layer of the fibrin sealing system was applied on the wound before adaption of skin grafts. For fibrin sealing the Tissucol system was used (Immuno GmbH 6900, Heidelberg, FRG) (Fisseler-Eckhoff et al. 1987).

Histological Methods

During additional skin transplantations, samples 1–2 mm in diameter of former sealed and/or nonsealed grafts from the first operation were taken at different intervals (days 1, 2, 4, 5, 6, 8, 9, 12 and 14 after the first operation). Bioptically obtained specimens were fixed in formalin and embedded in paraffin. To demonstrate histologically the fibrin components of the fibrin sealing sytem, the sensitive Martius scarlet blue stain (MSB-Lendrum) was used (Lendrum et al. 1962). This stain is supposed to be the most sensitive method for morphological demonstration and differentiation between fresh and older fibrin components (Dinges et al. 1979, 1980).

For **objective morphological evaluation** of wound healing in sealed and nonsealed grafts intensity of inflammatory reaction, connective tissue proliferation, capillary proliferation, adaptation and fixation of the grafts were evaluated according to a defined scale (see Fig. 3).

Aim of Investigations

The comparative morphological examinations of sealed and nonsealed skin grafts were guided by the following questions:

- Are there any morphological differences in regenerative and reparative changes of wound healing in sealed and nonsealed grafts?

- Are there differences in the intensity of capillary and connective tissue proliferation dependant on time after surgery?
- Is the fibrin glue detectable by histological staining?

Results

Intersection Zone Transplant/Wound Inflammation

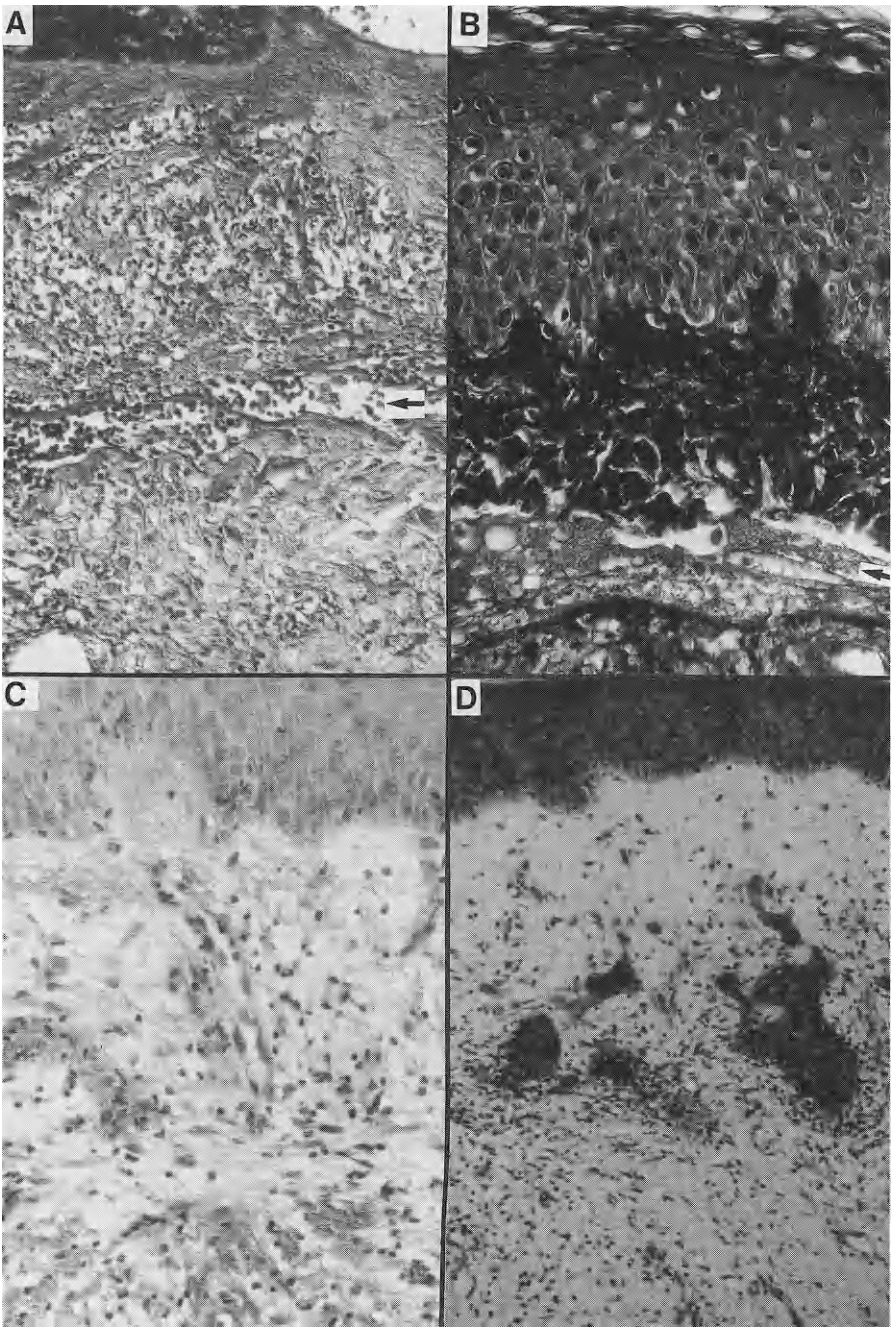
In the first 2 days after transplantation a different enlarged gap was demonstrated without any connections between the grafts and skin of the hosts. Within the transplantation zone erythrocytes, inflammatory cells, fibrinous and proteinous exudate were seen in both sealed and in nonsealed specimens (Fig. 1A). The fibrin glue was visible as a homogenous, linear, granular, red substance in the junction area without broadening of the tissue gap (Fig. 1B). Beside exogenous fibrin of the sealing system endogenous fibrin could be demonstrated in sealed and nonsealed specimens in regions of transplantation zones and in deeper layers of the corium.

In all specimens inflammatory reactions with lymphocytes and granulocytes – normally restricted to the region of transplant and gap – could be observed, but all other layers of the host skin could be involved, too. Different degrees of inflammation were observed between sealed and nonsealed specimens. In contrast to sealed grafts an enforced immigration of inflammatory cells was observed in nonsealed grafts in early time intervals after surgery (Fig. 1A). Only mild inflammation was observed on the first day in sealed grafts up to 14 days after operation. Inflammation was restricted to epidermal remnants left after necrectomy beyond the former transplantation zone (Fig. 1D).

Connective Tissue Proliferation

While inflammation and bleeding were prominent during the first 2 days, in sealed grafts mild proliferation of fibroblasts was observed even in the first 2

Fig. 1A–D. Comparative histological demonstration of wound healing in sealed and nonsealed skin grafts. **A** Nonsealed skin graft 1 day after transplantation without any connection between the graft and the skin of the host. Within the transplantation zone (*arrow*) erythrocytes, inflammatory cells and exudate; 69 year old woman, Ladewig stain, ×140. **B** Sealed skin graft 3 days after transplantation. Between the graft and the tissue of the host the fibrin glue is visible as granular, linear substance (*arrow*) in the junction area without broadening the tissue gap; 55 year old woman, Lendrum stain, ×350. **C** Sealed skin graft 6 days after transplantation. Proliferation of capillaries and fibroblasts. Loosened connection between transplant and host skin; 52 year old woman, Goldner stain, ×220. **D** Sealed skin graft 8 days after transplantation. Increased fiber proliferation with overwhelming of the gap. Demonstration of original epidermal islets with mild inflammatory reaction within the former zone of transplantation gap staining positive with keratin; 52 year old woman, keratin stain, ×140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days. From the fifth day onwards concomitant and increasing fibroblast and capillary proliferation could be observed (Fig. 1C). These regenerative changes were prominent from the sixth day up to the twelfth day after surgery. In nonsealed grafts regenerative and reparative changes took place with a delay of 2 days. Intensity of reactions increased up to the twelfth day. Production of matrix structures with proliferation in first weak and then stronger collagen fibers on the fifth day in sealed specimens and on the eighth day in nonsealed specimens increased with advancing time was seen. The results demonstrated an even looser connection between transplant and host skin at the fifth postsurgical day in sealed grafts. With concomitant increase of fiber proliferation a stronger connection between transplant and host skin, which finally led to closure of the gap, was seen in sealed grafts. In nonsealed grafts the gap was primarily crossed by capillary loops before fine fiber neosynthesis appeared (see Fig. 3). On the fourteenth day after surgery skin repair comparable to that of regular undamaged skin could be observed. The former transplantation gap could only be recognized by the original epidermal islets, which stained keratin positive immunohistochemically (Fig. 1D).

Staining of Exogenous Fibrin Due to the Fibrin Sealing System

For better differentiation of endogenous and exogenous fibrin components staining of fibrin was compared in different staining methods. Endogenous fibrin was demonstrated as fine cord-net or fiber-like structures whereas exogenous fibrin of the fibrin glue appeared as a more homogenous, granular substance, as stained by MSB-Lendrum staining. During the first 4 days the glue was demonstrated as different, wide, border-like lines of demarcation between the transplant and the original host tissue skin (Fig. 2A). In the course of early enzymatic digestion and resorption fibrin is dissected into small fragments. From the fifth day on only fragmented clots were found (Fig. 2B). Due to the different microscopic appearance of endogenous and exogenous fibrin, demonstrated by the sensitive MSB-Lendrum staining, the fibrin sealing system could be demonstrated from the first day up to the ninth day. After the ninth day no exogenous fibrin remnants were found within the former transplantation zone. By the MSB-Lendrum stain, a histological differentiation between collagen fibers, which were stained dark blue, and the exogenous fibrin clots (staining colour of the glue in early stage red, late stage blue) was possible. Endogenous fibrin was detected in low amounts in peripheral zones of the transplant and in the subcutis.

Discussion

Morphological results of histologically obtained biopsies 1–2 mm in diameter of sealed and nonsealed skin grafts of burns taken at defined time intervals

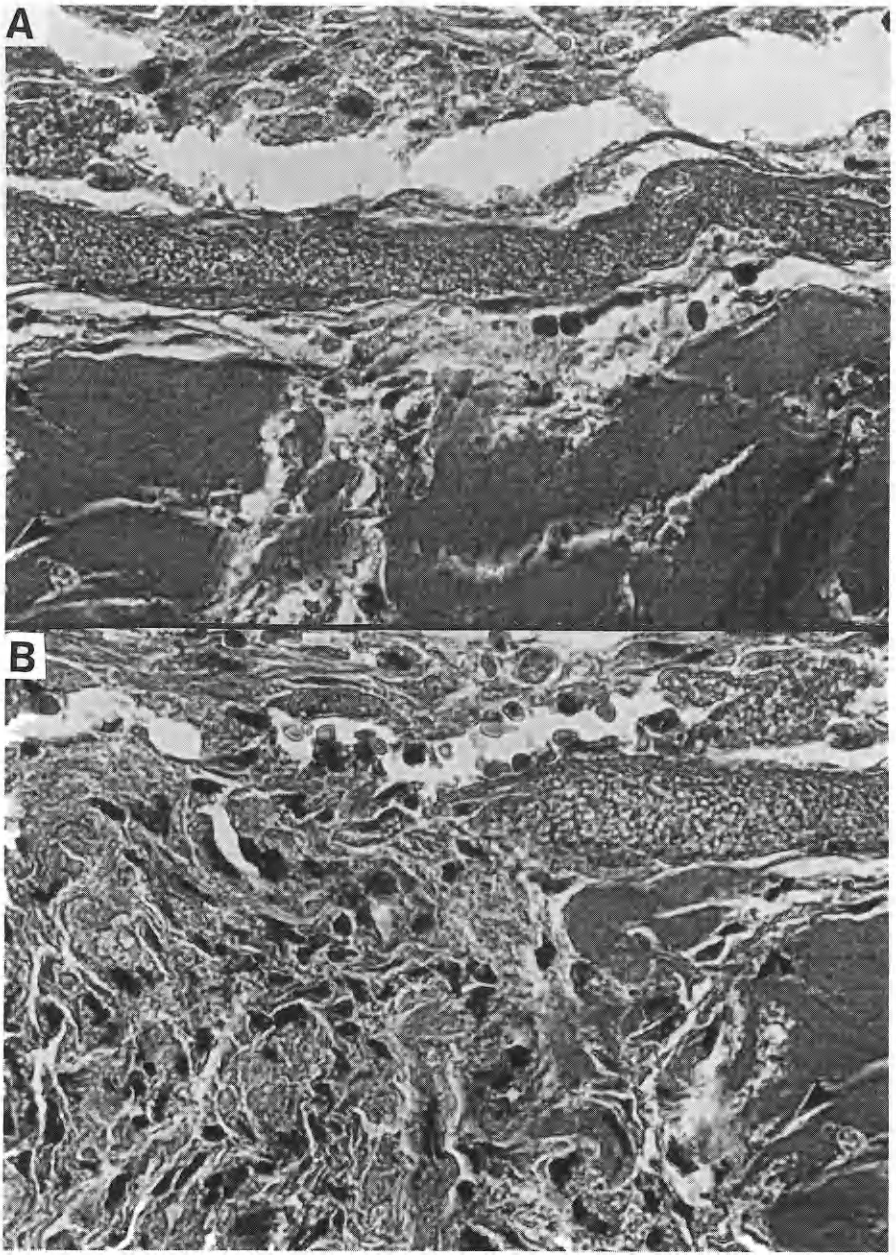

Fig. 2A,B. Histomorphological appearance of fibrin glue in Lendrum staining. **A** Fibrin glue visible as different wide border-like line of demarcation between the transplant and the original skin of host tissue 4 days after transplantation; 23 year old woman, Lendrum stain, ×560. **B** Histomorphological demonstration of fragmented rest clots of exogenous fibrin due to enzymatic digestion and resorption 5 days after transplantation. Overwhelming of the transplantation zone by proliferation of connective tissue fibers; 23 year old woman, Lendrum stain, ×560

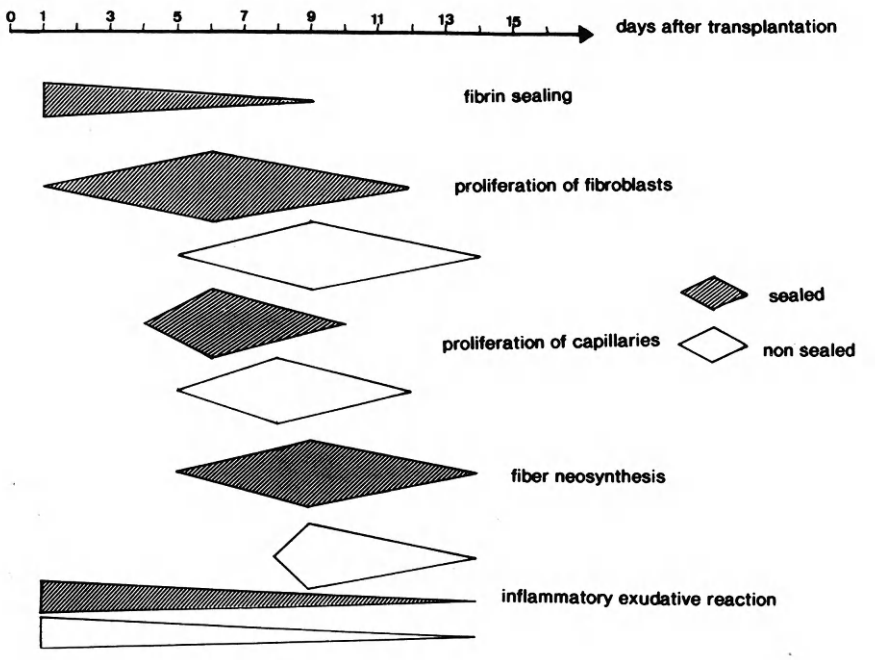

Fig. 3. Comparison of morphological results of wound healing from sealed and nonsealed skin grafts in relation to time

after first transplantation demonstrated differences in regenerative and reparative reactions in wound healing (Fig. 3). In biopsies which have been sealed, the early phase of transplantation is characterized by a discrete increase in fibroblast and capillary proliferation during the first days, followed by tight connection of proliferating granulation tissue from the fifth day on. In nonsealed grafts the early phases of wound healing were demonstrated not earlier than the sixth postoperative day. In the later phases of wound healing there were no essential morphological detectable differences (Fig. 4). Proliferation of fibroblasts in sealed grafts up to 4 days earlier than in nonsealed grafts (see Fig. 3) can be attributed to the fibroblast stimulating activity of fibrin and clotting factor XIII. In animal experiments Knoche and Schmitt (1976) could already demonstrate an inducing, accelerating effect of factor XIII on fibroblast proliferation. Beside clotting factor XIII, fibrin itself has a direct fibroblast stimulating effect that is essential for the presence of a fibrin network as a proliferative attraction of fibroblasts in culture (Beck et al. 1962; Bruhn et al. 1979; Turowski et al. 1979). A further positive influence on early fibroblast proliferation might be the tight and calm junction of transplant with host skin. Due to the loss of micromovements between graft and wound base, sheer forces will be much fewer. This might be a major advantage of fibrin sealing in adaptation and incorporation of the

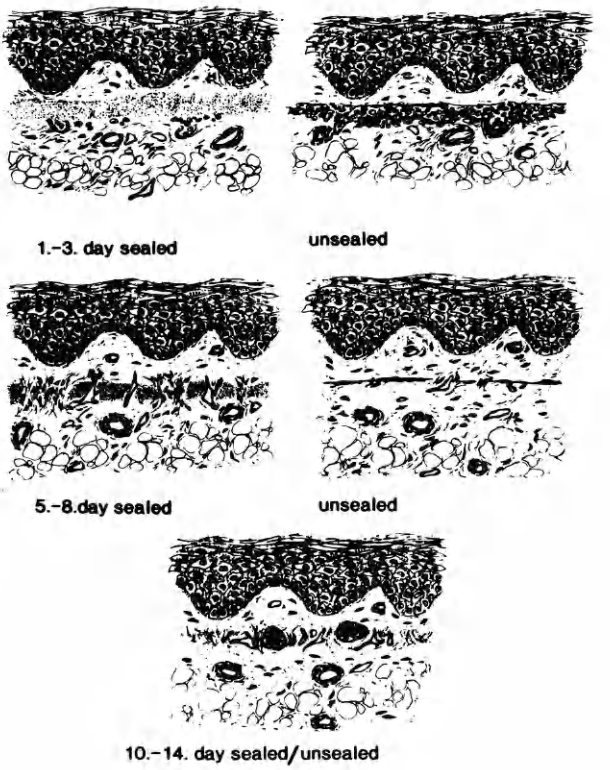

Fig. 4. Comparative demonstration of phases of wound healing in sealed and nonsealed skin grafts with morphological differences up to the ninth day after transplantation

graft especially in the early postoperative phase. In our investigations inflammatory reactions and the intensity of bleeding were fewer in sealed than in nonsealed grafts. By contrast, Edinger et al. (1982) observed severe inflammatory reactions in sealed grafts compared to nonsealed grafts in experiments in rabbits. This was suggested to be due to foreign body reactions in the transplant area. In our opinion this foreign body reaction can be explained by the use of a human cryoprecipitate as glue which is a heterogeneous protein component of animals. Therefore due to an antigenic effect an antigen-antibody reaction is induced. This was not the case in our investigations.

With increasing time intervals up to the ninth day after first transplantation, no substantial morphological differences were seen in the histological appearance of sealed and nonsealed specimens. Exogenous fibrin glue is demonstrated as a fresh red colored clot in the early postoperative phase. With an increasing postoperative time interval, a change in the staining color of fibrin, from red to blue, can be observed. The exogenous fibrin glue

is detectable up to the ninth day after surgery. MSB-Lendrum staining is the most sensitive staining method for demonstration of endogenous and exogenous fibrin (Dinges et al. 1979, 1980). After 9 days exogenous fibrin is resorbed completely.

According to histological examinations and clinical studies, the use of fibrin glue in skin transplantation of burned patients is not injurious. However, the demonstrated histological results concerning proliferation of granulation tissue, inflammatory reaction and bleeding are preliminary due to the small number of cases.

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Reepithelialisation of Wounds

I.M. Leigh

Introduction

Reepithelialisation of wounds involves a number of different processes affecting the biology of the keratinocyte. Following breaching of the epidermis the keratinocytes must first migrate across the surface of the denuded area, then mitosis must occur close to the edge to expand the population (Odland and Ross 1968; Krawczyck 1971; Clark 1985). Once keratinocytes have covered the defect they must establish a basement membrane zone, which aids keratinocyte attachment. Then stratification and differentiation must occur to normalise the newly regenerated epidermis. Subsequently the dermis is remodelled, which also involves the keratinocytes by keratinocyte-mesenchymal interactions mediated by cell contact, diffusible cytokines and extracellular matrix proteins. Replacement of a denuded area of skin by different forms of skin grafts, including keratinocyte sheets, will accelerate the epithelialisation process. These biological processes of keratinocytes will be discussed following an introduction to keratinocyte culture, which provides the basis for many observations applicable to wound healing.

Keratinocyte Culture as a Model of Wound Healing and for Grafting

Basal keratinocytes can be separated from a mixed epidermal cell suspension and will differentially attach to a tissue culture dish. In the optimal culture conditions, described by Rheinwald and Green (1975), both a feeder mesenchymal mouse 3T3 layer (Alitalo et al. 1982) and mitogens are provided in a serum containing nutrient medium (Rheinwald and Green 1975). The keratinocytes attach to the dish. Individual keratinocytes then divide to form colonies which migrate across the dish to form confluent stratifying

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sheets (Green et al. 1979). This process mirrors what happens at the edge of a healing wound and can therefore be used to investigate the factors stimulating keratinocyte migration and proliferation. Epidermal growth factor and transforming growth factor-α (TGF-α) increases the lateral migration of colonies (Barrandon and Green 1987). Other factors also stimulate growth including cyclic AMP elevating agents, such as cholera toxin (Green 1978), and transferrin which are routinely added to the culture medium. The keratinocytes can be removed by trypsinisation and repassaged for about 50 population doublings, before normal cells senesce. Following establishment in culture, the keratinocytes can be replated in different culture conditions. Low levels of extracellular calcium reduce the ability of keratinocytes to stratify by loss of desmosomal connections, so the cells grow as a monolayer (Hennings et al. 1980; Boyce and Ham 1985). Keratinocytes cultured on plastic are activated and produce a wide range of cytokines, which form complex autocrine loops of growth promoting and growth inhibiting signals (Kupper et al. 1988).

The stratification obtained in the 3T3 feeder system is incomplete, with no formation of a stratum granulosum and stratum corneum or expression of differentiation markers such as membrane coating granules (Holbrook 1989), so the factors influencing differentiation require different culture conditions. The addition of a collagenous substrate (to make a skin equivalent) increases morphological differentiation with the formation of a cornified layer, and the thickness and organisation of this terminal differentiation is increased by adding fibroblasts, either to the undersurface of the matrices or incorporated within them (Bell et al. 1983; Mackenzie and Fusenig 1983). Most matrices are based on collagen (human, rodent or bovine) often stabilised by the addition of other matrix proteins such as chondroitin sulphate (Burke et al. 1981; Boyce and Hansborough 1988). Deepidermalised live or cryopreserved dermis can also be used as a substrate for keratinocytes, which promotes good morphological differentiation (Pruneiras et al. 1983; Asselineau and Pruneiras 1984). An additional feature of keratinocytes cultured on matrices is that basement membrane components are synthesised, including hemidesmosomes. In all culture systems the keratinocytes continue to proliferate rapidly, the cell cycle in culture (Albers et al. 1986) being around 22 h. Keratinocytes express a pattern of differentiation characteristic of hyperproliferative skin. From these studies in vitro we can make a number of observations about wound repair: that the reepithelialisation of wounds is preceeded by keratinocyte activation, adoption of the hyperproliferative phenotype and keratinocyte migration.

Keratinocyte Migration

In vitro, the migration of keratinocytes in response to changes in substrate can be examined. Keratinocytes in low calcium culture conditions were

examined in a migration assay with a variety of substrates including albumin, types I, IV and V collagen, heparan sulphate proteoglycan and fibronectin. Migration of basal keratinocytes occurred when the cells were in contact with fibronectin and collagens type I and IV, but was inhibited when the keratinocytes were in contact with laminin. The keratinocyte migration index was maximal on type IV collagen and completely inhibited by laminin. Laminin may therefore act as a stop signal for keratinocytes to prevent them from migrating (Woodley et al. 1988; O'Keefe et al. 1984, 1985). In time lapse photography of keratinocyte culture, the keratinocytes which are migrating will have a flattened appearance with pseudopodia projecting in the direction of movement, and indeed individual keratinocytes may migrate away from the moving edge of keratinocyte colonies to patrol the advancing area. Interaction between keratinocytes and mesenchyme depends on cell surface receptors or integrins binding and directing the specific connective tissue components (Hynes 1987). In cultured keratinocytes $\alpha 2\beta 1$ and $\alpha 3\beta 1$ mediate adhesion to collagen and laminin, $\alpha 5\beta 1$ mediates adhesion to fibronectin and $\alpha v\beta 5$ is a vitronectin receptor (Adams and Watt 1991); Carter et al. 1990). Extracellular contact with fibronectin also inhibits terminal differentiation (Adams and Watt 1989).

In normal skin the dermis contains collagen, elastic fibres, endothelial cells, fibroblasts and extracellular matrix proteins including heparan sulphate proteoglycans. The basement membrane zone separates the epidermis from the dermis and mediates epithelial-mesenchymal interactions. In normal skin integrin expression is largely confined to the basal layer, expressing $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 4$ and $\alpha v\beta 5$. During wound reepithelialisation keratinocyte migration proceeds laterally both from the edge of a wound and from the hair follicle appendages correlating with changes in adhesiveness (Grinnell 1992). On wounding, the basement membrane is ablated and keratinocytes will have to migrate across the matrix which contains high levels of plasma and tissue fibronectin (Clark 1990). Keratinocytes from intact skin adhere poorly to fibronectin but keratinocytes from a wound bed adhere well to fibronectin (Takashima and Grinnell 1985). The wound bed also contains altered matrix components. Alternatively spliced fibronectin (III CS) is synthesised by fibroblasts in the upper dermis, but there is also induction of novel components. Tenascin is an extracellular matrix protein which is found in focal condensations at the tips of dermal papillae in normal skin but which is widely expressed in the dermis during wound healing, tumour invasion and epidermal hyperproliferation such as psoriasis. These proteins may also play a role in directing keratinocytes as they appear at invasive sites.

Keratinocyte integrin expression is altered in wound healing. The fibronectin receptor is expressed by keratinocytes migrating from explants (Guo et al. 1990), and following suction blister injury there was a striking alteration in the distribution of integrins, normally restricted to basal keratinocytes. At the time of wound closure $\alpha 3$ and $\beta 1$ integrins were found

suprabasally at 3 days following injury and $\alpha 6$ at 4 days then $\alpha 2$ (Hertle et al. 1992). Integrins may be up-regulated by cytokines and growth factors in the wound bed (Nathan and Sporn 1991).

Keratinocyte Proliferation

The normal epidermis comprises a proliferative basal layer of keratinocytes and suprabasal compartment of terminally differentiating keratinocytes (Fuchs 1990). In a steady state basal cells divide infrequently to replace corneocytes shed at the epidermal surface. The proliferative population appears to arise from a number of slow cycling stem cells in protected sites such as the hair follicle, and undergoes a limited number of amplifying divisions before becoming postmitotic (Potten and Morris 1988). There is a tremendous reserve capacity for increased numbers of amplifying divisions on demand. The epidermis becomes hyperproliferative in many skin diseases, but particularly psoriasis, when the cell cycle time is approximately 50 h and the epidermal turnover reduces to 24 h (Weinstein 1975). When epidermis is injured, for example by gentle sustained suction, there is minimal dermal damage as the suction splits the skin through the lamina lucida of the basement membrane zone, and the dermis is not overtly disrupted (Hunter et al. 1974). There are however subtle changes in dermal neuropeptides and vasoactive peptides (unpublished data). Reepithelialisation starts to occur by migration of the edge between 12 and 24 h. It is likely that alteration in cell shape and rigidity by altering the cytoskeleton to a more plastic form is a prerequisite for this migration to occur. Subsequently mitosis occurs away from the migrating edge. During repair from suction blister injury (Mansbridge and Knapp 1987), although the blister edge epidermis contributes to reepithelialisation, regeneration also proceeds from the appendages particularly the hair follicle (Lane et al., in preparation). A potent stem cell population has been known to occur in the hair follicle from work on epidermal regeneration following radiation (Withers 1967). Recent studies have suggested that this population occurs in the upper part of the deep outer root sheath at the site of attachment of the erector pili muscles: the bulge region (Cotsarelis et al. 1990). During the hair cycle, anagen follicle and secondary trichocyte differentiation proceed from this point. Following suction blister injury, the bulge region of the regenerating hair follicle becomes irregularly expanded in outline suggesting that this pluripotential region is contributing to the follicular regeneration.

Much of the knowledge about epidermal growth has come from studies in vitro where the effects of growth factors, hormones and nutrients in the tissue culture medium can be studied under controlled conditions. The growth requirements in vitro include nutrients, particularly amino acids, carbohydrates, vitamins and nucleic acids, lipids and low molecular weight trace elements. The addition of serum is necessary for growth of most

keratinocytes in vitro, but pH, oxygen tension and temperature of the media have to be considered also. Standard growth culture medium contains those factors known to stimulate keratinocyte growth, in particular epidermal growth factor, triiodothyronine, hydrocortisone, bovine brain extract, insulin, transferrin and trace elements. Keratinocytes can be cultured in the absence of serum in the presence of many growth factors including a bovine pituitary extract which contains a number of growth promoters (Boyce and Ham 1985).

Keratinocytes in vitro are activated and synthesise a range of cytokines, small peptides which act as biological mediators and intercellular messages to promote cell proliferation, alone or in concert by binding to specific cell surface receptors. They are multifunctional molecules acting on different cells in different ways; for example TGF-β promotes fibroblast growth whereas it inhibits keratinocyte growth. Hyperproliferative epidermal keratinocytes are therefore activated and secrete a large range of cytokines which promote keratinocyte growth via autocrine loops. These activated keratinocytes clearly differ in phenotype and behaviour.

As determined from keratinocyte studies in vitro, there are a large number of cytokines/growth factors which could potentially aid reepithelialisation in vivo either by direct effects on keratinocyte growth and migration or indirectly through effects on mesenchymal cells, such as enhancing vascularisation. The wide range of growth factors which have an effect on keratinocytes can now be produced by recombinant DNA technology and can be added to the surface of the wound. They may act singly, in concert with other growth factors or by initiating a cascade of growth factors (McKay and Leigh 1990). The growth factors which have been shown to enhance keratinocyte growth in vitro are shown in Table 1 and some of these have been shown also to enhance epithelialisation in animal models. Major growth factors likely to promote epithelialisation are TGF-α (Schultz et al. 1987), interleukin-1 (IL1) (Mackenzie and Sauder 1990) and interleukin-6 (IL6) (Grossman et al. 1989). However, other growth factors such as TGF-β (Sporn et al. 1983) and platelet derived growth factor (Lynch et al. 1987) affect the wound, predominantly through the mesenchymal component of the wound bed with enhanced angiogenesis and the promotion of granulation tissue which may then have a secondary effect on the epithelialisation. On the whole, however, the wound bed will contain a large number of growth factors and the addition of growth factors to the surface may not perhaps produce a consistent reproducible result. There is very little known about the expression and role of growth factors in normal and pathological wound healing, and these data are required before the interpretation of the growth factor responses. It is clear that the keratinocytes in vitro and in wound healing do secrete large amounts of TGF-α, IL1 and IL6, which form autocrine loops amplifying keratinocyte production of growth factors and growth factor receptors. Growth factors may potentially promote the growth of tumours if they were present in the wound, and of course there is a risk of

Table 1. The growth factors produced by keratinocytes (P) and those where biological response to growth factors (R) has been demonstrated

Growth Factors		Keratinocytes		
Families	Members	P		R
FGF	aFGF	?		+
	bFGF	+	A	+
	KGF	−		+
EGF	EGF	−		+
	TGF-α	+	A	+
	AR	+	A	+
	HB-EGF	−		+
PDGF	PDGF AA and BB	+		−
	VEGF	+		−
TGF-β	TGF-β1	+	A	+
	TGF-β2	+		?
	TGF-β3	+		?
TNF-α	TNF-α	+	A	+
	TNF-β	−		+
IL-6	IL-6	+	A	+
	ONCO-M	?		+
	G-CSF	+		−
SIG	MCAF	+		−
	MGSA	+		−
	IP-10	+		−
	IL-8	+	A	+
Small peptides	Endothelin	+		?
	EIP	+	A	+
	Epithelin-1	+	A	+
	Bradykinin	?		+
Others	IL-1	+	A	+
	SF	−		+
	GM-CSF	+		+
	M-CSF	+		−
	MULTI-CSF (IL-3)	+		−
	IL-4	−		+
	IL-10	+		?
	NGF	+	A	+
	IGF-1	−		+
	IFN-γ	−		+

tumour development in chronic ulceration, so it is clearly of utmost importance to exclude the presence of any tumour before any forms of growth factor treatment. Also there are potential harmful effects from the systemic absorption of growth factors and the promotion of distant tumours, as well as systemic effects such as on the acute phase response. Therefore growth factors need to be assessed cautiously before going into widespread clinical use.

Keratinocyte Growth Inhibition

Interferon-γ has many effects on keratinocytes which can be both immunomodulatory and growth regulatory (Nickoloff et al. 1991). Exposure of keratinocytes to interferon-γ caused the induction of class II HLA antigens on the cell surface and ICAM I expression (Barker et al. 1990). The presence of interferon-γ, however, is inhibitory to keratinocyte proliferation and reduces keratinocyte maturity, extracellular matrix production and responsiveness to growth factors. During cutaneous wound healing there has to be a phasic response once reepithelialisation is complete. As interferon-γ can inhibit keratinocyte migration and proliferation, it could be a trigger for decreased keratinocyte growth late in the wound healing response. Addition of gamma interferon exogenously to a wound will inhibit wound healing. Nickoloff et al. (1991) has suggested that when keratinocytes become activated following epidermal injury and disruption of the basement membrane, this activation is associated with ICAM 1 expression and low levels of production of chemotactic factors such as IL8 and NCAM. As the keratinocytes become exposed to the extracellular matrix, including fibronectin from platelets, the infiltrating cells provide those cytokines which stimulate keratinocyte movement, proliferation and inhibition of terminal differentiation. At this time there are low levels of growth inhibitory cytokines, such as interferon-γ, however, later, when there is an acute inflammatory response with activated lymphocytes and monocytes, these increase in amount and eventually produce an inhibition of keratinocyte proliferation and induction of differentiation.

A further keratinocyte growth inhibitor is TGF-β but this is detected early after wounding in suction blisters and in hypoxic wound edge; therefore it may have more effect on migration than growth. The promotion of granulation tissue and increased tensile strength in incisional wounds produced by TGF-β is also of importance (Mustoe et al. 1987).

Keratinocyte Attachment: Basement Membrane Components

In normal skin the basal keratinocytes are in contact with the basement membrane zone (Briggaman and Wheeler 1983), which comprises plasma membrane bearing hemidesmosomes, electron lucent lamina lucida, which contains particularly laminin and the extracellular portion of bullous pemphigoid antigens (BP Ag1 and 2). Below this lies the lamina densa comprising type IV collagen and heparan sulphate proteoglycan from which the anchoring fibrils stretch into the epidermis and loop into a pattern interdigitating with interstitial collagens. When the skin is wounded this basement membrane is damaged, as already discussed. Keratinocytes use fibrin and fibronectin as the framework on which they move. Many of the basement membrane zone proteins can subsequently be synthesised by

keratinocytes so when they have completed migration and filled a defect, basal keratinocytes will resynthesise and reorganise a basement membrane zone of components which then aid keratinocyte anchorage (McGrath et al. 1992).

Keratinocyte Differentiation

Normal skin undergoes a programme of terminal differentiation, which entails epibasal synthesis of differentiation specific keratins (1 and 10). Higher in the epidermis the precursors of the cornified envelope particularly involucrin can be detected beneath the cell surface membrane (Fuchs 1990). In hyperproliferation such as psoriasis and wound healing, the epidermis undergoes an alternative programme of differentiation, with a reduction in synthesis of differentiation related keratins and the expression of a new pair of keratins (6 and 16) (Weiss et al. 1984).

Extracellular factors can influence the stratification and differentiation of the epidermal keratinocytes in vitro. The lowering of extracellular calcium causes the suprabasal keratinocytes to lose their adhesion and shed from the culture leaving a monolayer of keratinocytes which lose their desmosomal interconnections and develop wide intercellular spaces. In human keratinocytes, stratification is inhibited but terminal differentiation is still initiated as larger cells express the envelope precursor involucrin. On restoration of 1.2 m*M* calcium these larger cells move suprabasally as the desmosomal connections reform. A calcium gradient exists in normal skin. Retinoids also have effects on squamous differentiation, both from effects on keratins and envelope proteins. There is a down-regulation of keratins 1 and 10 and an up-regulation of keratin 19 in vitro. Administration of retinoids to patients results in a mucous metaplasia.

Following wounding, such as suction blisters, the hyperproliferative keratins can be detected by immunohistochemistry by 12 h after wounding and persist for 7–10 days (Lane et al., in preparation). Following deeper wounding with ablation of the basement membrane, such as partial thickness and full thickness wounds, the hyperproliferation phenotype persists for longer: up to 6 months following grafting.

Keratinocyte Mesenchymal Interactions

In addition to the specific effects of extracellular matrix proteins on keratinocyte behaviour, there remain other well established but poorly understood effects of mesenchymal tissues on keratinocyte growth and tissue specific differentiation, both in development and in established adult tissues (Mackenzie and Hill 1984). These mesenchymal influences may be directive, to tell the keratinocytes how to differentiate, or permissive, to allow in-

trinsic differences between keratinocytes to occur. Early studies showed that corneal, oral and epidermal keratinocytes retained specific characteristics in vitro, but these were optimally expressed when grafted back into an appropriate tissue environment (Sun et al. 1983). Some of these directive effects can be observed in keratinocyte coculture when pieces of regional connective tissue or cultured fibroblast populations can to some extent direct keratinocyte differentiation (Mackenzie and Sauder 1990). The role of cell to cell contact with specific subpopulations of fibroblasts high in the papillary dermis is currently under investigation. Specific interactions between endothelial cells and keratinocytes may possibly occur, but so far studies have shown that keratinocytes induce angiogenesis by the release of specific factors, and little has been found concerning specific influences of vascular endothelium on keratinocytes, although endothelial activation occurs early in any inflammatory or wound healing response. In addition to general influences of mesenchyme on keratinocytes, the presence of keratinocytes will influence the pattern of dermal remodelling. Following keratinocyte grafting remodelling is slow (Compton et al. 1989), but where a dermal graft is used as the basis for keratinocyte attachment, dermal remodelling and reinnervation occur under the graft. This suggests that keratinocytes have directive inflences on dermal remodelling. Recent manipulation such as the use of neutralising antibody to TGF-β has shown that intervention in scarring can be successfully achieved. Scarring does not occur in foetal skin and this is providing a fruitful area for study (Whitby and Ferguson 1991).

Why Does Reepithelialisation Fail?

Chronic Nonhealing Wounds

Most incisional wounds will heal with adequate care particularly using the new synthetic dressings that provide a moist wound healing environment (Carver and Leigh 1992), but there are groups of chronic nonhealing wounds which provide a significant problem. These include chronic leg ulcers and pressure sores.

Pressure Sores

Pressure sores result from sustained compression over 30 mm Hg upon skin over bony prominences. The state of the patient's nutrition, infirmity and other factors influence susceptibility to necrosis. The problem concerns the elderly, the neurologically disabled and the very sick of all ages. Adequate pressure relieving measures early in an admission will prevent many cases, but 60% of patients are admitted with pressure sores. Reepithelialisation will not occur in the deep full thickness wounds until the dermal defect is

repaired and the vascular granulation tissue forms a good bed for keratinocyte migration, although some reduction in the area of the wound can be obtained by wound contraction. Failure to reepithelialise often results from failure to adequately relieve pressure by the provision of pressure relieving mattresses and wheelchair cushions. Extensive deep pressure ulcers should be treated surgically to expedite progress, but superficial wounds will usually respond to appropriate wound care.

Leg Ulcers

Chronic venous hypertension secondary to loss of deep vein valve function produces morphological changes in the cutanous vasculature, with thickening of the wall and the production of a fibrin matrix cuff around the vasculature, which together with white cell microthrombi produces necrosis of skin in the stasis areas above the malleoli. Leg ulcers increase in prevalence in the elderly and are aggravated by nutritional problems, obesity, immobility, and additional arterial disease. Reepithelialisation will readily occur in the majority of venous ulcers if adequate sustained external compression is provided. However reulceration is common and recurrent ulcers eventually become nonhealing, and fail to reepithelialise. A common reason for failure is the lack of external compression and this has been addressed in a large multicentre community programme, which showed that an enthusiastic approach to compression bandaging can successfully heal 85% of ulcers.

Why some chronic wounds do not heal is an underinvestigated field. It is clear that a holistic approach to wound healing is required. The whole patient and the environment have to be investigated, from the provision of bandages and pressure relieving devices, to the correction of malnutrition, anaemia and other systemic contributory factors. Even after correcting these factors there are still patients whose skin fails to heal. We need to know about the basic biology of this. Is the epidermis failing to regenerate because it has lost the capacity to respond to growth stimuli such as TGF-α? Is it exhausted or are there major growth or migration inhibitory factors being expressed such as TGF-β and interferon-γ? Is this an adynamic or hyperdynamic state? Is there excessive proteolysis or a failure of proteolysis? Until we know the answers to many of these basic questions we cannot plan rational therapy for the individual but have to use empirical general methods of management.

Replacement of Epidermis by Grafting

Once a patient has been hospitalised for the failure of the wound to respond to dressing regimes, split skin grafting, or flap grafts provide a rapid way of reepithelialising a wound and closing the defect to rehabilitate the patient

rapidly. Classical split skin or mesh grafts require a painful donor site and time in theatre. Pinch grafts involve taking 2–3 mm pieces of skin and planting them across the wound surface. This is done under local anaesthetic and provides a minor donor site. It can be performed at the bedside and in the community (Poskitt et al. 1985). Once the ulcer has healed following grafting, strenuous aftercare is needed to prevent recurrence: the wearing of fitted supporting stockings or pressure relieving devices for example (Blair et al. 1988b).

Since keratinocytes can now be grown into sheets in vitro, as discussed above, they can be used for keratinocyte grafting. Autologous keratinocytes were first used to graft burns patients in the early 1980s (O'Connor et al. 1981), and although this can clearly be used as a form of permanent skin replacement, the clinical results are still highly variable (Phillips 1988; Leigh et al. 1991). The take of keratinocyte grafts onto chronic wound beds is poor, probably around 20%–30%. The keratinocytes are very vulnerable to superinfection and to treatment with disinfectants, both of which are toxic to keratinocytes in vitro (Tatnall et al. 1990). Many factors have yet to be rigorously investigated which may enhance keratinocyte graft take, including the type of keratinocyte graft, the time from establishment in culture, the backing dressing and the role of the provision of the dermal component. Skin equivalents can be used for skin grafting but they have to be produced after expansion of the primary keratinocyte population so they take a long time to develop. One stage composite grafting has been used to treat leg ulcers (Moll et al. 1991), burns (Hull et al. 1990; Hansborough et al. 1989) and in animal studies (Bell et al. 1983), but the results are also rather variable. However, as pointed out earlier (Cuono et al. 1986, 1987; Heck et al. 1985), the take of keratinocyte autografts on a dermal bed which has been pretreated with cadaver skin, to provide an integrated dermis, is highly successful and increases the average take rate to 60%–90%. Therefore recent studies have been performed involving two stage keratinocyte grafting in both humans and pig, which do indeed show that the dermis is crucial in the take of keratinocyte autografts. In fact, many of the initial patients treated did receive the standard dressing of cadaver graft skin whilst awaiting expansion of the keratinocytes for autografting which may explain early accounts of successful results.

Keratinocyte allografts were suggested to be a successful means of transplanting keratinocytes from one patient to another and these were used clinically in burn patients and leg ulcers widely in the 1980s (Phillips 1988). The interpretation of the results was complicated by an effect on wound healing which appears to be due to growth factors produced by the keratinocyte grafts. It is clear from DNA based studies that keratinocyte allografts do not survive transplantation (Brain et al. 1989; Burt et al. 1989) and therefore the major effect of keratinocyte allografting is on the promotion of wound healing and possibly vascularisation of the dermal bed. Therefore there are two different results from keratinocyte grafting. Kera-

tinocyte autografts can be produced as stable grafts whereas keratinocyte allografts provide a wound healing dressing material. The investigation into the use of these materials in all types of chronic wounds is continuing and will be an interesting part of the 1990s.

Prevention Is Better Than Cure

There are many simple therapeutic measures which can be performed even at the present time to improve the healing progress and reepithelialisation of chronic wounds. These include the delivery of adequate compression support bandaging and stockings to the chronic venous ulcer patient and the provision of adequate pressure avoidance regimes to the patients who are at high risk of developing pressure ulcers. The community delivery of this type of care is probably more important than the development of new materials with marginal effects. Wound care services in the UK have been scattered across hospital and community services and a move is taking place to try and coordinate these into properly planned wound care services and wound care units. There is no doubt that in the Riverside Project (Blair et al. 1988a) where there was a considerable coordinated delivery of compression bandaging to a large number of venous ulcer patients, high performance indicators were set with approximately 85% of venous ulcers healing within 12 weeks. The administration of this effective community programme will also reduce the costs of health care substantially as well as improve patient management. So, fundamentally, the prevention and treatment of wounds in community based services needs to be dramatically improved, and pressure ulcer wounds, for example, need to be on the agenda at every point of patient care and hospitalisation, including the emergency room.

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Pathophysiology of Wound Healing in Venous Leg Ulcers*

J.R. Mekkes and W. Westerhof

Pathogenesis

Venous ulceration is a common problem in clinical practice. Although it is quite clear that venous insufficiency is the underlying cause, the exact pathogenetic mechanism is still not fully understood and definitively very complex.

Since 1868, when John Gay [1] described the relation between venous thrombosis and ulceration for the first time, several theories about the pathogenesis of venous ulceration have been developed, and new hypotheses are added each year. Some of these theories are listed in chronological order in Table 1.

Deep venous thrombosis, amongst many other primary or secondary conditions affecting the venous valves, may result in venous insufficiency. The flow of blood back to the heart is disturbed, leading to high blood pressure in the veins. Pitting edema will be the result, but edema is not a single cause of ulceration, since ulceration is rarely observed in pitting edema due to cardiac failure. Homans' [2] suggestion that stagnation of blood in tortuous and dilated veins causes tissue anoxia and skin necrosis was never proved using modern laboratory investigations. The same goes for the concept of arteriovenous shunting.

A major contribution to the problem of unraveling the pathogenesis was the work published by Browse and Burnand [6–9]. They suggested that the high ambulatory pressure is transmitted to the capillary bed and enlarges the pores between endothelial cells, thus allowing large molecules to escape into the interstitial fluid. Among these fibrinogen is supposed to be most important because it is deposited as fibrin around capillaries. They postulated

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Table 1. Theories about the pathogenesis of venous leg ulcers

Author(s)	Cause
Gay [1]	Deep venous thrombosis
Homans [2]	Stagnation of blood flow in superficial dilated veins
Linton [3]	High blood pressure in veins
Brewer [4]	Arteriovenous shunts
Fearnley [5], Browse et al. [6]	Decreased fibrinolytic activity
Browse [7], Burnand et al. [8], Browse and Burnand [9]	Fibrin cuff around capillaries
Fagrell [10]	Tissue microedema blocking normal nutritional pathways
Ernst et al. [11]	Blood viscosity hindering venous perfusion
Franzeck et al. [12]	Occlusion of capillaries due to microthrombi
Thomas et al. [13], Coleridge et al. [14]	Occlusion of capillaries due to leukocyte aggregation
Ackerman et al. [15]	Catalysis of free radicals and O_2 radicals by iron

that the fibrin cuff around the capillaries blocks the diffusion of oxygen. This theory is not completely satisfactory, because it is unlikely that the mesh-like structure of the large fibrin molecules forms a barrier to the small oxygen molecules.

We measured transcutaneous pO_2 in a series of patients with venous leg ulcers and noticed an immediate increase in pO_2 in the tissue when the patient was in a horizontal position and an even more pronounced increase during walking with nonelastic compression as compared to measurement in an edematous standing leg. This cannot be explained by a sudden disappearance of the fibrin cuff, nor by an instant drop in tissue edema. It can only be explained by an abrupt end to the venous congestion, a restoration of blood flow through the subepidermal capillary bed resulting in improvement of all conditions necessary for normal wound healing.

Disturbed rheologic conditions may lead to reduced blood flow and eventually may cause entrapment of white or red cells in the capillaries. Microthrombi can be visualized histologically in venous leg ulcers. They may give rise to rarification of blood vessels. A quantitative assessment of this phenomenon is still missing. Perivascular deposition of erythrocytes ultimately causes an increase of iron in the skin in chronic venous insufficiency. Hypothetically this could cause an increased release of free radicals, which would explain the tissue damage and eventually ulceration.

The numerous aforementioned pathogenetic mechanisms clearly demonstrate that there is no unifactorial explanation for the ulceration in venous insufficiency patients. It could well be that venous ulceration is caused by a multifactorial process. It is attractive to describe a sequential process based on various factors (Fig. 1), rather than to come up with a theory based on a single factor.

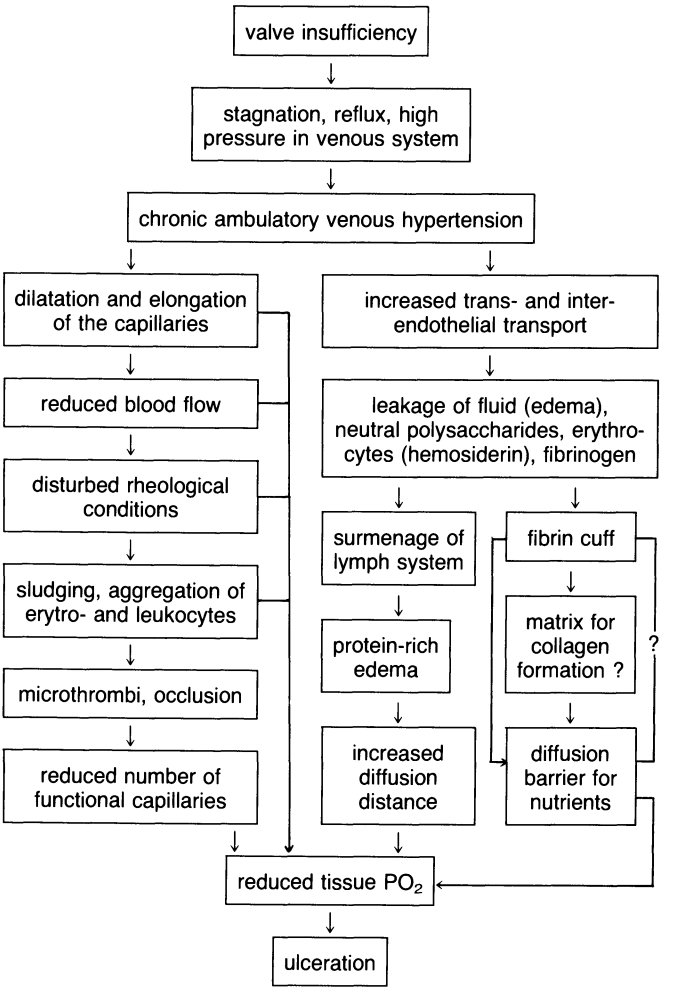

Fig. 1. The multifactorial concept of the etiology of venous ulceration

Most chronic leg ulcers are caused by insufficiency of the deep venous system, or the perforating veins. Superficial vein insufficiency (varicosis) is often associated, but does not lead to ulceration [16]. Normally the pressure in the venous system is low (approximately 30 mm Hg) [7]. When the deep or perforating veins are insufficient, the systolic pressure in the superficial veins is considerably higher (60–80 mm Hg), especially during contraction of the calf muscles [16]. This induces morphological changes in the capillaries, resulting in capillary leakage [17–19].

Due to the venous hypertension, which exceeds normal values by 40–60 mm Hg, there is increased transudation of serum in the capillary bed, resulting in pericapillary edema. Normally the transudation on the arterial

side of the capillary bed is counterbalanced by the resorption of serum at the venous side. An increased amount of protein-rich extracellular fluid is drained via the lymphatic vessels. In case of venous hypertension this system easily becomes overcharged and blocks. Consequently, the interstitial tissue fills with fluid, debris, lipids, and proteins [20]. This may interfere with oxygen (carbon dioxide) exchange at the cellular level in the tissues. Edema itself is a cause of nutritional impairment, because it lengthens the distance for diffusion. The same might hold for waste products. Hypoxia of the tissue is the result. In a chronic situation this is probably aggravated by fibrin transudation and the formation of a fibrin cuff around capillaries. After a short time fibroblasts grow into this cuff and form a fibrous tissue. The clinical situation is termed lipodermatosclerosis. When edema develops in this superficial and often circular rigid tissue, the tissue pressure can become so high that numerous capillaries become compressed, giving rise to flow through limited numbers of shunts that do not contribute to the oxygen supply of the epidermis.

The process of pericapillary fibrin formation, lipodermatosclerosis and fibrosis may continue for years without development of leg ulcers. But when the state of atrophy blanche is reached spontaneous ulceration may occur. Atrophy blanche must be considered as an extreme form of lipodermato-

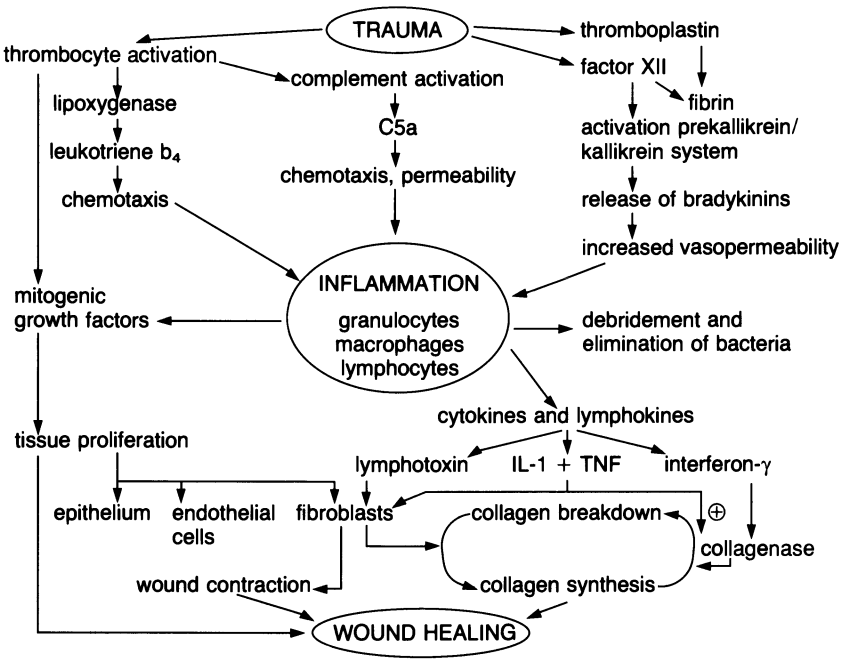

Fig. 2. Cellular and humoral interactions in normal wound healing

sclerosis. There is a progressive reduction of the capillary bed probably due to microthrombi resulting from sludging of the blood (red and white blood cells) [12, 21, 22]. In most instances a minor trauma initiates the ulcer (Fig. 2). The capacity of the tissue to repair this defect is simply absent. Infection consumes oxygen, initiates the complement cascade and tissue damage is the result. Necrosis itself might also sustain complement activation and maintain the downward spiral of tissue damage. Granulocytes are attracted to cause further damage. Macrophages, which play a key role in tissue repair, apparently are not able to migrate into the inflamed tissue around the defect in sufficient amounts.

Wound Repair

The process of wound repair can start when the patient is bed-ridden or when nonelastic compression is applied. We therefore assume that the temporarily disturbed wound healing is a local event. It is well known that in patients suffering from venous leg ulcers wound healing is not disturbed in other regions, e.g., above the calf.

The so-called disturbed fibrinolytic activity in patients with venous leg ulcers is an epiphenomenon of the infection in the ulcer rather than a pathogenetic factor. In our opinion preparations like stanazolol do not have a place in leg ulcer treatment and must be considered dangerous because of their side effects.

When the leg is raised, approximately to the level of the heart, or when nonelastic compression is applied, we see restoration of the wound healing capacity and apparently all the mechanisms involved in normal wound healing take place. The only thing we have to do as doctors is not to counteract natural processes but to create a favorable situation for wound healing and, where possible, speed up the different steps. These include:

- Antiseptics (cave cytotoxicity)
- Debridement (surgical, mechanical and enzymatic)
- Creation of an optimal wound environment
- Improvement of the general condition and nutritional state of the patient
- Growth stimulation
- Grafting methods
- Preventative measures

Antiseptics

Ulcers are full-thickness defects of the skin without any tendency for healing. Often the tissue damage increases due to infection. This becomes apparent as a red painful swelling of the surrounding skin. The ulcer exudate which is

abundant in venous leg ulcers is an excellent culture medium giving rise to pus formation.

Usually, wet dressings containing 10% povidone iodine, 1% chlorhexidine, 2% acetic acid or other antiseptics are sufficient to eliminate contaminating bacteria. Certain antiseptics are cytotoxic and delay granulation tissue formation. An example is hydrogen peroxide. The molecular oxygen is not only toxic to bacteria, but also to human cells.

Only *Streptococcus pyogenes* and some strains of *Staphylococcus aureus* require systemic treatment. The choice of antibiotics should be based on the resistance pattern of these bacteria. In general local treatment with antibiotics is contraindicated because of the risk of poor penetration of these drugs through the slough of the ulcer. Under these circumstances resistant bacteria may develop. Finally topical application of antibiotics may induce contact eczema. Wet dressings serve as a carrier for antiseptic solutions, absorb pus, bacteria and soluble constituents of debris. With every change of dressings (usually three times daily) some necrotic tissue adheres to the gauze and is mechanically removed.

Debridement

As already explained necrosis inhibits wound healing and needs to be removed. Acute hypoxia as in arterial insufficiency gives rise to black necrosis. This can be quickly removed surgically. There is a risk of removing healthy tissue and cutting into blood vessels so that bacteria can enter the bloodstream.

Yellow necrosis is caused by gradual and more chronic exerted hypoxia, as in venous leg ulcers. This necrosis consists of fibrin, elastin, collagen, cells and pus. Yellow necrosis cannot be removed by surgical methods without causing damage to the granulation tissue. Efficient debridement can be accomplished with proteolytic enzymes [23]. When the proteins are degraded into peptides and amino acids they become soluble and can be absorbed by wet gauzes or other absorbing materials.

Occlusion

Provided that there is no severe infection in the wound occlusive dressings can be applied. Their therapeutic effect is provided by the moist environment which is a favorable condition for wound healing, superior to wound healing with crust formation or dessication. Apart from this it is assumed that other physiological factors favoring wound healing are optimized. These factors include: (a) natural proteolytic enzymes; (b) granulocytes/macrophages; (c) growth factors.

It is important to stress that it does not matter whether these occlusive materials are permeable to oxygen or not. The molecular oxygen of our

atmosphere is of no importance to our tissue oxygenation as the gradual and safe energy transfer can only take place via the bloodstream (hemoglobin).

Growth Stimulation

When the infection is under control and necrosis is removed, all attention is focused on granulation tissue formation. Recently studies on the effect of various growth factors have been published [24]. Most investigations were in vitro or animal studies. Little is known about the physiological concentrations of these factors in serum or wound fluid or the sequence in which they exert their influence. We therefore do not know whether conditions with a reduced concentration of growth factors exist resulting in disturbed wound healing.

Frequently new growth factors are discovered; additionally it has been recognized that cytokines (more specific lymphokines) contribute significantly to the wound healing process (Fig. 1). Finally research has been done on chemical agents which recruit and stimulate macrophages, the pivotal cells in wound healing.

All of these substances are still used only experimentally. It is known that certain combinations of growth factors can shorten the process of normal wound healing by 1 day. The effect of cytokines and agents having an influence on macrophages will certainly be in the same range. In relation to a total healing period of 4–8 weeks, a reduction of only 1 day is not satisfactory, especially when considering the cost of these treatments (particularly growth factors).

Grafting Techniques

If ambulatory treatment of a venous leg ulcer with compression therapy fails or would take too long it is advisable to perform skin grafting. We compared split-skin mesh grafts to full-thickness punch biopsy skin grafts [25]. Venous ulcers grafted with the punch biopsy technique will heal within 2–3 weeks, regardless of their size. We found the punch biopsy method to be superior with regard to graft take, the speed of the healing process, the quality of the healed skin, and regarding the recovery time and the functional and cosmetic aspects of the donor site, especially when the split skin is too deep, which is often the case in the center. It is also superior considering the simplicity of the technique which does not require a sterile operating theatre, extra qualified nursing staff, expensive instruments, or sterilization facilities, because the punches are disposable.

Since 1981 attempts have been made to graft cultured skin of autologous origin. The results were poor due to the same reasons as with split-skin grafts. The keratinocyte sheets do not take well on these contaminated and exudative ulcers. When cultured heterologous epidermal sheets are used this

is even more pronounced, due to the rejection phenomenon. It was proved in several studies that no grafted cells could be demonstrated 7 days after grafting [26].

We were the first group to succeed in grafting cultured autologous full-skin equivalents in a series of leg ulcer patients [27]. We compared this method with our well established full-thickness punch biopsy method. In patients with leg ulcers sufficient donor skin is always available, which is not the case in patients with extensive burns, where large areas may be involved. The punch biopsy skin grafts took better, healed sooner and provided a stronger less atrophic skin. The skin equivalent consisting of cultured epidermis and dermis, rendered a smooth surface to the grafted wound (no cobblestone aspect) and the donor site consisted of only two linear scars, where the oval skin biopsies had been removed.

The methods involving skin culture can only be performed in well equipped laboratories and require both expensive materials and instruments and highly qualified lab personnel.

The development of universal cultured full-thickness skin, which can be stored at moderate freezing conditions of, e.g., −20 °C (freezing compartment in ordinary refrigerator), is our next goal, but problems concerning graft rejection and transmission of viral infections need to be solved first.

Prevention

All above mentioned measures and treatments are to no avail when the underlying cause cannot be prevented. At present nonelastic and elastic bandages and measured and made to measure elastic stockings are indicated. Apart from compression, which exerts its action in several ways, the bandages or stockings also protect against (micro-) trauma, which often initiates ulcer formation. Correction of venous insufficiency will be possible when valves can be implanted successfully. Easy accessible places for implantation of artificial venous valves are the groin (vena femoralis) and the knee (vena poplitea). The problem at present is the development of thrombosis after operative placement of the valve, due to the slow blood flow in the venous system, and the rough defective surfaces at the site of the sutures. With the therapeutic methods presently available for treating venous leg ulcers, this ailment does not represent a problem anymore, and healing of venous leg ulcers has become quite rewarding.

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Collagen Metabolism and Wound Contraction

G. Abatangelo, P. Brun, and R. Cortivo

Introduction

All wounds involve connective tissue and heal by the process called repair. The events occurring in the repair process are such that the original connective tissue architecture is lost. The new tissue, called repair tissue, restores continuity within the wound space and consists first of granulation tissue and later scar tissue. The latter shows several compositional and architectural differences from the tissue native to the site. In all of the repair phases, the connective tissue reaction is the principal event. Thus knowledge of connective tissue biology is essential to understanding the repair process.

The architecture of the skin consists of two layers: a thin top layer called the epidermis and a thicker inner layer called the dermis. With regard to skin wound healing it must be noted that reepithelialization is another crucial aspect of wound repair, since the epidermis is needed to reestabilish the physiological barrier for the underlying dermis. The epithelial cells of the wound edges detach themselves from the basal layer and become mobile, spreading over the injured area (Fig. 1). After this, the cells proliferate, deposit a new basement membrane and restratify to restore the original epidermis.

Although the succession of parallel events which occurs after wounding risks oversimplification, such modeling is necessary in trying to understand the fundamental steps of such a complicated phenomenon (Fig. 2). Wound repair starts with clot formation, followed by three fundamental phases: inflammation, granulation tissue formation and matrix deposition. Epithelialization is needed to cover the injured area of the skin, while maturation and modeling of the scar are essential to restore the functional properties of the dermal connective tissue. Many different cell types take part in all these events, including platelets, inflammatory cells, fibroblasts, mast cells, endothelial cells, and epithelial cells. Several factors are released by these different elements such as growth factors, cytokines, and chemoattrants.

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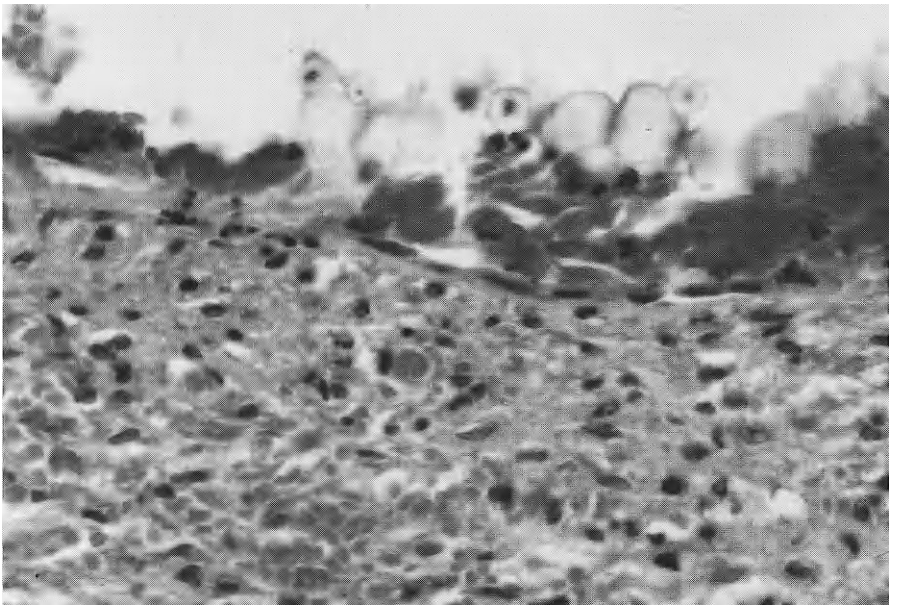


Fig. 1. Rat skin 24 h after wounding. Epithelial cells moving from the wound edges cover the underlying granulation tissue

A very characteristic phenomenon of the healing process is represented by wound contraction. This event is of great clinical importance in reducing the size of the wound and consists of a progressive centripetal movement of the edges of the wound by means of the retractile activity of the granulation tissue. Fibroblastic cells are responsible for such tissue contraction in that they undergo phenotypic modulation to become myofibroblasts.

Myofibroblasts and Wound Contraction

The contractile forces responsible for wound contraction were recognized in the granulation tissue by Carell and Hartmann in 1916 [1] and collagen was supposed to be the fibrillar component responsible for this property. Later the observation that fibroblasts present in granulation tissue exhibited morphological and functional properties intermediate between those of fibroblasts and smooth muscle cells suggested that they might represent the elements capable of generating the contractile force in wound movement [2]. To these phenotypically distinct cells the name myofibroblasts has been assigned. Many studies have been carried out in in vivo and in vitro models to better understand this phenomenon. Bell et al. [3] set up an in vitro equivalent of granulation tissue contraction consisting of fibroblasts dispersed

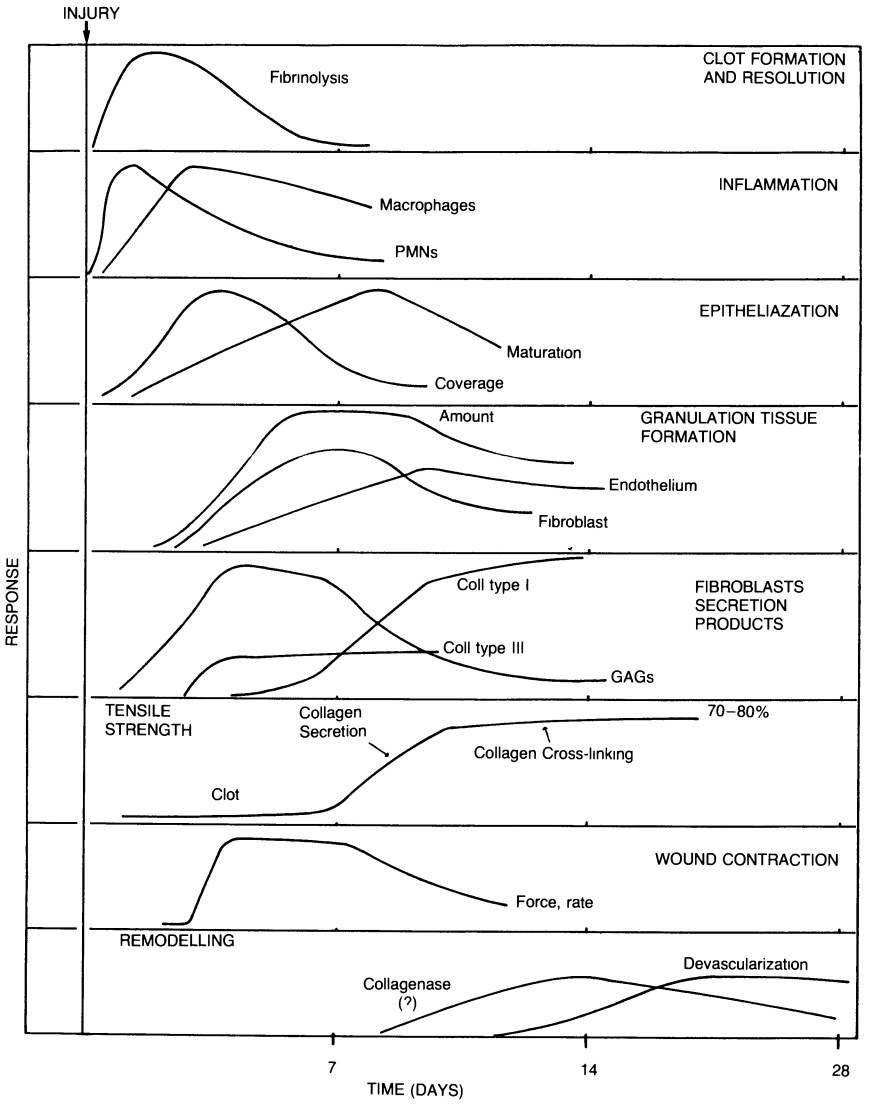


Fig. 2. Principal events in wound repair. [From 8]

within a hydrated collagen gel. After 24–48 h the fibroblasts were able to contract the gel as they extended and withdrew pseudopodia attached to collagen fibers. Thus the fibers were drawn together toward the cell body resulting in collagen condensation.

A peculiar and important connection between fibroblastic cells and extracellular matrix is represented by gap junctions [4] and by the so-called fibronexus described by Singer [5, 6]. The latter is a transmembrane complex

made by intracellular microfilaments and extracellular fibronectin fibers organized in close association with each other. In this case the movement of the filamentous apparatus of the cells is transmitted to the surrounding extracellular matrix.

Myofibroblasts represent the phenotypically defined cells which, by virtue of their cytoskeletal organization, are able to produce forces strong enough to contract wounds. Gap junctions between myofibroblasts represent the major pathway for synchronization of contractile activities passively transduced to the extracellular structures.

Recently, Gabbiani et al. [4], by evaluating cytoskeletal proteins in fibroblastic cells of different soft tissue specimens, have proposed a classification of four main phenotypes: (a) expressing vimentin (V cells); (b) coexpressing vimentin and desmin (VD cells); (c) coexpressing vimentin and α-smooth muscle actin (VA cells) and (d) coexpressing vimentin, desmin and α-smooth muscle actin (VAD cells).

During wound healing, fibroblastic cells modulate toward a phenotype containing vimentin and α-smooth muscle actin (VA cells) and this differentiation is instrumental in activities such as wound contraction. When the wound is closed there is a reversion to the quiescent forms (V cells). In pathological conditions such as hypertrophic scars, fibromatoses, and scleroderma lesions, all of the fibroblastic phenotypes are present. An external stimulus is necessary to induce synthesis of new cytoskeletal macro-

Table 1. Myofibroblasts in diseases

Injury and repair
Liver cirrhosis
Burn contracture
Liver nodular hyperplasia
Hypertrophic scars
Tenosynovitis
Fibrous capsule and granulomas
Sarcoidosis, pulmonary fibrosis
Scleroderma
Nonmalignant and malignant proliferation
Dupuytren's contracture
La Peyronie's disease
Cardiac and abdominal fibromatosis
Fibromatosis
Elastofibroma
Lederhose's disease
Desmoplastic reaction[a]

[a] This reaction is present in many epithelial tumors and is characterized by proliferating fibroblasts with excessive collagen production.

molecules, which under normal conditions can be physiologically reversed. In pathological conditions persistence of the stimulus makes expression of the myofibroblasts permanent. In Table 1, the main types of clinical disorders associated with persistence of myofibroblasts are listed. The factors which regulate the phenomena of differentiation and dedifferentiation of myofibroblasts are unknown. Several growth factors, extracellular components, and mechanical forces are good candidates for stimulation of myofibroblast production. Nothing is known about factors which regulate the disappearance of myofibroblasts during scar formation and work is needed to elucidate the mechanisms of scar function and pathological wound healing processes.

Granulation Tissue and Matrix Formation

After wounding many cells move into the wound area to constitute the so called granulation tissue. This term derives from the granular appearance of the tissue, which is very rich in newly formed blood vessels.

Fibroblasts, macrophages, and endothelial cells move into the wound space and, under appropriate stimuli, give rise to fibroplasia and angiogenesis. In particular fibroblasts are able, under such conditions, to proliferate, migrate, and synthesize and deposit new matrix. After cytoskeletal modification, fibroblasts are able to promote wound contraction.

All the granulation tissue cells are dependent on each other, fibroblasts being responsible for the formation of matrix on which macrophages can

Table 2. Cytokines and the fibrotic response (modified from [8])

Molecule	Proliferation	Collagen	Collagenase
EGF TGF-α	Mesenchymal cells Epithelial cells	No change	No change (except serum-starved cells)
bFGF	Mesenchymal cells Endothelium	No change	Increased
PDGF	Mesenchymal cells	No change	Increased
TGF-β	Context-dependent	Increased	Decreased
IFN-γ and IFN-β	Mesenchyme-inhibitory	Decreased	Decreased by TIMP
IL-1 TNF	Fibroblast	Decreased	Increased
GM-CSF	Mesenchymal cells Hematopoietic cells	Increased	No change

EGF, epidermal growth factor; TGF-α, transforming growth factor-α; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; TGF-β, transforming growth factor-β; IFN, interferon; TNF, tumor necrosis factor; GM-CSF, granulocyte/macrophage-colony-stimulating factor; TIMP, tissue inhibitor of metalloproteinases.

move and endothelial cells providing neovasculature necessary for oxygen and nutrient supply. Even though reepithelialization of injured tissue begins within the first 24 h, several days before granulation tissue formation, it is nevertheless considered to occur within granulation tissue development since the two processes are strictly related in rebuilding the protective barrier of the organ.

Many signals are implicated in the formation of new repair tissue. Table 2 lists some of the relevant intracellular signals. These cytokines possess common features: (a) they are peptides, (b) they interact with unique cell surface receptors that are able to transduce the message into cellular metabolic pathways such as tyrosine phosphorylation, cellular calcium mobilization, activation of protein kinase C.

The main growth factors are: EGF (epidermal growth factor), which stimulates proliferation of both epithelial and mesenchymal cells; TGF-α (transforming growth factor-α), which is homologous to EGF and is able to bind to EGF receptor; and TGF-β (transforming growth factor-β), which has a complex influence on cells and tissues, often reduces the growth rate of cells with a concomitant increase in production of secreted proteins, and induces collagen synthesis in vivo and in vitro [7]. Another class of polypeptides is represented by FGF (fibroblast growth factor), which is present in two forms, acidic (aFGF) and basic (bFGF). When these biochemically distinct polypeptides are injected into an experimental wound they stimulate formation of new blood vessels and increase the rate of granulation tissue formation, as confirmed by the accumulation of DNA and collagen [8].

In wound repair fibroplasia must be limited if excessive scarring is not to occur. To achieve this control, negative feedback regulation and the presence of other substances such as IFN-γ (interferon-γ) [9] and glucocorticoids [10] plays a significant role in limiting fibrogenesis and promoting organization of fibrous collagen. The other obvious mechanism for both regulation of collagen synthesis and remodeling of granulation tissue extracellular matrix is represented by controlled proteolysis. Most connective tissue cells, including fibroblasts and endothelial cells, produce very low levels of proteases. They can, however, be stimulated to synthesize relatively high levels of proteolytic enzymes (collagenases, gelatinase, stromalysin, etc.) by cytokines such as IL-1 (interleukin-1) and other factors. With regard to skin wound healing, the remodeling process takes place from a few days after injury to several months following the formation of granulation tissue. Among the most important enzymes are plasminogen activator, collagenases, hyaluronidase and elastase. Plasmin is not only responsible for clot lysis, but also degrades glycoprotein components of extracellular matrix such as fibronectin and laminin [11, 12]. Plasmin is also necessary to activate other proteinases secreted by the cells as inactive precursors (procollagenase, proelastase). Degradation of extracellular matrix constituents triggers a mechanism for recruitment of additional cells (macrophages, parenchymal cells) by degradation products of fibrin, collagen, elastin, fibronectin, and hyaluronan.

Collagen in Wound Healing

The major extracellular components in human skin are collagen fibrils, elastin, glycosaminoglycans, and glycoproteins (Table 3). In tissues collagen provides the necessary structural architecture and tensile properties. Elastic fibers are responsible for elasticity and proteoglycan complexes ensure hydration by means of their ability to bind large amounts of water. Glycoproteins play a role in the attachment of cells to the extracellular matrix (fibronectin, laminin, entactin/nidogen) and in the formation of a framework for deposition of elastin fibers. Collagen is a generic term which comprises a group of genetically distinct proteins consisting largely of the unique triple helix. Well over 20 different collagenous polypeptides (α-chains) have been described which constitute subunits of many distinct collagen types, 13 of which have been listed so far.

Type I collagen is the major structural component of skin, in association with type III collagen. These molecules represent 70%–80% of the dry weight of the dermis [13]. Collagen is initially synthesized in the cell as a precursor (procollagen), which contains noncollagenous extensions both in the NH_2- and COOH-terminal ends [14]. Once secreted in the extracellular

Table 3. Major structural components of extracellular matrix relevant to skin

Component	Functional role	Tissue distribution
Collagen		
Type I	Major component	Ubiquitous
Type I trimer	Unknown	Minor component
Type III	Provides tensile properties	Skin, blood vessel
Type IV	Major structural component of basement membranes	Basement membranes
Type V	Pericellular localization	Ubiquitous
Type VI	Not clear	Ubiquitous
Type VII	Structural components of anchoring fibrils	Skin, fetal membranes
Elastin fibers		
Elastin	Resilience and elasticity of the skin	Blood vessels, skin and lungs
Microfibrils	Scaffolding in formation of elastic fibers	Same as elastin
Hyaluronan proteoglycans	Maintenance of water and ion balance; regulation of growth, migration, and attachment of cells	Cartilage, skin
Fibronectin	Attachment of cells to the extracellular matrix	Cell surface, plasma
Laminin	Specific binding for the major components of basement membrane	Basement membrane
Entactin/nidogen	Form complexes with laminin, regulation of cell adhesion	Basement membrane

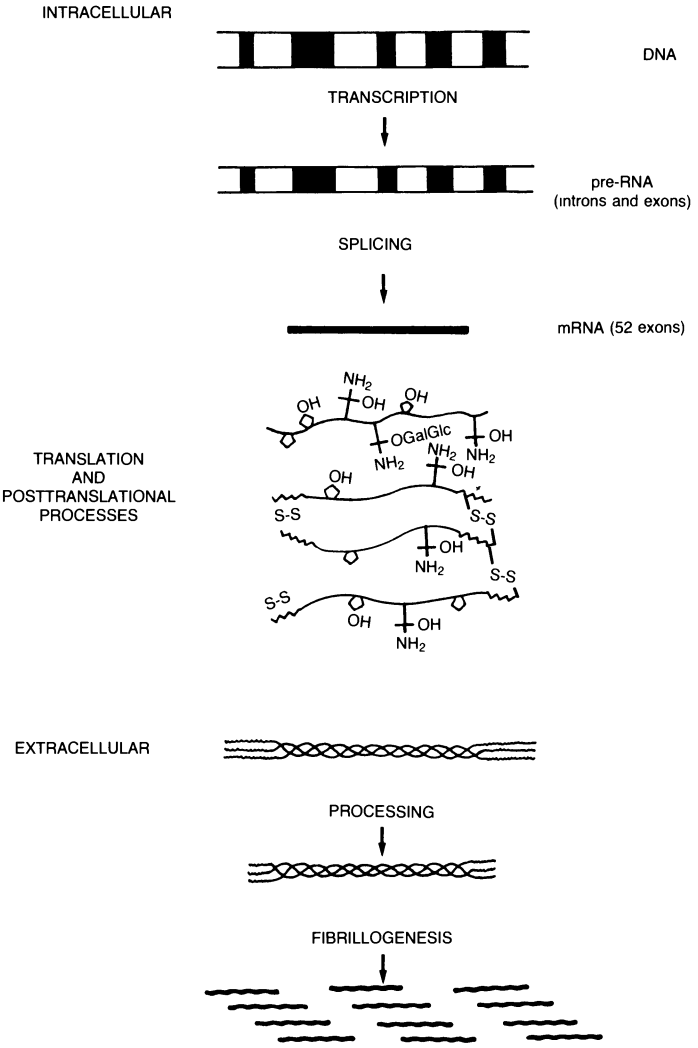


Fig. 3. The fundamental stages in collagen biosynthesis. [Modified from 54]

space, the polypeptide extensions are removed and the collagen molecules align themselves in the fiber structure (Fig. 3). This process is strongly affected by glycosaminoglycans and proteoglycans [15]. Errors can be introduced at several distinct levels into collagen structure or metabolism in a such way that they manifest phenotypically as a disease. The main genetic collagen diseases with cutaneous involvement are listed in Table 4.

Under normal physiologic conditions, the expression of collagen genes must be coordinated and well regulated to ensure that collagen synthesis is

Table 4. Genetic collagen diseases with cutaneous involvement

Cutis laxa	Homocystinuria
Ehlers-Danlos syndrome	Marfan syndrome
Epidermolysis bullosa	Menkes' syndrome
Familial cutaneous collagenoma	Osteogenesis imperfecta
Focal dermal hypoplasia	Tuberous sclerosis

in balance with the normal degradation and turnover of the fibers. In this way the normal level of collagen in tissues is maintained. If the balance is altered either an abnormal accumulation or a decrease of collagen could occur. During growth and development and in tissue repair processes, several observations suggest that both the absolute quantity and the ratio of the different collagen types synthesized by cells change. The synthesis can be modulated also by drugs and viral transformations and in some pathological conditions of wound repair. In fibrotic diseases there appears to be excessive deposition of collagen and perturbation of the balance of synthesis/degradation. Several different mechanisms could explain the excessive accumulation of collagen, namely: (a) increased production of collagen by cells; (b) reduced degradation of collagen polypeptides; (c) increased amount of local fibroblasts with a normal rate of collagen production. Studying fibroblast cultures obtained from several patients with dermal fibrotic conditions, the results indicate an increased collagen production [16–19]. This overproduction seems to be a common phenotypic aspect in fibroblasts of diseased skin, both in in vivo and in vitro conditions.

With regard to the wound healing process, collagen gene expression is increased with a concomitant increase in synthesis and accumulation of collagen for 3 weeks; thereafter accumulation ceases. These observations have been clearly confirmed in a rat wound healing model using in situ hybridization, which allows localization of collagen gene expression [19]. In hypertrophic scars and keloids, fibroblasts exhibit an extended period of proliferation and an elevated rate of collagen synthesis compared to normal wound healing. In addition cultured keloid fibroblasts respond abnormally to hydrocortisone or TGF-β [10]. In normal fibroblasts TGF-β and hydrocortisone inhibit growth and collagen synthesis, respectively; in keloids cells, TGF-β stimulates growth and hydrocortisone does not decrease collagen production. These observations are consistent with the view that conditions inhibitory to growth and collagen synthesis at the late stage of normal wound healing fail to inhibit keloid fibroblasts.

Extracellular Matrix Organization and Cell Movement

Orientation of the fibrous component of the granulation tissue matrix can influence entry of cells into a lesion. Although several cell types participate

in the wound healing process, fibroblasts are the unique elements responsible for the synthesis of extracellular matrix. Fibroblast entry into the wound area initiates the reparative phase of wound healing. In the earliest phase of granulation tissue formation fibronectin plays the leading role, since both stabilization and architectural maturation of the matrix are organized by this glycoprotein, which builds a scaffold for deposition of type I and III collagen [20] and collagen type VI [21]. Fibroblasts that emigrate into wounds arise from the undifferentiated mesenchymal cells in the surrounding tissue and undergo phenotypic changes to synthesize elevated levels of collagen. Fibroblast migration is subject to the control of many processes such as haptotactic influences and contact guidance mechanisms. In addition, the extracellular matrix must open up to provide space for the penetration of cells. Both macrophages and fibroblasts provide a variety of enzymes which are potential candidates for this function. These cells are also able to clear spaces by actively phagocytosing the extracellular matrix. Mechanical forces also play a role in opening up spaces within the matrix of the healing wound. In this respect it is interesting to note that granulation tissue is rich in hyaluronan produced by fibroblasts. The elevated capacity of this substance to bind water is responsible for the high pressure within the granulation tissue and so it could serve to create spaces in it. Orientation of the fibrous component of the wound matrix also changes as the wound heals. The early wound clot is very rich in randomly arranged fibrils and newly formed

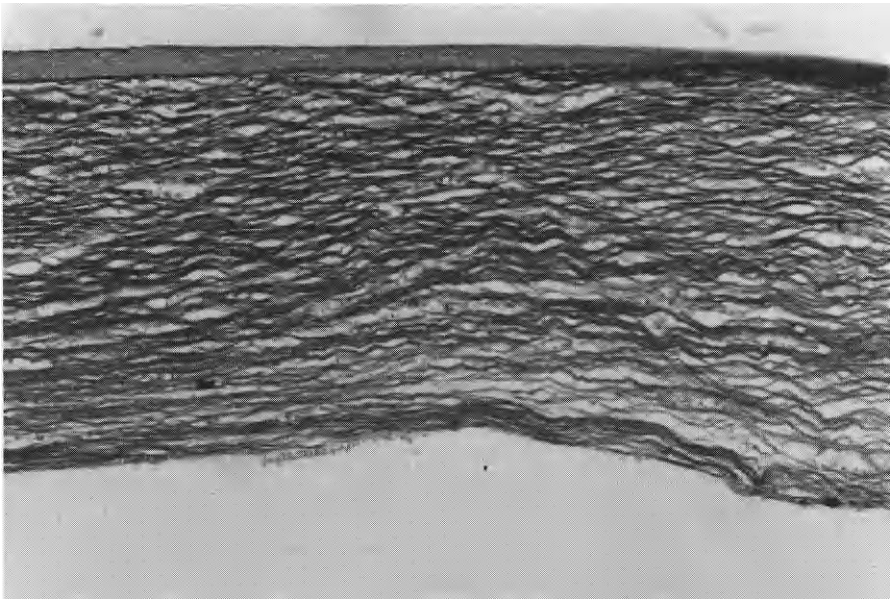


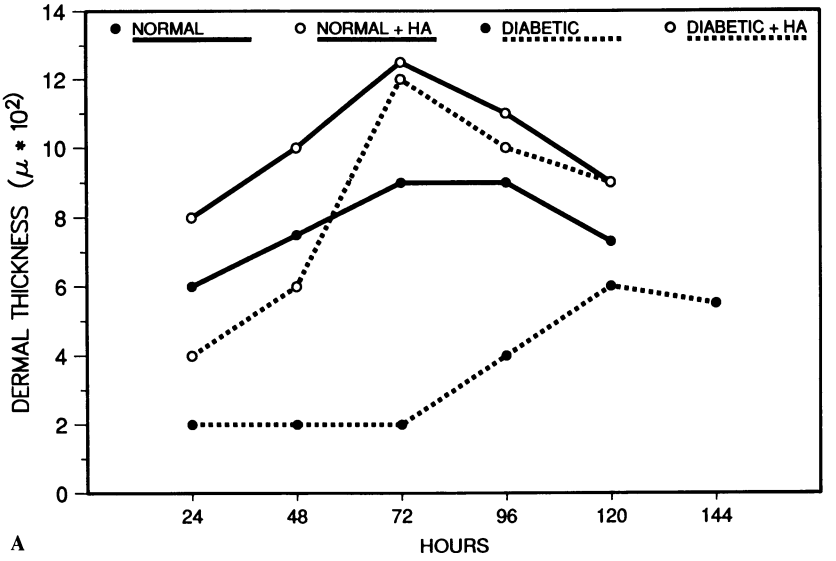
Fig. 4. Rat skin 7 days after wounding. The scar tissue is characterized by flattened epidermis. Collagen bundles are densely packed in parallel array, horizontal to the surface

collagen is laid down in a more or less random way. As fibroblasts invade the wound area there is a progressive change from this random orientation to a more orderly deposition of fibrils that quite often run parallel to the wound surface (Fig. 4). In all these processes of extracellular matrix remodeling, both glycosaminoglycans and proteoglycans play a key role in influencing the final architecture of the repair tissue.

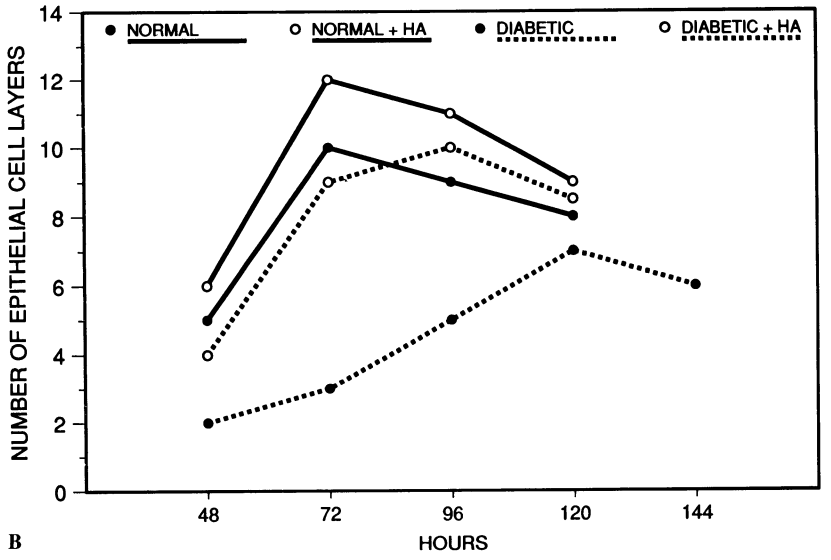
Proteoglycans and Glycosaminoglycans in Wound Healing

Proteoglycans are present in all connective tissues but can also be present as cell surface-associated components. Not only do they act as receptors for other matrix molecules such as fibronectin [22], they may also interact with the cytoskeleton via their intracellular domain or via membrane receptors as in the case of hyaluronan [23]. This nonsulphated glycosaminoglycan plays different roles in various biological processes. Hyaluronan (HA) has been shown to promote neural crest [24] and corneal mesenchymal cell [25] migration during development; it is able to facilitate migration of cardiac cushion cells [26], embryonic mesoderm cells [27] and wound fibroblasts [28]. HA influences, sometimes in a contradictory role, cell motility, phagocytosis, adhesion and detachment. For example Kujava et al. demonstrated that HA bound to cell culture substratum inhibits myofibroblast differentiation [29, 30] but promotes the differentiation of chondroblasts [31]. It has been demonstrated that HA promotes cell detachment in a dose-dependent manner when added to 3T3 fibroblast culture [32]. HA is able to promote chick embryo fibroblast and chondroblast expression in vitro [33]. In the diabetic rat wound model this nonsulphated glycosaminoglycan is able to improve healing by promoting both reepithelialization and fibroplasia (Fig. 5) [34]. In fetal wounds there is clear evidence that the prolonged presence of HA is a predominant characteristic of the healing process [35–37]. In contrast, in adult wounds, HA is present only in the initial phases of the repair process. The persistence of HA in fetal wounds may account for the prevention of scarring or fibrosis in the tissue.

Many experiments have been performed in humans and in animals to study the stimulatory effects of HA in wound healing processes [34, 38–42]. In the healing of tympanic membrane perforations HA improves the quality of the scar tissue [38–40] by reducing connective tissue formation [43]. Also, in flexor tendon repair HA has a beneficial effect by decreasing the peripheral inflammatory response and by influencing orientation of fibroblasts and collagen fibers [44]. In human skin and post-burn scar, HA has been shown to be associated with collagen and this association seems to function in controlling matrix deposition and the remodeling process [45]. In in vitro models, HA facilitates the movement of fibroblasts through three-dimensional collagen matrices [46] and through collagen based wound dressings [28, 47].



A



B

Fig. 5. **A** Epithelial cellularity as vertical cell counts of regenerated epithelial sheet in diabetic rat wounds. The values are the average of four counts in two histological preparations from two different rats. **B** Dermal thickness measurements in diabetic rat wound areas. The values are the approximated average of not less than four measurements in two histological preparations from two different animals

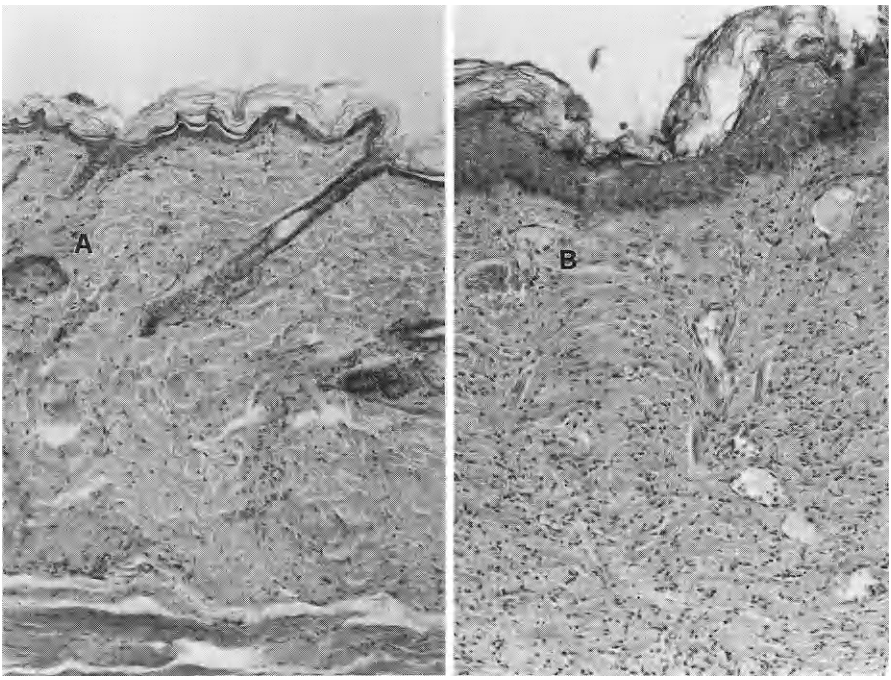


Fig. 6. **A** Normal rat skin (control) and **B** hyaluronan-treated skin after 72 h (see text). Note the hypertrophy of the epithelial layer and the density of the cell population in the dermis

Using an animal model we have studied the effect of HA on dermal cells by infiltrating the substance into the dermis of rats. The animals were treated with dermal infiltrations of 1 ml 2% HA (molecular weight 1.6×10^5). The injection was performed in a restricted area of the dorsal thoracic skin. The control group received only the vehicle. HA injection resulted in a localized swelling which persisted for 8–10 h. Control and treated rats were then killed at 1, 2, 3, 4, 5 and 10 days after treatment. The entire infiltrated area was removed and formalin fixed for histological observation. The HA treated animals showed a structural modification at the epithelial and dermal level. In particular there was a marked increase of both epithelial and dermal cells (Fig. 6). With automated morphometric analysis dermal cells present in the HA injected area were counted. The results shown in Table 5 clearly indicate an increased density of mesenchymal elements in the dermis of treated rats compared to the controls. The increase reached the maximum level on days 3 and 4 after treatment after which there was a progressive decrease. After 10 days there were no longer any detectable differences between HA treated and control animals.

With regard to the secretion of extracellular matrix during wound healing, HA has been shown to influence both deposition of collagen [28, 47] and remodeling of extracellular matrix [48, 41]. Using an experimental

Table 5. Number of dermal cells present in rat skin infiltrated with hyaluronan

Time (h)	Rat	Treated	Untreated	Increase (%)
72	1	577 ± 46*	227 ± 15	60
	2	697 ± 61*	256 ± 42	67
	3	484 ± 70*	211 ± 64	53
96	1	459 ± 30*	231 ± 13	51
	2	567 ± 49*	225 ± 23	60
	3	715 ± 22*	229 ± 6	68

* $p < 0.01$.
Results are expressed as mean values of three sections ± SD.
Differences between the mean values obtained from the sections of different rats were tested by Student's *t* test.
For each animal three histological sections were analyzed. In each section the number of cells was calculated in different areas for a total of 150 mm^2.

Table 6. Collagen content of scar tissue samples

Tissue sample	Total collagen content[a] (μg/mg dry weight)	Type III (%)
Normal rat skin	330 ± 40.1	14.8 ± 1.1
Normal rat scar		
72 h	50.3 ± 4.6	31.4 ± 3.4
96 h	54.2 ± 5.0	32.6 ± 3.6
Diabetic rat scar		
72 h	65.6 ± 4.8	15.2 ± 1.7
96 h	69.1 ± 5.1	16.1 ± 1.8
Hyaluronan-treated diabetic rat scar		
72 h	44.2 ± 3.2	24.1 ± 2.8
96 h	46.1 ± 4.1	26.2 ± 3.6

Average values obtained from three different animals in each group.
Type I and type III collagen content determined by SDS-PAGE.
According to Miller and Rhodes 1982; modified by Merkel et al. [48].
[a] As determined by hydroxyproline analysis.

wound healing model in the diabetic rat we studied both total collagen synthesis and the ratio of collagen types I and III. The shaven dorsal thoracic skin of normal and alloxan-induced diabetic rats was wounded by means of a scratch incision with a dermotome under aseptic precautions. The wounds were approximately 8–10 mm in length and no more than 0.7–1.0 mm deep. The wounded animals were allocated to two experimental groups of ten normal and ten diabetic animals each. The first group received

a local application on the wound area of 2 ml sterile 0.2% sodium hyaluronate solution every 8 h. The second group was used as a control and was treated with saline in a similar fashion. At fixed times of 24, 48, 72, 96, and 120 h after wounding the animals were anesthetized and the granulation tissue excised and extracted in ten volumes of ice cold 0.5 *M* acetic acid for 4 days at 4°C. The tissue pellets were resuspended in acetic acid with the addition of pepsin (Sigma) at a concentration of 20 mg/g wet tissue, according to the procedure described by Merkel et al. [48]. Total collagen content was determined on the basis of hydroxyproline content [49]. Collagen samples were analyzed by SDS-PAGE in the presence of 3.6 *M* urea and the gels were stained with Coomassie blue R-250 [49]. Table 6 shows the results of the collagen analysis. It can be seen that in HA treated rat scars there is a higher content of type III collagen, thus demonstrating that the presence of HA affects the expression of mesenchymal cells present in granulation tissue.

Conclusions

The healing process represents a very complex cascade of events in which many cells, moving either from surrounding tissues or from blood, cooperate in order to restore continuity to the injured tissue. Fibrin deposition and the synthesis of fibronectin in the early stages are followed by collagen secretion by mesenchymal cells present in granulation tissue. Deposition of collagen ensures the tensile properties of the scar tissue, while the presence of other extracellular matrix components seems to influence the final architecture of the repair tissue. Among the glycosaminoglycans, HA seems to play a fundamental role in reducing fibrosis, as is clearly the case in fetal wound healing, in which the predominance of HA is a characteristic feature in all stages of the repair processes [35–37]. In experimental wound healing models, in humans and in animals, many data support the importance of HA in influencing the repair process, either by facilitating cell movement through granulation tissue or by influencing the deposition and architecture of the scar's fibrous component [38–49]. The involvment of glycosaminoglycans and proteoglycans in the many stages of tissue repair, however, has not yet received sufficient attention; based on several experimental studies and studies on development and embryogenesis, our conclusions are that these molecules must play a definite role in influencing cell behavior. Our point of view is supported by data showing that these molecules are present on the cell surface, sometimes as integral membrane components. The recent discovery of a cell membrane receptor for HA in many mesenchymal cells [50–53] gives additional support to our interpretation that this glycosaminoglycan not only plays a role as a spatial organizer of proteoglycans in extracelluar spaces, but also is capable of promoting many cell activities (see [23] and references therein included).

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The Role of the Connective Tissue Matrix in Wound Healing: Fibroblast and Collagen Interactions

H.P. Ehrlich

Introduction

Regeneration and Repair

The damage and loss of skin initiates the repair process. In traumas in which there is only superficial loss of the epidermal surface, such as occurs in minor sunburn, the repair process is limited to regeneration of a new epidermal surface. The regeneration of the epidermis results from the migration and proliferation of epidermal cells residing in subepidermal appendages beneath the surface. The resurfacing of the injury site is rapid and complete with no structural changes occurring to the underlying dermis.

With deeper tissue loss caused by more severe trauma, such as a scald burn, the epidermal surface and structural portions of the dermis are destroyed. The resolution and repair of such an injury is not the regeneration of dermis but rather its replacement by scar. The loss and destruction of dermis lead to the deposition of a new connective tissue matrix with characteristics different from that of dermis. A major difference between dermis and scar is demonstrated in Fig. 1. With polarized light optics the birefringence of dermis shows a basket weave structure of dense collagen fibers (Fig. 1A). In contrast, the birefringence of scar shows less dense collagen fibers arranged parallel to the skin surface (Fig. 1B). Dermal collagen fibers are laid down in an orderly fashion during fetal development. Scar collagen fibers which are less orderly arranged are laid down as a consequence of the repair process.

The Connective Tissue Matrix of Repair

The volume of scar tissue depends upon the depth and area of the injury. A more severe trauma causing greater tissue loss requires more scar tissue to

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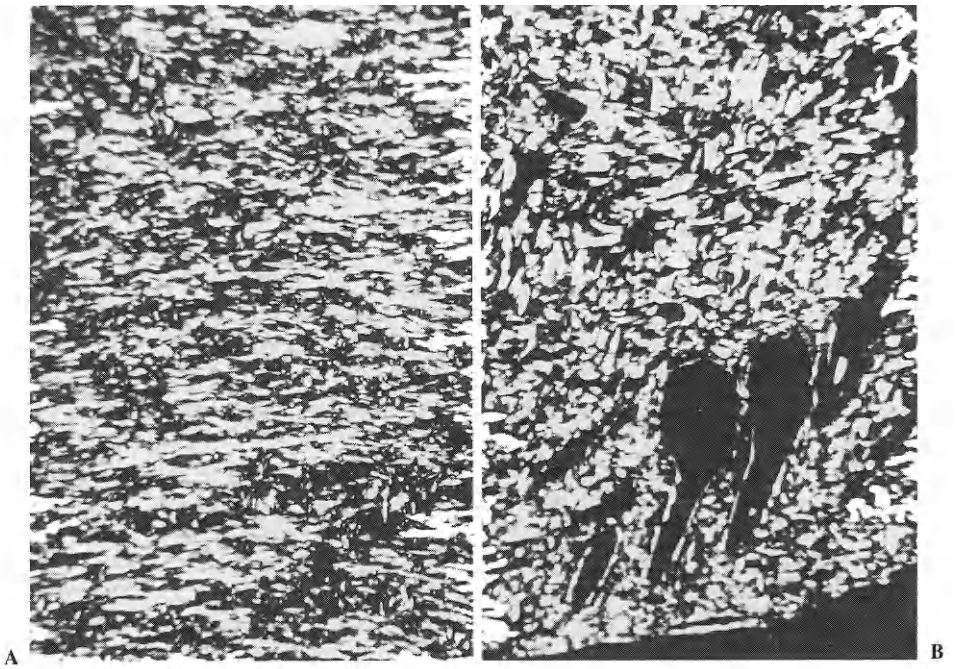

Fig. 1A,B. Polarized light view of normal dermis and scar. This is an histological section of normal rat skin as viewed by polarized light optic microscopy. **A** The birefringent collagen fibers show the basket weave pattern characteristic of normal dermis. The epidermal surface is located at the *top* of the figure. **B** The birefringent pattern of a 5 week rat scar reveals the parallel arrangement of collagen fibers and the absence of a basket weave pattern of collagen fibers

patch the defect. With an incision wound which is closed by surgical suturing, skin edges are brought together reducing the volume of tissue required to fill the defect. The bridging of the new scar tissue and residual dermis involves a splicing of old and new fibers. The fibroblasts splice the dermal collagen fibers with the granulation tissue collagen fibers. The further organization of newly deposited fibrils within granulation tissue by fibroblasts entails the translocation and reorientation of collagen fibrils. The end result of this process is the condensation of fine collagen fibrils and the formation of thicker collagen fibers. The final outcome of collagen reorganization is the restoration of the skin's integrity. Though the volume of new tissue deposited may be modest, the continuing organization of collagen produces a scar of increasing strength. The inclusion of covalent intermolecular cross-links between collagen fibrils adds significantly to the strength of that tissue (Dunphy and Udupa 1955).

With open wound repair, where a greater volume of tissue is needed to fill the void, the same requirements of integrating the new granulation tissue collagen with the residual collagen of dermis are essential. The volume of

granulation tissue to be reorganized is much greater in excisional wounds than in sutured closed wounds. This organization of fine collagen fibrils into thicker collagen fibers produces contractile forces which affect the skin surrounding the granulation tissue. If that surrounding skin is loosely attached to the underlying surface, such as found in rats and rabbits, the contractile forces generated produce wound contraction. During the process of wound contraction, normal skin is pulled over the defect, which covers portions of the defect with skin rather than scar. Wound contraction reduces the volume of tissue needed to fill the original defect (Abercrombie et al. 1954; Billingham and Russel 1956). The mechanism for the generation of these contractile forces within granulation tissue is the cellular translocation of collagen fibrils (Ehrlich and Rajaratnam 1990).

If the wound is in an area where the surrounding skin is tightly attached to its underlying surface, then wound contraction does not occur. This is the usual case with human skin, where defects fill in with scar. With deep second degree or full-thickness burns, the development of a residual scar is common. These burn scars can often generate into hypertrophic scars, where the deposited scar tissue is excessive and is elevated above the skin surface (Brody et al. 1981). The collagen fibers within hypertrophic scar are arranged in a random disorganized fashion. Histologically, the hypertrophic scar is immature retaining characteristics of granulation tissue. Cell density, numbers of vessels and the volume of collagen are elevated in hypertrophic scar compared to dermis. In normal scar the cell number and vessel density are less than in normal dermis. The granulation tissue-like character of hypertrophic scar extends the time for fibroblast-collagen interactions. One outcome of this prolonged period of collagen reorientation contributes to the nodules and distorted appearance of the hypertrophic scar surface.

Scar tissue, filling in a defect over a joint, often leads to scar contractures. Here the compaction of collagen reduces the volume of tissue over the joint which limits its extension. The occurrence of scar contracture is common in healed scald burns of children. The compaction of that scar tissue results from the prolonged ongoing translocation and compaction of collagen by resident cells. Physical manipulation of such a scar by the physical therapist using splints, elastic garments and exercise can modify these cell-collagen interaction forces. Physical manipulation and the eventual maturation of that scar by the stabilization of the connective tissue matrix will produce near normal physical motion of that joint. This type of therapy is long and tedious but effective.

Fibroblast-Populated Collagen Lattice Model

The fibroblast-populated collagen lattice (FPCL) was introduced by Bell et al. (1979). It is manufactured from freed monolayer cultured fibroblasts, culture medium containing serum, and soluble native collagen. When these

components are rapidly mixed together at 37°C, the collagen rapidly polymerizes and traps the fibroblasts in the fast forming matrix. Within 24 h of manufacture, the size of the FPCL is reduced. This process is referred to as lattice contraction. It is a model of collagen organization similar to that occurring in wound healing and scarring. In FPCL contraction, cells produce forces which reorganize the surrounding collagen fibrils. Fibroblasts, initially spherical in shape, spread and elongate producing a reduction in the thickness of the collagen lattice. Following the initial reduction in lattice thickness, there is the later reduction in the diameter of the collagen lattice, lattice contraction. The mechanism of lattice contraction is the translocation and reorientation of collagen fibrils by cellular forces. The collagen fibrils are organized into thicker collagen fibers which reduce the volume of the collagen lattice. The rate of this translocation of collagen fibrils and degree of collagen fibril packing determine the rate and degree of lattice contraction. The degree that fibroblasts can organize and increase the integrity of this collagen gel structure was demonstrated by Bell et al. (1981), who used it as a skin graft in rats. The FPCL model facilitates the investigation of cell-collagen interactions in a controlled environment.

Mechanism for Cell Translocation of Collagen Fibrils

Cell Contraction vs Cell Tractional Force

Cultured fibroblasts in monolayer cultures demonstrate both the generation of cell contraction forces and cell tractional forces. The question is: What is the mechanism fibroblasts employ to organize collagen fibrils within FPCLs?

A model of cell contraction (ATP-induced cell contraction) demonstrates the contractility of cytoplasmic microfilaments (Goldman et al. 1976). After 24 h in monolayer culture, cell preparations were treated with descending concentrations of glycerol (50%–5%) to permeabilize the cell membrane (Ehrlich et al. 1986). Adding ATP and cofactors Ca^{2+}, Mg^{2+}, and KCl to these permeabilized preparations induced cell contraction, resulting from the sliding of cytoplasmic actin-myosin filaments (Fig. 2). During ATP-induced cell contraction, the actin-myosin microfilaments of cells contract to form aggregates. These sliding filaments pull inward contracting the cell. If collagen lattice contraction occurs by a mechanism of cell contraction, then the appearance of compacted actin aggregates in the cytoplasm of fibroblasts might be expected. Contracted cells with aggregated microfilaments have not been observed in contracting FPCLs. It is argued that cell contraction is not the mechanism that is responsible for lattice contraction.

Harris et al. (1980) demonstrated fibroblast tractional forces. Fibroblasts plated onto a thin film of heat-polymerized silicone oil produce wrinkles on the surface when they attempt to move (Fig. 3). Fibroblast locomotion forces cause the thin membrane surface to wrinkle. Fibroblasts do not

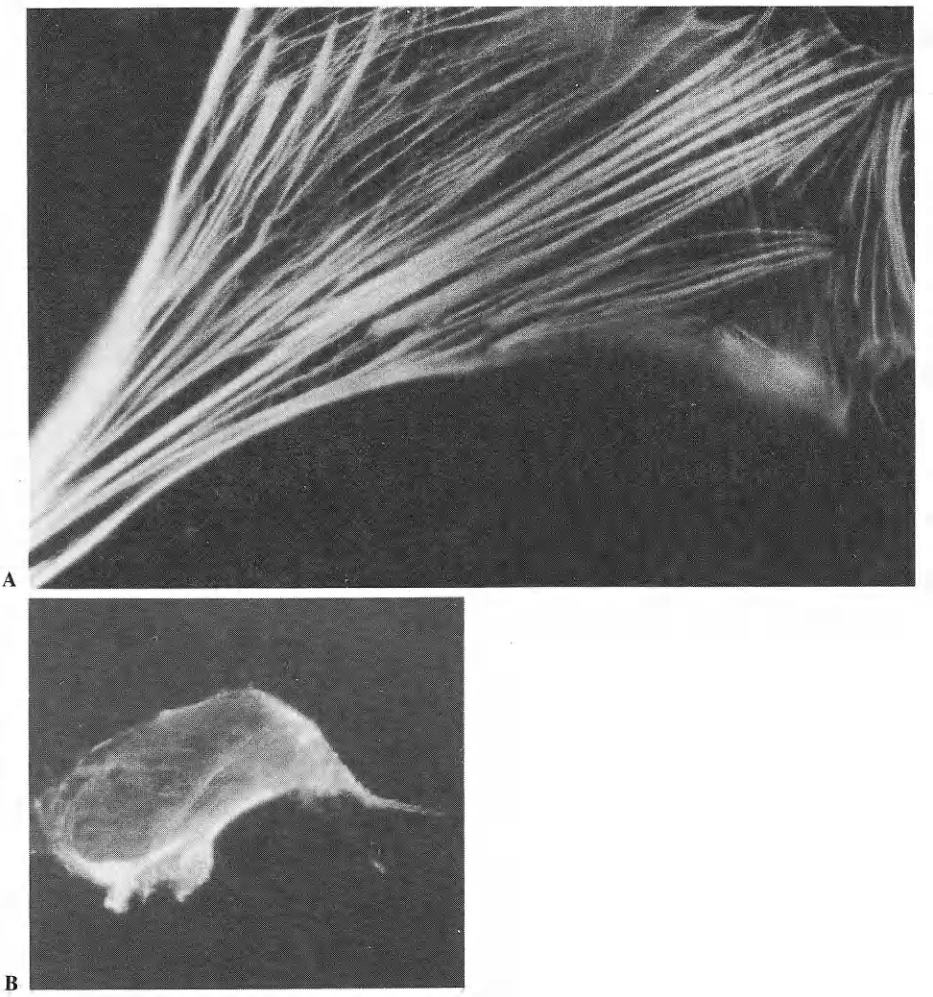

Fig. 2A,B. ATP-induced cell contraction. Fibroblasts were plated on glass coverslips for 24 h. The coverslips were subjected to 30 min incubations of glycerol in buffer at 50%, 25%, 10% and 5% concentrations at room temperature. **A** Some coverslips were fixed and stained with rhodamine-phalloidin. **B** Other coverslips were incubated with ATP for 10 min and then fixed and stained

contract as they wrinkle the surface beneath them. When wrinkles appear under a cell, the cell does not lose its flattened, elongated appearance. Populations of microfilaments within the cytoplasm contract pulling on attachment sites linked to the silicone film surface. In a FPCL, fibroblast microfilaments contract, moving the collagen fibrils which are attached to the cell surface by specific intergrins over the cell surface. There is no cell contraction or shape change during the translocation of collagen fibrils.

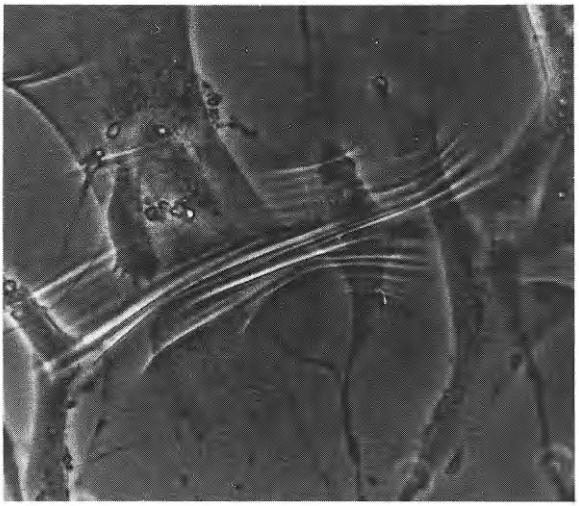

Fig. 3. Fibroblasts wrinkling the surface of a silicone rubber substrate. Silicone oil was layered on a glass coverslip and the surface briefly flamed in a Bunsen burner. A thin film was produced on the surface of the oil by heat polymerization. Human dermal fibroblasts were plated on that surface and viewed 6 h later. By phase contrast optics fibroblasts are shown wrinkling the film surface by tractional forces. Note the cells are spread and not contracted

These tractional forces are like the movement of the track on a military tank. The mechanism for collagen fibrils translocation requires no cell contraction.

The Wedge Experiment

Two distinct cell populations emerge during lattice contraction. These populations can be demonstrated by staining actin filaments with fluorescently tagged phalloidin (Weiland and Faulstich 1978; Burak et al. 1980). One population is located at the periphery or edge of the lattice. Cells are in close contact with one another, arranged in parallel arrays (Fig. 4A). Based upon the morphology of these cells (cytoplasmic stress fibers and cell-cell contacts), they are defined as myofibroblasts (Majno et al. 1971; Hirschel et al. 1971; Gabbiani et al. 1972). The second cell population is located in the central portions of the lattice. They are randomly orientated, have few cell-cell contacts, and their cytoplasmic microfilaments are composed of diffuse, fine, filamentous actin. Based upon their morphology, these cells are fibroblasts (Fig. 4B).

If myofibroblasts working through a mechanism of cell contraction are responsible for lattice contraction, then the outer edge of the FPCL should show greater contractile ability than the central region of the FPCL. One wedge (wedge a) is cut from a contracting FPCL which includes one outer

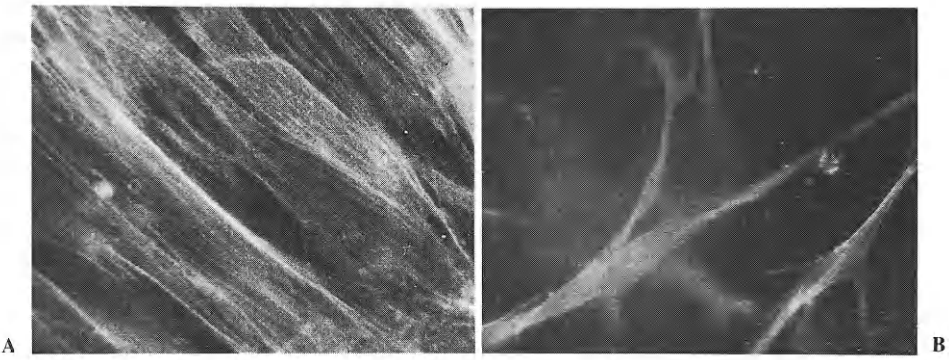

Fig. 4A,B. Actin filament patterns of cells in fibroblast-populated collagen lattices (FPCLs) at 24 h. FPCLs made with 100 000 fibroblasts were fixed at 24 h and stained with rhodamine-phalloidin. **A** Cells at the edge of collagen lattice show stress fibers and cell-cell attachments. **B** Fibroblasts in central locations of collagen lattices show random orientation and their cytoplasms have fine filamentous actin staining patterns

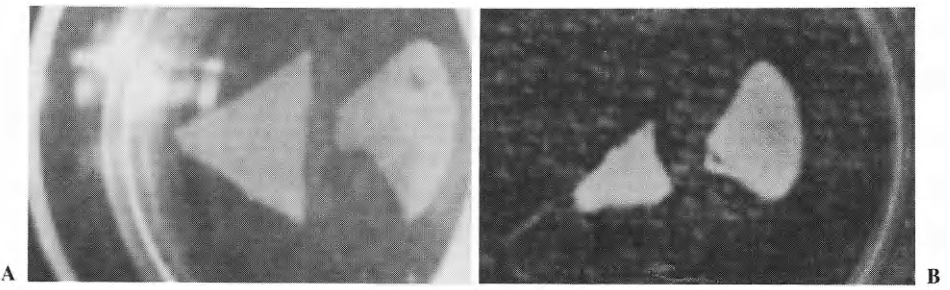

Fig. 5A,B. The wedge experiment: A fibroblast-populated collagen lattice (FPCL) was made and at 24 h the lattice was cut up into two types of wedges. **A** The cut wedge "a" (*right*) contains one peripheral edge of FPCL, while wedge "b" (*left*) contains three cut edges. **B** The two wedges are viewed after 48 h of incubation. Wedge "a" (*right*) has contracted but minimally at the peripheral edge of the FPCL. Wedge "b" (*left*) shows more uniform contraction on all edges

edge rich in myofibroblasts. That edge would be expected to show greater contraction capacity than the two other cut edges which are populated with fibroblasts (Fig. 5A). A second wedge (wedge b) was cut where the three edges contained fibroblasts and only one apex of the wedge contained myofibroblasts (Fig. 5A). The one edge on wedge a, rich in myofibroblasts, showed minimal contraction at 2 days compared to the other two edges of wedge a. In contrast, wedge b, with fibroblasts at all three cut edges, showed greater contraction which was equal on all three edges. The edge populated with myofibroblasts demonstrated the least contraction, while the cut edges with populations of fibroblasts showed greater contraction (Ehrlich and Rajaratnam 1990).

Fibroblasts, therefore, produce greater lattice contraction and organize collagen fibrils more efficiently than myofibroblasts. It is proposed that lattice contraction occurs by cell tractional forces rather than by cell contraction. Cell generated tractional forces, in which cells retain an elongated shape, are responsible for organizing collagen. The translocation of collagen fibrils over the cell surface, while linked to contracting cytoplasmic microfilaments, is the proposed mechanism of lattice contraction and collagen reorganization in wounds and scars.

The Tight Skin Mouse Experiment

An in vivo experiment examined the role of fibroblasts and myofibroblasts in wound contraction (Hembry et al. 1986). A full-excision wound in a tight skin mouse (TSK), a genetic strain of mouse which exhibits tight skin bound to the underlying musculature, showed a 3 week delay in the initiation of wound contraction (Ehrlich and Needle 1983). However, after that period, wound contraction began with a rate equal to that seen in normal mice, in whom closure began at day 1. With TSK mouse wounds, it took 6 weeks for complete wound contraction compared to 3 weeks with normal mice. At 1, 2, 3, 4 and 5 weeks after wounding, biopsies of TSK mouse wounds were taken and examined by histology. Actin stained cytoplasmic stress fibers characteristic of myofibroblasts were prominent throughout the granulation tissue at 1 and 2 weeks. At 4 weeks, when TSK mouse wounds are actively contracting, biopsies from contracting wounds showed no actin staining cytoplasmic stress fibers. Myofibroblasts containing cytoplasmic stress fibers were absent during active wound contraction in TSK mice. The resident cell population of contracting TSK mouse wounds was made up of fibroblasts. When wound contraction was almost completed at 5 weeks in TSK mice, myofibroblasts with prominent cytoplasmic stress fibers had reappeared. While the appearance of fibroblasts was indicative of wound contraction, the reappearance of myofibroblasts marked the termination of active wound contraction.

In rat wound contraction studies, the first appearance of myofibroblasts with prominent cytoplasmic stress fibers was at 7 days (Majno et al. 1971). In excisional rat wounds, wound contraction is about 50% completed by this time. It is proposed that fibroblasts, functioning as individual units within granulation tissue, organize collagen by cell tractional forces, which is the mechanism responsible for wound contraction.

Collagen Control of Cell Translocation of Collagen Fibrils

Fibroblasts produce the forces which organize collagen fibrils. The composition of the collagen influences the rate and degree fibroblasts can translocate

and reorient collagen fibrils. The degree whereby collagen can be translocated is dependent upon the structural stability of the matrix in which they reside. Collagen fibers arranged in an ordered state are not readily rearranged by fibroblasts (Ehrlich and Hembry 1984).

Collagen Types

Collagen is made up of a family of proteins. The major interstitial collagens of skin and scar are types I and III. Granulation tissue and hypertrophic scar have a 50% increase in the concentration of type III collagen (Bailey et al. 1975a,b). When fibroblasts were isolated from normal skin and compared to fibroblasts isolated from hypertrophic scar, only modest differences in lattice contraction were found (Ehrlich 1988a). However, when collagen extracted from hypertrophic scar was compared to collagen extracted from normal skin, significant differences were found. Hypertrophic scar collagen FPCLs were better at lattice contraction than normal dermal collagen FPCLs.

The possibility that the elevated level of type III collagen present in hypertrophic scar was responsible for enhanced lattice contraction was investigated (Ehrlich 1988b). FPCLs were made with either type III collagen, extracted and purified from a benign uterine tumor (leiomyoma), or type I collagen, extracted and purified from normal human dermis. FPCLs made with type III collagen contracted faster and to a greater degree than lattices made of type I collagen. It appears that fibroblasts can organize type III collagen fibrils faster than type I collagen fibrils. The location of the tissue from which the collagen was extracted influences lattice contraction. FPCLs made with type I collagen extracted from human placenta were faster at lattice contraction than FPCLs made with type III placental collagen (Trollier et al. 1990). The placental type I collagen was equivalent to leiomyoma type III collagen and the placental type III collagen was similar to dermal type I collagen. The reasons for these differences are unresolved.

The Length of Collagen Molecules

The contraction of a FPCL containing intact rat tail tendon (RTT) collagen is greater than one composed of RTT collagen shortened by limited pepsin digestion (Woodley et al. 1991). Intact collagen lattices had contracted by 79% at day 1, while the pepsin treated collagen lattices had contracted only 31%. By day 5 the intact collagen lattices had contracted by 92%, while the pepsin treated collagen lattices had contracted by 77%.

RTT collagen treated with other proteinases was studied to demonstrate how the length of collagen chains would influence lattice contraction. The size of RTT collagen treated with pepsin, trypsin or elastase was compared by SDS polyacrylamide gel electrophoresis. The electrophoretic mobility of

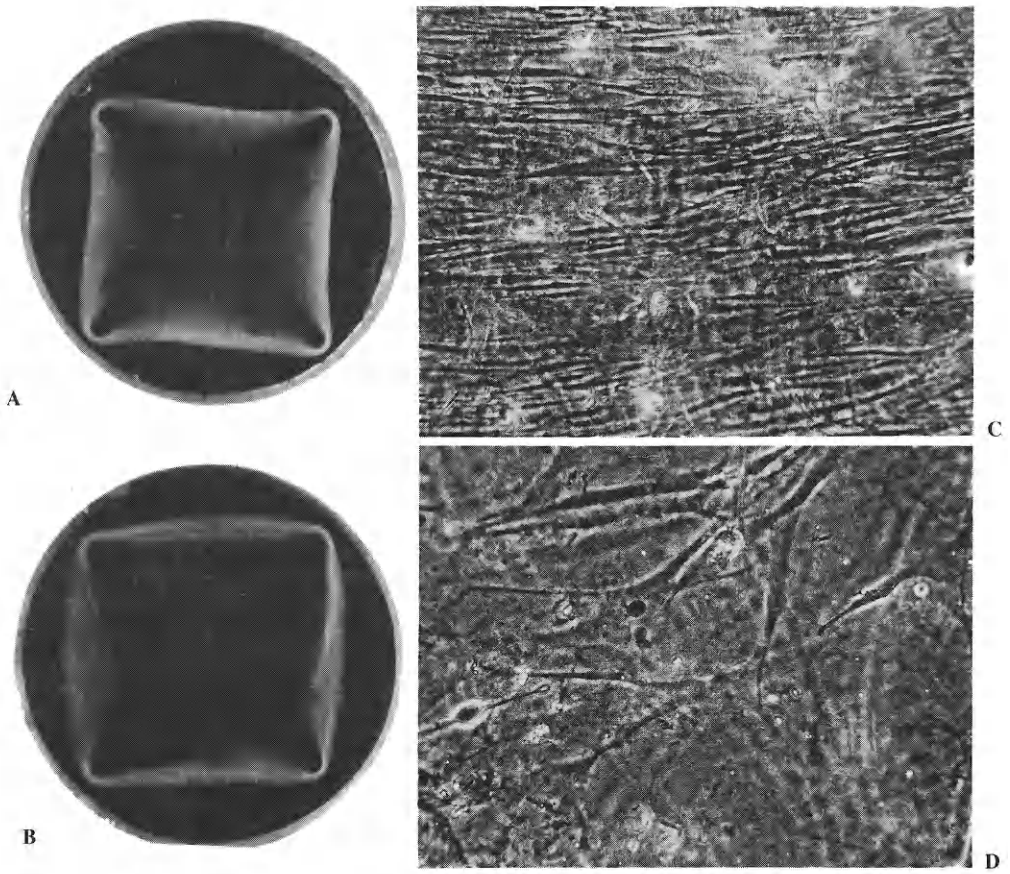

Fig. 6A–D. Splinted fibroblast-populated collagen lattices (FPCLs) at 7 days. A FPCL was cast in the presence of a splint which limited lattice contraction to an area of 100 mm^2 at 7 days. The splinted lattice in **A** was made with intact rat tail tendon (RTT) collagen and the one in **B** was made with elastase treated collagen. **C** The fibroblasts in the splinted lattice made with intact collagen (shown in **A**) are oriented in parallel arrays. **D** The cells in splinted lattice made with elastase treated collagen, (shown in **B**) are randomly orientated

α chains demonstrated collagen molecular size. It was found that limited pepsin digestion produced a 4% decrease in size, trypsin a 6% decrease in size and elastase a 15% decrease in size. It should be noted that elastase treatment had removed some of the helical ends of the collagen chains and all the nonhelical portions of the molecule.

FPCLs were made with 80000 fibroblasts and 2.5 mg of collagen. Intact RTT collagen lattices were reduced by 70% at day 1 and 95% at day 7. Trypsin treated RTT collagen lattices were reduced by 57% at day 1 and 90% at day 7. Pepsin treated RTT collagen lattices were reduced by 59% on day 1 and 90% at day 7. Lattice contraction was equally retarded with either

pepsin or trypsin treatment of collagens. FPCLs made with elastase treated RTT collagen were reduced by only 40% on day 1 and 74% on day 7. The treatment of RTT collagen with elastase, which removed a greater portion of nonhelical peptide ends, retarded lattice contraction the most. All four preparations of soluble collagen polymerized rapidly and equally. The retardation of collagen lattice contraction was not attributed to altered collagen gelling.

FPCLs were made with an included splinting device which limited lattice contraction to a square shape and a final size of 100 mm^2. FPCLs splinted for 7 days are presented in Fig. 6. In Fig. 6A the FPCL had 130000 human fibroblasts in intact RTT collagen and in Fig. 6B the splinted FPCL had 130000 cells in elastase treated RTT collagen. Both lattices had equivalent surface areas of 100 mm^2 at 7 days. Phase contrast microscopy showed the fibroblasts arranged within intact RTT collagen splinted lattice to be in parallel arrays. As shown in Fig. 6C the cells are orientated parallel to one another and arranged in sheets, one sheet near the top surface and the other sheet near the bottom surface. The splintering of FPCLs caused the fibroblasts to arrange themselves in an orderly fashion within the matrix. The cells in elastase treated RTT collagen splinted lattices (Fig. 6D) did not form distinct layers or sheets of cells. The cells in elastase treated collagen were arranged in a random fashion throughout the splinted lattice. The removal of globular peptides from the ends of collagen influenced the arrangement of fibroblast populations.

Woodley et al. (1991) reported that the telopeptide of collagen modulated FPCL contraction. Using pepsin solubilized collagens derived from bovine dermis or RTT, they showed that partially eliminating the telopeptide of collagen by limited pepsin digestion retarded lattice contraction. The rate of lattice contraction during the first 24 h was the most affected by decreasing the size of the collagen molecule. The rate of lattice contraction between 24 and 48 h was similar in the undigested and digested bovine dermal collagen preparations. We found with elastase treated collagen that the rate of lattice contraction after 2 or 3 days was similar to that of intact collagen preparations. The initial 2 days of lattice contraction demonstrated the differences seen with proteinase modified collagen lattices. Woodley's group demonstrated that the presence or absence of histidinohydroxylysinonorleucine, the hydrolysis product of a collagen cross-link, minimally affected lattice contraction.

One possibility for the inhibition of lattice contraction by removal of the nonhelical ends of collagen is the attachment of collagen to fibroblasts. Goldberg and Burgeson (1982) reported that eliminating portions of the telopeptide of collagen did not alter the binding of collagen to the surface of 3T3 fibroblasts in monolayer culture. They showed that specific cyanogen bromide digest derived peptides were effective at blocking collagen attachment to fibroblasts. These peptides were derived from regions located within the triple helical portions of the molecule and not in the non-helical regions.

The binding of collagen to the cell surface of fibroblasts in monolayer culture has been reported to be independent of the nonhelical ends of collagen.

Fibroblasts have been shown to utilize integrins for their attachment to collagen during lattice contraction (Gullberg et al. 1989). A non-RGD-dependent integrin was involved in lattice contraction. The inclusion of an antibody directed to the $\beta 1$ subunit of the collagen binding integrin effectively inhibited lattice contraction (Gullberg et al. 1990). There is the possibility that integrins exist which bind to the nonhelical regions of collagen. The enzymatic removal of collagen nonhelical telopeptides would eliminate binding to those sites. Reducing the number of collagen attachments on the cell surface would diminish the number of collagen fibrils translocated at any moment of time, reducing both the rate and degree of lattice contraction.

Another consideration is the packing of the collagen molecules. The compacting of collagen fibrils into thicker collagen fibers requires closer packing of collagen fibrils. The nonhelical end of collagen has been shown to be involved in the self-assembly and polymerization of collagen solutions (Helseth and Veis 1981; Gross and Kirk 1958). Though there were no obvious differences noted in the polymerization of collagen lattices with the partially cleaved collagen preparations, the eventual packing of collagen fibrils into thicker fibers may be altered by eliminating the nonhelical ends of collagen.

The orientation of fibroblasts in splinted lattices is greatly altered in elastase treated collagen. Splinted lattices produce a high degree of ordering of both collagen fibrils and cells. With the removal of nonhelical ends of collagen used to make splinted lattices, there is a reduction in the ordered arrangement of cells. This suggests that the composition of globular ends of collagen influences the degree to which collagen can be organized which may influence cell orientation. The globular ends of collagen appear to be involved with the cellular orientations and the translocation of collagen fibrils. The microinteractions between fibroblasts and collagen control the macrostructure of tissues. The nonhelical ends of collagen modulate those microinteractions.

The experiments presented show that a continuing interaction between cells and their extracellular matrix can effect the quality of the connective tissue in which they reside. Minor alterations of the protein composition of that matrix can alter cell functions. The nonhelical ends of collagen influence fibroblast cell-cell orientation and the physical rearrangements of collagen fibrils. The mechanism for these actions is not clear. Understanding how the termini of collagen can modulate fibroblast functions may have biological relevance in the development of bioengineered transplanted tissues.

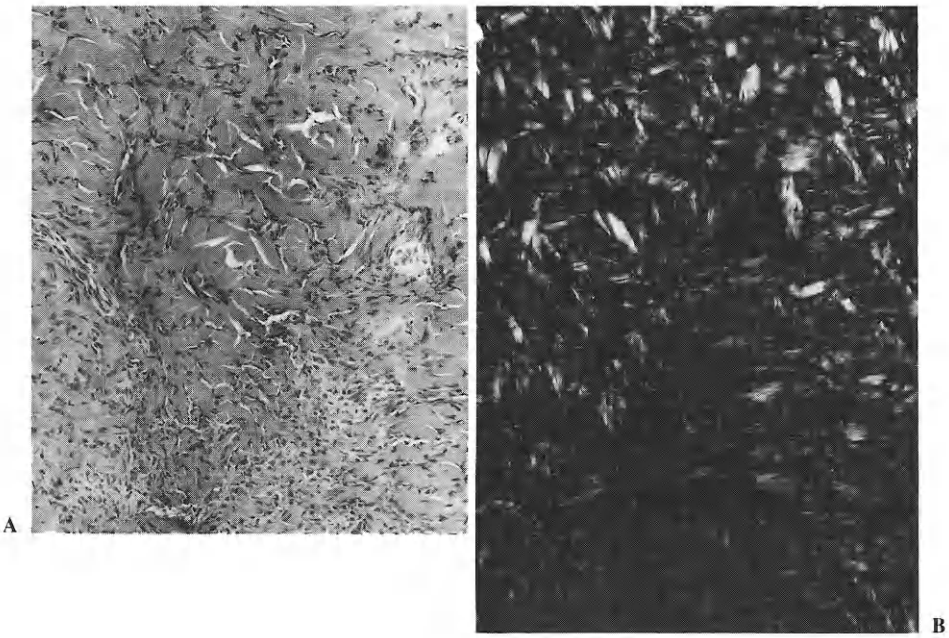

Fig. 7A,B. Histology of a 10 day freeze injury in rats. A biopsy from a 10 day old healing freeze injury in rats was fixed and stained. **A** A healing freeze injury is shown with surviving dermal collagen fibers at the *edges* of the photograph and a pocket of granulation tissue in the *middle*. **B** The same area of the healing freeze injury was viewed by polarized light optics. The birefringence at the *edges* of the photograph demonstrates collagen fibers of surviving dermis. The lack of birefringence in the *middle* demonstrates the presence of immature granulation tissue

Wound Contraction of Freeze and Burn Injuries

Full-thickness burn wounds in rats heal by wound contraction. In contrast, a full-thickness freeze injury does not heal by wound contraction. The depth of injury and the degree of the inflammatory response in both types of injury are similar. The histology of 10 day old burns made with a brass probe equilibrated in boiling water and applied to the skin for 20 s was compared to freeze wounds made with an identical brass probe equilibrated in liquid nitrogen and applied to the skin for 45 s (Ehrlich and Hembry 1984). Both types of injury had myofibroblasts. The absence of wound contraction in freeze injury could not be related to a cellular alteration. However, freeze injuries at 10 days revealed the presence of residual dermal fibers (Fig. 7A). Histologically, a 10 day healing burn exhibited granulation tissue uniformally filling the defect. Burn trauma killed cells and denatured the connective tissue matrix, which was completely replaced by granulation tissue. With freeze injury the cellular components of the dermis were killed

but the dermal collagen fibers survived. In a 10 day healed burn there was little birefringence demonstrated by polarized light optics in granulation tissue. In contrast, the 10 day freeze injury showed birefringent dermal fibers throughout the healing wound with pockets of nonbirefringent granulation tissue (Fig. 7B).

With burn injury, the heat energy destroys both the cells and connective tissue matrix. The devitalized tissue is completely replaced by granulation tissue and healing proceeds by wound contraction. With freeze injury the cellular component of damaged skin is destroyed, but the collagen dermal fibers are spared. The infiltration of granulation tissue repopulates the devitalized tissue. There is a marriage between the old residual collagen fibers and the newly synthesized granulation tissue. The residual dermis is quite stable and acts like an internal splint, preventing the contractile forces of granulation tissue from pulling in the surrounding skin. Hence, freeze injury inhibited wound contraction was not an alteration of the cellular composition of the healing wound, but rather an aberration of the dermal collagen fibers retained within the healing wound. This in vivo model shows that the collagen structure can control the organization of scar tissue.

Wound healing is essential for survival. The replacement of damaged skin by scar is a process composed of cellular proliferation, new connective tissue deposition and the further organization of that tissue. Much is known about the cell proliferation and the deposition of new connective tissue. The ongoing organization of that newly deposited matrix has not been studied as much. When an open wound is covered by an epidermis or the stitches are removed from a sutured closed incision, wound closure is complete. However, the remodeling phase of the wound healing process continues well after the completion of wound closure. The remodeling phase of repair is responsible for the continued gain in wound breaking strength and formation of hypertrophic scars in healed burn injuries. A major player in the remodeling phase of repair is the structure and composition of the connective tissue matrix. The physical arrangement of collagen fibers and chemical changes and modulation of those fibers are critical in determining the quality of wound healing and scarring.

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Modulation of Fibroblastic Cytoskeletal Features During Wound Healing and Fibrosis

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The mechanisms leading to the retraction of granulation tissue during wound healing have not been presently fully elucidated (for review, see Schürch et al. 1992). Several years ago, our laboratory described the presence within granulation tissue of fibroblasts having several ultrastructural features of smooth muscle (SM) cells, including the presence of microfilament bundles with dense bodies scattered within (Gabbiani and Majno 1972). These cells, called myofibroblasts, have been proposed to play a retractile role in several conditions such as granulation tissue contraction, parenchymal organ retraction, fibromatosis and stromal reaction to epithelial tumors (Sappino et al. 1988, 1990a; Skalli et al. 1989; Darby et al. 1990; Kapanci et al. 1990; Schmitt-Gräff and Gabbiani 1992; for review, see Sappino et al. 1990b). The coincidence of the presence of myofibroblasts with retractile phenomena has supported this hypothesis. However, direct proof of the presence and activity of contractile elements in myofibroblasts has been possible only after suitable techniques have been developed in order to localize and quantify cytoskeletal and contractile proteins within the affected organs. For this purpose, advances in the understanding of cytoskeletal and contractile element morphology and biochemistry in different cells have been of great help (for review, see Skalli and Gabbiani 1988, 1990).

The cytoskeleton of eukaryotic cells is composed of three filamentous systems (for review, see Rungger-Brändle and Gabbiani 1983): (1) microfilaments, made up mainly of actin and myosin; (2) intermediate filaments formed by at least six distinct classes of proteins; in mesenchymal cells, vimentin and/or desmin are expressed, lamins being present in all nuclear membranes; and (3) microtubules consisting mainly of tubulin. By two-dimensional gel electrophoresis (Garrels and Gibson 1976) and amino acid sequence analysis (Vandekerckhove and Weber 1978, 1981), six isoforms of actin (produced by different genes, Ueyama et al. 1984) have been described in mammalian tissues: the β- and γ-cytoplasmic isoforms which are expressed

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by all cells, and the α-cardiac, α-skeletal and α- and γ-SM isoforms, which are limited to specific cell types. Myosin is a polymeric molecule composed of two heavy chains and four light chains, each chain displaying isoformic variations. The analysis of cytoskeleton elements has been facilitated by the production of specific antibodies for the SM and nonmuscle myosin isoforms (Benzonana et al. 1988), for the intermediate filament proteins vimentin and desmin (Kocher et al. 1984), and of a monoclonal antibody against the α-SM isoform of actin (Skalli et al. 1986) which is typical of vascular SM cells (Gabbiani et al. 1981). The regulation of actin synthesis can be studied by means of specific mRNA probes (Kocher and Gabbiani 1987; Bochaton-Piallat et al. 1992).

The cytoskeletal characterization of stromal cells present in a variety of human and experimental soft tissue specimens known to contain myofibroblasts has revealed the presence of four main phenotypes: (1) expressing vimentin (V cells), (2) coexpressing vimentin and desmin (VD cells), (3) coexpressing vimentin and α-SM actin (VA cells), and (4) coexpressing vimentin, desmin, and α-SM actin (VAD cells). Among the SM cell markers expressed by fibroblastic populations in vivo, α-SM actin is the most common, followed by desmin while SM myosin is practically always absent (Eddy et al. 1988; Sappino et al. 1990b).

When we study normally healing granulation tissue with these criteria, we find that during granulation tissue contraction a high proportion of myofibroblasts develop the expression of α-SM actin (phenotype VA) but not of desmin and SM myosin, and hence acquire at least in part SM features. When contraction stops and the wound is fully epithelialized, myofibroblastic cells containing α-SM actin disappear probably by apoptosis (Darby et al. 1990) and the scar becomes less cellular, as classically described, and composed of typical fibroblasts with well developed rough endoplasmic reticulum and no more microfilaments, nor α-SM actin. In more permanent retractile conditions and in particular in kidney, lung or liver fibrosis, myofibroblasts expressing α-SM actin are constantly present and, in addition, a proportion of them expresses desmin (Skalli et al. 1989). However, at present, no myofibroblast expressing SM myosin has been described (Benzonana et al. 1988; Eddy et al. 1988). On the basis of these results, we propose that during the development of fibrocontractive diseases, fibroblasts acquire contractile features and produce the centripetal force leading to retraction. For this purpose, myofibroblasts have the capacity of developing connections to the surrounding extracellular matrix and hence to act on the whole tissue (Sappino et al. 1990b). Traction rather than contraction forces have been shown to be responsible for the retractile activity of cultured fibroblasts on their substratum (Harris et al. 1981). In analogy with these observations, we suggest that the retractile activity of myofibroblasts during fibrotic changes is more dependent on isometric contraction than on isotonic contraction. In human and experimental liver cirrhosis, myofibroblasts have first been visualized in fibrotic tissue surrounding parenchymal nodules

(Rudolph et al. 1979; Irle et al. 1980). Evidence was provided for the contractile potential of these cells (Irle et al. 1980). As in other cases of fibrosis and during wound healing the origin of myofibroblasts during fibrosis has not been fully elucidated. A likely possibility is that they derive at least in part from perisinusoidal or fat-storing cells (Ballardini et al. 1988; Schmitt-Gräff et al. 1991).

Several aspects of myofibroblast biology are still unknown, in particular their origin and the factors regulating their development and disappearance during pathological reactions. We (Skalli et al. 1986) and others (Vandekerckhove and Weber 1981; Leavitt et al. 1985) have previously observed that a subpopulation of primary and passaged fibroblasts in culture expresses α-SM actin. We had suggested that these α-SM actin positive cells present in cultures of fibroblasts correspond to contaminating SM cells or pericytes (Skalli et al. 1986); however, these cells could also represent fibroblasts modulated in culture: this last possibility is compatible with the temporary expression of α-SM actin in granulation tissue fibroblasts during wound healing in vivo (Darby et al. 1990).

We have investigated the presence of α-SM actin in fibroblast cultures, clones and subclones (Desmoulière et al. 1992a). In all cases the fibroblastic populations studied showed a proportion of α-SM actin expressing cells. Even after cloning, we never obtained populations negative for α-SM actin. We conclude that α-SM actin expression in fibroblastic cultures is not due to contaminant cells but is a feature of fibroblasts themselves. It is noteworthy that α-SM actin can be expressed by a proportion of cells in a population cultured from a single α-SM actin positive or α-SM actin negative cell. This observation suggests that most if not all fibroblasts have the potential of generating α-SM actin expressing cells; this is generally, but not always, masked in normal conditions and may become manifest when specific functional needs are required. The mechanisms leading to α-SM actin expression in fibroblastic cells both in vivo and in vitro clearly need further investigations.

Microenvironmental factors probably play an important role in the development of α-SM actin in a proportion of cultured fibroblasts. Previous reports have shown that α-SM actin is expressed by cultured fibroblasts from organs where in situ fibroblasts do not contain this protein. This finding has been described in lens cells (Schmitt-Gräff et al. 1990) and mammary gland stroma (Ronnov-Jessen et al. 1990) and has been correlated with the observation that during pathological conditions in vivo both lens cells and mammary gland fibroblasts can express α-SM actin (for review, see Sappino et al. 1990b). In a subset of bone marrow stromal cells, α-SM actin is expressed in normal and pathological conditions (Schmitt-Gräff et al. 1989) and in vitro (Charbord et al. 1990; Peled et al. 1991). Although the origin of these myofibroblastic cells remains unknown, it seems likely that the resident stromal cell phenotype can be modulated by stimuli produced, for instance, by neighboring epithelial or mesenchymal cells (Sappino et al. 1990b). Our

experiments show that all tested fibroblastic populations develop α-SM actin in a proportion of cells and are in agreement with the well accepted observation that myofibroblasts can develop in many organs and tissues (for review, see Schürch et al. 1992). However, fibroblast clones have expressed varying proportions of α-SM actin positive cells suggesting that intrinsic cell features determine the expression of this protein, at least in part.

Furthermore, our results support the view that fibroblastic cells are a heterogeneous population. During the last few years, evidence has accumulated suggesting that fibroblastic cells represent a heterogeneous population during normal conditions in vivo (for review, see Komuro 1990; Phipps et al. 1990). Some pathological conditions could be due to the recruitment or repression of fibroblast subtypes leading to the predominance of a particular population or alternatively to the modulation of phenotypic features of fibroblasts.

The mechanisms leading to the development, in vitro and in vivo, of cytoskeletal features similar to those of SM cells in fibroblasts including the factors which can regulate in vivo and in vitro the appearance of α-SM actin and desmin remain to be explored. The more likely candidates for these actions are cytokines, which can be locally liberated by vascular cells, inflammatory cells and fibroblastic cells themselves, and extracellular matrix components which have been shown to influence the shape, replication and development of cytoskeletal features in fibroblastic and SM cells (for review, see Sappino et al. 1900b). Working along these lines, we have observed that γ-interferon (γIFN), a cytokine mainly produced by T helper lymphocytes, is capable of inhibiting the expression of α-SM actin in both SM and fibroblastic cells (Hansson et al. 1989; Desmoulière et al. 1992a). When γIFN is applied to Dupuytren's nodules, it produces an improvement of the retractile conditions, and in hypertrophic scars, in addition to the reduction of the size of the lesion, it elicits the disappearance of α-SM actin in myofibroblasts (unpublished observation). Although further studies are needed to confirm these preliminary results, we feel that work in these directions can help not only the understanding of the pathogenesis of fibrocontractive diseases, but also suggest future directions for treatment. In this respect, we have observed in an experimental model in vivo that the application of granulocyte macrophage-colony stimulating factor (GM-CSF) to the rat subcutaneous tissue induces not only the proliferation of fibroblasts and the formation of ultrastructurally typical myofibroblasts, but also the expression of α-SM actin in a significant proportion of these cells (Rubbia-Brandt et al. 1991). GM-CSF is mainly known for its hematopoietic effect (Clark and Kamen 1987), but some extrahematopoietic activity has been attributed to this factor. Thus, GM-CSF stimulates migration of human endothelial cells (Clark and Kamen 1987) and proliferation of different nonhematopoietic cells of mesenchymal origin in vitro, such as endothelial cells (Bussolino et al. 1989), bone marrow fibroblast precursors, and several transformed cell lines (Dedhar et al. 1988). Moreover, in transgenic mice expressing GM-

CSF, fibrotic nodules developed in areas where macrophages accumulate (Lang et al. 1987). These lesions have been interpreted as following chronic macrophage activation induced by GM-CSF. However, another study did not detect any side effects in response to long-term GM-CSF treatment in mice (Pojda et al. 1989). Furthermore, GM-CSF does not induce a neosynthesis of α-SM actin in cultured fibroblastic cells, suggesting that its action in vivo is mediated by other, presently undetermined, substances. In order to clarify the possible mechanisms of GM-CSF action, we have studied chronologically the formation of granulation tissue after the application of GM-CSF delivered by osmotic pumps. Our results suggest that the appearance of α-SM actin expressing myofibroblasts is preceded by a characteristic cluster-like accumulation of macrophages, thus suggesting that such macrophages may be involved in the stimulation of α-SM actin synthesis by myofibroblasts (Vyalov et al., submitted). Clearly, these experiments need further studies in order to be confirmed, but they indicate that progress in the understanding of cytokine influence on fibroblastic cells may furnish explanations for the mechanisms leading to the development of a contractile phenotype in fibroblasts. It is well known that heparin and heparan sulfates inhibit SM cell replication (Clowes and Karnovsky 1977) and increase the expression of α-SM actin in these cells (Clowes et al. 1988; Desmoulière et al. 1991). We have also observed that heparin inhibits proliferation and increases the expression of α-SM actin protein on cultured fibroblastic cells (Desmoulière et al. 1992b). The analysis of [^{3}H]-thymidine incorporation in synchronized cells suggested that heparin produces a selection of α-SM actin expressing cells. For in vivo studies, osmotic minipumps filled with tumor necrosis factor-α (TNFα), without or with nonanticoagulant heparin, were implanted subcutaneously. The local application of TNFα resulted in the formation of a typical granulation tissue: immunofluorescence showed that accumulated fibroblastic cells express α-SM actin only in the presence of heparin derivatives. In TFNα-treated animals, electron microscopic examination established the presence of myofibroblasts, but α-SM actin was expressed in microfilament bundles only in the presence of heparin derivatives. These results show that heparin and its nonanticoagulant derivatives influence the expression of α-SM actin in fibroblastic cells both in vitro and in vivo and that this effect is probably related to the selection of a particular cell subpopulation. They suggest a possible role for heparin during the formation and evolution of granulation tissue.

In conclusion, the early observation that fibroblasts modify their phenotype during wound healing and fibrocontractive diseases has been implemented by several biochemical and functional findings which support the idea that myofibroblasts are a key cell for the understanding of retractile phenomena. Further studies on the factors regulating the phenotype of myofibroblasts will probably be useful for understanding their behavior in vivo and possibly modifying this behavior in the different clinical settings.

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III. Microcirculation and Angiogenesis

Measurement of Microcirculation

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Introduction

Microcirculation plays an important role in the total vascular system. However up to a few years ago the microcirculation was understood only with respect to angiology. This was mainly due to the fact that good investigative instruments were lacking. In the past decades several noninvasive instruments have become available for this purpose. In recent years more interest has been given to the relationship between venous diseases, microcirculation and venous leg ulcers. With more knowledge about the microcirculation, the need for investigational tools for daily practice will grow. Here, I will mainly focus on noninvasive techniques for measuring the skin microcirculation. Special attention will be given to the connection between the skin microcirculation, venous diseases and wound healing.

Although not all of the available techniques are noninvasive, for frequent use these techniques are preferable. All techniques discussed here have been used up to now mainly for research. In daily practice these techniques are not yet established. The major techniques for studying the skin microcirculation are summarized in Table 1.

We have to realise that all these techniques do not measure the same thing. Thus to get a good impression about what is going on in the superficial – capillary – plexus, we need, in general, to use more than one technique. By combining the results of these techniques it is possible to make conclusions.

The skin microcirculation can be divided in two major parts: the nutritional flow and the thermoregulatory flow. The capillary loops just under the epidermis filling the dermal papillae are the nutritional capillaries. Flow in this part is only $\leq 15\%$ of the total microcirculatory flow. The deeper capillaries have their main function in regulation of body temperature. This is called the thermoregulatory microcirculation. Flow here is $\geq 85\%$ of the total flow. As not all techniques can penetrate both parts of the microcirculation, some techniques measure only one part, others a combination.

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Table 1. Techniques for investigating the microcirculation

Noninvasive	Capillaroscopy
	Direct
	With dyes
	Transcutaneous oxygen tension
	Laser Doppler flux
	Plethysmography: capillary filtration rate
Invasive	Direct capillary pressure
	Micro(lymph)angiography
	Radioisotope techniques
	Immuno/histopathology

Disease in relation to the microcirculation can also be divided in two major groups: (1) a primary process at the level of the microcirculation and (2) diseases of circulation – arterial or venous – with secondary influence on the microcirculation.

Leukocytoclastic vasculitis is a typical example of a primary process in the microcirculation. Such a vasculitis will lead to skin changes such as purpura and sometimes even necrosis. Also, diabetic microangiopathy can in some cases be regarded as a primary process of the capillaries.

Especially in the second group of microcirculatory diseases, many skin changes will occur in the course of both arterial occlusive disease (AOD) and chronic venous insufficiency (CVI). Although the primary cause will be a dysfunction of the major circulation, all clinical signs (skin lesions) will present as alterations of the skin microcirculation.

Skin circulation can change from one point to another as has been shown in patients with CVI. By use of transcutaneous oxygen tension measurements ($tcPO_2$) it was proven that circumscribed hypoxia occurs in venous leg ulcers, white atrophy and lipo- and dermatosclerosis areas. In contrast, a more gradually diminishing $tcPO_2$ is found from proximal to distal in AOD patients. Also a parallel can be made between retinal angiopathy and a low $tcPO_2$ of the skin in patients with diabetes mellitus. Special stress tests are of value for this purpose.

The introduction of video capillaroscopy makes it possible to differentiate between primary and secondary Raynaud phenomena. In the near future several skin diseases such as necrobiosis lipoidica and Martorell's ulcus will be interesting conditions to study at the level of the microcirculation.

New techniques, with a combination probe for $tcPO_2$, laser Doppler and capillaroscopy are already available. This makes it possible to study the microcirculation at one spot with three major noninvasive tools.

Some functional measurements on the microcirculation can be made with a capillary filtration rate technique. This can be done with several types of plethysmography with or without additional radionuclide methods. The skin microcirculation is closely connected to the lymph system. New pos-

sibilities such as microlymphangiography and lymph capillaroscopy are now being used.

Noninvasive Techniques

Transcutaneous Oxygen Measurements

The $tcPO_2$ measurement is a polarographic method using a Clark electrode [1]. In 1951 Baumberger and Goodfriend [2] reported that they were able to measure arterial oxygen pressure through intact human skin.

The measurement of oxygen tension at the surface of the skin has been shown to yield values closely corresponding to those of arterial oxygen tension, provided the skin at the measuring site is sufficiently heated. This increases tissue perfusion to such high levels that no significant diffusion gradient can develop between the oxygen "source," i.e., small arterioles, and the oxygen electrode [3]. The PO_2 so measured is referred to as the transcutaneous PO_2, abbreviated $tcPO_2$.

Continuous transcutaneous oxygen monitoring has become routine practice in neonatology, where it provides a reliable and instantaneous measure of arterial oxygenation [4–6].

In general practice a commercially available polarographic analyzer of transcutaneous oxygen with a Clark-type electrode [1], according to Huch et al. [4], is used. To obtain reliable results it is necessary to heat the skin [7]. Measuring at normal skin temperatures yields $tcPO_2$ values in normal subjects of approximately 10 mm Hg [8]. In general measurements are done in the supine position (Fig. 1) at room temperature. Before measurements are performed the subject rests in the supine position for 15 min. A period of 15–20 min is necessary to obtain a good diffusion equilibrium [7].

The $tcPO_2$ method is, in contrast to capillaroscopy and laser flux technique, a reflection of the overall function of the skin microcirculation. The

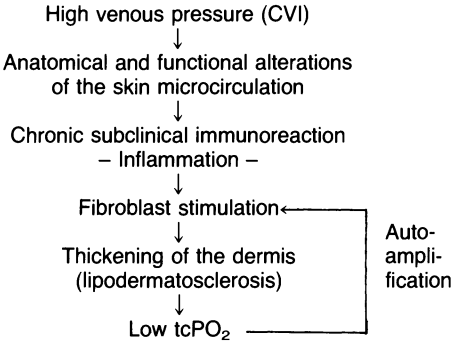

Fig. 1. A model of low PO_2 and CVI

signal is generated from both the nutritive and thermoregulatory parts of the microcirculation, arteriovenous shunts and the afferent-efferent arterial and venous networks.

A significantly lower $tcPO_2$ was found by us in legs of patients with CVI syndrome compared to the value of a normal control group without known vascular disease [7, 9]. This finding has been confirmed by several other authors [10–16].

Tønnesen introduced, already in 1978, measurement of $tcPO_2$ for patients with AOD [17]. Further studies were performed by Matsen et al. [18]. There exists a clinical correlation between the $tcPO_2$ value and the classification of AOD according to Fontaine. Especially stages III (pain at rest) and IV (necrosis) show significantly low to very low $tcPO_2$ values [19, 20]. For AOD the value of $tcPO_2$ measurement can be enhanced by stress, such as before and after occlusion, supine and standing position or walking [21]. All these investigations show what seems to be logical: the $tcPO_2$ will diminish when the arterial influx diminishes. However, also in cases of CVI but without any sign of arterial disease very low $tcPO_2$ values can be measured [7]. This technique can thus be misleading regarding the surgeon's decision to perform an amputation [20]. In these cases it is very important that CVI be ruled out as the cause of the low $tcPO_2$.

$tcPO_2$ measurement is of special value for monitoring treatment schedules for vascular patients. For CVI it has been proven that compression therapy, i.e., edema reduction [22], drugs such as *O*-(β-hydroxyethyl)-rutosides and stanozolol [23, 24], surgical treatment of lipodermatosclerosis [25] and urokinase treatment [26] will enhance $tcPO_2$. In AOD, $tcPO_2$ technique is also used for this purpose. Infusion therapy with prostaglandin E1 has been done in patients with Fontaine stage III and IV [27].

In diabetic children and adults [28], $tcPO_2$ measurements will only be of value when a postocclusive hyperemic test is used [21, 29]. During 4 min a suprasystolic occlusion is given. After releasing the occlusion a postocclusive hyperemia will occur. The time necessary to reach the preocclusion $tcPO_2$ is measured. In diabetes patients a significant elongation as compared to normal controls exists [29].

Up to now it is not clear if this test is predictive for development of diabetic ulcers. No comparable studies are available between this hyperemic test and the alterations observed in the retina.

A totally different application of the $tcPO_2$ technique is described by Prens et al. [30], who introduced this technique in dermatology. Epicutaneously applied known contact allergens are widely used for detecting allergic eczema. These tests are usually called patch tests. Prens et al. have used $tcPO_2$ for quantification of these patch test reactions [30].

Several factors can cause a lower than normal $tcPO_2$. The most important of these have been listed in Table 2. In general, a lower than normal $tcPO_2$ can be caused by an insufficient supply of oxygen to the tissues; by "instrumental" problems, including an abnormally small vasodilatory reac-

Table 2. Possible causes for a lower than normal $tcPO_2$ [7]

Low arterial PO_2
Local circulatory impairment (arteriosclerosis)
Systemic circulatory impairment
Respiratory disease
Other
Impeded diffusion from arterial blood to measuring electrode
Instrumental
Insufficient hyperemic reaction
Diminished vasodilatatory response?
Insufficient heat application
Consumption
Increased tissue metabolism
Utilization
Decreased tissue perfusion
Low perfusion pressure, e.g., arterial disease, chronic venous insufficiency
Increased resistance to flow of (sub)cutaneous microvasculature: edema, microstructural changes
Others
Diffusion limitations:
Decreased capillary density
Diffusion barriers
Others

tion to heat causing a greater than normal PO_2 diffusion gradient from oxygen source (i.e., the small arterioles) to oxygen "sink" (the measuring electrode); and finally, by factors related to the utilization of oxygen by the intervening tissues. In AOD, lower than normal $tcPO_2$ values are found because arterial influx of oxygen to the tissue exists. In other diseases, such as erysipelas, lower than normal $tcPO_2$ values are due to a high consumption by the inflammatory process and, later on, maybe also to edema formation. We have shown that edema in the course of CVI has a strong influence on $tcPO_2$ [22]. As oxygen will be soluble in water the main reason for low $tcPO_2$ in edema is decreased capillary density (Table 2). In CVI the decreased $tcPO_2$ is not so easy to explain. There is no a priori reason why, in CVI, tissue metabolism should be increased compared to normal, and to such an extent that, even with the supposedly large hyperemic flow, a significant oxygen gradient would be caused. If it could first be established that in CVI the same degree of heat-induced hyperemia can be elicited as in normal skin, the possibility of increased oxygen consumption (over and above that caused by the local hyperthermia) could be tested, as described by Thunstrom et al. [31]. For the time being the most likely cause of the lowered $tcPO_2$ in CVI legs would seem to be an impaired tissue perfusion by the separate or combined effect of an increased capillary filtration rate, an increased back pressure and an increased resistance to flow of the (sub) cutaneous microvasculature by edema and, in long-standing cases, morpho-

logic changes of microcirculation and/or skin. Particularly relevant to this discussion is the microangiopathy, a prominent feature in CVI [32, 33]. Bollinger et al. [34] have combined the techniques of intravital capillaroscopy and fluorescein angiography with video densitometry. This enabled them to quantitate the transcapillary and interstitial diffusion of a dye (Na-fluorescein) in the skin of healthy subjects and in patients with CVI [34]. This work is important in that it correlates morphologic and functional parameters of local microcirculation. The data are very suggestive of diffusion-limited transport in those areas of skin which have a decreased capillary density. Admittedly, the diffusional properties of fluorescein and oxygen are likely to differ, but this is a quantitative, rather than a qualitative, matter. The link between capillary density and local oxygen availability could presumably be made by means of the new transparent electrode of Huch et al. [35], which permits $tcPO_2$ readings to be made simultaneously with intravital (fluorescence) video microscopy of the cutaneous capillaries. Their reported results are stated as indicating a good correlation between $tcPO_2$ data and cutaneous capillary density. In low density capillary networks the $tcPO_2$ is reported to approach zero over a distance of 100 μm from a capillary [35].

Browse and Burnand [36–38] postulated that venous ulceration may be due to the deposition of pericapillary fibrin. This theory was later called the "fibrin cuff theory". It is proposed that high venous pressure in CVI causes leakage of large molecules, such as fibrinogen, through capillaries out into the pericapillary space. Recently it has been shown that the cuff consists of fibrin and not fibrinogen [39]. Fibrinogen polymerizes to insoluble fibrin, forming a pericapillary fibrin cuff that could act as an oxygen diffusion barrier. A significant increase of the collagen IV layer in or around the capillaries has also been found [40]. We suggested that the fibrin may function as a matrix for the formation of collagen.

There are, however, severe doubts about the role of fibrin as a diffusion barrier [41]. For that reason we studied patients with porphyria cutanea tarda, which is a rare skin disease in which strong pericapillary fibrin deposits (cuffs) are present [42]. As controls we used healthy persons, patients with CVI with and without fibrin deposits and nonvenous ulcers (Table 3).

Table 3. $tcPO_2$ values (mm Hg) in patients with and without fibrin cuffs

Patients	Normal	PCT	CVI	CVI	Ulcers
n	10	4	8	6	4
Fibrin deposits	0	4	8	0	3
Clinical CVI	0	0	8	6	0
Leg ulcer	0	0	0	0	4
Mean $tcPO_2$	68	74	48	50	71

PCT, porphyria cutanea tarda; CVI, chronic venous insufficiency.

The results of this study indicate that porphyria cutanea tarda patients with fibrin cuffs have normal $tcPO_2$ values. Patients with CVI with and without fibrin cuffs have a lower $tcPO_2$, but no difference could be observed between the groups. Thus it seems likely that fibrin does not act as a barrier for oxygen.

The fibrin deposits will be more of an indicator for microcirculatory disturbances than the cause of low $tcPO_2$. From compression therapy studies [22] and capillary density studies [10] at least the capillary density seems to play a role in the oxygenation in CVI. Up to now insufficient knowledge has been available for the explanation of the lower than normal $tcPO_2$ in CVI. We have postulated a model, as outlined in Fig. 1, to explain the lower than normal PO_2 in CVI patients.

Laser Doppler Flux Measurements

The same Doppler principle is used for laser Doppler as for the widely used ultrasonic vascular techniques. Only the wavelength is different. Usually a helium neon laser is used with a wavelength of 632.8 nm and a power of 2 mV. This emitted light is reflected by the moving erythrocytes and, due to the shift in wavelength after reflection, this light can be detected by the probe. As erythrocytes move in all directions in the capillaries it is not possible to measure flow, but only flux. The laser beam light penetrates the skin for about 1 mm. Most probes will sample a volume with a radius of 1 mm [43, 44]. The contribution of the thermoregulatory capillaries is 95% of the signal [45]. That means that the most interesting part of the skin microcirculation, i.e., the nutritive capillaries, is rather poorly studied with laser Doppler. Laser Doppler flux measurements are mainly used in investigating diabetics and AOD.

Laser Doppler flux measurements do not correlate with $tcPO_2$ measurements [45]. Laser Doppler flux measurements include signals from the venous plexus and shunts and are therefore an indicator of thermoregulatory flow [43]. The value of combining $tcPO_2$ and laser Doppler flux in one and the same probe is for this reason not proven [46], as $tcPO_2$ measures more the overall function of the skin microcirculation.

Lancaster et al. [47] studied microcirculatory flux with the laser Doppler in patients with CVI. They found a change in the variability of the waveform and changes in the absolute level. The observed changes correlated well with a simultaneously performed photoplethysmographic assessment of venous reflex.

In an erect position the efficiency of the venoarteriolar response is reduced in diabetics [48], in patients with CVI [47, 49], and in those with peripheral vascular disease [50].

Up to now it is not clear if laser Doppler flux measurement will have a predictive value in assessing peripheral vascular disease, especially in

diabetics. Besides fundoscopy it will be useful to have a skin model for detecting "high risk" patients. This is especially important because diabetic ulcers comprise an important part of the medical care necessary in elderly people. A good noninvasive and easy to perform test in calculating the risk for diabetic ulcers will advance daily care of diabetics.

A totally new and promising development in laser Doppler technology is laser Doppler perfusion imaging [51]. With this technique a scan of a skin surface can be made, which gives a good impression about the investigated part of the microcirculation. The resolution of the system is high enough to provide information about special areas of interest, e.g., ulcers, vascular malformation, and obstructions. In contrast to the classical laser Doppler flux technique, not only a pinhole, but a real surface of skin can be investigated.

Capillaroscopy

In 1700 von Marshall described a microscopic technique by which he was able to visualize the microcirculation in a fishtail [52]. In the last few decades capillaroscopy has been used for many microvascular studies. This technique is extensively reviewed by Bollinger and Fagrell [53]. Improvements were made by adding a video camera and TV monitor to the system [54]. Later, the use of fluorescense dyes, e.g., Na-fluorescein, provided new possibilities.

The nailfold capillaries are relatively easy to investigate, due to the fact that the capillary loops run parallel to the skin surface. Other skin areas are also amenable to investigation, but the capillaries will not be seen as loops, as is the case in nailfolds, but as dotted or comma-like structures.

In CVI the capillaries in the affected skin areas become tortuous and they will be visualized as glomerulus-like structures. CVI is a good condition for studying the microcirculatory alterations with the use of fluorescence video microscopy [55, 56]. In these studies microthrombi and a large pericapillary halo have been observed. The major parameters for capillaroscopy are summarized in Table 4.

Table 4. Capillaroscopy of the skin microcirculation

Vascular microcirculation
Video microscopy
Capillary morphology
Erythrocyte flow rate
Fluorescence video microscopy
Na-fluorescein as tracer
Indigo green [57]
Lymphatic microcirculation
Fluorescence microlymphography [58]

Table 5. Nailfold capillaroscopy

With good results:
Scleroderma
Mixed connective tissue disease
Dermatomyositis
With poor results:
Eosinophilic fasciitis
Lupus erythematosus
Rheumatoid arthritis

Capillaroscopy gives a good anatomical impression of alterations of the microcirculation. Also some information can be obtained about the flow in the system. By using dyes this technique will give some functional data, e.g., regarding leakage of the capillaries. Values obtained about halo formation as result of capillary leakage are, however, different from the data obtained from capillary filtration rate.

Capillaroscopy is not only used in assessing vascular diseases, as in the cause of CVI, AOD and diabetes, but also in scleroderma, and Raynaud's phenomenon [52]. The major indications for nailfold capillaroscopy are summarized in Table 5. Although it is a complicated technique it is a very interesting research tool for all who are interested in skin microcirculation.

Studying the skin microcirculation is not very easy. Due to the fact that all the above described techniques measure a special part of the microcirculation, it is useful to combine the different techniques. Other techniques can also be useful. Direct pressure measurements will give information about the relationship between macro- and microcirculation. Especially in CVI this technique can be useful for calculating the effect of the high walking venous pressure on the capillaries (Neumann, unpublished observation).

Capillary leakage can be measured with the filtration rate method. There are mainly two different types of capillary filtration rate measurements. A plethysmographic method and a combination of this technique with a radionuclide technique [59]. A very useful technique was described by Mostbeck, who used a simultaneous measurement of leg and regional blood volume with plethysmography combined with Tc 99m-labeled erythrocytes [60]. Labeling with technetium is easier to use than the classical labeling with $_{51}Cr$ [61] because the half-life of $_{51}Cr$ is 28 days compared with 6 h for Tc 99m. The technique is mainly used for pharmacological studies [60, 62].

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Spatial Pattern of Cutaneous Perfusion in Wound Healing

M. Stücker, T. Auer, K. Hoffmann, and P. Altmeyer

Introduction

Cutaneous perfusion is characterized by a marked inhomogeneity [17, 22]. Strongly hyperperfused areas can be localized next to weakly perfused areas. To assess the spatial pattern of perfusion several one-point measurements of adjoining measuring points are necessary. Noninvasive measurement techniques, such as one-dimensional laser Doppler fluxmetry [LDF, 11], capillaroscopy [4] and $tcPO_2$ measurement [12], demonstrate small, point-like skin areas only. For the evaluation of subclinical changes in perfusion, noninvasive objective measuring methods are indispensable [1]. For the first time it is now possible to display a two-dimensional perfusion image of the skin by laser Doppler scanning (LDS).

For the exact evaluation of two-dimensional perfusion patterns, both the extent and the intensity of cutaneous perfusion have to be determined. In this study we tried to answer the question whether there are advantages in using LDS for the evaluation of skin perfusion in comparison to clinical evaluation and one-dimensional laser Doppler fluximetry. Furthermore, the possibility of using LDS for predicting the healing course of surgical wounds was evaluated.

Material and Methods

Laser Doppler Scanning in a Model of Inflammation

The reactions of 20 healthy subjects (26–76 years, mean 48 years) to defined amounts of recall antigens were examined. Seven antigens and one control substance were applied to the skin of the back with the help of a test stamp (Multitest Merieux, Institut Merieux, Germany). The following were applied: tetanus, diphtheria, *Streptococcus*, tuberculin, *Proteus*, *Trichophyton*

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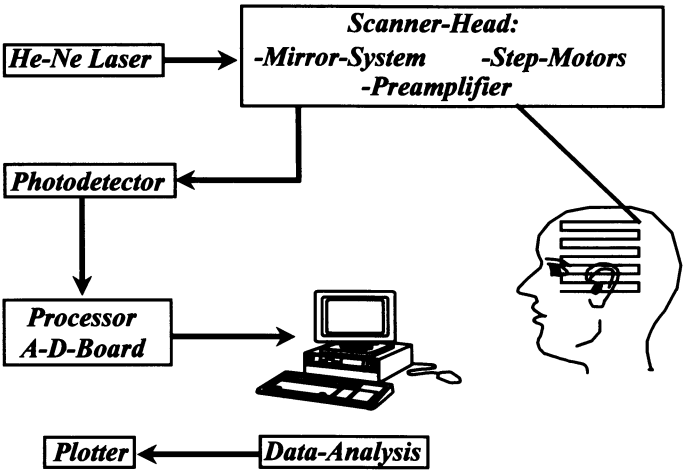

Fig. 1. A laser Doppler scanner

and *Candida*. Evaluation of the inflammatory reaction was performed before the application of the test stamp and 10 min, and 24, 48 and 72 h afterwards. All measurements were performed at a room temperature of 23°–24°C after the patient had acclimatized him/herself to this temperature for half an hour in a prone position. The reactions were examined in a defined sequence of techniques: two-dimensional LDS, one-dimensional LDF and planimetry.

As with the conventional unidimensional LDF, the principle of the two-dimensional LDS (laser Doppler perfusion imager PIM 1.0, Lisca Developement, Linköping, Schweden) is the Doppler effect of monochromatic light on moving erythrocytes. The measuring distance between the scanner head and the region of interest is between 15 and 20 cm; the measurement takes place without direct skin contact. The area of examination can be as large as 12 × 12 cm, depending on the density of measurement points and the examination distance. With the help of a mirror system controlled by two stepping motors a helium-neon laser (wavelength 632.8 nm) is guided in a meandering pattern over the examination area. Signal deviations caused by irregularities on the skin surface are compensated for by a correctional algorithm. The resulting perfusion image after processing is made up of 4096 measurement points (Fig. 1). Perfusion values are coded with a six-color scale, the flux values increasing from blue to green, yellow, orange and red.

A color graduation of three or more was defined as a hyperemic reaction, equivalent to a perfusion intensity of ≥ 3.2 AU. The expansion of this hyperemic area was compared with the clinical findings. The median flux for the hyperperfused areas was calculated.

The visible outlines of the erythema were transferred to a piece of cellophane and determined planimetrically by image analysis (Microscale, Digithurst, UK). The intensity of the cutaneous perfusion in the center of

the erythema was detected by one-dimensional laser Doppler (MBF 3D, Moore Instruments, UK) calculating the mean value of three subsequent measurements.

Laser Doppler Scanning of Traumatic Lesions

The wounds of ten patients (48–63 years) after different surgical techniques were examined: cryosurgery in the closed contact method (three patients), simple excision (four patients) and rotation flaps (three patients). After the operation the perfusion pattern was monitored daily in the first week and every 2 days in the second week using LDS. The perfusion patterns were analyzed according to hyper-, hypo- and unperfused areas.

Results

Laser Doppler Scanning in a Model of Inflammation

Erythema could be quantified by planimetry and by LDS. The planimetrically determined size correlated significantly with the hyperperfused areas established with LDS (10 min: $r = 0.76$, $p \leq 0.0001$; 24 h: $r = 0.82$, $p \leq 0.0001$; 48 h: $r = 0.89$, $p \leq 0.0001$; 72 h: $r = 0.75$, $p = 0.0010$). The mean size of the hyperperfused areas was larger than that of the visible erythema (10 min: 6.28×; 24 h: 1.33×; 48 h: 1.8×; 72 h: 3.2×). LDS revealed an elevation in perfusion in nine cases in which the skin demonstrated no clinical changes. In one case, erythema which was visible clinically did not display an elevated flux. In healthy skin the flux values measured by the conventional one-dimensional laser Doppler did not correlate with those measured with LDS ($p = 0.5779$, $r = 0.13$). In the erythemateous areas the two measurement techniques correlated significantly ($p \leq 0.05$).

Laser Doppler Scanning of Traumatic Lesions

In patient 1, a basal cell carcinoma of the left cheek was excised and the defect closed by a rotation flap (Fig. 2). On the second postoperative day a marked perfusion gradient from the base to the end of the flap, as expressed in the color code as the transition from yellow to green to blue, could be seen (Fig. 3). The suture was not perfused (gray). The suture material itself appeared as not perfused (gray) horizontal lines. The peripheral part of the flap was much more perfused than the center.

The surrounding healthy tissue showed an increase in perfusion towards the margin of the suture. Four days postoperatively the healthy skin around the flap was still as hyperperfused as it was on the second postoperative day

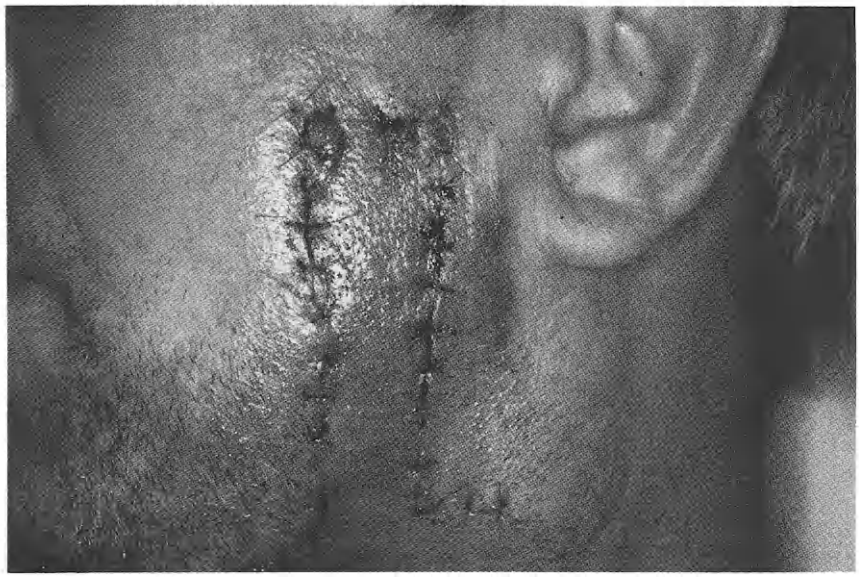

Fig. 2. Rotation flap on the left cheek of a patient operated on for basal cell carcinoma: second postoperative day

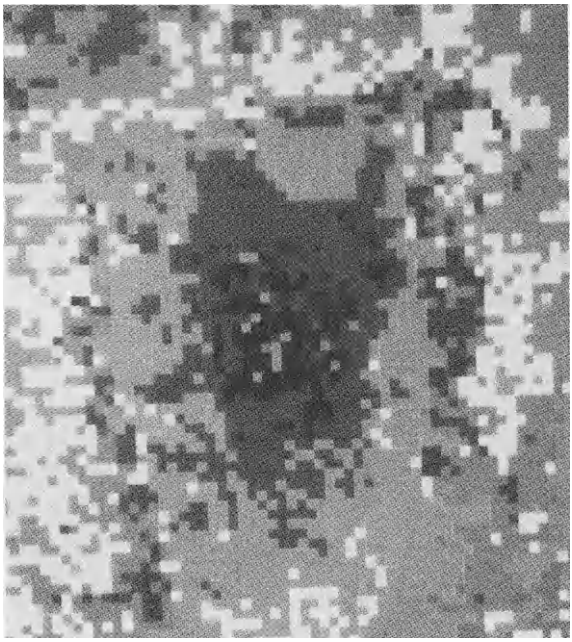

Fig. 3. Same patient as in Fig. 2, corresponding laser Doppler scanning (LDS) image: second postoperative day. Suture (*gray*), flap, and adjoining tissue can be well differentiated

(Fig. 4, blue, upper part). However at the suture margin the perfusion increased even further on day 4. The rotation flap was distinctly more perfused than on the second day with the exception of the end of the flap (blue). The perfusion pattern, however, still showed a gradient from the base to the end and from the center to the borders of the flap.

The gray-coded area of the upper left corner of the flap correlated with a necrosis that became demarcated during the next few days. The hyperperfused areas (red and yellow) which were in direct contact with the incision doubled in size. On the 12th postoperative day in the gray code area perfusion was still not measurable (gray). The suture was now strongly perfused (red) (Fig. 5).

In patient 2 (39 years old), a Kaposi sarcoma was treated by cryosurgery using the closed contact method. Clinically, on the first postoperative day, the round impression of the cryostamp was clearly visible; the tumor was hemorrhagic and necrotic. In the LDS image the round cryostamp could be recognized (Fig. 6). According to the typical, even clinically visible, inflammatory reaction the healthy surrounding tissue developed a marked hyperperfusion (yellow and red). The tumor itself was not perfused (gray). On the fourth postoperative day the necrotic tumor was demarcated. The increase in perfusion in the region surrounding the cryosurgically treated area had diminished (from yellow and red to blue). The border between the nonlesional skin and the treated area was also hyperperfused (yellow). The whole lesion was hypoperfused, while the necrotic tumor showed no perfusion at all (gray, Fig. 7).

Discussion

Since its first use by Stern [18] in the field of skin perfusion, LDF has been broadly applied [16]. Skin perfusion is influenced by several parameters such as changes in skin temperature and position of the patient [9]. Since even measurements at rest are difficult to compare with each other, using one-dimensional measuring methods the reactive hyperemia after 1–3 min of arterial occlusion is used for the detection of pathological alterations in perfusion [6]; not the absolute values but relative temporal changes in perfusion are assessed. Apart from these temporal changes skin perfusion reveals a spatial inhomogeneity [17, 22]. To guarantee standardized conditions during continuous measurements, the probe has to be placed in direct contact with the skin [10]. On the one hand the results can be falsified by the probe-induced pressure, on the other hand skin contact implies the risk of microbial transfer.

LDS allows examination of the perfusion patterns of the skin without any skin contact [24]. Our study shows that the elevated perfusion as verified with LDS extends substantially beyond the region of the planimetrically quantified erythema. There is a certain correlation between the

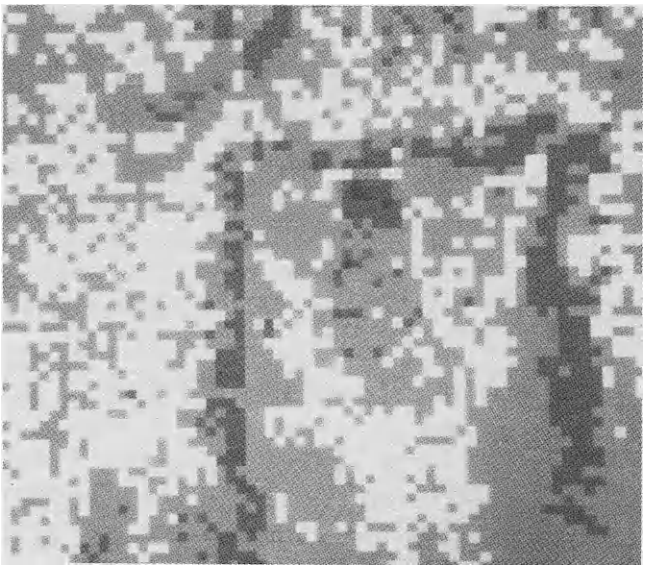

Fig. 4. Same patient as in Fig. 2: fourth postoperative day. The whole flap is hyperperfused. The adjoining tissue shows a marked increase in perfusion towards the margin of the suture

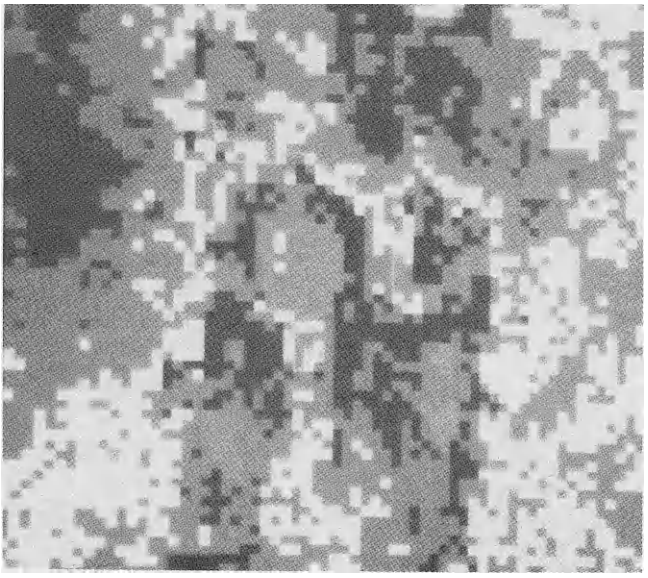

Fig. 5. Same patient as in Fig. 2: 12th postoperative day. Replacement of the previously unperfused suture by strongly perfused tissue

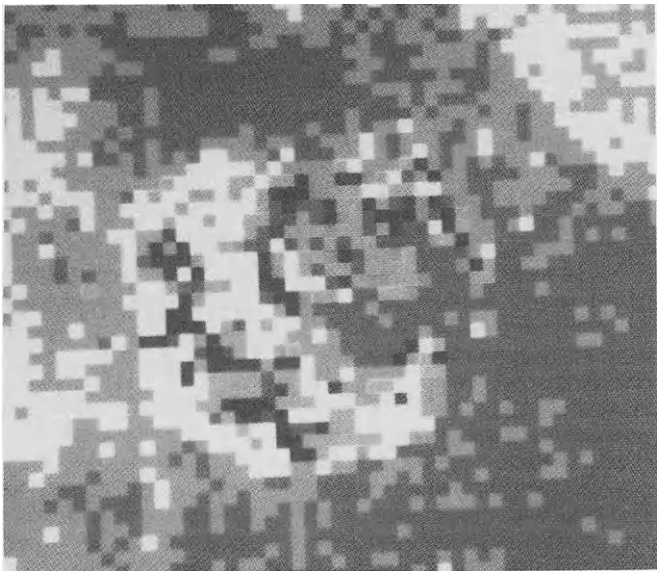

Fig. 6. Cryosurgery of a Kaposi's sarcoma, corresponding laser Doppler scanning (LDS) image. The round impression of the cryostamp can be recognized. The necrotic tumor is not perfused (*gray*)

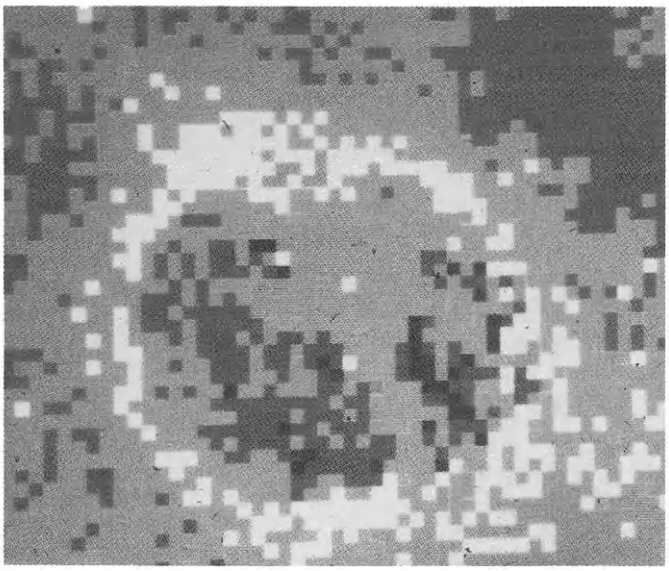

Fig. 7. Same patient as in Fig. 6: fourth postoperative day after cryosurgery, corresponding laser Doppler scanning (LDS) image. Increased perfusion of the margin of the wound, still no perfusion of the necrotic tumor

dimensions of the erythema and the hyperperfused area, although no constant factor for this relationship can be established. Clinical determination of the expansion of an erythema as the basis for planimetry is subjective. The differentiation of reds and browns can be especially difficult in sunburned skin [8]. Evaluation of the expansion of a skin reaction by one-dimensional fluximetry (LDF) has been discussed previously [20]. In clinically healthy skin the flux values measured with the LDF did not correlate with those measured by LDS. When the erythema was due to the reaction to trauma or to the antigens of the test stamp, the flux values of both techniques correlated significantly. It appears that subclinical inhomogeneities in cutaneous perfusion can be detected less exactly with one-dimensional LDF than with LDS. For this reason the variance of the LDS measurement is smaller than that of the LDF [14]. Obviously LDS allows a much more objective assessment of two-dimensional perfusion patterns than LDF or clinical evaluation. With one-dimensional LDF perfusion changes can be detected during exercises, e.g., intraoperative stretching of a flap [13]. When controlling skin perfusion for a longer period the examination of exactly the same location with one-dimensional measuring methods is problematic. LDS reveals additional points for orientation because of the two-dimensional perfusion image. Thus development of increased, decreased or nonperfused areas of a wound can be evaluated.

The influence of different suture materials and techniques can be evaluated also. According to our first studies it seems to be possible to identify necrotic areas of a rotation flap by LDS prior to their clinical demarcation.

In cryosurgery one benefits from the cold sensitivity of tumor cells [2]. Decisive for the success of cryosurgery is the quick freezing and slow defrosting of the treated tissue [5]. Increased perfusion of the surrounding area slows the freezing and accelerates the defrosting [15]. Therefore the hyperemia of the surrounding, not of the treated, skin caused by the cold is an important potential source of disturbance. Already during treatment, perilesional perfusion is increased [3]. The laser Doppler scanner shows that this reactive hyperemia includes a large area of the surrounding tissue for several hours or days. This is in accordance with the clinical experience of a posttraumatic exsudative inflammatory reaction. In the cryosurgical lesion only the tumor area was without perfusion, while the rest of the lesion showed weak perfusion. This might support the observation of an increased sensitivity of the tumor to low temperatures. Blood flow through the capillaries stops nearly completely during cryosurgical treatment [25]; that of large vessels remain unchanged [7]. The LDS signals are caused by the flow of red blood cells through the capillaries of the papillae and the deeper, subpapillary thermoregulatory plexus [23]. The weak remaining perfusion of the scanner image results from blood flow in the deeper dermal vessels.

Apart from being influenced by perfusion, skin temperature is determined by the heat conduction of the tissue and by metabolic heat production [21]. In exudative processes skin perfusion, as the determining

factor of skin temperature, takes a back seat in relation to evaporation [19]. Therefore thermography seems to be less suited for the objective evaluation of cutaneous perfusion in wound healing than does LDS.

To summarize, LDS is a new method for the evaluation of perfusion patterns of skin without skin contact. The two-dimensional image allows the examination of various single measurement points discontinuously and for a longer period. Through this the effects of different surgical techniques and drugs on perfusion during wound healing can be quantified.

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The Vascular Architecture of the Keloid: A Scanning Electron Microscope Study on Vascular Corrosion Casts

A. Lametschwandtner[1] and O. Staindl[2]

Introduction

Healing of skin lesions is a complex process in which intracellular and extracellular factors interact. Morphologically the entire healing process consists of three stages: (1) the exudative stage, (2) the proliferative stage, and (3) the reparative stage.

In the *exudative stage* fibrinogen/fibrin is the key element. It seals the wound edges and provides the basis for the initiation of wound healing. In the *proliferative stage* fibroblasts are the dominating cell type. Fibroblasts proliferate into cross-linked fibrin and synthesize collagen. Both collagen synthesis and the entire wound healing process is highly dependent upon factor XIII (fibrin stabilizing factor, FSF). This factor, a β-globulin, catalyzes fibrin cross-linkage which leads to a clot of considerable mechanical strength. This process is essential for normal wound healing [1]. Factor XIII inhibits proteolysis by preventing the proliferation of connective tissue cells and the incorporation of α-antiplasmin in the fibrin matrix [17]. Fibronectin also plays a specific role in the wound healing process. It mediates the adhesion of fibroblasts to collagen. Fairly recently, immunohistochemical evidence has shown a relationship between disturbed wound healing and resulting pathological scar formation, on the one hand, and the blood plasma level of factor XIII, the fibronectin concentration in intercellular compartments, and the formation of pathological fibroblasts – termed myofibroblasts – on the other hand [4, 8, 9, 11].

Keloids

Keloids are a dreaded complication occurring in the *reparative stage* of wound healing. It is still not fully understood which mechanical factors

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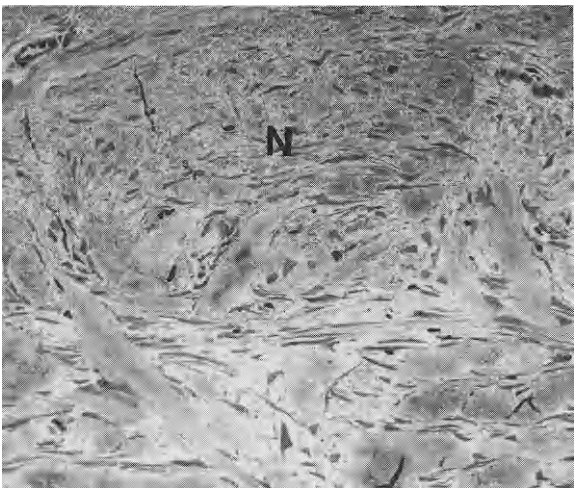

Fig. 1. Histology of the keloid. The nodular accumulation of fibroblasts (*N*) becomes evident. Semithin section; azure-II/methylene blue, ×175

ultimately cause keloid formation. Clinically, a keloid is defined as scar tissue which outgrows the original wound area and, similar to a tumor, proliferates into adjacent healthy tissue. A major difference between keloids and hypertrophic scars is the absence of spontaneous regression in the keloid. Usually, keloids also have a more delicate surface texture and contracture is less frequent than in the case of hypertrophic scars.

There is a familial disposition to keloid formation. As a rule, young persons are affected and keloids have also been observed more often in dark-skinned people. Certain regions of the body, such as the presternal region, the shoulders, the neck, and the external regions of the upper arms are predisposed to the development of keloids [14].

The above-mentioned biological and clinical points of differentiation between keloids and hypertrophic scars are not reflected in the histology of the keloid (Fig. 1). Both lesions are characterized by an excessive increase of collagen forming discrete nodules. Increased levels of fibronectin, immunoglobulins, histamine and chondroitin-4-sulfate have been found both in hypertrophic scars and in keloids. PO_2 values are lower than in normal skin, whereas PCO_2 values are higher [11].

In both keloids and hypertrophic scars, Lehmann and Gabbiani [13] observed myofibroblasts. The latter term has been used by the authors to denote pathologically altered fibroblasts of the same type as demonstrated on electron micrographs of Dupuytren's disease and various forms of tracheal stenosis. The presence of these myofibroblasts and the increase in fibronectin appear to be attributable to a disorder in collagen metabolism.

In addition to the above-mentioned intracellular and extracellular parameters, the vascular structure of keloids and hypertrophic scars shows a number of characteristics which so far have only been described histologically. Compared with normal skin, an inordinate hypervascularity has been observed in keloids [10].

While there exists an abundance of literature on the microstructure of keloids, no detailed analysis of scar tissue in general, or of keloids in particular, has yet been done. This may be due to the insufficient depth of focus of light microscopes, which do not allow one to visualize the three-dimensional course of blood vessels over longer distances. Reconstruction of the course of blood vessels by means of serial sections is laborious and time-consuming.

In contrast, the microvascular corrosion cast/scanning electron microscopy (SEM) method [15, 16], due to its high depth of focus and the positive identification of cast arteries and veins [18], can provide information on both quality and quantity of blood vessels within healthy and diseased tissue. It is the objective of this chapter to demonstrate that the microvascular corrosion cast/SEM method enables one to replicate the blood vascular system of tissues harvested during surgery. The keloid, whose behavior is essentially determined by its vascular structures, provides a worthwhile study object.

Material and Methods

Three keloids obtained during corrective surgery were prepared for histology and the microvascular corrosion cast/SEM method. Sufficient filling of the vascular bed was achieved in one specimen only. This was a keloid (12 cm by 7 cm) removed from the pectoral skin of a 22 year old male patient (Fig. 2).

Light Microscopy

Keloid tissue samples (1 cm^2 in size) were fixed by immersion in buffered 2.5% glutaraldehyde (0.15 *M* cacodylate buffer, pH 7.35), dehydrated in a graded series of ethanol and propylene oxide and embedded in Epon 812 (Serva). Semithin sections (1 μm) were stained with azure II/methylene blue.

Microvascular Corrosion Casting

Excised keloids were immediately immersed in heparinized saline (15 IU/ml) and transferred into the preparation laboratory. Here an artery was dissected free under stereomicroscopic control, a fine glass cannula was in-

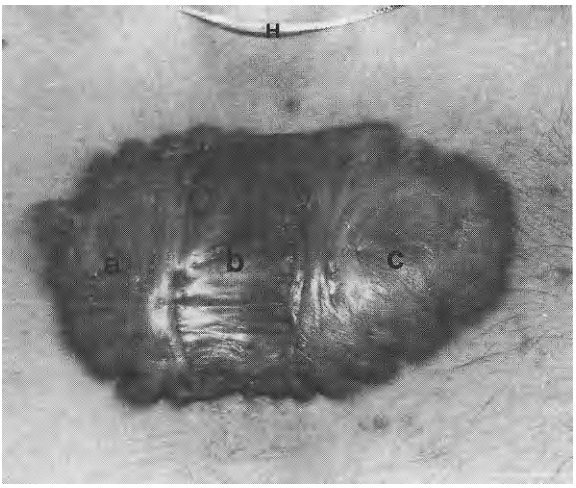

Fig. 2. Keloid of the pectoral skin in a 22 year old male patient. *H*, necklace

troduced and securely tied in place. Then larger vessels cut by the surgical removal were carefully ligatured with suture material and the blood vascular system was rinsed with heparinized saline. Using the same route Mercox-Cl-2B, diluted 4:1 with monomeric methylmethacrylate (FLUKA, Buchs) [5], was injected by manual pressure. After polymerization of the injected casting material the surrounding tissue was macerated with 15% potassium hydroxide (40°C), rinsed in several passages of distilled water, submerged for 15 min in 5% formic acid (20°C), and finally deep-frozen in bidistilled water. Freeze dried corrosion casts were mounted to specimen stubs using the "conductive bridge method", sputtered with gold, and examined in a scanning electron microscope at 5 kV accelerating voltage. In order to study the internal vascular architecture of the keloid, the corrosion cast was sectioned into 5 mm thick slices while refrozen in distilled water. For further details on the method, see references in [12, 18].

Results

Figure 3 shows the examined vascular corrosion cast in toto. It can be seen that the microvascular bed is not entirely filled all over the specimen. While in zone c subpapillary capillaries are well cast, areas marked a and b reveal predominantly the subpapillary venous plexus with fewer capillary loops entirely cast (Fig. 4) than in normal skin (Fig. 5). Capillary loops are simple (Fig. 6) and lack arteriovenous anastomoses shunting ascending and descending portions of the loops at different levels in normal skin. In the keloid capillary loops ascending and descending portions are farther apart than in

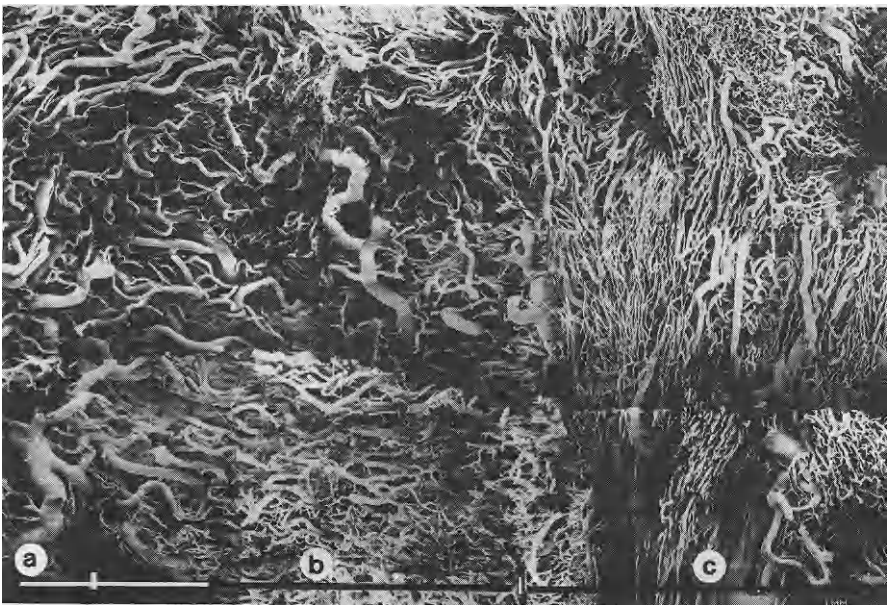

Fig. 3a–c. Cast of the microvascular bed of the keloid demonstrated in Fig. 2. Scanning electron micrograph. Photomontage consisting of 13 single micrographs. *a*, *b*, *c* Correspond with areas labeled in Fig. 2. Note the longitudinally running capillaries in **c**

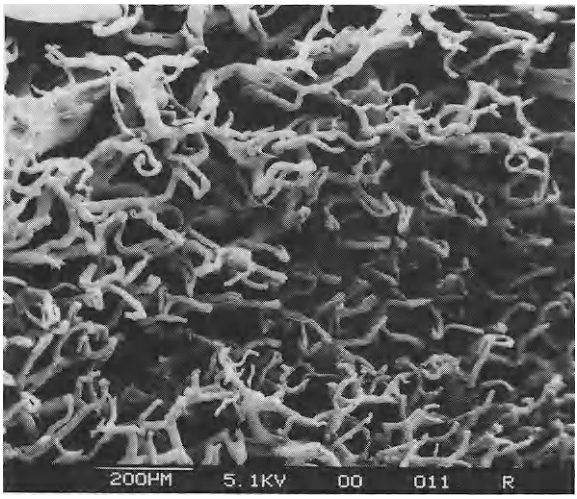

Fig. 4. Vascular bed of the papillary layer of the keloid. Microvascular corrosion cast

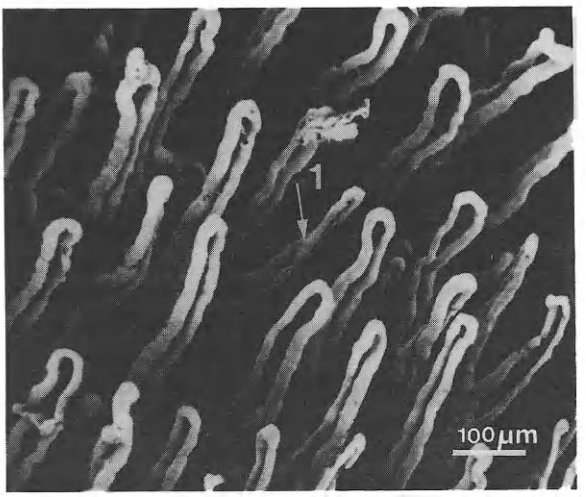

Fig. 5. Hair-pin capillary loops of normal skin. Note the thin ascending and the somewhat thicker descending loop of the capillary. Sometimes capillary loops are twisted (*arrow*)

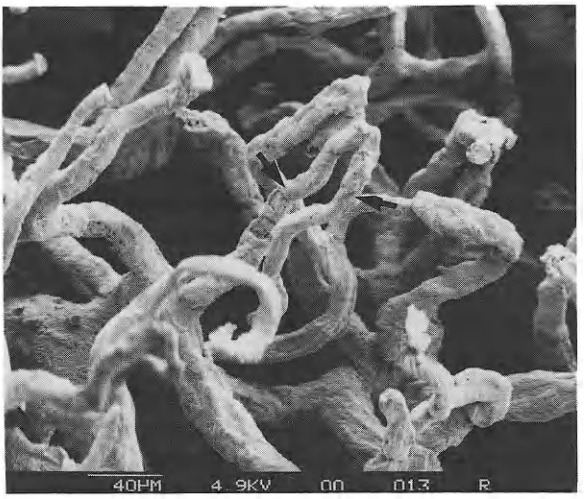

Fig. 6. Close-up view of papillary capillary loops of the keloid. Note the uniform thickness of ascending and descending capillary loops (*arrows*)

normal skin (Fig. 7). Twisted capillary loops as seen in normal skin (Fig. 5) are absent. Sections through the cast keloid microvascular bed reveal densely vascularized areas, but also avascular patches (Fig. 8). In the densely vascularized areas, capillaries and small venules are most common. Larger vessels, if present, have a highly tortuous course.

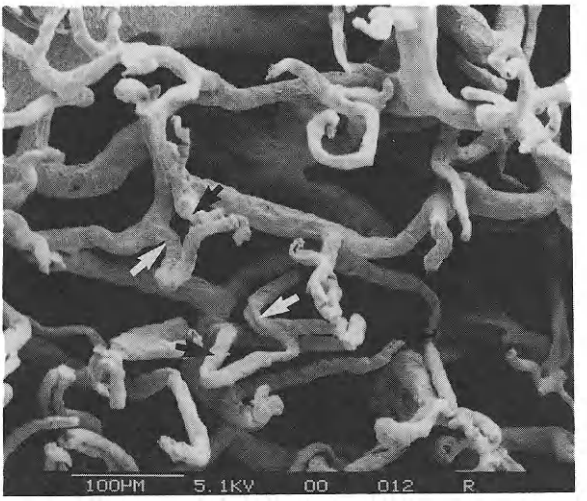

Fig. 7. Capillary loop of the papillary layer of the keloid. Note the wide distance between ascending and descending loop (*arrows*)

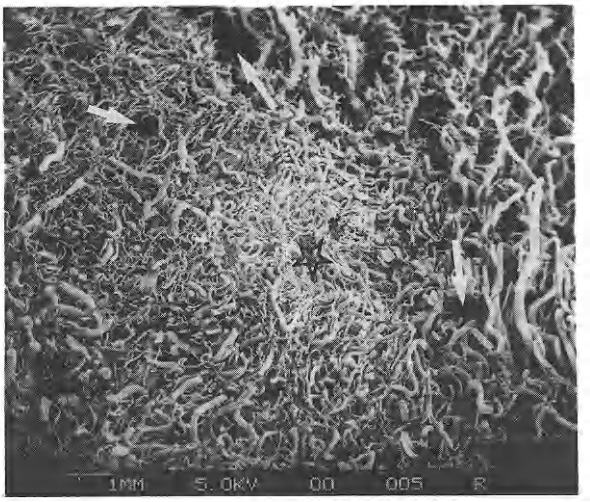

Fig. 8. Cut surface of the keloid microvascular cast revealing densely vascularized areas (*star*) with avascular patches between (*arrows*)

When viewing normally the papillary and subpapillary zones of the vascular bed it is seen that large vessels are aligned either longitudinally (Fig. 3; upper half) or transversely (Fig. 3, lower half; parallel to right or left margins of the micrograph). In the papillary layer, when viewed from

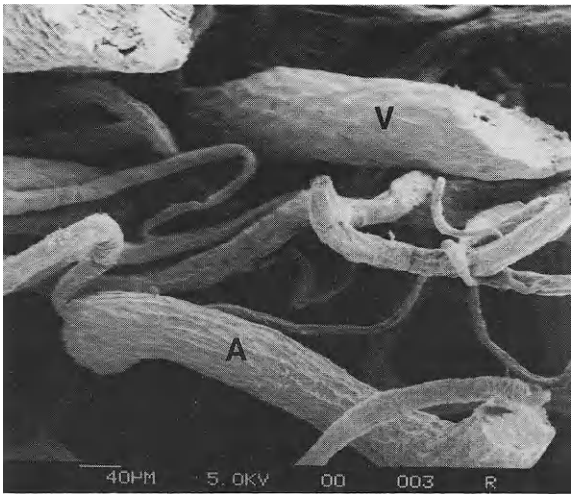

Fig. 9. Detail view of a cast arteriole (*A*) and venule (*V*). Note the round profiles of both vessels and the prominent endothelial cell nuclei imprints

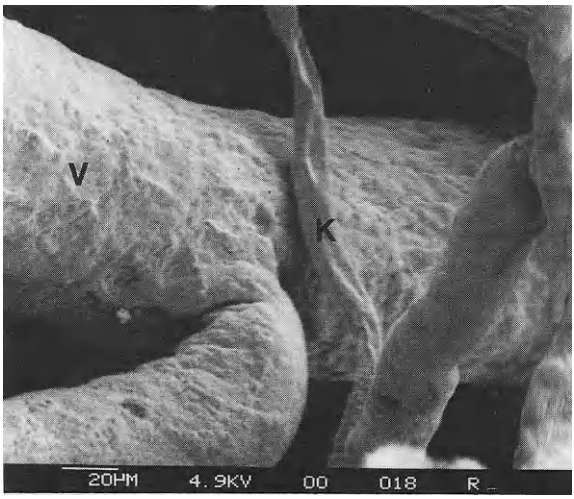

Fig. 10. Endothelial cell nuclei imprints on a cast venule (*V*) and capillary (*K*)

above (normal), capillaries overlaying the subpapillary venous plexus run in a longitudinal direction (Fig. 3c).

Details from the casts of arterial, venous and capillary vessels show pronounced imprint patterns of endothelial cells or endothelial cell nuclei. This indicates that in keloid vessels endothelial cells or their nuclei con-

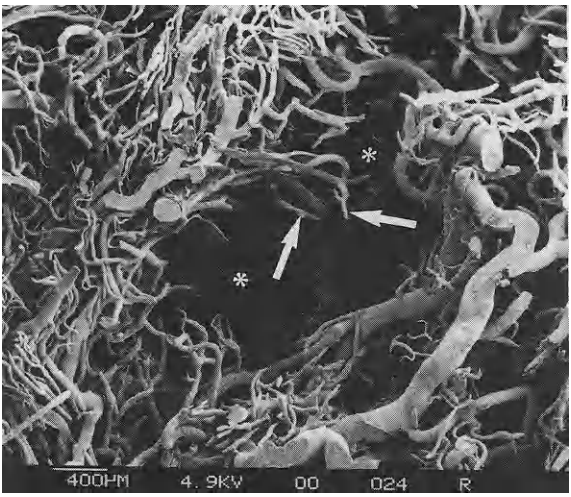

Fig. 11. Avascular area (*asterisks*). Note the vessels radially projecting into the avascular space (*arrows*)

spicuously bulge into the vessel lumen (Figs. 9, 10), a finding not so prominent in normal skin vessels (Fig. 5).

Surprisingly, all cast vessels have a rather round profile (Fig. 9). Tapered or flattened vessels indicative of compression by surrounding keloid tissue are absent. This is also true for vessels adjacent to avascular areas with radially protruding blind-ending branches (Fig. 11). Some of these structures are without any doubt artifacts, especially the round-ending ones, whereas the pointed-ending ones probably represent vascular sprouts proliferating into the avascular areas (Fig. 10).

On closer inspection the vascular casts reveal structures in venules indicative of sphincters (Figs. 12, 13). Since the keloid has a compact connective tissue parenchyma, the casts not only replicate the luminal structures, but also deeper elements of the vessel wall, such as spirally arranged smooth muscle cells of the vessel media (Fig. 14), or even collagen fibers adjoining the vessels external layers (Fig. 15).

Generally, the endothelial cell nuclei imprint patterns are more pronounced on venous vessels (Fig. 16). These also exhibit a large number of tipped, blind-ending branches. Some of them have "horns" or sprouts, doubtlessly a sign of incipient vascular proliferation (Fig. 17). It should also be noted that venules opening into larger vessels vary greatly in size and caliber (Fig. 18).

Moreover, attention has to be drawn to "plastic strips" (Fig. 19). These were first described by Anderson and Anderson and by Castenholtz et al. [2] and since then have been a long-lasting subject of discussion.

Fig. 12. Venule (*Ve*) draining into a larger vein (*V*). Note the circular constriction at the entrance of the venule (*arrows*). The capillary (*K*) reveals obliquely arranged, spindle-shaped imprints (*arrowheads*)

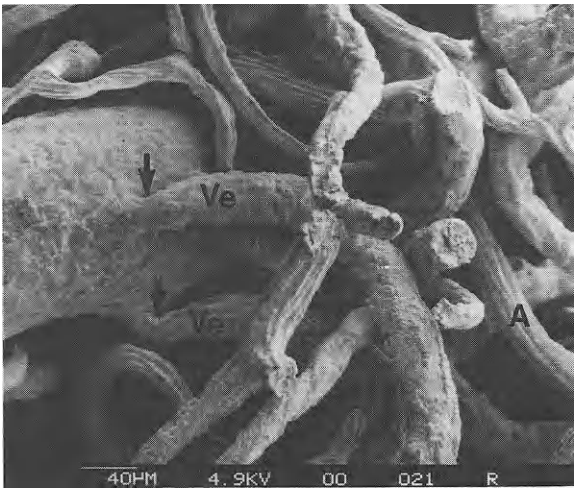

Fig. 13. Two venules (*Ve*) join a larger vein. Note the caliber changes at the entrance sites (*arrows*); *A*, arteriole

Furthermore, circular constrictions of various degrees have been found immediately at or somewhat away from the sites where small venules empty into larger ones (Figs. 12, 19). These constrictions probably respresent sites of sphincters.

Fig. 14. Venule with spirally arranged, spindle-shaped imprint patterns (*arrows*)

Fig. 15. Extravasal structure with proposed imprints of nearby collagen fibers (*arrows*); *A*, arteriole

Discussion

In all three keloids removed during surgical correction vessels large enough for cannulation were dissected without any difficulties. Despite the fact that all three vascular corrosion casts were prepared by the same method, only one specimen was sufficiently cast to be studied in the SEM. In the well

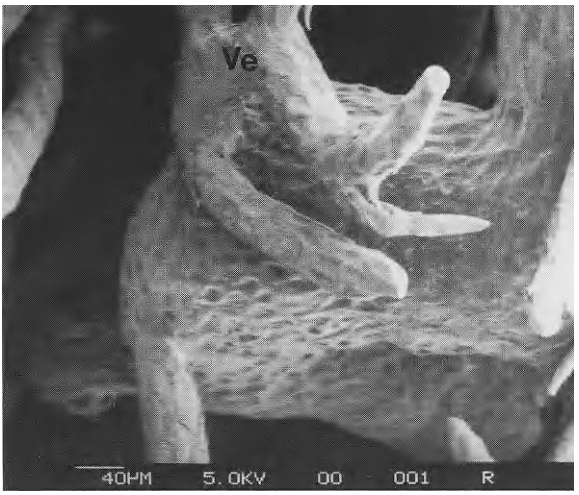

Fig. 16. Venule (*Ve*) with tapered, blind-ending structrues

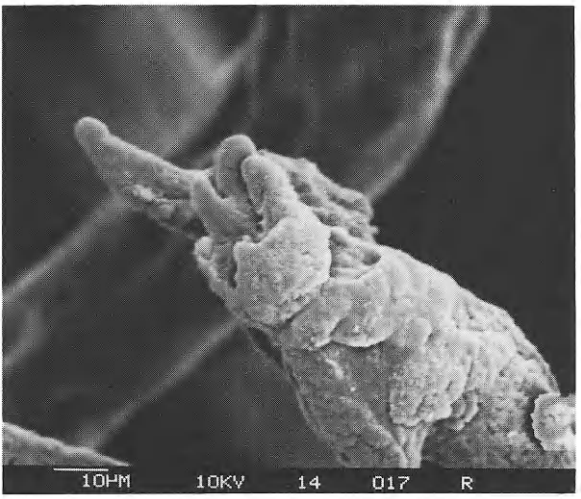

Fig. 17. Tapered-ending blood vessel interpreted as sprout

filled specimen the vascular bed obviously was supplied by a large vessel, whereas in the other two specimens the supply was probably via small collaterals and the injected resin bypassed the microvascular bed through larger arterial branches of the cannulated vessel. It becomes evident that the success of casting intraoperatively harvested tissue cannot be predicted with certainty and to a large degree depends on how the vascular bed has been

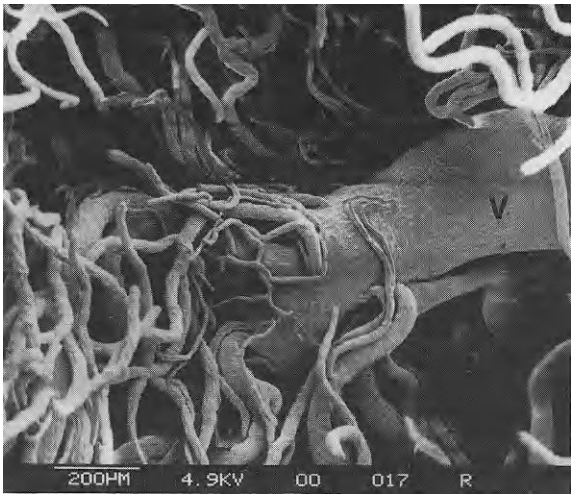

Fig. 18. A vein (*V*) receives many venules of differnet calibers

Fig. 19. Venule with "plastic strips" (*asterisks*). Note the prominent constriction of a venule (*arrow*)

severed by the excision. If precautions such as coating cut surfaces with low melting point paraffin improve the casting outcome still has to be tested.

Compared with normal skin the keloid examined is much more densely vascularized [6, 7]. In particular, the subpapillary venous plexus is much more developed. Moreover, in the keloid bulging of the endothelial cell lining was prominent in arterial and venous vessels. Occluded vessels, des-

cribed in immature keloids [11], were not found. Since many small vessels were not ligatured thus serving as efflux pathways, and since several capillary loops of the papillary layer were not cast thoroughly, it seems very unlikely that too high injection pressure explains the lack of compressed or occluded vessels. Furthermore, the cast studied was a mature one. The lack of compressed vessels indicates that the tissue pressure in the keloid was not increased. On the contrary, vessels were found to be exceptionally wide with round profiles. This observation fits very well with the hyperemia found at macroscopic examination of the keloid. How far the treatment of the excised tissue prior to casting caused vasodilation cannot be stated.

Finally, it should be mentioned that the vascular system of the keloid showed no prominent damage or leaks. Extravascular structures that do not represent normal vessels (Fig. 3) are rare and have been found also in normal skin and were considered to represent microlesions.

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Growth Factor Control of Wound Healing Angiogenesis

D.R. Knighton, V.D. Fiegel, and G.D. Phillips

Introduction

Angiogenesis is a fundamental biologic process which plays a pivotal role in development, pathologic states and physiologic responses. In the developing embryo, blood vessels form by two mechanisms: (1) vasculogenesis, in which mesoderm-derived angioblasts migrate to specific sites in the embryo and form a network of vascular cords, and (2) angiogenesis, which occurs primarily during organogenesis, and is characterized by the sprouting of new vessels from the established cords into previously avascular organ rudiments [1, 2]. Soluble angiogenesis factor or factors deposited in the extracellular matrix may act to stimulate and direct this neovascularization.

Angiogenesis is also a significant component in a multitude of pathologic states. Solid neoplastic growth requires angiogenesis to provide the oxygen and nutrients to sustain the rapid growth of a tumor [3]. A number of factors associated with, or isolated from tumors, including basic fibroblast growth factor (bFGF) [4, 5], tumor necrosis factor-α (TNF-α), vascular permeability factor (VPF) [6], prostaglandins of the E series (PGE) [7], endothelial cell stimulating angiogenesis factor (ESAF) [8] and angiogenin [9] could act as the stimuli and/or regulators for tumor vascularization.

Examples of physiologic angiogenesis occur during pregnancy and wound healing. Early in pregnancy, the corpus luteum produces angiogenesis factors which induces its own vascularization [10, 11]. In the later stages, the maternal placenta elaborates angiogenesis factors implicated in the modulation of placental vascularization [12–15].

The formation of capillaries is also crucial for the successful healing of wounds. The angiogenesis which accompanies wound repair provides for the nutritive and oxygen requirements of the new tissue and plays an equally important role in removing the waste products of metabolism. When angiogenesis is impaired due to disease, age, irradiation, or steroid treatment, wound healing is adversely affected. Macrophages, neutrophils,

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platelets, fibroblasts, endothelial cells and smooth muscle cells within the wound environment are capable of releasing a variety of growth factors that have been implicated in the control of angiogenesis [16]. Although each isolated factor has influenced endothelial cells in vitro, neovascularization in vivo, or some facet of angiogenesis in a variety of assays, their exact role in the regulation of wound healing angiogenesis remains to be determined.

The goal of our laboratory has been to explore the role of those factors derived from the platelet α-granule in wound healing angiogenesis. Specifically, we have studied the effects of platelet α-granule releasate on the chronology and morphology of wound healing angiogenesis, and the mechanisms of transforming growth factor-β (TGF-β)- and platelet-derived growth factor-BB (PDGF-BB)-induced angiogenesis.

The Morphology of Angiogenesis

The morphology of new capillary growth has been described in detail. Autoradiographic and scanning electron microscopy studies demonstrate that new capillary growth is initiated with endothelial cell movement [17–19]. First, the venular capillary endothelial cell produces proteases to dissolve the basement membrane. A new capillary sprout is drawn away from the parent capillary or venule and forms a bud. The capillary endothelial cell then migrates toward the stimulus. The bud becomes a capillary sprout, and the trailing capillary endothelial cells are stimulated to proliferate in order to elongate the new sprout. This process continues, forming a capillary loop, and a capillary network which eventually is remodeled into a mature capillary bed. As they mature, they produce a new basement membrane and are surrounded by pericytes. In short, angiogenesis is characterized by at least five processes; (1) endothelial cell chemotaxis, (2) endothelial cell proliferation, (3) endothelial cell protease production, (4) the formation of endothelial cell tubes, and (5) production of a new basement membrane. The chemotactic process provides the initial signal for capillary growth and also the direction of growth, protease production allows the migrating cell to dissolve its basement membrane and connective tissue matrix, cell division provides the additional cellular mass, and tube formation represents endothelial cell differentiation.

Although the sequence of events in angiogenesis has been described, the regulation of these events is unclear. Since angiogenesis plays such a pivotal role in many biologic processes, understanding the biochemical regulators of the process has received a wealth of experimental attention. A vast number of studies have dealt with "angiogenesis" factors, but the answers they have provided about the biologic regulation of angiogenesis are confusing.

A series of experiments was performed to examine the effect of platelet growth factors on the chronology and morphology of the angiogenic response during wound repair [20]. Collagen sponges (1 cm diameter) were saturated

with either 0.9% NaCl or an α-granule releasate from 5×10^9 platelets/ml, known as platelet-derived wound healing formula (PDWHF) [21]. The sponges were subsequently implanted subcutaneously in the hind limbs of rats, superficial to the gluteus maximus muscle. The surrounding tissue treats the sponge as a wound space and attempts to fill it with granulation tissue, of which capillaries are a component. At time intervals ranging from 6 h to 14 days the vasculature of the hind limbs was perfused with Mercox polymer via the abdominal aorta. Once the polymer hardened, the tissue surrounding and subjacent to the sponge was digested away with alternating emersions in KOH and distilled water. The resulting casts of the blood vessel lumina were sputter-coated with gold and viewed with the scanning electron microscope at 8–20 kV.

In the vessels beneath the sponges treated with saline, hemispherical depressions representing the marginating sites of leukocytes were apparent in the replicas of venules by 6 h after the implantation. The number of marginating leukocytes increased through 24 h. Vessel sprouts were visible in the same venules by 48 h and were the predominant structure between 72 and 96 h. Initially the sprouts were singular, with second order divisions occurring in the later time intervals. Between 7 and 14 days, a network of capillaries formed in and around the sponge, presumably by end-to-end, side-to-side, and side-to-end anastomoses. This process of wound healing angiogenesis was accelerated and augmented by treating the sponges with PDWHF. Maximum margination of leukocytes was observed at 6 h instead of 24 h after surgery. The first vascular sprouts were seen at 24 h, with their numbers peaking at the 48 h time point. Between 7 and 14 days, the number of vessels forming in response to PDWHF dramatically surpassed that stimulated by saline alone. Thus, it appears that PDWHF accelerated the time course of normal wound healing angiogenesis and also enhanced the magnitude of the angiogenic response.

TGF-β-Stimulated Angiogenesis

Transforming growth factor-β is a known component of PDWHF and has been reported by other investigators to stimulate angiogenesis [22]. To confirm the angiogenic capacity of TGF-β we incorporated the protein into Hydron, a slow release polymer, and examined its angiogenic activity in the rabbit corneal assay [23]. The rabbit cornea is normally avascular and provides an excellent model for visualizing blood vessel growth stimulated by an implanted growth factor. The angiogenesis can be graded visually on a 0–4 scale, with +1 representing only slight blood vessel growth from the blood vessels at the corneal-scleral limbus toward the implant, +2 representing vessel growth one half the distance to the implant, +3 representing capillaries extending to the proximal base of the implant, and +4 representing vascular growth out to and surrounding the implanted factor. TGF-β

Table 1. TGFβ-induced angiogenesis in the rabbit corneal assay

Test substance	Positive Tests/total	Average test score
Control (BSA/HCl)	0/8	0
0.01 ng/implant TGF-β	0/6	0
0.1 ng/implant TGF-β	0/6	0
1.0 ng/implant TGF-β	1/8	1
10 ng/implant TGF-β	7/8	3
50 ng/implant TGF-β	8/8	4
100 ng/implant TGF-β	4/4	4

Corneas were graded on 0–4 scale and scores were determined on day 7. Bovine serum albumin in 4 m*M* HCl (the carrier for the TGF-β) served as the negative control. All corneas which exhibited angiogenesis showed dose-dependent inflammatory responses on day 2 as determined histologically.

elicited angiogenesis in a dose-dependent manner, with +4 capillary formation observed at the 50 and 100 ng doses (Table 1). However, TGF-β induced corneal opacification between 2 and 5 days post implantation, with this opacification disappearing by day 7. Routine H&E histological analysis demonstrated the opacification to be an influx of inflammatory cells. Nonspecific esterase staining of cryostat sections suggested that many of these cells were macrophages. Transmission electron microscopy revealed that the majority of the inflammatory cells at 2 days were neutrophils. By 5 days the neutrophils were gone, and macrophages were the predominant inflammatory cell. Scanning electron microscopy of vascular corrosion casts showed that these inflammatory cells reached the corneal stroma via the limbal venules.

Seven days after implantation, a large number of capillaries were observed between the vessels at the corneal-scleral limbus and the implant. Histological examination showed that the new capillaries had grown out to and around the implanted TGF-β and that the inflammation had subsided. Transmission electron microscopy demonstrated "typical" capillaries within the corneal stroma between the collagenous laminae [24]. Scanning electron microscopic evaluation of vascular corrosion casts showed capillaries extending only from the venules at the limbus, forming an extensive network conforming to the curvature of the cornea.

These studies suggested that TGF-β-induced angiogenesis was indirect in nature. That is, TGF-β recruited inflammatory cells which in turn produced factors which acted on the endothelial cells causing them to form capillaries. To determine if this indeed was true, the following experiments were carried out [25]. In experimental group 1, 50 ngTGF-β was implanted in the cornea. In group 2, the implantation of TGF-β was immediately followed by subconjunctival injection of 16 mg methylprednisolone acetate (MPA) to supress

the inflammation. In group 3, TGF-β was implanted, but the MPA injections were delayed 2 days to allow the inflammation to occur. Corneas were analyzed on days 2 and 7. All of the corneas from group 1 exhibited inflammation followed by intense angiogenesis. All of the corneas in group 2 lacked an inflammatory reaction, and no angiogenesis occurred. In group 3, after the inflammatory infiltration was allowed, blood vessel formation followed even in the presence of the MPA.

These experiments showed that inflammation was a required component of TGF-β-induced angiogenesis. Since in vitro studies of wound-derived endothelial cell chemotaxis were negative and TGF-β was found to inhibit wound-derived endothelial cell proliferation, we concluded that TGF-β is an indirect angiogenesis factor.

PDGF-BB Stimulated Angiogenesis

Studies were undertaken to investigate the role of PDGF-BB in wound healing angiogenesis. The results demonstrate the ability of PDGF-BB to induce a chemotactic response in wound-derived capillary endothelial cells and the presence of B-type receptors on these cells. In vivo studies of angiogenesis demonstrate that PDGF-BB induces neovascularization which appears to be direct angiogenesis at low doses (100 ng or less) but is associated with an inflammatory response at higher doses.

Rabbit wound capillary endothelial cells (RWCECs) were isolated from sponge implants undergoing active wound healing angiogenesis. To confirm their identity, the RWCECs were examined for two endothelial cell markers. Uptake of fluorescently labeled acetylated-low density lipoprotein was performed on all endothelial cell cultures used in these studies. In addition, RWCECs were positive when stained for the presence of immunoreactive angiotensin converting enzyme.

The ability of the three isoforms of PDGF to induce chemotaxis in RWCECs was examined and the data shown in Fig. 1 demonstrates that PDGF-BB causes a dose-dependent chemotactic response in these wound-derived endothelial cells. PDGF-AB has minimal activity and PDGF-AA had no activity for RWCECs. The results of checkerboard studies indicated that the migratory effect of PDGF-BB is predominantly chemotactic with a chemokinetic component. None of the three PDGF isoforms were able to induce mitogenesis or proliferation of RWCEC.

Scatchard analysis of ^{125}I-PDGF-BB binding to RWCEC demonstrated the presence of 35 000–45 000 receptors per cell and a K_d value of 0.1–0.3 nM. These values are comparable to that typically found on fibroblasts and other cells responsive to PDGF-BB. When the various forms of PDGF were tested for their ability to compete with ^{125}I-PDGF-BB for the cell surface receptor on RWCECs, PDGF-AA was unable to compete for ^{125}I-PDGF-BB binding sites, while PDGF-AB and PDGF-BB were able to displace the

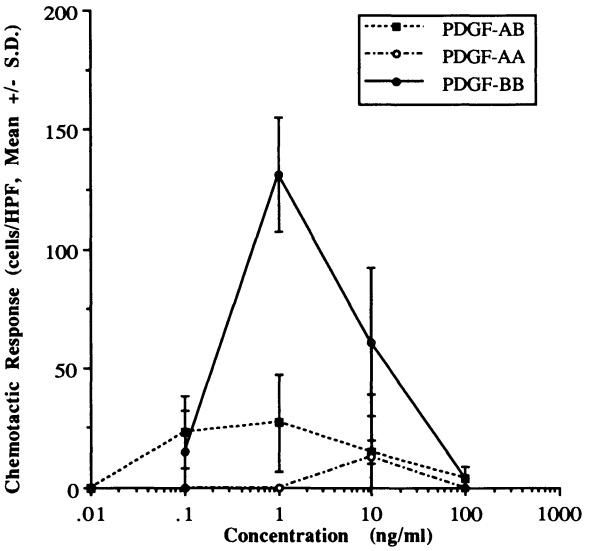

Fig. 1. Effect of various concentrations of PDGF-AB, AA, and BB on rabbit wound capillary endothelial cell (RWCEC) chemotaxis. Doses from 0.01–100 ng/ml were tested. Migrating cells were enumerated microscopically after staining with Diff-Quik

Table 2. PDGF-BB-induced angiogenesis in the rabbit corneal assay

Material	Number of positive corneas/total	Average score	Inflammation	
			Day 2	Day 7
PBS/HSA	0/8	0	–	–
PDGF-BB				
10 ng/implant	1/4	0.25	–	–
50 ng/implant	4/6	1.5	–	–
100 ng/implant	5/6	2.5	–	–
250 ng/implant	2/2	2.5	+	–
500 ng/implant	6/6	3.8	++	+

Corneas were graded on 0–4 scale and scores were determined on day 7. Human serum albumin (HSA) in phosphate-buffered saline (PBS, the carrier for the PDGF-BB) served as the negative control. Inflammation at days 2 and 7 was determined histologically.

radiolabeled ligand. These results confirm the presence of the PDGF-BB receptors on RWCECs.

Various concentrations of PDGF-BB were tested in the rabbit corneal assay of angiogenesis. PDGF-BB was implanted into the cornea and examined daily for 7 days. On days 2 and 7, representative corneas were removed and examined histologically for the presence of inflammation.

The results of these studies are summarized in Table 2. PDGF-BB induced a dose-dependent angiogenic response which appeared to be noninflammatory at doses of 50 and 100 ng/implant. Doses of 250 and 500 ng/implant induced significant inflammation as determined histologically at 2 days. These results demonstrate that PDGF-BB is a direct angiogenesis factor at low doses since it produces new capillary formation in the absence of any inflammatory reaction and has a direct chemotactic effect on wound-derived capillary endothelial cells. At higher doses PDGF-BB induces significant inflammation which is followed by intense angiogenesis.

Discussion

The regulation of angiogenesis is a critically important, fundamental biologic component of many normal and pathologic processes. The five main events in the process of angiogenesis have been studied in vitro and described in detail, and the morphology and chronology of angiogenesis is being examined with transmission and scanning electron microscopy, but confusion arises when the growth factor control of angiogenesis is evaluated. Many factors have been labeled as angiogenic on the basis of one in vivo or in vitro assay. Is it possible that all of the these factors directly stimulate the production of new capillaries? If so, should they all carry the label "angiogenesis factor?" If a factor, such as TGF-β, stimulates inflammation followed by angiogenesis in an in vivo assay, is it a proinflammatory factor or an angiogenesis factor? If a factor stimulates proliferation of a large vessel lining endothelial cell, is it an aortic endothelial mitogen or an angiogenesis factor?

As the regulation of angiogenesis by growth factors continues to attract significant research attention, a standardization of nomenclature and in vivo and in vitro assays will certainly assist in the interpretation of results and the understanding of capillary formation. Identical in vivo assay procedures with careful monitoring of the assay for inflammation, and chronology and morphology of new capillary formation, should be employed to determine whether a factor has in vivo angiogenic activity, and if it does, whether the activity is direct or indirect. This is important clinically because the inflammation associated with indirect angiogenesis could be accompanied by detrimental protease production causing tissue destruction or excessive fibrosis in addition to new capillary formation. Furthermore, the location of the endothelial cell type affected and the chronology and initiation of proliferation, migration and/or basement membrane destruction by protease production should be determined. Standardized in vitro assays using a variety of different endothelial cells would determine the presence of any heterogeneity in endothelial cell response to a given growth factor. When combined with in vivo histologic and electron microscopic morphologic data, the potential role of the specific factor in the overall biology of angiogenesis would start to emerge.

The orderly analysis of the results from comparative in vivo and in vitro assays would result in a comprehensive matrix of angiogenesis activities and begin to elucidate the mechanism by which many different factors in concert, or as a progressive cascade, stimulate and regulate this fundamentally important biologic process. These data would also significantly affect future applied basic and clinical scientific investigation by directing attention toward a single factor or defining a cellular and/or biochemical cascade.

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Blood Flow in Ulcerated Tissue and Surrounding Skin: Healing Correlates with Return of the Venoarteriolar Reflex

D.W. Pryce[1], P.S. Friedmann[1], R. Walmsley[2], and M.G. Rippon[2]

Introduction

Assessment of leg ulcer healing is usually made by measuring the area of ulceration. Measurement of blood flow by laser Doppler flowmetry may be a more physiological assessment of wound healing. Blood flow in skin can be quantified with a laser Doppler flow meter. This instrument measures the Doppler shift in wavelength produced when laser light is scattered by moving red blood cells within the skin and computes an output known as "flux", in arbitrary units, which is derived from the product of the concentration of red cells (within a hemisphere of tissue of 1 mm radius beneath the probe head) and their average velocity [1]. When the probe is attached to the lower leg the flux value is highest in the supine position and falls on standing upright. This effect is known as the venoarteriolar reflex [2]. We have reported recently that this reflex is attenuated in the base of venous ulcers when compared with adjacent nonulcerated skin [3]. The venoarteriolar reflex is the arteriolar constriction that occurs in the skin of the lower limb on standing upright. It is postulated that it acts via an axon reflex and has the effect of protecting dermal capillaries from the increasesd hydrostatic pressure associated with the upright posture. The receptor site for the afferent side of the reflex may be located in small veins while the efferent limb acts on the smooth muscle of arterioles [4]. We wished to see whether healing is associated with changes in either resting blood flow (expressed as flux) or the venoarteriolar reflex in the ulcerated area.

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Methods

Patients

There were 15 patients with a total of 20 small (<3 cm in greatest diameter) venous ulcers who were recruited for the study from the leg ulcer clinic in our dermatology department. The state of the arterial supply to the lower leg was assessed, in the supine position, by measuring the systolic blood pressure in the arm and ankle with the aid of a Doppler ultrasound probe. The ratio was calculated ("ankle/brachial systolic pressure ratio") and patients with values <1 were excluded.

Blood Flow Measurement

We used a laser Doppler flowmeter Perimed PF3 with a Perimed integrating probe. The integrating probe consists of seven efferent fibres and 14 afferent fibres (two close to each efferent fibre). The instrument processes an integrated signal which greatly reduces variability due to non-homogeneity of local microvasculature. The laser Doppler flowmeter and probe were calibrated according to the manufacturers instructions.

Blood flow (flux) readings were taken from nonulcerated skin over the lower leg and from directly over the ulcer. The laser probe holder was attached to the skin or ulcer base using double-sided adhesive tape. Each subject was required to lie supine for at least 15 min before the study commenced. All measurements were made at the same time of day (10.00 am–12.30 am) and at a room temperature maintained between 21° and 24°C. On each subject three sites on nonulcerated skin and one or two sites on the ulcer were studied. At each site a 3 min recording was made onto a chart recorder and the mean value obtained. After measurements had been taken in the lying position the subject was asked to stand for 3 min. The procedure was then repeated after this interval with the subject standing upright. The entire procedure was repeated at intervals of 4 weeks until the ulcer had healed completely. The resting blood flow (flux) and the postural drop for each month, up to and including the month in which complete healing occurred, were analysed.

The venoarteriolar reflex at each site was measured as the drop in blood flow (flux) on standing expressed as a percentage of value in the lying position:

$$\text{Venoarteriolar reflex} = (\text{flux lying} - \text{flux standing})/\text{flux lying} \times 100$$

During the study all the ulcers were dressed in an identical manner with a layered bandage comprising a hydrocolloid pad, a layer of padding extending from the proximal end of the toes to the knee and an elasticated bandage also extending from toes to knee. The dressings were changed twice weekly by the same experienced nursing sister.

Statistics

Paired data were analysed using the Wilcoxon signed rank test and unpaired data were analysed using the Mann-Whitney *U* test.

Results

Of the 20 ulcers studied five had healed after 1 month, 11 after 2 months, 12 after 3 months, 13 after 4 months, 14 after 5 months and 17 after 6 months. At the time of writing one had failed to heal completely after 18 months and two patients (two ulcers) have been lost to follow-up. When the 17 ulcers were healed we were able to look back at the results obtained in the preceeding months to see how the blood flow and the venoarteriolar reflex had changed.

Data were available from 17 ulcers in the month when they were fully healed, 14 ulcers 1 month prior to healing, 11 ulcers 2 months prior to healing, 6 ulcers 3 months prior to healing, five ulcers 4 months prior to healing, four ulcers 5 months prior to healing and three ulcers 6 months prior to healing.

Throughout the observation period the blood flow (flux), in the lying position, in the bases of the ulcers was significantly greater than in adjacent nonulcerated skin ($p < 0.01$) and there was no significant trend as healing progressed (Fig. 1).

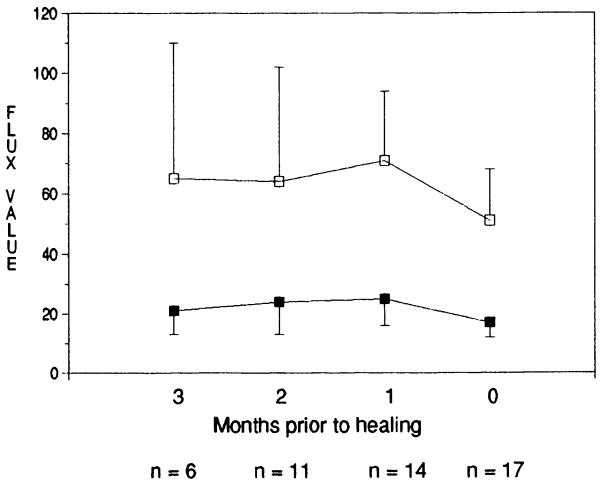

Fig. 1. Blood flow (flux) in the lying position in ulcer bases (*open squares*) and in adjacent nonulcerated skin (*filled squares*) when fully healed and 1, 2 and 3 months prior to complete healing. Mean values (arbitrary units) ±95% confidence intervals. Reprinted with permission from Pryce DW et al. Hemodynamics of leg ulceration assessed by laser doppler flowmetry. J Am Acad Dermatol 29(5):708–714

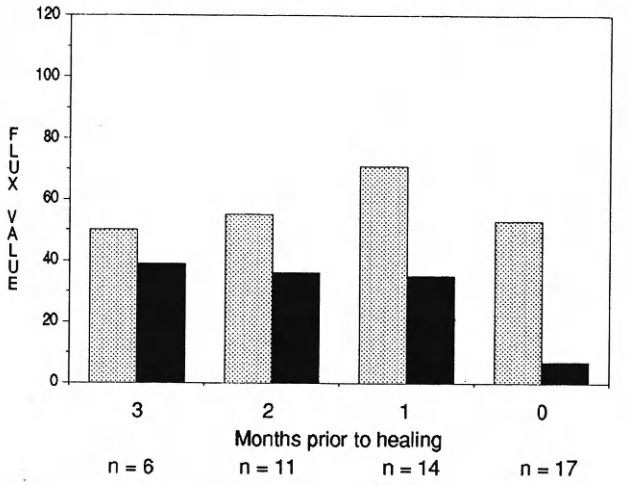

Fig. 2. Blood flow (flux) from the ulcer bases when fully healed and 1, 2 and 3 months prior to complete healing. *Grey bars*, lying position; *black bars*, standing position; Mean values (arbitrary units). Reprinted with permission from Pryce DW et al. Hemodynamics of leg ulceration assessed by laser doppler flowmetry. J Am Acad Dermatol 29(5):708–714

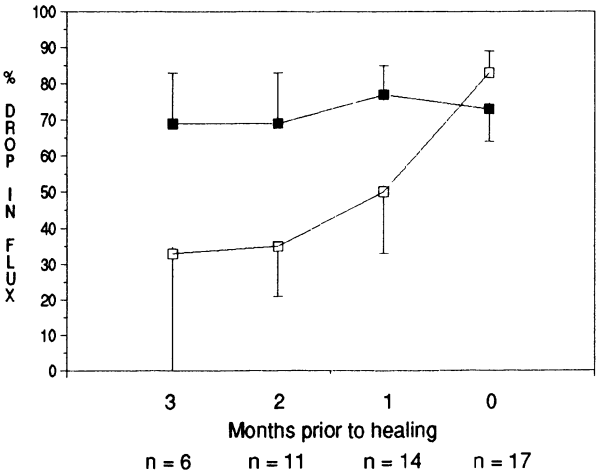

Fig. 3. The postural drop in blood flow (flux) in the ulcer bases (*open squares*) and in adjacent nonulcerated skin (*filled squares*) when fully healed and 1, 2 and 3 months prior to complete healing. Mean values + ±95% confidence intervals. Reprinted with permission from Pryce DW et al. Hemodynamics of leg ulceration assessed by laser doppler flowmetry. J Am Acad Dermatol 29(5):708–714

Analysis of the postural drop in blood flow (flux) in the bases of the ulcers did reveal a trend as healing progressed (Fig. 2). The difference between the blood flow (flux) in the lying and standing positions increased.

This is represented (Fig. 3) as a positive correlation between the postural drop in blood flow (flux) in the bases of the ulcers and healing. The postural drop increased from 35% (95% CI 21–49) 2 months prior to complete healing to 83% (95% CI 77–90) when fully healed ($p < 0.01$). In contrast the postural drop in adjacent nonulcerated skin remained at approximately 70%.

Discussion

Resting blood flow in the bases of small venous ulcers, as assessed by laser Doppler flowmetry, did not change as healing progressed but we have shown a correlation between healing and the return of the venoarteriolar reflex. The venoarteriolar reflex has not been studied previously in relation to wound healing.

As angiogenesis proceeds perhaps new vessels become innervated and respond to the axon reflex that is considered to be the basis of the venoarteriolar reflex. This is not to say that vascular innervation is necessary for ulcer healing, as the return of the venoarteriolar reflex could be an effect of ulcer healing rather than a cause. An alternative explanation might be that the reflex is related to the depth of the ulcer and therefore to the type of blood vessel present.

Whether the reflex is related to the presence of innervated blood vessels or to the anatomical depth of the ulcer, the associaton between the venoarteriolar reflex and healing may be useful. Venous leg ulcers heal slowly and it is sometimes difficult to know from month to month whether there has been any objective improvement or whether any change of treatment has improved the chances of healing. Measurement of the venoarteriolar reflex can be used to monitor the healing of leg ulcers because the reappearance of the reflex in an ulcer base indicates that healing is progressing.

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Three-Dimensional Reconstruction of Terminal Blood Spaces in the Proximal Tibia Metaphysis of the Growing Rat – A Model to Study Normal Angiogenesis

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Introduction

Since Albrecht von Haller's (1763) suggestion that the vascular system is responsible for osteogenesis, anatomists have tried to elucidate the vascular pattern in the metaphysis of the growing bone. Our primary interest was the development of capillary sprouts and their conversion into new capillary loops. Since the tibia grows unidirectionally, one would expect that its capillaries follow a similar pattern. In order to study all vessel-like structures, including sprouts without an active hemoperfusion, we used serial sectioning instead of corrosion cast techniques. To understand the growth and hemoperfusional pattern it is important to visualize both cartilaginous cavities and intertrabecular spaces (ITS), and the capillaries and capillary sprouts.

Vessel growth in the growing bone is a particularly interesting model to study normal angiogenesis. In contrast to wound healing, capillary growth and architecture follows a structured pattern depending on the location of the vessels in relation to the epiphysis.

Material and Methods

The reconstruction is based on 4-μm sections of a plastic embedded proximal tibia metaphysis of a growing 23-day-old Wistar rat. The tibia was cut perpendicularly to the longitudinal axis. All sections were stained with cresylviolet. Approaching from the epiphysis, we started our reconstruction at the level of calcifying cartilage. Shortly below this plane we observed the first extravasations preceeding the capillary sprouts to come. We chose a region which showed such extravasations only at one spot. By doing so we had the opportunity to observe and to reconstruct the vascularization of an area as a whole. In total 35 successive sections were photographed and

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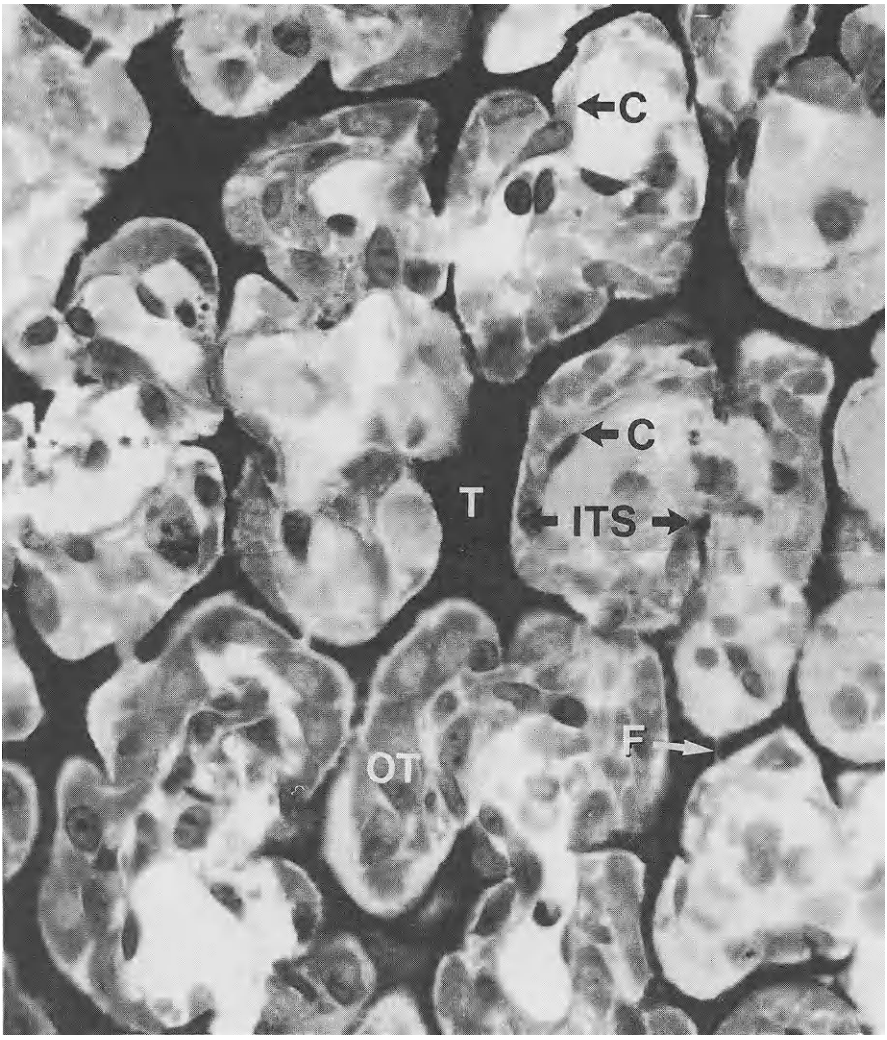

Fig. 1a,b. Terminal blood spaces in the proximal tibia metaphysis of the growing rat. *T*, trabecula; *ITS*, intertrabecular space; *C*, capillary wall; *OT*, osteogenous tissue. **a** Light microscopic section at the level of the bulbous dilation (*arrow* in Fig. 3a). Some intertrabecular spaces communicate through fissures (*F*). **b** A few sections further we were able to observe an erythrocyte passing through a fissure

enlarged to a final magnification of 1080. In a second step the paper prints were manually preadapted for maximum congruency within the section pile, allowing two centering points to be passed through all sections.

Structures of interest were redrawn on tracing paper and named by a system of indices. Special attention was given to structure branching and

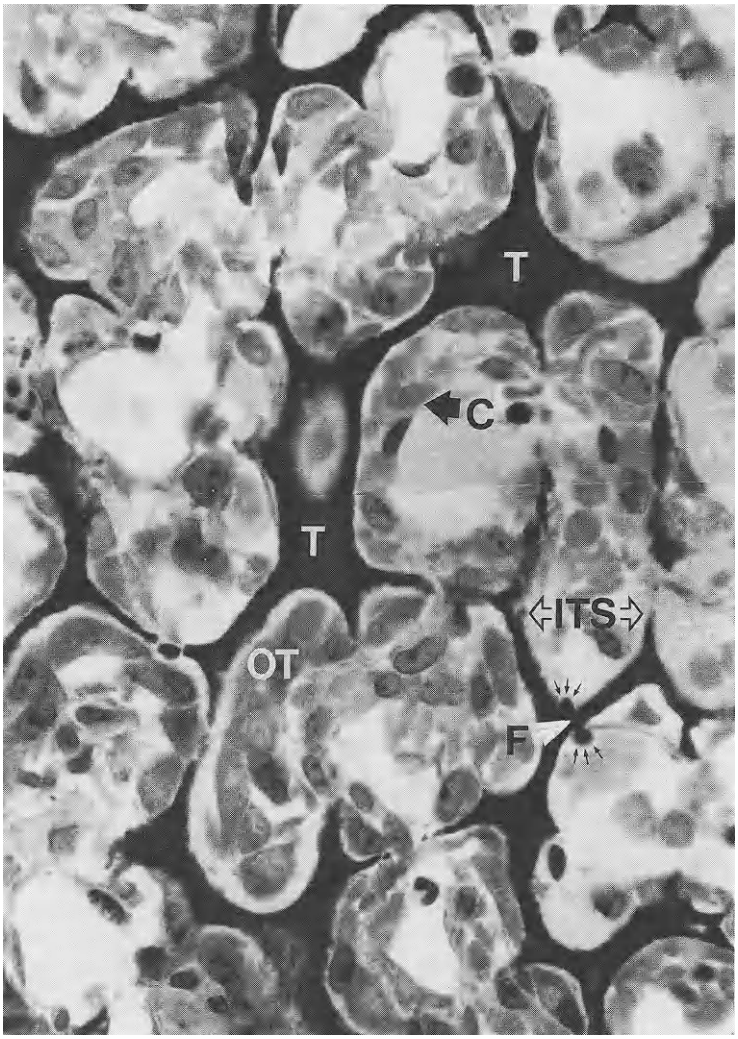

Fig. 1b

fusion. Using a digitizing tablet, the structures were entered as a stream of points (X/Y coordinates) into an Atari Mega 2 Computer (Atari Corp., Sunnyvale, California, USA). The program ANAT3D (el Gammal 1987) is written in 68000 Assembler (Digital Research, USA) and embedded in a Pascal shell (Pascal ST+, CCD and OSS, Eltville, FRG) supporting GEM (Digital Research, USA).

The sections were adapted interactively within the section pile by matching the centering points. For this purpose, every section was rotated and translated relative to its preceeding section on a monitor screen. For the

final reconstruction all structures of interest within the structure list and all sections necessary within the section pile can be chosen. For every structure the following reconstruction modes are available: shaded surface reconstruction, wire frame model (shaded or plain), shaded contour lines, and point cloud. The reconstruction is moved/rotated/scaled in real time while the user observes the three-dimensional red–green stereo pictures or color pictures in line mode. Once the optimal view is found, the objects are redrawn as realistically illuminated hidden surface models. If there is superposition, the foreground structures can be made partly transparent by changing their presentation mode so that foreground and background are seen at the same time. By suppressing one or more sections, tubular structures appear to be partly discontinous and it is possible to see the inner and outer surface simultaneously.

Results

The inner lining of a cartilaginous cavity or intertrabecular space (ITS) exhibits capillary walls (Fig. 1). Sometimes the trabeculae (T) separate the intertrabecular spaces or these spaces and a cartilaginous cavity incompletely, thus forming fissures (F) in their lining. All these structures were entered section by section into the computer (Fig. 2). Figure 3 shows that the upper part of the neighboring capillary complex (NC) is in close contact with the capillary C3. The trabecula separating both intertrabecular spaces belonging to these capillaries shows such fissures, which allow a substantial noncapillary mediated transport between both sides.

The fissures are represented as small crosses lying at the trabecular lining (Fig. 2), which turn into diamonds during surface reconstruction (Fig. 4). Some fissures were up to 90 μm long and formed a long opening with a narrow, but changing gap. It must be postulated that a plasma exchange takes place through them, as we found blood cells within these gaps (Fig. 1b).

The branching of capillaries is complex (Fig. 3a) and originates from a large bulbous dilation (BD). Four capillaries point towards the diaphysis (C4–C7) and three towards the epiphysis (C1–C3). They show marked differences in diameter. Two of the three capillaries heading for the epiphysis are very short (C1 + C2). Both have just penetrated the cartilaginous capsule by vertical arrosion and both sprouts finish at the upper limitation of the cartilaginous cavity, which they have just arroded. These sprouts are supplied by the bulbous dilation (BD) through a wide oval opening. The third capillary (C3) is much larger in diameter and extends about 60 μm towards the epiphysis. At its very top the diameter has diminished to a size comparable to the two other sprouts mentioned before (Fig. 3a). Similarly, the sprout C3 is about to enter a new cartilaginous cavity. The confining capsule (K, Fig. 4) of this cavity, which is represented in line mode, shows

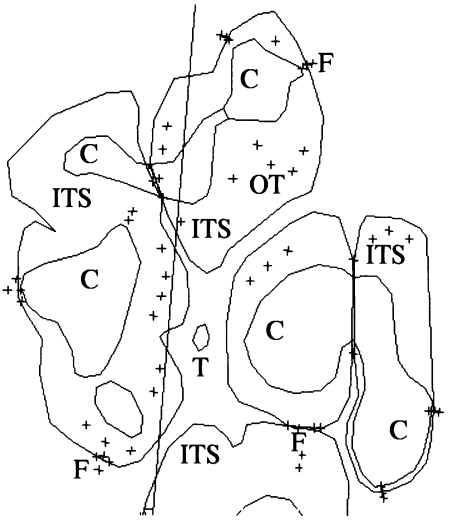

Fig. 2. A single section with all structures entered into the computer. The location of cell nuclei and fissures (*F*) is marked by *crosses*. *ITS*, intertrabecular space; *OT*, osteogenous tissue; *C*, capillary wall

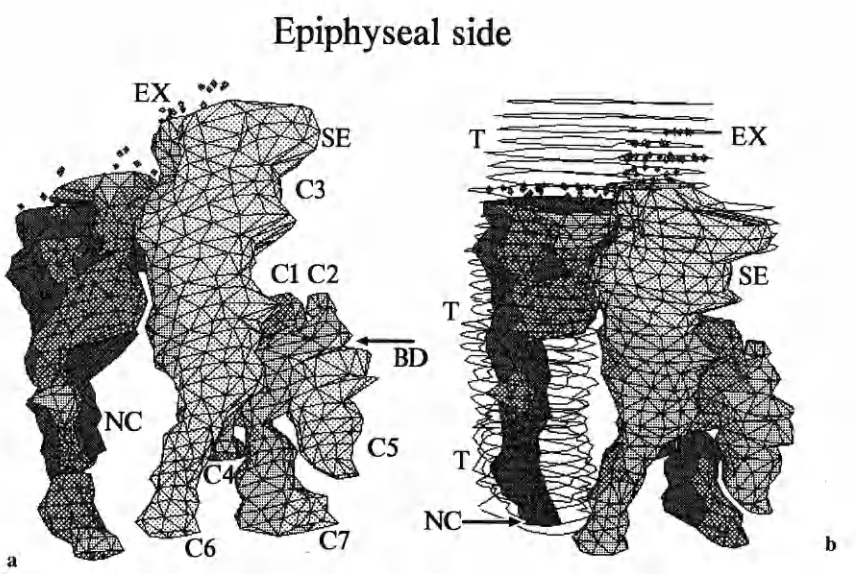

Fig. 3a,b. Surface reconstruction of the terminal blood spaces in the proximal tibia of the growing rat. *BD*, bulbous dilation; *SE*, sinusoidal enlargement; *NC*, neighboring capillary complex; *EX*, extravasates. Some capillaries point towards the epiphysis (*C1–C3*), some towards the diaphysis (*C4–C7*). a The capillary complex (*NC*) is in close contact with C3 through a long fissure. **b** The shape of the trabecular lining (*T*) is almost cylindrical, whereas its capillaries taper towards the diaphysis, providing increasing space for osteogenous tissue

the extravasates which, at this stage of development, penetrated the cavity preceeding the capillary sprouts to come. As has been reported by several authors (Clark and Clark 1940; Anderson et al. 1966; Schoefl 1963; Truetter 1963; Schenk et al. 1967), such extravasations are caused by discontinuities of the endothelial lining.

Shortly beneath the peak we can observe a sinusoidal enlargement (Figs. 3, 4) of the capillary, directly below the junction of its intertrabecular space and three cartilaginous cavities. This junction actually represents a transititonal zone between the wide intertrabecular space and the narrow, partly arroded cavities. After arrosion and resorption, these cavities may become part of a new intertrabecular space.

A similar situation must have existed at an earlier time in the bulbous dilation beneath (Figs. 3a, 5). One sprout grew further towards the epiphysis (C3). Another sprout penetrated the separating lateral trabecular bar by transversal arrosion, entered the neighboring intertrabecular space, and fused with an adjacent sprout, thereby forming a new capillary loop (C6 + C7). From the four capillaries of the bulbous dilation (BD) heading for the diaphysis, only two (C6, C7) are seen in all sections below the branching. Gaining distance from the dilation, the capillaries of the loop become smaller in diameter. In contrast to this observation, the trabeculae (T) run almost perpendicularly (Figs. 3b, 4). The increasing space between both is filled by a growing amount of osteogenous tissue in the perivascular space.

Sometimes small connections between different intertrabecular spaces or cartilaginous cavities develop into wide gaps, through which even capillaries may pass, giving rise to syphon-like structures. In Figs. 4 and 6, a capillary (C6) leaves its original trabecular housing to continue into another intertrabecular space.

Discussion

Although there have heen many attempts to visualize the development of capillaries in the growth plate of the proximal metaphysis, details of the three-dimensional architecture of the capillaries and their interaction are still unknown.

Using computer-aided three-dimensional reconstructions we were able to study this network in its natural environment. In contrast to the corrosion casts, even unperfused long sprouts could be visualized.

The cartilaginous portion of the growth plate is divided into various zones according to function and morphology. Moving from the epiphysis towards the metaphysis, three zones are distinguished: the resting ("germinal") zone, the proliferative zone, and the hypertrophic zone. The last zone is often subdivided into the zone of maturation, the zone of degeneration, and the zone of provisional calcification (Brighton 1987).

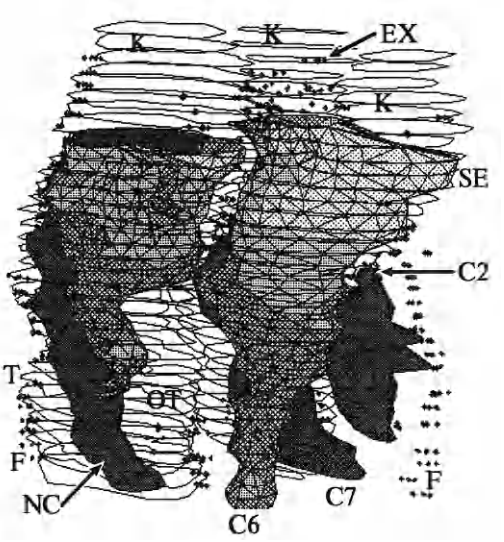

Fig. 4. Surface reconstruction of the terminal blood spaces in the proximal tibia metaphysis of the growing rat. *F*, fissures in the trabecular lining; *T*, trabecular lining; *OT*, osteogenous tissue; *SE*, sinusoidal enlargement of *C3*; *C2*, capillary sprout just having arroded a cartilaginous cavitity; *C6*, capillary leaving its original housing towards the fore plane; *K*, cartilaginous cavities in the zone of provisional calcification; *EX*, extravasates preceeding the capillary sprouts to come

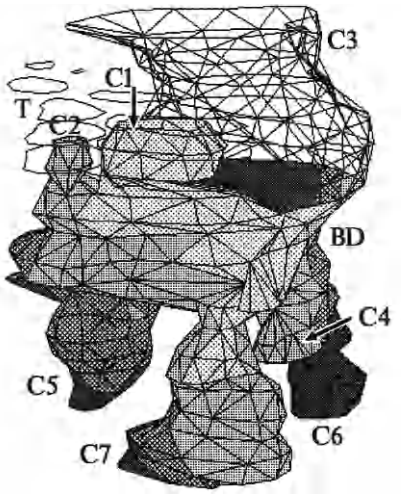

Fig. 5. Surface reconstruction (back plane) of the terminal blood spaces in the proximal tibia metaphysis of the growing rat showing the bulbous dilation with branching capillaries (*C1–C7*). *C6* and *C7* form a capillary loop. The capillary *C3* (wire frame mode) ends in a sinusoidal enlargement, which was omitted in this picture. *BD*, bulbous dilation; *T* trabecular lining

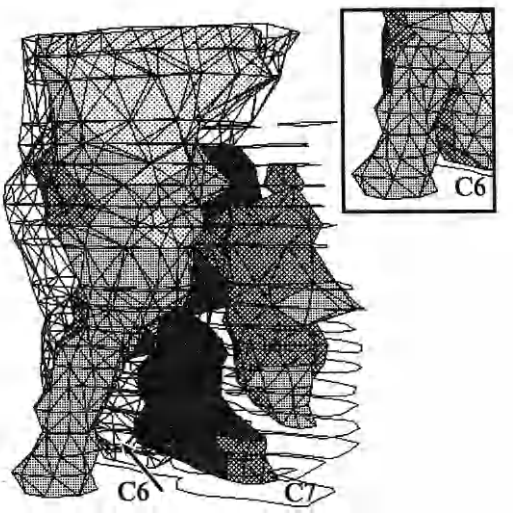

Fig. 6. Surface reconstruction of the terminal blood spaces in the proximal tibia metaphysis of the growing rat. *C6* leaves its original trabecular housing (represented in wire mode) to continue in another trabecular cavity. Refer also to the *inlet* (surface mode)

In the metaphysis, terminal branches of the nutrient and metaphyseal arteries pass vertically towards the bone–cartilage junction of the growth plate and end in vascular loops or sprouts just below the last intact transverse septum at the base of the cartilage portion of the plate. At this level the vessels turn back and fuse to form venous branches descending towards the diaphysis.

Apart from the sprouts, the loops are closed and do not penetrate the bone–cartilage junction (Brighton 1987). In the zone of provisional calcification, the longitudinal septa are calcified, whereas the last intact transverse septum is nonmineralized (Schenk et al. 1967).

In light microscopy we often observe a rouleaux formation of erythrocytes in capillary sprouts, indicating vascular stasis. We noticed that the last lacuna of the cartilaginous column is not completely empty, but contains one or several red blood cells. Figure 1b shows a red blood cell passing through a fissure in the trabecular lining.

Schenk et al. (1968) reported in an electron microscopic study that capillary sprouts lined with endothelial and perivascular cells invade the base of the cartilaginous portion of the plate. Cytoplasmic processes from these cells push into the transverse septa and, presumably through lysosomal enzyme activity, degrade and remove the nonmineralized transverse septa. Electron microscopy of ultrathin sections (Schenk et al. 1967; Schoefl 1963) indicated that in these blind endings the endothelium is partly discontinous.

We believe that the sprouts invade the former cartilaginous cavity and eventually occupy the entire cavity space. These processes are repeated as the

vascular invasion progresses. The shape of a terminal part of a sprout is therefore just a function of the shape and number of arroded cavities (SE, Figs. 3, 4).

Our reconstructions showed that those sprouts that manage to penetrate several adjacent cartilaginous cavities often have a remarkably wide base. Thus diffusion is facilitated. The irregular shape of the capillary loops causes turbulences, propagating into the bottom part of the sprouts. Both factors improve blood exchange, nutrition, and the removal of waste products. Thus, sprouts can grow to an extent which cannot be achieved solely by diffusion. The calcified longitudinal septa that separate different cartilaginous cavities at the zone of provisional calcification are of varying thickness (Anderson et al. 1966; Schenk et al. 1967). Sometimes when a sprout has arroded several cartilaginous cavities in succession, it is far ahead of the other sprouts. In such a situation, thinner bars separating an arroded cavity from an unarroded one are the predilection zone for transversal arrosion. Consequently, this sprout penetrates the septa and develops a transversal extension into the neighboring cavity. This mechanism contributes to the formation of the bulbous dilation (Figs. 3a, 5).

If the hydrostatic pressure within the two branches of the capillary loop differs, an oriented circulation of blood develops. Even if both branches originate from the same capillary loop but from different parts of it, different intraluminal pressures will be present. Active hemoperfusion will improve the nutritious situation at the front of vascularization.

This environment activates metabolic processes essential for the degradation of cartilaginous material and for the remodeling of bone. We would like to emphasize that the diameter of capillaries visualized in the reconstruction can be misleading concerning their flow rate and their relevance for the vascular supply.

Smaller capillaries with active hemoperfusion are far more efficient than wide capillary sprouts by means of diffusion. As explained above, the wide sprouts do not have an oriented circulation, and the vascular stasis will worsen the disastrous rheological situation.

Developmental physiology has taught us that, except for remodeling, once such a circulation exists, the capillary has a tendency to become well established, thus promoting further differentiation and histogenesis of the surrounding tissue. The terminal capillaries of the metaphysis form a complex three-dimensional system of communicating vessels, where capillary loops with an active hemoperfusion are the prerequisite from which sprouts discharge to extend into the surroundings (Fig. 7).

The combination of standard techniques of light or electron microscopy with computer-aided surface reconstruction and deliberate suppression of certain structures gives rise to unique, synthetic three-dimensional views of the interaction between different invading capillaries in neighboring chondrocyte capsules. The reconstruction revealed a complicated labyrinth with enlarged sinusoidal vessels towards the epiphyseal plate. This finding is

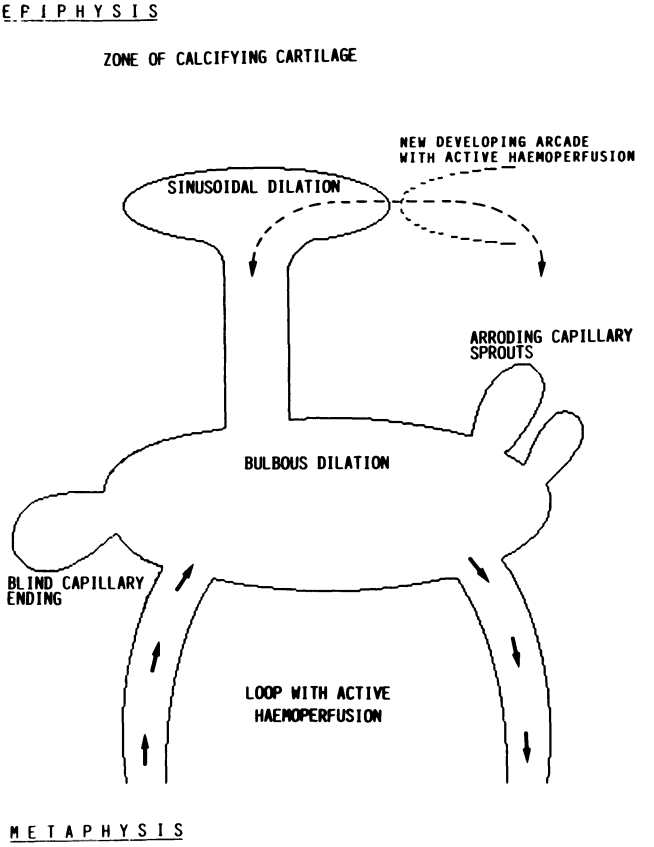

Fig. 7. New capillary arcades with active hemoperfusion develop by vertical and transverse arrosion of cartilaginous cavities

in contrast to the commonly assumed finger-like capillary sprouts or capillary loops (Dodds 1932; Urist et al. 1980) in the growing bone.

Summary

Using three-dimensional computer-reconstructions from the proximal tibia metaphysis of the growing rat, we were able to study and visualize the capillaries and their relationship to the surrounding trabeculae. This method allowed us to examine even those capillary sprouts, which were not part of a loop with active haemoperfusion.

Our results show that capillary loops have a bulbous dilation at their tip. Sprouts disengage from these bulbous dilations. Most of them grow in

vertical direction towards the epiphysis arroding the last transversal septum in the zone of provisional calcification. Later, some of them also penetrate the calcified longitudinal bar of their own intertrabecular space, thus getting in contact with a sprout from a neighbouring capillary loop. If those sprouts fuse and their hydrostatic pressure differs, this is the onset of a new capillary loop with an active hemoperfusion.

Vessel growth in the growing bone is a particularly interesting model to study normal angiogenesis. In contrast to wound healing, capillary growth and architecture follow here structured pattern.

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IV. Biochemistry and Immunology

Trace Elements in Normal and Impaired Wound Healing

T.A. Söderberg

Introduction

For several decades trace elements have been receiving wide attention from biomedical investigators. Though present only in minute amounts, they have been shown to perform biochemical functions indispensable to life or, on the contrary, to interfere with vital processes. The nutritional importance of trace elements has recently grown rapidly due to better understanding of their vital biological functions. In wound healing the most important trace element is zinc, which probably is one of the most important and interesting inorganic elements related to health sciences. In addition to zinc, copper, chromium, selenium, and manganese are essential for normal growth and development in humans.

Recent estimates indicate that most tissues of a living organism contain as many as 50–80 inorganic chemical elements. Although the bulk of living matter consists mainly of the five elements, hydrogen, nitrogen, carbon, oxygen and sulphur, most mammals cannot survive without the inorganic chemical elements called the trace elements. They were conveniently called that because they could not until recent years be quantified accurately.

Hydrogen and oxygen (water) alone constitute 60%–65% of an adult human body. Inorganic chemical elements were earlier classified as "bulk" and "trace" substances of living matter depending upon their concentrations. Between the bulk and trace elements, a third group of elements such as sodium, potassium, calcium, magnesium, chlorine and phosphorous is sometimes termed the macrominerals.

Definition

In medicine there is no exact definition of a trace element but chemically an element is usually considered as a trace element when occurring at a level of $< 0.01\%$ (i.e., $<100\mu g/g$). Iron and iodine, though appearing in these

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concentrations, are usually considered as distinct from the trace element group because their biomedical significance was established long before other elements were shown to have a biological function.

Classification

Trace elements are often classified into essential and nonessential. If depletion results in a deficiency syndrome and repletion reverses the abnormalities a trace element is considered as essential. The following trace elements are generally considered as essential: copper, zinc, cobalt, manganese, molybdenum, fluorine, silicon, vanadium, chromium, nickel, selenium, arsenic and tin. Nonessential trace elements include the other trace elements present in living matter but for which no proof of essentiality is known today. All trace elements are potentially toxic if the limits of safe exposure are exceeded and this is true even for essential trace elements.

Metabolism

It is relevant to consider the factors regulating the metabolism of the trace elements, and the most important factors are bioavailability, solubility and permeability. The bioavailability depends on the chemical form of the element and, generally speaking, salts of the elements which are easily soluble in water and in the acidic environment of the stomach are more available for absorption than the insoluble ones. Once the inorganic elements are made available for absorption, they usually form complexes with endogenous chelating agents and are transported across the gut membrane. The mucosa of the small intestine largely regulates the absorption of trace elements and once absorbed they are transported by the blood and taken up by different organs and tissues. In the blood or serum the trace elements are bound by specific proteins, albumins and globulins and a fraction is carried in the form of amino acids or small peptide complexes. The amounts of the elements in the tissue are regulated by the control of absorption in the alimentary canal. The routes of excretion are mainly urine and bile.

Effects

The association of trace elements with enzymatic catalysis and their role in oxidation-reduction and transport processes, membrane permeability, muscle contraction and the function of subcellular organelles such as mitochondria are very important. Trace elements also have a role in the synthesis and structural stabilization of both proteins and nucleic acids. Metalloenzymes contain a metal as an integrated part of their molecule in a

fixed amount per molecule of protein. The specific and unique chemical nature of the metal-protein interactions apparently confers both stability and reactivity on the molecule. Zinc is probably the most widely distributed metal among metalloenzymes, which participate in a wide variety of metabolic processes including carbohydrate, lipid, protein, and nucleic acid synthesis or degradation. Zinc is present in several dehydrogenases, aldolases, peptidases and phosphatases. Other metalloenzymes containing copper, manganese, selenium and molybdenum have been isolated. Metal-enzyme complexes compose a group of enzymes more loosely associated with metals in which the criterion of association is the activation of catalysis. During the chemical reaction the metal acts as a temporary link between the enzyme and the substrate, and besides stabilizing the enzyme-substrate complex, the metal can also stabilize the reaction products, thus facilitating the reaction. Activation of metal-enzyme complexes has been found in trace elements like copper, zinc, manganese, nickel and many others.

Trace elements may cause toxicity in excess and these effects on biological systems appear to occur at similar loci as do essential functions. Metals may hereby cause enzyme inhibition, alter membrane permeability, and impair protein synthesis or distort nucleic acid structure (Vallee and Ullmer 1972).

Zinc

Zinc is a mineral essential for normal growth of plants, animals and humans (Underwood 1977). Zinc is one of the oldest forms of therapy noted in Ebers' papyrus, 1550 BC. It was topically used by the ancient Egyptians in the form of calamine for promotion of wound healing (Failla 1977). Since then zinc compounds have been used in a large variety of powders, lotions and ointments for the topical treatment of skin and eye lesions. Zinc oxide, the most frequently used compound containing zinc, is used as a vehicle with mild astringent and local drying properties. It is a cocomponent of many formulations which are applied repeatedly to extensive areas of diseased skin in chronic disorders such as psoriasis and eczema. In the last decades the effect of zinc in treatment of various skin disorders has been subject to extensive research. For wound treatment both zinc sulphate and zinc oxide are most commonly used. Zinc sulphate is highly soluble in water (965 g/l), whereas zinc oxide is almost insoluble (0.0016 g/l). Zinc oxide is the most appropriate zinc compound for topical use as opposed to zinc sulphate which is usually given per os.

The importance of zinc as an essential trace element for humans was not fully realized until Prasad et al. (1961, 1963) described a group of Iranian dwarfs whom they suspected to be zinc deficient. The knowledge of the features of zinc deficiency in animals such as growth retardation, testicular atrophy, skin changes, and poor appetite led Prasad's group to suspect that

the dwarfism and hypogonadism present in the Iranian subjects were due to the zinc deficiency. Later studies in Egyptian men who exhibited similar symptoms helped to confirm this hypothesis and emphasized the essential role of zinc in normal human growth and development.

The total zinc amount in adults ranges between 0.8 and 2.3 g with an average of 2.0 g. An average diet provides 8–15 mg of zinc daily. Most ingested zinc is excreted in the feces (Vallee 1959; Cousins 1979; Hsu 1980). The amount excreted increases with the amount ingested. The uptake of zinc from the gut is dependent on the nutritional state of the organism, i.e., when a deficiency exists the absorption is increased and excretion reduced. On the contrary, in case of an excessive intake of zinc, the absorption is decreased and excretion increased. Urinary excretion averages less than 0.6 mg/day. Significant zincuria may occur after surgery, burns, and multiple injuries. The extent and the duration of the increase seems to relate to the severity and nature of the injury. In serum, zinc is almost entirely found in bound form: approximately 50%–60% binds with albumin, 30%–40% to an α2-macroglobulin and a small fraction to various amino acids (Henkin 1974; Hsu 1980).

In the assessment of zinc status the most commonly used method is measurement of serum or plasma zinc levels. It is assumed that with zinc depletion the circulating content will decline progressively. This is generally true but it is not always correct to imply the reversed situation; that is that a low concentration signifies zinc deficiency. There are certain states (infection or inflammatory stress) in which serum zinc is lowered not necessarily due to lower body stores but because of the redistribution of zinc from the serum to the liver. Circulating levels of zinc can also be depressed by the use of corticosteroid or oral contraceptive agents. In situations in which deficiency does exist serum concentrations will fall below the normal range of 70–110 μg/100 ml.

A significant association with venous leg ulceration and lowered plasma zinc concentration has been noted (Ågren et al. 1986). Low plasma zinc also occurs in chronic decubitus ulceration (Abbott et al. 1968), ulceration due to vascular insufficiency (Halstead and Smith 1970) and due to ulcers as a result of sickle cell anemia (Serjeant et al. 1970). Serum zinc has also been shown to be diminished in patients with leprosy with or without trophic ulceration (Oon et al. 1974).

Zinc and Wound Healing

Zinc is recognized as an essential factor in wound healing. This was suggested by Strain et al. (1953) after observing that wounded and burned rats that were maintained on diets accidentally enriched with zinc healed more rapidly than animals on control diets. Since then the need for zinc in wound healing has been confirmed in many studies.

Oral Zinc Treatment of Ulcers

A number of studies concerning the healing capacity of orally administered zinc on different kinds of wounds have been performed. As there is no standard treatment today, the choice of control treatment for clinical evaluation is arbitrary.

In 1967, Pories et al. reported that oral administration of zinc sulphate to otherwise healthy males undergoing excision of pilonidal sinuses was attended by a twofold increase in the rate of epithelialization. The 20 men were randomly assigned to control and treatment groups. Ten of them were given zinc sulphate (220 mg three times daily) and ten were controls. No placebo was given. Healing was calculated both by time and wound volume. The primary differences between zinc treated and control persons occurred during epithelialization. No great differences in healing rate were seen in the first 15–20 postoperative days. The patients given zinc supplement appeared to have wounds containing pinker, cleaner, healthier looking granulation tissue. Healing rates were lower in the untreated than in the treated subjects and the mean time taken for complete healing in the control group was almost twice that of the treatment group. In this trial, however, the mean initial size of the wounds in the treatment group was bigger than in the untreated. Bigger wounds heal relatively faster in terms of the area reepithelialized in a given time. Days for complete healing was biased in favor of the control group since their lesions were smaller. The fact that days for complete healing were significantly less for the treatment group in spite of the bias supports the conclusion of the study that zinc therapy was effective.

Hallböök and Lanner (1972) in a double-blind study using patients suffering from venous stasis leg ulcers observed significant differences in healing time between oral zinc sulphate (200 mg three times per day) treated and placebo treated patients in a group (14 patients) with individual serum zinc levels lower than 110 μg/100 ml, but not in a group (13 patients) with serum zinc levels of 110 μg/100 ml or higher. They concluded that zinc sulphate increased the rate of healing for patients with low serum zinc.

In a study by Haeger and Lanner (1974) zinc sulphate in the form of effervescent tablets containing 200 mg zinc sulphate was given three times daily to patients with ischemic leg ulcers. The treatment group consisted of 14 patients and the serum zinc level was low in seven of them and normal in the other seven. These patients were compared with 16 nontreated controls. A significant difference in ulcer healing was noted between the patients with low serum zinc levels receiving zinc sulphate and the patients of the control group ($p < 0.01$). The average healing quotient (healing area in mm^2/day) increased by more than 100% with zinc.

There are several other controlled studies reporting on the effect on leg ulcer (Greaves and Boyd 1967; Greaves and Skillen 1970; Husain 1969; Serjeant et al. 1970) and burn wound (Henzel et al. 1970) healing of zinc

sulphate given per os in combination with meals. These and studies on treatment with oral zinc in other, different conditions (Flynn et al. 1973; Ruggles and Linqvist 1976; Sedlacek et al. 1976; Frommer 1975; Wallace et al. 1978; Gang 1980; Hallböök and Lanner 1972; Strömberg and Ågren 1984; Suomalainen 1983; Söderberg et al. 1983; Gang and Lengh 1982) favor the conclusion that oral zinc sulphate is a valuable adjunct in the treatment of ulcers for patients with subnormal serum zinc levels. Carruthers (1973) and Henkin (1974) have independently reviewed the clinical evidence and concluded that administration of zinc sulphate to patients who are zinc deficient is one of the factors which accelerates the healing of cutaneous wounds.

Topical Zinc Treatment of Ulcers

Zinc oxide is the most appropriate zinc compound for topical use but zinc sulphate is topically used in lotions to promote granulation of indolent ulcers and in aqueous solutions to relieve chronic inflammation in conjuntivitis. In a recent study by Ågren et al. (1991), pigs with normal zinc status were locally treated with a gauze containing zinc oxide and the healing was promoted by more than 30% compared with control treated wounds. Zinc sulphate at three different concentrations had, however, no beneficial effect on the healing of the porcine wounds. These findings indicate that not only the administration route but also the type of zinc compound is important in achieving the positive effect. It has also been shown (Ågren 1990) that the delivery from zinc oxide results in fairly constant zinc concentrations over time in wound fluid and wound tissue after a single application on open wounds, whereas with zinc sulphate, the zinc concentration in wound fluid declines after one day. Topically administered zinc is absorbed into the blood stream through intact skin and from open wounds (Hallmans 1977a,b, 1978; Hallmans and Lidén 1979; Söderberg and Hallmans 1982; Wetter et al. 1986; Ågren 1990; Ågren et al. 1991). The amount of zinc absorbed depends, among other factors, on the size of the treated area and the concentration gradient.

In a randomized, double-blind, placebo controlled study Strömberg and Ågren (1984) investigated the effect of local treatment with gauze containing zinc oxide on arterial and venous leg ulcers in 37 patients. The patients were matched on the basis of ulcer type and time of admission. In each pair one patient was treated with a sterile dry cotton gauze and the other with a similar gauze containing zinc oxide. A total of 18 patients received local zinc therapy and 19 were given placebo. During a period of 8 weeks the ulcers were photographed and ulcer area determined using a planimeter once weekly. The treatment effects were evaluated by two independent doctors. The result was recorded to be successful (as determined before trial start) if: (1) there were visible granulations in the ulcer or (2) when visible granula-

tions were present before treatment, the initial ulcer area should be reduced by 25% for arterial and by 50% for venous leg ulcers and (3) the ulcer debrided in 8 weeks. The result was regarded as unsuccessful if: (1) the initial ulcer area increased by 50% or more during the treatment period, (2) antibiotics were needed or (3) the ulcers did not comply with a successful criteria. Treatment was noted as successful in 15 of the 18 zinc treated patients and in eight of the 19 placebo treated patients. This result was statistically significant ($p < 0.05$). In the placebo group the average ulcer area was 4.2 cm^2 and after treatment 2.7 cm^2. Corresponding values for the zinc group were 3.6 cm^2 and 0.4 cm^2, respectively. In the placebo group six ulcers were infected during treatment whereas infection only occurred in one patient in the zinc oxide treated group.

In a randomized single-blind investigation (Ågren and Strömberg 1985) Varidase was compared with gauze containing zinc oxide. A total of 28 patients was included in the study. Of these, 14 were treated with Varidase and 14 with zinc oxide gauze (0.4 mg zinc oxide/cm^2). The result was considered successful if the ulcers were cleansed after 8 weeks of treatment. Evaluation was based on photographs of the ulcers and carried out by an independent surgeon. Necrotic tissue disappeared in the zinc oxide treated ulcers of seven patients and in six Varidase treated patients. It was concluded that the two treatments are about equally effective in the removal of necrotic tissue. Desloughing time was 23 days (range: 7–56) for the zinc oxide group and 21 days (range: 7–42) for the Varidase group.

Zinc Tape Treatment of Wounds

The wound healing effect of adhesive tape treatment is an old observation and the use of adhesive tape in the treatment of leg ulcers is well known all over the world. It is said to have been used already around 1550 by Mariano Santo. Baynton's tape therapy introduced in 1779 is better known. The adhesive tape was applied as a compressive bandage. Subsequently taping has been employed with various modifications.

Natural rubber (latex) together with ordinary rosin and fillers like zinc oxide are the main components of the adhesive mass in adhesive zinc oxide tapes. Zinc oxide is incorporated primarily as a reinforcing agent, simultaneously enhancing the cohesive strength and extending the longevity during storage (Grove 1971).

Gilje (1949) described his method of treating venous leg ulcers with the use of adhesive tapes. He concluded that the favorable effects of the adhesive tape treatment on healing of leg ulcers may be due to a combination of many factors:

1. The significance of compression for preventing edema
2. A stimulating effect of mechanical pressure on the healing process

3. The protective effect against other outer injuries and irritation
4. The "moist chamber" effect
5. The chemical irritation from the plaster substance
6. The bacteriostatic effect

His observations can still be considered as the basis for the current research on the actions of adhesive tape treatment of various ulcers.

Healing of full-thickness excisions is the sum of wound contraction and reepithelialization (McGrath and Simon 1983). Söderberg and Hallmans (1982) have clearly shown that if a zinc tape is applied also on the adjacent undamaged skin, wound contraction is inhibited and retarded wound closure is obtained. Hallmans and Lasek (1985) could demonstrate a quicker wound closure in both zinc-deficient and zinc-sufficient rats treated with an adhesive tape containing zinc oxide. The time taken for complete healing of full-thickness open wounds in rats after local zinc treatment has been studied in different investigations. In the study by Hallmans et al. (1979), the wound healing time was 33% faster with adhesive zinc tape treatment which retains the moisture in wounds (water vapour permeability 100 g/m^2/24 h) compared with open treatment with dry gauze in normal rats. These results can be explained rather by the moisture retaining effect of the tape than the therapeutic effect of zinc at least in animals with normal zinc levels. However, the authors found a shorter healing time with zinc tape than with an allograft (frozen pig skin) which also prevents dehydration of the wound.

Söderberg et al. (1982) performed an open comparative study on plantar ulcers in leprosy patients in India. The effects of zinc oxide adhesive tape and an Eusol soaked dressing (1.25% boric acid; 1.25% chlorinated lime) were compared. The investigation was carried out at two hospitals in India and included 90 patients with a total of 128 ulcers. All the wounds were located on the plantar side of the feet or on the toes. In all cases the numerical values of the wound healing time were lower (approximately 30%) in the zinc tape treated groups than in the Eusol treated group. However, the differences were statistically significant ($p < 0.01$) only concerning the small wounds in one hospital.

In a study by Hallmans (1977a) eight patients with second and third degree burns were treated with zinc tape on 5%–20% of the total body area. Subnormal serum zinc levels were found before treatment was initiated. Normal serum zinc values were reached during the first treatment week, and in six patients supernormal values of >124 μg/100 ml (>19 μmol/l) were found during the treatment period.

Gang (1980) reported on a comparative study of zinc tape treatment in burns. A total of 40 patients, 26 with superficial burns and 14 with full-thickness burns, were studied. Half of the patients were treated with zinc tape and the other half with the exposure method (the burned area was cleaned with Savlon and saline, and Flamazine was applied every day). Serum zinc was determined in ten patients before and after 1 and 5 days of

tape treatment. The mean duration for complete healing of the superficial burns was significantly shorter with the zinc tape than with the exposure method; 9 days compared to 12 days. The complete separation of necrotic eschar in full-thickness burns took place within an average of 10 days with zinc tape, whereas with the exposure method surgical desloughing was needed after 13.17 days. Hence, skin grafting was done much earlier in the zinc tape treated patients. Serum zinc values were subnormal in most cases before start of treatment. A rise in serum zinc was observed in all patients after 24 h of zinc tape treatment. The author concluded that adhesive zinc tape treatment promotes healing not only in superficial burns. In full-thickness burns it results in a gradual dissolution and separation of necrotic tissue enabling an earlier skin transplantation.

Brandrup et al. (1990), in a prospective, randomized trial of a hydrocolloid and zinc tape over a period of 8 weeks, determined their healing ability and effect on pain for venous and arterial leg ulcers. The initial ulcer areas decreased with zinc tape by 64% and by 48% after treatment with the hydrocolloid. A similar analgesic effect was seen.

An open, randomized, controlled study (Apelqvist et al. 1990) was carried out on 44 diabetic patients with necrotic ulcers treated with adhesive zinc oxide tape (Mezinc) or with a hydrocolloid (DuoDERM). Of the 21 patients treated with Mezinc, 74% had their necrotic ulcers improved by at least 50% compared to six out of 21 with the hydrocolloid dressing ($p < 0.025$). Fifteen patients showed an increase in the area of necrosis during the course of the 5 week study, and of these ten had been treated with the hydrocolloid dressing.

Zinc and Collagen Metabolism

Collagen and protein production was decreased in polyvinyl sponges implanted subcutaneously in zinc-deficient rats (Fernandez-Madrid et al. 1973). These results indicate that in zinc deficiency there is a general depression of protein synthesis rate rather than a specific effect on collagen synthesis; this is presumably due to a depressed activity of RNA polymerases. However, enzymes involved in different steps of the formation of collagen molecules are also influenced by zinc. Prolyl hydroxylases, an iron-dependent enzyme, is inhibited by zinc supplementation. The activity of lysyl oxidase, a copper-dependent enzyme, is reduced both in zinc deficiency and excess of zinc (Chvapil and Misiorowski 1980). Lysyl oxidase is required for an adequate cross-linking of collagen. The decreased breaking strength of 3 week old incised wounds in zinc-deficient rats could be due to a reduced lysyl oxidase activity (Ågren et al. 1991).

Hydroxyproline measurements (a measure of collagen) in wounds treated with zinc either locally (Hallmans et al. 1979; Wetter et al. 1986) or systematically (Elias and Chvapil 1973; Tengrup et al. 1981) suggest that the

accumulation of collagen in granulation tissue is not reduced after zinc treatment. On the contrary, three studies showed increased collagen concentrations in zinc treated wounds (Elias and Chvapil 1973; Hallmans et al. 1979; Tengrup et al. 1980). This could be attributed to a reduced collagenolytic activity as a result of a depressed leakage of hydrolytic enzymes from inflammatory cells because of the membrane stabilizing effects of zinc (Tengrup et al. 1980).

Zinc and Inflammation

Zinc is one of the substances which regulate the activity of various inflammatory cells (Söderberg 1990). Feeding rats with a high zinc content diet has been shown to decrease the phagocytic activity and the rate of migration of macrophages (Chvapil 1976). Macrophages taken from zinc-deficient animals migrated more than those from controls. By using light microscopy and scanning electron microscopy it was shown that the cell surface of the macrophages from guinea pigs given a diet with a high zinc concentration (2000 mg/kg) was smooth and round while there were extensive protrusions in cells from control animals (40 mg/kg zinc diet). It has also been shown in vitro that the oxygen consumption and phagocytosis by polymorphonuclear leukocytes (PMNs) are inhibited by higher concentrations of zinc ions (83 μmol/l) (Chvapil 1976). Zinc supplementation (i.p) also inhibits the penetration of granulocytes in a dose-dependent manner into a pleural inflammatory exudate in rats (Yatsuyanagi et al. 1987). The conclusion of these studies has been that zinc immobilizes inflammatory cells by stabilization of lysosomal and cell membranes.

The anti-inflammatory effect of zinc has further been studied in vivo, using the carrageenan-induced edema in rats, and artificial abscesses in rabbits measuring the inflammatory area (Singla et al. 1986; Guillard et al. 1987). The inflammation was reduced in both models after zinc administration, i.e., a decreased edematous reaction and a decreased infiltration of leukocytes in the inflamed area were demonstrated.

Treatment of excisional wounds with adhesive tape resulted in fewer PMNs in the granulation tissue, measured indirectly by the alkaline phosphatase activity (Wetter et al. 1986).

Recent studies have shown that zinc treatment blocks tumor necrosis factor (TNF)-mediated cytolysis of the target cells (Flieger et al. 1989). $ZnSO_4$ (0.1 and 1.0 mM), and $ZnCl_2$ (but not $CaCl_2$, $MgSO_4$ or $CuSO_4$) in the same concentration range, prevent cell death indicating that it is the zinc ions that are mediating the effects. Even if the zinc ions are added 2 h after TNF treatment the cell lysis is effectively inhibited, and this indicates that it acts at a late stage after binding of TNF to its receptor.

Burn injuries cause an imbalance in immunological functions and thereby increase the leukocyte membrane permeability. The cells lose their

lysosomal enzymes which appear in serum in high concentration; extracellularly they play an important role in cell destruction in burns. Zinc oxide inhibits the oxidation of membrane lipids in vitro preventing the increase of acid phosphatase, a marker of lysosomal enzymes, leaking from leukocytes taken from burn patients. If those lytic effects of lysosomal enzymes in burn patients might be prevented in vivo this can possibly improve the burn prognosis (Haberal et al. 1987).

Recently it has been shown that cytotoxic effects of resin acids to polymorphonuclear cells in vitro are inhibited by zinc (Söderberg 1990) and the protective action of divalent cations probably occurs via the closing of pores in the membranes (Bashford et al. 1986, 1988).

Zinc and Infection

It has been demonstrated that zinc inhibits bacterial proliferation in vitro (Turkheim 1953; Paetzold and Wiese 1975; Hallmans and Elmros 1980; Moorer and Genet 1982; Söderberg et al. 1990, 1991; Söderberg 1990) and in vivo (Sunzel et al. 1990). Gram-positive bacteria appear to be more sensitive to the zinc ion than gram-negative bacteria.

It is a well established fact that zinc is an essential trace element which is required for replication and growth and plays a key role in many fundamental molecular processes involved in host defense. Immune response of the host may be changed if there are alterations in zinc status. The cellular biochemistry is dependent on the zinc concentration as zinc is a constituent of more than 100 metalloenzymes. The metalloenzymes become active when zinc is incorporated (Failla 1977). The cells aquire zinc from a variety of zinc salts or from zinc bound to different proteins. Zinc readily binds to membranes and in this manner the cell membranes are stabilized. Zinc at physiologic levels decreases functioning of phagocytes within minutes and zinc deficiency decreases T cell functions but has little or no effect on B cell functions. Zinc protects against endotoxins and bacterial infections perhaps by stabilizing lysosomal membranes that may be involved with the development of shock. Both different acute noninfectious illnesses and infections are associated with a decrease in serum levels of zinc of 10%–60% after some hours and this may last for days depending on the course of the sickness. Chronic zinc deficiency is often associated with a pronounced susceptibility to infections but it is not clear if this is a primary or secondary phenomena.

Microbial cellular functions such as cell division and protein production require zinc. Zinc is a component of the outer layers of many microorganisms and it probably stabilizes the ribosomes and membranes by a mechanism similar to that by which it stabilizes mammalian membranes and thus may protect the organisms from penetration by toxic compounds. If the zinc concentrations are higher than the normal physiologic range there

is a subsequent inhibition of enzyme function in bacteria. Production of bacterial toxins requires the presence of near physiologic concentrations of zinc in several species and adherence of bacteria to mammalian cell surfaces is considered part of the pathogenesis of different infections. There are a lot of data suggesting that zinc is not a virulence factor and that it will kill or inhibit bacteria, inhibit viral replication, and at millimolar or higher concentrations it can clump bacteria under certain incubation conditions, thus offering phagocytic cells an easier target (Sugarman 1983).

Different types of infectious diseases induce acute phase response: fever, leukocytosis, depressed plasma zinc concentrations, increased hepatic zinc concentrations and altered synthesis of acute-phase plasma proteins. The alterations are supposed to be mediated by the protein hormone endogenous pyrogen-leukocyte endogenous mediator, which has been equated with a factor released from macrophages called interleukin-1. The redistribution of zinc that occurs during infections may influence different aspects of cellular metabolism, membrane stabilization and other cellular functions. In previous studies the effects of treating rats with zinc during endotoxemia and infection have been studied. A fivefold increase in plasma zinc levels to what is a supraphysiological concentration (10 μg/ml) prevented death in rats injected with *Salmonella typhimurium* endotoxin (Sobocinski et al. 1977). Treatment in excess of this seems to be toxic and provides diminished protection. The same tendency has been shown when infected mice have been protected against lethality induced by *Salmonella typhi* endotoxin (Snyder and Walker 1976).

There seem to be different opinions about the protective role of supranormal plasma zinc levels in animals infected with bacteria. Tocco-Bradley and Kluger (1984) have shown that preventing the fall in plasma zinc during infection enhanced rather than hindered the survival rate, although raising plasma zinc concentration beyond the normal physiological level was not beneficial to the host. If hepatic zinc concentration was elevated beyond that of infected controls without altering the infection-induced fall in plasma zinc concentration no enhancement of survival rate in *Salmonella*-infected rats was noted. Loading tissues such as liver and small intestine with more zinc than that obtained with an infusion of 30 μg zinc/hour enhanced survival if plasma zinc was maintained at noninfection levels, but did not enhance survival if plasma zinc was raised to supranormal levels. Their conclusions were that an elevated plasma zinc concentration at the onset of the infection is protective, but this protection is completely negated if plasma zinc levels are maintained at supranormal concentrations over the course of the infection. One has to remember that these experiments were performed using only one gram-negative endotoxin-producing bacterium and the results may be different if other bacterial strains or combinations of bacteria are tested.

Chvapil (1976) reported that increasing plasma zinc levels to 20 μg/ml in rats and guinea pigs was associated with a decrease in polymorphonuclear

leukocyte and macrophage mobilization and phagocytosis. It has been hypothesized that these effects occur because zinc stabilizes membranes. As many diverse cytotoxic agents damage cells by rendering them leaky to the passage of ions and low molecular weight metabolites this can be prevented by raising extracellular zinc concentrations. The protection is through a direct action at the extracellular side of the plasma membrane and zinc probably affects some endogenous component of the plasma membrane. The phospholipids of the outer leaflet appear to be the most likely site (Bashford et al. 1988).

Other Metals

The role in wound healing of the minerals called macrominerals such as sodium, calcium, potassium, chloride, magnesium, and phosphorus is beyond the scope of this review. They are all essential for a wide variety of tissue functions many which are necessary for a normal wound healing process. Magnesium is required in almost all stages of wound healing, but particularly in collagen formation as this metal activates a large number of enzymes involved in energy-producing cycles and protein synthesis. Deficiency is not common but it has been reported.

Although iron is considered as distinct from the trace element group its importance in wound healing is mainly due to the fact that it is required for the hydroxylation of lysine and proline and therefore it is necessary for effective collagen synthesis. Iron deficiency is one of the most likely forms of single micronutrient deficiency to occur in the abscence of any other accompanying form of malnutrition. It is very important for the immune system and in excess there is a risk of infection. Deficiency gives a functional abnormality of iron deficient phagocytic cells and the primary defect is in bacterial killing. Although iron deficiency has no effect on the rate of epithelialization of wounds there are some conflicting papers on the effect of iron deficiency on the tensile strength. A possible solution to the controversy has been offered by Hunt and Zederfeldt (1969) who stressed that hypovolemia, vasoconstriction, trauma, and elevated blood viscosity rather than anemia impair oxygen transport.

Copper is essential for normal erythropoiesis. It is a constituent of many vital enzymes and is vital for connective tissue metabolism. The amount of copper in the body is 20–50 times lower than that of iron and zinc. The plasma concentrations of copper increase during healing of extensive wounds. The effects of copper are antagonized by many other components especially zinc and cadmium.

Copper is part of lysyl oxidase, a copper-containing enzyme that catalyzes the conversion of lysyl and hydroxylysyl residues in collagen to the corresponding aldehydes, the first step for formation of stable covalent collagen cross-links. These covalent cross-linkages strengthen scars (Page

and Benditt 1967; Prockop and Guzman 1977). No clinical cases of impaired wound healing due to copper deficiency alone have been reported.

Manganese is essential for growth, reproduction, skeletal development, and brain functions (Underwood 1977). It is required for the activation of many different enzymes such as phosphatases, kinases, decarboxylases, and glucosyl transferases (Levenson et al. 1977). It is most concentrated in tissues which have a large number of mitochondria in their cells and manganese appears to concentrate in mitochondria. Some of its specific roles in wound healing include activation of the enzymes responsible for glycosylation of procollagen molecules and those required in the synthesis of proteoglycans.

In addition, chromium, selenium, and molybdenum are necessary for growth and development, but their relation to wound healing is not clear. As they have an influence on some cells and tissues in the body, a deficiency might interfere with wound healing.

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Plasmin-Mediated Pericellular Proteolysis by Keratinocytes: Extracellular Matrix Reorganization vs Tissue Damage

M.D. Kramer, R. Batrla, G.M. Hänsch, and J. Reinartz

Introduction

Plasminogen activators are specific proteolytic enzymes which convert the inactive proenzyme plasminogen to plasmin (Danö et al. 1985; Mayer 1990). Plasmin is a potent and nonspecific protease which cleaves fibrin clots (Collen 1980) and other extracellular proteins (Danö et al. 1985), including the glycoproteins of extracellular matrices (Cohen and Shuman 1990). The "plasminogen activator system" is complex; it consists of proenzymes of the plasminogen activators (PAs) (tissue-type PA (tPA), urokinase-type PA (uPA)), the PA substrate plasminogen, the active enzymes (uPA, tPA, and plasmin), several natural inhibitors of plasminogen activators and plasmin, and the cellular receptors that bind the proenzymes, enzymes and enzyme/inhibitor complexes (Figs. 1, 3). The coordinated interactions of these components control location, timing, and extent of plasmin activity.

The epidermal basement membrane, a highly organized structure of the extracellular matrix, exerts adhesive forces for the basal keratinocytes of the overlying epidermis; its integrity is pertinent to dermo-epidermal cohesion (Katz 1984, 1985). Morphological studies revealed that during wound healing the basement membrane zone adjacent to the wound bed "loses definition" prior to epithelial migration (Stenn and Depalma 1988). Proteolytic dissolution of basement membrane constituents is thought to contribute to this morphological alteration. The notion that plasminogen activation by keratinocyte-associated PAs participates in this process has been derived mainly from two lines of evidence. Firstly, in vitro studies have indicated that both expression of PAs (Morioka et al. 1987) and the cell surface receptor for uPA (McNeill and Jensen 1990; Del Rosso et al. 1990) are associated with migrating rather than with sessile keratinocytes. Secondly, detailed immunohistological studies on murine and human skin wounds revealed that uPA was present in keratinocytes of the epithelial outgrowth

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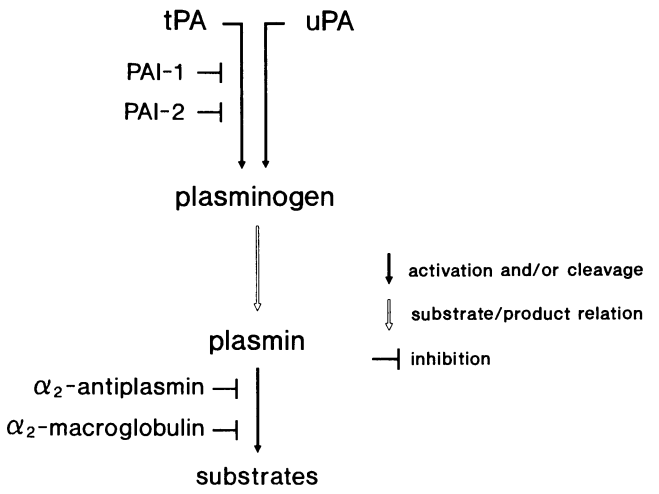

Fig. 1. The plasminogen activator (*PA*) system. Two types of plasminogen activators are known: tissue-type plasminogen activator (*tPA*) and urokinase-type plasminogen activator (*uPA*). They convert the precursor enzyme plasminogen to plasmin by limited proteolysis. The plasminogen activators are regulated by specific inhibitors, plasminogen activator inhibitor type-1 (*PAI*-1) and *PAI*-2. Active plasmin is regulated primarily by its specific inhibitor α_2-*antiplasmin* and by α_2-*macroglobulin*. Plasmin has a trypsin-like specificity and may degrade a variety of protein substrates, including the classical substrate fibrin and glycoproteins of the extracellular matrix

(Grohndahl-Hansen et al. 1988), which covers the primary wound substratum, consisting of fibrin and other plasma-derived compounds. In normal epidermis no uPA-specific immunoreactivity was found.

Expression of PAs by keratinocytes has been observed in various skin diseases (Jensen et al. 1990a; Baird et al. 1990), including nonblistering disorders such as psoriasis vulgaris (Grohndahl-Hansen et al. 1987; Lotti et al. 1988) but also blistering skin diseases such as bullous pemphigoid (Jensen et al. 1988; Baird et al. 1990). The latter is characterized by subepidermal cleft formation at the level of the epidermal basement membrane zone. Although both the etiology and the histopathology of the diseases display a great variability, destruction of extracellular structures is a common feature (Farmer 1985a,b; Dubertret et al. 1982). It has been suggested that expression of PAs is tissue-damage related rather than disease-specific (Kramer et al. 1992a).

In the following we will present evidence for a cell surface-associated pathway of plasminogen activation in human keratinocytes and for local enrichment of plasmin(ogen) and conversion of plasminogen to plasmin under pathological conditions in the skin.

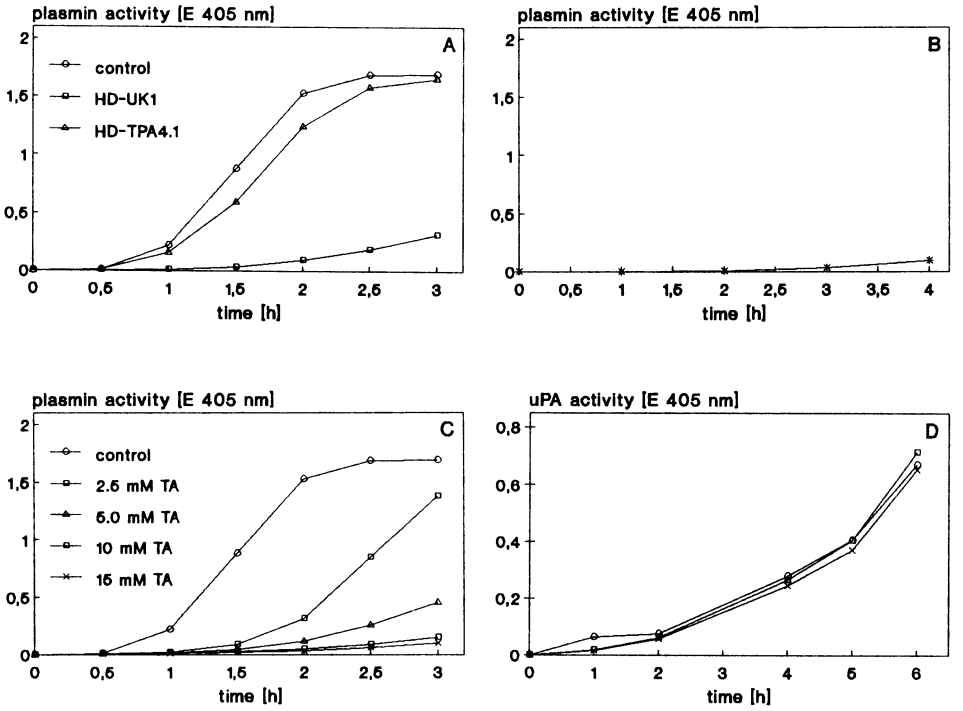

Fig. 2A–D. Mechanism of plasminogen activation by cultured keratinocytes (HaCaT cell line, Boukamp et al. 1988). **A** HaCaT cell monolayers were incubated with plasminogen and the plasmin substrate S-2251 under serum-free conditions. The time course (*abscissa*) of plasmin generation was monitored by absorbance measurement (*ordinate*). The enzyme reaction was allowed to proceed in the absence (*control*) or presence of anticatalytic anti-uPA (*HD-UK 1*) or anti-tPA (*HD-TPA 4.1*) monoclonal antibody (Kramer et al. 1992b). **B** HaCaT cell monolayers were incubated with serum-free HL-1 medium. After 2 h of incubation the conditioned media were harvested and tested for plasminogen activator activity by addition of plasminogen and substrate S-2251. Absorbance at 405 nm (*ordinate*) was measured after different intervals of time (*abscissa*). **C** Analogous setup as in **A**, except that the enzyme reaction was performed in the absence (*control*) or presence of varying concentrations of tranexamic acid (*TA*). **D** A total of 20 ng/ml of human high molecular weight uPA were mixed with serum-free HL-1 medium. Plasminogen activator activity was determined by addition of plasminogen and the plasmin substrate S-2251 in the absence (*circles*) or presence of tranexamic acid (10 *rectangles* and 20 m*M crosses*)

Plasminogen Activation by Human Keratinocytes

Keratinocytes can produce uPA and tPA in vitro (Birkedahl-Hansen and Taylor 1983; Hashimoto et al. 1983; Isseroff et al. 1983; Jensen et al. 1990a) and in vivo (Morioka et al. 1985; Grohndahl-Hansen et al. 1987, 1988; Lotti et al. 1988; Reinartz et al. 1993a). uPA is synthesized and secreted by keratinocytes as a proenzyme (pro-uPA) with little or no intrinsic activity

(Hashimoto et al. 1988; Buessecker et al., unpublished results). uPA of cultured keratinocytes is localized to the surface by specific cell surface receptors (McNeill and Jensen 1990; Del Rosso et al. 1990), possibly in an autocrine manner (Stoppelli et al. 1986). tPA secretion by keratinocytes (Jensen et al. 1990a,b) has so far not been studied in great detail.

Plasminogen is produced primarily in the liver and is present in plasma and the interstitial fluid (Miyashita et al. 1988). There is no evidence for synthesis of plasminogen by keratinocytes. However, by immunohistochemistry plasmin(ogen) has been localized to the basal and suprabasal layer of normal human epidermis (Isseroff and Rifkin 1983; Justus et al. 1987), indicating that plasminogen of the interstitial fluid can bind to keratinocytes. In fact, binding of exogenous plasminogen to the basal and suprabasal layers of epidermal cryosections has recently been demonstrated (Burge et al. 1992). Binding was found to be mediated by the kringle 5AH site and the lysine binding sites of kringle 1–3 of the plasminogen molecule.

We have explored in vitro whether there exists a cell surface-associated pathway of plasminogen activation, which could provide plasmin for pericellular proteolysis in the vicinity of the PA-expressing keratinocyte. A basic experiment in that respect is shown in Fig. 2. Human keratinocytes in culture were incubated with plasminogen and a chromogenic plasmin substrate under serum-free conditions. Plasminogen was efficiently activated already within the first 2 h of incubation (Fig. 2A). Plasminogen activation was counteracted by anticatalytic anti-uPA, but not by anticatalytic anti-tPA antibodies (Fig. 2A). To explore whether secreted or cell-associated PAs were responsible for plasminogen activation, serum-free cell culture supernatants were harvested after 2 h of culture. The supernatants were then tested for PA activity by adding plasminogen and a chromogenic substrate for plasmin: no significant PA activity was seen in the supernatants after incubation for 2 h (Fig. 2B); even after prolonged reaction time (6 h) only weak (E405 nm = <0.1) activity was observed (Fig. 2B). The finding argues against a significant contribution of secreted PAs to plasminogen activation within the first 2 h of keratinocyte cell culture.

Plasminogen and also plasmin bind to proteinaceous surfaces, such as the surface of a fibrin clot (Collen 1980) or to cell surfaces (Plow et al. 1991) via "lysine binding sites." The lysine analogue tranexamic acid interferes with the lysine binding site-mediated interactions of plasmin(ogen). We explored plasminogen activation by intact HaCaT cells in the presence of tranexamic acid. The presence of tranexamic acid caused a dose-dependent inhibition of plasminogen activation by HaCaT cell monolayers (Fig. 2C). In control experiments, activation of plasminogen by soluble uPA was not affected by the presence of tranexamic acid (Fig. 2D). The findings indicated that plasminogen activation by intact HaCaT cells was uPA-dependent, required binding of plasminogen to the cell surface, and was not mediated by secreted PAs. In more refined studies we could demonstrate that plasminogen binding to keratinocytes is saturable and proceeds in a

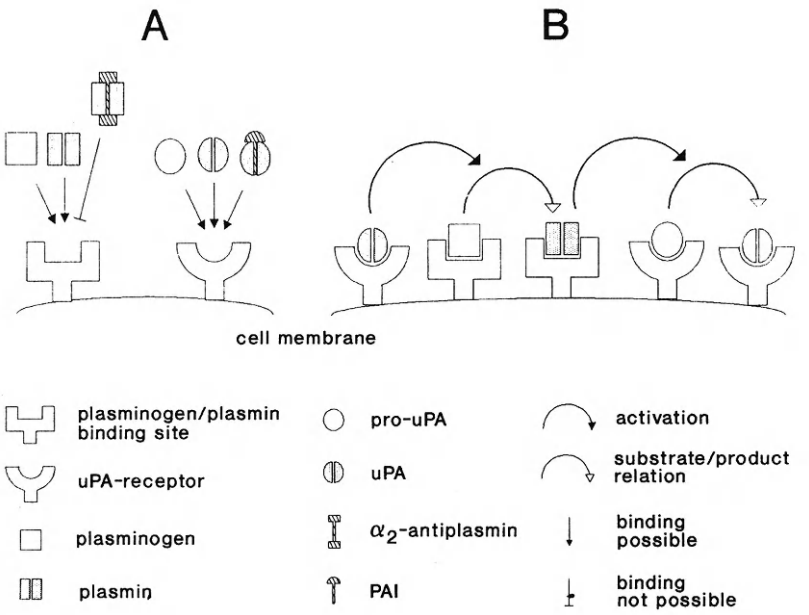

Fig. 3A,B. The plasminogen activator system on the keratinocyte surface. Keratinocytes express binding sites for plasminogen and surface receptors for uPA. **A** Plasminogen binding sites can bind plasminogen and plasmin, but not the inactive plasmin/α_2-antiplasmin complex. Receptors for uPA can bind pro-uPA, active uPA or inactive uPA/PAI complexes. **B** Cell surface bound uPA can activate cell surface bound plasminogen, and – vice versa – receptor-bound pro-uPA may be activated by cell surface bound plasmin. Once bound to its binding site, plasmin is not accessible for its specific inhibitor α_2-antiplasmin. The series of interactions provides cellular surfaces with the proteolytic potential of plasmin. Due to the low affinity interaction of plasmin with its cell surface binding sites, cell bound plasmin may diffuse away from the surface, bind to and cleave glycoproteins in the pericellular space

time- and concentration-dependent manner (Reinartz et al. 1993b). Moreover, in contrast to plasmin in solution, cell surface-bound plasmin was protected against inhibition by its specific inhibitor α_2-antiplasmin. Our present view on the organization of the plasminogen activator system at the surface of human keratinocytes is summarized in Fig. 3.

Possible Consequences of Pericellular Plasmin Generation

Epidermal cells under physiological conditions exhibit strong intercellular cohesion and adhesion to the subepidermal basement membrane. During wound healing cells adhere first to a primary extracellular substratum which consists mainly of fibrin and other plasma constituents (Stenn and DePalma 1988; Donaldson and Mahan 1988). When isolated extracellular matrix constituents became available their adhesive interactions with keratinocytes

could be explored. Adhesion of keratinocytes to multiple extracellular matrix proteins, including fibrin, fibronectin, laminin, vitronectin, and collagens, was observed. Similar to other cells, the binding interactions were mediated by cell membrane-inserted receptors of the integrin family (Albelda and Buck 1990; Kaufmann et al. 1990).

We have tested whether plasmin can interfere with adhesion to the isolated extracellular matrix constituents collagen type I or fibrin. Plasmin interferes with keratinocyte adhesion to fibrin but not to collagen type I (Fig. 4).

Evidence for Plasminogen Activation In Vivo

Bullous pemphigoid, an acquired disease of the elderly (Farmer 1985a), was chosen in order to explore a possible role of the PA system in a blistering skin disease. The disease is characterized by deposition of autoantibodies at the epidermal basement membrane zone of lesional skin. The autoantibodies are specific for a structural component of the hemidesmosomes, the "bullous pemphigoid antigen." The hemidesmosomes mediate adhesion of basal

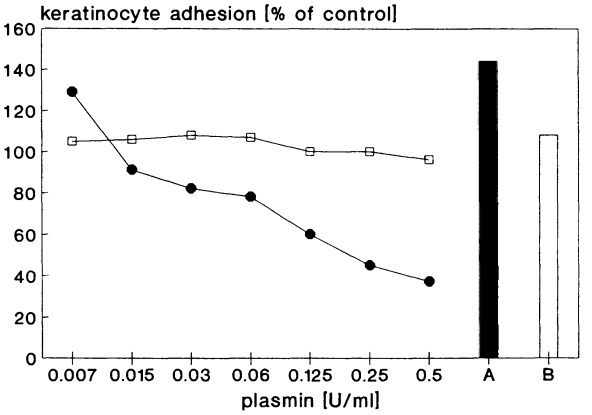

Fig. 4. Adhesion of keratinocytes (HaCaT) to fibrin (*closed circles*) or collagen type I (*open rectangles*) in the presence of varying concentrations of plasmin (*abscissa*). Microtiter plates were coated with fibrin (250 μg/ml) or collagen type I (10 μg/ml). A single cell suspension of HaCaT cells was added to the plates (2×10^5 cells/well) together with varying concentrations of plasmin (0.5 – 0.007 U/ml). After 60 min nonadherent cells were removed by washing with phosphate-buffered saline. Bound cells were fixed and stained by using methylene blue. Cell associated methylene blue was eluted and quantified by absorbance measurement at 595 nm. The extinction values are dependent on the number of cells adherent to the plate. The whole method is a modification of the original method described by Goldman and Bar-Shavit (1979). *Bars* represent adhesion of keratinocytes to fibrin (*A*, *closed bar*) or collagen type I (*B*, *open bar*) in the presence of 0.5 U/ml plasmin and the plasmin inhibitor aprotinin (100 U/ml). Under the experimental conditions HaCaT cells did not adhere to noncoated microtiter plates. (From Batrla et al., unpublished)

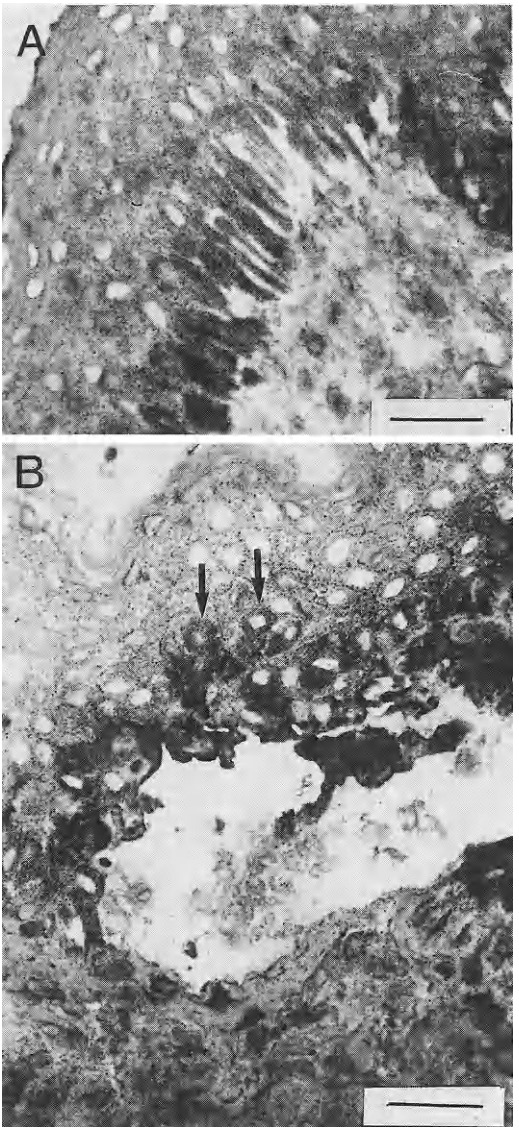

Fig. 5A,B. Deposition of plasmin(ogen) at the site of cutaneous tissue damage in lesional skin of bullous pemphigoid. Frozen sections of perilesional skin with **A** incipient blister formation and **B** a fully developed subepidermal blister were stained with a murine monoclonal antibody (clone HD-PG 2) to human plasmin(ogen). Bound antibody was visualized as dark color by the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique (compare with Gissler et al. 1992; Kramer et al. 1992a). Arrows in **B** indicate staining of single keratinocytes. Scale bars: **A** 15 μm, **B** 20 μm

keratinocytes to the supporting basement membrane (Mutasim et al. 1989; Stanley et al. 1988).

By immunohistochemistry we observed a conspicuous deposition of plasmin(ogen) in lesional skin, apparently associated with basal and suprabasal keratinocytes (Fig. 5). Plasmin(ogen)-specific immunoreactivity was seen already in early incipient blisters (Gissler et al. 1992). The prominent deposition of plasmin(ogen) in lesional as compared to nonlesional skin was taken to suggest a participation of plasmin(ogen) in the development of the skin lesions. While the immunohistochemical data do not allow us to distinguish between plasminogen and plasmin (Justus et al. 1987), the presence of PAs in the keratinocytes of lesional skin areas of bullous pemphigoid (Jensen et al. 1988, 1990b; Baird et al. 1990) would strongly suggest the occurrence of plasminogen activation.

For functional analyses fluid was taken from skin blisters of bullous pemphigoid patients. By using a plasmin-specific chromogenic substrate assay, plasmin activity was detected (Fig. 6). Control fluid aspirated from subepidermal suction blisters (Volden et al. 1983) produced on clinically normal skin did not contain measurable plasmin activity (Fig. 6).

Concluding Remarks

We demonstrate a cell surface-associated pathway of plasminogen activation on cultured keratinocytes. The pathway provides plasmin activity for proteolysis of pericellular substrates. Plasmin cleaves the extracellular matrix

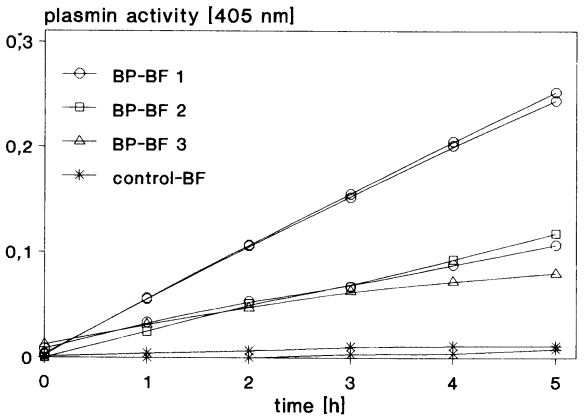

Fig. 6. Evidence for plasmin generation in lesional skin of bullous pemphigoid. The fluid of subcutaneous blisters generated spontaneously in two bullous pemphigoid patients or the fluid of subepidermal suction blisters raised experimentally on clinically normal skin were tested for plasmin activity using the plasmin-specific chromogenic peptide substrate S-2251. Activity was found in the fluid of bullous pemphigoid blisters but not in the fluid of suction blisters. The finding provides evidence for generation of plasmin at some stage during formation of bullous pemphigoid skin lesions

protein fibrin, resulting in reduced keratinocyte adhesion to fibrin. Moreover, evidence is provided that plasminogen binding to keratinocytes and activation of plasminogen may occur also under pathological conditions in vivo. The expression of plasminogen binding sites on keratinocytes and their colocalization with uPA provides keratinocytes with a basic mechanism for harnessing plasmin activity. Whether there are mechanisms, e.g., induced by inflammatory stimuli (Kramer et al. 1992a), which up-regulate cell bound plasmin activity of keratinocytes either by increasing the rate of plasminogen activation or by increasing plasmin(ogen) binding of keratinocytes has to be addressed in future experiments.

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Reduced Migration Potential of Epithelial Cells from Explants of Chronic Decubitus Ulcers: The Cause of Impaired Wound Healing?

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Introduction

Decubitus ulcers of older patients are representative of impaired wound healing: chronic ischemia, recurring infections, necrosis, fibrin persistence with reduced fibrinolytic activity, a tendency to heal poorly with inability to epithelialize. Lack of epithelialization is the visible characteristic of impaired wound healing. The causes of impaired wound healing of chronic ulcers are only partially known. A clear, overall picture of how all the known factors interact is still missing. Certain is that infections (Brook 1991; Seiler et al. 1979), insufficient microcirculation (Ardon et al. 1991; Ek et al. 1984; Parkhouse and LeQuesne 1988), chronic tissue hypoxia (Dowd et al. 1983; Hauser et al. 1984; Seiler et al. 1979; Xakellis et al. 1991) and reduced fibrinolytic potential in the ulcer tissue (Larsson and Risberg 1977; Ljungnér and Bjergqvist 1985; Seiler et al. 1980) are, among other factors, important contributors. Since the epithelial cell initiates epithelialization, and since absence of epithelialization is actually the result of all factors interfering with wound healing, the migration pattern and mitosis of epithelial cells are of particular interest. In the search for causes of the missing epithelialization, tissue cultures were examined for their migration potential and for the mitosis pattern of both epithelial cells and fibroblasts from explants derived from the edges of chronic decubitus ulcers of geriatric patients. It was noted that the epithelial cells taken from the edges of decubitus ulcers showed a significantly reduced migration potential (Seiler et al. 1989).

In order to verify these unusual results once more, new explants were taken from four chronic decubitus ulcers of three geriatric and one young paraplegic patient and the migration pattern of the epithelial cells of these explants was studied using modern techniques. The following report briefly addresses the results of both studies.

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Patients and Methods

The subject of the first study was the migration potential and mitosis pattern of epithelial cells and fibroblasts from explants taken from eight chronic decubitus ulcers of six geriatric (ages 77–91 years, median age 84 years) and of one 21 year old paraplegic patient, to be compared with six explants of healthy skin from breast reduction surgery (control). All biopsy samples were removed during plastic surgery procedures as transverse section from the ulcer edge and radiating across surrounding ulcer tissue; sample areas were labeled by their distance from the ulcer edge; 0–1 cm from the ulcer edge is area 0/1; distance of 2–3 cm, area 2/3; distance of 4–5 cm, area 4/5. Some 40–60 explants from each area 2 mm^2 in size were started in a tissue culture bottle (Corning) with MEM (minimal essential medium of Eagle) with 10% fetal calf serum and 0.2% Polybactrim, then covered with cellophane foil, and allowed to cultivate for 14 days. The same collection principle was applied to the explants of the second study. Here three biopsy samples were taken from two geriatric patients (median age 81 years) and a 42 year old paraplegic patient. Again, skin from breast reduction surgery was used for control purposes. For this study, ten 1 mm^2 explants per area were washed in MEM containing 3% fetal calf serum and gentamicin (as in the first study) was added. The explants were allowed to cultivate for 14 days in Petri dishes coated with Vitrogen 100.

Outgrowth rates were counted every second day. Histomorphometrical examinations were conducted on the explants from all areas. For this purpose, the tissue was fixed with Davidson's solution (formaldehyde 38% w/v, ethyl alcohol 95% v/v, acetoacetic acid 98% v/v), distilled water in proportions of 2 : 3 : 1 : 3, and placed in paraffin. Thin, 5 μm vertical skin cuts were tinted with hematoxylin/eosin and observed under a microscope (Leitz Orthpan) with ×40 magnification. At this time, epithelial cells, fibroblasts, cell-rich and cell-poor connective tissues were individually circled with a felt tip pen and the markings copied onto transparent graph paper. The area of these markings (appearing in square millimeters on the graph paper) were calculated and the percentages of the total surface area of each colony for each specific cell type was established.

Results

Outgrowth Rates

In the first study, the maximum outgrowth of epithelial cells (percentage of outgrowth compared to the initially existing explants) in the transverse section was seen at the ulcer edge with increasing outgrowth when approaching area 4/5. The maximum outgrowth of explants in area 0/1, actually the edge of the ulcer, was 8.3% ± 7.2%, it was 35.4% ± 14.0% for area

2/3, and 62.9% ± 14.2% for area 4/5. Explants of the control skin had an outgrowth area of 87.7% ± 7.8%. A comparison shows that the values for area 0/1 are significantly lower than those for area 4/5 ($p < 0.05$). No difference existed in the behavior of fibroblasts between the various areas; they will not be considered further in this study. The results of the second study, utilizing different and more modern laboratory techniques, resulted in similar readings. The individual outgrowth area for explants of area 0/1 were 4%–21%, for area 2/3 35%–42%, and for area 4/5 60–80%. Explants of the control skin reached outgrowth areas between 78% and 88%.

Histomorphometry

The histomorphometry of the normal (control) skin of both studies showed little cell growth, considerable fiber-rich connective tissue, and less than 5% growth of epithelial cells. The scatter range of the epithelial cells from skin of the ulcer edge, area 0/1, showed a larger proportion of epithelial cells, between 1.9% and 48.2%. While the skin from areas 2/3 and 4/5 contained a larger percentage of epithelial cells (3.9%–14.6%) than the control skin (<5%) their share remained smaller than that from area 0/1.

Discussion

Already 12 h after a trauma (injury) mitotic activity of epithelial cells and fibroblasts within at least 12 mm around the ulcer area can be observed. A short time later, these cells start their migration in the direction of the ulcer or wound and reepithelialize the ulcer in a normal wound healing process (Morhenn 1988; Pierce et al. 1991). Mitosis and migration of a cell do not occur simultaneously. It can be said that migration is an active process which occurs independent from mitosis (Marks and Nishikawa 1973). It is unknown what causes separation and migration of cells during normal wound healing and why these are largely absent during impaired wound healing. Chronic decubitus ulcers do not heal or do so at a very slow rate. In this study, the migration potential of epithelial cells from the ulcer edge (maximum outgrowth of area 0/1 = 8.3% ± 7.2%) of chronic decubitus ulcers was greatly reduced in comparsion with epithelial cells and fibroblasts of the ulcer region (maximun outgrowth for area 4/5 = 62.9% ± 14.2%) and normal skin (maximum outgrowth of 87.7% ± 7.8%). Such significantly reduced maximum outgrowth could be the result of reduced mitosis and reduced migration potential. The maximum outgrowth surface of colonies depends more on migrating ability than on mitotic activity. Therefore, the significantly reduced maximum outgrowth surface of the epithelial cell colonies (results are not shown here) are based on the impaired migration potential rather than on defective mitosis. Further clarification is derived from the histomor-

phometry which is an in vivo representation of the situation. It indicates a significantly larger percentage of epithelial cells in the area 0/1 (1.9%–48.2%), where the maximum outgrowth is lowest (8.3% ± 7.2%), than in areas 2/3 and 4/5 (3.9%–14.6%) and in the control skin (< 5%) where, by contrast the maximum outgrowth is highest. It appears that the epithelial cells separate in vivo (histomorphometry) but remain backed up near the ulcer edge as if, for some reason, they are unable to migrate across the ulcer base (epithelial cell congestion at the ulcer edge). The only explanation for the contradictory evidence in the behavior of epithelial cells is the reduced migration potential of these cells. Most of the epithelial cells are found in vivo (histomorphometry) near the ulcer edge where they, due to their inability to migrate (lowest outgrowth area in vitro in tissue culture), cannot escape the area of the ulcer edge (epithelial cell congestion). The causes for this defective migration potential of the epithelial cells are not known. All factors which delay wound healing (ischemia, infection, necrosis, fibrin persistence, etc.) can, initially, contribute to this. However, one question occurs: Is there one central factor or the absence of such a factor responsible for all or maybe some of the interference in the wound healing process? Perhaps the search for the cause of defective migration could lead to this factor. It seems important that the epithelial cells of the ulcer edge do not regain their ability to migrate past the edge even under the optimum conditions of a culture. One seems forced to assume that the epithelial cells of area 0/1 lack in vivo one or more factors which would encourage migration. In vivo, the cause may be ischemia, infection, fibrin persistence, etc. However, this defect (one or more missing factors) has to have been brought into the culture by the particular cell in which in vitro migration is either inhibited or not stimulated. This defect (or lack of one or more factors) does not seem to depend on the ulcer or the area surrounding the ulcer because it continues to be exhibited even in the optimum conditions of the culture. Is the factor in the epithelial cells (or in the fibroblasts) no longer paracrine or later induced as autocrine? This means that the factor strongly affects the chemotactic properties of the epithelial cell.

An absence of the factor could explain the poor migration of the epithelial cell even in the culture. In which direction does the pathophysiology of wound healing point? Macrophages are at the center of wound healing. Together with thrombocytes and monocytes they supply the wound with important growth factors, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factors α and β (TGF-α and TGF-β), and others (Assojan and Sporn 1986; Dijke and Iwata 1989; Hebda 1988; Wahl et al. 1987). Due to the poor vascularization of the ulcer base and the ulcer edge (Witkowski and Parish 1982), a reduced supply of the ulcer tissue and especially of the ulcer edge with growth factors by way of microcirculation is conceivable. Evidence for the existence or absence of these growth factors, especially of TGF-β in

the ulcer tissue, could explain the defective migration potential of epithelial cells from the ulcer edge.

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Fibronectin and Its Role in Wound Healing

H. Hörmann

In a healing wound fibronectin appears in various stages. It is present in the fibrin clot which closes and protects the wound and it is synthesized in the granulation tissue. Consequently, it plays a role in several processes which is possibly due to its multifunctional nature.

Fibronectin (for reviews see Ruoslahti 1988; Hynes 1990) exists in fibrous form mostly in the pericellular space of fibroblasts and some other cells. In addition, there is a plasmatic form circulating in blood. This plasmatic form also can be incorporated into fibrils (Oh et al. 1981).

Plasma fibronectin (molecular weight approximately 500 kDa) is a thread-like molecule with a length of approximately 120 nm and a diameter of about 2 nm (Engel et al. 1981). It consists of two very similar subunits of about 61 nm length connected close to their COOH-terminal ends by disulfide bonds. In solution the flexible thread is coiled to a more compact conformation exhibiting a disk-like shape (Marković et al. 1983). In fibrils there is evidence that stretched molecules overlap on each side with regions comprising about half of the subunit length with preceding or subsequent dimers giving rise to long linear strands (Hörmann and Richter 1986). Figure 1 shows a tentative model of a fibronectin fibril in which three strands are arranged in a staggered way. The fibrils are stabilized mainly by intermolecular disulfide bridges which, however, have not yet been located (Hynes and Destree 1977).

The elongated structure of the molecule results from a sequence of structural repeats in the subunit peptide chains each homology expressing a globular shape (Fig. 2; Kornblihtt et al. 1985). Three types of homologous sequences can be discriminated (Petersen et al. 1983). Most type I repeats are concentrated in the NH_2-terminal third which also contains two type II repeats. It follows a sequence of 15–17 type III repeats, the number varying in the different isoforms. The last type III repeat is separated from the preceding ones by a structureless connecting segment (III-CS) also termed V-region. At the COOH-terminal again three type I repeats are located.

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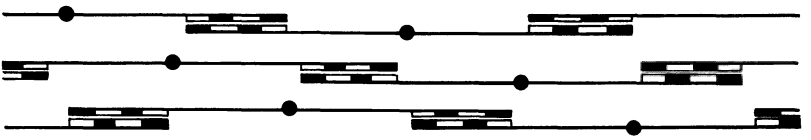

Fig. 1. Tentative model of fibronectin assembly in pericellular fibrils. Subsequent fibronectin molecules overlap in broad NH_2-terminal regions comprising four domains of alternating net charge (Hörmann and Richter 1986). Three infinite strands assemble in a staggered way to final fibrils. *Filled circles*, disulfide bonds connecting fibronectin subunits at their COOH-terminal; *filled and open boxes*, domains with positive or negative net charge, respectively

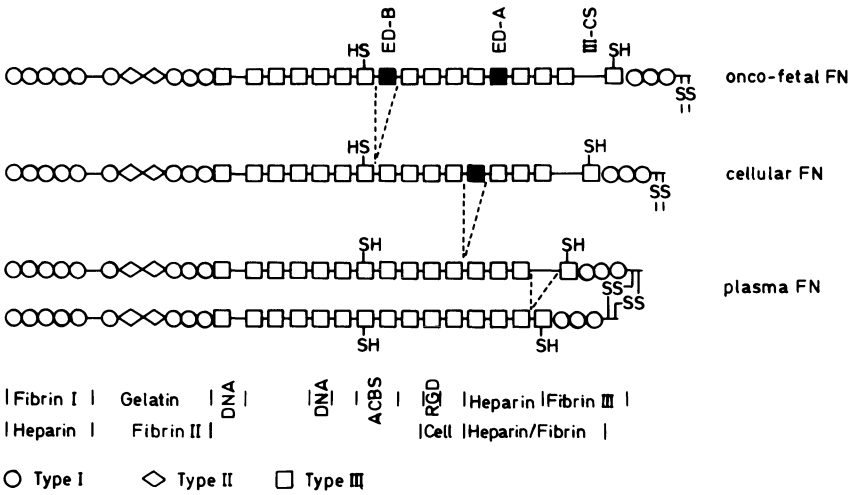

Fig. 2. Fibronectin peptide chains containing a sequence of homologous repeats of three different types. Isoforms differ in the deletion of special repeats. Domains with affinity for matrix substances or cell receptors are indicated. *ACBS*, alternative cell binding site; *RGD*, cell-binding sequence Arg-Gly-Asp; *III-CS*, connecting segment between type III homologies, also termed V-region

Within the fibronectin peptide chains the various homologous sequences are grouped to functional domains which express binding sites for matrix substances, cell receptors and complementary sites involved in self-association (Fig. 2). Well characterized are domains with affinity for fibrin, heparin and gelatin. A type III repeat with the sequence Arg-Gly-Asp (RGD) is recognized by a fibroblast receptor of the integrin type (Ruoslahti and Pierschbacher 1986). Integrins of other cells bind to the V-region (Humphries et al. 1986) and unknown receptors have affinity to alternative cell binding sites (ACBS) in the central region (Obara et al. 1987). Also the heparin-binding domain may interact with cell-surface components (Rogers et al. 1985). Recently, a self-association site was characterized in a basic region subsequent to the gelatin-binding domain (Morla and Ruoslahti

1992). Data from Hörmann and Richter (1986) suggest that the gelatin-binding domain might provide the complementary site. In the model in Fig. 1 these two sites are in a juxtaposition within the overlap region. Another site necessary for fibronectin assembly exists in the NH_2-terminal fibrin-binding domain (McKeown-Longo and Mosher 1985; McDonald et al. 1987). Close to the amino ends of the two chains there is a transamidase-sensitive site (McDonagh et al. 1981) which allows covalent cross-linking of fibronectin with fibrin or with other fibronectin molecules (Mosher 1975).

There exists a number of isoforms of fibronectin, the most important being presented in Fig. 2 (Kornblihtt et al. 1985; for review see Hynes 1985). All variants are coded by the same single gene. The differences are due to splicing events at the mRNA level giving rise to deletion of special sequences. The complete information of the peptide chains is expressed in an oncofetal variant primarily found in fetal and tumor tissue (Zardi et al. 1987) but also present in the aorta (Takasaki et al. 1992). In contrast, fibronectin fibrils in the pericellular space of fibroblasts are composed of another variant termed cellular fibronectin. In this form one type III repeat termed extradomain B (ED-B) is spliced out. The peptide chains of the oncofetal and the cellular fibronectin predominantly exist in fibrils of infinite length stabilized by covalent cross-links.

The plasmatic form of fibronectin consists of two similar but not identical peptide chains which are connected close to their COOH-terminal ends by a pair of disulfide bonds. In both peptide chains ED-B and a further type III repeat, extradomain A (ED-A), are deleted. In addition, in one of the two chains the connecting segment or V-region in the COOH-terminal half is missing.

Within a wound different fibronectin variants are distributed over several locations (Fig. 3; Ffrench–Constant et al. 1989). In the fibrin clot the plasmatic variant A−, B−, V+/− is closely associated with the fibrin net-

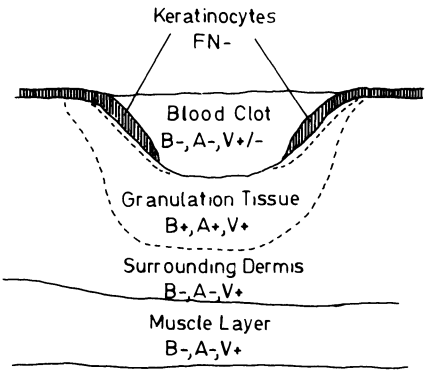

Fig. 3. Distribution of fibronectin (*FN*) variants in a healing rat skin wound. (From Hynes 1990)

work and with platelets. Most likely, it provides there attachment sites for invading cells, for instance macrophages and neutrophils which ingest wound debris and kill bacteria. At the bottom of the wound, however, synthesis of new fibronectin is activated. High levels are found in the granulation tissue particularly beneath the tongue of invading epidermal cells. Analysis of the fibronectin mRNA yielded all alternatively spliced segments indicating the variant A+, B+, V+. It is believed that this variant forms at the interface to the clot with fibrin a special structure which functions as a provisional matrix for the invading keratinocytes (Clark et al. 1982). Later, these cells replace the matrix by synthesis of their own basement membrane. Minor fibronectin synthesis is also induced in the surrounding dermis and muscle layer, where the type A−, B−, V+ is expressed.

In the granulation tissue, synthesis of novel connective tissue, collagen and proteoglycans, takes place. At this stage pericellular fibronectin fibrils generally show a codistribution with collagen, mainly collagen type III (Furcht et al. 1980), and there is some evidence that fibronectin fibrils function as a scaffold for the assembly of collagen precursors and their processing to fibrils. This theory is based on the finding that Fab' fragments of antibodies which inhibit the assembly of fibronectin to fibrils on a fibroblast surface also block the expression of collagen fibers by those cells (McDonald et al. 1982).

To investigate the cellular factors required for the assembly of fibronectin on a cell surface, model experiments with macrophages were helpful. These cells express fibronectin receptors but are unable to induce a polymerization of fibronectin once nucleation molecules are bound by the receptor. Fibronectin receptors had been shown to be essential for the expression of fibronectin fibrils as demonstrated on fibroblasts which contain receptors of the integrin type $\alpha_5\beta_1$ (McDonald et al. 1987). Although macrophages appear to have fibronectin receptors of another type, most likely $\alpha_4\beta_1$ recognizing the V-region, the experiments given below suggest that the type of receptor is of minor importance for fibril formation at a cell surface.

The retention of soluble fibronectin by macrophages is considerably increased in presence of heparin (Fig. 4, dashed curve; Hörmann and Jelinić 1980). This result is explained by the formation of fibronectin aggregates at the cell surface. On fibroblasts the heparin effect is less pronounced, most likely because these cells already contain heparin-like compounds at their surface which had been characterized as heparan sulfate proteoglycans. Therefore, it is suggested that heparin-like membrane components are essential for the aggregation of fibronectin at a cell surface. Actually, heparan sulfate proteoglycans codistribute with fibronectin in pericellular fibrils of fibroblasts (Hayman et al. 1982; Hedman et al. 1982).

Another cell-associated factor involved in fibril formation was recognized when the heparin-dependent fibronectin aggregation was compared on macrophages of young and old guinea pigs (Hörmann et al. 1989). The

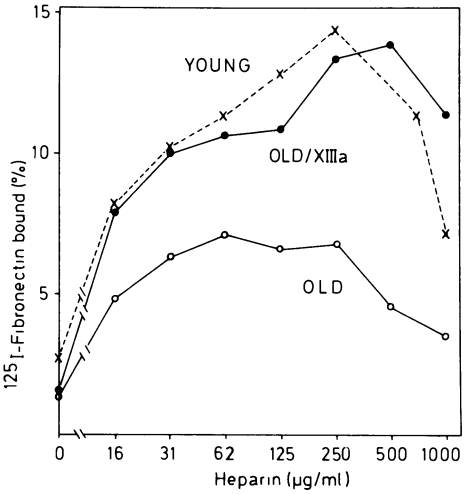

Fig. 4. Heparin-dependent accumulation of plasma fibronectin on macrophages of 12 week old (*dashed curve*) and 33 week old (*solid curves*) guinea pigs. The *upper solid curve* represents data obtained with macrophages pretreated with thrombin-activated coagulation factor XIII (260 µg fibronectin, 400 ng ^{125}I-fibronectin, 10^7 cells per ml). (From Hörmann et al. 1989)

dashed curve in Fig. 4 shows the results obtained with macrophages of 12 week old animals. The lower solid curve demonstrates that on cells of 33 week old animals the heparin effect was considerably reduced. If, however, the macrophages of the older animals had been pretreated with plasma transamidase (thrombin-activated coagulation factor XIII), the heparin-dependent accumulation was elevated up to the level of younger animals. It is suggested that plasma transamidase attaches to the surface of the macrophages and promotes there the aggregation of fibronectin by cross-linking. Most likely, in the absence of the transamidase only weak reversible fibronectin aggregates can form. In the presence of cell-attached transamidase, however, they are stabilized by cross-linking so that fibril formation becomes irreversible.

Principally any cross-linking enzyme should be able to stabilize fibronectin aggregates at a cell surface. There are also some doubts whether plasma transamidase or a related enzyme is the real factor as pericellular fibronectin fibrils are mainly stabilized by disulfide bonds rather than by transamidase-mediated bonds (Hynes and Destree 1977). However, cell associated disulfide isomerases are not yet characterized. Further investigations are therefore necessary to clarify whether, in the special situation of cell surface, transamidases, which are SH-enzymes, also can catalyze disulfide rearrangements. There is also evidence that tissue transamidases are closely associated with fibronectin in pericellular fibrils (Birckbichler and Patterson

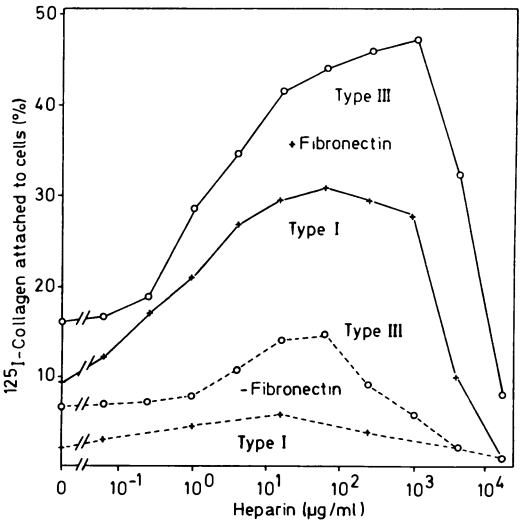

Fig. 5. Fibronectin-mediated binding of soluble ^{125}I-collagen type I and III in dependence of heparin by macrophages (260 μg fibronectin, 1 μg ^{125}I-collagen, 10^7 cells per ml). (From Hörmann 1982)

1978). In addition, the requirement of transamidases in wound healing is well known.

The data obtained with the macrophage model system suggest that assembly of fibronectin on a cell surface requires the cooperation of fibronectin receptors with heparin-like membrane components, most likely heparan sulfate proteoglycans, and cell-associated transamidase or any other cross-linking enzyme.

Further experiments demonstrate that a heparin-promoted fibronectin association on macrophages also improves the binding of radiolabeled native soluble collagen type I and type III by those cells (Fig. 5; Hörmann and Jelinić 1981; Hörmann 1982). Obviously, the soluble collagen binds to the cell-attached fibronectin aggregates. In the absence of fibronectin heparin only had a marginal effect on collagen binding.

These data support the idea that cell-associated fibronectin aggregates are capable of binding soluble collagen as a first step of cell-promoted collagen fibril formation.

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Mechanisms of Action of Collagenase in Wound Repair

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Introduction

Specific collagenases possess the unique capability of degrading native collagen which is otherwise resistant to breakdown by other known tissue proteases [1]. Reports suggest that degradation products of collagen released after cleavage by collagenase may in turn control the migration and activity of important inflammatory cells such as wound macrophages and, therefore, at a very early stage in wound repair substantially influence the healing process [2–4]. Abnormal collagen deposition as a result of alterations in collagenolysis may be seen in various pathologic conditions such as hypertrophic scars and keloids [5]. Excessively high collagenase activity is found in rheumatoid arthritis, local tumor invasion, excessive bone resorption following bone injury, anastomotic insufficiency following bowel surgery and post-burn granulation tissue [6–11] (Table 1). Selective therapeutic enhancement or blockage of the collagenolytic system may therefore prove a valuable tool in treatment or prevention of these conditions.

Mammalian Collagenases

Mammalian collagenase is an endoprotease (proteinase) (Fig. 1) which cleaves the collagen molecule triple helix at physiologic (neutral) pH and temperature. There is exactly one susceptible sequence which predetermines decomposition, placed at two thirds along the molecule [12]. The specificity with which mammalian collagenases cleave their substrate at a defined site is truly remarkable [13]: The target amino acid sequence is gly-ile in collagen type I, which appears three times in the $\alpha 1$ chain, and gly-leu in collagen type III, which appears 18 times in the $\alpha 1$ chain. The cleavage of

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Table 1. Collagenase activity

Condition	Activity	Source
Healing wounds	↑	Grillo and Gross [10]
Post-burn granulation tissue	↑	Kishi et al. [9]
Keloids	↓	McCoy
Hypertrophic scar	↑	Cohen et al. [44]
Bone resorption	↑	Harris and Krome [1]
Anastomotic insufficiency of the bowel	↑	Hawley
Peridontitis	↑	Fullmer et al. [20]
Diabetic rats	↑	Black et al. [11]
Rheumatoid arthritis	↑	Evanson

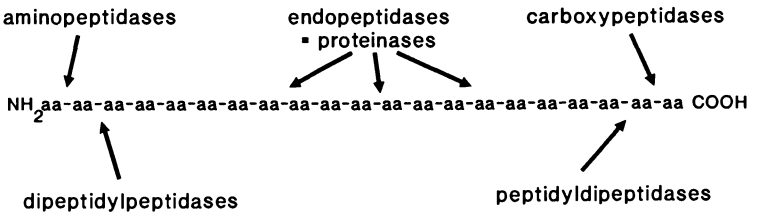

Fig. 1. Different classes of proteolytic enzymes and their sites of cleavage

collagen type I always takes place between residues 775 and 776 of the $\alpha 1$ chain. This seems to be a weak point in the triple helix structure, resulting in typical lengths of cleaved fibrils that are three fourths and one fourth of the helix. Collagen types II and III have comparable results after cleavage. Consequently, chains appear to be parted into long NH_2 -terminal and short COOH-terminal fractions.

Localization

Only two biochemically distinct interstitial collagenases have been descibed to date: the human neutrophil collagenase (HNC) [14] and the human fibroblast collagenase (HFC) [15]. They are capable of degrading interstitial types I–III collagens. Two type IVcollagenases(gelatinases) in 72 and 92 kDa forms have been described. However, their biological function has not yet been clearly defined. They degrade type V, VII and X collagens and gelatin. As to the ubiquitous presence of connective tissue, the two interstitial collagenase types HNC and HFC are produced by a number of mesenchymal cells. Collagenase is not an intracellularly stored enzyme nor is it constantly released. Its synthesis can only be induced [16], making

collagenase extraction from tissue very difficult. Therefore, knowledge as to the different sites of collagenase production is still incomplete. As a matter of fact, fibroblasts in the upper papillary dermis produce more collagenase than those in deeper dermal areas [17]. In wound healing two types of collagenase have been detected, one synthesized by epithelial cells, the other by granulation tissue [18]. Large amounts of collagenase have been found in supernatants of synovial cell cultures, derived from patients suffering from primary chronic arthritis [19]. Its production is triggered by a mononuclear cell-derived factor, in this case leading to the destruction of collagen-containing structures of the joint. Collagenase activity has been confirmed by examination of the collagen fractions. Another known location for collagenase production is gingival tissue, as it has been detected near erosions which are found in gingivitis/peridontitis [20]. The characteristic breakdown of connective tissue is initialized by bacterial plaques on teeth, triggering mononuclear cells which in turn release factors activating collagenase. But most important, considerable amounts of tissue breakdown and remodeling take place at the site of classic inflammation. Therefore, it was suspected early on that hematopoietic cells invading these sites are capable of producing collagenase, which was confirmed later: azurophilic granules of granulocytes contain collagenase [21] aside from elastase and chymotrypsin [22].

Lymphocytes, monocytes and macrophages [23] produce collagenase and at the same time secrete lymphokines and monokines triggering collagenase synthesis by fibroblasts [24]. There are two known mechanisms of collagenase mediated breakdown of collagen by neutrophilic granulocytes: (1) Extracellularly partly cleaved fibrils are taken up by the phagocyte via endocytosis and completely digested by intracellular lysozymes (collagenase and other granule derived proteases). (2) Most of the cleavage takes place in pocket-like structures on the surface of phagocytes which largely protect collagenase from serum inactivators [16]. These pockets can be identified in electron microscopic preparations and have been called "ruffled borders." The importance of the intracellular location of collagenase activity is further underlined by the fact that collagenase secreted into the extracellular matrix is immediately degraded by serum-derived inhibitory factors.

In conclusion, there are two main cell groups responsible for collagenase mediated collagen breakdown: de novo synthesizing mesenchymal cells releasing collagenase into extracellular matrix, where it takes effect, and phagocytic cells storing the enzyme in intracellular granules, lysing collagen either on their surface or intracellularly. The interaction of these two types might play a key role in the regulation of rapid tissue breakdown.

Regulation of Synthesis and Activation

How collagenase production is regulated has been studied most intensively in synovial cells of patients with rheumatoid arthritis. By adding peripheral blood monocytes to cell cultures of dendritic cells, collagenase production

is triggered in a reciprocal manner. The reason for this was attributed to a 14–24 kDa protein produced by monocytes which was found to be interleukin-1 (IL-1). Several laboratories have shown IL-1's responsibility for the induction of fibroblast collagenase synthesis [25]. Bauer et al. could show that platelet-derived growth factor (PDGF) stimulates human skin collagenase expression in vitro [26]. Other factors influencing collagenase synthesis are transforming growth factor-β1 (TGF-β1) [27], basic fibroblast growth factor (bFGF) [28], prostaglandin (PG) E_2 (increase), indomethacin (decrease by inhibiting PGE_2 synthesis [29]) vitamin A [30], corticosteroids (decrease) [31], and the Fc portion of IgG (increase by stimulating macrophages) [32].

What mechanism activates the "sleeping" ubiquitous enzyme? Since the consequences of tissue destruction in the case of a control defect may be fatal, it has to be a highly sensitive pathway. In vivo experiments have shown several factors, e.g., cathepsin B, plasmin and kallikrein to activate collagenase directly. Unnamed factors have been detected which promote collagenase synthesis in macrophages of the rabbit lung and rat uterus postpartum. Also it was found that progesterone indirectly inhibits collagenase secretion by suppressing the activating factor.

Indirect activation has been found in bone tissue: an endogenous activation system including at least one protease, which in turn had to be activated itself, was responsible for the onset of collagenase action.

In vitro activation can be elicited by a number of reagents reacting with thio- or disulfides and by proteases such as trypsin, plasmin, chymotrypsin, cathepsin B, kallikrein, thermolysin and pepsin. The exact biochemical process taking place in activation is as yet unknown. It is most probably an enzyme-linked inhibitor which is decleaved or the zymogen is activated by cleavage at some part of the molecule. The activating mechanism in human neutrophilic granulocytes is more defined and might be transferable to other sites: latent collagenase appears to be a mixed disulfide consisting of active collagenase (65.5–67 kDa) and an inhibitor (20–25 kDa). The enzyme is activated by reduction of the disulfide linkage. Interaction with the glutathione redox system provides regulation and continuation of metabolic events. Collagenase can also be activated by a conformation change without any measureable molar weight loss/gain (as an effect of chaotropic reagents in vitro and human serum factors, human skin elements and rat uterus elements in vivo).

The presence of divalent metal ions (Ca^{2+} and Zn^{2+}) is of high importance with respect to possible activation, stability and effectiveness of the enzyme. Chelators such as EDTA inhibit collagenase completely. The two structurally distinct interstitial collagenases differ in their reactivity towards various collagen types: HFC for example lyses both type I collagen and type III [15, 33], whereas HNC attacks type I much quicker than type III [34]. Type II generally seems to be more resistant to collagenolytic influence than the other types. This has to be ascribed to different three-dimensional

factors, especially to the amount of fibril cross-linkings. The reason for the considerable speed of collagen degradation in postpartum uterus might be a relatively small amount of cross-links due to its young age. The resulting collagen fractions denature at lower temperatures than the native molecule, e.g., type I at 32°C, which results in instability of the triple helical structure. Subsequently, susceptibility to nonspecific proteases increase and degradation is accelerated.

There are several factors which terminate collagenase activity: the most potent plasma-derived inhibitor is α2-macroglobulin [35], due to its molecular size only active when transported out of blood vessels together with other blood components or in the case of vessel disruption. Thus, inhibitors of smaller molecular size play a major role, such as a specific β1-collagenase inhibitor (40 kDa) and α1-antitrypsin (54 kDa). The latter is known to inhibit collagenases of skin fibroblasts, neutrophilic granulocytes, thrombocytes and human synovium. Interestingly, it has been found that parts of the procollagen peptide, released into the extracellular matrix during collagen synthesis, act as inhibitors of collagenase thereby protecting the newly built molecule from inactivation.

Bacterial Collagenases

Bacterial collagenases were discovered long before mammalian ones. They have been mostly used experimentally in laboratories and as pharmacological agents. The best characterized bacterial collagenase is produced by *Clostridium histolyticum*, easily available since it is secreted by the bacterium into the culture medium in large quantities. In contrast to mammalian collagenases bacterial collagenase activity results in small peptides at an approximate size of five amino acids. Some larger residues have also been detected. It was demonstrated that short segments are clipped off sequentially from each end of the molecule [36, 37]. The initial digestion reactions seem to be of the first order; with increasing amounts of cleaved particles, second order reactions predominate.

Clostridium histolyticum-derived collagenase cleaves all five collagen types at nearly the same rate [38]. Differences in digestion rate have been found (at least in collagen type I) when using substrate from individuals of various ages. Collagen derived from diabetic patients was determined to behave like that of elderly people. Like mammalian, the clostridial collagenase is a metalloenzyme each containing one zinc atom, demands calcium for stability and is inactivated by EDTA, cysteine or 1,10-phenylanthraline. The molecule possesses functional groups, such as carboxyl tyrosine or lysine; blocking them inactivates the enzyme at once.

Collagenase as a Therapeutic Tool

Advancements in the understanding of the biology of healing wounds have permitted the developement of surgical techniques and pharmacological agents to support and expedite the repair process. Skin wounds may be partial-thickness or full-thickness depending upon the depth of the wound. The former will heal by reepithelialization starting from the epithelial margin. The full-thickness wound involves the subcutaneous tissue and will heal either by primary or secondary intention.Healing by primary intention is simply achieved by suturing the two edges of the wound together. Healing by secondary intention is a step by step process involving the formation of granulation tissue, collagen formation, wound contraction and reepithelialization.

Inhibition of the repair process may result in chronic nonhealing wounds and as a consequence abnormal scar formation. Retardation of tissue repair may be a result of systemic factors such as diabetes, circulatory impairment, generalized immunosuppressed states (transplant recipients, AIDS, tumor patients) or local factors such as bacterial infection and surface debris. The primary goal in the treatment of problem wounds healing by secondary intention should be rapid wound closure. In order to achieve good functional and cosmetic results, thorough wound debridement is the mandatory first step within a cascade of treatment modalities (Fig. 2). If wound infection is present this may be done in combination with the application of disinfecting agents. However, such agents should not be used over prolonged periods of time since most of them (e.g., polyvinyliodine) demonstrate cytotoxic activity inhibiting fibroblast proliferation necessary for ultimate wound closure and mature scar formation. Wound debridement means prompt removal of non-viable debris and pus from the wound surface. This can be done by careful surgical excision and the additional application of proteolytic enzymes (Table 2). Although a variety of enzymes (e.g., trypsin-chymotrypsin,

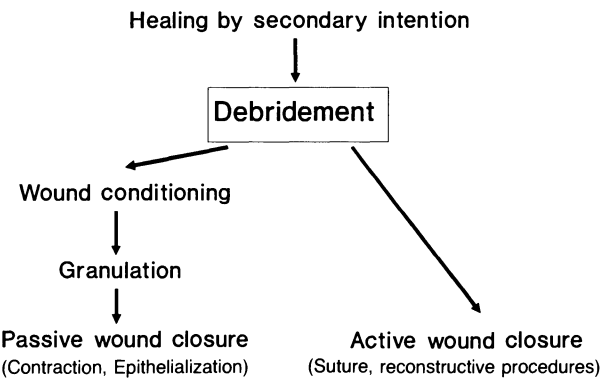

Fig. 2. Local wound treatment

Table 2. Enzymes used in wound debridement

Enzyme	Use
Cathepsin B	Hydrolyzes proteins with a specifity resembling that of papain
Collagenase	Degradation of helical regions of native collagen to small fragments; cleavage: -Gly in the sequence-Z-Pro-X-Gly-Pro-X
Deoxyribonuclease	Endonucleolytic cleavage to 5′-phosphodinucleotide and 5′-phosphooligonucleotide end-products; preference for double stranded DNA.
Elastase	Cleaves bonds involving the carbonyl group of amino acids bearing uncharged nonaromatic side chains; hydrolysis of elastin.
Fibrinolysin	Preferential cleavage: Lys-, Arg-; converts fibrin into soluble products; formed from plasminogen.
Hyaluronidase	Random hydrolysis of 1,4-linkages between 2-acetamido-2-deoxy-β-D-glucose and D-glucuronate residues in hyaluronate
Papain	Preferential cleavage: Arg-, Lys-, Phe-X-
Streptokinase	Preferential cleavage: Arg-Val in plasminogen; converts plasminogen to plasmin
Streptodornase	Endonucleolytic cleavage to 3′-phosphodinucleotide and 3′-phosphooligonucleotide end-products; prference for double stranded DNA.
Trypsin	Preferential cleavege: Arg-, Lys-

streptokinase-streptodornase, desoxyribonuclease-fibrolysin, ficin, papain) have been clinically tested, bacterial collagenase has proven most successful. The reason for this is that collagease may specifically hydrolyze native collagen, whereas the other enzymes can not. The collagenase available for clinical use is obtained from *Clostridium histolyticum* (Santyl, Iruxol). Its action on native collagen has been discussed earlier. Extensive clinical studies using bacterial collagenase as a debriding agent have been undertaken by several investigators especially in patients with burn wounds, decubitus ulcers, peripheral vascular disease and diabetic ulcers [39–42]. All have shown a significant acceleration of wound healing on the basis of enhanced granulation tissue formation and epithelialization. Interestingly, especially in third degree burn wounds, hypertrophic scarring and scar contracture were markedly reduced in collagenase treated wounds [43]. Although at the moment no conclusive experimental data exist, bacterial collagenase may not only expedite debridement by attacking native collagen of all types but also by enhancing macrophage chemotaxis and activation within the wound itself, thus acting to some extent as an immunomodulating agent and wound conditioner. Postlethwaite and Kang [2] have reported that macrophages and precursor monocytes which are key promoters in the repair process demonstrate enhanced chemotaxis upon contact with collagen-derived peptides generated by bacterial collagenase. This same effect was shown on

fibroblasts but not neutrophils. Collagen-derived peptides seem to promote granulation tissue formation by the above mechanism. Furthermore, an increase in macrophage numbers and their activation will lead to enhanced cytokine secretion in these wounds. This represents a cascade of events potentiating the immunostimulatory effect with substantial influence on collagen formation, thus conditioning the wound for further procedures such as skin grafting.

The reason why a remarkable reduction in hypertrophic scarring is encountered in collagenase treated wounds is not known [39, 40]. Hypertrophic scars show an increase in collagen deposition with an increase in the ratio of type III to type I collagen. This may be due to excessive production by activated fibroblasts or the decreased degradation by tissue collagenase. Although collagenase activity in hypertrophic scars is increased there is evidence that the level of collagenase inhibitors such as α2-macroglobulin and tissue inhibitor of metalloproteinases (TIMP) is also elevated [44]. This may lead to an overall net decrease in collagen degradation with preference for certain collagen types as has been shown in post-burn wound tissues, where increased collagenase activity leads to enhanced type I collagen breakdown [9]. Bacterial collagenase degrades all types of collagen with almost the same affinity and may therefore prevent certain pathologic collagen ratios from becoming established, although at the moment there are no reports to support this hypothesis. Direct intradermal injection of clostridial collagenase could be promising in the treatment of hypertrophic scars and keloids. Friedman et al. [45] have shown, in an experimental pig model, that intradermal injection of collagenase in combination with hyaluronidase may cause dramatic degradation of dermal collagen in a dose-dependent manner, although no clinical trials have been reported to date. Further studies are needed to clarify the effect collagenase treatment of wounds has on remodeling and scar formation. However, it is an intriguing thought that selective degradation of native collagen may be a useful therapeutic tool in conditions associated with abnormal scar formation.

Conclusions

Recent advances in our understanding of basic mechanisms of wound repair shed new light on clinical wound healing agents such as collagenase, raising new questions concerning their mode of action and possible therapeutic application. A new concept is that cells of the immune system play a dominant role in the repair process by releasing potent mediators such as cytokines and growth factors which influence fibroblast function and angiogenesis dramatically. The first clinical trials concerning the direct application of such cloned pure growth factors have been disappointing as that they have not yet proven superior to established treatment regimens. However, currently many studies are being undertaken to clarify their possible role

as clinical wound healing agents. It might also be worthwhile to study old and new pharmacological substances with respect to their actions on promoter cells such as wound macrophages, which are naturally occurring production sites of those growth factors now being tested as pure preparations. It could prove more useful to modulate macrophage function, since it stands at the starting point of a whole cascade of mediators representing an intricate network of control mechanisms which are only poorly understood.

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Studies of Growth Factor and Matrix Proteinase Activities During Early Stages of Wound Healing

W.Y.J. Chen and A. A. Rogers

Introduction

The hydrocolloid dressing (HCD) DuoDERM$_{TM}$ (ConvaTec, Bristol-Myers Squibb) has been shown to promote the dissolution of eschar at the wound surface and promote angiogenesis in granulation tissue [1]. Wounds dressed with HCD are characterized by the accumulation of wound fluid which has been shown to contain growth factor activities and factors that stimulate fibroblast urokinase production [2]. Urokinase-type (uPA) and tissue-type (tPA) plasminogen activators (PAs) are proteinase activators and are implicated in the biology of wound healing. Despite the implicated importance of PAs, direct demonstration of PA activities in dermal wound healing, their spatial distribution and temporal relationship with stages of wound healing have not been reported. We have used a recently developed technique to demonstrate PA activities during the healing of HCD-dressed wounds.

Materials and Methods

A full-thickness porcine wound model (Yorkshire White piglets, 20–30 kg) was used in these studies. Wound fluid samples were collected on days 1, 2 and 3 after initial wounding. The samples were dispersed in cell culture medium (Dulbecco's MEM), centrifuged (4500 $\times$g, 20 min), filter sterilized and used directly on fibroblast cultures or after various treatments as indicated in the "Results" section prior to cell culture experiments. Porcine tissue samples were collected from full-thickness dry-dressed (gauze) or HCD-dressed wounds, 2, 6 and 12 days postwounding. Tissues were embedded and cryosectioned (10 μm). Normal human dermal tissue samples were prepared in a similar manner. PA activities were demonstrated by histological zymography [3].

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Results

Figure 1 shows casein plasminogen zymographs of culture supernatant samples supplemented with HCD wound fluid. Stimulation of urokinase accumulation in culture supernatants by wound fluids is shown to increase with wound age. Prior heat treatment (90°C, 5 min) abolishes the stimulation of urokinase accumulation by cultured cells. Wound fluid from HCD-dressed wounds does not contain detectable PA activity (data not shown).

In Fig. 2, prior to culture, test medium samples containing HCD wound fluid (day 2) were preincubated for 1 h with an anti-bFGF (basic fibroblast growth factor) antibody at a variety of concentrations. Fibroblasts were cultured in the medium samples for 72 h. The presence of the anti-bFGF antibody is shown to neutralize the accumulation of urokinase stimulation activity of wound fluid.

Figure 3 shows histological zymographs of days 2, 6 and 12 porcine full-thickness HCD-dressed wounds. By day 6, PA activity is found both in the granulation tissue and in the adjacent subcutaneous tissue. In Fig. 3c,d, a large part of PA activity in the granulation tissue was inhibited by amiloride, suggesting that the major PA activity in the granulation tissue is uPA. By day 12 reepithlialization of the wound has commenced; uPA in the granulation tissue appears to be confined to the peripheral area adjacent to

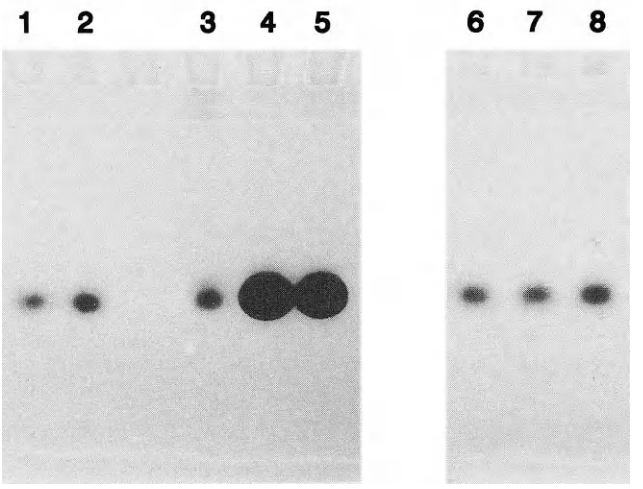

Fig. 1. Casein plasminogen zymographs of culture supernatant samples supplemented with hydrocolloid (HCD) wound fluid collected on days 1–3 after wounding (*tracks 3–5* respectively). Prior heat treatment (90°C, 5 min) abolishes the stimulation of urokinase accumulation by cultured cells (*tracks 6–8* respectively). Urokinase activity from the supernatant of control cultures in serum-free medium and medium supplemented with 5% FCS are shown in tracks 1 and 2 respectively

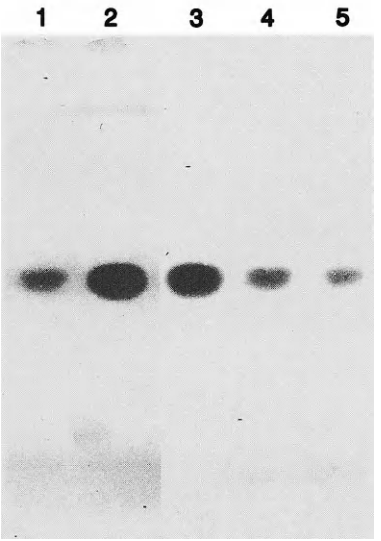

Fig. 2. Neutralization of urokinase stimulation activity of HCD wound fluid by anti-bFGF antibody. *Track 1*, culture medium only; *tracks 2–5*, culture medium with wound fluid and anti-bFGF at 0, 10, 20 and 50 μg/ml respectively

the subcutaneous tissue and beneath advancing "tongues" of epidermis (Fig. 3e,f). tPA activity is prominent beneath the granulation tissue (Fig. 3f). Controls show no protease activity (not shown).

Figure 4 shows histological zymographs of normal human skin. As with porcine skin (not shown), human dermal skin shows both uPA and tPA activities in the dermal and subcutaneous tissue of the skin in a punctate pattern (Fig. 4a,b). Negative controls show no areas of caseinolysis (Fig. 4c).

Discussion

PA activities have not been detected in wound fluid collected from under HCD-dressed porcine full-thickness wounds. However, factors have been found within wound fluid which stimulate the synthesis of uPA by human dermal fibroblasts [2] (Fig. 1). Furthermore, studies have suggested that one of these factors is bFGF [2] (Fig. 2).

By histological zymography, PA activities were characterized in porcine wounds and human skin. In general, initial (day 2) PA activities (uPA and tPA) are associated with the surrounding subcutaneous tissues with distribution similar to that of normal skin (Fig. 3a,b). By day 6, when significant amounts of granulation tissue have formed, PA activity (uPA and tPA) are found in granulation tissue and the surrounding subcutaneous tissue (Fig.

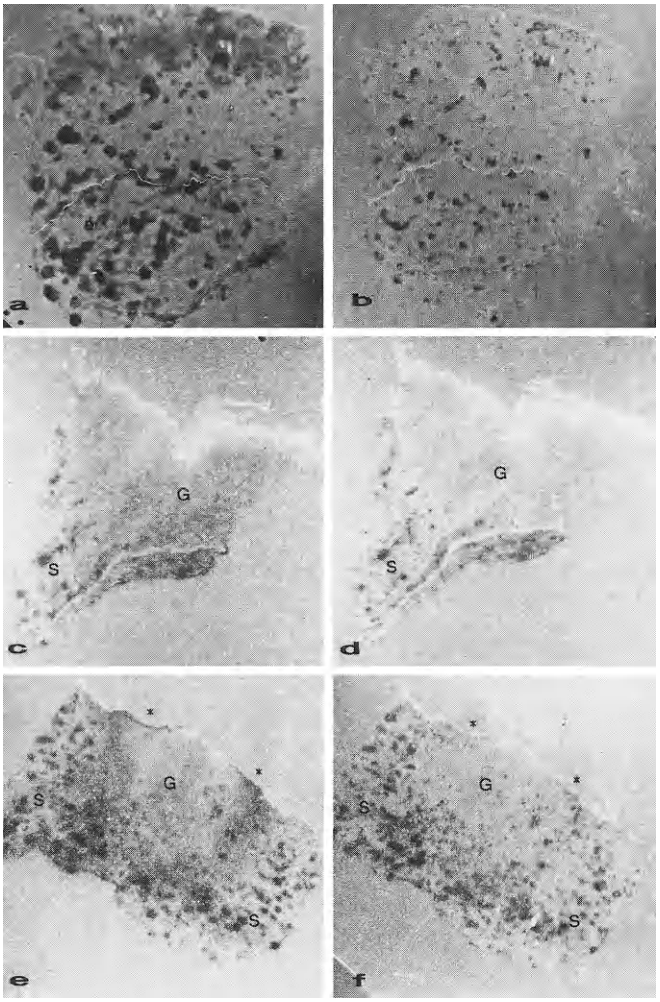

Fig. 3. Histological zymography of days 2 (a,b), 6 (**c,d**) and 12 (**e,f**) porcine full-thickness HCD-dressed wounds. *a,c,e* Cryosections incubated with plasminogen-containing agar-casein overlay, showing the distribution of total PA activity. **b,d,f** Cryosections incubated with plasminogen and amiloride, showing tPA activity. *G*, granulation tissue; *S*, subcutaneous tissue; *asterisks*, advancing "tongues of epidermis". For further details, see text

3c,d). On day 12, reepithelialization and revascularization of the wound are well under way and tPA activity is localized in the tissue beneath the granulation tissue (Fig. 3f) and may represent tPA activity from the ingrowing vasculature. uPA activity is also pronounced in the region between the granulation tissue and the subcutaneous tissues (Fig. 3e,f). uPA, and to a lesser extent tPA, activity are found beneath the advancing epidermis (Fig. 3e,f), suggesting their possible involvement during epithelialization.

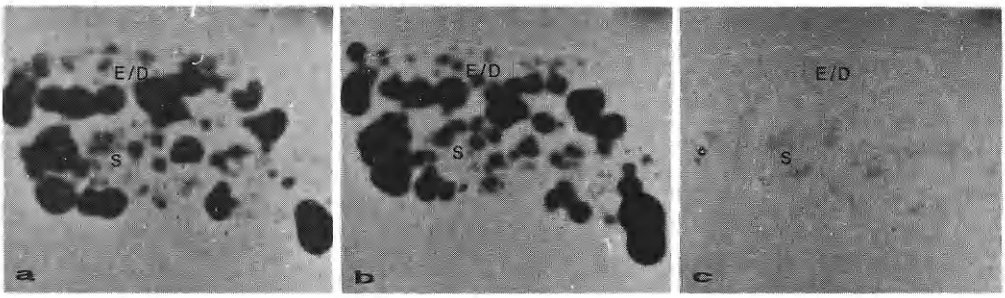

Fig. 4. Histological zymography of normal human skin. *a* Cryosection incubated for 21 h at 37°C with a plasminogen-containing agar-casein overlay, showing the distribution of total PA activity. *b* Cryosection incubated with plasminogen and amiloride, showing tPA activity. **c** Cryosection incubated with overlay without plasminogen (-ve control). *E/D*, dermal tissue; *S*, subcutaneous tissue. For further details, see text

Our results presented here suggest that the absence of significant PA activity in wound fluids may be due to these enzymes being tissue-bound. The spatial and temporal changes of uPA and tPA activity within the wound during healing are currently being investigated in acute and chronic wounds.

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Regulatory Aspects of Collagen Synthesis in Fibroblasts from Human Colon and Skin

T. Hendriks, K.M.L.C. Huyben, and M.F.W.C. Martens

Introduction

Wound healing is defined as a highly regulated sequence of various cellular events leading to reconstitution of tissue integrity following injury [1]. Fibroblasts play an important role in this sequence by producing extracellular matrix components such as the different types of collagen. Collagen deposition is the major determinant of tensile strength in primarily closed wounds. In fact, the number of fibroblasts participating in wound repair is often an indirect measure of the development of wound strength [2]. Collagen metabolism is closely controlled during the process of wound repair. Regulation is probably achieved both by the interaction of fibroblasts with the surrounding extracellular matrix and by cytokines and growth factors known to regulate specifically collagen gene expression and collagen metabolism [3, 4].

Most studies on wound healing have used skin, and although it seems likely that the basic repair sequence is similar for all wounds, it remains to be proven that data from experiments on skin repair may be extrapolated to the healing of other soft tissues like intestine [5]. We have found indications that intestinal wounds and skin wounds react differently to in vivo administration of various drugs. For instance, administration of methylprednisolone does not affect the strength or collagen content of intestinal anastomoses while impairing healing in skin [6]. Also, administration of D-penicillamine, a lathyrogen which inhibits collagen cross-linking, leaves anastomotic strength and collagen solubility unaffected while it lowers strength and increases collagen solubility in skin wounds [7]. Although various explanations for these phenomena are possible, the question arises if production and metabolism of collagen in wounds of intestine and skin are under different control.

Since fibroblasts are the primary collagen-producing cells in healing wounds, we have studied the regulation of the collagen production rate in

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fibroblasts from human colon. No quantitative data are available as yet regarding collagen synthesis in these cells. We have compared the results with those obtained with fibroblasts from human skin.

Materials and Methods

Materials

All supplies for cell culture were purchased from Life Technologies (Breda, The Netherlands). Transforming growth factor-β (TGF-β) from bovine bone was a gift from Dr. G. Ksander (Celtrix Labs, Palo Alto, USA). Interferon-γ (IFN-γ) was obtained from Boehringer (Ingelheim, Germany). Both human recombinant interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα) were a gift from Prof. Dr. J.W.M. van der Meer (University Hospital Nijmegen). L-[2,3-^{3}H] Proline (1.63 Tbq/mmol) was purchased from Amersham International, England. Collagenase (type VII), calcium ionophore (A23187) and dexamethasone were obtained from Sigma (St. Louis, USA). All other reagents were of analytical grade (Merck, Darmstadt, Germany).

Cell Culture

Normal human colon fibroblasts (HCF) were obtained from the American Type Culture Collection (CRL-1459). Human skin fibroblasts (HSF) were obtained from explants of skin biopsies of a healthy adult. Both the HSF and HCF were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) and 10% fetal calf serum (FCS) at 37°C in a 5% CO_2, 95% air humidified atmosphere. Cells were used between the third and tenth passage.

Assay of Fibroblast Collagen Production

Assay of collagen production by steady state, visually confluent fibroblasts was assessed over a 24 h period by [^{3}H]proline incorporation into collagenous protein.

Freshly trypsinized fibroblasts were plated in six-well plates at a density of approximately 1.5×10^5 cells/well in 2 ml DMEM plus 10% FCS. Three days after plating the medium was removed and replaced by the same

medium or with DMEM without serum. In the latter case the wells were first washed twice with phosphate buffered saline (PBS). Some 24 h later the medium was replaced by the same medium plus ascorbic acid (50 μg/ml), β-aminopropionitrile (50 μg/ml) and 2 μCi/ml [2,3-^{3}H]proline for the final 24 h of culture. IL-1β, TNF-α, IFN-γ, TGF-β, dexamethasone or calcium-ionophore A23187 were added during the labeling period. However, IL-1β and TNF-α were also added during the 24 h culture period prior to the labeling period. If the calcium ionophore was given, 1.5 m*M* $CaCl_2$ was added to the culture medium simultaneously.

After the labeling period the cells and medium were scraped from the wells and the wells were washed twice with 1 ml of 50 m*M* Tris-HCl pH 7.6 containing 25 m*M* ethylenediaminetetraacetic acid (EDTA), 10 m*M* *N*-ethylmaleimide (NEM), 1 m*M* phenylmethylsulfonylfluoride (PMSF) and 1 m*M* proline. The wash solution was added to the suspension which contained cells and medium. The final suspension was freeze/thawed three times and the proteins were precipitated with trichloroacetic acid (TCA; final concentration 10%). The radioactive protein was separated from free [^{3}H]proline by repeated (3x) washes with 5% TCA containing 1 m*M* proline at 4°C.

The final sediment was dissolved in 0.75 ml 0.2 *M* NaOH and neutralized by the addition of 0.3 ml 1*M* HEPES and 0.3 ml 0.15 *M* HCl. Aliquots from this solution (0.1 ml) were counted to determine the incorporation in total protein. In order to determine proline incorporation into collagen 0.2 ml 20 m*M* Tris-HCl, pH 7.6, containing 50 m*M* $CaCl_2$ and 0.1 ml collagenase (chromatographically purified on a G200 gel filtration column) were added to a 0.5 ml aliquot of the solubilized sample and the mixture was incubated for 5 h at 37°C. The digestion was terminated by the addition of TCA and tannic acid up to final concentrations of 0.6 *M* and 3 m*M*, respectively. After centrifugation (10 min; 14.500 × g) a 1.0 ml aliquot of the supernatant was counted in a liquid scintillation analyzer. The same procedure was followed without the addition of collagenase. Subtraction of the counts released in this blank incubation from those released in the presence of collagenase yielded the collagen specific incorporation, which will be referred to as collagenase-digestible protein (CDP). Subtraction of the radioactivity in the CDP fraction from that in total protein yields the incorporation into non-collagenous protein (NCP). Incorporation into CDP and NCP is quantified per well. The relative collagen synthesis was calculated with the formula [8] that takes into account the enrichment of proline in collagen compared to other proteins:

$$\text{Percent relative collagen synthesis} = \frac{\text{CDP}}{(\text{NCP} \times 5.4) + \text{CDP}} \times 100\%$$

Differences between cells cultured under the various conditions were tested for significance using a two-sided Wilcoxon test ($^*p \leq 0.05$).

Results

Collagen synthesis in the two fibroblast strains was dependent on the presence of fetal calf serum during culture. If serum was omitted, synthetic capacity decreased (Fig. 1). The absolute collagen synthesis was lowered by 63% and 74% in colon and skin fibroblasts, respectively. However, the synthesis of noncollagenous protein was affected far more strongly in colon fibroblasts. As a result, serum-free conditions caused the relative collagen synthesis to be more than halved in skin fibroblasts and to be increased by nearly 50% in colon fibroblasts.

Collagen synthesis was always measured both in the presence and in the absence of 10% serum. With respect to the effects of the various regulatory compounds, only those results will be shown here in which clear differences were observed between colon and skin fibroblasts. This was not the case after addition of IFN-γ or TNF-α to the culture medium. IFN-γ (50 U/ml) suppressed collagen synthesis, while synthesis of NCP remained unaffected. The same was also true for TNF-α (2 ng/ml), although the inhibitory effect was more explicit in the colon fibroblasts.

In colon fibroblasts cultured with serum the absolute collagen synthesis was not affected by IL-1β (Fig. 2). Since the synthesis of NCP increased with

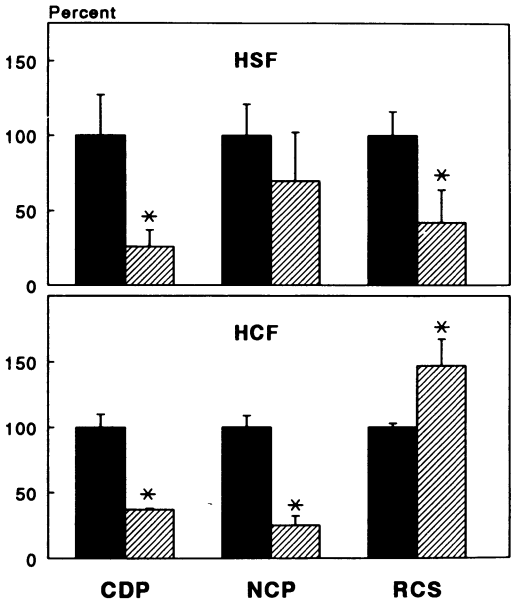

Fig. 1. Serum effects on protein synthesis in fibroblasts from human skin (*HSF, upper panel*) and colon (*HCF, lower panel*). Cells were grown in 10% fetal calf serum (*black bars*) or serum-free culture medium (*striped bars*). Collagenase-digestible protein (*CDP*), noncollagenous protein (*NCP*) and relative collagen synthesis (*RCS*) measured in the presence of serum are given as 100%. Results represent average values ± SD from five separate experiments

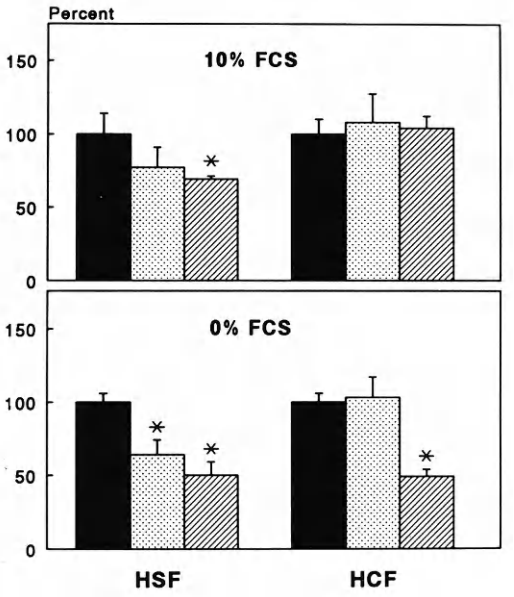

Fig. 2. Effect of interleukin-1β (IL-1β) on absolute collagen synthesis in fibroblasts from human skin (*HSF*) and colon (*HCF*). Cells were grown with (*upper panel*) or without (*lower panel*) fetal calf serum. Data are expressed as percentile values with regard to control cultures without IL-1β (*black bars*) and represent the mean ($\pm$ SD) of four cultures. The IL-1β concentrations tested are 1 (*dotted bars*) and 50 (*striped bars*) units/ml

the higher concentration of IL-1β used, the relative collagen synthesis was lowered (not shown). In the absence of serum, the absolute collagen synthesis was inhibited by 50 units/ml IL-1β. However, the collagen synthesis in skin fibroblasts appeared far more susceptible to the cytokine: the lower concentration suppressed synthesis independent of the presence of serum.

Growth factors, and especially TGF-β, are thought to stimulate fibroblast collagen synthesis. This was indeed the case in skin fibroblasts, regardless of culture conditions (Fig. 3). In both cases, the absolute and relative collagen synthesis were increased, although the latter less so in 10% serum since here the synthesis of NCP was also significantly enhanced. Colon fibroblasts reacted in just the opposite way, at least in the presence of serum. Both collagen and NCP synthesis were suppressed significantly. In the absence of serum, collagen synthesis in colon fibroblasts was enhanced by TGF-β, particularly at a concentration of 5 ng/ml. However, the synthesis of other proteins was stimulated to the same extent and thus the relative collagen synthesis remained unchanged.

The synthetic activity in colon fibroblasts was hardly affected by the glucocorticoid dexamethasone: in the presence of serum neither the collagen synthesis nor the synthesis of NCP changed after addition of the drug. Both activities appeared to be stimulated in serum-free cultures. As a result, the

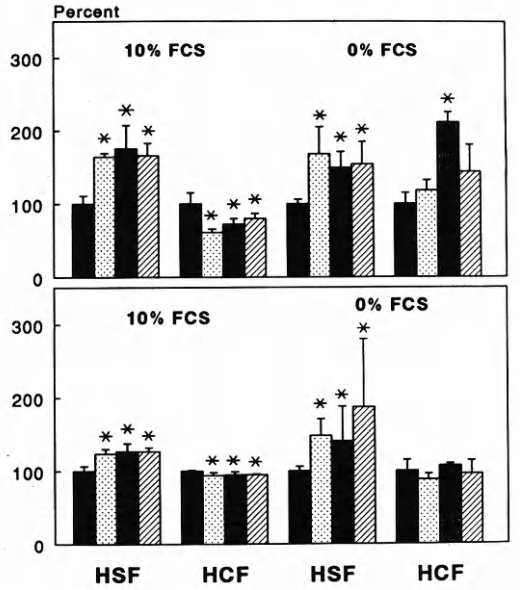

Fig. 3. Effect of transforming growth factor-β (TGF-β) on collagen synthesis in fibroblasts from human skin (*HSF*) and colon (*HCF*). Results are given for both absolute (*upper panel*) and relative (*lower panel*) collagen synthesis, measured in the presence and absence of serum. Data are expressed as percentile values with regard to control cultures without TGF-β (*black bars*) and represent the mean ($\pm$ SD) of four cultures. The TGF-β concentrations tested are 1 (*dotted bars*), 5 (*gray bars*) and 10 (*striped bars*) ng/ml

relative collagen synthesis remained unaffected (Fig. 4). Skin fibroblasts reacted differently: the synthesis of NCP was doubled by dexamethasone while the absolute collagen synthesis was not influenced. Thus, the relative collagen synthesis decreased significantly, from concentrations of 10^9 *M* dexamethasone upwards.

Another way to regulate collagen synthesis in colon fibroblasts is the addition of the calcium ionophore A23187. While no effects were observed in the presence of serum (results not shown), culturing cells in the presence of 0.6 μM A23187 suppressed the collagen synthesis by more than 75% (Fig. 5). Although skin fibroblasts were also found to be susceptible to the presence of the ionophore the picture seemed somewhat different: the significant decrease in the relative collagen synthesis appeared more a result from an increase in synthesis of NCP than from a lowered absolute collagen synthesis.

Discussion

Collagen synthesis is an essential and universal feature of wound repair. After construction of an anastomosis in the intestine the synthetic capacity is strongly, and specifically, elevated in the wound area [9, 10]. Since collagen

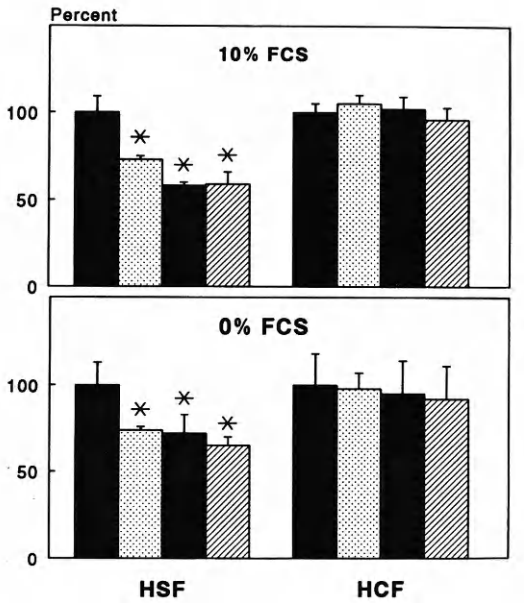

Fig. 4. Effect of dexamethasone on relative collagen synthesis in fibroblasts from human skin (*HSF*) and colon (*HCF*). Cells were grown with (*upper panel*) or without (*lower panel*) fetal calf serum. Data are expressed as percentile values with regard to control cultures without dexamethasone (*black bars*) and represent the mean (± SD) of four cultures. The dexamethasone concentrations tested are 10^{-9} (*dotted bars*), 10^{-7} (*gray bars*) and 10^{-5} (*striped bars*) *M*

synthesis is thought to be crucial for the development of anastomotic strength, the study of its regulation is of great potential interest. Leakage of colonic anastomoses is a phenomenon that occurs rather frequently [11] and insight into regulation of the processes which are essential in the repair sequence could contribute to the development of measures to avoid this complication. The cells most likely to be responsible for collagen synthesis are the fibroblasts which migrate into the wound and display great proliferative activity. Thus, we investigated collagen synthesis in an established line from human colon fibroblasts [12] and compared the results with those obtained with fibroblasts from human skin.

It has been shown that addition of serum to fibroblast cultures stimulates collagen synthesis [13]. Although colon fibroblasts behave similarly in this respect they seem to be unique in the sense that the synthesis of NCP is stimulated to a greater degree, resulting in a lower relative collagen synthesis under serum-supplemented conditions.

IL-1β inhibits collagen synthesis in colon fibroblasts cultured in the absence of serum. We found similar results for skin fibroblasts, an observation which is in agreement with those reported by others [14]. The fact that in colon fibroblasts such inhibition was not seen in the presence of serum confirms a similar observation by Duncan and Berman [4], although

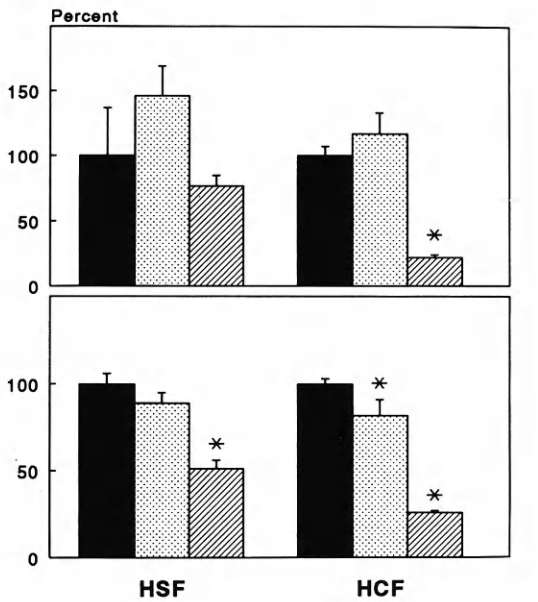

Fig. 5. Effect of calcium ionophore A23187 on collagen synthesis in fibroblasts from human skin (*HSF*) and colon (*HCF*). Results are given for both absolute (*upper panel*) and relative (*lower panel*) collagen synthesis, measured in the presence of serum. Data are expressed as percentile values with regard to control cultures without A23187 (*black bars*) and represent the mean (± SD) of four cultures. The A23187 concentrations tested are 0.15 (*dotted bars*) and 0.60 (*striped bars*) μM

our skin fibroblasts are also responsive under these conditions and display a suppressed collagen synthesis.

Growth factors, and especially TGF-β, are known to stimulate collagen synthesis in fibroblasts from a variety of tissues [15]. Our results with dermal fibroblasts confirm earlier results reported for cells from this tissue. In contrast, synthetic activity in human colon fibroblasts is significantly inhibited in the presence of serum. While there has been a report on negative effects of TGF-β on collagen synthesis in hepatocytes [16], this is the first evidence that TGF-β may inhibit collagen synthesis in fibroblasts. Fukamizu and Grinnell [17] described reduction of collagen synthesis by TGF-β in long-term fibroblast cultures, possibly as the result of lowered fibroblast activity concomitant with spatial reorganization of the extracellular matrix. In the absence of serum, absolute collagen synthesis in colon fibroblasts is indeed stimulated by TGF-β in a concentration of 5 ng/ml.

Neither the absolute nor the relative collagen synthesis in colon fibroblasts is significantly affected by dexamethasone. Most studies with skin fibroblasts indicate that glucocorticoids reduce collagen synthesis [18]. In the present study, a reduction in the absolute collagen synthesis is only

apparent, and not significantly so, if dermal fibroblasts are grown without serum. The decreased relative collagen synthesis is, certainly in serum-supplemented cultures, the result of a stimulation in the synthesis of NCP. In animal studies we have found that colonic wound healing is unaffected by administration of corticosteroids at doses which impair dermal wound healing [6]. Therefore, it is interesting to observe that collagen synthesis in fibroblasts from colon seems refractory to corticosteroids. Possibly, a difference in regulation of this fibroblast function may (partly) explain the divergent healing patterns in both tissues.

Next to investigating the behavior of collagen synthesis in colon fibroblasts added various regulatory compounds, the aim of this study was to establish if fibroblasts from colon and skin behave differently in this respect. This indeed appears to be the case:

- The relative collagen synthesis increases in dermal fibroblasts and decreases in colon fibroblasts upon the addition of serum.
- In the presence of serum IL-1β inhibits collagen synthesis in skin fibroblasts but not in colon fibroblasts.
- Dexamethasone suppresses the relative collagen synthesis in skin fibroblasts but not in colon fibroblasts.
- TGF-β stimulates the collagen synthesis in dermal fibroblasts cultured in the presence of serum but inhibits the process in colon fibroblasts.

The other differences observed are less explicit and may fall within the variations observed by us (results not shown) and others [4] for different cell lines from the same tissue. However, those mentioned above, and most particularly the discrepancy measured in the presence of TGF-β, suggest that repair activities of fibroblasts in colon and skin may be under different control. Wound fibroblasts obtained from implanted sponges differ from normal dermal fibroblasts with regard to their capacity to synthesize collagen and remodel collagen lattices [19]. Thus, the wound environment may alter fibroblast phenotype. Our results could mean that fibroblasts from skin and colon can exhibit divergent reactions to cytokines or growth factors produced during the inflammatory part of the healing sequence. Such a phenomenon may cause wounds in skin and intestine to behave differently under certain conditions.

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Platelet Released Factors Stimulate Rat Burn Wound Contraction

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Introduction

After thermal injury fluctuations in blood platelet levels have been reported. Eurenius et al. (1972) found that when 30% full-thickness burn wounds were induced in rats, a thrombocytopenia occurred within the first few hours of injury followed by a thrombocythaemia which reached a maximum between 3–5 days. Similar blood platelet level fluctuations have been observed in humans, albeit with a more prolonged time course (Baxter 1974).

Blood platelet levels depend upon the loss of platelets from the circulation and the rate of platelet production. Both of these changes may contribute to the observed changes in platelet numbers after thermal injury. Using chromium-labelled platelets, Eurenius demonstrated a decrease in platelet survival time after thermal injury.

Further analysis of the tissue distribution of the chromium-labelled platelets demonstrated that the burn wound site contained in the order of 50 times the amount of radioactive label as compared with an equal amount of unburned skin from either thermally injured or uninjured animals. This local sequestration accounted for 4%–5% of the total amount of labelled platelets. Platelet sequestration within the burn wound has also been demonstrated by Johansson (1960), who measured the local rise in hydroxytryptamine, a product released during the degradation of platelets, at the burn wound site. Platelet sequestration is also compatible with the observed progressive formation of venous microthrombi in burn wounds (Boykin et al. 1980).

Within the thermally injured area platelets adhere to insoluble components of the subendothelial matrix (Baumgartner et al. 1980). This process of adherence, and local platelet agonists such as thrombin, stimulate

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a platelet release reaction flooding the wound bed with biologically active substances (Holmsen et al. 1981). This study was concerned with the effect that these platelet-released substances may have on wound contraction.

It is proposed that the large number of platelets that deposit and degranulate at a burn wound site stimulate burn wound contraction. In order to investigate this, the effect of PDWHF on the contraction of partial-thickness burn wounds in the rat was studied.

Method

Preparation of Platelet Releasate

A platelet releasate was prepared according to a protocol described by Knighton et al. (1986). In outline fresh whole blood was collected into a transfusion bag containing anticoagulant and centrifuged to remove the red and white blood cells. Further centrifugation was performed to form a platelet pellet and a supernatant of platelet poor plasma. The supernatant was discarded and the platelet pellet resuspended in a platelet washing buffer (PWB) (Knighton et al. 1986) to form a platelet suspension at a concentration of 1×10^9 platelets/ml. Thrombin was added to this platelet suspension to degranulate the platelets. Spent platelets were then removed by further centrifugation, and the supernatant collected. This supernatant is a platelet releasate that has been termed platelet-derived wound healing formula (PDWHF) (Knighton et al. 1982).

The Rat Thermal Injury Model

The rat thermal injury model, approved of by the United Kingdom Home Office Inspectorate, was based on one described by Spector and Willoughby (1959). The model consisted of a heater and stirrer which maintained a water bath at a constant temperature (Fig. 1). Water was pumped into the hollow brass cylinder through an inlet by a pump. The water heated a solid contact surface of diameter 0.9 cm onto which the anaesthetised rat was placed, with the body weight supported by the cork platform. The temperature of this contact surface was measured by a thermister. Water left the hollow cylinder via an outlet.

Experimental Protocol

A total of 24 male Wistar rats of weights ranging between (220–275 g) were anaesthetised and the dorsal area of the neck shaved. Using the wound template (Fig. 2), the outer circles of points were tattooed onto the skin

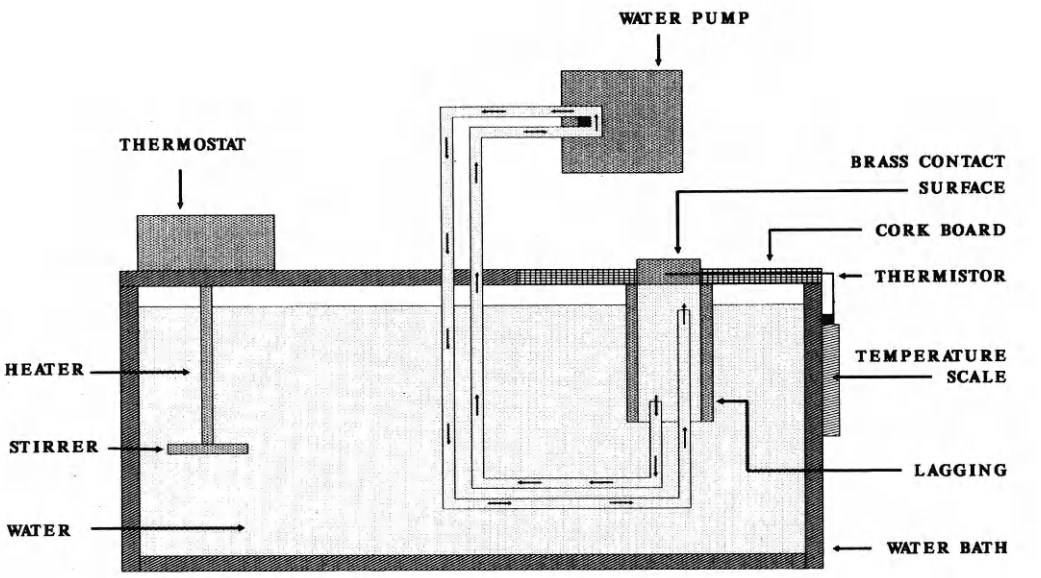

Fig. 1. Apparatus for the rat thermal injury model

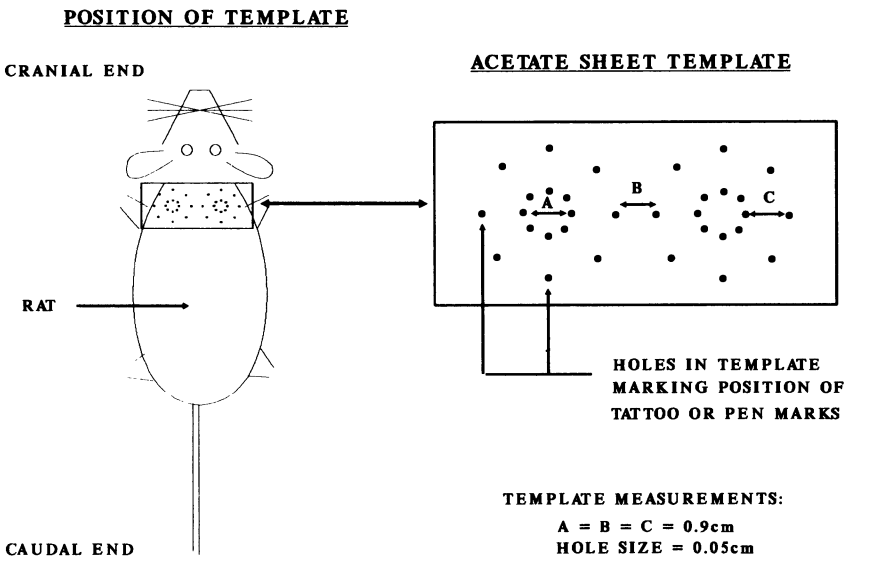

Fig. 2. The burn wound template

with India ink while the inner circles were marked out with pen. Within the inner circle on one side of the neck of each animal, a partial-thickness burn wound was induced by setting the rat thermal injury model at 56°C for 120 s.

Some 24 h later the rats were anaesthetised again, and both the wound and the control area along with a calibration scale were photographed. With six rats in each group 50 μl of either 0%, 1%, 10% or 100% PDWHF stock solution diluted in platelet washing buffer (PWB) containing 1 U bovine thrombin/ml of buffer, was injected under the burn wound eschar. These injections of PDWHF were repeated daily up to 7 days, and the tattooed areas photographed every other day. In addition the rats were weighed daily. On day 14, the final day of the experimental period, the rats were killed by an overdose of anaesthesia. Although none of the rats appeared distressed all rats received analgesia (buprenorphine 0.5 mg/kg/day) for the first 48 h after thermal injury.

Method for Determining Wound Contraction

The photographs were processed to film negatives, which were then placed in front of a light source. The image on the negative was then "captured" by a Kodak Videk high resolution camera and transferred to a computer screen by the image analysis software Optimas (Bioscan). Optimas was then used to calibrate each photograph using the photographed calibration scale. The outer circle of tattoo points surrounding the burn wound was identified, and the individual points connected by a line to form an octagon. The enclosed area of this octagon was then calculated by Optimas. A similar procedure was then performed on the control side.

The area enclosed by the tattoo marks that surrounded the wound was expressed as a percentage of the control area. This was in order to control for any growth of the rat over the experimental period.

Results

The wound contraction data are presented (Table 1) with a bar chart (Fig. 3) and statistical analysis (Table 2).

There was no significant difference in percent wound contraction between each group of rats at day 1, 24 h after injury but before administration of PDWHF. At 3 days, however, there was a significant ($p < 0.05$ Wilcoxon rank test) increase in contraction in the group that received 100% PDWHF as compared with the group that received no PDWHF. This significance was present throughout the experimental period except at day 11. No other significant difference was present between the other groups, except for two isolated points at days 3 and 5 between 0% and 10% PDWHF. There did, however, appear to be a trend of increasing contrac-

Table 1. Summary of burn wound contraction data

PDWHF (%)	Time (day)	*N*	Average wound contraction (%)[a]	S.D.	S.E.
0	1	6	89.20	6.642	2.711
	3	6	94.90	5.007	2.044
	5	6	91.08	4.538	1.853
	7	6	83.25	7.566	3.089
	9	6	77.79	6.813	2.782
	11	6	79.60	6.310	2.576
	13	6	71.36	8.021	3.275
1	1	6	92.87	5.624	2.296
	3	6	89.00	7.106	2.901
	5	6	88.28	6.863	2.802
	7	6	80.55	7.045	2.876
	9	6	77.79	6.813	2.781
	11	6	72.55	7.577	3.093
	13	6	71.36	8.021	3.275
10	1	6	91.05	8.891	3.630
	3	6	84.20	5.500	2.245
	5	6	84.44	4.084	1.667
	7	6	77.56	6.516	2.660
	9	6	75.66	5.545	2.264
	11	6	73.73	4.990	2.037
	13	6	71.36	8.021	3.275
100	1	6	93.15	5.207	2.126
	3	6	82.39	7.248	2.960
	5	6	82.75	3.717	1.518
	7	6	85.48	5.050	2.062
	9	6	83.25	7.566	3.089
	11	6	72.53	8.556	3.493
	13	6	67.12	8.290	3.384

PDWHF, platelet-derived wound healing formula.
[a] Expressed as a percent of the control side.

tion with increasing dose of PDWHF between days 3 and 13, again with the exception of day 11.

Of note was the timing of the sloughing of the burn wound eschar, which was between days 9 and 11 in the experimental groups 0% and 1% PDWHF, and between days 11 and 13 in the other two groups.

After a short lag phase that lasted up to 3 days all the rats gained weight steadily over the experimental period with no statistical difference (Kruskal-Wallis Analysis) apparent between groups throughout the experiment. This suggested that the rats were not distressed by the experiment, which was consistent with their observed behaviour. Further, none of the wounds appeared on daily inspection to have been traumatised by the rats.

Table 2. Statistical analysis of the burn wound contraction data using the Wilcoxon rank test

Day	0% with 1% PDWHF	0% with 10% PDWHF	0% with 100% PDWHF	1% with 10% PDWHF	1% with 100% PDWHF	10% with 100% PDWHF
1	0.261	0.471	0.173	0.936	0.936	0.810
3	0.128	0.013	0.020	0.172	0.230	0.689
5	0.378	0.030	0.013	0.173	0.128	0.689
7	0.297	0.128	0.013	0.575	0.128	0.575
9	0.128	0.066	0.045	0.575	0.173	0.575
11	0.128	0.093	0.093	0.471	0.810	0.810
13	0.173	0.128	0.045	0.378	0.173	0.230

PDWHF, platelet-derived wound healing formula.

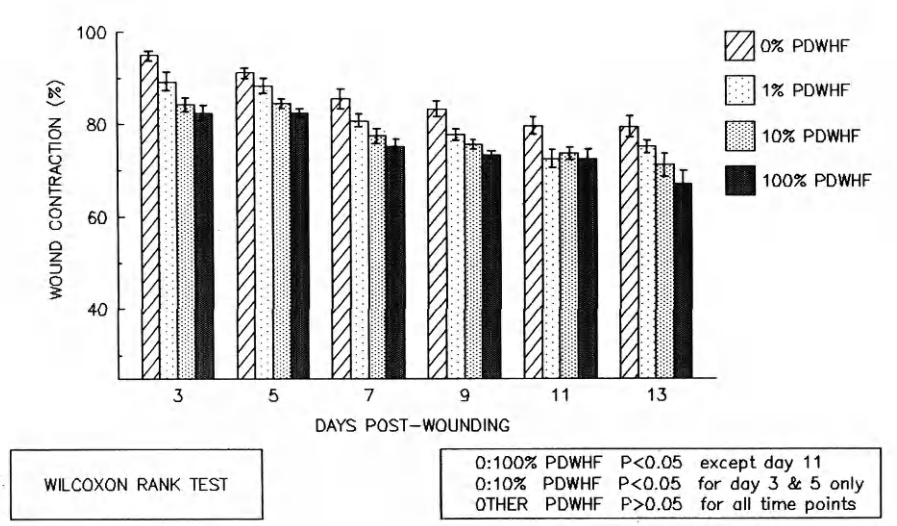

Fig. 3. The effect of platelet-derived wound healing formula (*PDWHF*) on the contraction of partial-thickness burn wounds in the rat

Experimental Errors and the Technique

The major problem with the chosen technique was found to be related to the position of the burn wound and contraction tattoos. The back of the neck of the rat was the site chosen, as it is a site that was both well tolerated by the rats and relatively inaccessible, which reduced interference to the wound site. Difficulty however was experienced in placing the rat in exactly the same position prior to photography and this may account for some of the experimental error. Due to the slight curvature of this region the area within the tattoo marks as seen on the photographs may not exactly correspond to the area within the tattoo marks on the skin surface. This emphasises the importance of photographing the control site on each rat and calculating the percent wound contraction as compared with the control site. The control was also required to correct for an increase in size of the rat throughout the experimental period.

Difficulty also arose over the formation of the burn wound eschar, as this has been reported to transiently effect contraction (Snowden et al. 1982). Two alternatives were considered, either to remove the eschar manually on a chosen day, or to wait until the eschar fell off as wound healing progressed. The latter was chosen as there was concern that the wounds may be traumatised by manual removal of the eschar. This appeared to lead to a transient error in the contraction data as the eschar fell off on different days postwounding in the different groups (Fig. 3). In groups treated with no or 1% PDWHF there appeared to be a transient

splinting effect of the burn eschar at day 9 which was lost by day 11 with a sudden step reduction in wound contraction between these two days. This reduction in these two groups is probably responsible for the loss of a significant difference between groups treated with no PDWHF and 100% PDWHF at day 11. Similarly, between day 11 and 13 the eschar fell off the other two groups, and there was again a step reduction in wound contraction. This restored the significant difference between the 0% PDWHF and 100% PDWHF groups.

Another area of concern was the administration of the PDWHF. This was injected under the burn wound eschar, because if it was placed on the surface of the dead nonvascular tissue it was considered that it would not reach the wound bed. Injection in this manner was not ideal as the exact depth of injection is difficult to determine, and a certain amount of trauma will be incurred. It was therefore important to have a control group (0% PDWHF) which was treated in a similar manner throughout the experimental period.

Discussion

Wound contraction can be defined as the diminution in size of an open wound, as a result of the centripetal movement of the whole thickness of the surrounding skin (van Winkle 1967). It is been described as a predominantly fibroblast-mediated activity (Gabbiani et al. 1971).

Two main opposing theories of fibroblast-mediated wound contraction have developed, the cell contraction theory (Gabbiani et al. 1971) and the cell traction theory (Ehrlich 1988). The cell contraction theory is based upon the concept that microfilaments contract individual spindle-shaped fibroblasts to a rounded shape simultaneously approximating cell surface-attached collagen fibres. The traction theory, however, proposes that fibroblast migration through the wound generates forces that approximate collagen fibres. The mechanism by which platelet releasate stimulates burn wound contraction may therefore be via an effect on fibroblast activity.

It has been shown that PDWHF stimulates the migration of human dermal fibroblasts in vitro (Kendall et al. 1992) and that thrombin-activated platelets injected into rabbit corneas stimulate the formation of collagen in vivo (Knighton et al. 1982). These observations suggest that in combination platelet factors may affect fibroblast activity.

There are also a number of studies which describe the effect of individual platelet-released substances on the activity of fibroblasts. For example, β-thromboglobulin, platelet factor 4 (Senior et al. 1983), fibronectin (Postlethwaite et al. 1981) and arachidonic acid metabolites (Reiger et al. 1990) all stimulate fibroblast migration in vitro. Platelet-derived growth factor is both a potent chemoattractant and mitogen for fibroblasts in vitro, and also has peak levels in wound chambers in vivo during the fibropro-

liferative phase of repair (Grotendorst et al. 1988). Transforming growth factor-β increases the number of fibroblasts within wounds as compared with vehicle alone (Pierce et al. 1988). Basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) are both rat granulation tissue fibroblast chemoattractants and mitogens in vitro (Davidson et al. 1988). Platelet-derived endothelial cell growth factor stimulates human dermal fibroblast chemotaxis in vitro and the proliferation of some fibroblast cell lines (Pierce et al. 1991). The mechanism by which platelet releasate stimulates burn wound contraction may therefore be due to individual factor activities. The action of individual factors may however be augmented by synergistic effects between factors (Newman and Marcone 1991).

Platelet releasate may also affect burn wound contraction by affecting the inflammatory phase of burn wound healing. PDWHF has been found to contain many proteins (Newman and Marcone 1991) which may be immunogenic when applied to the burn wound bed. An enhanced inflammatory response will attract macrophages and lymphocytes into the wounded area which in turn release a host of inflammatory mediators. Many of these mediators may also recruit fibroblasts and stimulate their proliferation (Wahl 1985).

Platelet-released components may stimulate burn wound contraction by causing a deeper burn wound to develop with a greater degree of tissue damage and loss of dermal collagen. As it has been reported that the degree of wound contraction is related to the amount of structurally intact dermal collagen (Brown et al. 1990), this would lead to a greater degree of wound contraction. Amongst the factors within PDWHF which may be responsible for increasing the depth of the burn wound are those that affect the local burn wound microcirculation. Components such as thromboxane, a potent platelet aggregator and vasoconstrictor, may increase microvascular occlusion. This along with the prostaglandins and prostacyclins which increase vessel permeability may encourage microvascular stasis. This increase in the depth of the zone of stasis would be expected to lead to an increase in the zone of ischaemia (Zawacki 1974) forming a burn wound that extends to a greater depth (and possibly also a greater width).

There are therefore a number of possible mechanisms by which platelet-released factors may stimulate burn wound contraction, and additional work is required to elucidate this further.

Conclusions

PDWHF appears to stimulate the contraction of partial-thickness burn wounds in a rat thermal injury model when compared with a control group. There are many possible mechanisms by which PDWHF may exert an effect on wound contraction, some of which include an effect on the burn wound fibroblast population. Further work is required to determine by which mechanism(s) the formula stimulates burn wound contraction.

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V. Experimental Studies

Biologic Activity of Tissue Flaps in the Treatment of Complicated Wounds

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Introduction

Optimal, uncomplicated wound healing requires a clean wound bed, free of damaged tissue and germs. Extensive and complete debridement of necrotic and infected areas, however, is not always achievable. In particular this applies to radiation damage to tissue and irradiation ulcers that are located in tissues covering internal organs, large vessels, nerve bundles and structures of functional importance, because of the risk of life threatening complications or severe functional impediment [1, 2]. In these cases one has to cope with the situation of being forced to cover nonradically excised tissue defects.

Materials and Methods

Between 1981 and 1991 we performed 114 repair operations using various flap techniques in 79 patients with irradiation defects. The cases in which radical debridement of the irradiated tissue could (n = 89) or could not (n = 25) be accomplished are shown in Table 1, where we grouped the patients according to the location of the damage.

We covered the wound bed using various flaps (Table 2). These were tissue flaps rotated on a vascular pedicle such as the latissimus dorsi musculocutaneous, flaps that included one of the rectus abdominis muscles with overlying vertical skin island rotated on a superior or inferior vascular pedicle, flaps that included the tensor fascia latae muscle, fascia latae and overlying skin, and the fasciocutaneous flap of the forearm that was supplied by the radial artery, the so-called Chinese flap. Another technique used was the transfer of a free flap with microsurgical anastomosis, such as the greater omentum, the latissimus dorsi musculocutaneous flap, the scapular

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Table 1. Number of cases of nonradical excision grouped according to location

Group	Location	Total operations (*n*)	Nonradical excision	
			n	%
I	Head and neck	37	3	8
II	Chest wall	26	6	23
III	Inguinal and gluteal regions	6	2	33
IV	Hand	21	8	38
V	Lower extremity	24	6	25

Table 2. Number and variants of flaps in nonradical operations in the different cases

Group	Surgical method	Cases (*n*)
I	GO free	2
	LD mc tr[a]	1
II	GO free	2
	LD mc tr	1
	VRA mc tr[a]	3
III	VRA mc tr	1
	TFL fasc tr	1
IV	Sc free	1
	1-WS free	5
	Morrison free	1
	Rad fasc cut free	1
V	GO free	1
	LD mc free	3
	LD m free and skin graft	1
	VRA mc tr	1
	Sc fasc free	1

GO, greater omentum; free, transfer with microvascular anastomosis; tr, transposition on the vascular pedicle; mc, musculocutaneous; LD, latissimus dorsi flap; TFL, tensor fasciae latae flap; Sc, scapular flap; 1-WS, first web space of the foot flap; Morrison, wrap around flap of the great toe; Rad fasc cut, fascia cutaneous radialis flap; m, muscle split.

[a] Flaps with secondary healing.

fasciocutaneous flap, the latissimus dorsi split muscle with immediate skin grafting, cutaneous flaps of the first foot web space and first toe, including nail, the so-called Morrison's flap and isolated scapular fascia.

We administered systemic and, if it was necessary, local antibiotics in each case before, during and after the operation. Except for the transfer of

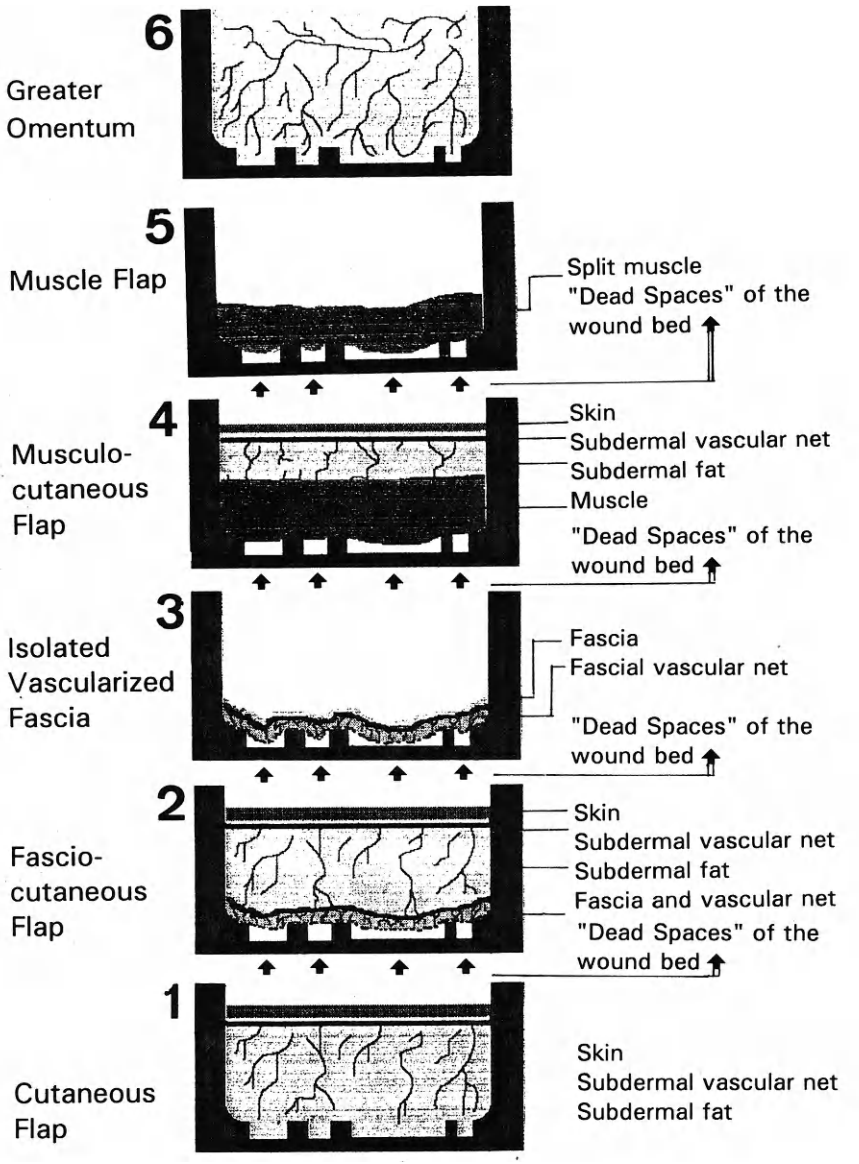

Fig. 1. The various flaps grouped according to their biologic activity

the greater omentum we used prolonged tube drainage of the space under the flap in for average of 7 days.

To evaluate the outcome, we classified the flaps (Fig. 1) according to what we call "biologic activity" (BA). This is the density of the vascular net in the donor tissue, its ability to neovascularize, plasticity and immunological

capacity. BA thus concerned the reaction of the deepest flap tissue layer that had been placed on the wound bed. Our concept was based on clinical observation and features known from experimental studies. The flaps are listed according to their increasing level of BA:

1. *Cutaneous flap*: This commonly includes subcutaneous fat, which covers the wound bed. It may fill small "dead spaces." The density of the vascular network is, however, poor and its ability to undergo angiogenesis is low [3].
2. *Fasciocutaneous flap*: Its deep surface fascia which covers the wound bed has a higher vascular density and ability to undergo angiogenesis [4], but its surface is slippery and cannot fill "dead spaces" appropriately.
3. *Vascularized fascia*: This is thinner and more pliable and thus may have a higher BA.1
4. *Musculocutaneous flap*: Circulation in muscles is commonly better than in fascias in the first hours after surgery [5]. The ability for ingrowth and angiogenesis is high [5, 3] and, despite its inability to obliterate all "dead spaces," the total BA. is considered to be higher than in the preceding flaps.
5. *Split muscle*: Plasticity and pliability are much higher than that of musculocutaneous flaps which increases the BA.
6. *Greater omentum*: The rich microvascular network, the excellent plasticity, active angiogenesis [6–8] and specific immunological properties [9, 24] may provide the greatest rate of BA.

Results

Of the 25 patients, 21 presented with uncomplicated primary wound healing (Table 3). In three the results were satisfactory: complete healing, but after a prolonged period and additional procedures. These were chemical wound debridement and secondary suture of the latissimus dorsi musculocutaneous flap transposed to the irradiation ulcer of the neck in one patient of group I; several sequestrectomies from the space under the flap in transposition of the vertical rectus abdominis flap on the vascular pedicle to an irradiation

Table 3. Results of the operation: wound preparation inappropriate in 25 of the 114 cases

Site	*n*	Percent	Primary healing	Satisfactory healing	Unsatisfactory healing
Head	3	8	2	1	0
Chest	6	23	4	1	1
Groin	2	33	2	0	0
Hand	8	38	8	0	0
Leg	6	25	5	1	0

ulcer of the upper sternum in one patient of group II; an ulcer formation near the flap edge followed the transposition of the same kind of flap applied on an irradiation defect of the thigh in one patient in group V. In one patient of group II who had a vertical rectus abdominis flap transposed to the full-thickness irradiation chest wall defect the results were unsatisfactory. Subtotal lysis of the flap enlarged the wound area and prolonged hospitalization up to 4 months. Finally the wound could be covered with a split-thickness skin graft.

Discussion

Surgical management in the irradiated field has been considered to be a great problem for many years [10–12]. Irradiation tissue damage has not only a high rate of contamination, but is also characterized by a large surrounding zone of fibrotic, badly nourished tissue with indistinguishable borders and a low rate of regeneration [13]. It is clear that when such severe damage is located in areas of vital or functionally important structures – even if these organs or structures are often involved in the degeneration process – the possibility of complete debridement is poor. The claim "the ulcer and surrounded area should be excised with wide margins out to and down to healthy tissues" [12] may not be realizable. As early as 1935 Gilles and McIndoe [1], who operated upon more than 100 patients suffering from complicated irradiation defects, recommended the application of skin flaps on the wound bed in case radical debridement is impossible: "It is sometimes wise to leave the deep scar in important organs such as thyroid gland or in important tendons and trust to the good effect of a healthy full-thickness skin flap." In 1967 Marino [2] stated that when ". . . a resection might risk irreparable damage, or serious danger for the patient, or when distinction between what can remain and what must be removed deserves the most astute clinical sense . . . the surgeon must resort to other physiological means. Undoubtedly, this can be achieved by living cellular elements and specific enzymes, ferments and antibodies provided by a flap and its vascular supply."

The introduction of flaps with axial blood supply in plastic surgery supported Marino's concept [14–18]. By then the importance of both the deep surface of the flap, which contacts the wound bed, and of the blood flow, especially in this layer, in determining the antimicrobial activity of the flap [20] had been emphasized.

Our clinical experience presenting a 84% primary healing rate in non-radically excised wounds supports this supposition. We, however, consider the anti-infection activity of the flap as being more complex. For example, when we compare the volume blood flow in greater omentum after its free transfer [19] and the literature data of the blood flow in axial muscle or fasciocutaneous flaps [21] the first appeared significantly low. At the same

time we observed what has been described in the literature [9, 22], that the outcome using the greater omentum in radiated wounds was far better.

There is also the necessity that the flap fill small dead spaces (bacterial incubators) of the wound bed; thus its ability for ingrowth [5] and its neovascularisation activity are also of significance. The last two factors are closely related to the ability of the flap's deep surface to produce myofibroblasts – very important active cells of wound healing [23]. Monocytes, macrophages and adventitial cells of small vessels are considered to be involved in the formation of myofibroblasts [23]. This implies that the density of the microvascular net in the deep surface of the flap is very important. In this context one has to admit that the greater omentum has the most extensive vascular network of all flaps, the most angiogenesis factors and is an excellent source of macrophages and lymphocytes [6, 7, 9, 24].

The compilation of the various factors involved directed us to range the different flaps which we used to cover the wound bed according to the BA of the deep surface. We feel that unsatisfactory results are due to improper selection of the flap regarding its BA. Tissue defects in which the chances for radical debridement are very poor need the highest BA level in the flap for reconstruction.

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Studies on Wound Healing in Axial Pattern Flaps of the Diabetic Rat

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Introduction

With increasing life expectancy, the number of diabetic patients requiring surgery increases. Micro- and macroangiopathic lesions are responsible for infections and prolonged wound healing periods. Therefore we decided to do a standardized experimental study on wound healing in pedicled skin flaps in streptozocin treated diabetic Wistar rats. Streptozocin specifically destroys the β-cells of the pancreas (Gerritsen and Dulin 1967; Junod et al. 1967; Tomita et al. 1986). The severity of the experimentally produced diabetes corresponds strictly to the dose of streptozocin administered (Junod et al. 1969; Tancrede et al. 1983; Ehrenfeld et al. 1988).

Materials and Methods

Experimental Diabetes

A total of 150 animals (average weight 170 g) were made diabetic by intraperitoneal injection of streptozocin (Serva). The injection was done under general anesthesia with ether at a dose of 70 mg/kg body weight.

The blood sugar level was periodically determined using the combination glucose test (GOD-Perid-Method, Boehringer Mannheim) from blood of the tail vein. We determined the concentration of glucose in the urine and the presence or absence of ketone bodies (Combur-9-Test, Boehringer/ Mannheim).

After 15 months of diabetes, skin and kidney specimens from five rats were taken for ultrastructural investigation.

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After 2 1/4 years of diabetes 23 out of 150 diabetic rats were still alive. As a control we used a group of 20 healthy nondiabetic animals.

Surgical Procedure

The rat, as a so-called loose skinned animal, is characterized by a cutaneous type of skin vascularization although there are some musculocutaneous perforators. A flap of 7×2 cm was designated on the rat's abdomen (Fig. 1). A long vascular pedicled skin flap containing a cutaneous artery was elevated. The flaps were elevated completely from the underlying surface. The superficial epigastric artery, running centrally, is the main vessel of this vascular pedicled epigastric skin flap (Fig. 2). The flaps were sutured back using 5-0 atraumatic sutures.

General anesthesia was performed using a combination of 16 mg/kg Rompun (Bayer) and 100 mg/kg Ketanest (Parke Davis).

On days 1–7, 10, and 14, the flaps of the diabetic and healthy animals under general anesthesia were measured. Necrotic areas and healthy areas were recorded and analyzed planimetrically.

Results

After injection of 70 mg/kg streptozocin we observed a typical triphasic course of the blood sugar levels. An initial increase after 3–4 h, due to adrenaline excretion caused by anesthesia, was followed by a sudden decrease of blood sugar levels after 5–10 h, caused by insulin secretion from necrotic islet cells. This period is followed by a persisting hyperglycemia as

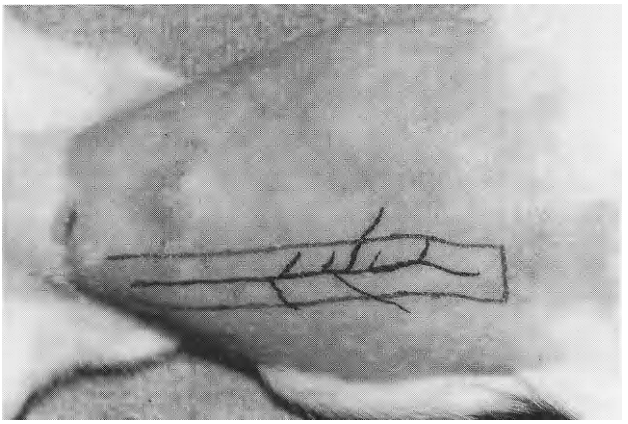

Fig. 1. The epigastric axial pattern flap. The superficial epigastric artery is marked

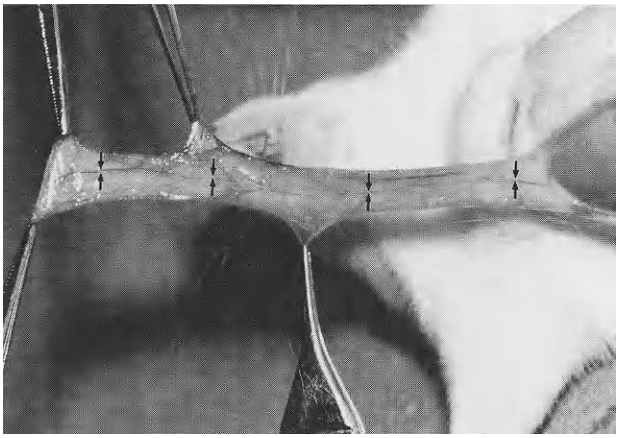

Fig. 2. The flap is completely elevated from the underlying surface. The superficial epigastric artery runs in the center of the flap (*arrows*)

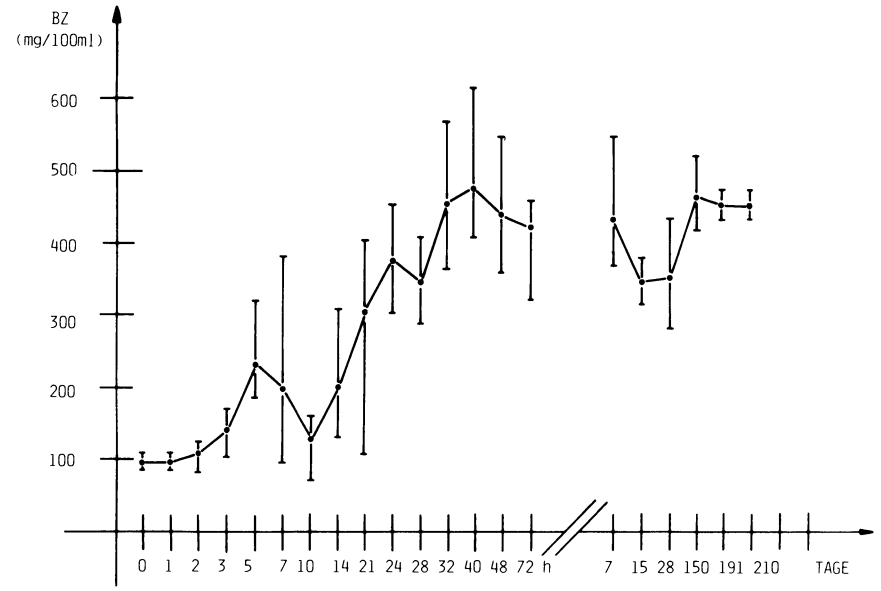

Fig. 3. The blood sugar (*B2*) concentration shows a triphasic course after the injection of 70 mg/kg streptozocin

destroyed islet cells could no longer react to increased blood sugar levels by secretion of insulin. The blood sugar level was between 300 and 500 mg% (Fig. 3). The animals were glucosuric; ketone bodies could not be found in any of the animals. Clinically the animals showed polydipsia and polyuria.

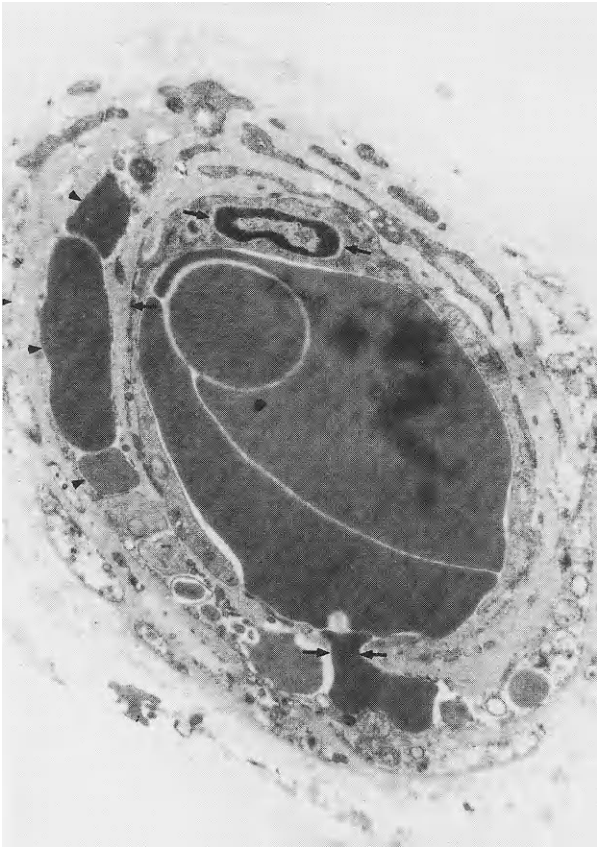

Fig. 4. Transmission electron micrograph of a skin capillary. Note the nucleus of the endothelial cell (*arrows* top) and three erythrocytes (*arrowheads left*) lying in the subendothelial space (*arrows left*). The endothelium is fenestrated with an erythrocyte migrating through the wall (*arrows, bottom*)×8000

After 4 months we noticed the formation of cataracts. After 15 months of diabetes angiopathic changes in the capillary walls of the kidneys could be observed in all animals examined, but in only one animal could we find angiopathic changes of the skin. These results are consistent with a diabetic microangiopathy (Fig. 4).

Figure 5 shows the ratio of necrotic area to the total flap area in 17 epigastric axial pattern flaps. The necrotic area of the diabetic Wistar-rats was 14%–15% compared to the total flap area. Maximal necrosis occurred on days 5–7. The differences in the size of the necrotic areas between the third and tenth days are statistically significant compared to the control group with $p = 0.05$. The location of the necrosis was at the end of the flap or at its edge. In healthy rats of the control group we did not observe any

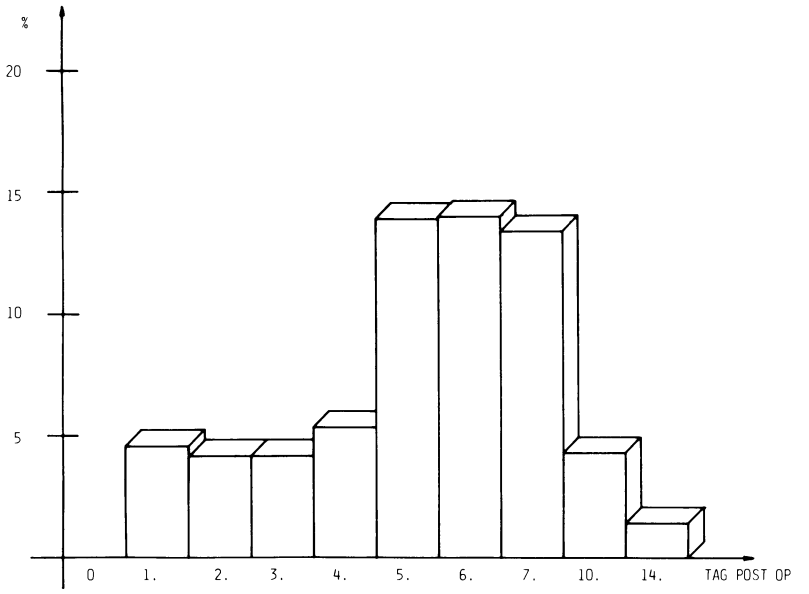

Fig. 5. The necrotic area as a percentage of the total flap area. A maximum of necrosis occurs after 1 week in streptozocin diabetic rats. For details, see text

necrosis. After 14 days the flaps healed in toto except for a small area at the end (Fig. 6a,b).

Discussion

We present an experimental rat diabetes model with long-lasting disease. After 2 years of diabetes vascular, pedicled, epigastric skin flaps were compared to those of healthy controls. The differences in the necrotic area compared to the total flap area were statistically significant in relation to the controls. We can thus say that in our comparative experimental study, we showed that the vascular pedicled skin flaps of diabetic Wistar rats healed in toto except for a small area of necrosis on the end and edge.

Injection of streptozocin causes a non-insulin-dependent diabetes. The destruction of the β-cells of the islets of Langerhans causes an acute deprivation of insulin (Rakieten et al. 1963; Gonet and Renold 1966; Eichhorn 1992). Basic pathophysiologic aspects of juvenile onset diabetes, such as sudden onset, total lack of insulin, sensitivity to insulin, glucosuria and reduction of fatty tissue, are present in this diabetes model. But there are also aspects of diabetes of the elderly patient (non-insulin dependent), e.g., stability of the diabetic situation and insulin independence.

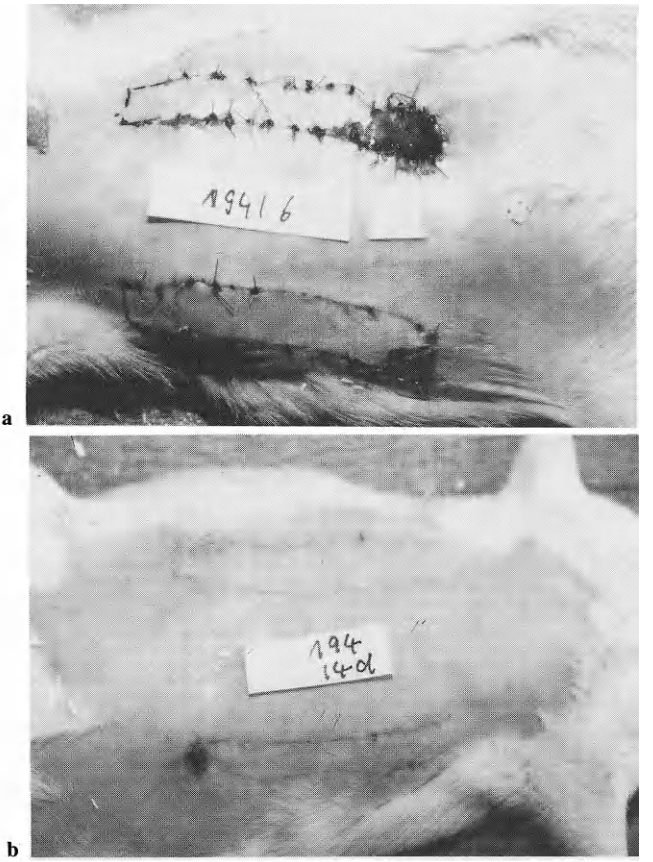

Fig. 6a,b. Result at the sixth (**a**) postoperative day with necrotic areas at the flap's end and edge. (**b**) On day 14 the same flap is healed in toto except for a small area of necrosis at the end

One aspect of this experiment is that the model of diabetic microangiopathy of the rat is not directly comparable to the pathophysiologic and pathologic changes in humans due to the rat's relatively short life span. Although juvenile onset type diabetes is not the most common one that the surgeon faces, it is the basic model for experimental diabetes research (Goodson and Hunt 1979).

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Endogenous Growth Factor Pathways May Regulate Epidermal Hyperplasia in Chronic Venous Wounds: Modulation by Hydrocolloid Dressings

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Perspective

While qualitative aspects of dermal repair differ in wounds of different types, duration, and depths, all wounds must be resurfaced by epidermal keratinocytes before they can be deemed "healed." Epidermal keratinocytes undergo a series of activation steps in acute wound healing, which are likely to be regulated by autocrine growth control mechanims. The state of epidermal keratinocyte activation in chronic venous wounds is examined in this study in relationship to the correct expression of these changes in acute wound healing. The expression of endogenous growth factor pathways in chronic wounds is presented and changes in the growth activation associated with healing of chronic wounds are studied. The results of this study establish that growth activation of the epidermis is not defective in chronic wounds. The activation of epidermis is likely to be regulated by endogenous keratinocyte cytokine and receptor pathways, suggesting that addition of exogenous epidermal mitogens to chronic wounds is unlikely to further alter epidermal healing. In contrast, some of the therapeutic benefits of hydrocolloid dressings in promoting healing of chronic wounds may be related to their ability to suppress excessive keratinocyte proliferation and activation in chronic wounds.

Background and Introduction

The Regenerative Phenotype of Epidermal Keratinocytes

A series of changes in epidermal keratinocytes are observed within 24 h after creation of an epidermal wound (Fig. 1) [1]. The altered epidermal phenotype associated with the onset of acute wound healing has been termed

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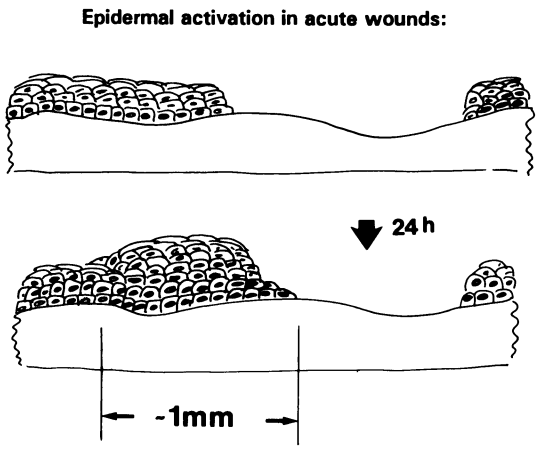

Fig. 1. Cellular and molecular alterations in epidermal keratinocytes during repair of epidermal wounds. Within 24 h of creation of a suction blister wound, regenerative epidermal growth is induced [1]. Molecular markers of regenerative growth are listed as are probable growth factor pathways which regulate endogenous epidermal growth and wound hyperplasia

"regenerative maturation" by Mansbridge [1] and these changes may be summarized as follows: After the creation of a purely epidermal wound using a suction-induced blister, changes in the epidermis that can be observed by routine histopathology include increased epidermal thickness (acanthosis) at the wound edge, migration of epidermal keratinocytes as a contiguous sheet, and keratinocyte hyperplasia [2]. Proliferating keratinocytes are usually found in a pool of cells located adjacent to the migrating cells, but migratory cells display a much lower proliferative rate [3]. These changes in tissue morphology and keratinocyte migration are accompanied by altered differentiation of keratinocytes and by transcriptional activation of new gene products in regenerating keratinocytes [1]. Keratinocytes synthesize keratin proteins 6 and 16 (K6/K16), proteins associated with epidermal hyperplasia, and psi-3, a nonkeratin protein also associated with epidermal hyperplasia

[1]. In the normal maturation of epidermal keratinocytes, filaggrin, involucrin, and transglutaminase proteins are normally expressed in the granular layer of the epidermis. The onset of regenerative maturation in wound healing is characterized by premature synthesis of filaggrin, involucrin, and transglutaminase by keratinocytes in the lower- to mid-spinous epidermal layer. Each of these changes, and the increased keratinocyte proliferation associated with epidermal wounds [4], returns to the pre-wounded "resting" epidermal phenotype within 7–14 days postwounding [1, 4]. A somewhat later marker of regenerative epidermal growth is increased expression of $\alpha_3\beta_1$ and $\alpha_6\beta_4$ integrins by suprabasal keratinocytes within 3–5 days postwounding [5]. The altered integrin expression reverts to that of normal skin by 14 days postwounding [5]. The epidermis in acute wounds thus undergoes a progressive series of morphological and biochemical changes that mark cellular activation induced in wound healing in a transient and reversible fashion. It should also be noted that, if a superficial skin wound also destroys the basement membrane zone of the epidermis, this epidermal structure will also need to be reformed. One of the latest events in repairing epidermal injury might be resynthesis of the anchoring fibril portion of the basement membrane zone. In some forms of burn wounds, reformation of anchoring fibrils can take 6 months or more after injury [6]. Thus, there may be late changes of epidermal "remodeling" that are analogous to those of dermal remodeling occurring over months to years following wounding [7].

Growth Factor Pathways Regulating Epidermal Regeneration and Wound Repair

One form of skin injury that induces both epidermal regenerative maturation [1] and proliferative activation of epidermal keratinocytes [4, 8] is removal of the epidermal stratum corneum by tape stripping. This is a superficial form of injury which does not visibly damage keratinocytes below the granular layer and which produces no bleeding or visible vascular injury. A reasonable inference from these data is that regenerative epidermal growth may largely be regulated by endogenous growth controls, instead of growth factors released from platelets or traumatized blood vessels. Over the last several years, considerable progress has been made in understanding how growth factors produced locally in the skin regulate normal epithelial cell growth and tissue hyperplasia related to wound healing.

Polypeptide Regulators of Epidermal Keratinocyte Growth

A number of growth factors or immunologically relevant cytokines regulate the proliferation of epidermal keratinocytes. Table 1 summarizes positive and negative polypeptide regulators of human keratinocyte growth based on

Table 1. Cytokines regulating epidermal keratinocyte growth

Mitogenic factors
EGF
TGF-α
amphiregulin
IGF-I
IGF-II
Insulin (at supraphysiologic concentrations)
aFGF
bFGF
KGF (FGF-7)
IL-1
IL-3
IL-6
GM-CSF
Inhibitory factors
TGF-β
IFN-γ
No direct effects
PDGF AA, BB, or AB
IL-2

EGF, epidermal growth factor; TGF, transforming growth factor; IGF, insulin-like growth factor; aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; KGF, Keratinocyte growth factor; IL, interleukin; GM-CSF, granulocyte/macrophage-colony stimulating factor; IFN, interferon; PDGF, platelet-derived growth factor.

their activity in in vitro assays of keratinocyte proliferation. Epidermal growth factor (EGF), transforming growth factor-α (TGF-α), and amphiregulin are proteins in the EGF family which act as keratinocyte mitogens through binding to the EGF receptor [9]. The proliferative stimulus provided by each of these EGF-like molecules also requires costimulation of the insulin-like growth factor-I receptor by an appropriate ligand, i.e., insulin-like growth factor-I or -II (IGF-I/IGF-II) or supraphysiologic concentrations of insulin [10]. At least three members of the fibroblast growth factor (FGF) family can also stimulate keratinocyte proliferation: basic FGF (bFGF), acidic FGF (aFGF), and keratinocyte growth factor (KGF, also termed FGF-7) have been shown to increase keratinocyte proliferation in culture [11, 12]. Like the action of EGF and related mitogens, induction of cell proliferation by FGF family polypeptides also appears to require costimulation of the IGF-I receptor [13]. Platelet-derived growth factor (PDGF), which is though to be the major polypeptide mitogen for connective tissue cells, is not a mitogen for human epidermal keratinocytes, because this cell type lacks receptors for PDGF [14]. An important distinction between epidermal and dermal responses to tissue injury (wounding) may be the

selective responsiveness of connective tissue cells to PDGF, whereas epidermal keratinocytes respond primarily to other mitogens. Conversely, fibroblasts and other connective tissue cells appear not to express receptors for KGF/FGF-7 and this molecule does not serve as a connective tissue cell mitogen [12]. The contrasting distributions of KGF/FGF-7 receptors and PDGF in different tissue compartments in skin may thus serve to allow selective amplification of epithelial vs connective tissue cells in the wound repair response. Transforming growth factor-β (TGF-β) inhibits the growth of epidermal keratinocytes in culture [11] and might thus serve to limit proliferative responses in some circumstances.

A number of immunologically relevant cytokines also regulate the growth of human epidermal keratinocytes in culture. Interleukin-1 (IL-1), interleukin-3 (IL-3), interleukin-6 (IL-6), and granulocyte-macrophage colony stimulating factor (GM-CSF) can all act as keratinocyte mitogens in tissue culture [15]. Interferon-γ (IFN-γ) inhibits the proliferation of keratinocytes in response to other mitogens in vitro. Although these factors are not usually considered as primary mediators of soft tissue wound repair, their importance in healing of cutaneous wounds should not be underestimated. Both acute and chronic inflammatory cells appear in skin wounds within hours to days after wounds are initiated [7], and interleukins, colony stimulating factors, and interferons are not only synthesized by activated inflammatory cells, but also regulate the local influx of these cell types. For example, GM-CSF can increase macrophage trafficking in human skin and it accelerates the healing of acute skin wounds in humans [16].

Cellular Sources for Growth Factors in Wounds

The faster healing of experimental wounds produced by application of a variety of growth factors has firmly established a biological role for these molecules in regulation of wound healing [17, 18]. As platelets accumulate and degranulate at sites of acute vascular injury, it has been proposed that much of the cellular activation associated with wound healing results from the release of PDGF and other growth factors from platelets at wound sites [19]. This scheme fits reasonably well with the conversion of largely inactive connective tissue cells in uninjured dermis into activated cells soon after dermal injury. In contrast, this model does not serve well to explain the sustained proliferation of epidermal keratinocytes in normal skin or their conversion to an activated phenotype (regenerative growth) without concomitant vascular injury. Instead, there is emerging evidence that normal and hyperplastic growth of epidermal keratinocytes may be largely regulated by autocrine growth factors and/or by growth factors supplied in a paracrine fashion from dermal connective tissue cells. TGF-α is produced by human epidermal keratinocytes and its secretion can be self-amplified [20]. In normal epidermis, TGF-α is produced predominantly by basal keratinocytes,

whereas in hyperplastic epidermis, TGF-α production is remarkably increased and it is synthsized by both basal and suprabasal keratinocytes [15, 21]. EGF receptors, which are capable of being activated by TGF-α, are also modulated in epidermal hyperplasia so that they display increased ligand binding [22]. Likewise, IGF-I receptors are expressed by basal keratinocytes in normal epidermis [10], and these receptors are quantitatively increased in hyperproliferative epidermis, with marked increase in tyrosine kinase activation over resting epidermis [23]. IGF-I is presumably supplied to epidermal IGF-I receptors from dermal fibroblasts or from high concentrations of IGF-I in blood plasma [23]. IL-1 and IL-6 are synthesized by normal keratinocytes and can serve as mitogens [24]. These molecules may regulate some aspects of epidermal hyperplasia, since IL-1 receptors and IL-6 ligand are increased in hyperplastic human epidermis [24, 25]. The synthesis of KGF/FGF-7 is rapidly increased in experimental wounds [26], and it seems likely that human wounds might display similar induction of this growth factor which would be targeted in a paracrine fashion to epidermal keratinocytes. While human epidermal keratinocytes do not express PDGF receptors, a rather surprising observation is that keratinocytes apparently synthesize PDGF A- and B-chains [14]. In this fashion, epidermal-derived PDGF might regulate the growth of dermal fibroblasts and vascular elements via PDGF receptors that are modulated on these cells in normal vs hyperplastic human skin [27]. Most of the information available on the expression and modulation of epidermal growth factor ligands and receptors comes from study of normal (unwounded) human skin and from stable tissue hyperplasias such as psoriasis. Data are presented in the next section on expression of some of these growth factor ligand/receptor systems in chronic venous wounds, but little information is available on modulation of these pathways in healing of acute human wounds.

Characterization of Cellular Activation and Growth Factor Activity in Chronic Human Wounds

Chronic (nonhealing) leg ulcers which are not attributable to arterial insufficiency present as clinically apparent superficial wounds. Unlike pressure necrosis (decubitus) ulcers located elsewhere and unlike leg ulcers due to arterial insufficiency, there is not usually a loss of deep dermal tissue in venous ulcers, unless these wounds are highly infected with bacteria. Healing of venous wounds appears to involve ingrowth of epithelium from ulcer margins more than visible alteration of the ulcer bed. Sometimes, more vascularization (redness) is clinically evident in healing responses of venous wounds, but usually the overall abundance of granulation tissue changes little with healing. Thus, our approach to analysis of cellular activation in the chronic venous ulcer involves a more extensive analysis of the adjacent epidermis.

Pathological Characterization of Nonhealing Venous Ulcers

Incisional biopsies of more than 30 venous ulcers ranging in duration from 2 months to more than 15 years were analyzed for expression of markers of regenerative epidermal growth and for expression of TGF-α, EGF receptors, IGF-I receptors, PDGF receptors, and IL-6, all of which are increased in stable epidermal hyperplasias relative to normal skin [15, 21–24]. As a direct measure of cell proliferation in these wounds, expression of the Ki67 cyclin protein was also assessed [28]. The expression of each marker protein was assessed by immunohistochemistry using specific antibodies on cryostat-cut or paraffin-embedded tissue samples.

Epidermal Activation in Chronic Wounds

Figure 2 displays expression of regenerative epidermal growth markers in epidermis at the edge of chronic wounds. The psi-3 protein is not expressed in unwounded human skin (Fig. 2A), but is abundantly present in lower spinous keratinocytes of acanthotic epidermis adjacent to a chronic wound (Fig. 2B), similar to its appearance in acute skin wounds [1]. Keratin 16, which is only expressed in hyperplastic epidermis, is present in all viable keratinocyte layers of periulcer epidermis (Fig. 2C). Filaggrin and involucrin expression was also examined in epidermis adjacent to chronic wounds (not shown). Each of these proteins was expressed by keratinocytes in the lower spinous epidermal layers, recapitulating the differentiation pattern seen in acute epidermal wounds. Without a single exception in more than 30 different wounds studied, markers of epidermal regenerative maturation were expressed in epidermis immediately adjacent to chronic wounds. One variable, however, was the extent of epidermal acanthosis associated with different wounds. This varied from three to 15 times the thickness of normal epidermis on uninvolved portions of the lower extremities. In acute wounds, epidermal regenerative activation occurs in epidermis within about 1 mm of the wound edge (Fig. 1). In chronic wounds, the zone of regenerating epidermis was much broader, extending at least 4–6 mm from the wound edge. It is likely that regenerative epidermis extended considerably further from the wound bed in some cases, but typical biopsies extended only about 6 mm from the wound margin and the further extent of regenerative epidermal maturation could not be assessed.

The regenerative phenotype of the epidermis adjacent to chronic wounds suggests that the epidermis is in an activated prohealing state. As a further assessment of this activation, expression of the Ki67 protein was analyzed in periulcer epidermis. The Ki67 antigen is a cyclin-like protein that is expressed in the nucleus of cells in mid-S, G_2, and M phases of the cell cycle and marks only cells which are actively proliferative [28]. The pattern of Ki67 expression in acanthotic epidermis adjacent to a venous

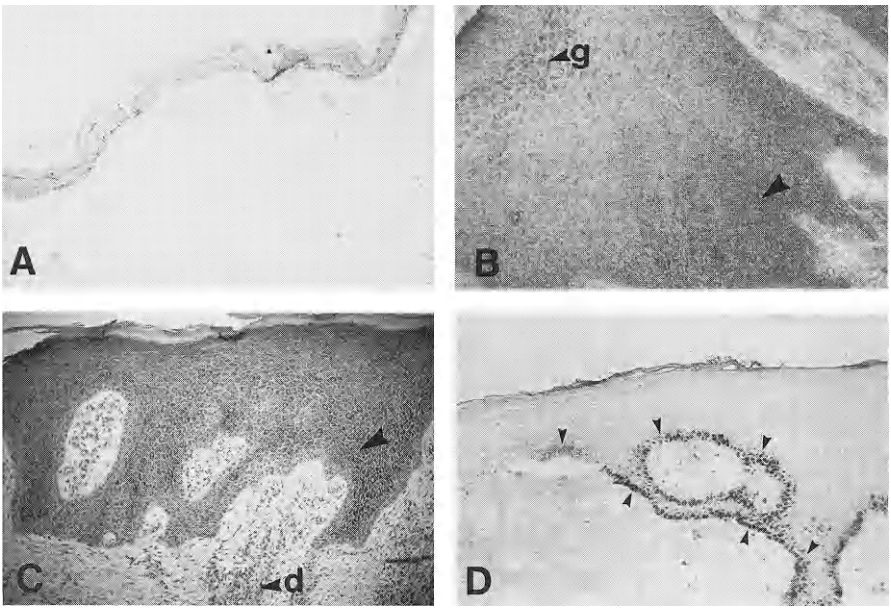

Fig. 2A–D. Immunohistochemical detection of regenerative epidermal growth or proliferation markers in epithelium immediately adjacent to chronic wounds. Normal skin (**A**) or periulcer epidermis (**B**) reacted with psi-3 antibodies. In **B**, psi-3 staining is indicated by *arrowhead*; the granular layer (*g*) is stained only by a hematoxylin counter stain and does not react strongly with psi-3 antibodies. Normal epidermis (**A**) does not express psi-3 protein. In **C**, expression of keratin 16 in suprabasal keratinocytes of periulcer epidermis is indicated by an *arrowhead*; nuclei of dermal cells (*d*) are stained only by the hematoxylin counter stain. In **D**, the reaction of periulcer epidermis with Ki67 antibodies is shown; *small arrowheads* point to some of the positive nuclear staining. No counter stain is used in **D** and all cells with dark nuclei express the Ki67 protein. 400×

ulcer is shown in Fig. 2D, where it is abundantly expressed by basal and immediately suprabasal keratinocytes. In unwounded human epidermis, the Ki67 is expressed maximally by one in ten basal keratinocytes (not shown). This establishes that epidermis adjacent to chronic wounds displays phenotypic markers of regenerating epidermis and a high rate of keratinocyte proliferation.

Growth Factor Activity in Chronic Wounds

The expression of several growth factor ligand/receptor systems was examined in epidermis and dermis adjacent to chronic wounds. EGF receptors are expressed at the surface of keratinocytes in the basal and spinous epidermal layers in periulcer skin (Fig. 3B). Figure 3A shows a control for the EGF receptor staining – there is no reaction of epidermal keratinocytes

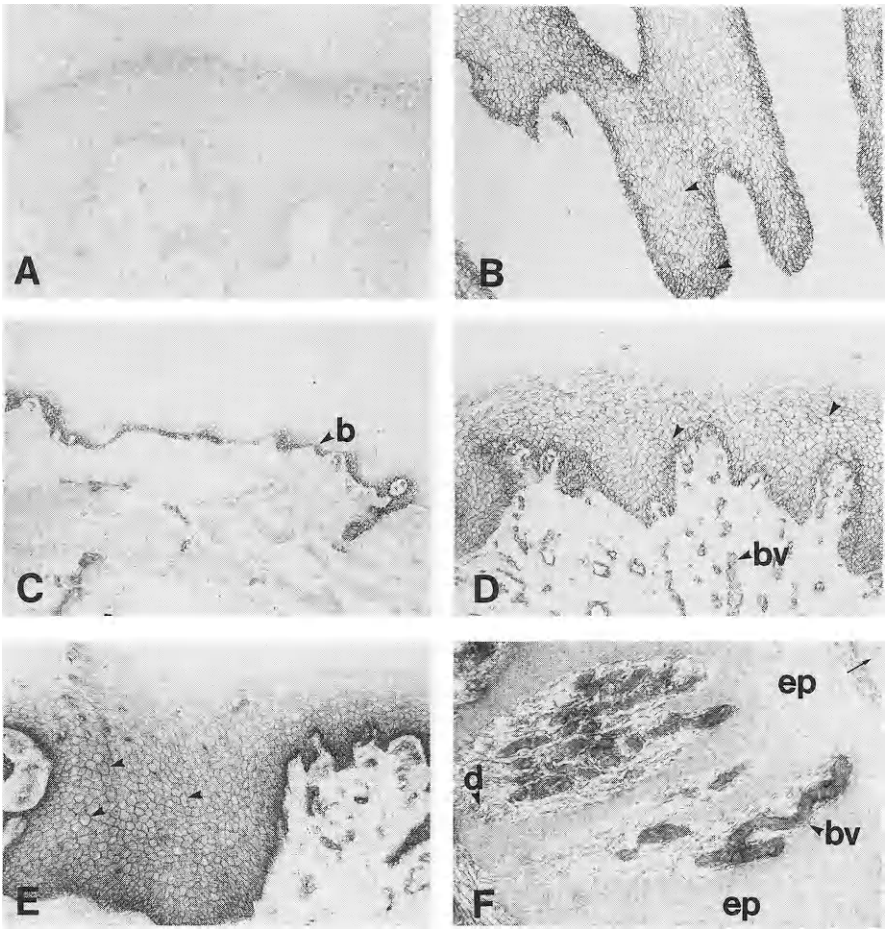

Fig. 3A–F. Immunohistochemical analysis of endogenous growth factor ligands/receptors by epithelium adjacent to chronic wounds. In **A**, there is no reaction of periulcer epidermis with IL-2 (interleukin-2) receptor antibodies. **B** Reaction of periulcer epithelium with epidermal growth factor (EGF) receptor antibodies; cell surface staining indicated by *small arrowheads*. Reaction of normal skin (**C**) or periulcer epidermis (**D**) with monoclonal antibodies to transforming growth factor-α (TGF-α); *b*, reaction of basal epidermal keratinocytes with TGF-α antibodies in **C**; in **D**, *small arrowheads* show staining of suprabasal keratinocytes and (*bv*) indicates staining of blood vessels for TGF-α. **E** Reaction of insulin-like growth factor-1 (IGF-1) receptor antibodies with suprabasal keratinocytes (*small arrowheads*) in periulcer epidermis. **F** Reaction of platelet-derived growth factor (PDGF) receptor (β-subunit-specific) antibodies with periulcer skin. The epidermis (*ep*) is not stained by the antibody; *arrow* indicates direction of the stratum corneum. Blood vessels (*bv*) and dermal fibroblasts (*d*) express abundant PDGF receptors. 400×

with antibodies to the IL-2 receptor (which is expressed only on activated T lymphocytes). The expression of TGF-α in normal epidermis and periulcer epidermis is shown in Fig. 3C,D. In normal skin, TGF-α is predominantly expressed by basal keratinocytes in the epidermis, whereas TGF-α is abundantly expressed by both basal and spinous keratinocytes in acanthotic epidermis adjacent to the wound bed (Fig. 3D). TGF-α is also predominantly associated with blood vessels in the dermis of periulcer skin. IGF-I receptors are also abundantly expressed at the cell surface of basal and spinous keratinocytes in acanthotic epidermis adjacent to venous wounds (Fig. 3E), in sharp contrast to the basal distribution of these receptors in normal skin (not shown [10]). The expression of EGF receptors and the increased expression of TGF-α and of IGF-I receptors in acanthotic epidermis adjacent to chronic venous wounds are similar to activated expression of these growth regulating molecules in hyperplastic epidermis of psoriasis [23]. The activation of these two growth factor systems by autocrine TGF-α and paracrine IGF-I would presumably be sufficient to stimulate keratinocyte hyperplasia in epidermis adjacent to chronic wounds [10, 23]. IL-6, which is also increased in epidermis of periulcer epidermis (not shown), might also contribute to epidermal hyperplasia as an autocrine factor [24]. Finally, the expression of PDGF-β receptors is shown in periulcer skin (Fig. 3F). PDGF receptors are prominently displayed in dermal blood vessels and dermal fibroblasts, but no receptors are detected on epidermal keratinocytes. This

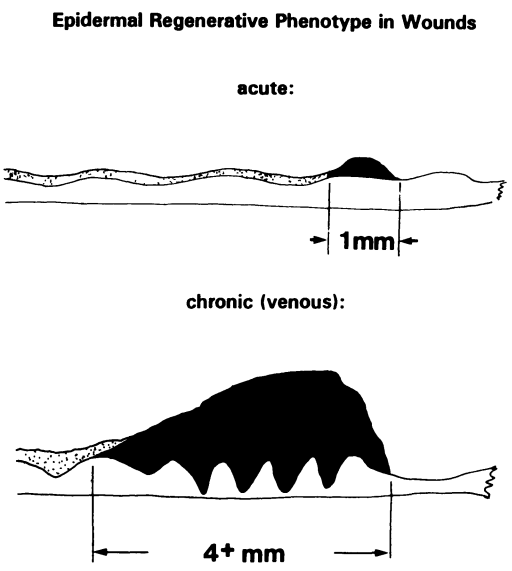

Fig. 4. The regenerative epidermal zone in acute vs chronic skin wounds. The epidermis in chronic wounds displays more acanthosis (thickness), a broader lateral spread, and more profound rete formation. Figs. 2 and 3 display the expression of proteins induced within the regenerative zone of chronic venous wounds

suggests that PDGF would selectively activate only dermal cells if it were present in chronic wound tissue.

Figure 4 illustrates the epidermal activation zones in acute wounds and in nonhealing venous ulcers. The activation zone in chronic wound epithelium is much broader and there is more extensive epidermal acanthosis compared to acute wounds. The changes in growth factor ligand/receptor systems described above occur throughout the activated zone illustrated in this figure.

Characterization of Growth Factor Activity and Cellular Activation in Venous Wounds Treated with Hydrocolloid Dressings

Hydrocolloid dressings accelerate the healing of acute and chronic skin wounds [29, 30]. The ability of this form of treatment to modulate growth factor accumulation and cellular activation in chronic wounds has been examined.

Analysis of Wound Exudate Under Hydrocolloid Dressings for Growth Factor Activity

In a previous study, it was established that wound exudates which were formed in acute human wounds treated with hydrocolloid (DuoDERM) dressings displayed mitogenic activity for human keratinocytes in culture

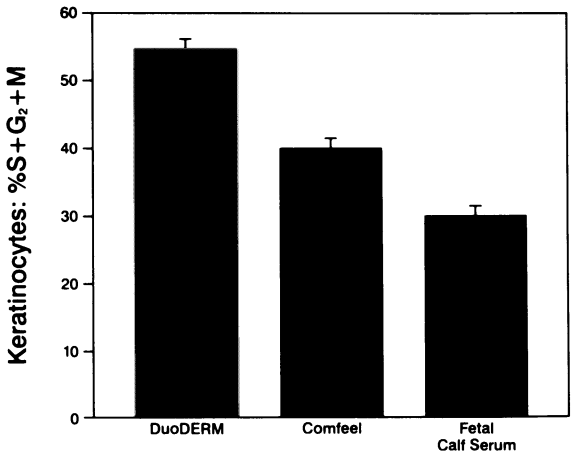

Fig. 5. Human keratinocyte proliferation induction in vitro by chronic wound exudates accumulating under DuoDERM or Comfeel hydrocolloid dressings. Control cultures contained only fetal calf serum as a mitogen. Proliferation was assessed by flow cytofluorometry, as previously described [31]

[31]. Using flow cytofluorometry to measure the cell cycle distribution of stratified cultures of human keratinocytes, an analysis of exudates of chronic venous wounds was performed by the same methods used for acute wound exudates [31]. Nine patients with chronic leg ulcers were enrolled into a crossover study which compared mitogenic activity of exudates accumulating under two different hydrocolloid dressings. The results of this study are illustrated in Fig. 5. Compared to control cultures maintained in fetal calf serum, exudates accumulating under Comfeel dressings increased the fraction of cycling keratinocytes ($S + G_2 + M$ phases of cell cycle) by an average of 9%, whereas exudates accumulating under DuoDERM dressings increased cell proliferation by an average of 24%. The epidermal mitogen(s) which accumulate in chronic wound exudate under occlusive dressings might mediate increased activation of wound margin keratinocytes, a finding which is described in the next section.

Changes in Cellular Activation in Chronic Wounds During Healing Induced by Hydrocolloid Dressing Therapy

A study is in progress to elucidate cellular changes which occur when chronic venous ulcers begin to heal through therapeutic intervention. In this study, patients with venous ulcers that are not healing on standard therapies, e.g., Unna boot or wet to dry dressings, are treated for a period of 4 weeks with one of two different occlusive dressings: hydrophilic polyurethane sheets without adhesive backing (Allevyn) or a new formulation hydrocolloid dressing with adhesive backing (DuoDERM CGF). Three incisional biopsies are obtained from each ulcer during the study: one before application of the occlusive dressings, one after 2 weeks of application, and another at the conclusion of 4 weeks of treatment. The degree of cellular activation in each biopsy was assessed by the expression of regenerative, proliferative, and growth factor ligand/receptor markers that were described above.

The results from one patient treated with a hydrocolloid dressing will be presented to illustrate that significant changes in cellular activation occur as healing commences in a chronic wound. Figure 6A illustrates the clinical appearance of a venous ulcer which had been present for 2 years in a 75 year old man. He had previously been treated with Unna boots, surgical debridement, topical antibiotics, vein stripping, and wet to dry dressings without improvement. The ulcer was largely healed after 4 weeks of treatment with hydrocolloid dressings (Fig. 6B). Before therapy, the epidermis adjacent to the ulcer showed a regenerative epidermal phenotype over the 5 mm of epidermis adjacent to the ulcer bed that was present in the tissue biopsy (not shown). Analysis of epidermal keratinocyte proliferation by expression of the Ki67 protein was performed on tissue sections from pretreatment, day 14, and day 28 treatment biopsies. In pretreatment epidermis adjacent to the ulcer, a relatively high rate of keratinocyte proliferation was

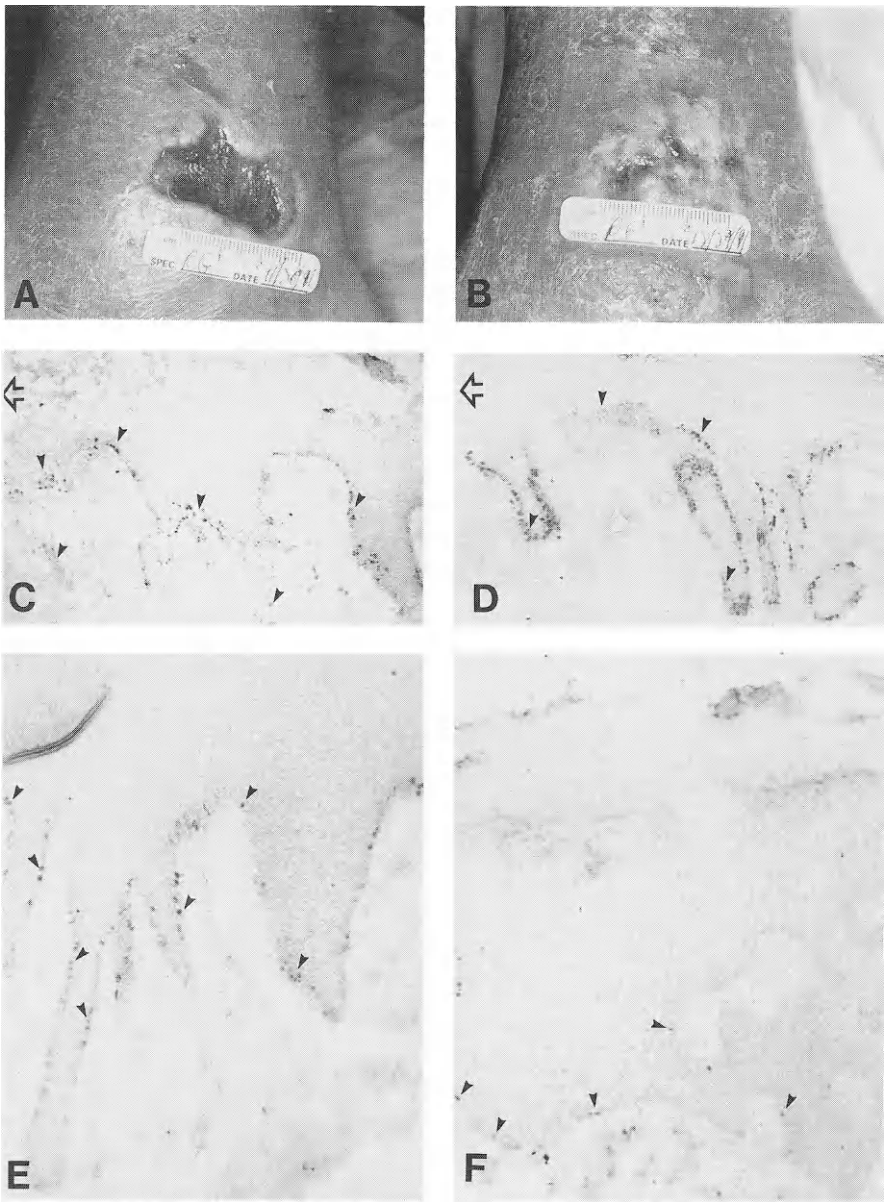

Fig. 6A–F. Evaluation of epidermal activation in chronic venous wounds during healing induced by hydrocolloid dressing therapy. The clinical appearance of a leg wound before (**A**) and after (**B**) 4 weeks treatment. Epidermal areas within 1 mm of this wound edge are shown in (**C** and **D**), where *open arrows* point towards the ulcer margin, while epidermis removed approximately 3 mm from the ulcer edge is shown in (**E,F**). These epidermal areas before treatment (**C,E**) and after 2 weeks of treatment with hydrocolloid dressings (**D,F**) are shown after reaction with Ki67 antibodies and immunohistochemical detection. *Small arrowheads* in **C–F** point to prominent nuclei expressing the Ki67 protein. Note the reduced proliferative rate in **F** compared to that in **E**

observed in epidermis immediately adjacent to the ulcer bed (Fig. 6C) and in epidermis 3 mm from the ulcer edge (Fig. 6E). In normal (unwounded) epidermis, the Ki67 protein is expressed only by one in ten (or fewer) basal layer keratinocytes. Thus, there is marked epidermal proliferative activation in the epidermis adjacent to the ulcer edge before hydrocolloid dressing application. The biopsy obtained from this ulcer after 2 weeks of treatment represents approximately the midpoint in healing of this ulcer. Epidermis within 1 mm of the ulcer edge shows marked proliferative activity of keratinocytes (Fig. 6D), which appears to be somewhat increased over the comparable area before treatment (see Fig. 6C,D). In sharp contrast, epidermis removed 3 mm from the wound edge (Fig. 6F) shows a remarkable reduction in keratinocyte proliferation compared to pretreatment epidermis at a comparable distance from the wound edge (see Fig. 6E,F). The rate of keratinocyte proliferation in the juxtamarginal (3 mm removed) epithelium has been reduced to a rate very similar to that of unwounded normal skin.

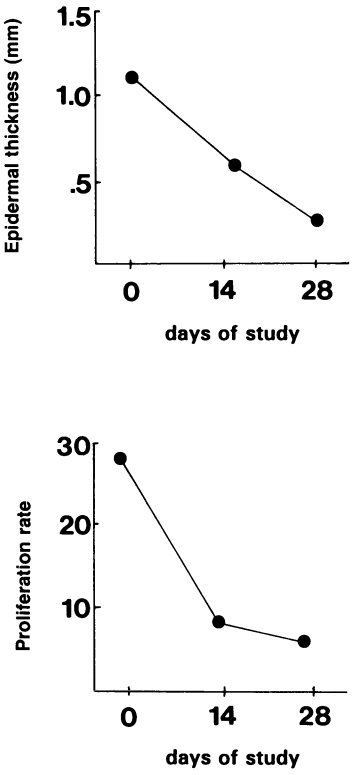

Fig. 7. Reduction in epidermal thickness (*top*) and rate of epidermal proliferation (*bottom*) in juxtamarginal epithelium during wound healing induced by hydrocolloid dressing therapy. These data are derived from the patient illustrated in Fig. 6. Epidermal thickness is measured from the granular layer to the basal epidermal layer. The proliferation rate is the number of Ki67+ basal cell nuclei per 100 basal cells

Figure 7 displays changes in epidermal acanthosis (thickness) and the abundance of Ki67-positive keratinocytes in juxtamarginal epidermis as a function of healing under a hydrocolloid dressing. The progressive reduction in epidermal acanthosis during the healing phase parallels a reduction in the density of proliferating epidermal keratinocytes. Also accompanying these changes were reductions in keratin 16 staining and suprabasal expression of IGF-1 receptors and TGF-α by keratinocytes (not shown). Thus, a considerable reduction in keratinocyte activation occurs in the juxtamarginal epithelium under hydrocolloid dressings. There is also some proliferative stimulation in epidermis immediately adjacent to the ulcer bed. The overall changes in cellular activation during this healing process appear to convert a broad zone of keratinocyte proliferation in chronic wounds (illustrated in Fig. 4) to a much narrower zone, more consistent with that found in acute wounds (see Fig. 4).

Discussion

Chronic venous wounds display profound regenerative and proliferative activation of the immediately adjacent epidermis. Why do these wounds fail to heal? Certainly there is no lack of proliferative signals to the epidermis; the extent of cellular activation in the epidermis exceeds that even in acute wounds [1]. The defect in healing thus appears to relate to lack of keratinocyte migration across a relatively cellular wound bed.

The explanation for defective keratinocyte migration across the venous ulcer wound bed is not immediately apparent from the present studies. From studies of epidermal migration in healing wounds [2], it appears that suprabasal keratinocytes are activated to migrate over basal keratinocytes at the wound edge which are attached to the basement membrane. From in vivo studies of epidermal migration in acute wounds, it appears that two functional pools of keratinocytes contribute to this process: a pool of keratinocytes which is relatively deficient in proliferating cells migrates or advances over the wound bed, while a second pool of keratinocytes that is rapidly proliferative appears to replenish the migrating pool [2, 3]. In this way, keratinocytes in a stratified epidermis might alternatively exist as either (1) resting keratinocytes which would contribute to low level epidermal renewal, (2) highly activated proliferative keratinocytes, or (3) actively migrating cells. If this is the case, then the failure of chronic venous wounds to heal could be related to pathologically excessive proliferative activation over a broad zone adjacent to the wound. Occlusive dressings, including hydrocolloid (DuoDERM) dressings, have been shown to rapidly downregulate excessive keratinocyte proliferation associated with epidermal wounds [4] or psoriasis [32]. Thus, one possible interpretation of the beneficial effects of hydrocolloid dressings in wound healing is that some or all of their therapeutic mechanism relates to their ability to suppress excessive

keratinocyte proliferation, perhaps thereby increasing the pool of migratory keratinocytes. In fact, the healing wound pictured in Fig. 6D had a narrow zone of keratinocytes in the advancing epithelial margin which was devoid of proliferating keratinocytes.

The accumulation of growth-promoting exudate under occlusive dressings might also contain factors which stimulate epidermal migration across the wound bed. Thus, another possibility for failure of chronic wounds to heal is that migration-associated molecules are defective in long-standing granulation tissue. Keratinocyte migration is related to the expression of specific integrin receptor types and to the availability of acceptor ligands for these receptors [33–35]. A deficit in either integrin receptor expression in chronic wound keratinocytes or appropriate acceptor ligands, e.g., intact molecules of fibronectin, laminin, or collagen, might prevent effective epidermal migration across a wound bed. Clinical studies which indicate that healing of chronic wounds can be accelerated by addition of exogenous fibronectin [34, 35] support the concept that migration-related ligands might be defective in chronic wounds. A potential benefit of hydrocolloid dressings in this regard could be release of collagen on the wound surface, as this class of dressings is formulated with gelatin (denatured collagen) in the base.

Although the present investigations do not distinguish between excessive keratinocyte proliferation or defective migration-related molecules in chronic venous wounds, the results strongly suggest that further addition of epidermal mitogens to chronic wounds would add little to the high endogenous proliferation present in affected epithelium. Thus, disappointing results from addition of high concentrations of recombinant EGF to chronic venous wounds might have been anticipated [36]. Even so, addition of exogenous growth factors which have no direct effects on epidermal keratinocytes, e.g., PDGF, or factors which increase synthesis of extracellular matrix components, e.g., TGF-β, might have greater therapeutic potential.

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Immunohistochemical Effect of a Hydrocolloid Occlusive Dressing (DuoDERM E) in Psoriasis Vulgaris

I.M.J.J. van Vlijmen-Willems, A. Chang, J.B.M. Boezeman, and P.C.M. van de Kerkhof

Introduction

Topical application of the hydrocolloid occlusive dressing DuoDERM has been shown in various studies to have an anti-psoriatic effect as monotherapy, but especially in combination with a topical corticosteroid.

Baxter and Stoughton (1970) reported that an occlusive dressing applied on psoriatic lesions for 24 h every day for 4 days decreases the mitotic index. This was confirmed by Fry (1970), who also found reformation of the granular cell layer.

Shore (1985) demonstrated that waterproof tape was more effective than topical emollients or other less occlusive tape and that application of tape for 1 week or longer was clearly superior to repeated daily applications. Friedman (1987) and Fiskerstrand (in press) evaluated both different adhesives and almost impermeable hydrocolloid dressings (HCDs) such as DuoDERM and compared these with other treatments of psoriasis. They reported that weekly treatment of psoriasis with corticosteroids and an HCD is superior to the steroid alone or to ultraviolet B treatment, and ultraviolet B was comparable with HCD alone.

Although many investigators reported clinical improvement by the application of HCD, the precise mechanism is still unknown. The aim of this study was to assess the effect of HCD as monotherapy at the immunohistochemical level. In particular, the questions to what extent does weekly HCD as monotherapy have an anti-psoriatic effect and how is it tolerated were addressed.

Material and Methods

Punch biopsies were taken from psoriatic lesions of ten patients before treatment and after 3 weeks treatment with DuoDERM E. Biopsies were embedded, snap frozen in liquid nitrogen and sections were cut and fixed.

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Immunohistochemical stainings were carried out with markers for epidermal growth (Ki-67), keratinization (Ks8.12), inflammation (T11 and anti-elastase) and endothelium (Pal-E and anti-ICAM-1).

Results

When we examined the psoriatic lesion before and after treatment with DuoDERM E we saw that there was an improvement of the lesion, but total clearance was not achieved after 3 weeks of treatment. After 1 week of treatment the severity scores were already significant with respect to induration and scaling. The improvement of these continued during the 3 weeks of treatment. Erythema showed less improvement. There was no sign of irritancy and the treatment was well tolerated by all patients.

The effect of DuoDERM E on parameters of epidermal growth, keratinization, inflammation and endothelium are summarized as follows: The number of Ki-67-positive nuclei showed a slight decrease during the 3 weeks of treatment, but this decrease was not statistically significant ($p = 0.16$). A similar decrease of suprabasal expression of keratin 16, assessed by Ks8.12 staining, was observed. The intensity of this staining decreased from pronounced to moderate after treatment with DuoDERM E.

In untreated psoriatic plaques polymorphonuclear leukocytes (PMN), as shown by the antibody anti-elastase, were present as microabscesses in the stratum corneum and dispersed in the epidermis. After treatment with DuoDERM E the number of PMN had decreased and no microabscesses were found ($p = 0.2$). Dermal PMN did not change. T lymphocytes (T11-positive cells) were numerous in the dermis and epidermis of untreated psoriatic lesions, comprising 51%–71% of the dermal infiltrate. There was a slight decrease ($p = 0.07$) of T lymphocytes in the dermis. The T11 staining in the epidermis did not decrease after 3 weeks treatment. The endothelium markers PAL-E and anti-ICAM-1 tended to stay the same after 3 weeks of treatment.

Conclusion

Topical application of HCD as monotherapy results in clinical improvement of the psoriatic lesion. Markers for inflammation, epidermal proliferation and keratinization showed only a tendency to improve. By clinical and immunohistochemical assessment it was clearly demonstrated that HCD as monotherapy was well tolerated and did not cause irritancy. Thus, HCD provides an attractive approach to incorporate topical antipsoriatic drugs, permitting weekly applications and encompassing optimal patient compliance.

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The Treatment of Malum Perforans Pedis: Operative and Conservative Therapy

R. Siebel, R. Baumgartner, B. Greitemann, and Th. Junker

Introduction

Malum perforans pedis frequently occurs as a complication in diabetes mellitus and neuropathic diseases. It typically appears as a nonpainful chronic ulcer of the sole of the foot, caused by bony projections (Fig. 1). As a result an infection of the underlying bone is often seen.

Especially in a foot suffering from disturbed circulation and immune defense, severe consequences of diabetes may occur. The development of malum perforans is due to a combination of several factors. In the diabetic the following factors are of importance:

1. Diabetic microangiopathy leads to atrophy of the skin and an increased vulnerability due to decreased circulation through the tissue.
2. At the same time, neuropathy develops due to disturbance of the circulation of the vasa nutritia of the nerve.
3. The diabetic neuropathy and the neuropathy in general cause the patient not to feel the pain, and the diabetic often does not see the wound because of accompanying disturbances in vision.
4. The osteoarthropathy leads to bone lesions with a collapse of the longitudinal arch of the foot and destruction of the metatarsalia.

A consequence is protrusion of the bony projections and a perforation of the skin. Generally this process sooner or later leads to amputation of the affected foot. In order to avoid this operation but to cure the ulcer, resection of the prominent bone is carried out in our clinic if conservative methods such as orthopedic shoes have failed. If the circulation is viable, usually a two of three resection is carried out. The resection of all metatarsalia equals a functional amputation of the terminal portion of the foot (Fig. 2).

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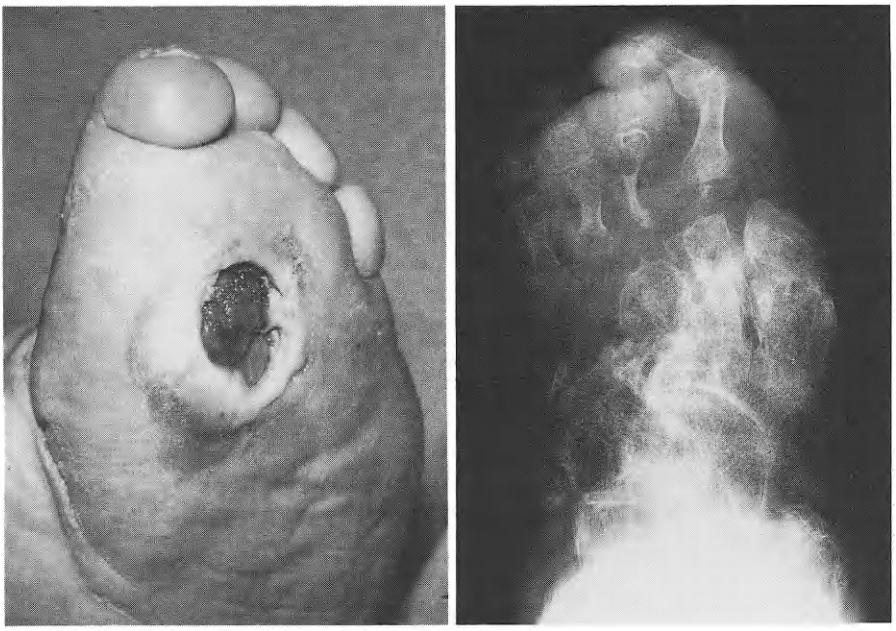

Fig. 1. Malum perforans with the corresponding X-ray

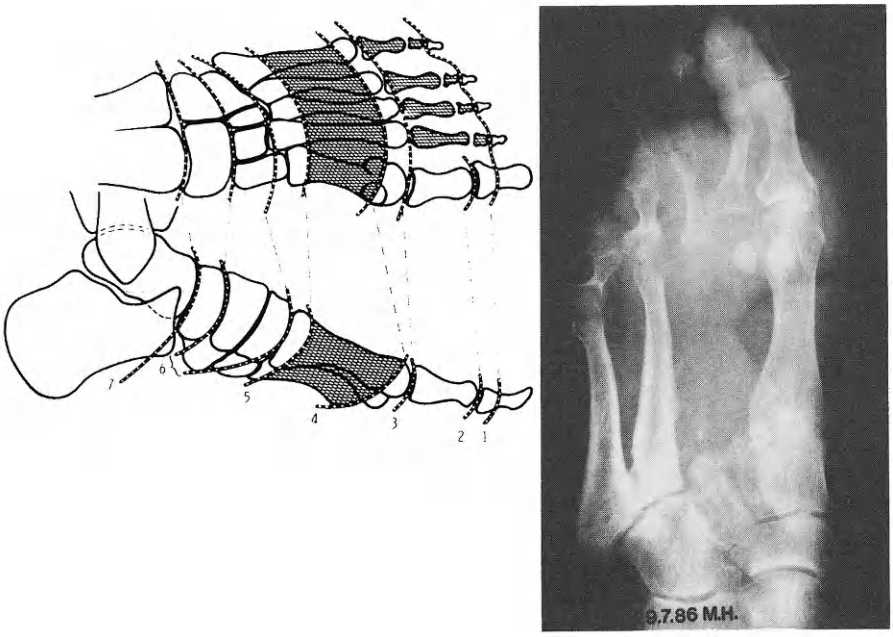

Fig. 2. Amputation scheme with corresponding X-ray

Toes are also very important for patients. They do not consider themselves amputees, in part because no phantom sensations or neuroma pain are felt.

Patients and Methods

A total of 44 patients (49 operated feet) who were treated between 1986 and 1991 were examined. Many of the patients had been operated on before. As expected, the typical basic diseases dominated.

Reexaminations were carried out on average 22 months after the operation. Some 94% of the patients could be reexamined.

In all cases the operation took place in longitudinal dorsal cuts to avoid scars of the foot sole. A precise preparation to avoid injury of the plantar vessels was necessary.

The mobilization took place on average 9 days postoperatively with the help of therapeutic footwear or handmade interimorthoses (Fig. 3).

After the operation had taken place and the ulcer was almost healed, enzymatic ointments and hydrocolloid dressings were used to support wound

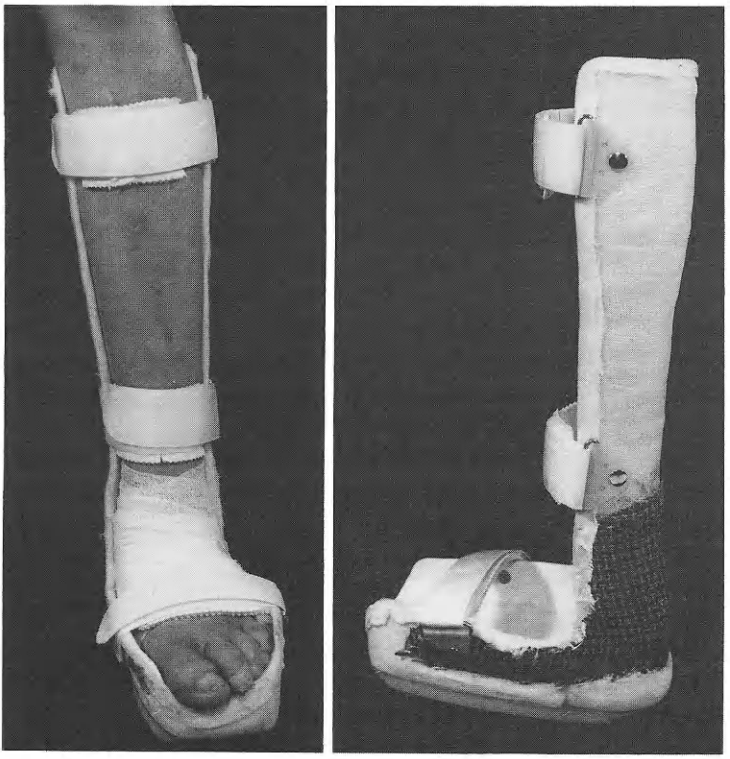

Fig. 3. Adapted shoe and interimorthosis

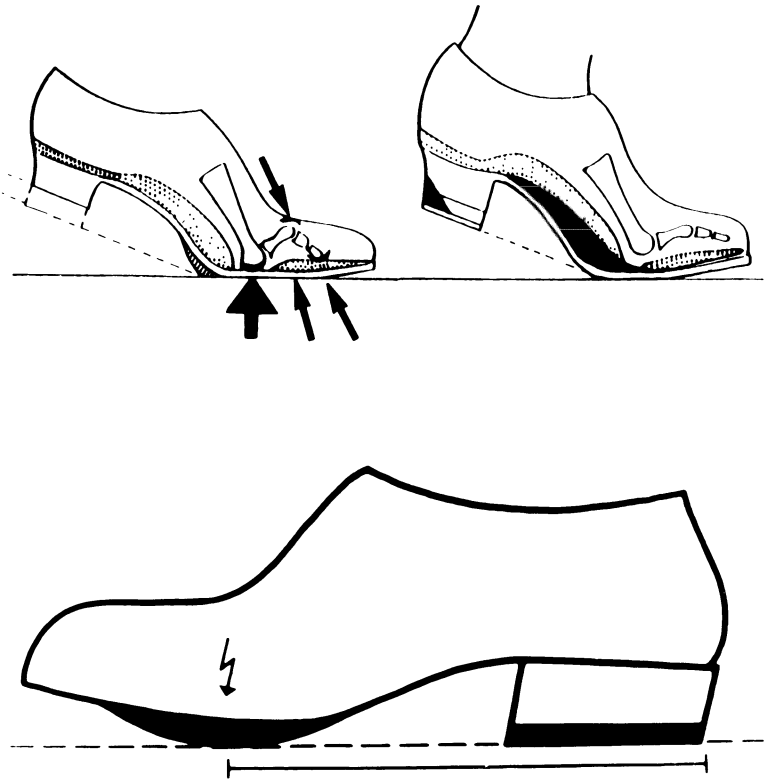

Fig. 4. Arch support and sole stiffening

healing. In fact, these methods are used even in ulcers not requiring surgery. Two months later, after wound healing, the patient received adapted shoes including orthopedic arch support (Fig. 4). Most important is the stiffening and flattening of the sole.

The crosswise support and the bedding sole are designed to avoid any pressure on vulnerable parts of the foot, all in relation to the X-ray.

Results

Resection of the metatarsalia proved to be very successful. All ulcers healed. Only four patients needed orthopedic custom shoes. In only nine patients was there the necessity to amputate afterwards; thus, in 85% amputation was avoided.

The judgement of the patients concerning the operation was impressive: 94% were very pleased, 71% evaluated the operation as good or quite good.

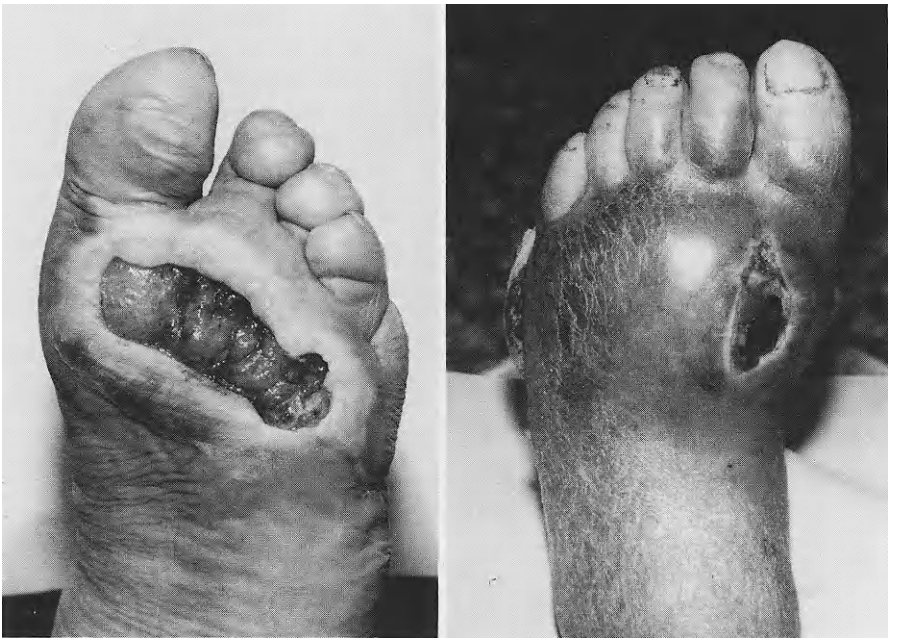

Fig. 5. Extensive malum perforans

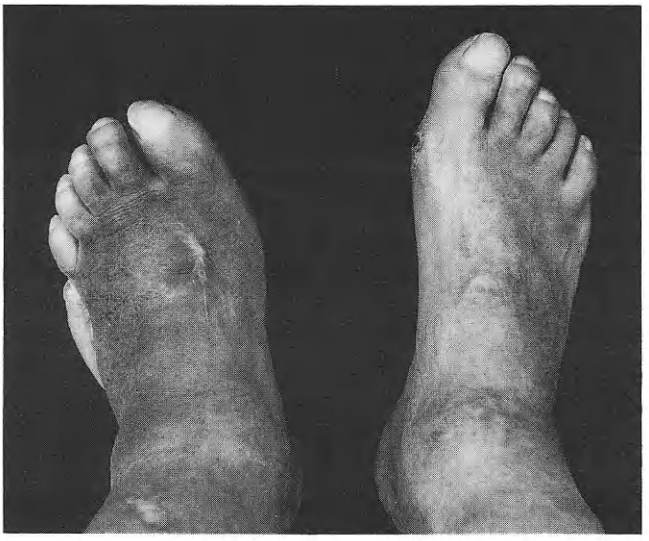

Fig. 6. Result 2 years after the operation (same patient as in Fig. 5)

As an example an extensive malum perforans is shown in Fig. 5. The postoperative development shows an extensive swelling of the foot and secondary wound healing due to a previous infection. However, even secondarily healing wounds lead to a satisfying functional result, shown in Fig. 6, 2 years after the operation.

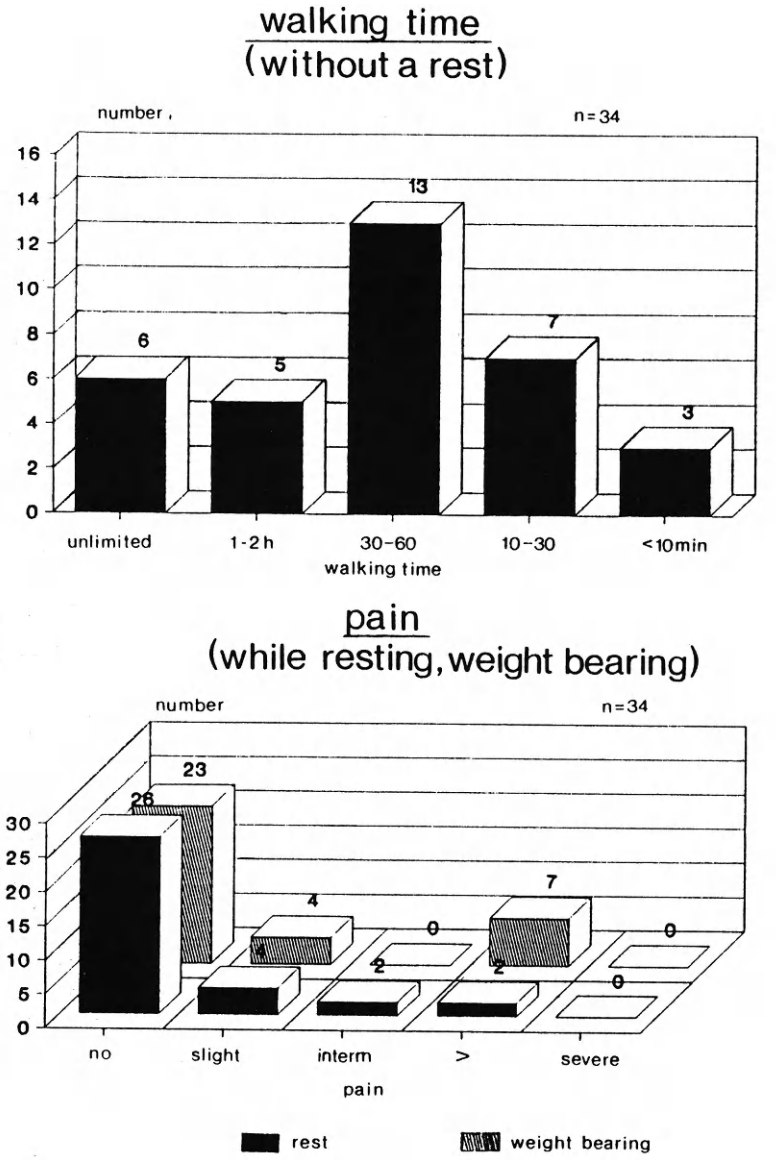

Fig. 7. Walking time without a rest; pain while resting, weight bearing

Clearly visible is a shortening of 1–2 cm after the resection of the metatarsalia. The shortening was mainly mentioned as a cosmetic problem. However, only in cases with total resection does shortening of the foot normally appear postoperatively.

Some 70% of the patients reported walking for half an hour without any problems; 80% admitted no or only little pain while weight bearing (Fig. 7).

The main problem was sufficient postoperative treatment and follow-up medical care. Even though extensive information about the operation had been supplied to home doctors, and although the patients had received primary adapted foot wear, 74% appeared at reexamination without appropriate footwear. In fact 14% of these wore shoes without any adaptation at all. This undermines the effect of the operation, as far as we are concerned, and points to problems in prophylaxis.

The care provided in clinics which deal specifically with problems of diabetics is more successful.

Conclusions

In conclusion it can be said that successful therapy of malum perforans pedis is a resection of the bony projection, in this case an extensive resection of the affected metatarsalia. Secondarily the application of enzymatic ointments and hydrocolloid dressings is useful; as a precaution orthopedic arch supports and adapted foot wear are absolutely necessary. Only departments trained to deal with these problems should treat this special group of patients.

Phosphocreatine Ameliorates Structural Integrity of Ischemic Skin Flaps

M.T. Stepke[1], W. Eichhorn[2], N. Schwenzer[3], and E.D. Voy[2]

Introduction

Plastic surgery, since its inception, has been concerned with the restoration and reconstruction of body structures that are defective or have been damaged by injury or disease. Central to the armentarium of the plastic surgeon is the reconstructive skin flap. Recently the use of simple skin flaps has yielded increasingly to the use of composite flaps, which contain any combination of muscle, bone, or fascia in addition to skin and subcutaneous tissue.

The earliest flaps on record appeared in India around 700 BC. These flaps, as described in the Susruta Samhita, employed cheek tissue to reconstruct the nose and appear to have been, in fact, full-thickness skin grafts rather than true pedicled flaps. The use of a true pedicled flap in India dates from the fifteenth century, when forehead flaps were employed in nasal reconstruction. In 1597 Gaspare Tagliacozzi described in great detail the use of a distally based upper arm flap for nasal reconstruction. Unfortunately, his methods soon slipped into obscurity and were not received until the nineteenth century. The next major advance in reconstructive techniques occurred in 1916 with the description of the tube pedicled flap. Despite the great versatility attained with these early procedures. all of these flaps were random pattern flaps, and an increase in the length of the flap could be attained only by widening its base or performing a delay procedure. The importance of the vascular anatomy of the raised flaps had gone largely unrecognized. The contribution of the immediated post-World War II period was the recognition of the significance of vascular anatomy in flaps, compounded by the use of regional flaps in the area of the head and neck. These flaps proved increasingly useful after the progressively more radical ablative procedures being developed. Not until 1963, however, did McGregor

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and Morgan (1963) coin the term "axial pattern flap," and clarify the distinction between the axial and the random pattern flap.

Following the discovery of the importance of flap vascular anatomy, there came a period of rapid expansion of reconstructive surgery with the ingenious description of flaps from scalp, forehead, neck, chest, and upper back. There followed a move away from the traditional concept of flaps as consisting of skin only and the development of the musculocutaneous flap. Improvements in microsurgical techniques led to the first human free flap transfer in 1971.

Strategies for Improving Flap Viability

The time honored method for improving flap viability is the delay procedure. Surgical delay is the process of raising a random pattern flap in 2 or more stages separated by a period of 2–3 weeks. Its positive effect is today explained as follows:

1. Resulting from the transection of the sympathetic nerves is a hyperadrenergic state which peaks over the initial 16–30 h after flap elevation. This state results in small vessel constriction which affects particularly the precapillary sphincters and reduces flow in the nutrient capillary bed. Reversal of the hyperadrenergic state occurs over several days, and thus, upon flap elevation, minimal adverse constriction occurs and a reliable flap results.
2. Cormack and Lamberty (1986) showed that surgical delay does not only increase the vascularity of the skin but also causes a significant degree of vessel realignment so that the dominant axis of blood flow becomes aligned with the long axis of the flap.

Multiple investigators (Pearl 1981; Jansson et al. 1975; Griffiths and Humphries 1981; Hendel 1983) tried to mimic the effect of surgical delay by a pharmacological delay using vasodilators, vasoconstrictors, sympathetic terminal depletors, and centrally acting sympathomimetics. The best results were reached using propanolol and prostacyclin. Propanolol is a β-blocker which acts by preventing β-mediated increases in metabolism and thus oxygen demand. Prostacyclin (PGI_2) increases flap survival in the dorsal cephalic based rat flap, most likely by its antiplatelet and vasodilating properties, directly antagonizing the deleterious effects of thromboxane. Some of the newest approaches to enhance flap viability make use of high energy phosphate compounds such as ATP, fructose-1,6-diphosphate and phosphocreatine (PCr).

With impaired circulation in the distal, failing portion of a flap, there are nutrient and oxygen deficits. Energy production is also compromised under these conditions. In 1981 Jurell and Friedholm were able to demon-

strate dramatic drops in tissue levels of ATP following flap elevation: to 50% of normal after 2 h and to 7% after 6 h. In addition there was an increase in the cAMP/ATP ratio in all parts of the flap between 6 and 18 h postoperatively. Consequently they concluded that during flap elevation there exists a simultaneous rapid depletion in energy stores and an enhancement of metabolism. Several investigators have shown a clear correlation between ATP levels or total adenine nucleotide content in predicting organ survival (Nichida et al. 1987).

We hypothesize that maintenance or early restoration of phosphometabolite balance within the cells of skin flaps may be an important step in preventing ischemic injury. This hypothesis is based on previous work by Cuono et al. (1988b), who demonstrated that depletion of PCr correlates with flap necrosis.

The aim of this study was to determine the efficacy of PCr-containing perfusates in preservation of structural integrity of skin flaps. We decided to use the transmission electron microscope to study the tissue deterioration. The time sequence of appearance of ultrastructural changes as the tissue sustains ever more severe hypoxic injury have been summarized by Trump (1976).

Materials and Methods

Under anesthesia (sodium pentobarbital, 30 mg/kg I.P.), the abdomen of 400 g male Sprague Dawley rats (Harlan-Sprague/Dawley, Charles River, MA. $n = 10$) was shaved. A 6 × 3 cm skin flap weighing 1.0 ± 0.25 g was elevated and dissected deep to the panniculus carnosus until the superior epigastric vessels were identified. Under the dissecting microscope, the superior epigastric vessels were carefully isolated, and the artery was separated from the vein. The artery was ligated with a 4-0 silk tie as proximally as possible, nicked distally to the ligature and quickly cannulated with a blunted 30-gauge needle attached to a polyvinylchloride catheter (Tygon-54-HL, A.H. Thomas, Philadelphia, PA). The cannulating needle was secured with a 4-0 silk tie tightened about the artery. The superior epigastric vein was then opened, and the flap was gently flushed with 2 ml of perfusing solution. Once the flap had been securely cannulated and flushed, the pedicle was transected, completely freeing the flap from the animal. The arterial cannula was connected to a peristaltic pump (Peristaltic Pump P-2, Pharmacia), and the flaps were perfused at a rate of 2 ml/h. In no case did more than 10 min elapse from interruption of arterial inflow to initiation of perfusion. Biopsies were obtained with a 1 mm tissue punch (Baker/Commins, Miami, FL) from the distal margin of the flap at 0, 4, 6, 8, and 12 h. after flap elevation. Biopsy tissue was immediately placed in modified Karnovsky's fixative (4% formaldehyde, 3% glutaraldehyde in a 0.1 *M* cacodylate buffer) and allowed to fix by immersion at 4°C for a minimum of

Table 1. Composition of the isoosmolar perfusate used

Compound	Concentration (g/l)	Molarity (m*M*)
Sodium citrate	19.00	65.0
Potassium phosphate (dibasic)	3.40	25.0
HEPES buffer	1.19	10.0
Glutathione	0.46	3.0
Mannitol	4.50	25.0
Magnesium sulfate	1.00	8.3
Bovine serum albumin	9.60	0.5

4 h and a maximum of 12 h. To ascertain the adequacy of perfusion, a single pulse of 0.01 ml/g of tissue of undiluted carbon black (Pelikan, biological ink Gunther Wagner, Hannover) was injected into the pedicle promptly after the 12 h biopsy was obtained. Some 5 min after the injection of colloidal carbon, the flaps had turned uniformly gray, and a second biopsy was obtained. Flaps were divided into five groups consisting of five flaps each. Each group was subjected to the following conditions as the biopsies were obtained: group 1: ischemia without perfusion at 4°C; group 2: ischemia without perfusion at 24°C; group 3: ischemia without perfusion at 37°C; group 4: ischemia, perfusion without PCr at 24°C; group 5: ischemia, perfusion with PCr (1 mg/ml) at 24°C.

In order to perfuse elevated skin flaps with the minimum amount of damage to tissues, an isoosmolar perfusate was designed (Table 1).

Our rationale for selecting the above materials was based on the writings of Pegg (1981). This perfusate was stored at 4°C and allowed to reach room temperature before being utilized in an experiment. Before each experiment, the osmolality of the perfusate was rechecked with an Advanced Wide Range Osmometer (3W2, Advanced Instruments, Needham Heights, MA) and if needed, readjusted to 290–300 mOsm by addition of sodium citrate. PCr disodium hydrate, 98%–100% (Sigma Chemical Co., St. Louis, MO), was added for the appropriate experiments at a concentration of 1 mg/ml, after which the osmolality was remeasured and readjusted.

The stability of PCr under the regimen required by our experiments was confirmed according to the method of Cuono and coworkers (1988b); 1 *M* solutions of PCr were prepared in perfusate.

Results

The ultrastructural changes occurring in the vascular endothelium for flaps subjected to various regimens can best be described as they appeared chronologically. For each time period from the different experiments, at least 20 vessels were closely examined. The vessels most representative of the changes occurring after that particular period of ischemia are presented. One of the most characteristic aspects of the ischemic damage to endothelial

cells was the focal nature of the lesions. In general, the proportion of normal to damaged vessels increased with the duration of ischemia, but individual differences between vessels were marked for periods up to 4 h of ischemia for flaps incubated at warm temperatures (24°C and 37°C), and up to 12 h for flaps incubated at 4°C.

Group 1: Flaps Subjected to Ischemia Without Perfusion at 4°C

Nonperfused flaps incubated at 4°C exhibited a relatively slow progression of ischemic changes. Tissue obtained at the beginning of the ischemic period contained for the most part normal appearing capillaries and small vessels. The endothelium of these vessels forms a complete, uninterrupted sheath around the lumen, with no evidence of intracellular swelling. The mitochondria show little or no evidence of damage, and cristae are plainly visible in several of these organelles. Likewise the endoplasmic reticulum shows minimal evidence of dilatation or other damage (Fig. 1).

The first clearly recognizable and prevalent signs of injury in flaps incubated at 4°C occur after 8 h of ischemia. At this time, diffuse, low grade swelling of the endothelium is readily apparent. Nevertheless, the endothelium exhibits no discontinuities or gaps. Several mitochondria are

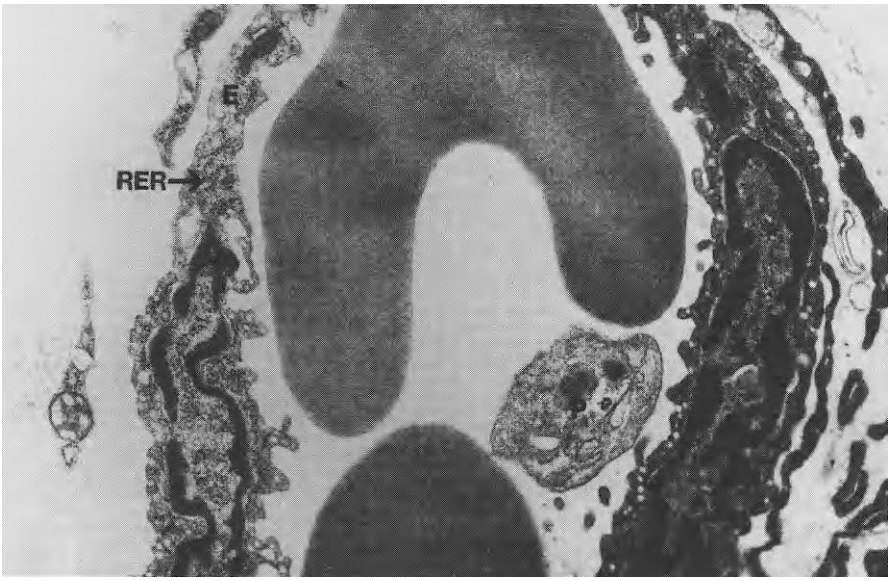

Fig. 1. Capillary from flap subjected to ischemia without perfusion at 4°C. The endothelium forms a complete, uninterrupted sheath around the lumen of the vessel and exhibits no evidence of intracellular swelling. Cristae are plainly visible within the mitochondria (*M*). The rough endoplasmic reticulum (*RER*) shows minimal evidence of dilatation or other damage. An erythrocyte (*RBC*) is visible in the lumen. ×26 800

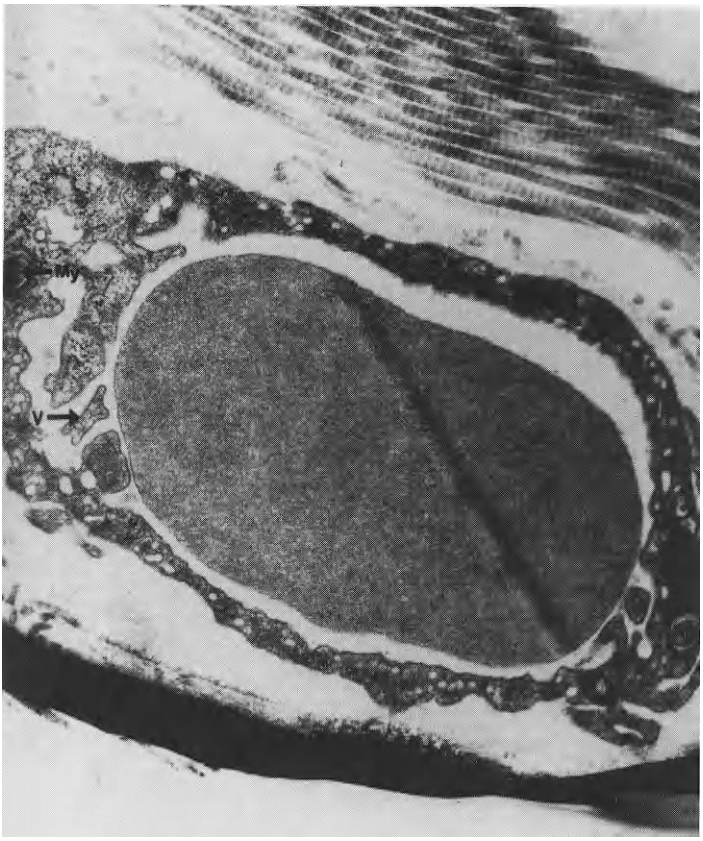

Fig. 2. Capillary from a flap subjected to 12 h of ischemia without perfusion at 4°C. Circular vacuoles (*arrows*) have now become ubiquitous. Microvilli (*V*) can often be seen pinching off as intraluminal membrane-bound vesicles. A myelin figure (*My*) is clearly visible within the endothelium. ×135 000

visible, all showing mild to moderate swelling but with cristae still plainly visible. The endoplasmic reticulum (ER) also shows mild swelling, though in the case of the rough ER (RER), ribosomes clearly remain attached to the membrane. Of note is the relatively normal appearance of the chromatin, which does not appear marginated or condensed.

By 12 h of ischemia, the circular vacuoles have become ubiquitous. Likewise, microvilli can frequently be seen projecting from the luminal surface, in various places seeming to pinch off into membrane-bound structures lying in the lumen. For the first time myelin figures can be detected. The state of the mitochondria and the ER does not appear to have changed significantly (Fig. 2).

After 24 h of ischemia the injuries have progressed dramatically. The endothelium appears extremely swollen, and no delineating plasmalemma

can be identified. The cytoplasm has become extremely light staining (rarefied), and exhibits few recognizable organelles. Highly condensed and marginated chromatin is also clearly evident in the nucleus of the endothelial cell.

Group 2: Flaps Subjected to Ischemia Without Perfusion at 24°C

The progression of ischemic injury at higher temperatures follows the same basic pattern as that occuring at 4°C. At 0 h of ischemia, the endothelium, mitochondria, and ER appear largely normal. By 4 h of ischemia notable changes have started to occur. The endothelium is moderately swollen with slight rarefication of the cytoplasm. Microvilli are projecting into the lumen of the vessel. The mitochondria are likewise extremely swollen, and in one example it appears that the cristae are gradually deteriorating. Membrane-bound structures are visible in the vessel lumen.

By 8 h of ischemia, extensive damage has resulted. The cytoplasm is extremely rarefied and light-staining and numerous vacuolated structures representing destroyed mitochondria are present. The luminal plasmalemma appears to have disappeared, and, in some cases, gaps have begun to appear in the endothelium.

Group 3: Flaps Subjected to Ischemia Without Perfusion at 37°C

These flaps exhibited the most rapid evolution of changes. After 4 h of ischemia, there is no recognizable ultrastructure. The endothelium appears to be unbound by any delimiting membranes. The cytoplasm appears as a homogeneous mass of necrotic material and vacuolated spaces.

Group 4: Flaps Subjected to Ischemia at 24°C, Perfused Without Phosphocreatine

In these flaps, the first biopsy was taken as soon as the perfusion was begun (t = 0 h). In no case did more than 5 min elapse between the onset of ischemia and the beginning of perfusion. As expected, the tissue obtained at the onset of the ischemic period exhibits the characteristics of normal endothelium.

After 6 h of ischemia, dramatic changes have taken place. Microvilli are very prominent, in many cases blebbing into the lumen of the vessel. Several circular, vacuolated areas are observed, most likely representing destroyed mitochondria. At this time point, it is practically impossible to find, in any of the examined blood vessels, evidence of mitochondria with visible

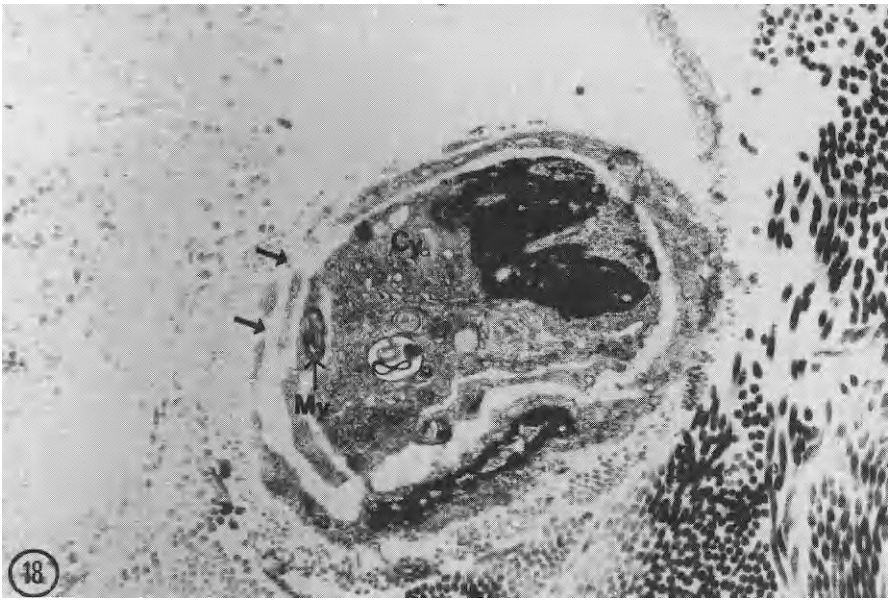

Fig. 3. Capillary illustrating dramatic changes in a flap perfused without phosphocreatine (PCr) after 6 h of ischemia. In this vessel, the lumen is completely occupied by cytoplasmic contents (*Cy*), which appear to have involuted into the lumen of the capillary. Myelin figures (*My*) and endothelial gaps (*arrows*) can easily be appreciated. ×16 280

cristae (Fig. 3). By 12 h of ischemia, the endothelium appears necrotic. The cytoplasm is devoid of normal detail, and myelin figures abound. The nucleus appears highly condensed. Microvilli are abundant, and multiple membrane-bound vesicles can be seen in the lumen (Fig. 4).

Group 5: Flaps Subjected to Ischemia at 24°C Perfused with Phosphocreatine

In these flaps, the most surprising result was the relative absence of notable ultrastructural changes after 4 h of ischemia. The ER appears to be of normal caliber with ribosomes prominently attached to the membrane of the RER. The nucleus exhibits normal appearing chromatin. Microvilli and intraluminal membrane-bound structures are not present in any of the examined capillaries. By 8 h of ischemia, the endothelium appears moderately swollen, though it still reveals readlily recognizable organelles. The nucleus of the cell appears normal. The most dramatic change in this example is the spilling of cytoplasmic structures into the lumen of the vessel (Fig. 5). By 12 h of ischemia, it is readily obvious that severe damage has occurred to the

Fig. 4. Severely damaged endothelium in a small venule perfused without phosphocreatine (PCr) after 12 h of ischemia. The endothelium (*E*) appears necrotic, devoid of recognizable ultrastructure. Many myelin figures (*My*) can be seen. The nucleus (*N*) appears highly condensed. Microvilli are abundant and many membrane-bound vesicles can be observed in the lumen (*arrow*). ×9000

endothelial cell. The extent of the damage is best illustrated by the presence of extravasated carbon particles.

Discussion

In our experiments we studied the effect of three factors on the resistance of the dermal vasculature to ischemic anoxia. The first variable studied was temperature. Several studies have estimated that the basal metabolic rate of skin decreases 7% for each degree Fahrenheit that the tissue is cooled. It was thus not surprising to find in our studies that cooling had remarkable effects in preserving the integrity of the vascular endothelium in the dermis. Even after 12 h of ischemia, only moderate damage was detected by electron microscopy in flaps maintained at 4°C. Comparable damage to the integrity of the endothelium and the organelles it contained was present by 4–6 h in tissue incubated at 24°C and by 4 h in tissue incubated at 37°C. Severe damage, with gaps in the endothelium and almost complete lack of recognizable ultrastructure, was present at 24 h in flaps kept at 4°C, by 8 h in flaps at 24°C and by 6 h in flaps at 37°C.

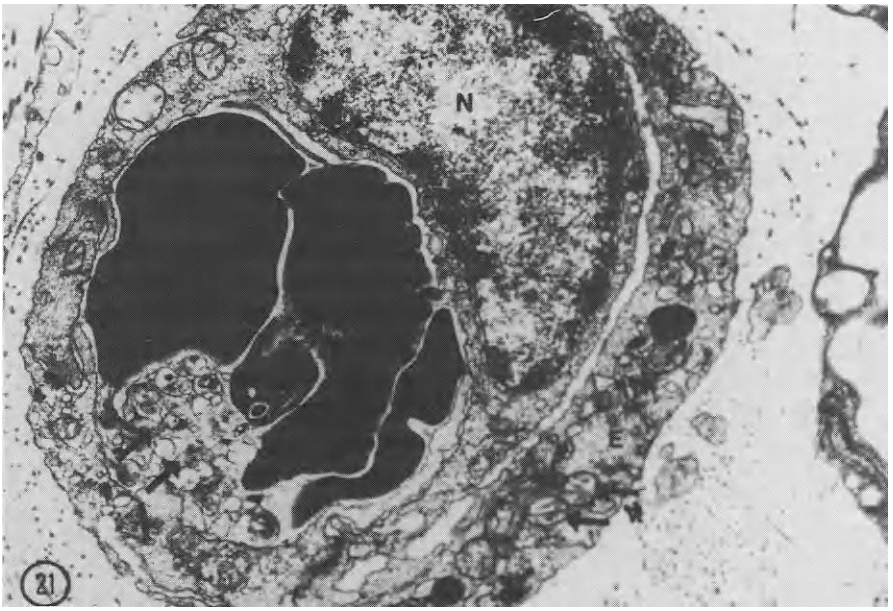

Fig. 5. Capillary from a flap subjected to 8 h of ischemia, perfused with phosphocreatine (PCr) at 24°C. The endothelium (*E*) appears moderately swollen, but nevertheless reveals many recognizable organelles, particularly mitochondria (*M*). In one region cytoplasmic contents appear to be spilling into the vessel lumen (*arrows*). The nucleus still appears normal at this time, showing no evidence of chromatin condensation. ×18200

Damage to the endothelium comparable to that seen by 8 h in nonperfused flaps at 24°C would in all certainty preclude the reestablishment of blood flow through these vessels. Although our experiments did not directly address the problem of the nonreflow phenomenon, the studies of Willms-Kretschmer and Majno (1969) indicate that, after 6–8 h of ischemia in rat skin, blood flow cannot be successfully reestablished. They speculated that, although reflow occurred at first, the damage of the vessels had been too severe to enable them to recover from the ischemic insult. Marzalla and colleagues (1988) expanded on these invetigations by means of an abdominal skin flap in the rat in which blood flow was occluded using vascular clamps. They were able to demonstrate increased blood reflow to flaps if superoxide dismutase was administered to the flap just prior to the release of the vascular clamp (Trump 1976).

In our studies we have been able to substantiate further the findings of these investigators. Unlike the previous studies, however, our flaps were not subjected to periods of arterial reflow before tissue was obtained for analysis. Nevertheless, our model allowed us to document that severe ultrastructural deterioration occurs in the endothelium before blood flow is reestablished and neutrophil-derived free radicals have had an opportunity

to attack the vasculature. We observed that with increasing periods of ischemia there is an increased number of vessels exhibiting of the endothelium and pinching off of what appears to be cytoplasmic contents into the vessel lumen. This process eventually occludes the lumen of the vessel. One extreme example is shown in Fig. 3, in which the vessel lumen appears totally occluded by cytoplasmic contents. Even in the absence of a visible occlusion in the vessel lumen, significant changes due to ischemia will occur in the endothelium and will eventually render the vessel nonfunctional. Trump and colleagues have summarized the time sequence of appearance of ultrastructural changes after hypoxic injury (1976). Our studies point out that these ultrastructural changes occur at or around 4 h in nonperfused flaps at 37°C, 8 h at 24°C and after 12 h at 4°C. Flaps perfused without PCr reached this point after 6 h, and flaps perfused with PCr at 8–12 h. The second factor addressed by our study is that of the possible beneficial effects of washing out stagnant blood elements by means of perfusion. The role of perfusion washout in microvascular surgery has long been a subject of controversy. Early studies by Harashina and Buncke (1975), and Chait (1978), utilizing simple crystalloid solutions such as normal saline, lactated Ringers or modified Hartman's solution, pointed to a detrimental effect of washout procedures.

In our experiments, we attempted to perfuse the vasculature of the flaps with an isotonic solution of previously specified composition. Our rationale for selecting the above materials was based on the writings of Pegg (1981) and Belzer et al. (1983). In working with organ preservation by cold storage, Belzer has defined several conditions to be met by a solution used to flush out stagnant blood prior to cold storage: (1) minimization of hypothermic-induced cell-swelling; (2) prevention of injury from free radicals, and (3) provision of substrates for regenerating high-energy phosphate compounds during reperfusion.

We believe that these principles for cold storage preservation of whole organs should also apply to the continuous perfusion of skin flaps utilized in our experiments. The third of Belzer's postulates constituted the provision we aimed to test by the use of PCr.

Our results are in accordance with the investigations of Cikrit, Heckler (1984) and Cuono et al. (1988b), all which demonstrated the efficacy of high energy phosphometabolite replacement in augmenting flap survival. This improvement can best be explained by the concept of the PCr energy shuttle (Fig. 6). Skepticism over favorable results obtained via the use of high energy phosphometabolite replacement to ischemic flaps has in part been based on the vasodilatory properties of these compounds. In our system the flap was completely disconnected from the donor rat, and the vasculature only came into contact with the compounds in the perfusing solution. The dramatic improvement in the condition of the vascular endothelium upon inclusion of PCr in the perfusate thus points to PCr as the factor responsible for our observations.

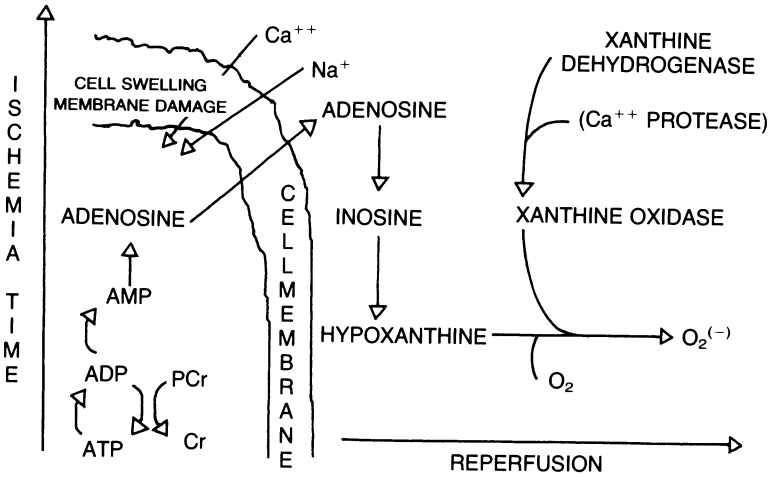

Fig. 6. As ischemic time increases, ATP is progressively diphosphorylated to AMP; once AMP is dephosphorylated to adenosine, adenosine leaks out of the cell. Adenosine is then metabolized to inosine and hypoxanthine, which in the presence of xanthine oxidase will react with reperfused oxygen to create oxygen free radicals. In addition, the cell membrane becomes incompetent due to electrolyte pump failure, permitting the influx of both sodium and calcium ions

In conclusion, our results support the clinical finding that cooling skin flaps may prolong viability, probably through the dramatic effect that cooling has on preserving the integrity of the vascular endothelium. The dramatic effect that PCr may have in delaying the onset of ischemic changes in flaps perfused at room temperature is due to its ability to buffer the sharp loss of ATP that occurs soon after the onset of anoxic ischemia. Since in our system, the flaps were not connected to the circulation of the animal, the vasodilatory properties of PCr cannot be invoked as the mechanism for the preservation of the endothelium.

In the future it would be of great interest to verify the mechanism of action by which PCr exerts its beneficial effects. This could be done by collecting the venous eluent from a perfusion experiment and analyzing its content by high performance liquid chromatography (HPLC) of phosphometabolites such as PCr, ATP, etc. If PCr acts indeed by buffering losses of ATP, this should be detected in such procedures. But whether PCr acts by this or another mechanism is subordinate to the fact that it has repeatedly appeared to be of benefit to tissues undergoing acute ischemia and undoubtedly deserves further attention as a possibly invaluable clinical tool.

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Granulocyte Activity Is Enhanced by Culture Supernatants of Mononuclear Leukocytes Incubated with Tetrachlorodecaoxide

R.E. Schopf, G.A. Hermann, J. Hinz, and B. Morsches

Introduction

Granulocytes (polymorphonuclear leukocytes, PMNs) and monocytes/macrophages are important in wound healing. Within the first few hours PMNs and monocytes begin to infiltrate sites of injury. The major function of PMNs is to kill microbes and phagocytose cellular debris so that healing can proceed [1]. Monocytes/macrophages invading the wound are also active in phagocytosis. In addition they release a number of cytokines including growth factors, interleukin-8 (IL-8), and tumor necrosis factor (TNF-α) needed for new tissue formation and shown to stimulate PMNs [1–3].

Normally, wounds heal by themselves. Treatment is sought, however, for wounds that fail to heal, i.e., ulcers. Recently, the novel agent tetrachlorodecaoxide (TCDO, Oxoferin) has been demonstrated to both increase host defense and to exhibit therapeutic effects in difficult wounds [4–6]. In particular, epithelialization and formation of granulation tissue have been found to be enhanced under treatment with TCDO [6]. Biochemically, oxygen activation is thought to be the basis for the physiological action of TCDO [7].

To better understand the therapeutic mechanisms of action of TCDO, the part of the oxidative metabolism of PMNs termed "respiratory burst" was examined. Upon appropriate stimulation such as by zymosan particles or TNF-α, PMNs respond by an enhanced uptake of oxygen and the production of highly reactive oxygen intermediates including superoxide anion, hydrogen peroxide, and singlet oxygen that can be measured by chemiluminescence [8, 9]. To measure the direct effects of TCDO on PMNs, the generation of chemiluminescence of PMNs incubated with TCDO was studied. In addition, the effects of supernatants of mononuclear leukocytes (MNLs) incubated in the presence of TCDO were examined both on resting PMNs and PMNs stimulated with the chemotactic peptide formyl-Met-Leu-Phe (FMLP).

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Our investigation reveals that the chemiluminescent response of PMNs remains stable under the direct influence of TCDO. Moreover, supernatants of MNLs cultured in the presence of TCDO are able to enhance the chemiluminescent activity both of resting and FMLP-stimulated PMNs.

Methods

Isolation of Mononuclear and Polymorphonuclear Leukocytes

These cells were separated from the peripheral blood of healthy individuals by standard density gradient centrifugation and sedimentation in dextran [10].

Mononuclear Leukocyte Cultures

Under sterile conditions, 10^6 MNLs/ml were incubated for 24 h at 37°C, 5% CO_2 in 24-well microplates using minimum essential medium (MEM) supplemented with 1% penicillin/streptomycin (Gibco) and 1% bovine serum albumin. TCDO (Oxoferin, Gist Brocades, Heidelberg) was added as indicated. After 24 h the cell suspensions were collected, spun for 10 min at 200 × g, and the supernatants were pipetted off and passed through a 22 μm sterile filter (Millipore) and stored at −40°C until used to incubate PMNs. Previous experiments have demonstrated that the stimulating activity for PMNs in cell cultures of MNLs was mainly generated by monocytes, not lymphocytes (data not shown).

Measurement of Chemiluminescence

In order to determine luminol-enhanced chemiluminescence 5×10^5 PMNs were incubated as described [9], except that Dulbecco's MEM for chemiluminescence (Boehringer Mannheim) was used. TCDO was added as exhibited in the results; in these samples 5% autologous serum was added as a source of catalytic hemoproteins [7]. As further shown, PMNs were also stimulated with the chemotactic peptide FMLP ($10^{-4}M$). When supernatants of MNLs were added the medium for PMNs was supplemented with 20% autologous serum. Chemiluminescence was recorded every 10 min for 10 s over 1 h employing a Berthold LB 9500 luminometer (Wildbad, FRG). Results are given as the counts of sums of the means of duplicate samples.

Statistical Methods

Data were compared using the two-tailed Wilcoxon test; p values were considered significant if <0.05.

Results

Direct Effects of Tetrachlorodecaoxide on Polymorphonuclear Leukocytes

At first, PMNs were directly incubated with TCDO to measure the chemiluminescent activity. TCDO was used at concentration starting from 100 μM (100% strength) and dilutions thereof, as indicated in Fig. 1. The findings reveal that TCDO has measurable effects on PMNs; however, the differences failed to reach statistical significance. Likewise, when TCDO was added to PMNs stimulated with zymosan particles the chemiluminescent response was not enhanced either (data not shown). From these results it was concluded that the chemiluminescent response of PMNs directly incubated with TCDO remained stable. Next, it was of interest to measure indirect effects of MNLs on PMNs, i.e., the effects of cell culture supernatants generated in the presence of TCDO.

Effects of Supernatants of Mononuclear Leukocytes on Polymorphonuclear Leukocytes

When cell culture supernatants of 1×10^6 MNLs incubated for 24 h in MEM supplemented with 1% bovine serum albumin/ml and various concentrations of TCDO were added to resting PMNs to measure chemiluminescence the data, as depicted in Fig. 2, emerged. The findings indicate that the respiratory burst of PMNs elicited by supernatants of MNLs can be augmented if MNLs were incubated for 24 h in the presence of TCDO. Next, it

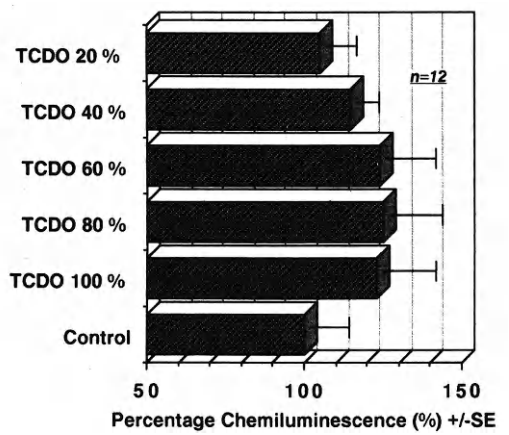

Fig. 1. Chemiluminescent activity of 5×10^5 polymorphonuclear leukocytes (PMNs) incubated with tetrachlorodecaoxide (*TCDO*). A reading of 100% chemiluminescence corresponds to 35 385 counts (sums of means of duplicate samples measured every 10 min for 10 s over 60 min) of resting, unstimulated PMNs using 5% autologous serum; *TCDO 100%* corresponds to 100 μM

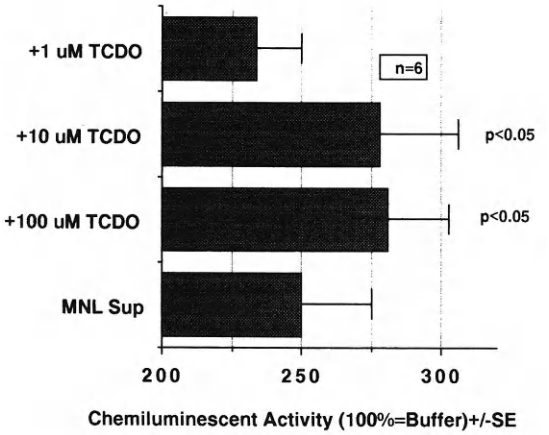

Fig. 2. Chemiluminescent responses of 5×10^5 polymorphonuclear leukocytes (PMNs) stimulated by culture supernatants of 1×10^6 mononuclear leukocytes (*MNLs*) incubated for 24 h with different concentrations of tetrachlorodecaoxide (*TCDO*). A reading of 100% chemiluminescent activity corresponds to 200 546 counts of resting, unstimulated PMNs employing 20% autologous serum (see Fig. 1). The effects on PMNs of the MNL supernatants (*Sup*) with supernatants of MNL cultured in the presence of TCDO are compared by *p* values

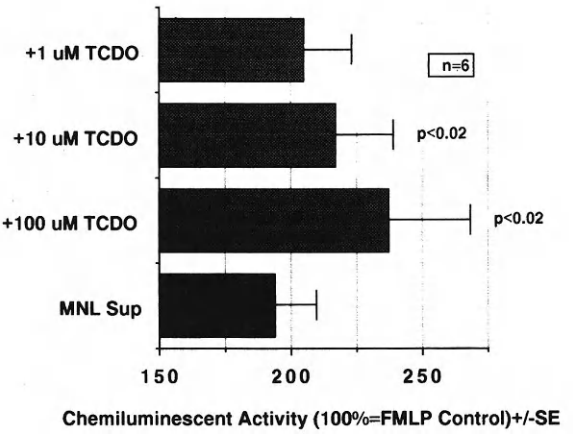

Fig. 3. Chemiluminescent response of formyl-Met-Leu-Phe (*FMLP*)-stimulated polymorphonuclear leukocytes (PMNs) by culture supernatants of MNL incubated with tetrachlorodecaoxide (*TCDO*); 100% corresponds to 585 771 counts (see Fig. 2)

was of interest to find whether the chemiluminescent response of FMLP-stimulated PMNs could further be enhanced by supernatants of MNLs incubated in the presence of TCDO. Figure 3 exhibits the results. The data indicate that the incubation of MNLs with TCDO leads to cell culture supernatants with enhanced capacity to stimulate PMNs.

Discussion

Our findings first of all demonstrate that the chemiluminescent response of PMNs remains stable if TCDO at the concentrations used is added directly to PMNs. These results are in accordance with those of others [11], showing no change in chemiluminescence of resting and zymosan-stimulated PMNs after the addition of TCDO to short-term PMN cultures. This indicates that TCDO has a different mechanism of action compared to known stimuli of the respiratory burst such as zymosan particles, chemotactic peptides, cytokines such as TNF-α, or phorbol myristate acetate [2, 8]. In addition, these findings indicate that TCDO does not act as an opsonin for zymosan such as serum or complement split products like C3b in the elicitation of a respiratory burst in PMN [12].

When added to MNL cultures, TCDO was able to augment the stimulating activity of the cell culture supernatants for PMNs. This indicates that cytokine production in MNLs was enhanced under the influence of TCDO. Among MNLs, monocytes are the most likely source of the PMN-stimulating cytokines [3, 13]. Monocytes are able to produce TNF-α, a potent stimulator of the respiratory burst [2]. It remains to be demonstrated to what extent TNF-α production in monocytes was responsible for the PMN-stimulating activity found. Granulocyte/monocyte-colony stimulating factor (GM-CSF), produced in T cells and monocytes, is another likely source of PMN-stimulating activity [13, 14]. Other cytokines such as IL-8 may also be involved [3].

The mechanisms responsible for enhancing cytokine production in MNLs are unknown. The following explanations exist: (1) TCDO may have been contaminated by bacterial lipopolysaccharide (LPS), a well-known potent stimulus of monocytes. This can be excluded since the preparation of TCDO was free of LPS as tested by the manufacturer. (2) It seems likely that TCDO acts on MNLs by biochemical oxygen activation as reported [7]. Moreover, tumor oxygenation has also been found to be improved after treatment with TCDO; measurements of oxygen tension (P_{O_2}) values with O_2-sensitive microelectrodes have revealed a distinct enhancement of oxygenation of tumor cells after the addition of TCDO into the cell culture medium [15]. The probability of enhanced oxygenation of MNLs in our investigation is supported by the report of an increased host defense against experimental infections after TCDO treatment of rodents [4]. (3) At this time, other, as yet unknown, factors may also have been responsible for the augmented activity of supernatants of MNLs on PMN chemiluminescence.

Our findings are in accordance with the clinical effects of improved wound healing after TCDO treatment [5]. Since wound healing consists of a coordinated effort of the body involving redundant mechanisms in tissue repair including activation of platelets, blood coagulation, endothelial cells, adhesion molecule receptors, phagocytes, fibroblasts, and epidermal cells [1], it seems likely that TCDO may, in addition, exert either direct or

indirect effects on cells other than phagocytes resulting in improved wound healing.

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Skin Reactions and Sensations Induced by Intradermal Injection of Substance P into Compound 48/80 Pretreated Skin

G. Heyer[1] and W. Magerl[2]

Introduction

Besides local effects, intracutaneous injection or iontophoretical application of histamine is known to induce a flare reaction in the surrounding skin [13, 14, 16]. This flare apparently depends on small afferent nerve fibers excited by histamine [8]. This neurogenic vasodilatation is probably induced by the release of vasoactive neuropeptides from nerve endings, in particular of substance P.

On the sensory side, intracutaneously applied histamine produces mainly itch, probably by exciting unmyelinated afferent nerve fibers [7, 8, 17]. This is in striking contrast to other irritant substances which induce mostly burning pain [15]. However, substance P also produces pure itch when intracutaneously injected and a flare reaction develops in the surroundings [10].

In this study we tried to find out if substance P has an independent pruritugenic and flare inducing potency or if it merely acts via the release of histamine from mast cells, as conjectured by some authors [3, 6, 18]. To this end we injected various amounts of substance P into normal skin of human volunteers and in skin sites pretreated with compound 48/80, which is known to deplete the histamine stores of cutaneous mast cells. To establish the actions of substance P and the efficacy of repeated iontophoretic application of compound 48/80, we carefully evaluated skin reactions with laser Doppler flowmetry (LDF) and planimetry of the flare and wheal responses. Intensity of itching was repeatedly assessed on a rating scale.

Material and Methods

Ten healthy volunteers, six female, four male, age 21–57 years (median 26 years), took part in a first series of experiments. In a second series we

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investigated 20 healthy volunteers, nine female and 11 male, age 24–59 years (median 26 years). All subjects gave informed consent to participate in this study, which was approved by the Ethics Committee at the Medical Faculty of the University of Erlangen-Nurenberg.

Constant room temperature (21°–22°C) and air humidity (60%–65%) were provided. The tests were performed between noon and 4 P.M. The right or left forearm was placed on a temperature controlled arm rest at 34°C.

Compound 48/80 Iontophoresis

Compound 48/80 (Sigma, Deisenhofen, FRG) (1%) was dissolved in a gel of 2.5% methylcellulose in double distilled water. The gel was placed in the cavity of an acrylic applicator with 5 mm diameter containing a silver-silver chloride electrode for current delivery. The reference electrode, in the form of a larger silver plate in a moistened sponge, was held by the subject in her/his ipsilateral hand. An identical method has been used before for the application of histamine and other polar substances [9, 16]. For delivery of compound 48/80 from an anode constant current pulses from an isolated stimulator (World Precision Instruments, New Haven, CN, USA) were used. A stimulus of 1 mA lasting 20 s (20 mC) was applied at three different locations on the right or left volar surface of the forearm. Some 4 h after the first three applications of compound 48/80, the test was repeated in the same three areas to further release remaining histamine from cutaneous mast cells. In a second series of experiments we applied four different stimuli of compound 48/80 (5, 10, 15 and 20 mC) in 20 healthy controls to assess the dose-response function and to compare it with that of histamine [9].

Substance P Injection

Some 24 h after the second compound 48/80 application 0.01 ml of substance P (Serva, Heidelberg, FRG) in three different concentrations (10^{-5}, 10^{-6} and 10^{-7} *M*) in sterile physiological saline was injected intradermally in each of the three pretreated areas in alternating sequence. Substance P doses were therefore 10^{-10}, 10^{-11} and 10^{-12} mol. The effect of substance P injection after compound 48/80 pretreatment was compared with the reactions to substance P injections in normal skin, results from our former study [10]. Both wheal and flare areas were marked and redrawn on translucent paper for planimetric analysis 10 mins after each compound 48/80 or substance P application.

A laser Doppler flowmeter (Periflux/PF2, Perimed, Stockholm, Sweden) was used to assess the relative increase in blood flow as the product of velocity and number of blood cells (flux) to a depth of approximately

0.6 mm from the skin surface, i.e., in a half sphere of tissue encompassing about 1 mm^3 [11]. The sensor was placed at a distance of 10 mm from the compound 48/80 applicator.

All data were read into a laboratory computer through a 12-bit IO converter and displayed on a chart recorder. Baseline skin blood flux was determined over a 40 s period prior to compound 48/80 or substance P application. Stimulus induced changes were expressed as net flux increase over baseline averaged during an 8 min observation period.

Rating of Itching or Burning Pain Sensations

Compound 48/80 and substance P induced itching, stinging or burning pain was repeatedly assessed by an open ended category partitioning scale, from 0 (no response), 1 (very low), 2 (low), 3 (medium), 4 (strong), 5 (very strong) and 6 (extreme). Each category was further subdivided into 10 parts [5]. On this scale, for example, a rating of "medium" itch (3), grade 6 on the subscale is expressed as 3.6. The subjects reported ratings verbally over an 8 min period after drug application on acoustic in 10 s intervals. Average and maximum ratings were assessed as in previous studies [10].

Data Processing

Data evaluations were performed using the following commercial software packages: Reflex (Borland, Scotts Valley, CA, USA) for the database, Dadisp (DSP Development Corporation, Cambridge, MA, USA) for editing the LDF recordings, the graphic package Sigmaplot (Jandel, Corte Madera, CA, USA) for graphic and Complete Statistical Software (Stat Soft, Tulsa, OK, USA) for all statistical analyses.

Results

Compound 48/80 and Histamine Iontophoresis

Wheal and Flare Reactions

Iontophoretically applied compound 48/80 elicited wheals of an average size of 226 ± 60 mm^2. (mean ± SEM), the smallest wheal was 127 mm^2, the largest wheal 325 mm^2. Flare sizes were between 530 mm^2 and 1610 mm^2, the average was 1134 ± 273 mm^2 (mean ± SEM). The second application of compound 48/80 in pretreated areas elicited significantly smaller wheal and flare reactions (Fig. 1). On average, the wheal areas were only 8.8% ±

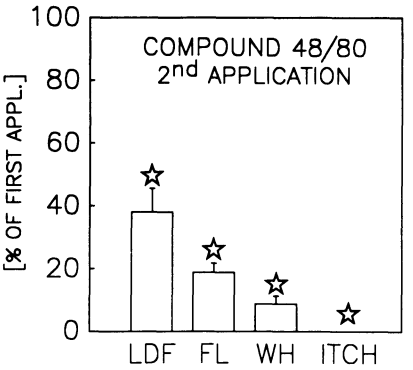

Fig. 1. Average laser Doppler flowmetry fluxes, flare sizes, wheal sizes and itch intensity after the second compound 48/80 application as a percentage of the results of the first compound 48/80 application. *LDF*, laser Doppler flux increases; *FL*, flare sizes; *WH*, wheal sizes; *white star*, $p < 0.0001$

2.6%, the flare areas only 18.8% ± 3.0% (mean ± SEM) ($p < 0.0001$) of the first responses.

Laser Doppler Flowmetry

Generally the iontophoresis of compound 48/80 induced flux increases similar to those observed after histamine iontophoresis, albeit after a longer latency. The plateau of the reaction was reached after approximately 3 min compared to 2 min after a comparable histamine stimulus (Fig. 2). The stimulus response curves show a rightward shift of compound 48/80 by a factor of 20 compared to histamine (Fig. 2B). At equipotent doses, responses to compound 48/80 also exhibited a delayed onset of vasodilatation (delay approximately 40 s) (Fig. 2A). The second application of compound 48/80 elicited only 38.1% ± 7.5% of the LDF response to the first application (mean ± SEM) ($p < 0.0001$).

Ratings

The sensation induced by first application of compound 48/80 was always itching. In contrast, the second application 4 h later in no case induced itch (Fig. 1). The maximum itch ratings during the first experiment were between 0.6 and 3.2 on the rating scale, i.e., between "very low" to "medium." The average rating was 1.8 ± 0.73 (mean ± SEM). The duration of the itching sensations ranged from 2 min (shortest duration) to the whole observation period (8 min). The onset of itching was reported 10–40 s after

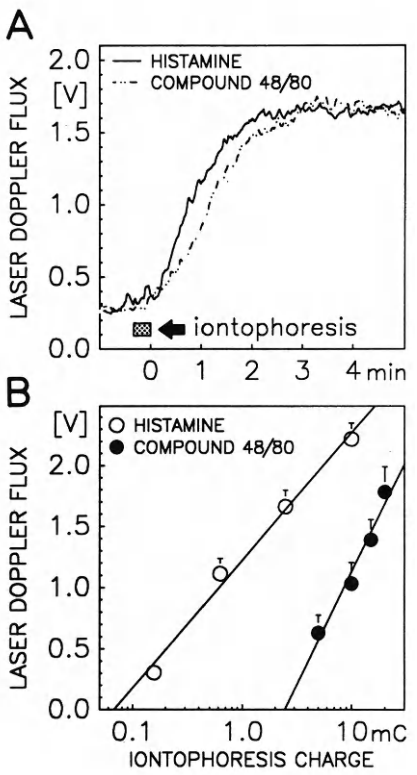

Fig. 2. **A** Average blood fluxes of 20 controls in flare areas induced by histamine (0.625 mC stimulus) (*continuous line*) and a equipotent dose of compound 48/80 (10 mC stimulus) (*dashed line*). Recordings were corrected for baseline fluxes before averaging, subtracting the means of a 40 s prestimulus period. **B** Average blood fluxes (*V*) after iontophoresis of four different histamine stimuli (0.156, 0.625, 2,5 and 10 mC) and four different compound 48/80 stimuli (5, 10, 15 and 20 mC)

starting the compound 48/80 stimulation, compared to a generally 10–20 s latency period of itch perception after histamine application.

Substance P Injection

Wheal and Flare Reactions

Differences in wheal and flare sizes in untreated skin [8], and after compound 48/80 pretreatment are shown in Fig. 3. The diminution of the substance P induced wheals and flares was highly significant at all doses. In particular, the lowest dose of 10^{-12} substance P hardly induced any wheals

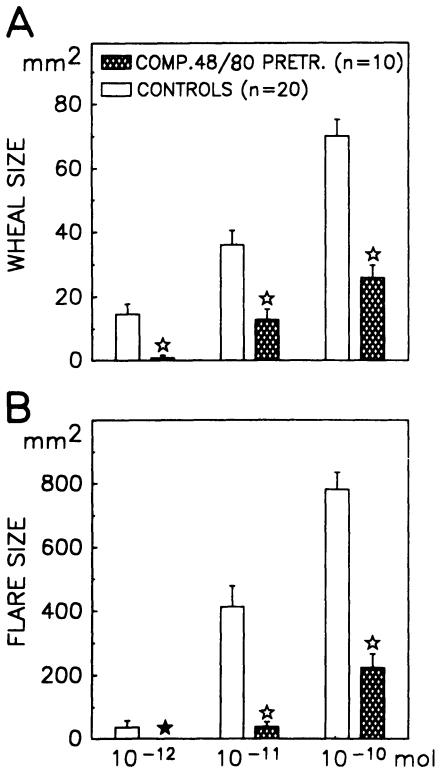

Fig. 3. **A** *Open columns* show mean wheal sizes in mm^2 after three different substance P injections (10^{-10} mol 10^{-11} mol, 10^{-12} mol). *Shaded columns* show mean wheal sizes after substance P in compound 48/80 pretreated skin; *white star*, $p < 0.0001$. **B** *Open columns* demonstrate mean flare sizes in mm^2 after the three different substance P injections, as compared to substance P induced mean flare sizes in compound 48/80 pretreated skin (*shaded columns*); *black star*, $p < 0.05$

and flares after pretreatment, in contrast to wheals of $15 \pm 11.6\,mm^2$ and flares of $50 \pm 19.2\,mm^2$ (mean ± SEM) on average, in healthy skin.

Laser Doppler Flowmetry

The LDF baselines, which were always documented for 40 s prior to the substance P injection, were 0.47 ± 0.08 V (mean ± SEM) in skin pretreated with compound 48/80 compared to 0.28 ± 0.02 V (mean ± SEM) in unpretreated skin (Fig. 4A), and this difference was highly significant ($p < 0.0001$, t test). On this elevated baseline substance P induced smaller flux increases. However, when the total fluxes measured with and without compound 48/80 pretreatment were taken into account, no significant differences were encountered between normal and pretreated skin (Fig. 4B).

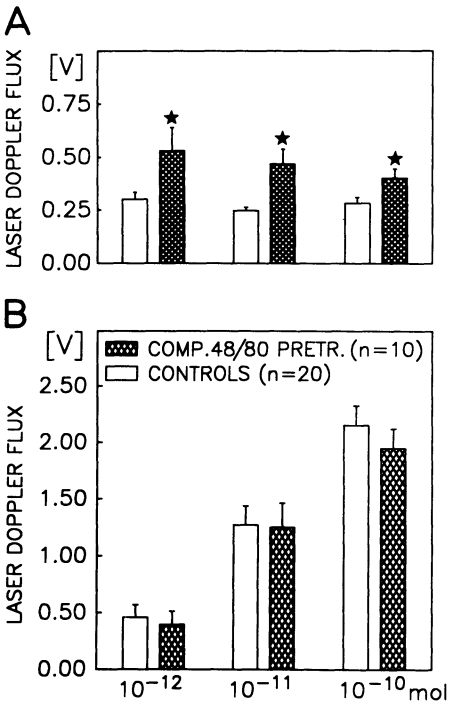

Fig. 4. AThree mean baseline fluxes recorded in 40 s period prior to each of the three identical but differently located stimuli in normal skin (*open columns*) and in compound 48/80 pretreated skin (*shaded columns*); black star, $p < 0.05$. **B** Comparison of blood flow increase in the erythematous area from baseline (40 s) over an 8 min period after three different substance P injections in normal skin (*open columns*) and compound 48/80 pretreated skin (*shaded columns*). An increase of 100% is equivalent to an average doubling of fluxes in the 8 min observation period

Ratings

In untreated skin the substance P injections (concentrations 10^{-9}–10^{-12} *M*)-generally elicited the sensation of itch. Only four of the 20 controls felt, in addition, burning pain during the first 2 min after substance P injections of 10^{-9} and 10^{-10} *M*. It is unclear if these early sensations were due to the small injection trauma. Substance P induced itch sensations were dose-dependent, on average 1.1 ± 0.16 ("low") after 10^{-10} mol injection, 0.84 ± 0.15 ("very low") after 10^{-11} mol injection, and 0.13 ± 0.05 ("very low", "almost no sensation") after 10^{-12} mol (see Fig. 5). Latency between substance P application and the first positive itch rating was also dose-dependent. At the highest dose (10^{-9} mol) itch was generally reported within 20–30 s.

In contrast, after pretreatment with compound 48/80 no pure itch ratings were reported. Even after the highest dose of 10^{-10}, one of the ten subjects

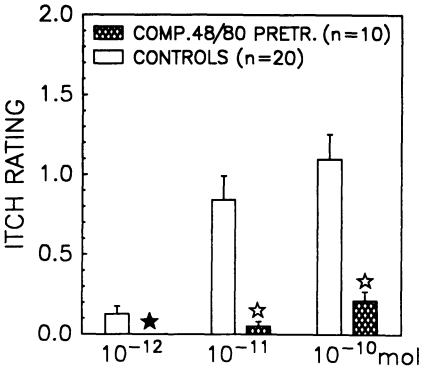

Fig. 5. Mean itch ratings over an 8 min observation period, given on the category partitioning scale (*1*, very low; *2*, low; *0.1–0.9*, subscale ratings) after substance P injections in normal skin (*open columns*) and in compound 48/80 pretreated skin (*shaded columns*); black star, $p < 0.05$; *white star*, $p < 0.0001$

Table 1. Number of volunteers reporting pure itch, itch and pain or pure pain after injection of substance P (10^{-10} mol) in untreated skin ($n = 20$) and after the same dose of substance P in compound 48/80 pretreated skin ($n = 10$)

	Pure itch	Itch and pain	Pure pain
Substance P	17/20	3/20	–
Substance P after compound 48/80	–	3/9	6/9

reported no sensation at all , the remaining nine subjects reported stinging (one of nine), burning (five of nine) and burning with itching (three of nine). At 10^{-11} mol six subjects felt nothing, and 10^{-12} mol in no case induced a sensation related to the substance P application (Table 1).

The pattern of stimulus quality thus significantly shifted towards a sensation of burning or stinging pain following compound 48/80 pretreatment (Fisher's exact probability test, $\chi^2 = 0.84$, $p < 0.0001$). The quantitative ratings of the substance P induced sensations were significantly lower after compound 48/80 at the injection levels.

Discussion

Compound 48/80, a mixture of oligomers, is known to deplete histamine stores from mast cells [1]. In this study we have shown that it can successfully be applied to small skin patches by means of iontophoresis. As shown before with histamine iontophoresis [9] the size of compound 48/80 induced

flares was closely related to the log of the electrical charges applied in the iontophoresis procedure.

A second application 4 h later induced wheal and flare responses having only about 9% and 19%, respectively, of the sizes of those initially induced. These results indicate that the superficial histamine stores were largely depleted by the first application. More important, no itch was induced by the second application indicating that the amount of histamine released was too small to excite a sufficient proportion of nerve fibers mediating itch sensation.

When substance P was injected after two applications of compound 48/80 wheal and flare sizes were diminished by about the same amount as the wheals and flares to the second compound 48/80 application. This indicates that indeed both reactions are mainly indirect effects of substance P mediated by histamine. This is somewhat surprising in the case of the wheals, since substance P has also been shown to be potent in inducing plasma extravasation [4, 12]. However, similar to Wallengren's findings [18], wheal sizes were not as strongly affected by pretreatment of compound 48/80 as were flare sizes. This is also in accordance to Barnes' results [2], showing no effect of H_1-antihistamines on substance P induced wheal sizes.

LDF appears to have been, in this case, a poorer indicator of a diminished flare reaction than the planimetry of flare sizes since:

1. Blood fluxes in the skin pretreated with compound 48/80 were still elevated 24 h later when substance P was applied, so flux increases from different baselines can be compared only with difficulty. In the present study the flux increase from baseline was significantly diminished (not shown in figures) but not the absolute magnitude of the fluxes after substance P application.

2. As the LDF probe was placed directly next to the application area, LDF measures only a small field of the entire neurogenic inflammation. Therefore, the blood flux results obtained by LDF can be discussed as a direct local vasodilating effect of substance P, which was not affected as much by pretreatment of compound 48/80 as flare sizes. To distinguish between local vasodilation and neurogenic flare reaction a second LDF probe should be placed more distant to the application area in further studies.

Even at the higher doses substance P no longer induced pure itching after compound 48/80 and rarely any sensations mixed with itching. At lower doses no sensations at all were elicited and at higher doses mainly burning and stinging sensations were reported. These findings indicate that the itching responses to substance P are an indirect effect, induced by histamine release from cutaneous mast cells, whereas substance P directly excites a few receptor units which induce painful burning or stinging rather than itching sensations.

This is a further indication for the existence of partly or entirely different populations of chemosensitive small afferent fibers mediating itch and pain sensations [5].

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Effect of Wound Fluid on Cultured Keratinocytes

R.M. Soehnchen, E. Hilbert, C.C. Zouboulis, and C.E. Orfanos

Introduction

Wound occlusion leads to an accleration of the healing time in chronic wounds. Winter [16] already showed that occlusive dressings speed epithelialization of superficial wounds. Modern wound healing technology takes advantage of this. Faster removal of necrotic tissue, formation of healthy granulation tissue and quick epithelialization are the effects of occlusive dressings. The accumulated wound fluid contains elements for a provisional matrix out of fibronectin and vitronectin which is used by migrating keratinocytes as a track during tissue remodeling [12]. Furthermore the accumulated wound fluid causes a stimulation of the cell populations. Positive effects of wound fluid on the proliferation of fibroblasts and endothelial have been shown [10]. The study presented here examined the effect of wound fluid on attachment and proliferation of cultured human keratinocytes.

Materials and Methods

Human keratinocytes were isolated from human foreskins and grown on fibroblast feeder layer under serum-containing conditions [6]. Second passage keratinocytes were used for the experiments.

Wound fluid (WF) from five different donors was collected on day 1 or day 2 after surgery under sterile conditions from spilt skin graft, centrifuged at 10000 rpm for 10 min, filter sterilized and stored at −80°C until used.

For attachment experiments 10 μl WF was pipetted into 96-well plates and dried overnight at 37°C. Second passage human keratinocytes were plated at 10^4 cells/well in Dulbecco's modified essential (DME) medium without serum. After 24 h incubation the plates were washed and attachment was visualized after staining with toluidine blue.

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Growth experiments were carried out with DME medium in 24-well chambers seeded at 2×10^5 cells/well. Cells were seeded with the addition of 10% fetal calf serum (FCS) and left for 24 h at 37°C, medium was then changed and either 10% WF from different patients or 0%, 5% or 10% FCS as controls were added to the medium.

Proliferation was determined by numerical cell counting on days 2, 4 and 6. In addition proliferation studies with 5% or 10% WF as medium supplementation were done in 96-well plates by methyl umbiliferone heptanoate (MUH) assay [15] after 5 days of culture.

Results

Cell attachment on 10 μl adsorbed wound fluid after 24 h incubation in medium without serum was increased compared to that on uncoated tissue culture plastic or to coating with 10 μl FCS. The staining with toluidine blue revealed a comparable intensity for WF from different donors and an increased attachment compared to FCS controls and controls without supplementation.

Numerical cell counts on days 2, 4 and 6 indicated a steady increase in cell number for WF treated and control cultures. The population doubling time was 2.1–3.1 days in the logarithmic growth phase for keratinocytes

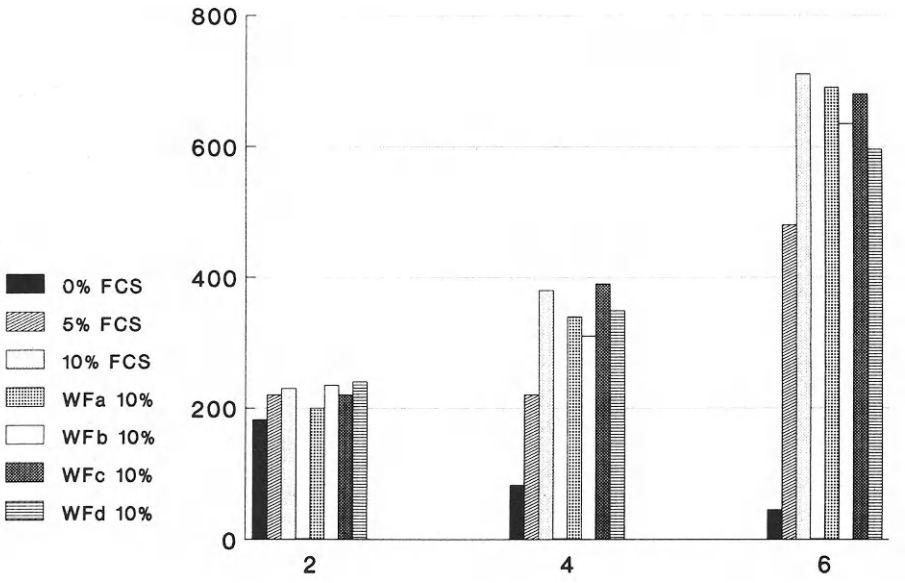

Fig. 1. Keratinocyte growth with wound fluid (*WF*): numerical counts days 2, 4, and 6; *FCS*, fetal calf serum

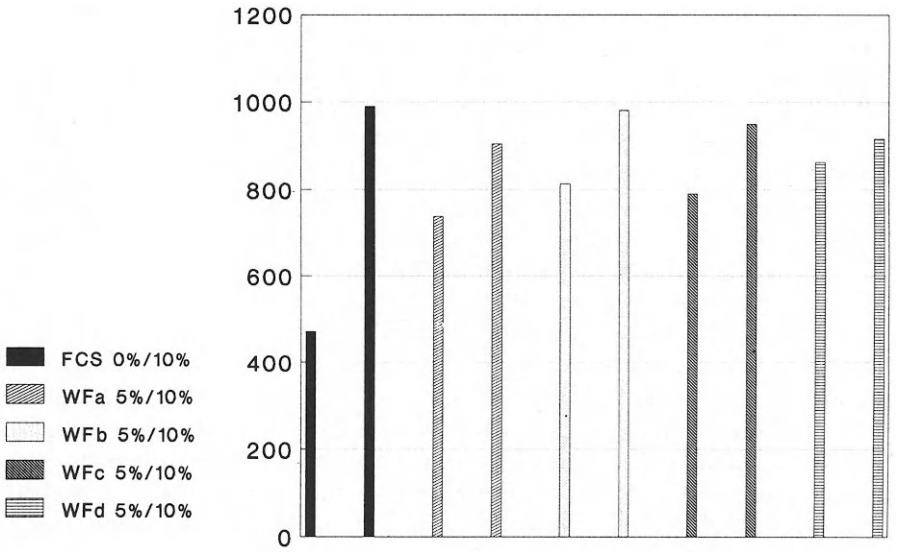

Fig. 2. Keratinocyte growth with wound fluid (*WF*): methyl umbiliferone heptanoate (MUH) proliferation assay (AFU), day 5; *FCS*, fetal calf serum

treated with the different wound fluids and 2.3 days for FCS treated control cells.

On day 4 of culture 10% WF led in all tested batches to a similar level of growth stimulation as 10% FCS did, whereas on day 6 the cell number in the 10% FCS control cultures was slightly higher than in the WF treated cultures (Fig. 1).

Proliferation experiments with the MUH assay revealed a stimulatory effect after 5 days for cells treated with 5% WF, similar to that in cells treated with 10% WF and to that of the 10% FCS control. Supplementation of medium with 10% WF did not induce a markedly elevated level of cell proliferation when compared to 5% WF supplementation. Control cultures with 0% FCS resulted in a steady decrease in cell number (Fig. 2).

Discussion

Wound fluid generated under occlusive wound conditions has a positive effect on the healing time. We have shown here that WF in vitro, as a medium supplement, can increase cell attachment and also has mitogenic activity in human keratinocytes.

The growth of keratinocytes is dependent on substrates and matrices on which keratinocytes attach, for example, during wound healing. Collagen fibrils [14] and also fibronectin molecules [5] serve as primary adhesives and

as a temporary track for ingrowing keratinocytes, migrating from the wound edges. Elevated levels of fibronectin occur in granulation tissue and also in inflammatory states [7]. Fibronectin contains the RGD tripeptide sequence, which mediates cell attachment of various cell types [13]. The RGD sequence has recently been detected in fresh WF. The attachment promoting effect could be blocked by antibodies to the RGD tripeptide [8]. WF from chronic wounds does not show increased attachment capacities, most probably due to proteolytic enzyms which degrade the fibronectin molecules [4]. Precoating of culture dishes with fibrin and fibronectin enhances epidermal cell motility and cell attachment [12], most probably due to RGD.

In our study the coating with fresh WF led to increased keratinocyte attachment on tissue culture plastic. In comparison to uncoated tissue culture plastic wells, cells in WF coated wells had more aggregated attachment, without major variation between the WF samples collected from different donors. We did not observe disruption and detachment of the cell monolayer in the flask, as has been described for fibroblast monolayers exposed to chronic WF [10], probably because we used fresh WF generated only 1 or 2 days after surgery. As has been shown recently [4, 8], WF of chronic wounds contains proteases which degrade cell adhesion proteins such fibronectin with the consequence that cells detach and round up.

Fresh WF reveals stimulatory effects on fibroblasts and endothelial cells [10]; even in concentrations of 2% WF the effects were similar to 2% FCS. We observed mitotic activity in keratinocytes cultured in medium supplemented with WF when added instead of FCS. Only minor variations between the different tested samples of WF were observed. Keratinocyte proliferation was steady during the testing period of 6 days, with about a threefold increase in the absolute cell number for treated and also control (10% FCS) cultures. The observed stimulation was similar for 5% and for 10% WF, indicating that minimal amounts of WF are sufficient to stimulate growth and that this effect is not dose-dependent.

As with FCS as a medium supplement the growth stimulation by WF may be due to the release of mitogenic factors from platelets and macrophages [11]. Platelet-derived growth factor (PDGF) has been shown to be responsible, at least in part, for the observed growth stimulation in fibroblasts [10]. The addition of an antibody against PDGF partly inhibited the growth stimulation by WF in fibroblasts; thus beside PDGF additional factors are responsible for the observed stimulation.

Angiogenic activity has also been observed in WF [1, 3]; endothelial cell migration and angiogenesis in vitro were stimulated. It has been proposed that WF is equivalent to serum [2]. Similar as for the previously reported growth stimulation in fibroblasts and endothelial cells [10], our data indicate that WF from fresh wounds stimulates growth of cultured human keratinocytes comparable to growth stimulation by FCS as a medium supplement. This gives further in vitro evidence for the beneficial effect of occlusive wound dressings in order to accelerate healing time.

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Strategies of Keratinocytes and Fibroblasts in Wound Closure Observed in an In Vitro Model

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Introduction

One of the most important functions of the integument is to maintain internal homeostasis and protect the organism from the entry of pathogens. So it is essential for any organism to repair this basic structure after an injury. Experimental investigations on wound closure are rendered difficult by technical and structural problems related to the complex microenvironment of a healing wound. Various models have been established; these reduce the complexity but consider only a few of the aspects in wound healing, for example, stripping of epidermis (e.g., Christophers 1973), suction-induced subepidermal blisters (Krawczyk 1971, 1973), or the rabbit ear chamber (Clark and Clark 1953).

The system presented here is an in vitro model of wound closure which avoids animal tests. It is possible to observe both the behavior of different cell species isolated from each other in wound repair and the mutual interactions of different cell species in this process.

The Model

The basis of this wound model is a culture dish that is divided into two different growth areas by a teflon strip (Fig. 1). The teflon strip can be removed in order to initiate a gap in the culture. This gap should simulate an incision, as it occurs, for instance, during surgery, in which undamaged cells form a boundary around a cell-free space. In this model different parameters during wound closure can be investigated. We worked with primary fibroblasts as representatives of the dermis and with keratinocytes in order to consider wound closure of epidermal cells. By using an image analysis system, changes in morphology were observed. With indirect im-

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Fig. 1. The wound closure model. Fibroblasts were seeded at a density of 6×10^4 cells/ml in multi-well dishes (Nunc, Wiesbaden, Germany). The density for keratinocytes was 8×10^4 cells/ml. Every dish was divided in two areas by a teflon spacer of 3 mm thickness. The spacers were manufactured in our workshop. Some 24 h after seeding the cells, the spacers were removed with sterile forceps

munofluorescence we examined collagen I synthesis in fibroblasts after addition of vitamin C to the culture medium. In vivo, vitamin C is essential for collagen synthesis and supports the functions of connective tissues (Pinnel et al. 1987).

Materials and Methods

Cell Culture

Keratinocytes

Investigations were performed with the keratinocyte line HaCaT, which was kindly provided by Prof. N. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany). The cultures were grown in Dulbecco's medium supplemented with 10% fetal calf serum (FCS), 1% penicillin-streptomycin (10 000 U/10 000 μg/ml) and buffered by sodium bicarbonate (3.7 g $NaHCO_3$/l; pH 7.0) at 37°C in an atmosphere of 10% CO_2 and 100% relative humidity. Media were exchanged twice a week. The cells were harvested by washing with 0.2% EDTA followed by 0.125% trypsin/0.2% EDTA treatment.

Fibroblasts

Fibroblasts were obtained from surgical material from our hospital. The skin samples were incubated for 2 h at 4°C in RPMI 1640 culture medium with 2% penicillin/streptomycin. The medium was changed every 30 min. Subsequently, the fatty tissue was dissected and removed. The skin sample was cut into small pieces (about 1 cm^2) and fixed with the dermis to a culture dish moistened with FCS. The skin pieces were incubated at 37°C and 5% CO_2 in RPMI 1640 with 10% FCS and 2% penicillin/streptomycin. The medium was changed twice a week. After about 14 days the fibroblasts had emigrated from the skin sample and colonized the whole dish. The rest of the skin sample was withdrawn and the fibroblasts were trypsinized (trypsin/EDTA: 0.05%/0.02%) and subcultured for the experiments.

Indirect Immunofluorescence

Fibroblasts were washed with phosphate-buffered saline (PBS) and then fixed with a methanol/acetone solution (vol/vol: 1/1) for 3 min. After washing the cells with PBS-BSA (PBS supplemented with 1% bovine serum albumin, Serva), collagen I was labeled using a primary anti-collagen I-antibody (goat, Institut Pasteur de Lyon).

The cells were washed again with PBS-BSA. A TRITC-coupled anti-goat IGE antibody (Sigma) served as the secondary antibody. After six washes in PBS the samples were embedded in Mowiol (Hoechst, Frankfurt, Germany).

Image Analysis System

After removing the teflon spacers, the cells were fixed at different points of time (0 h, 24 h, 72 h) and dyed with May-Grünwald/Giemsa solution. Microscopic images were scanned by a video camera (Proxiscan, Proxitronic) and computerized by an image analysis system (Optimas, Bioscan). Cells were counted at different well positions and the cell area was determined and related to distance to the wound gap.

Results

The results are shown in Figs. 2–6.

Discussion

Our results suggest that in a primary reaction to an incision wound, both keratinocytes and fibroblasts show increased motility into the gap. Fibro-

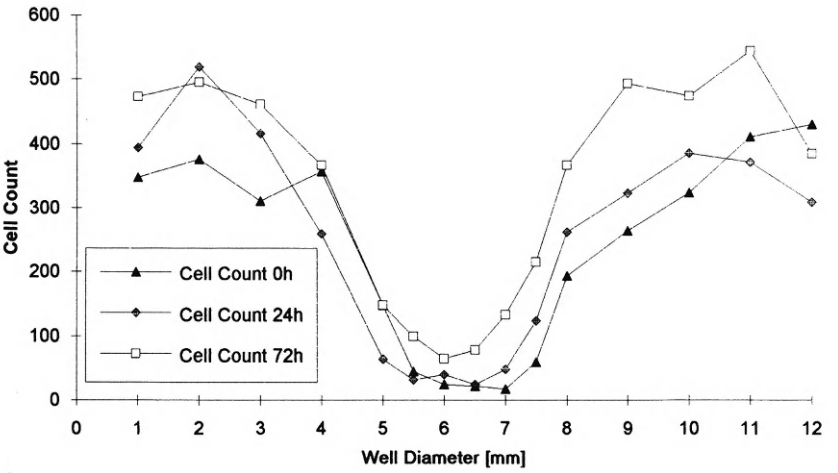

Fig. 2. Wound closure by fibroblasts 1. Cell count vs well diameter relative to different fixation times. The cell density is minimal at the gap, but increases in time, whereas the gap width decreases. Density maxima could be observed on the left and right side of the gap at 2–3 mm and 10–11 mm, respectivly. The teflon spacer did not fit exactly so there were already a few cells in the gap area at 0 h

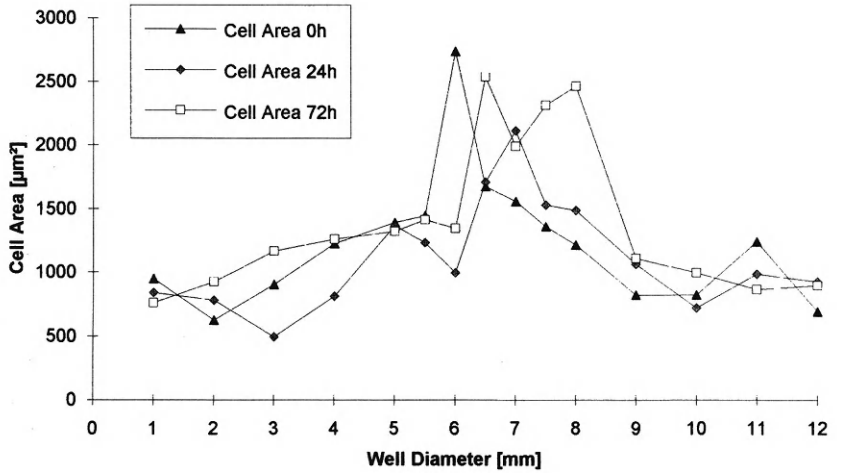

Fig. 3. Wound closure by fibroblasts 2. Cell area vs well diameter as a function of time. An increase in cell area in the wound region can be detected. The maximum areas were reached in the range between 5 and 8 mm. The teflon spacer did not fit exactly so there were already a few cells in the gap area at 0 h

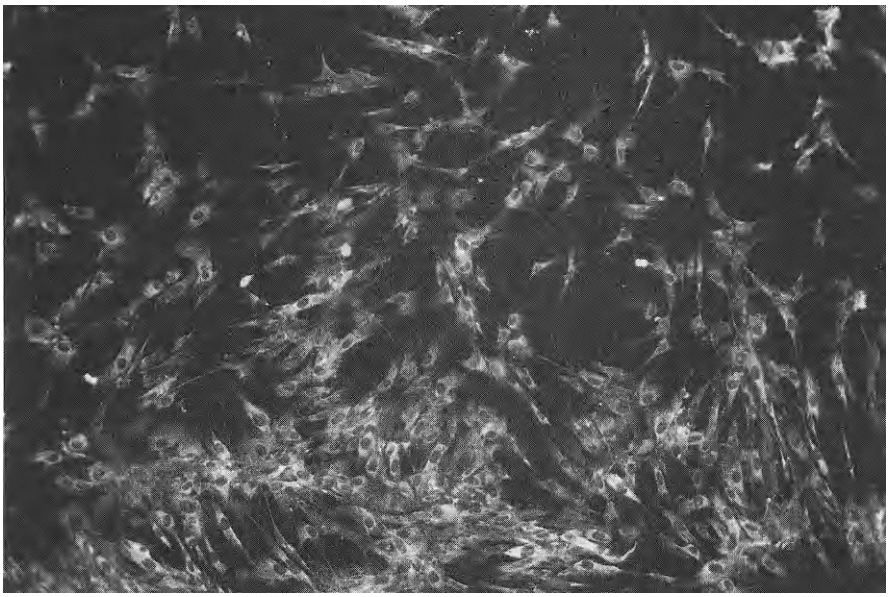

Fig. 4. Indirect immunofluorescence of collagen I. Fibroblasts cultured in absence of vitamin C. Fibroblasts in the gap area show many dendritic cell branches which were not uniformly directed

Fig. 5. Indirect immunofluorescence of collagen I. Fibroblasts cultured with 50 μg/ml vitamin C. Cells were enhanced in polarity, length and their axis points to the wound gap

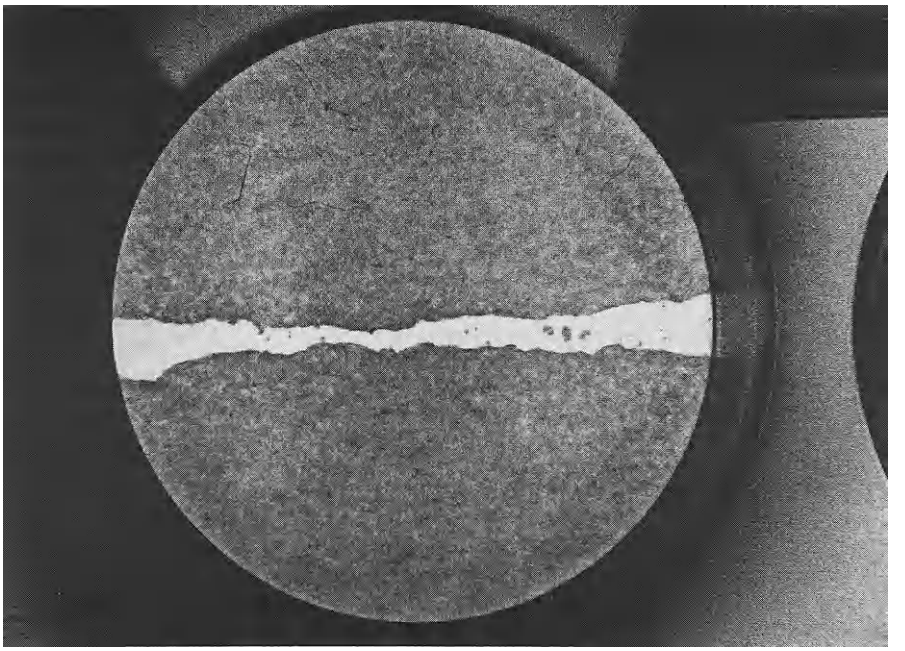

Fig. 6. Keratinocytes at the wound gap (overview image). Keratinocytes close the gap as a total front but connected sheets of keratinocytes can also be seen in the gap area

blasts migrate as single cells whereas keratinocytes migrate as a total front or as connected colonies. Fibroblasts seem to occupy the area by enlargement of their cell shape and by building up a kind of network between each other via dendritic plasma seams (Fig. 4). Vitamin C supplementation in the culture medium leads to a more directed spreading of fibroblasts into the gap. Vitamin C is known as a coenzyme of proline hydroxylase and is indispensable for synthesis of procollagen (Pinnel et al. 1987). Extracellular collagen seem to be a kind of orientation marker for migrational processes in skin (Fig. 5). The vitamin C-free cultured cells were able to synthesize α-chains of collagen I but these chains remained intracellular and could not form collagen molecules (Pruckop et al. 1979). Measurements of the fluorescence with image analysis show an increased collagen I content in fibroblasts cultured with vitamin C.

Keratinocytes seem to be not so flexible in shape. At the wound borderline keratinocytes were increased in their mitotic activity. This was observed by BrdU staining (not shown).

This two-dimensional model is suitable for the investigation of different strategies in wound closure by different cell species. It is also possible to

observe mutual interactions of different cell species by using a coculture model. This model is not suitable for simulating "burn wounds." In these cases the killed cells were left in position, and no free surface appeared.

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Interleukin-2 Improves Transfusion-Suppressed Repair of Experimental Intestinal Anastomoses

T. Tadros, T. Wobbes, and T. Hendriks

Introduction

Blood transfusions have become a common treatment for correction of anemias and for the replacement of significant losses resulting from trauma or operation. During the last decade, however, increasing evidence has emerged that blood transfusions carry certain risks, previously unrecognized, for the patient, in particular with respect to immunosuppression [1]. For instance, clinical and experimental studies have clearly demonstrated an association between blood transfusions and the occurrence of infectious complications [2, 3]. We have shown recently that the development of early anastomotic strength and the accumulation of anastomotic collagen in the intestine are inhibited if rats receive a blood transfusion immediately after operation. In addition, we observed an increased susceptibility to intra-abdominal septic complications [4]. Although the mechanisms underlying this phenomenon still need to be elucidated, it seems reasonable to suggest that impaired healing is caused by a suppressed cell-mediated immune response [5]. Cell-mediated immune reactions depend primarily on two cell types, macrophages and T lymphocytes. The essential role of macrophages in wound healing is well established [6] and the importance of lymphocytes in this process has been recognized recently [7].

Among the reported effects of blood transfusion on macrophage and T lymphocyte function is suppression of interleukin-2 (IL-2) production [8]. In addition to being the principal growth factor for activated T cells, IL-2 supports the growth and function of other immune effector cells, including macrophages [9]. Since it has also been reported that exogenous IL-2 enhances the healing of skin wounds in rats [10], we have investigated if administration of IL-2 would improve transfusion-impaired healing of experimental intestinal anastomoses.

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Materials and Methods

Animals

Sixty young adult male Lewis rats, with a weight between 225 and 275 g, were divided into three experimental groups of 20 rats each: a control group, a blood transfusion group (BT) and a group which received IL-2 after blood transfusion (BT + IL-2). Adult male Brown Norway rats were used as donor animals. The animals were housed in separate cages and had unlimited access to food and water. They were observed for a minimum of 1 week before start of the study to discover the presence of any preexisting diseases.

Operation and Transfusion

The animals were anesthesized with intraperitoneal sodium pentobarbital and underwent a median laparotomy. One centimeter of both ileum and colon were resected at 15 cm proximal to the ileocecal junction and 3 cm proximal to the rectal-peritoneal reflection, respectively. Inverted one-layer end-to-end anastomoses were constructed microsurgically with 8 × 0 monofilament suture material.

Blood was obtained from anesthesized Brown Norway rats by cardiac puncture. The blood was heparinized and given through the dorsal penile vein. Immediately after operation the rats in the transfusion groups received 3 ml blood, while animals in the control group received 3 ml saline.

Interleukin-2

Highly purified recombinant human IL-2 (specific activity 18×10^6 IU/mg) was provided by EuroCetus, Amsterdam, The Netherlands. The lyophilized material was reconstituted immediately prior to use and diluted in 5% dextrose. From operation onwards animals in the transfusion/IL-2 group received 250 μl subcutaneously once a day, in a dose of 2×10^6 IU/kg. Rats in the control and transfusion groups received dextrose only.

Investigations

Either 3 or 7 days after operation, the animals were anesthesized with intraperitoneal sodium pentobarbital. A second laparotomy was performed and the abdomen was inspected for the presence of abscesses. Both ileal and colonic anastomoses were prepared free, leaving surrounding tissue adherent to the anastomoses untouched, and removed. Thereafter, the animals were killed by an intraperitoneal overdose of sodium pentobarbital.

Approximately 5 cm intestinal segment containing the anastomosis was carefully cleaned from fecal content and attached to an infusion pump filled with methylene blue-stained saline solution. The pressure was increased by infusing 2.5 ml/min and recorded graphically. Both bursting pressure and site of rupture were measured. Subsequently, the anastomotic segment was cleaned from surrounding tissue and a 5 mm segment, containing the anastomosis, was collected for analysis. The samples were frozen immediately and stored in liquid nitrogen until processing. After weighing, the samples were pulverized and lyophilized and the hydroxyproline content was measured as described before [11].

Results

One animal in the transfusion/IL-2 group died prematurely. All results are thus derived from data in ten animals, except those obtained 3 days after operation in the transfusion/IL-2 group where nine rats were left for analysis.

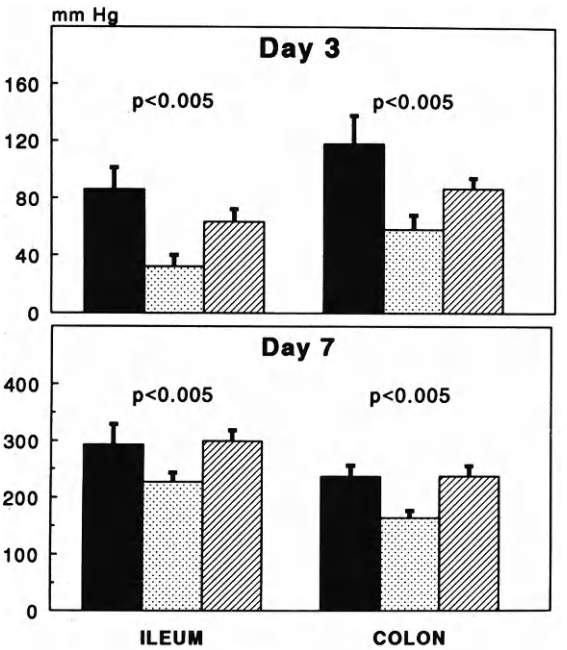

Fig. 1. Bursting pressure of anastomotic segments 3 (*upper panel*) and 7 (*lower panel*) days after operation. Data are expressed as average value ± SD. *Black bars*, control group; *dotted bars*, transfusion group; *striped bars*, transfusion/interleukin-2 group. Differences between groups are tested for significance using a two-sided Wilcoxon test; *p* values depicted concern differences between the transfusion group and both other groups

Blood transfusions consistently reduced anastomotic strength (Fig. 1). Three days after operation, at which time point all anastomoses ruptured within the suture line, the average bursting pressures in the transfusion group were lowered by 63% in the ileum and by 51% in the colon, if compared to those in the control group. Treatment with IL-2 could partly negate this loss of strength. Average values in the transfusion/IL-2 group were significantly higher than those in the transfusion group. However, at this time there was no complete restoration of strength since the mean bursting pressures remained 25% ($p = 0.0051$) lower than those in the control group. At 7 days after operation, a similar picture emerged. Here, IL-2 appeared to completely restore anastomotic strength to the transfused animals. At this time, however, the bursting pressure in the control group did not reflect anastomotic strength, because the bursting site was always outside the anastomotic area (Fig. 2). Thus, the actual average anastomotic strength must have been even higher than depicted in Fig. 1. Blood transfusion induced a statistically significant shift in bursting site: a majority of the anastomoses in the transfusion group ruptured within the suture line, again indicating loss of anastomotic strength. This phenomenon was completely prevented in the transfusion/IL-2 group.

The hydroxyproline content is taken to be a measure of the anastomotic collagen level. Again, blood transfusion exerted a negative effect on this

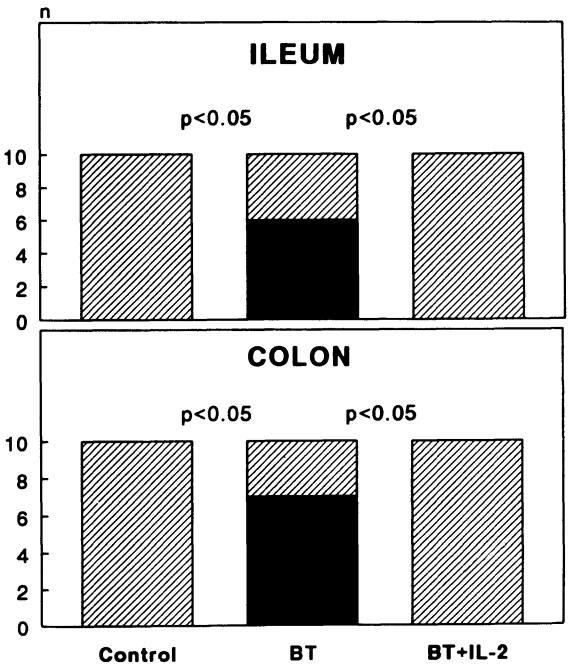

Fig. 2. Bursting site in ileal (*upper panel*) and colonic (*lower panel*) anastomotic segments 7 days after operation. *Striped bars*, rupture outside suture line; *black bars*, rupture within suture line. The significance of shift in bursting site between groups is calculated with a χ^2 test

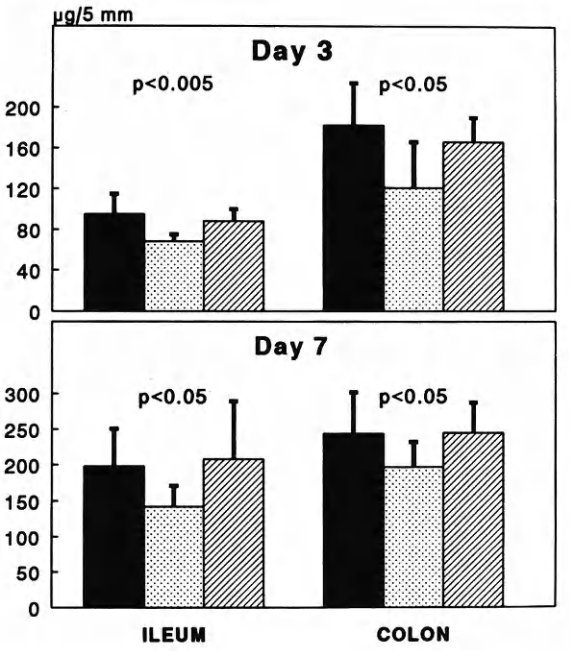

Fig. 3. Anastomotic hydroxyproline content 3 (*upper panel*) and 7 (*lower panel*) days after operation. Data are expressed as average value ± SD. *Black bars*, control group; *dotted bars*, transfusion group; *striped bars*, transfusion/interleukin-2 group. Differences between groups are tested for significance using a two-sided Wilcoxon test; *p* values depicted concern differences between the transfusion group and both other groups

parameter of repair. The anastomotic hydroxyproline content in the transfusion groups was significantly lower than in the control groups (Fig. 3). At 3 days after operation wound hydroxyproline levels were reduced by approximately 30%, both in ileum and colon. While a quantitatively similar reduction was found after 7 days in the ileum, the decrease was somewhat less, though still significant, in the colon. Administration of IL-2 after transfusion resulted in increased accumulation of wound hydroxyproline. Anastomoses in the transfusion/IL-2 groups contained significantly more hydroxyproline than those in the transfusion groups. No further differences were found between the transfusion/IL-2 and control groups, neither in ileal nor in colonic anastomoses.

Discussion

During the past decades, the immunological consequences of blood transfusion have been a topic of numerous studies and the fact that transfusions suppress the immune response seems now well established [1]. Although

transfusion can clearly improve transplant survival rates, it is becoming increasingly clear that it may exert detrimental effects in other respects [12]. For instance, an increased incidence of tumor recurrence and metastasis and, as a consequence, a decreased survival rate have been reported in cancer patients after blood transfusion [13, 14]. Also, blood transfusion appears to be an independent factor responsible for an enhanced frequency of postoperative infections [2, 3]. The latter effect is also evident from our first experiment with the current model: the number of infectious complications, i.e., anastomotic abscesses, is significantly enhanced in the transfusion group ([14], Tadros et al, manuscript in preparation). It is also clear that transfusion impairs the healing of intestinal anastomoses in rats. Both the development of early anastomotic strength and the accumulation of wound collagen are inhibited. At present, the exact mechanisms causing this effect are unknown, but we suggest that transfusion affects healing by interfering with macrophage and/or lymphocyte function. Blood transfusions are known to alter macrophage migration and function [15] and to diminish T cell responses to inflammatory stimuli [8, 16].

Wound healing is a complex and intricate sequence of events in which macrophages and lymphocytes play a pivotal and regulatory role [6, 7]. Probably, cytokines and growth factors produced by these cells regulate the process that must ultimately restore integrity and strength to the healing tissue and IL-2 might be one of the cytokines involved. Although a direct involvement of IL-2 in the healing sequence has not yet been demonstrated, it has been reported that addition of this compound can improve the healing of normal [10] and adriamycin-impaired [17] skin wounds in rats. There is increasing evidence that blood transfusion suppresses the IL-2 generation. Transfusion has been shown to reduce the number of lymphocyte helper cells which are known to synthesize IL-2 [16]. Also, transfusion appears to greatly decrease IL-2 production in splenocytes from rat [8] and mouse [18].

Taken together, the findings discussed above led us to speculate that a suboptimal presence of IL-2 might be one of the factors contributing to the negative effect of blood transfusion on anastomotic healing, observed before [4] and confirmed in the present experiment. Indeed, it appears that daily administration of IL-2 has a clearly positive effect on transfusion-suppressed anastomotic healing. Wound strength is partly restored after 3 days, and 7 days after operation strength in the transfusion/IL-2 group appears to be completely back to normal. At the same time, the reduced accumulation of wound hydroxyproline, induced by transfusion, is fully negated by the IL-2 treatment. It thus seems that administration of exogenous IL-2 eventually results in increased production of collagen, presumably by wound fibroblasts. Since IL-2 is unable to directly stimulate fibroblast collagen synthesis (Hendriks, unpublished observations), it could very well be that IL-2 stimulates the functions of immune effector cells thereby inducing the production of growth factors which in turn enhance fibroblast activity. While these results by no means prove that IL-2 is intrinsically involved in the healing

sequence, they offer a means to prevent negative effects of transfusion on intestinal healing. In view of the frequent use of blood transfusions in gastrointestinal surgery and the severity of the complications which may follow disturbed healing – such as anastomotic leakage – further investigations into the mechanisms involved are certainly warranted.

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Neuropeptides and Merkel Cells in Tissue Expansion

U. Wollina and U. Berger

Introduction

Tissue expansion has become a widely used method in plastic and reconstructive surgery (for overview see: Baker 1991; Mackay et al. 1989; Table 1). A silicone prosthesis is implanted under the skin. After 1 or 2 weeks, the expansion is started by controlled injection of saline solution. Particular attention must be paid to the pO_2 and to the dermal blood flow (Berger and Hyckel 1989; Berger et al. 1991). The dermal tissue becomes rearranged under stretching conditions. The epidermis responds with a temporary thinning of the papillary layer and a decrease in the length of rete ridges (Johnson et al. 1988; Leighton et al. 1988). The surplus of skin which is achieved at the end of treatment can be used to cover defects or reconstruct tissue.

Some questions, however, remain to be answered: (1) What is the impact of expansion on dermal vasculature? (2) How is the mechanical stress translated into a mitotic response? (3) Is there a normal epidermal differentiation in expanded skin?

Certainly there is no single mechanism responsible for the complex rearrangement and surplus production of tissues during expansion. In wound repair, factors such as cytokines (Rothe and Falanga 1991), proteases/-protease inhibitors (Penneys et al. 1991) and direct distorting effects on the cell membrane have been considered (Ryan 1989). Tensile forces and hydration of the connective tissue might also have some impact on skin response during expansion (Kibblewhite et al. 1992). On a closer view, it seems that tissue responses to controlled expansion are somewhat different from those of common wound healing (Table 2).

This chapter will concentrate on some examples of our recent work on tissue expansion in vivo and in vitro. Since the skin of domestic pigs shows an intimate correlation in both structure and function to human skin

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Table 1. History of soft tissue expansion

year	Author	Approach	Reference
1908	Magnuson	Experimentally lengthen limbs	Versacci and Balkovich (1984)
1957	Neumann	Skin expansion with an inflatable ballon (ear)	Neumann (1957)
1976	Radovan	Expansion of an arm flap with a temporary tissue expander	Radovan (1976)
1982	Radovan	Breast reconstruction after mastectomy	Radovan (1982)
1989	Mustoe et al.	Rapid tissue expansion	Schmidt et al. (1982)
1989	Logan and Hayden	Continuous tissue expansion device	Schmidt et al. (1982)

Table 2. Differences between normal wound healing and tissue expansion

Normal wound healing	Tissue expansion
Closing of dermal and epidermal defects	Surplus of skin
Initiation of clotting mechanisms	Stimulation of blood flow
Prominent inflammatory response	Absence of inflammation
Formation of a hypovascularized scar	Formation of a hypervascularized dermal capsule
Depletion of neuropeptides	Generation/liberation of neuropeptides?
First step in reepithelialization: migration	Proliferation
Skin texture disturbed	Skin texture unchanged
Loss of skin adnexes	Preservation of skin adnexes
Scars (hypertrophic) often itching	No pruritus

See also: Clark 1988; Bernstein 1991.

(Archambeau and Bennett 1984; Meyer et al. 1978; Wollina et al. 1991a), it was possible to investigate some aspects in this in vivo model. Other problems have been investigated by the use of cell culture systems in vitro.

Skin Effects of Controlled Tissue Expansion

In the following we will discuss selected aspects of tissue response to a controlled expansion. The more clinical aspects and questions of mechanical properties of expanded skin have been discussed in excellent reviews recently (Baker 1991; Mackay et al. 1989).

Dermal Vascularity

Tissue expansion, though causing an increased pressure on dermal vessels, has a significant positive effect on flap survival and on survival length of random pattern skin flaps in pigs (Cherry et al. 1983; Wollina et al. 1991b).

Sasaki and Pang (1984) were able to demonstrate an increased total capillary blood flow in expanded random pattern flaps and Sasaki and Krizek (1983) measured by means of microsphere perfusion an enhanced dermal blood flow and a marked vascularity in the capsule surrounding the tissue expander. Their results gained further support by angiographic and histological investigations of Leighton et al. (1988), who found that the capillary counts were highest within the papillary layer. By contrast, Wollina et al. (1992a) studied the vascular counts in papillary dermal layer of Minilewe pigs after controlled tissue expanion over 6–9 weeks. The expanders were filled once within 2 weeks to reach final expander volumes of 350–500 cm^2. This was followed by a retention period of 10–12 weeks. Skin biopsies were taken within 3–7 days after an additional distention. Capillary counts per visual field were evaluated on frozen sections stained with fluorescein isothiocyanate-labeled lectins (ConA, PSA, WGA, UEA I). Surprisingly, we found a mean decrease of vascular counts of 45.1% ± 12.5% in expanded samples ($p < 5\%$) (Wollina et al. 1992a; Fig. 1). We assume that in the case of our study design, stretching of skin by the final expansion had overwhelmed neovascularization. Indirect evidence came from the observation of a decreased length of rete ridges and thinning of the papillary layer. Additionally, the use of frozen sections might have avoided formalin induced shrinkage of tissue.

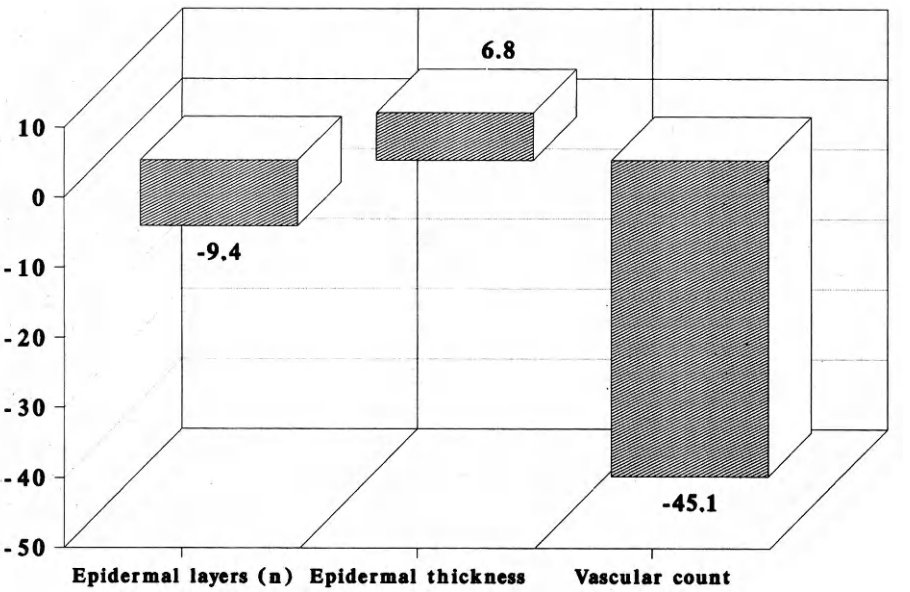


Fig. 1. Relative change (in percent of controls) of quantitative skin parameters in porcine skin due to controlled skin expansion. Significance reached only the decrease of papillary vascular counts. (From Wollina et al. 1992a)

Direct evidence for a practical, important, functional enhanced vascularity has been gained by creation of extreme skin flaps. Recently, Wollina et al. (1991b) reported on randomized porcine skin flaps with a length/width ratio of 6:1 (24 cm × 6 cm). By comparision to nonexpanded, acute randomized flaps, the survival rate increased 230% in the thoracic and 170% in the pelvic region. These excellent in vivo results are thought to be related to a delay phenomenon (Fig. 2).

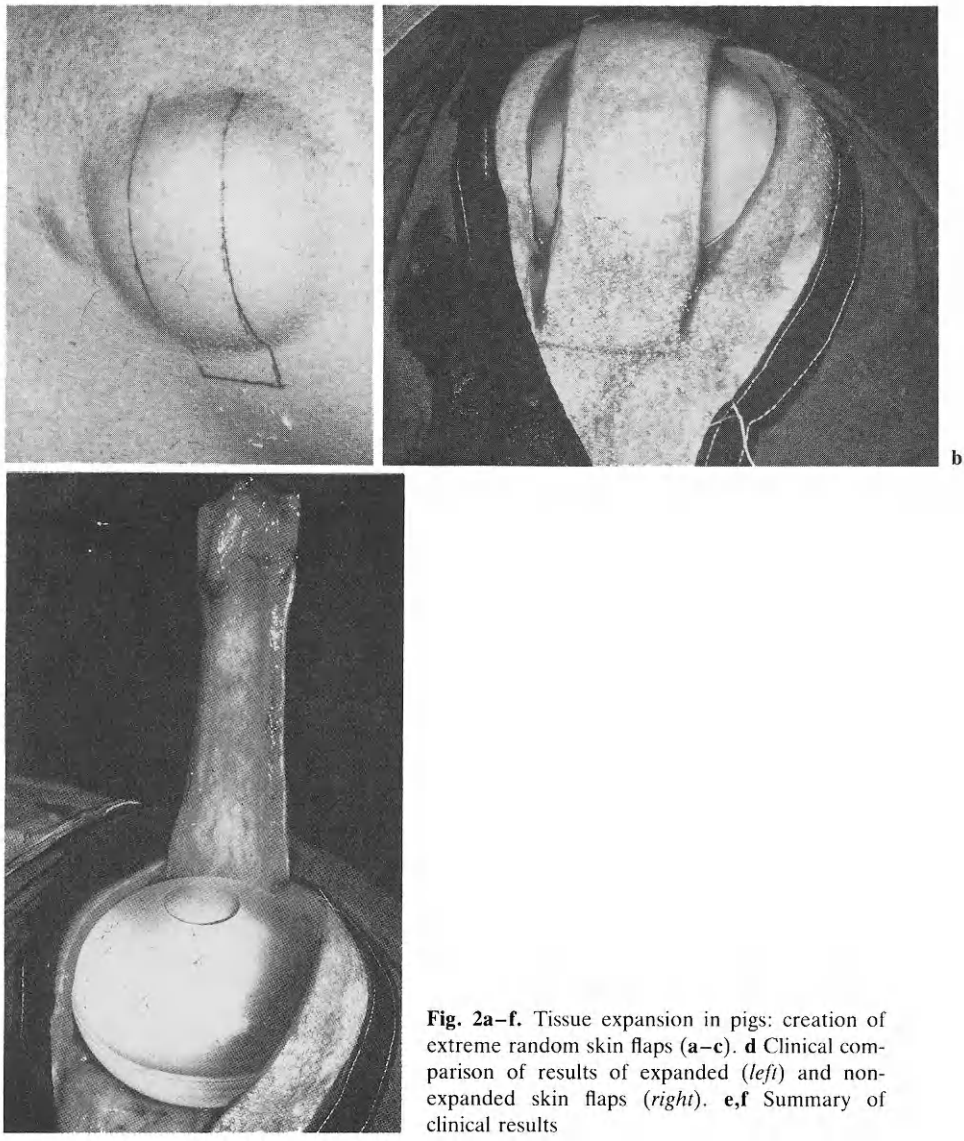
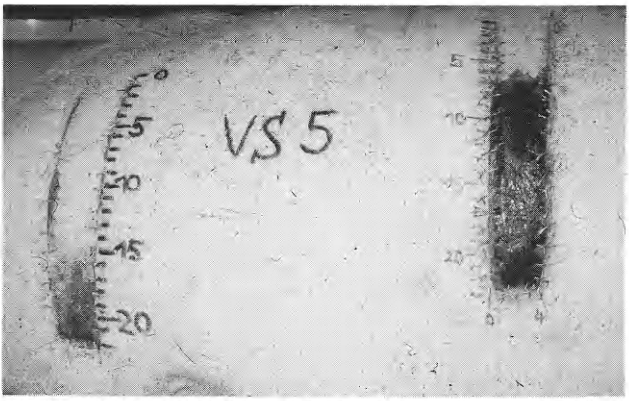


Fig. 2a–f. Tissue expansion in pigs: creation of extreme random skin flaps (**a–c**). **d** Clinical comparison of results of expanded (*left*) and nonexpanded skin flaps (*right*). **e,f** Summary of clinical results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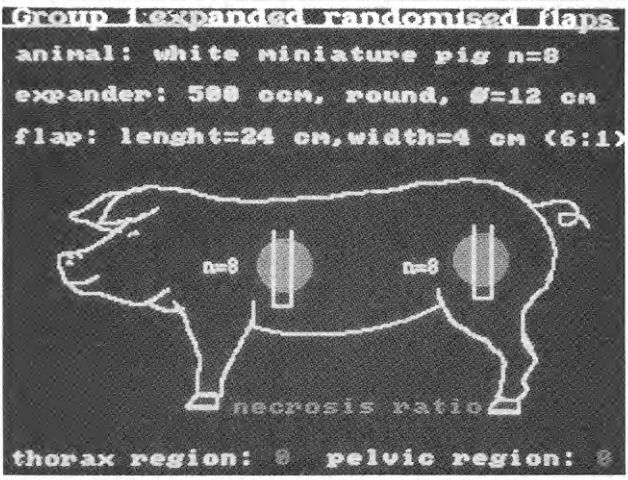
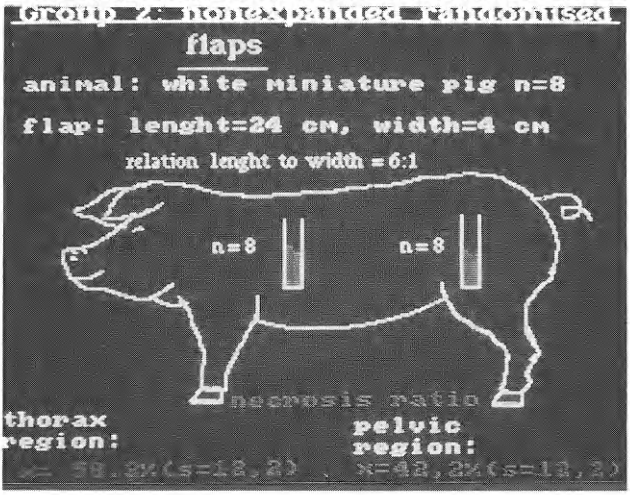

Group 1:expanded randomised flaps
animal: white miniature pig n=8
expander: 500 ccm, round, Ø=12 cm
flap: lenght=24 cm,width=4 cm (6:1)
n=8
n=8
necrosis ratio
thorax region: 0 pelvic region: 0

e


Group 2: nonexpanded randomised flaps
animal: white miniature pig n=8
flap: lenght=24 cm, width=4 cm
relation lenght to width = 6:1
n=8
n=8
necrosis ratio
thorax region:
pelvic region:

f

Fig. 2d–f.

Positive effects on blood flow cannot be explained by mechanical forces alone, which are mainly responsible for dermal pressure on blood vessels. Rather, mediator mechanisms have also to be considered, which counteract.

Epidermal Response

When skin becomes stretched during expansion a thinning of the dermal and epidermal layers above the prosthesis seems conceivable. By contrast, studies in humans, pigs and beagle-mixed dogs reported either a significant increase of epidermal thickness or no significant changes in epidermal thickness (Pasyk et al. 1988; Johnson et al. 1988; Schmidt et al. 1991; VanderKolk et al. 1988; Wollina et al. 1992a; Fig. 1). A relative increase of the horny layer was observed in pigs and guinea pigs (Austad et al. 1982; Leighton et al. 1988; Wollina et al. 1992; Fig. 1).

Histomorphologic examination of expanded epidermis at the light microscopic level revealed no dramatic change (Leighton et al. 1988; Austad et al. 1982; Pasyk et al. 1987; VanderKolk et al. 1988). Ultrastructural investigations showed that the intercellular spaces of living cells layers became narrowed (Kivanc et al. 1992).

In pigs and humans a regular sequence of expression of glycoconjugates in expanded epidermis is evident (Wollina et al. 1991c, 1992a). The epidermal differentiation pathway obviously remains unchanged.

Since no marked thinning develops during expansion, trophic factors must be present in or near by the dermis and the epidermis. Austad et al. (1986) measured proliferative bursts of epidermal keratinocytes by thymidine labeling. The proliferative reponse reaches a maximum within 24 h after expansion. These findings raise the following questions: How do the keratinocytes realize an enhanced proliferative activity when the actual blood flow temporarily decreases and the tensile strength of both the dermis and the epithelium is elevated? What are the mediators/transducers of stressing biomechanics into: (a) a stimulated vascularity and (b) a prompt but sensitive adaption of epidermal mitotic activity? Recent work suggests involvement of neuroendocrine cells and peptides in wound repair and probably also tissue expansion.

Neuropeptides and Merkel Cells in Tissue Expansion

Epidermal Merkel Cells

Epidermal Merkel cells (MCs) are neuroendocrine epithelial cells with a closer relationship to cutaneous nerve endings and a very low if any proliferative activity (Mahrle and Orfanos 1974; Narisama and Hashimoto 1991; Hartschuh et al. 1983). Table 3 summarizes possible MC functions. As one

Table 3. Presumptive functions of Merkel cells in mammalian skin (from Wollina and Mahrle 1992)

Mechanoelectrical transducer
Neuromodulator of the response generated directly in the nerve endings by mechanical stimuli
Topic element for cutaneous (terminal) nerve endings
Trophic element for cutaneous (terminal) nerve endings
Paracrine activity on adjacent cutaneous cells

Table 4. Epidermal Merkel cells (number per square millimeter surface area) in expanded and control skin)

Skin	Expanded skin	Controls	p
Human skin			
Transverse sections[a]	21.3 ± 19.9	14.4 ± 21.5	>0.05
Upside down preparations[b]	7.5 ± 17.5	12.7 ± 10.4	<0.05
Porcine skin			
Transverse sections[a]	5.5 ± 5.5	1.6 ± 2.1	>0.05

[a] Estimation according to Moll et al. (1991).
[b] The differences between transverse and upside down preparations are not significant. The lower values of the latter seem to be caused by underestimating the infundibular Merkel cells.

can see, epidermal MCs might be of particular interest in the generation of an epidermal response by either paracrine activity on adjacent epidermal cells or indirect mechanisms such as targeting cutaneous nerve endings.

The detection of sparsely distributed MCs is facilitated by immunohistochemistry with monoclonal antibodies against simple type keratins (Moll et al. 1984; Narisawa et al. 1992). We used two different approaches to enumerate MCs in expanded skin and controls: (1) Serial frozen sections were made and the number of epidermal MCs was estimated as a rough calculation according to Moll et al. (1991). (2) Human frozen skin samples were incubated in 4 N sodium bromide (30–60 min at 37°C), which separates epidermis and dermis. Then the former was carefully slipped over glass slides upside down. In both approaches, APAAP technique with monoclonal antibody Cam 5.2 (Becton Dickinson) against simple type keratins was performed according to standard protocols. The epidermal MC number per square millimeter of expanded skin and controls was estimated in both humans and Minilewe pigs (Table 4, Fig. 3). In several human specimens the proliferative activity was assesed by use of a monoclonal antibody against Ki67 nuclear antigen (Dakopats).

The MC density in Minilewe pigs was lower than that of human beings. However, the standardized surgical technique and localization in pigs de-

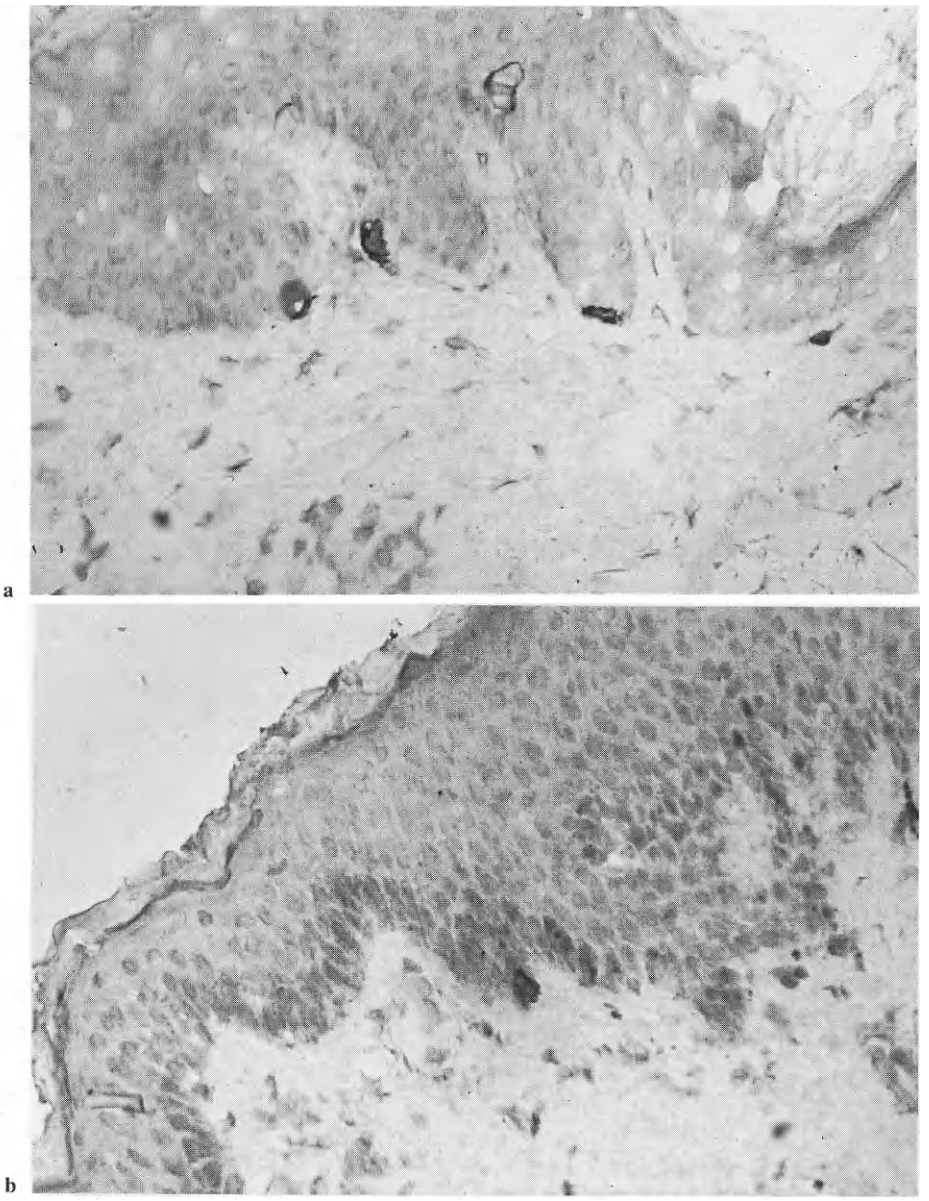


Fig. 3a,b. Human skin. The effect of tissue expansion on Cam 5.2-positive epidermal Merkel cells (MCs). The longitudinal sections suggest a tendency to MC clustering. **a** Expanded skin; **b** control

monstrates more clearly that the MC count does not significantly vary due to expansion, though a marked surplus of skin was achieved. In light of a largely increased skin area due to expansion, one would expect either a marked decrease of MC density (indicative of nonproliferating MCs) or an increase in the proliferative activity of MCs. Obviously, none of the alternatives can be supported by our in vivo studies.

In accordance with our findings, Compton et al. (1990) observed MCs in skin from cultured keratinocyte grafts. They called the phenomenon "MC regeneration." The experimental protocol made immigration of dermal MCs into the graft impossible. We suppose that MC regeneration in skin grafts and in randomized expanded flaps is due to direct transdifferentiation of basal keratinocytes. Transdifferentiation is a term used for cells changing their differentiation program without necessarily changing their proliferative activity (see also: Beresford 1990). Transdifferentiation is probably the major pathway of epidermal MC generation in general (Narisawa et al. 1992; Narisawa and Hashimoto 1991).

Neuropeptides in Tissue Expansion

Numerous neuropeptides have been detected in skin (Brain and Edwardson 1989; Bernstein 1991; Wollina 1991). Most of them have been localized to cutaneous nerve endings, mast cells and MCs (Table 5).

It is generally accepted that neuropeptides regulate cutaneous blood flow (Brain and Edwardson 1989; Bernstein 1991). Sann et al. (1988) proposed that neuropeptides could be liberated from vascular nerve endings

Table 5. Neuropeptides of mammalian skin

Atrial natriuretic peptide
Bradykinin
Calcitonin gene-related peptide
Galanin
Leu-enkephalin
Met-enkephalin
Neurokinin A
Neuropeptide Y
Neurotensin
Pancreatic polypeptide
Peptide histidine-methionine
Somatostatin
Substance P
Vasoactive intestinal peptide

According to Bernstein 1991; Brain and Edwardson 1989; Wollina 1991; Wollina and Mahrle 1992.

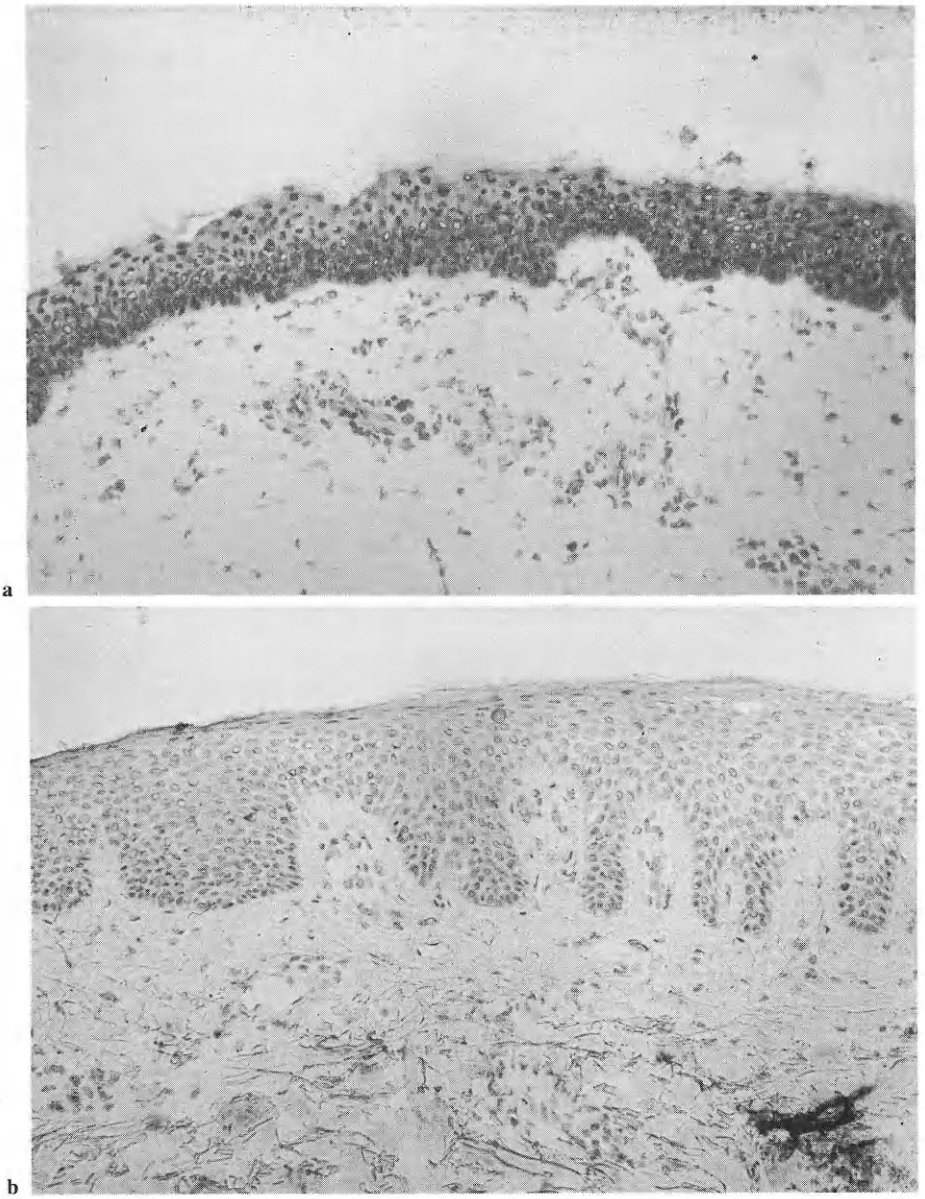


Fig. 4a,b. Human skin. Expression of epidermal synaptophysin-like immunoreactivity in **a** expanded vs **b** nonexpanded skin. Note the basal diffuse staining in the expanded sample

even by mechanical stimulation – an interesting feature for tissue expansion. Some neuropeptides, such as substance P, stimulate DNA synthesis of skin fibroblasts in vitro (Nilsson et al. 1985). Experimental studies on dermal wound healing suggest a trophic role (Senapati et al. 1986).

In vitro studies with human keratinocytes have recently shown that, in particular, vasoactive intestinal peptide is capable of enhancing epithelial proliferation (Haegerstrand et al. 1989). The peptide facilitates survival and proliferation even under conditions of stress (Wollina et al. 1992b,c), making it an interesting mediator for biomechanical stress as well.

As demonstrated recently, MCs occur in hyperproliferative epidermal disease, e.g., psoriasis, in which they show unusual increased expression of neuropeptides (Wollina and Karsten 1988; Wollina and Mahrle 1992). We investigated the expression of neuropeptides by human MCs in expanded skin but failed to detect any immunoreactivity for the following MC-related peptides: somatostatin, pancreatic polypeptide, neuron-specific enolase and chromogranin A. However, an increased diffuse immunoreactivity with a polyclonal antibody against synaptophysin SY 38 [1] (Dakopatts) was shown in the lower epidermal layers and was not restricted to particular cells (Fig. 4).

Conclusions and Outlook

Tissue expansion is increasingly used in plastic and reconstructive surgery. The underlying mechanisms responsible for the transduction of mechanical forces caused by distention of the silicone prosthesis into a proliferative response are poorly understood. However, some progress has been made in this field, suggesting the involvement of neuropeptides and neuroendocrine cells of the skin.

Neuropeptides, which can be liberated from cutaneous nerve endings even by mechanical stimuli, regulate cutaneous blood flow, which becomes markedly increased in expanded skin. In relation to epidermal proliferation, vasoactive intestinal peptide, which has been detected in cutaneous nerve endings and epidermal MCs (Hartschuh et al. 1983), became of particular interest. In vitro, the peptide exerts positive effects on cell survival and growth for human keratinocytes under stress conditions (Wollina et al. 1992a,b). Special attention has to be paid to the de novo formation of epidermal MCs in the expanded skin. These cells with paracrine activities and an intimate relationship to cutaneous (peptidergic) nerve endings might play a crucial role in the transduction of tissue expansion into a proliferative epidermal response. Though we are far away from completely understanding tissue expansion in its biological terms, the present data are challenging our understanding of the involvment of neuroendocrine cells and neuropeptides.

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Congenital Factor XIII Deficiency as Cause of Therapy-Resistant Burn Wounds

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Case Report

Actual history as reported by the patient's father and the hospitals involved: On February 23, 1991, a 10 year old healthy boy contracted second degree burns of 12% BSA on both legs while pouring gasoline into an open fire. He was admitted to a hospital in Libya where conservative treatment was initiated. Failure of wound healing caused the transfer of the patient to a hospital in Cyprus where a skin grafting was done. After his return to Libya the wound condition deteriorated due to loss of transplants and secondary infection. The patient was readmitted in Cyprus with multiple infected wounds and raw areas on both limbs. After an initial conservative wound treatment a secondary transplantation of split skin, taken from abdomen and right arm, was carried out. Again there was an extensive loss of grafts. The donor sites did not heal. The patient's condition deteriorated physically and psychologically. By September 3, 1991 he had undergone 20 procedures under general anesthesia. On October 29, 1991 the patient was transferred to our institution.

Status on Admission

An 11 year old boy,weighing 30 kg 1.57 meters tall, with raw, red, granulating wounds on both thighs and parts of the legs surrounding islands of surviving skin grafts. Red bluish marginal zones were observed between healthy skin and wound surface. In addition, there were areas of non-epithelialized skin at the donor sites on abdomen and right upper arm and very fragile epithelium at the healed areas. Contractures of both knee joints were partially fixed and partially maintained active to avoid pain. Mental status: very anxious. Nutritional status was fair, accounting for the concurrent muscle atrophy after 8 months in a mostly recumbent position.

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Laboratory Status

On admission normal serum results were obtained for the following parameters: Na, Ca, Crea, BUN, CHE, AP, glucose, copper, Mg, PTT, and TT. Zinc and potassium levels were at the lower border of normal. Blood count: Hb 10.6 g/dl; HK 32%; HbE 27.8 pg; MCV 84 fl; thrombocytes 175/pl; leukocytes 7.4/pl; lympho 43%, mono 7%, granulo 50%; complement C3 172 mg/dl; C4 26.7 mg/dl. Total serum protein: 7.2 g/dl; serum electrophoresis: Alb 53.9%; α-14.6%; α-2 12.7%; β 10.2%; γ 18.4%.

Therapy at Our Institution

The patient was put on a high caloric diet; potassium, zinc and vitamins were substituted. On November 11 the wounds on both legs and open donor sites on abdomen and right upper arm were debrided and grafted with meshed (1:1.5) split skin taken from the scalp, left proximal thigh and lower abdomen. After mobilization in general anesthesia both legs were immobilized by splints to prevent shearing forces on the grafts. Perioperative prophylactic antibiotics were given. The postoperative course was uncomplicated in the beginning. On the fifth postoperative day, all grafts were vital and there was no sign of infection. The grafts were unsuspicious until the 14th postoperative day. Then a piece by piece loss occurred with each change of dressings. The wounds looked moth-eaten. The remaining epithelium was glossy and thin. The borderline areas between unburned healthy skin and the older grafts were fragile and displayed a bluish red color which did not disappear with time. On the abdomen the donor sites did not epithelialize. Instead, the partially healed donor areas started to lose their epithelium. On December 12th, a last attempt was started to graft the open donor sites on abdomen, right upper arm. Donor area was the right lateral trunk. Again only a fraction of the grafts took permanently.

Repeated agglutination assays of serum factor XIII activity had determined a persistent reduction to 30%. On January 7, a substitution of factor XIII was initiated. The patient received a total of 20 000 units (fibrogammin-HS) i.v. over a period of 20 days, maintaining a serum level of 100%. The other treatment modalities were not changed. The patient was bathed daily and the wounds were dressed with fatty gauze.

Within 1 week a dramatic change of the clinical picture took place. There was no additional loss of epithelium. Spontaneous epithelialization set in at all wounds and donor sites. The fragility and redness ceased continuously. Within 5 weeks all wounds were healed except for a small 1 × 2 cm area at a donor site at the left temporal scalp. The patient was discharged. He returned in very good physical condition for a final check-up 6 weeks later.

Determination of the patient's healthy father's factor XIII also revealed a level of 30%. The patient is the sibling of a healthy girl and brother of 4 additional children of the family, all of them healthy as is their mother. There was no history of hereditary illness or coagulation disorders in the family. The only hint given by the patient's father was the information that minor wounds had always taken a very long time to heal in his son.

Discussion

We have no reason to doubt that the surgical procedures at the pretreating hospitals were carried out lege artis since they were done in specialized departments. By chance, the desperate parents sought the boy's transfer to our country.

In accordance with the experience of the pretreating surgeons, the specific complication in our patient, the piece by piece shedding of the grafts, occurred delayed postoperatively at a time when skin grafts, as a rule, would gain normally sufficient stability. In contrast, graft loss due to infection or insufficient debridement is usually evident within 5 days. In vitro studies have determined the importance of factor XIII for fibroblast activity in wound healing. This effect is mediated by a cross-linking of collagen by a transpeptidase activity of the A-subunit. Mechanical instability of living epithelium in skin grafts and donor sites, the predominant symptom in our patient, seems to be caused by the hereditary deficiency of factor XIII.

Conclusion

Using the more precise assay kits currently available, controlled trials should determine the relationship between factor XIII serum activity and clinical effects to establish guidelines for substitution and dosage. Unexplained loss of skin grafts or loss of epithelium from uninfected wounds which are not exposed to mechanical stress and missing wound contraction should prompt an assay of the patient's factor XIII activity, even if the patient's history is hitherto uneventful.

VI. Microbiology and Wound Infection

The Microbiology of Wounds

B. Gilchrist

Introduction

For any clinician involved in the management of healing wounds, the possibility of infection and the disruption of healing that follows are a matter of constant concern. Although the mechanisms of this disruption are only partly understood [1], it is clear that it may not be possible to apply one rule to all wounds. There is increasing evidence that not all bacteria are harmful and that the way in which they interact with the wound may be different, according to the type of wound being considered.

The Problem of Definition

Much of the present difficulty in any discussion about infection is a result of a misunderstanding about what is actually being discussed, and the loose use of terminology which has led to many precise concepts being confused. The first problem is that not all wounds are the same, and both the aetiology and the way in which they are healing will influence the mechanisms of infection.

In a surgical wound healing by primary intention, the presence of bacteria is not a normal event; however currently accepted definitions of wound infection for this type of wound point out that the presence of a positive swab is not a necessary condition for that wound to be defined as infected [2, 3]. In contrast, a chronic wound healing by secondary intention is virtually never sterile [4] and bacterial swabs will almost always reveal growth.

The second point of confusion is the use of the word "infection". It is important to remember that the presence of an organism does not necessarily mean that the wound is infected, and that any discussion of bacterial presence should clearly differentiate between the following three possibilities [5]:

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1. Contamination: Bacteria are present, but are not multiplying
2. Colonisation: Multiplication is occurring, but there is no host reaction.
3. Infection: Deposition and multiplication of organisms in tissue, with an associated host reaction.

It follows from this that the decision about whether or not a wound is infected is a clinical one, and is based largely on the presence of clinical manifestations of infection such as heat, redness, swelling, pain, cellulitis, increased exudation or abnormal granulation tissue, for example. While the swab result may be helpful in the treatment of an established infection, it may also be misleading, especially if the culprit organism is an anaerobe, which may not be easy to culture [6].

In a chronic wound, the surface swab will almost always reflect colonisation rather than infection, and there is increasing evidence that these wounds will heal with bacteria present: in an experimental pig wound it was shown that wounds healed despite the presence of large numbers of bacteria [7]; some investigators [8] have concluded that as a swab gives no useful prognostic information, there is no need to take one at all, while others [9] have demonstrated that there is no role for prophylactic antibiotics, which do not significantly alter the flora.

It can also be seen that it follows from these definitions that in order to treat an infection, the therapy must treat the bacteria present in the tissues, not just the ones found superficially on the wound. Antibiotic therapy must therefore be systemic, and there would appear to be no justification whatsoever for the routine use of topical antibiotics on a chronic granulating wound. Indeed, the possibility of skin sensitivity reactions, and the promotion of bacterial resistance would appear to be positive contraindications to their use. There may be a case for their use immediately prior to grafting to assist graft take, but this should be seen as a special case.

Infection and Moisture

Recently, there has been increasing interest in the role of the "moist wound healing" concept as a means of increasing the healing rates in granulating wounds. This concept follows from experimental work which showed that wounds which are kept wet heal 50% faster than similar wounds left exposed to the air [10]. Clinical acceptance of this concept has, however, been slowed by the genuine concern that promoting a moist surface on a wound would lead to increasing clinical wound infection, a concern first raised by Winter, the investigator responsible for the initial development of the actual concept [11]. Initial attempts to promote a moist environment with total occlusion appeared to bear out this concern [12], and experiments on intact skin showed that occlusion lead to a very rapid increase in the numbers of bacteria present [13]. The development of semipermeable polyurethane

dressings did, however, allow this principle to be utilised successfully in a clinical situation [14].

The advent of a new type of dressing, known as a hydrocolloid, provoked much further interest. After it had been shown experimentally that this type of dressing did not have any effect on the bacterial flora of intact skin or the numbers present [15], it was also tried in the treatment of chronic wounds such as venous ulcers with some success [16].

The bacteriology of such wounds treated clinically with hydrocolloids has been investigated [17]. The results confirmed that there was very little change in the flora of an ulcer over time and that the presence of many types of bacteria, including anaerobes, did not hinder wound healing or lead to clinical infection. It was also noted that the dressing under investigation (DuoDERM, Bristol-Myers Squibb, also known as Granuflex) appeared to have an anti-pseudomonal effect, and it has been conjectured that this is a result of the exclusion of oxygen [18] as well as the possibility that, as the dressing suspends water, there may also be a dessicant effect [19].

The effect of dressing type on wound infection has been reviewed [20] and appeared to show that under conventional dressings such as gauze or paraffin tulle the reported infection rate was 6.9%, while under occlusive dressings such as films, foams or hydrocolloids it was 2.5%. This type of approach is open to criticism, however, as such reviews rely for a definition of infection on whatever criteria individual papers choose to use, and as we have already seen this can be misleading. To counter this criticism, a randomised prospective trial comparing hydrocolloid with conventional dressings, and including a very tight definition of infection was carried out in a multicentre trial [21]. The results are surprisingly similar, with an infection rate of 5.7% in the control group of nonoccluded wounds compared to a rate of 2.1% in the hydrocolloid group.

Importantly, careful quantitative bacteriology also showed that the numbers of bacteria were not increasing under the hydrocolloid, thus bacterial proliferation did not appear to be a problem.

The evidence therefore does not appear to support the proposition that occlusion leads to infection [22], but rather appears to suggest that occlusion may actually have a role in reducing infection. Although the mechanism for this is not fully understood, it seems reasonable to suggest that the following are the most important features:

1. Promotion of the normal immune response: In an occluded wound the leukocytes do not dry out, die and become incorporated in the scab, but rather remain on the wound surface and remain functional [18, 23]; thus bacterial proliferation is kept in check.
2. Suspension of water. As much of the water present under a hydrocolloid is suspended in the hydrocolloid matrix [24] it is not available for bacterial growth and, as has been previously suggested, this may lead to dessication of bacteria.

3. pH effects. Low pH under such dressings may contribute to retardation of growth of some bacteria [18].
4. Bacterial barrier properties. Some (although not all) of the new occlusive dressings are bacteria impermeable [25]. In these cases, bacteria are not able to pass through the dressing, as would be the case with wet gauze, for instance. The bacteria being sampled thus reflect the normal flora, rather than superinfection.

Despite this evidence, there may still be some cause for concern in the case of one specific organism, the β haemolytic Streptococcus group A, and it is currently thought prudent to search for this in any new ulceration [26] and to treat vigorously, whether or not clinical infection is apparent.

Much still needs to be done before the exact nature of the relationship between bacteria and wound healing is fully understood. It is clear, however, that dressing protocols which attempt to remove bacteria from chronic wounds are not only likely to be unsuccessful, but are also unnecessary. Use of the newer occlusive dressings, and minimum disruption to the wound will lead to faster healing. They will also have the benefit of being cheaper and of reducing the need for painful dressing removals, thus having the added benefit of increasing patient satisfaction.

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Pathogenesis of Wound Infections

T. Wadström and Å. Ljungh

Introduction

The introduction of antibiotics has caused a decline in surgical infections, and gram-negative organisms replaced *Staphylococcus aureus* as the major nosocomial pathogen during the 1960s and 1970s in the industrialized world. However, during the last decade we have encountered a revival of *S. aureus* as a major wound pathogen, partly because of the emergence of multiple antibiotic resistant strains and methicillin-resistant *S. aureus* (MRSA). In biomaterial-associated infections, i.e., infections in intravascular and intraperitoneal catheters, joint and vascular prostheses, coagulase-negative staphylococci, mainly *S. epidermidis*, are the most important pathogens [9, 106]. They may also cause wound infections, and multiple antibiotic resistant strains are commonly isolated in hospital environments. In burn wound infections *Pseudomonas aeruginosa* is well established as a primary pathogen, as is the fungus *Candida albicans* [33]. Groups A, C and G streptococci have, throughout the decades, been the primary pathogens of different kinds of wound infections.

Classification of Wounds

Surgical wounds are by tradition classified as clean or contaminated depending on the initial load of bacterial contamination. Most important is, however, that a poor blood supply and the presence of nonviable tissue in a wound will permit colonizing aerobic and anaerobic bacteria to proliferate and establish an infection.

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Source of Infection

Group A streptococci never form part of the normal microbial flora but may be transmitted from the throat or infected wounds of the patient or from other persons. *S. aureus*, *S. epidermidis* and other coagulase-negative staphylococcal species belong to the normal human flora of the skin and nares, which are sources of infection from the patient and from personnel and other contacts. *C. albicans* may form part of the normal skin flora. *P.aeruginosa* is commonly isolated in the environment, including suction flasks and water sinks. The intestinal microbial flora with both aerobic and anaerobic organisms is a source of infection of abdominal and mainly distal wounds. None of these organisms can cause infection of intact skin.

Pathogenesis of Wound Infections

By definition, in a wound, the first line of defense, i.e., the normal skin lining, is broken. In burn wounds, diabetic and arteriosclerotic wounds, and in immunocompromised patients the second line of host defense mechanisms is severely impaired. These are dealt with elsewhere in this volume, and only those which are the target for microbial factors will be highlighted.

All wounds become, with time, colonized by bacteria from the patient's own microbial flora, or from other persons or the environment. Microbes have different growth requirements but, in general, nutrients are available in wounds. *P. aeruginosa* and *C. albicans* cannot grow under anaerobic conditions, whereas streptococci, staphylococci and many species in the intestinal microflora are facultatively anaerobic, i.e., they can grow in both aerobic and anærobic environments. The vast majority of bacteria in the intestine are strictly anærobic microorganisms.

These microbes produce enzymes and toxins which: (a) enable rapid spread in the tissue, (b) degrade tissue to obtain nutrients, or (c) inactivate host defense mechanisms to ensure survival and proliferation in the tissue. However, mere enumeration of bacteria in a wound is not equal to an established wound infection (see below). The next step for the microbe to initiate an infection is adhesion to underlying tissues. In all wounds extracellular matrix (ECM) proteins like fibronectin, vitronectin, collagens and laminin are exposed together with glycosaminoglycans like dermatan sulfate and heparan sulfate. Microbes can adhere to these ECM components by hydrophobic and charge interactions and by receptor-like specific binding. Binding to these proteins and proteoglycans has been proposed as a first step of tissue colonization [101, 102, 108], and the microbial agents discussed here express a number of surface structures which mediate this process. By binding to these ECM components the microbes appear to be more protected from host defense mechanisms and can proliferate and exert local tissue damage with clinical symptoms of infection.

Microbial Spreading Factors

Staphylococcal and streptococcal cytolytic toxins were purified and most extensively studied for their mode of action in the 1970s. The extracellular protein profile of group A Streptococcus is presented in Table 1. Lysis of erythrocytes by haemolysins provides the organism with an iron source. Hyaluronidase is an important tissue spreading factor, and streptokinase degrades fibrin and enhances tissue spread. Interestingly, corresponding toxins and enzymes can also be produced by *S. aureus* (Table 2). In addi-

Table 1. Extracellular proteins produced by group A streptococci

Toxins
Streptolysin O and S
Erythrogenic toxins[a]
Nephritogenic toxin
Spreading factors
Hyaluronidase
Streptokinase
Streptodornase
Proteases[b]
Deoxyribonuclease and ribonuclease

[a] Three antigenic variants have been isolated.
[b] Proteases with broad and narrow substrate specificity.

Table 2. Extracellular proteins of staphylococci

Toxins
α-Toxin
β-Toxin[a]
τ-Toxin I[a]
δ-Toxin
Toxic shock syndrome toxin-1
Enterotoxin A, B, C_1, C_2, D, E[a]
Coagulase
Spreading factors
Hyaluronate lyase
Staphylokinase[a]
Proteases (at least three)[b]
Lipases
Deoxyribonuclease and ribonuclease

[a] Not produced by coagulase-negative staphylococci.
[b] The most recently recognized protease, the second component of τ-toxin is a protease, but not a spreading factor.

tion, *S. aureus* produces coagulase and a few other factors which induce fibrin formation and enhance abscess formation and well localized infections. Whether *S. aureus* will induce a wound infection or an abscess depends on which enzymes and toxins are produced locally and is strain dependent [43].

Very little is known about how the wound environment affects production of various extracellular proteins. Staphylococcal α-toxin has been detected in wound fluid [20], and antibodies to α-toxin were shown to protect mice against lethal experimental infections [8]. However, it is well established that streptococcal dermal infections induce formation of anti-nuclease (anti-DNAse) antibodies to a larger extent than anti-streptolysin (AST) antibodies [29]. In *S. aureus* two superregulatory genes have been identified: (1) *agr*, which regulates production of protein A and several extracellular proteins [82]; if production of protein A is down-regulated, the production of α-toxin and several enzymes is up-regulated; (2) *hld*, which regulates several other toxins and enzymes [37]. How growth conditions affect the expression of agr and hld genes has not been elucidated [75].

In addition to coagulase, staphylokinase and β-haemolysin have not been detected in coagulase-negative strains [75]; all other toxins and enzymes of *S. aureus* can be produced by coagulase-negative staphylococci (Table 2).

Several other bacterial species which can cause wound infections are known to produce potent haemolysins and tissue-degrading enzymes, e.g., *Clostridium perfringens*, the most important etiological agent of gas gangrene. *Aeromonas* and *Vibrio sp.* can cause serious infections of water-exposed wounds, and produce a wide range of extracellular proteins, including haemolysins [60]. By contrast, *Cl. tetani* never induces local reaction and does not produce tissue-degrading enzymes or toxins, nor does *Bacillus anthracis*.

The dominating extracellular enzymes produced by *C. albicans* and *P. aeruginosa* are proteases with different substrate specificity which degrade ECM proteins such as collagens or elastin [42, 76, 113, 114]. In addition, *C. albicans* strains produce phosphatases and some other enzymes which may be of importance for the organism to obtain nutrients, and *P. aeruginosa* produces a haemolysin with broad cell specificity and detergent-like properties. *P. aeruginosa* also produces exotoxin A, which

Table 3. Immunomodulating toxins produced by wound-associated microbes

Staphylococcal enterotoxin A
Toxic shock syndrome toxin-1
Staphylococcal erythrogenic toxin A and B
Streptococcal erythrogenic toxins
Pseudomonas exotoxin A
Corynebacterium diphtheria toxin

is a potent toxin resembling diphtheria toxin [110, 113] (Table 3). The production of exotoxin A is inhibited by increased levels of iron [110].

Finally, *P. aeruginosa* and *S. aureus* strains produce leukocidin, a cytolytic toxin which only affects leukocytes. *S. aureus* leukocidin was recently shown to induce dermal and vascular necrosis after intradermal injection and was suggested to play a role in cutaneous infections [17].

Microbial Factors and Host Defense Mechanisms

Beneficial Effects

The mere presence of bacteria in a wound attracts leukocytes, macrophages, fibroblasts and other cells which stimulate wound healing and phagocyte bacteria. Several of the bacterial products which are chemotactic have been identified. This set the basis for the proposal by Beebe that $<10^5$ bacteria/g tissue represented colonization and was beneficial, but $>10^5$ bacteria/g tissue indicated that an infection was established [6]. It should be emphasized that the figure was calculated from studies in normal, vital skin. In patients with immunosuppression, arteriosclerosis and poor blood supply, the limit is significantly lower. Furthermore, with group A streptococci there is no threshold number, since also colonization by very low numbers can lead to clinical infection.

Inoculation of living cells of *S. aureus*, but not of coagulase-negative staphylococci or *P. aeruginosa*, stimulated induction of production of collagen hydroxyproline [52, 54]. This effect was attributed to production of staphylococcal enterotoxin A, whereas enterotoxin B, C_1, C_2, D, E and toxic shock syndrome toxin-1 were ineffective. Similarly, inoculation of wounds with *Micrococcus varians*, an apathogenic bacterial species, induced accelerated tissue repair as measured by collagen hydroxyproline, hexosamines, uronic acids, nitrogen, and granulation tissue deoxyribonucleic acid [53]. Staphylococcal lipoteichoic acid (LTA) and probably streptococcal LTA are potent mitogens [1].

Immunomodulating Toxins

Staphylococcal enterotoxin A (SEA), B and E, epidermolytic toxin A and B, and toxic shock syndrome toxin-1 (TSST-1), and δ-toxin are potent mitogens for T_1 cells. SEA and TSST-1 further induce production of tumor necrosis factor (TNF)-α and -β, interleukin-1 (IL-1) and IL-2 [2, 3, 22]. The staphylococcal enterotoxins belong to a family of immunomodulating toxins, together with the streptococcal erythrogenic toxins (Table 3). At low doses these toxins probably will exert a beneficial effect in tissue repair, but at higher doses they will impair host defense mechanisms. Exotoxin A from *P.aeruginosa* and diphtheria toxin are also immunomodulating toxins. They

are potent inhibitors of protein synthesis and highly cytotoxic for a wide variety of cells [110]. The resemblance between these toxins could suggest that they play a role in the pathogenesis of wound infections since *Corynebacterium diphtheriae* is a significant cause of wound infections in tropical/subtropical climates.

Adverse Effects

P. aeruginosa protease and elastin have been shown to inactivate τ-interferon and TNF [78]. Only limited proteolysis was necessary for biological inactivation. Other bacterial products have not been investigated for ability to inactivate cytokines. Nor has the effect of bacterial enzymes and toxins on transforming growth factor-β (TGF-β) and epidermal and fibroblast growth factors (EGF, FGF), which are essential in wound healing, been studied. This may represent an important field for future research.

Most bacterial elastases are serine-or metalloproteases. However, the elastases produced by *S. aureus* and *S. epidermidis*, which differ in molecular weight, are cysteine proteases. Recently, an elastase produced by coagulase-negative strains, was shown to cleave immunoglobulin M and A, fibronectin, fibrinogen and serum albumin [92]. This represents a novel mechanism of inactivation of host factors and may have been overlooked with other bacterial enzymes.

Adhesion to Wounded Tissue

Microbial adhesion to wounded tissues is mediated by hydrophobic and charge interactions, and/or specific binding to proteins and glycosaminoglycans exposed in the wound.

Cell Surface Hydrophobicity

A large number of wound pathogens have been shown commonly to express cell surface hydrophobicity (CSH), such as streptococci, *S. aureus* and *Candida* species [27, 87, 103]. In group A streptococci M proteins and LTA contribute to the CSH [107]. The growth conditions profoundly influence the expression of CSH by bacteria and fungi [27, 87, 103]. The majority of bacteria recovered from wounds express high or moderate CSH, and few such pathogens are encapsulated [58]. The polysaccharide capsule of bacteria such as *Klebsiella pneumoniae*, pneumococci and certain strains of *S. aureus* confers low CSH to the pathogen [40]. However, measurement of the CSH of microbes grown under in vivo-like conditions has yet to be performed [47].

The successful treatment of wound infections by a hydrophobic dressing, Sorbact 10^5, supports the finding that microbes growing in wounds commonly express a hydrophobic cell surface [24, 63, 105]. Proteolytic enzyme treatment of wound pathogens has been shown to reduce cell surface hydrophobicity, indicating that surface proteins confer the CSH [58, 87]. It is then reasonable to assume that proteases produced in the wound will decrease the CSH of microbes growing in the wound. This assumption is supported by the clinical observation that treatment of wounds with hydrophobic dressings is beneficial during an initial, limited time, but is less efficient for long-time treatment of inflammation of chronic wounds.

Staphylococci and streptococci grown in the presence of subinhibitory concentrations of antibiotics were less hydrophobic than cells grown in the absence of antibiotics and expressed a more negative net surface charge [100]. Bacteria and eukaryotic cells to which they attach are both negatively charged, so a repulsion between microbe and cell has to be overcome in the initial adhesion process. Most often this is overcome by hydrophobic interactions [31].

Microbial Binding to Extracellular Matrix Proteins

The discovery in 1978 by Kuusela that *S. aureus* strains bind fibronectin [51] initiated research to explore binding of fibronectin and other ECM proteins, e.g., collagens, laminin and vitronectin, by various pathogenic bacteria, fungi and parasites. Subsequently, microbial binding to ECM proteins has been suggested to represent an important mechanism of tissue adherence [101, 102, 104, 108]. The protein binding profile of *S. aureus* strains has been most extensively studied (Table 4).

Table 4. Cell surface proteins of *Staphylococcus aureus* mediate binding to some proteins and glycosaminoglycans

Fibrinogen (three different proteins)
Fibronectin (at least two proteins)[a]
Laminin[a]
Collagens[a]
Vitronectin[a]
Thrombospondin
Elastin
Heparan sulfate[b]

[a] Proteins mediating binding to these proteins have been detected also on coagulase-negative staphylococci but appear to differ from those produced by *S. aureus*.
[b] Also other glycosaminoglycans [55].

S.aureus strains bind to the 29 kDa NH_2-terminal of fibronectin, and not to the cell attachment domain which recognizes the RGD sequence [68]. One fibronectin binding protein was purified from *S. aureus* and identified as a high molecular weight protein degradable into smaller fibronectin binding fragments [88]. At least two genes encoding fibronectin binding proteins have been isolated, FnBP A and B [41]. Coagulase-negative strains have been shown to express binding of several of these proteins as well, but the fibronectin, collagen and vitronectin binding proteins of coagulase-negative strains differ from those of *S. aureus* strains from a physicochemical point of view, and coagulase-negative strains expressing fibronectin binding do not hybridize with the gene encoding FnBP A [79, 80]. Thus, we face the existence of a family of staphylococcal fibronectin binding proteins.

Streptococci of groups A, C and G commonly bind fibronectin, whereas groups B and D strains rarely do [93, 97]. A new cell surface fibronectin binding protein, protein F, has recently been identified [26]. Pancholi and Fischetti described a 35.8 kDa surface protein of group A streptococci mediating fibronectin binding [77]. This protein is distinct from protein F. In addition, LTA and M protein have been implicated in fibronectin binding by *S. pyogenes* [90]. It thus appears that *S. pyogenes* may have evolved a number of different mechanisms to interact with fibronectin.

Fibronectin is a predominant ECM protein in wound healing and is certainly the most extensively studied. Degraded fibronectin and vitronectin were detected in chronic wound fluids and shown to inhibit cell adhesion [25]. The 170–210 kDa gelatin binding fragments of fibronectin were shown to retain the opsonic activity for macrophages [86], although to a lesser extent than intact fibronectin. During wound healing processes, a fetal fibronectin appears in wounds [21] which is more glycosylated than adult fibronectin and is more resistant to proteolytic enzymes [16].

These findings support earlier studies that microbial binding of fibronectin is an important initial tissue adhesive mechanism but of little importance during a wound healing process. Supporting this are also reports that treatment with fibronectin-substituted gels enhances wound healing in a wound infection model in young pigs [59]. The number of bacteria recovered from fibronectin-treated wounds decreased significantly (Fig.1). The effect of exogenous fibronectin can be dual: (1) to bind microbes which express fibronectin binding proteins, and hence immobilize these microbes, and (2) to stimulate wound healing, as shown in treatment of, e.g., corneal ulcers [73].

Cell surface protein A of *S. aureus* was once suggested to mediate fibronectin binding but later studies have clearly shown that this is not the case. Protein A, however, mediates binding of immunoglobulins via the Fc portion. Since studies on the regulation of staphylococcal proteins by the *agr* gene have shown that up-regulation of synthesis of extracellular toxins and enzymes, important to establish an infection, is accompanied by

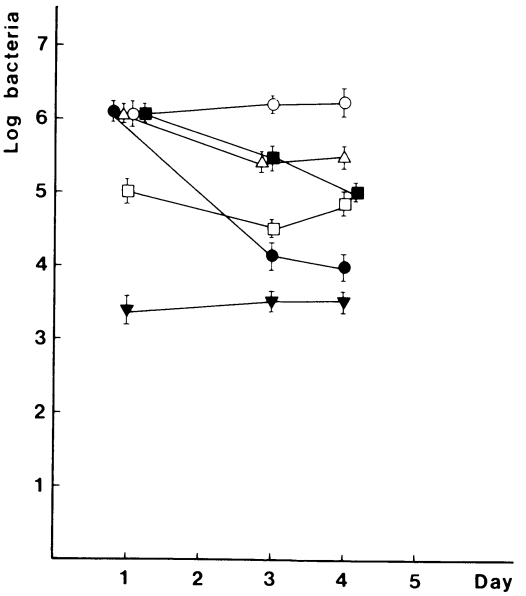

Fig. 1. Treatment of experimental *Staphylococcus aureus* infections in pigs with fibronectin-substituted gels. Pigs were treated during days 1–5 with daily mechanical cleansing and changing of dressings. Quantitative bacterial cultures were taken on days 1, 3 and 4. The wounds were treated with: fibronectin-substituted Sepharose 6B (●), unsubstituted Sepharose 6B (○), fibronectin-substituted DEAE Sephadex A25 (■), unsubstituted DEAE Sephadex 25 (□), Dekylagarose C14B (△), and Dextranomer (▼). Results are expressed as mean log bacteria recovered from triplicate wounds. (From [59])

down-regulation of protein A [82], it is likely that protein A is of less importance in the initial pathogenesis of staphylococcal wound infections.

S. aureus have further been shown to bind soluble collagens, laminin, and vitronectin [15, 34, 62, 95] and these proteins immobilized on latex beads [79, 81].

Group A streptococci bind collagen type IV [50], albumin, immunoglobulin G (IgG), fibrinogen and β_2-microglobulin [10]. The affinity of streptococcal protein G for the Fc portion of IgG is even higher than that of staphylococcal protein A [116]. The presence of M protein did not influence the fibrinogen binding but the fibrinogen mediating structure on streptococcal strains has not been identified [10]. The receptor for β_2-macroglobulin was shown to be located on the M protein of group A streptococci [10]. Most recently, group A and C streptococci were shown to bind vitronectin [49].

Both germ tubes and yeast cells of *C. albicans* also bind a number of these ECM proteins, e.g., fibronectin, vitronectin, laminin, fibrinogen, collagen, and serum complement factors (iC3b) [12–14, 35, 36, 91].

Escherichia coli can express binding of fibronectin, various collagens, laminin and vitronectin [15, 56, 57, 94], and *Aeromonas hydrophila* binds fibronectin, fibrinogen and collagens [4, 5]. Apart from mediating cell attachment, vitronectin binding by microbial pathogens may interfere with complement activation by virtue of interfering with the vitronectin binding of complement factors C5–C9.

Fibrinogen binding by *S. aureus* was earlier called "clumping factor" or "cell-bound coagulase."At present three fibrinogen binding proteins have been identified, with molecular weights of 87, 60 and 19 kDa [11]. Of these, the 87 kDa protein has coagulase activity, and the 60 kDa protein also binds prothrombin. Fibrinogen binding is of great importance in establishing localized infections and abscesses by inducing a fibrin barrier.

S. aureus was recently shown to bind heparan sulfate and heparin [55], which opens up new perspectives of tissue adherence and interference with host factors. Also, expression of ECM binding surface proteins are known to be influenced by growth conditions [41, 56, 57]. Finally, some microbes bind lactoferrin, e.g., *S. aureus* [69]. Lactoferrin binding may influence the level of available iron in wounds, which in turn affects production of some extracellular toxins and enzymes, like the *Pseudomonas* exotoxin A [110].

Successful wound pathogens thus express a number of cell surface proteins which enable the pathogen to colonize in tissue lesions. Furthermore, a number of interactions between these proteins have been characterized, such as collagen-collagen, fibronectin-collagen, and collagen-vitronectin bindings, and *S. aureus* vitronectin binding was enhanced in the presence of collagen [81].

Complications of Wound Infections

After successful colonization of underlying tissues the infection may spread locally or systemically. One serious complication of wound infections is when the organisms invade the blood stream. Necrotizing fasciitis and gas gangrene are well-known complications of anaerobic wound infections. Similar symptoms may also arise from infections with group A streptococci and *Aeromonas* sp., and gas-producing *E. coli*, respectively. Absorption of endotoxin, other pyrogenic substances, and of toxins such as TSST-1 and other staphylococcal and streptococcal toxins may cause the serious symptoms. It is to be emphasized that TSS may develop through adsorption of TSST-1 toxin from *S. aureus* growing without causing signs of a local infection. It appears that focal growth in the presence of low magnesium levels enhances toxin production [44, 98]. These conditions can be produced, e.g., by absorption of magnesium from cellulose fibers such as found in tampons. The majority of nonmenstrual TSS cases have been described in tamponated wounds in otorhinolaryngeology, and in occluded surgical wounds [7].

Late sequelae of group A streptococcal infections, i.e., glomerulonephritis and rheumatic fever, may arise from localized streptococcal infections and from tonsillitis.

Methods to Assess Wound Infection

The threshold of 10^5 bacteria/g tissue for colonization-infection was determined by biopsies. This is indeed a method which aims at distinguishing surface localized bacteria from invading bacteria, in which the latter should represent infection (Table 5). However, the method is painful, may in itself delay wound healing, requires multiple biopsies to overcome the variability of the method [89, 96], and generates both false-negative and positive results [83]. Williamson and Kligman introduced a noninvasive method, scrubbing [112]. After mechanical removal of loosely surface-adherent bacteria, a cylinder is placed on the wound and 1 ml of buffer with detergent is inoculated. A rubber policeman or similar nondamaging plastic device is used to gently mechanically loosen surface bacteria for 1 min. Surface bacteria are aspirated in the inoculated fluid and quantitated. The addition of detergent should overcome hydrophobic bondings between bacteria and tissue and bacteria-bacteria. While this is still a valid method, simplifications have been developed, e.g., the contact plate [111] and the velvet contact plate by Raahave, which permits detection of aerobic and anaerobic bacteria [85].

In catheter-associated infections, the semiquantitative rolling method of extirpated catheter tips, described by Maki [64], has gained widespread acceptance. If the number of bacteria cultured from the tip exceeded 15 colony-forming units (CFU) per plate the risk of catheter-associated septicaemia increased significantly. This method was recently applied to surgical wounds by rolling a catheter tip in the wound and then on an agar plate [115]. The authors studied 53 wounds by daily cultures. From 44 wounds, <15 CFU were recovered, and these wounds did not give clinical infection. By contrast, from nine wounds >15 CFU grew from the whole tip (1.5 cm), and these developed purulent discharge.

Table 5. Methods to quantitate bacteria in wounds

Method	Reference
Homogenized tissue biopsies	[54, 89, 96]
Surface scrubbing using a buffer with detergent	[112]
Contact plate	[111]
Velvet contact plate	[85]
Rolling of a catheter tip in the wound	[115]

Fewer methods have been developed to measure wound healing objectively. One promising method is the computerized wound image analysis [19] described elsewhere in this volume. Areas of black and yellow necrosis, and red granulation tissue are measured by a white-gray-black scale. This appears to be more discriminative than methods such as the heat camera and may be a valuable tool to evaluate new treatment regimens.

Animal Models for Wound Infections

Numerous animal models have been used to study wound infections [47, 48]. It was shown early that normal skin is fairly resistant to staphylococcal infections, but that the introduction of a foreign material, e.g., cotton dust or a suture, significantly reduced the required infectious dose [74]. Furthermore, rodents are quite resistant to staphylococcal infections, and pigs to streptococcal infections [59], whereas pigs are highly susceptible to *Staphylococcus hyicus* infections, which commonly progress into septicemia [96]. This reflects the fact that there are multiple host factors involved in the development of wound infections. To overcome this limitation, different approaches have used foreign materials to induce staphylococcal infection after occlusive dressings, thermal injury or systemic depression of host defense mechanism (e.g., by corticosteroids or cytostatic agents).

To be able to evaluate new treatment strategies to prevent or treat wound infections, we certainly need standardized animal models for these kinds of infections.

New Treatments of Wounds in Relation to Microbes

Dextran polymers (Debrisan, Pharmacia, Uppsala, Sweden) have been applied to wounds to allow flow of wound fluid containing bacteria into the covering surface layer. We took advantage of this finding and replaced dextran by a polymer which permitted both wound fluid flow and binding of bacteria and bacterial products. The resultant hydrophobic wound dressing Sorbact 10^5 (Cyanamid, Sundbyberg, Sweden) was shown to enhance wound healing in experimental burn infections in pigs [105] and in patients [24, 63].

A number of s.c. occlusive wound dressings were introduced during the 1980s [30]. These provide a moist environment, enhance wound healing, and prevent wounds from secondary colonization by environmental bacteria [29, 65, 66]. The dressing material influences the proliferation of different bacterial species and the barrier function [66]. Bacteria may proliferate underneath the occlusive dressing [45, 61, 65], so this kind of dressing should be used with caution in contaminated wounds. Sugar paste dressings are likewise based on an adsorptive principle, but the high osmosis created in the sugar environment may inhibit bacterial proliferation [67].

Fibronectin and fibrinogen are well known as substrates for epidermal cell migration during wound closure [18]. The beneficial effect of exogenously administered fibronectin was shown in eye infections [73, 99], but fibronectin-substituted matrices may exert their beneficial effect by binding fibronectin-binding bacteria and by providing a source of exogenous fibronectin [59]. Furthermore, an increased precipitation of fibronectin and fibrin(ogen) was observed during the use of semiocclusive dressings [38]. Along these lines are also recent clinical trials with collagen-substituted dressings. Growth factors such as TGF-β stimulate wound healing and modulate extracellular matrix gene expression [28, 84]. So far, growth factors have not been shown to be inactivated by bacterial products, and clinical experience with wound infections treated with regimens including growth factors are awaited with great interest. Hyaluronic acid, exogenously administered, was shown to enhance wound healing [46]. The mechanism for this beneficial action is so far unknown.

The mechanical cleansing of wounds and surgical debridement are still fundamental in wound care. However, a number of enzymes have been employed as adjuncts to degrade necrotic tissue and may be quite useful in the initial phase of wound infections. One of the most recently introduced enzymes is the krill enzyme mixture from arctic krill [109].

The recent findings that protease inhibitors and immunoglobulins prevent systemic spread of *C. albicans* and *P. aeruginosa* from burn infections open up new perspectives for treatment of wound infections in immunocompromised or burn patients [32, 70–72]. It may also suggest that proteolytic inactivation of host defense mechanisms is more common than envisioned [71, 92].

Early grafting of burn wounds and late grafting of leg ulcers and decubitus ulcers represent new treatment schedules in which the threshold of 10^5 bacteria/g tissue is probably significantly lower.

Conclusions

Only a few studies have been performed on bacteria grown under in vivo-like conditions. A number of methods have been developed in which microbes are inoculated in tissue cages, plastic cylinders or silicone tubing implanted in the peritoneum (see [47]). These will be useful tools to study the expression of presumptive virulence factors by microbial agents during growth. Also, the construction of genetically defined mutants which lack single virulence factors [23, 39] will be important to study in different animal models to increase our understanding of the pathogenesis of wound infections.

Thorough elucidation of the pathogenesis of wound infections, including exploring how pathogens interact with ECM and growth factors, is of great

importance in view of the introduction of new concepts of wound treatments involving their exogenous administration.

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Interaction of Bacteria with Host Defense Cells: Mechanisms of Burn Wound Sepsis

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The Burn Trauma

Among many functions, the skin serves as an effective mechanical and physicochemical barrier against most microorganisms present in our environment. Whenever this protective barrier is impaired, usually by trauma or other destructive influences, an immediate and predictable response will occur, i.e., inflammation. The typical signs of the immediate inflammatory response, redness, heat and swelling, and the clinical signs of local vasodilation or leukocyte accumulation at the sites of injury are obligatory and important mechanisms of the host defense system to attack invading microorganisms and to repair the affected tissue if the extension of the skin damage is locally restricted. Obviously, this sequence of events does not occur when extended and deep burns destroy the skin.

Severe burns are characterized by the loss of the protective skin and the occurrence of extended open wounds. The burn wounds provide a vast area for the entry of microorganisms. Burn injuries still remain one of the most life-threatening traumata (Pruitt 1986). Burn victims commonly develop microbial colonization. These patients are at high risk that bacterial colonization will turn into bacterial invasion; this may then lead to burn wound sepsis with a resulting multiorgan failure (Deitch 1992; Marshall and Sweeney 1990). Epidemiological data clearly demonstrate that burn wound sepsis and consecutive multiple organ failure are predominant causes of late deaths after major thermal trauma (Cerra 1991; Winkler et al. 1987, 1989). The late morbidity and mortality as the consequences of septic complications are thought to be the result of an inadequate host immune response. This commonly leads to a depressed immunological reactivity which includes impairment of both the nonspecific and the specific immune system (Arturson 1985; Bone et al. 1989; Shires 1991).

Severe burn patients represent the most impressive group for studying a suddenly occurring trauma-induced alteration of the host defense system. In

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addition, the underlying biochemical processes and the inter- and subcellular regulatory circuits are currently being intensively investigated.

Posttraumatic Alterations of the Immune System

Our studies were directed to analyzing the influence of microbial pathogenicity factors on the cellular function in severely burned patients, e.g., the reactivity of peripheral blood granulocytes (neutrophils, basophils), lymphocytes (T and B cells), and monocytes. Recent studies have provided evidence that microbial pathogenicity is due to multiple factors such as adherence, chemotaxis, invasion, resistance against host defense mechanisms, and the production of a variety of toxins (König et al. 1990a, 1992; Schlüter and König 1990). During microbial colonization and invasion, the synthesis and secretion of microbial toxins seems to be important for bacterial survival, growth, and persistence in the host. Toxins also exert profound effects on host defense mechanisms (Bremm et al. 1987; Ventur et al. 1990; König et al. 1991).

The first cellular defense line against colonizing and invading bacteria is mainly represented by neutrophil granulocytes. It has been shown that the neutrophil responds to different signals in its environment and produces a variety of lipid mediators and cytokines (König et al. 1990b).

Granulocyte Dysfunction

One of the early responses after granulocyte activation is the release of biologically active mediators such as leukotrienes. Among these leukotriene (LT) B_4 exerts potent biological activities, modulates the induction of inflammatory processes and is involved in immunoregulation (König et al. 1990b). LTB_4 is highly chemotactic for other phagocytes; it serves as a positive signal for lymphocyte functions (Yamaoka et al. 1989).

We studied the capacity of neutrophils from severely burned patients to produce LTB_4 by reverse phase-HPLC analysis. The granuloctes from burn patients showed a reduced capacity to generate chemotactically active LTB_4 which returned to normal values when the patients recovered from their injuries. In contrast, granulocytes from two patients who finally succumbed to burn wound sepsis showed a pronounced suppression which resulted in cellular anergy (Köller et al. 1988, 1989a,b). Similar data were obtained when histamine release from patients' basophil granulocytes was studied (Bergmann et al. 1989, 1990). In addition, the metabolic conversion of the released lipid mediators was altered. The conversion of LTB_4 to biologically less active metabolites was enhanced in patients' cells (Brom et al. 1987). This was not observed for the metabolism of platelet-activating factor (PAF), which showed decreased activity (Schönfeld et al. 1990). Furthermore,

we noted reduced expression of LTB_4 receptors on these granulocytes, as analyzed by radioligand binding (Brom et al. 1988).

This decreased capacity to generate LTB_4 and to express LTB_4 receptors by the patients' neutrophils preceded or accompanied invasive bacterial colonization, as was confirmed by quantitative bacterial count within respective bioptic tissue (Köller et al. 1988, 1989a).

Studies with clinical isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, which in our patients were the major pathogens in burn wound sepsis (Winkler et al. 1987), provide evidence that cell-bound and soluble pathogenicity factors (hemolysins) induce mediator release from mast cells and granulocytes (Bergmann et al. 1989). Thus, cellular deactivation may occur as a consequence of permanent stimulation in vivo by microbial pathogenicity factors. In addition to the generation of lipid mediators neutrophils also produce a series of cytokines such as interleukin (IL)-1, IL-6, IL-8 or tumor necrosis factor (TNF)-α. IL-8 belongs to the group of small inducible cytokines and is one of the most potent chemotactic cytokines for neutrophils. Thus, similar to LTB_4, IL-8 is necessary for the coordinated recruitment of additional neutrophils to the inflammatory site (Strieter et al. 1992).

As shown in Fig. 1, IL-8 production by neutrophils from patients with sepsis syndrome is clearly decreased in comparison to that of healthy donor cells. These results are obtained with either stimulated or nonstimulated cells. The functional alterations of patients' polymorphonuclear leukocytes (PMNs) are, in part, correlated with the occurrence of immature band cells

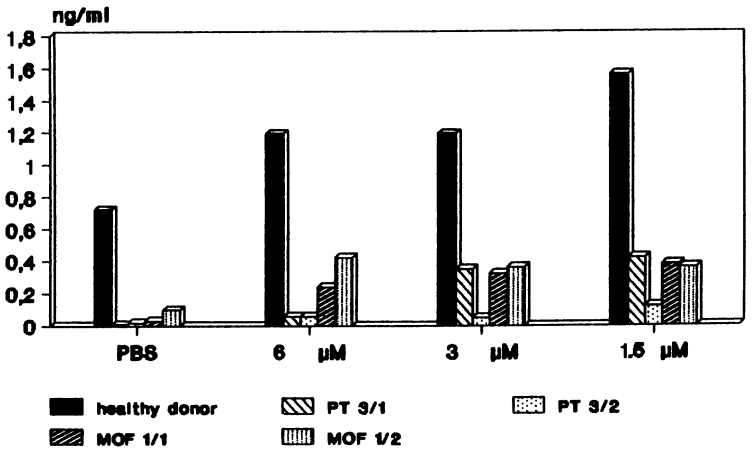

Fig. 1. Interleukin (IL)-8 release from neutrophil granulocytes of patients with septic syndrome. A total of 1×10^7 granulocytes/500 μl buffer were stimulated with buffer (*PBS*) or different concentrations of the calcium ionophore A23187 (*6 μM, 3 μM, 1.5 μM*). Supernatants were analyzed for IL-8 content by ELISA. *Closed bars*, granulocytes from healthy donors; *hatched bars*, granulocytes from patients PT and MOF

in the posttraumatic course (Köller et al. 1989b, 1992). However, additional mechanisms have to be considered as well (Hasslen et al. 1991). As we also observed, the patients' granulocytes were refractory towards both receptor-mediated and nonreceptor-mediated stimuli. Thus, it is clear that elements of the signal transduction pathways must be altered.

It was observed that receptor expression for chemotaxins and the activity of GTP-binding proteins (G-proteins) were modulated. An enhanced GTPase activity in membrane fractions of PMNs from burned patients has been shown, suggesting a short-lasting activation of GTP-binding proteins by rapid metabolism of the GTP-bound, i.e., "switch on," status (Brom et al. 1993).

Additionally, patients' PMNs express a reduced amount of 5-lipoxygenase. This enzyme initiates the conversion of arachidonic acid into LTs (Köller et al. 1992). Whether this reflects a cellular differentiation with regard to the occurrence of immature granulocytes has yet to be elucidated.

Lymphocyte Dysfunction

The posttraumatic changes of granulocyte functions fundamentally reflect impairments of the nonspecific host defense system. However, nonspecific and specific immune functions are intimately connected by intercellular signals such as inflammatory mediators and cytokines. Thus, trauma-induced general immunosuppression also includes specific host defense mechanisms.

T cell proliferation is a prerequisite for the clonal expansion of selected T cells and the coordination of cell-mediated immunity. As was observed, mitogen-induced T cell proliferation was decreased in both survivors and in nonsurvivors. T cell proliferation reached normal values or was even increased in the late phase postburn. T cell proliferation of the nonsurvivor was suppressed shortly before the patient succumbed to sepsis (Schlüter et al. 1991a). Alterations in T cell-expressed surface receptors were also observed. CD25 is the 55 kDa subunit of the heterodimeric IL-2 receptor (IL-2R), which mediates the pleiotropic biological effects of IL-2. Our data showed that spontaneous, IL-2-, or mitogen-induced expression of CD25 by patients' T cells is markedly enhanced in comparison to that of T cells from healthy donors (Schlüter et al. 1991a). This indicates that patients' T cells are highly activated in vivo, which is also suggested by the increase of soluble IL-2R levels in burned patients' sera. However, the concomitant number of CD25+ T cells is significantly reduced. In addition, antigen- or mitogen-induced T cell proliferation is depressed and cannot be fully restored to control values by substitution of exogenous IL-2, providing evidence for alterations of signal transduction elements.

Antibody production from B cells (plasma cells) is essential for host defense against invading bacteria. This is due to the properties of antibodies such as toxin neutralization, opsonization, complement activation, and en-

hancement of bacterial cell lysis. In severely burned patients these functions are impaired. Furthermore, activation, proliferation, and differentiation processes of B cells are altered (Schlüter et al. 1990).

The membrane-bound CD23 activation antigen appears at early stages of B cell maturation and is rapidly lost after isotype switching by proteolytic cleavage into its soluble form (sCD23), which has growth promoting activities on B cells. It was demonstrated by Schlüter et al. (1990) that cytokine (IL-4, IL-2)-induced CD23 expression was decreased during the second to fourth week postburn, although the absolute B cell numbers were within normal range. In parallel, the release of sCD23 was reduced. These data indicate an impairment of B cell activation towards T cell derived helper signals. The mitogen-induced polyclonal synthesis of immunoglobulins was decreased, especially during the second, third, and fourth week postburn (Schlüter et al. 1990). However, evidence was obtained that also B cell differentiation into Ig-secreting plasma cells is impaired after major thermal trauma.

Excessive Cytokine Release

Cytokines such as IL-6 and TNF-α are responsible for many effects observed during sepsis and the sepsis syndrome and account in part for altered lymphocyte functions (Akira et al. 1990). Under physiological conditions IL-6 or TNF-α were clearly suppressed. During the course of sepsis high levels of these cytokines were measured in patients' plasma (Takayama et al. 1990). Thus, the adverse host responses leading to the sepsis syndrome may be mediated by high systemic concentrations of TNF-α or IL-6 over a prolonged time period. Extremely high amounts of IL-6 were detected in sera of severely burned patients (Takayama et al. 1990; Guo et al. 1990). A positive correlation between high IL-6 levels and mortality was obtained. Almost all of the patients with high plasma IL-6 values did not survive, whereas the IL-6 level in sera of survivors decreased continously (Schlüter et al. 1991b; Marano et al. 1990). Additionally, the spontaneous expression of specific mRNA for IL-6 was enhanced in mononuclear cells of burned patients as was measured by RNase protection analysis. It may be suggested that nuclear DNA-binding proteins involved in IL-6 gene expression might be continuously activated by as yet unknown mechanisms. The overproduction of IL-6 may not simply explain the multitude of immunological alterations during the sepsis syndrome. Certainly, cytokines with similar and overlapping biological functions, e.g., IL-1 or TNF-α, contribute to the sepsis syndrome (Akira et al. 1990). The biological effects of TNF are mediated by two different TNF-α receptors, R1 and R2, expressed on the majority of all TNF-sensitive cell types. They differ in molecular weight and in the induction of the TNF response (Tartaglia and Goeddel 1992). The extracellular parts of these receptors are shed into the circulation after

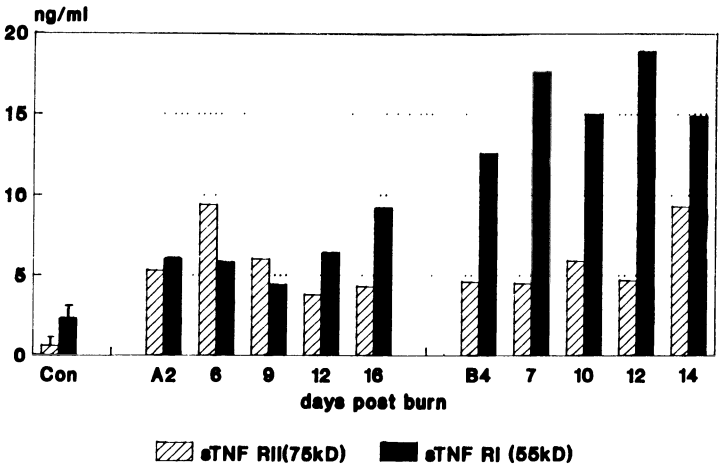

Fig. 2. Soluble tumor necrosis factor (sTNF) receptors (*RI and RII*) in the sera of severely burned patients. TNF receptors were measured by ELISA. *Closed bars*, R1; *open bars*, R2; *Con*, granulocytes from a healthy donor; *A*, burn patient, female, 40 years old, 51% TBSA, inhalation injury; *B*, burn patient, male, 50 years old, 75% TBSA, sepsis syndrome after day 7 posttrauma

proteolytic cleavage. These soluble TNF receptors are able to bind soluble TNF (Dayer 1991). As is shown in Fig. 2 in the sera of severely burned patients enhanced concentrations of either soluble type 1 or type 2 receptor were observed. These elevated soluble receptors could counteract overwhelming TNF-α released into the circulation. The binding of TNF by these molecules may decrease the pathophysiological effects of TNF by acting as an extracellular protective system comparable to the elevation of acute phase proteins within the circulation.

Heat Shock Proteins

In all cells adverse physiological conditions lead to the expression of specific but highly conserved subsets of cell proteins known as heat shock proteins (HSPs) or stress proteins (Dubois 1989; Morimoto 1991).

The heat shock response is induced not only during hyperthermia but also during infection and inflammatory processes (Köller and König 1991; Köller et al. 1989a,b). As we observed, a variety of bacterial toxins induce HSPs (Hensler et al. 1991). This process may be biochemically mediated by distinct lipid mediators and cytokines (Köller et al. 1993). We have demonstrated that IL-6 and TNF-α arëpotent activators of HSPs, as determined by Western blotting using anti-HSP72. IL-6 is a potent inducer of HSP72 in mononuclear leukocytes whereas TNF-α induced pronounced HSP72 expres-

sion in PMNs. Thus, an increased HSP synthesis protects cells from noxious molecules and bacterial toxins (Skowyra et al. 1990). As a consequence, HSP synthesis may enable cells to resist higher and normally lethal toxin concentrations. These results were obtained experimentally by analyzing the effect of the staphylococcal toxin leukocidin (Köller et al. 1993).

Conclusion

The acquired postburn alterations of the host defense system are not restricted to a single cell type or cellular function, but rather involve virtually all cellular components in a distinct manner. The interaction of microorganisms and microbial toxins – either bacterial bound or soluble – with the cells of the host defense system has provided new insights into the pathophysiology of microbial infections. Multiple factors such as microbial colonization and bacterial endo- and exotoxins modulate cellular functions. The exaggerated mediator release or the paralyzed cell function favors microbial invasion and sepsis. Future therapeutic strategies should be directed to coordinate the inflammatory response in such a way as to achieve normal cellular functions or to up-regulate the impaired host defense.

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Streptococcus pyogenes Induced Necrosis and Ulcers of the Skin: The Possible Role of Streptococcal Exotoxins

M. Buslau and P. Schmidt

Introduction

Infections with β-hemolytic streptococci may be harmful to the skin in different ways. Milder forms may cause a contagious superficial pyogenic infection of the skin characterized by blisters and erosions (impetigo contagiosa), an inflammation of the dermis and upper subcutaneous tissue without necrosis (erysipelas), or a pyogenic infection of the skin characterized by the formation of adherent crusts, beneath which ulceration occurs (ecthyma). Acute ulcers, often on the legs and feet, are one of the more frequent forms of streptococcal pyoderma under humid tropical conditions [2]. Chronic ulcers of streptococcal origin may follow insect bites or abrasions. They may persist for many months but heal rapidly with antibiotic treatment [21].

Previously severe forms of streptococcal skin infections (necrotizing erysipelas, necrotizing fasciitis) were among the rarest complications, associated with reduced immunity of the host. However, since the late 1980s, we have observed the emergence of sporadic, severe, group A streptococcus (GAS) infections, reminiscent of those of the early decades of the twentieth century. Their course is rapid, often requires amputation and may be fatal in spite of the immunocompetent status of the host, antibiotic therapy and intensive care medicine. Children may be affected as well [39]. Most commonly, these infections begin at the site of minor even trivial local trauma of the skin or mucous membranes. Clinically these infections are characterized by necrotizing erysipelas, necrotizing fasciitis, necrotizing myositis, bacteremia or a streptococcus-induced toxic shock-like syndrome (toxin-strep-syndrome, strepTSS).

The pathogenesis of streptococcal-induced necrosis and ulcers of the skin is not well understood. Virulence factors of *Streptococcus pyogenes* (Table 1) and possibly synergistic bacterial invaders (e.g., *Staphylococcus*

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Table 1. Possible virulence factors of *Streptococcus pyogenes*

M proteins
C5a peptidase
Immunoglobulin-binding proteins
Hyaluronic acid (capsule)
Fibronectin-binding protein
Erythrogenic toxins
Hemolysins (streptolysin S and O)
Streptokinase and plasmin receptor
Nucleases (NADase, DNase)
Hyaluronidase
Proteinases
Serum opacity factor

aureus), and reduced immunity, deficient nutrition or predisposing diseases of the host have to be considered.

This chapter will summarize the current state of understanding of the pathogenesis of invasive GAS infection as far as the skin and the adjacent tissue are concerned. For a thorough understanding of the clinical picture, epidemiology and the problem of extracutaneous involvement of invasive group A streptococcal infection we call the reader's attention to some of the excellent reviews [24, 26, 28, 50].

Erysipelas and Necrotizing Erysipelas

Erysipelas is a predominantly streptococcal infection of the dermis and and upper subcutaneous tissue. Severe erysipelas can progress to dermal necrosis and uncommonly to fasciitis or myositis. Other possible complications are subcutaneous abscesses, nephritis, and septicemia, which may be fatal especially in infants or debilitated immunosuppressed patients. Local trauma, chronic venous insufficiency and systemic immunodeficiency seem to be important factors. Recurrent streptococcal erysipelas is attributed to lymphatic damage, which, though sometimes initially clinically inapparent, predisposes to further infection and further lymphatic impairment manifesting as lymphoedema.

There are conflicting reports about impaired phagocytic and antimicrobial functions of polymorphonuclear leukocytes (PMNs) in erysipelas [29, 35, 45, 46, 61] Pronounced association between multiple relapses of erysipelas and HLA A2, B5, B12 and Bw35 antigens has been reported and suggests an immunogenetic predisposition [23]. Dochez and Stevens [13] proposed the possibility of a hypersensitivity reaction in erysipelas rather than its occurrence as a function of strain specifity. Immunological investi-

gations have never revealed increased levels of circulating immune complexes, the presence of fixed immune complexes and of complement C3 in the affected skin and the participation of IgE-mediated allergy against *Str. pyogenes* in the pathogenesis of recurrent erysipelas [4, 11, 18].

Due to the difficulty in isolating putative pathogens from erysipelas, it has been suggested that the disease is produced both by a small number of residual bacteria and by fragmented bacterial remnants and amplified by lymphokines that are secreted in response to antigenic challenge [42]. Recently, we investigated cytokine levels in suction blister fluid obtained from lesional skin and the corresponding sera from patients with erysipelas. High levels of interferon (IFN)-γ and interleukin (IL)-2 in suction blister fluids but not in the corresponding sera suggest that IFN-γ and IL-2 are produced locally in lesional skin of erysipelas, most likely by activated T cells. Moreover, we found high levels of soluble IL-2 receptor (IL-2R) in suction blister fluids and in the corresponding sera of patients suffering from erysipelas. Our data support the speculation of an underlying T lymphocyte-dependent hypersensitivity in erysipelas [9]. Whether this pathomechanism may be important for the development of necrotizing erysipelas is unknown.

Future research has also to take into account the role of highly mitogenic ("superantigenic") streptococcal exotoxins [3, 15]. Besides their T cell activating properties, they induce mononuclear cells to synthesize tumor necrosis factor (TNF)-α, IL-3 and IL-6 [26]. We found significant levels of TNF-α, IL-1, IL-6 and IL-8 in the skin of erysipelas patients (Table 2). Only recently, we could demonstrate that human epidermal cells are potent antigen presenting cells for streptococcal exotoxins [10]. Therefore, the skin may serve not only as an important entry point for exotoxin-producing strains, but also as an important target for toxin binding and presentation. Bierhaug [6] reported on a soluble exotoxin produced by β-hemolytic streptococci cultured from lesional skin in erysipelas. This exotoxin was different from the toxin identified in scarlet fever. Current research focuses on

Table 2. Cytokine levels in suction blister fluids of erysipelas

Cytokine	Level (pg/ml)[a]
IL-1β	365
IL-2	336
IL-6	1409
IL-8	2930
TNF-α	52
IFN-γ	2114

IL, interleukin; TNF, tumor necrosis factor; IFN, interferon.

[a] ELISA, mean values of ten patients.

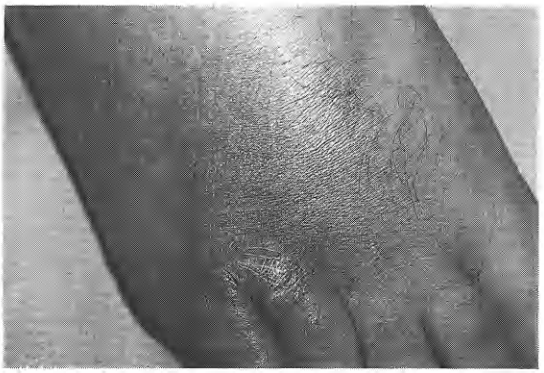

Fig. 1. Bullous erysipelas with petechiae

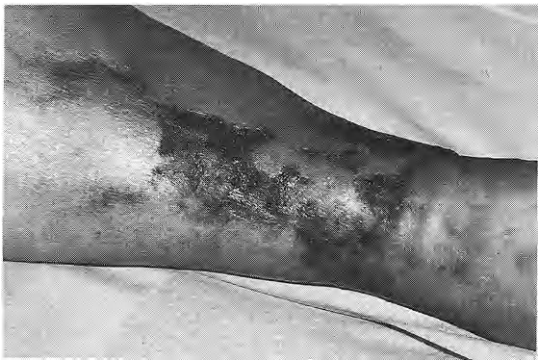

Fig. 2. Erysipelas with superficial hemorrhage and progress to dermal necrosis

the physicochemical and immunological properties of this toxin (Fleischer, personal communication).

Another point of interest is the observation of early circulatory disturbances in erysipelas patients. Unna [56], in his fundamental work on the histopathology of skin diseases, mentioned dermal fibrin thrombosis and clot formation in capillaries, small arteries and lymphatic vessels as an early histopathological sign of erysipelas. Patients suffering from erysipelas sometimes exhibit a dusky blue color of the skin and purpura-like lesions (Fig. 1). We suggest that the increased redness in erysipelas may follow increased blood flow through superficial dilated vessels, resulting from obstruction in deeper vessels causing a diversion or shunting of blood through the more superficial vessels. Partial stasis and congestion may be responsible for the more dusky color in erysipelas. Erysipelas and its necrotizing variants (Figs. 2, 3) probably exhibit a common underlying pathogenesis. The clinical picture seems to depend on the degree of circulatory damage to the skin caused by the occlusion of dermal vessels.

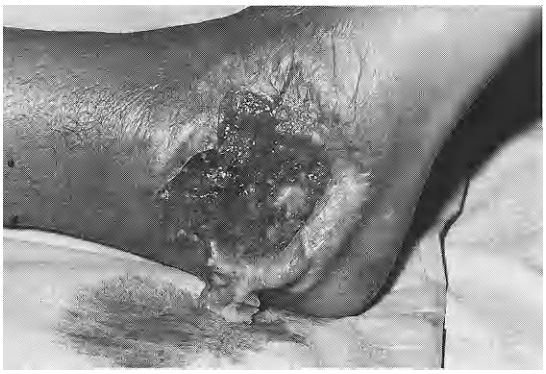

Fig. 3. Erysipelas with early dermal necrosis: development of a deep ulcerative lesion of the skin within 5 days; 33 year old otherwise healthy woman

It is still unknown what causes early thrombus formation in erysipelas but a *Str. pyogenes*-derived exotoxin would be a possible candidate. By immunogold labeling it has been demonstrated that PMNs and thrombocytes exhibit significant binding of erythrogenic toxin A [48]. Therefore it would be important to know whether this is also true for the recently characterized erysipelas toxin.

Necrotizing Fasciitis

Necrotizing fasciitis is a rare but rapidly progressive and often lethal disease. The principal focus of disease lies within the deep dermis, adipose tissue and subcutaneous fascia, where the hallmark of infection is extensive necrosis accompanying cellulitis [30, 31]. Hemolytic streptococci are by far the most frequent causative organisms, though bacteria other than streptococci, such as *Clostridium perfringens*, *C. septicum* and *S. aureus* can produce a similar pathologic process. Hemolytic streptococci are capable of causing the disease independent of copathogens. Of the microaerophilic and true anaerobic streptococci, some species can colonize wounds and are important in some cases of necrotizing fasciitis [30]. Other infections are polymicrobial and usually include an anaerobe [20]. Sometimes the onset may be indolent, giving rise to a false sense of urgency [54]. In men, Fournier's gangrene occurs where there is infection around the lower abdominal fascial plane, with tracking of the infection into the scrotum (Fig. 4). It may be caused by group A streptococci or multiple organisms. In a series of 29 patients with necrotizing fasciitis of the vulva, many of the infections were initially thought to be labial cellulitis. Of 15 women with a delay greater than 48 h between presentation and treatment, 11 died. Twenty of 29 were diabetic, accounting for 11 of the 14 deaths [49].

Fig. 4. Fournier's gangrene in 44 year old man without predisposing underlying disease

Well before the antibiotic era the mortality rate was as low as 20%, whereas subsequent reports written between 1950 and 1980 suggest that mortality rates >50% were common, even with antibiotics and aggressive surgical debridement [50]. Most of the patients described by Rea, Aitken and Quintiliani had predisposing diseases, e.g., diabetes, cachexia, peripheral vascular disease, or cirrhosis, or were receiving corticosteroids [1, 38, 40]. In contrast, young and otherwise healthy people were affected, according to the reports by Meleney and Wilson [32, 60]. Fournier's gangrene may occur abruptly in healthy young men, as originally described by Fournier [17] and recently by others [36, 43], but most of the patients had an underlying immune defect [47, 53]. Fulminant lid necrosis due to group A streptococcus infection may develop in previously healthy children [52].

Necrotizing fasciitis due to β-hemolytic streptococci occurs when the organisms spread through tissue above the fascial plane, causing a thrombosis of vessels that results in gangrene of the dermis and subcutaneous fat. The only apparent difference between the acute and subacute types of necrotizing fasciitis is the higher incidence of thrombi in some blood vessels of patients with acute disease [5]. Microvascular thrombosis may be an important factor in the rapid spread of infection along the fascial plane. Noninflammatory intravascular coagulation of vessels was found at all levels of tissue. The microorganisms had no clear anatomic relationship to the vessels or thrombosis [55]. Bacterial toxins of various origin may trigger a cascade of events characterized by the interaction of inflammatory cells and mediators resulting in microvascular thrombohemorrhagic changes [50].

Disseminated intravascular coagulation may complicate the course of the disease [43].

The pathogenesis of necrotizing fasciitis is also thought to be related to the relatively poor blood supply to the fascia. Animal studies provided some insight into the poor ability of the fascia to clear bacterial inoculum and pointed out the potential vulnerability of this layer to necrotizing fasciitis [44].

The inflammatory infiltrate may consist of neutrophilic granulocytes and lymphocytes [22]. A perivascular, predominantly lymphohistiocytic, infiltrate with plasma cells and lobular and septal panniculitis was noted in the papillary and reticular dermis in one series. The reticular dermis, subcutaneous fat, and fascia were edematous, with a dense inflammatory infiltrate composed of neutrophils [55].

A promising therapeutic regimen would be the combination of heparin, given intravenously in therapeutic doses, and of systemic antibiotics and early debridement and excision of necrotic tissue [22]. Novel approaches targeted toward improving survival have centered on two aspects: the delivery of adequate oxygen and the administration of immunosuppressive agents to diminish immune-mediated microvascular thrombosis.

Hyperbaric oxygenation (HBO) is a useful adjunct therapy in necrotizing infections [7, 41] and other acute and chronic disturbances of wound healing [8]. This effect is based on a combination of factors: (1) facilitation of oxygen diffusion and associated improvement of oxygen supply from the surrounding intact capillary network; (2) reduction of edema following vasoconstriction as a result of the effect of oxygen on the vessels; (3) reduction of infection; (4) improvement in phagocyte function; (5) improvement in collagen synthesis [7]. The structural genes of the vir-regulon of *S. pyogenes*, which controls important virulence factors of *Str. pyogenes* like the antiphagocytic M protein, the complement factor-inactivating C5a peptidase and the immunoglobulin-Fc-binding proteins, are expressed at a high level under anaerobiosis [37]. Thus, the administration of HBO may reduce the virulence of *Str. pyogenes* by raising the tissue oxygen level.

Van der Meer et al. [57] treated a patient with perineal necrotizing fasciitis with high doses of prednisolone. The authors based this unusual treatment on the supposition that inflammatory mediators, rather than the infection per se, were responsible for tissue necrosis [57]. They cite clinical parallels with the Shwartzman phenomenon as support for this hypothesis [33].

Streptococcal Toxic Shock Syndrome

β-Hemolytic streptococci group A (*Str. pyogenes*) may cause a toxic shock-like syndrome, as first described in 1987 [12, 59]. To date more than 800 cases have been published [26]. Predominantly, such patients are between

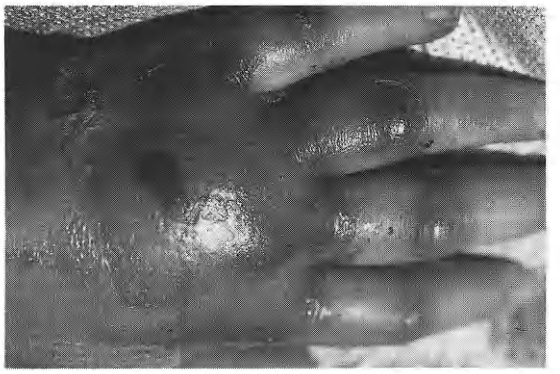

Fig. 5. Toxin-Strep-Syndrome with necrotizing fasciitis of the left arm and hand in a 14 year old previously healthy girl. Course: amputation of the arm, multiorgan failure, death

20 and 50 years old and frequently did not have predisposing underlying diseases. Reports indicate an overall high mortality rate, in spite of aggressive modern treatments, because of multiorgan failure (e.g., renal and respiratory insufficiency, disseminated intravascular coagulation) [51].

The main portals of entry of streptococci are the skin, mucous membranes or lower respiratory tract, although a definite entry point cannot be ascertained in all cases [50]. Most commonly, the streptococcal infection begins at the site of minor local trauma with subsequent development of soft-tissue infection, localized swelling, and erythema. Abrupt and severe pain is the most common initial symptom of strepTSS. The pain most commonly involves an extremity and most of these patients present with deep-soft tissue infection. A characteristic sign is the progression of soft-tissue swelling to formation of vesicles and bullae, which take on a violaceous or bluish coloration. Soft-tissue infection evolves to necrotizing fasciitis or myositis and in these cases surgical debridement, fasciotomy, or amputation is required (Fig. 5).

M protein types 1, 3, 12 and 28 are the most frequently isolated toxins from such patients [50]. M protein contributes to invasiveness through its ability to impede phagocytosis of streptococci by human PMNs [26]. In contrast to previous reports, Fleischer et al. could show that M protein has no superantigenic properties [16].

Studies involving strepTSS have demonstrated that erythrogenic toxins (ETs) were found in the majority of patients [25]. In others, isolated streptococci, negative for one of the "classical" ETs, produced low molecular weight mitogens [19]. More than two thirds of the patients with strepTSS were caused by strains of two related clones [34]. The associated toxins may play a central role in the pathogenesis of strepTSS: ETs induce human mononuclear cells to synthesize not only TNF-α but also IL-1β, IL-2, IL-3, and IL-6, thereby suggesting that TNF-α could mediate the fever, shock,

and tissue injury observed in patients with strepTSS [14, 27]. Direct stimulation of helper T cells can occur in the absence of classic antigen processing by the ability of ET to bind to both the class II major histocompatibility complex of antigen-presenting cells and the V_β region of the T cell receptor [15]. Other biological properties of ETs that in part may play a role in the pathogenesis of strepTSS are: reticuloendothelial system blockade, alteration of the synthesis of IgM or other isotypes, and alteration of cell-mediated immune responses. Moreover, there is an enhancement of host susceptibility to lethal shock by bacterial endotoxins [3].

The interaction between these microbial virulence factors and an immune or nonimmune host determines the epidemiological factors, clinical syndrome, and outcome of infection. It was hypothesized that once invasion of mucosal or epithelial barriers by GAS has occurred in a host without specific antibody to M protein, tissue invasion and possibly bacteremia will occur. If the invading strains also produce ETs, then their expression results in shock, multiorgan failure, and tissue destruction in patients who lack specific antibody to ET produced by the invading strain [50]. If large quantities of toxin have been produced and absorbed to induce shock-producing quantities of cytokines, the mortality may be high even in young, previously healthy patients, who seem to be most susceptible.

Conclusion

There is striking evidence that *Str. pyogenes*-derived exotoxins may play an important role in the pathogenesis of erysipelas, necrotizing fasciitis and strepTSS, possibly via T cell activation, cytokine induction and thrombus formation. The skin is an important entry point for toxin producing strains and an important target organ as well [58]. Inflammation of the skin may result in severe tissue necrosis and ulcer formation. One may speculate whether erysipelas and strepTSS represent different ends of a spectrum of diseases.

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Inhibition of Wound Healing by Topical Anti-microbial Agents

R. Niedner

Introduction

Antimicrobial agents are widely used in the treatment of chronically infected wounds. Although infection inhibits the wound healing process, one should keep in mind that antimicrobial substances will equally inhibit wound healing. This latter effect was studied by treating experimental wounds with different agents.

Wounds

The granulation process can be examined in full-thickness wounds on guinea pigs. For this purpose we used a model, originally developed by Barbara Rudas (1960), to test corticosteroids. We incised the back skin of guinea pigs down to the muscular fascia. We then placed a teflon ring with an inner diameter of 2 cm into the wound, thereby inhibiting wound contraction and epithelialization (Fig. 1). The different wound healing substances were applied to the fascia within the ring and the ring itself was covered by cotton gauze without any occlusion. Some 24 h later, both the substance and the covering material were exchanged. After 5 days, when the granulation tissue had formed, the animals were killed. The granulation tissue was excised down to the muscular fascia, and microscopic sections were made to measure the thickness of the granulation tissue. In total we made ten sections through each histological slice (Fig. 2), resulting in ten values. The mean of these ten values was taken as a single value for the statistical analysis.

The second and third types of wounds were strictly epithelial. We produced blisters with the poison cantharidin or with the suction blister technique (Kiistala 1968). The cantharidin blister is very superficial, leaving the stratum basale of the epidermis intact (Fig. 3). The suction blister is slightly deeper, with a raised stratum basale (Fig. 4). Removal of the

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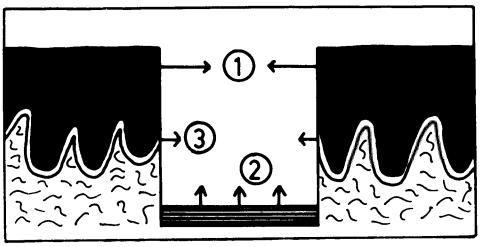

Fig. 1. Full thickness wound: *1*, wound contraction; *2*, granulation process; *3*, epithelization

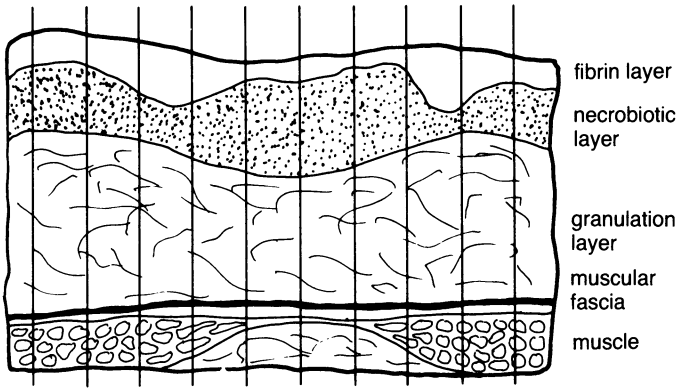

Fig. 2. The different layers of the granulation tissue within the teflon ring. Only the granulation layer was measured. The mean of all ten sections was taken as a single value for statistical analysis

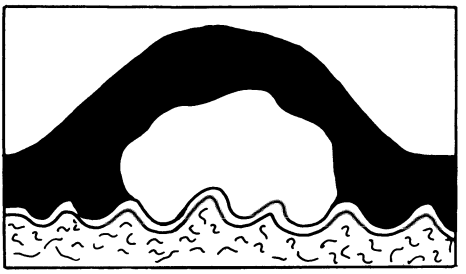

Fig. 3. Suprabasal blister caused by cantharidin

respective blister roof from cantharidin treated skin leaves a suprabasal wound which will be closed by differentiation of the epidermis. In the case of the suction blister a new stratum basale must be formed with an epithelialization from the margin.

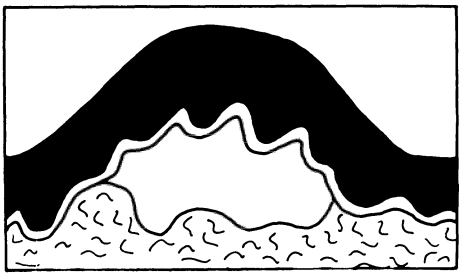

Fig. 4. Subepidermal blister caused by suction technique

Materials

The advantage of locally applied antibiotics is that both their site of application and their site of action are identical. We can therefore achieve very high local drug concentrations at the site of infection, without any systemic side effects (60- to 600-fold minimal inhibition concentration).

We examined dyes, antiseptics, antibiotics and one antimycotic agent. All the substances were used in the current clinical concentrations (Table 1). The triphenylmethane dyes used were gentian violet B, brilliant green and eosin. The antiseptics used were povidone-iodine, chloramine-T, chlorhexidine, silver nitrate, thimerosal, and the brand Dibromol, a mixture of propylene glycol, isopropanol, bromochlorophene and bromhydroxybenzosulfonate. As antibiotics we applied tetracycline, chloramphenicol, gentamycin and neomycin. Finally we investigated the effect of miconazole, an antimycotic agent.

Results

Granulation

Dyes

The triphenylmethane dyes gentian violet B (pyoktanin) and brilliant green profoundly inhibit wound granulation, when used in a concentration of 0.5 g/100 ml. The thickness of the granulation layer (TGL) was 0.12 ± 0.12 mm (gentian violet B), whereas the control had a thickness of 2.18 ± 0.14 mm. The corresponding value for brilliant green was 0.13 ± 0.10 mm, control 2.32 ± 0.15 mm. The differences were statistically highly significant ($p < 0.01$) (Table 1).

Eosin (0.5 g/100 ml) caused no inhibition of the wound healing process. The TGL was 83.0% of the control (Table 1).

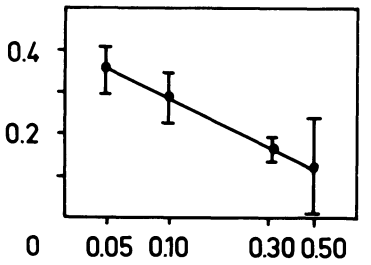

Fig. 5. Suppression of the granulation caused by gentian violet B: *x-axis*, concentration of gentian violet B (g/100 ml); *y-axis*, thickness of the granulation layer (mm)

Due to the great value of gentian violet B in dermatological therapy, especially in the treatment of leg ulcers, we looked for a relationship between the extent of inhibition of wound granulation and the concentration of the dye (Fig. 5). Here one could see a linear relationship between both parameters ($y = 0.037 - 0.244 \log x$), with a correlation coefficient $r = 0.530$, differing significantly from zero ($p < 0.05$). It could be seen that even with the lowest concentration of 0.05 g/100 ml, the reduction of the TGL (0.354 ± 0.061 mm) corresponded to 15.3% of the control.

Antiseptics

All examined antiseptics caused an inhibition of the wound healing process (Table 1). A slight inhibition was seen with povidone-iodine (5 g/100 ml); the TGL was 2.01 ± 0.09 mm, 81.4% (2.47 ± 0.15 mm) of the control. The difference was statistically significant ($p < 0.05$).

Chloramine-T (1.0 g/100 ml) had a TGL of 2.02 ± 0.20 mm, corresponding to 81.8% of the control and silver nitrate had a TGL of 1.71 ± 0.14 mm, 75.0% of the corresponding control. The differences compared to the control were statistically not significant. This was not the case for Dibromol (0.1 ml per wound), a mixture of different antiseptic agents. A decrease of the TGL to 1.74 ± 0.05 mm, 70.5% of the control, was statistically significant ($p < 0.02$) (Table 1).

A significant inhibition could be seen with chlorhexidine in a concentration of 0.5 g/100 ml (Table 1). The TGL was only 31.8% of the control. Due to the marked effect on wound granulation, a dose-effect relationship was estimated (Fig. 6). There it can be seen that even a tenfold dilution inhibited the TGL (43.3% of the control). The mathematical relationship between the concentration and inhibiting effect was $y = -0.26579 \log x - 0.00079$, with a correlation coefficient of $r = 0.575$, which differed significantly from zero ($p < 0.05$).

The negative influence of antiseptics could be examined by the treatment of leg ulcers. During the treatment of leg ulcers with streptokinase and

Table 1. Thickness of the granulation layer after treatment with different substances

Substance	Concentration (g/100 ml)	TGL (mm)	SD (mm)	Percent of control	*p*
Dyes					
Gentamycin	0.6	0.12	0.12	5.5	<0.01
Control	–	2.18	0.14	100.0	
Brilliant green	0.5	0.13	0.10	5.6	<0.01
Control	–	2.32	0.15	100.0	
Eosin	0.5	1.81	0.70	83.0	>0.10
Control	–	2.18	0.14	100.0	
Antiseptics					
Povidone-iodine	5.0	2.01	0.09	81.4	<0.05
Control	–	2.47	0.15	100.0	
Chloramine-T	1.0	2.02	0.20	81.8	> 0.10
Silver nitrate	1.0	1.71	0.14	75.0	>0.10
Dibromol	0.1[a]	1.74	0.05	70.5	<0.02
Chlorhexidine	0.5	0.77	0.21	33.8	<0.01
Control	–	2.28	0.32	100.0	
Antibiotics					
Tetracycline	1.0	0.93	0.12	46.1	<0.01
Control	–	2.01	0.22	100.0	
Gentamycin	0.6	0.82	0.05	51.6	<0.02
Chloramphenicol	1.0	0.92	0.15	58.0	<0.02
Control	–	1.59	0.10	100.0	
Neomycin	0.67	1.05	0.14	63.5	>0.10
Control	–	1.65	0.43	100.0	
Bacitracin	770[b]	2.11	0.20	115.9	>0.10
Control	–	1.82	0.11	100.0	> 0.10
Antimycotics					
Miconazole	1.0	1.59	0.10	102.6	>0.10
Control	–	1.55	0.07	100.0	

TGL, thickness of the granulation layer.
[a] 0.1 ml per wound.
[b] 770 IU/ml.

streptodornase (varidase) we used the original solution with thiomersal as preservative agent in one panel and without it in the other panel. Thereby we could show that the granulation process was markedly inhibited by thiomersal (Niedner 1990) (Fig. 7).

Antibiotics

Antibiotics are frequently used for wound treatment. The strongest inhibitory effect on wound healing could be seen with tetracycline (10 mg/ml)

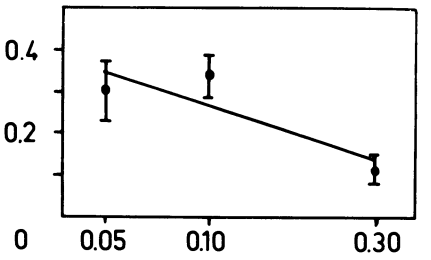

Fig. 6. Suppression of the granulation caused by chlorhexidine: *x-axis*, concentration of chlorhexidine (g/100 ml); *y-axis*, thickness of the granulation layer (mm)

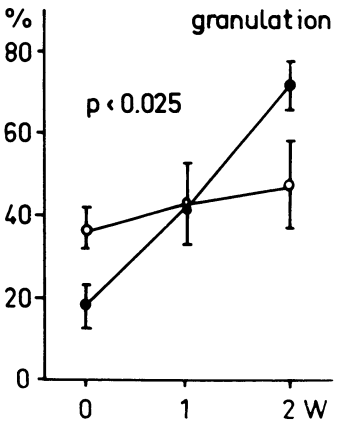

Fig. 7. Enhancement of the granulation process by removing thiomersal from streptokinase and streptodornase preparation: *open circles*, with thimerosal; *closed circles* without thimerosal; *x-axis*, weeks; *y-axis*, percentage of wound granulation

(Table 1). The TGL was only 46.1% of the control. Gentamycin (6 mg/ml) and chloramphenicol (10 mg/ml) also diminished the height of the TGL significantly. Compared to the control we could find only 51.6% with gentamycin and 58.0% with chloramphenicol. The results with neomycin (6.7 mg/ml) demonstrated an inhibition of the TGL, but this difference could not be proved statistically because of the great differences in the single values (Table 1). In contrast to all other antibiotics, bacitracin (770 IU/ml) led to a slight enhancement of the granulation process (TGL 2.11 ± 0.20 mm; TGL of the control 1.82 ± 0.11 mm) but this difference was statistically not significant.

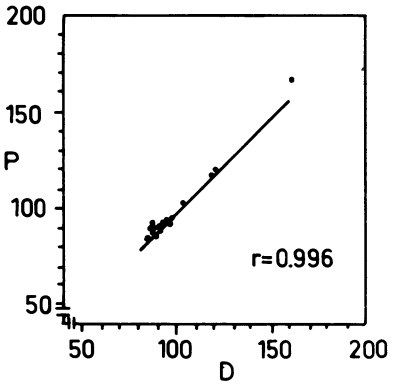

Fig. 8. Healing time of the epithelialization process measured by diametry (*x-axis*) and planimetry (*y-axis*). For details see text

Antimycotics

As a representative agent of topical antimycotics, miconazole (1 g/100 ml) was investigated. This azole caused a TGL which was similar to the control (Table 1).

Epthelialization

As described above (see "Methods"), measurement of the epithelialization of subepidermal wounds produced by the suction blister method was achieved by two procedures. The area of the wounds was calculated either by planimetry with serial photograms, or by diametry using the minimal and maximal diameters. The two procedures were compared with each other. It could be shown that the two methods were absolutely comparable. In Fig. 8 the healing time (HT) was noted, that is the time from the initial setting of the wound until its complete closure.

We calculated the HT by regression analysis, and all the values were measured simultaneously by planimetry and diametry. We used the results of 18 series of experiments each time with 10–30 pairs of values. The correlation coefficient was 0.996, which differed from zero by $p < 0.01$; the curve followed the equation $y = 4.443 + 0.940x$.

Dyes

The very intense coloring of the complete wound and surrounding skin by the triphenylmethane dyes made it impossible to measure their effect, with

Table 2. Healing time of epithelial wounds after treatment with different substances

Substance	Concentration (g/100 ml)	Healing time (h)	SD (h)	Percent of control	p
Control	–	90.9	2.6	100.0	
Eosin	0.5	89.8	1.7	98.8	>0.10
Control	–	94.5	2.6	100.0	
Chloramphenicol	1.0	102.1	2.8	109.2	<0.01
Control	–	93.5	2.9	100.0	
Gentamycin	0.6	120.2	3.2	126.7	<0.01
Control	–	89.9	2.6	100.0	
Miconazole	1.0	96.2	2.4	107.0	<0.01

the exception of eosin, which was not too dark. The HT of eosin was 89.8 ± 1.7 h ($y = 31.418 - 0.350x$; $r = 0.877$; $p < 0.01$), which was nearly identical with the control time (HT = 90.9 ± 2.6 h; $y = -23.847 - 0.262x$; $r = 0.956$; $p < 0.01$) (Table 2).

Antibiotics

As already shown by the influence on the granulation process, chloramphenicol and gentamycin inhibited the epithelialization as well. The HT for chloramphenicol was 9.2% longer than the control (Table 2), and the HT of gentamycin was 26.7% longer than the control.

Antimycotics

The azole agent miconazole prolonged the HT slightly, by 7.0%. The HT of the control was 89.9 ± 2.6 h, and with miconazole 96.2 ± 2.4 h.

Discussion

Dyes, antiseptics, and antibiotics are applied very frequently in dermatological therapy. However the use of antibiotics is controversial (Daschner 1981, 1984; F. Daschner 1986, personal communication). The triphenylmethane dyes were mainly applied in impetiginous eczemas, leg ulcers and other wounds. Gentian violet B and brilliant green have well known anti-eczematous and antimicrobial effects (Paetzold 1964). Clinically relevant damage with necrosis of the skin and the mucosa have often been reported (John 1968; Björnberg and Mobacken 1972; Meurer and Konz 1977; Sommer and Happle 1977). The intense cytotoxicity could be demonstrated by an

almost complete inhibition of the wound healing process upon administration of the dyes in the usual concentrations of about 0.5%. The heights of the granulation tissue were only 5.5% (gentian violet B) and 5.6% (brilliant green) of the control. Even in the dose relationship study using 0.05 g/100 ml gentian violet B, the TGL was only 15.3% of control.

The cause for this definitive inhibition is the strong binding of the dyes to DNA (Noeske 1966; Rosenkranz and Carr 1971). It could be demonstrated by Hagedorn and coworkers (1979) in cell cultures that gentian violet B inhibited the growth of fibroblasts by damaging the cell during the S, G_1 and the G_0 phases (Rosenkranz and Carr 1971). The defect was illustrated by diminished oxygen consumption and protein and RNA synthesis (Mobacken et al. 1974).

Consequently, one should avoid treating open granulating wounds and even the epithelializing margins with the triphenylmethane dyes. Furthermore, it must be taken into consideration that gentian violet B is not an ideal antiseptic agent. The desirable antimicrobial results quoted in the literature (Paetzold 1964) are valid only for gram-positive bacteria and not for the more problematic gram-negative ones. The minimal inhibition concentration for pseudomonads (0.05–0.10 g/100 ml; Möhlenbeck 1970) will also lead to a strong inhibition of wound healing. We must consider that in the clinically used solution of 0.5% gentian violet B, viable gram-negative bacteria could be found (F. Daschner 1986, personal communication). This dye is able to penetrate into the cells of the macroorganism which leads to an inhibition of the proliferation process. Probably this antiproliferative effect may be the real mechanism for the well known antieczematous efficacy of the triphenylmethane dyes.

In contrast to the other triphenylmethane dyes, eosin does not cause inhibition of wound granulation. Also, epithelialization is not disturbed; the HT is comparable to the control. Eosin is the typical dye for dermatologic therapy in France. It is less widely used in Germany. An inhibition of bacteria can only be observed with relatively high concentrations of about 2%, but eosin in combination with UV light leads to a better antimicrobial effect (Aron-Brunetière 1982).

Increasingly, antiseptics are preferred to topical antibiotics. Antiseptics which are used in dermatology are povidone-iodine, chloramine-T, chlorhexidine, silver nitrate, Dibromol, and thimerosal.

Povidone-iodine causes only a slight inhibition of wound granulation. The reduction of the TGL is 18.6%, a value consistent with the examinations of Hagedorn et al. (1979), who could demonstrate this effect in vitro. A similar cytotoxic effect on fibroblasts was observed by Kallenberger (1979) and by Faddis et al. (1977). In vitro studies on rats and the treatment of split-graft donor sites of humans showed no pathological signs, either microscopically or macroscopically (Gruber et al. 1975). These results can be regarded only with reservations, as the authors made no statements on the concentrations.

Chloramine-T is a substance which was introduced to dermatological therapy decades ago. The clinically used concentration is 0.5%–1.0% for the treatment of impetiginous eczemas and for cleaning wounds. The bactericidal efficacy is caused by the formation of sodium hypochloride. The very old studies of Dakin et al. (1916), Dold (1921) and Fetcher (1924) pointed out a good antibacterial efficacy (dilution 1:1 000 000 to 1:50 000). A cytotoxic effect on the macroorganism could not be proved in our own studies. The very small difference to the control was statistically not significant.

In contrast to chloramine-T, the antiseptic agent chlorhexidine is toxic for the macroorganism. The granulation process was markedly inhibited; the TGL was only 33.8% of the control, corresponding to an inhibition of 66.2%. The inhibition of wound healing is dose-dependent. Even with the lowest concentration of 0.05%, inhibition was 56.7%. These data correspond very well to those of the literature. After the implantation of sponges (Paunio et al. 1978) or of teflon cylinders (Heldén et al. 1974), the cytotoxic effects could be demonstrated by a decrease of the amount of DNA and of hydroxyproline in the cells. In vitro data with cultured cells demonstrated this effect, with a degeneration of cells (Heidmann et al. 1980), reduction of protein synthesis (Goldschmidt et al. 1977) and a dose-related damage of cells or even death (Kallenberger 1979). Therefore the application of chlorhexidine for the treatment of wounds has to be rejected. Furthermore the antibacterial efficacy is not very marked. Actually, *Staphylcoccus aureus* will be killed in vitro by concentrations of 0.01%, but *Pseudomonas aeruginosa* remains uninfluenced (Hennesey 1977).

Silver nitrate showed an inhibition of wound healing of about 25.0%, corresponding to a TGL of 75.0% of the control. This difference could not be proved statistically significant because of the varying single values. Wuite and Van der Meer (1974) found only slight disturbances of wound healing when silver nitrate was used in the treatment of leg ulcers (application of 2%). The epithelialization in burn wounds (application of 0.5%) was not influenced. The HT of the donor site of split-graft wounds (0.5%) was delayed (Bellinger and Conway 1970), whereas no negative effect could be seen by setting deeper defects on the rabbit ear (Scapicchio et al. 1968). Thus, application of a 0.2% solution is harmless for wounds. It must be pointed out that silver nitrate has a good antiseptic efficacy which is more pronounced against gram-negative than against gram-positive bacteria (Sykes 1965).

Dibromol, a mixture of different antiseptics, is used very frequently as a disinfectant. The very pragmatic use of this agent (0.1 ml directly onto the wound) led to a decrease of the granulation tissue (70.5% of the control). This effect is mainly due to the alcohol and the halogenated substances included in the mixture. The application of Dibromol for the treatment of open wounds is not recommended, since even a very small volume (0.1 ml)

markedly affected the healing process. Also, the subjective symptom of burning caused by the alcohol should be considered.

A pyogenic infection of the wound has to be treated, otherwise wound healing will be inhibited (Eisele 1981; Laurance 1983). However, one must keep in mind that because of the real effect – inhibition of the ribosomal protein synthesis by the antibiotic (Buss et al. 1984) fibroblasts and basal cells could also be damaged, which causes an inhibition of the wound healing process. In order to examine such an influence, the antibiotics were applied topically to open granulating and epithelializing wounds. It was shown that both tetracycline and chloramphenicol and the aminoglycosides gentamycin and neomycin caused a thinning of the TGL. Only bacitracin did not lead to a worsening of the granulation or the epithelialization process.

The observed inhibition of granulation by tetracycline (by 53.9%) and by chloramphenicol (42.0%) agrees with the results of Struck (1981), who examined the tensile strength of wounds under the influence of antibiotics. Similiar inhibiting effects were found even with bacitracin, in contrast to our own results. However this could be caused by different dosages, since Struck did not give any data on the concentration and period of application.

Bacitracin has no influence on epithelialization, which is in accordance with data of Petroutsos et al. (1983), who looked for the reepithelialization of corneal epithelium. This is valid at least for the applied concentrations (500 IU/ml by Petrousos and 770 IU/ml by our own experiments). Petroutsos et al. saw a prolongation of the epithelialization time only upon increasing concentrations up to 10 000 IU/ml.

The use of gentamycin showed that both granulation and epithelialization were inhibited by 6 mg/ml gentamycin when applied to the wound over a period of 24 h daily. Half the concentration of gentamycin (3 mg/ml) and the dropwise application into the conjuctival sack over a shorter period left epithelial regeneration undisturbed (Petroutsos et al. 1983).

Epithelialization with 10 mg/ml chloramphenicol is undisturbed. Actually the value of the HT is prolonged, but this is only apparent because of the different starting points of the wounds. We could see a comparable slope of the curve ($b = -0.218$ of chloramphenicol and $b = -0.213$ of the control); thus there is only a parallel shift of the curve.

In the case of neomycin, inhibition of wound granulation was very clear (36.5%), but because of very different single values the statistical proof could not be effected. The applied concentration of neomycin was 6.7 mg/ml, leading to a tissue concentration of 300 μg/g granulation tissue. This corresponds to a 60- to 600-fold minimal inhibition concentration (Niedner 1987). No statement could be made about the actual intracellular concentration of the antibiotic that caused its cytotoxic effect.

The use of topical antibiotics is completely rejected by Daschner (1981, 1984, 1990), because of the possibility of a selection of germs and because of

superinfection of the wound by resistant germs caused by uncontrolled swaying of the effective dose. Furthermore Daschner rejects them because of the risk of an epicutaneous sensitization. Schöpf (1981) pointed out that the risk of sensitization is real, but the risk of developing resistance is not too high, because very high concentrations can be reached on and in the wound, depending furthermore on the galenical form.

These above mentioned very high concentrations can only be reached on open wounds, where there is no stratum corneum as a resistant layer against penetration. So it is questionable whether the development of resistance during administration of local antibiotics is really as frequent as with systemic treatment. Due to the concentration gradient in the depth of the wound, only small effective amounts of the antibiotic can be expected, but these relatively low concentrations lie below a higher level which never can be reached by systemic administration. The only exceptions are wounds covered by fibrin layers. In these cases the concentration of the antibiotic will be diminished to a greater extent and the development of resistance is more likely.

Independent of the important discussion regarding the risk of an epicutaneous sensitization the problems with local antibiotics have to be seen more from the point of view of their potential to inhibit the proliferation process, thus disturbing the wound healing process. Such an inhibition can be observed with dyes (exception eosin), antibiotics (except bacitracin), and nearly all antiseptics.

In all cases in which we can observe infection of wounds by gram-positive bacteria, administration of bacitracin is well indicated. All other germs must be treated by other agents. Neomycin is effective against gram-negative bacteria and its wound healing inhibition is not too distinctive, but because of its high risk of sensitization the respective problems will arise. The local application of gentamycin must be rejected completely because it is a reserve antibiotic and its effectiveness with respect to resistant organisms should not be jeopardized.

Of all antiseptics and dyes, eosin has the lowest cytotoxicity; its germ inhibition efficacy in vivo begins with a concentration of about 2%. Chloramine-T and silver nitrate can be used without any reserve because they have only a modest cytotoxicity, even though they are not the least effective antimicrobic agents. Povidone-iodine is a good alternative but we must remember that a certain degree of inhibition of the granulation process can be observed, which is lower than the inhibition by chloramphenicol and tetracycline.

Antimycotics are used only rarely, as, in general, wounds are more often infected by bacteria than by fungi. In our experiments miconazole did not disturb either the granulation or the epithelialization process. Actually the healing time differed by 6.8 h as compared with the control, but, as already described with chloramphenicol, the regression was only shifted parallel to the curve of the control experiment. So miconazole can be used

in all cases of fungal superinfections without any risk of an inhibition of wound healing.

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Intra-arterial Application of Ceftizoxime Supports Wound Healing in Infected Ischemic Lesions of the Limbs

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Introduction

The treatment of polyinfected lesions in arterial occlusive disease is based on a multimodal approach, in which several procedures are applied in concert to avert the impending loss of a limb. In addition to the various techniques for improving blood perfusion in the ischemic areas, combating infections plays a major role. Initial measures comprise local surgical treatment with necrectomy, lancing of abscesses, border zone amputation and local antiseptic treatment. This local treatment must be supplemented by systemic control of infections using a potent antibiotic with the broadest possible spectrum of activity. The efficacy of this antibiotic therapy depends above all on the concentration of active drug at the site of infection which can be considerably increased by administering the antibiotic by intra-arterial, instead of intravenous, route (Amendt and Hild 1990; Gottlob and Hugeneck 1982; Widmer and Hürlimann 1966).

In view of the favorable pharmacological properties of ceftizoxime, with its low plasma protein binding in association with its relatively long half-life, this drug was considered to be particularly suitable for intra-arterial treatment, especially since animal experiments had demonstrated that intra-arterial infusion is well tolerated by the tissues (Hashimoto et al. 1989). The aim of this study was to determine the tolerance and efficacy of the intra-arterial administration of ceftizoxime in polyinfected ischemic lesions.

Method

During February 1990 to April 1991, 35 patients (16 female, 19 male with an avearge age of 71.6 years) with infected ischemic lesions in arterial occlusive disease were treated with intra-arterial ceftizoxime. 24 patients had an

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ischemic ulcer and 11 patients had gangrene, which was moist in six cases. Bone involvement in the form of osteomyelitis was detected in 15 patients.

The treatment of the patient population was multimodal. Patients with arterial occlusive disease of the pelvic type and patients with a stenosis of the exit of the deep femoral artery accompanied by an occlusion in the thigh were treated primarily by surgical measures and therefore without intra-arterial infusion. All other patients with arterial occlusive disease received intra-arterial infusion therapy, for which in most cases prostaglandin E1 was administered in addition to ceftizoxime. Recent arterial thromboses were subjected to local fibrinolysis with streptokinase. When blood flow was significantly improved, chemical lumbar sympatholysis was performed with a peridural catheter. Nonresponders underwent surgical revascularization whenever possible. In some patients, however, it was attempted to clear up the infection prior to scheduled surgical revascularisation..

The intra-arterial infusion was administered by means of a direct puncture of the femoral artery with a cannula 0.9 mm diameter. A total of 2 g ceftizoxime dissolved in 50 ml of NaCl 0.9% was infused in 50 min by means of a motor-driven pump. To maintain a sufficient active drug level over 24 h, a further infusion of 2 g ceftizoxime was given intravenously after an interval of 12 h, usually into the cubital vein. The mean treatment period was 9.2 days, the shortest treatment being concluded after 4 days, and the longest after 24 days.

Results

The microbiological determination of the bacterial flora in the ischemic lesions of 29 patients revealed a pattern of mixed colonization, mainly with gram-positive bacteria (Table 1). Staphylococci and streptococci were found most frequently. Instead of waiting for the results of the microbiological investigation, antibiotic treatment was initiated immediately. This explains why in five patients (17%) ceftizoxime-resistant germs were also treated with ceftizoxime until the microbiological findings became available. The infections were mainly due to *Pseudomonas aeruginosa*. In all of these, in spite of the resistance to ceftizoxime, a marked improvement of the local findings was observed which was most likely due to the efficacy of the local antiseptic treatment and the local surgical measures. Local surgical measures were administered in about two thirds of our patients (Table 2). In 31 cases, rheological treatment with prostaglandin E1 was given, in most cases also by intra-arterial infusion. Chemical sympatholysis controlled by computer tomography was performed in five patients. Six patients who exhibited an insufficient healing tendency had to undergo surgical revascularization. In nine cases, it was attempted to clear up the infection before the scheduled surgical revascularization.

Table 1. Bacteriological findings in 29 patients with infected ischemic lesions

Bacteria	Percent of patients
Staphylococcus aureus	52
Staphylococcus epidermidis	7
Streptococci	31
Enterobacteria	27
Pseudomonas aeruginosa	10
Other bacteria	7
No bacterial growth	10

Table 2. Concomitant therapy in 35 patients with intra-arterial infusion treatment with ceftizoxime

Therapy	n
Local surgery	23
Revascularization	15
Prostaglandin E1	31
Sympatholysis	5

Table 3. Regression of the inflammatory reaction in 35 patients with infected ischemic lesions undergoing intra-arterial treatment with ceftizoxime

Regression of inflammation	*n*
Complete	15
Marked	10
Slight	6
None	4

To determine the local tolerance of the intra-arterial administration of ceftizoxime, the first 20 patients of the study received a questionnaire containing a visual analogue scale for self-rating of any increase in pain experienced during or after the infusion of ceftizoxime. In a total of 198 intra-arterial infusions of ceftizoxime, an increase in pain at the infusion site occurred in only two cases. This was probably caused by incorrect positioning of the infusion cannulae. No other signs of local intolerance such as reddening and swelling were observed. In one patient the antibiotic treatment with ceftizoxime had to be discontinued due to gastrointestinal symptoms.

The regression of the inflammatory reaction at the ischemic lesion was assessed after completion of the intra-arterial therapy. Altogether 25 lesions (71%) showed a good healing tendency with complete (n = 15) or extensive regression (n = 10) of the inflammation. Since six patients with only slight regression of the inflammatory reaction subsequently underwent successful vascular reconstruction, it was possible to preserve the extremity in altogether 31 patients (88%). Four patients had to undergo knee joint exarticulation (n = 3) or above-knee amputation (n = 1) due to progression of the infected lesions.

Discussion

In addition to improving the blood flow, combating infections is the most important aspect in the treatment of infected ischemic lesions. Pain at rest frequently subsides when the infection has been brought under control, as the relaxation of the tissue tension, which is increased due to the inflammation, allows improved perfusion of the ischemic tissue. The impending loss of a limb at stage IV arterial occlusive disease can only be prevented by adopting a multimodal approach in which the different procedures complement each other. Antibiotic therapy plays an important role in this therapeutic scheme. Its purpose is to kill germs that are inaccessible to local treatment. Due to the deficient perfusion of ischemic tissue, however, there is a risk that only inadequate drug levels may be achieved in the tissue, which promotes the development of resistance. The concentration of active drug at the site of infection can be increased by intra-arterial infusion of the antibiotic, which provides tissue drug levels up to four times higher than those achievable with intravenous administration (Amendt and Hild 1990). Moreover, the attainable concentration of drug in the tissue depends on the diffusion properties of the antibiotic in ischemic and necrotic tissue. This behavior is essentially determined by the plasma protein binding of the drug (Klaus 1989; McNamara et al. 1988). The unbound and therefore active portion can diffuse into ischemic tissue, whereas the portion bound to protein remains within the vascular system. In view of the favorable pharmacological properties of ceftizoxime, this antibiotic appeared especially suitable for intra-arterial treatment of ischemic lesions. The results of this clinical study have confirmed its good local tolerance. However, the multimodal approach of treating ischemic lesions cannot be used to assess antimicrobial efficacy of ceftizoxime. This will require further studies with determinations of drug concentrations in the tissue. Since the infection was successfully controlled in 71% of the patients without the need for vascular reconstruction measures, the drug can nonetheless be assumed to have high antimicrobial efficacy.

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VII. Noninvasive Measurement Techniques

Noninvasive Measurement of Wound Healing of the Skin

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Introduction

Clinical research has tried to speed up wound healing for more than a century. In order to develop appropriate medication for this purpose, we need to have an exact understanding of the physiology of wound healing. The development of wound healing models and the capacity to evaluate wound healing in an objective way are additional, important prerequisites. So far, there has been a lack of highly standardized or generally recognized wound healing models developed in human beings which meet strict scientific and ethical requirements. Investigations referring to wound healing models therefore have largely been restricted to animal experiments or to observations of the healing process on the surfaces of wounds.

Popular models such as leg ulcers can be challenged on the grounds of various well-founded scientific arguments; factors complicating informative, controlled, and randomized clinical examinations of therapeutic agents for wound healing include the following:

- Heterogeneity of the patient population (concerning additional underlying diseases, nutritional state, age, sex etc.)
- Variations in the localization and extent of lesions
- Variations in wound genesis (determinants!)
- Variations in the treatment regime (e.g., positioning method, dressings, ointments)
- Difficulty in defining end points and objective target variables for wound healing

The precise quantification of the biological processes affecting wound healing is a significant problem in the two in vivo models mentioned above. Unfortunately, we still know very little about these models and about other aspects of wound healing. However, the difficulties posed by these models

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would substantially decrease if we were to succeed in the swift and sufficient quantification of all the important factors determining the genesis and subsequent healing of wounds. We are faced with the dilemma that a clear characterization of "important influencing factors" in terms of physiology and biochemistry is not always possible or requires extremely complex, costly, highly precise, and noninvasive measurement methods. This often results in a vicious circle: We need measuring methods to characterize influencing factors, but in order to develop measuring methods we need to have a precise understanding of the influencing factors. The quantification of wound healing in follow-up studies must be achieved by noninvasive methods. In fact, the noninvasive character of the investigations is more important than in most other areas of medical research, because this is the only way to quantify a wound from its genesis (detection) to its complete healing. We distinguish between primary and secondary target parameters in the evaluation of the wounds: first-order characteristics can be described in mathematical terms, e.g., surface area/circumference, diameter, volume, surface structure, and depth range, while second-order characteristics reflect the biology and physiology of a wound, e.g., tissue structure, tissue perfusion, and genesis. The various factors affecting wound healing modify these target parameters in quantity as well as in quality, which other chapters of this book will describe in more detail. In the following, we will primarily present noninvasive measurement methods for the objective evaluation of wound healing. All the methods mentioned require the definition and observance of stringent practical standards, which should be described when establishing the study protocols. The more widespread use of these techniques (which include very costly, complex, and complicated methods) will only make sense if this good laboratory practice is carefully observed.

Sonographic Evaluation of Wound Healing

The visual inspection of a wound is almost invariably restricted to its surface, while underlying structures cannot be observed. Researchers have therefore in a number of cases resorted to sonography for the noninvasive monitoring of repair processes taking place in the skin. We have primarily used high-frequency sonography for the evaluation of wound healing processes in cryosurgical lesions resulting from the treatment of basal cell carcinomas (Altmeyer and Luther 1989; Hoffmann et al. 1989, 1990a,b). We saw that the observation of cellular repair processes in poorly reflecting ("echo-poor") necrotic areas is restricted by the resolution of the scanning equipment ($\approx 80\,\mu$m; Hoffmann et al. 1993a). Granulation tissue cannot be clearly delimited using 20-MHz sonography (Fig. 1). Both granulation tissue and fresh cicatricial tissue exhibit a low echogenicity and can only be distinguished through the comparison of sonograms taken at successive points in time (Hoffmann et al. 1993a). This is of particular importance since echo

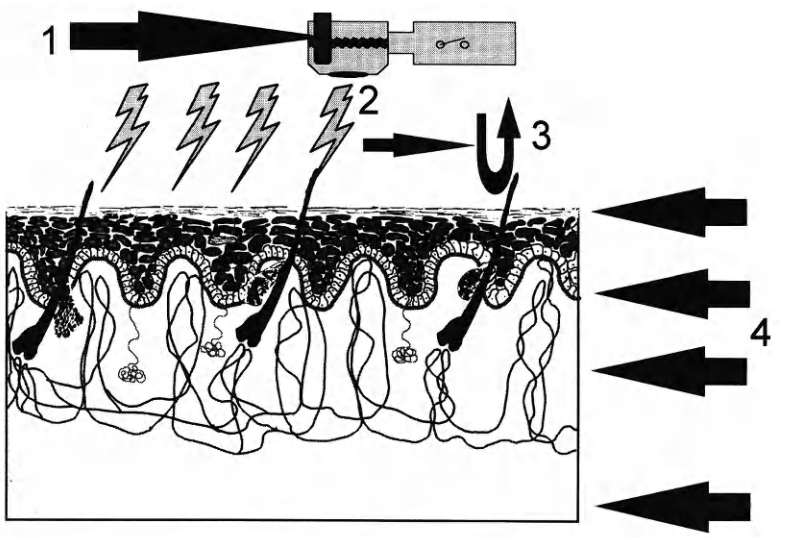

Fig. 1. Principle of 20-MHz sonography. The transducer (*1*) emits echo pulses (*2*). These pulses are reflected (*3*) by certain tissue formations within the skin (*4*), e.g., the stratum corneum and collagen bundles. The transducer also receives the reflected pulses and an image is generated by the connected computer unit. In granulation tissue, the sonographic image exhibits only limited internal echoes within an area of reduced echogenity, as the deposition of collagen bundles is very limited. Irregularities in the deposition pattern of collagen bundles in scar tissue is also responsible for low echogenity

poor areas can be observed in the cryolesion both at the beginning and at the end of the healing process. In the early postoperative stage (2–4 days after cryosurgery), the echo poor inhomogenously distributed internal echos may be interpreted as evidence for a conglomeration of residual cutaneous appendages and vessels closed by fibrin-rich clots, a view supported by histological examinations (Luther et al. 1989). By the end of the healing process, the internal echos in the lesion probably indicate newly formed finely fibrillar connective tissue, whose irregular cross-link structure presents surfaces reflecting the ultrasound signals (Winkler et al. 1992).

The size of the wound can be continuously ascertained by the computer system implemented in the scanner. Thus, increases or decreases in the dimensions of the wound can be precisely measured during the healing process. It is often difficult to delimit the lower boundary of the corium from muscle fascia or subcutaneous bands of connective tissue and adipose tissue if the corium is edematous and significantly expanded. However, the fact that the boundary between the dermis and the subcutis often remains indistinct up to the 14th postoperative day is not sufficiently accounted for by the persistent dilation of the edematous corium. A similar phenomenon involving the thermal conversion of initially poorly reflecting adipose tissue

Fig. 2. DUB 20S 20-MHz sonography unit. Both commercial available system (DUB 20 and Dermascan C) are comparable and do not differ very much in the technical possibilities for routine diagnostics

into echo-rich structures might also be conceivable and was described by Brink (1986). The reduction in the sonographic density is caused by the reduction of the interfaces in the exudatively softened corium. On the other hand, absorption in the collagen fascicles pushed apart by the edema is significantly intensified. In some cases, the wound healing defect is evident in a prolongation of the clinical phases of wound healing and in a persistent reduction of the sonographic echo in the area of the inflammatory infiltration.

On the whole, sonography is a suitable method for ascertaining the depth of a lesion and for evaluating the depth of a wound in the course of healing. So far, it has not been possible to ascertain tissue characteristics with adequate reliability (Hoffmann et al. 1993b). The newly developed advanced DUB 20-S Scanner (Fig. 2) or the Dermascan C, however, now enables us to carry out extremely extensive and efficient image analyses. Therefore, we have reason to hope that true textural analysis (histodifferentiation) will also be possible in future, at least to a limited extent. We may also expect that the three-dimensional reconstruction of images will supply additional information. Our experience has shown that in these investigations the volume of the lesion should be used as a valid parameter instead of the wound surface.

Interferometry

Topographic on-line measuring and testing technologies based on holographic interferometry have for a number of years been applied in mechanical engineering to analyze the deformation and vibration behaviour of components, aggregates, and machines (Breuckmann et al. 1991). The term "on-line topometry" comprises methods that use structured light and allow the pictorial representation of three-dimensional measurement data as well

as the computer-assisted processing of the parameters recorded (Thieme 1987; Seitz and Tiziani 1986). For approximately 3 years we have been working with a new method derived from on-line topometry, which was developed by our department in cooperation with the Dr. Duwe firm of consulting engineers (Tegernsee, Germany). This method allows us to quantify the surface, height/depth, or volume of ulcerative skin lesions and enables us to carry out quantitative measurements of the wound healing process.

Technical Principle

The newly developed "Holon-View" interferometry system consists of a projector, a video camera, an object support frame, and a computer-assisted digital image processing system (Figs. 3, 4). The computer-controlled projector system (projector and filter) allows us to project a software-driven line grid onto an object. The pattern of the sectional light lines on the examination object is recorded by a video camera, digitized, and read into the image processing computer. We project a number of different line patterns onto each examination object. The so-called "gray code projection method" is used for the detection of line lengths. The "phase shift method" is additionally employed to optimize resolution; in this case, line grids projected in a slightly defocused arrangement result in sinusoidal line patterns. The calculation of the phase angle for each image point in combination with the gray code sequence technique results in definite intensity and height values. Computer-assisted image analysis then, in only a few seconds, provides contour information. After an image has been recorded, the digitized measurement data is converted to a three-dimensional line image. The measuring accuracy is in the range of 100 μm to 1 mm, depending

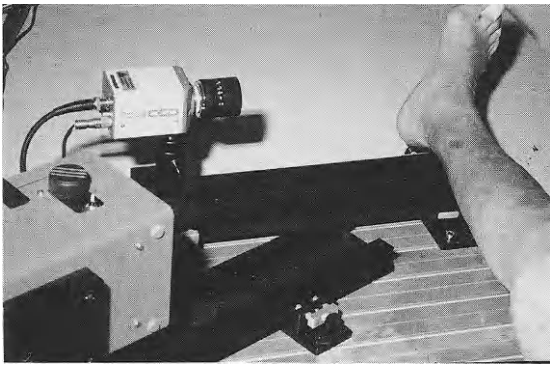

Fig. 3. Holon-View interferometry system. This special system is a prototype and was developed 3 years ago. The method has now been evaluated for study purposes

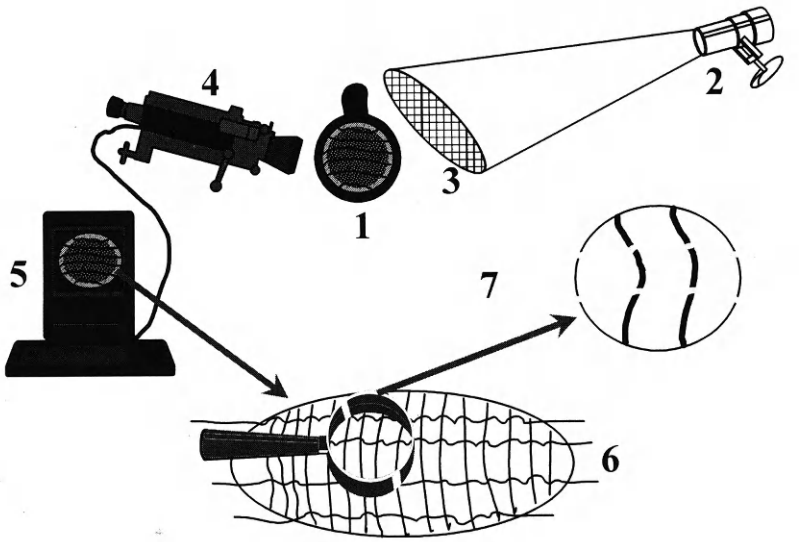

Fig. 4. Holon-View, technical principle. The examination object (*1*) is illuminated by a projector (*2*), projecting a grid line pattern onto the object (*3*). The object is recorded by a video camera (*4*) and transferred to the computer for further processing (*5*). By using the gray code sequence technique, each image point is converted into intensity and height values (*6,7*)

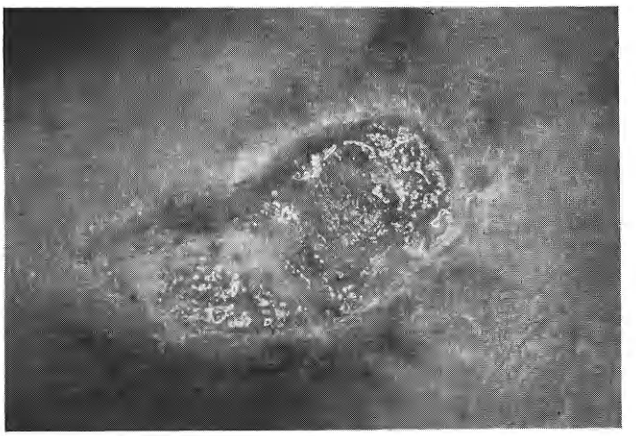

Fig. 5. Venous stasis ulcer at the lower leg. In vivo examination by the Holon-View unit is possible; however, we prefer the use of replicas to obtain standard conditions

upon the size and depth of the object. As a rule of thumb, the measuring resolution is equivalent to 1/1000–1/5000 of the object size. Apart from the measurement of object heights, interferometry also allows volume and surface measurements. In principle, the investigations can be carried out in

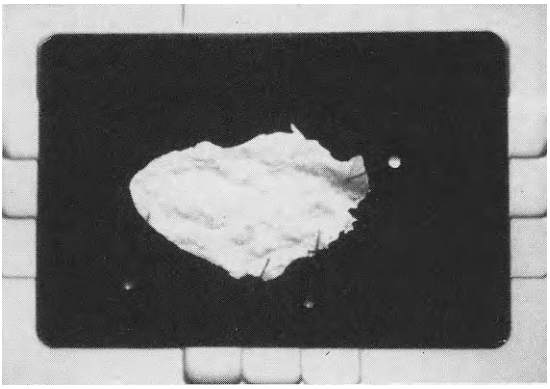

Fig. 6. Replica of venous stasis ulcer at the lower leg (see Fig. 5). For producing replicas, dental impression material (Silflow, Flexico Ltd., England) is used

vivo or in vitro (Figs. 5, 6), though we prefer the latter method because it is easier to handle.

Examination Procedure in Interferometry

After the test object has been secured in the image plane and the distances and angles between all the measuring directions and objects have been standardized, a combined gray code and phase shift sequence is recorded. The recorded data is then translated to absolute values on the basis of fixed-programmed calibration parameters. A section of the object is delimited using the cursor so that the volume, surface, and maximum height of this region of interest can be ascertained. The border area of this region is used to calculate a base area (0 level).

Validation of Interferometry

Objects of known volumes, heights, and surfaces as well as impressions (silicone replicas) of leg ulcers were measured to ascertain the validity of interferometry. The ulcer volumes were determined by weighing the silicone replicas before and after filling them in with water. Serial measurements with varying orientation of the objects in the image plane were carried out to confirm the reproducibility of interferometry. A UBM 20 laser profilometry system (UBM, Ettlingen, Germany) with a measuring accuracy of more than 1 μm was also used to validate measurement results. However, although this system supports highly accurate structural measurements, the measurement procedure is very complicated and extremely time consuming for the interferometric comparison of our samples (>10 h).

Initially, serial measurements of objects with defined volumes were made to confirm the validity and reproducibility of the measurement results. Two cuboids of known volumes (194.4 mm^3 or 1940.5 mm^3), surfaces (367.2 mm^2 or 21443.7 mm^2), and heights (0.6 mm or 1 mm) were subjected to 20 measurements each. The maximum standard deviation of the mean values amounted to 1.6% in the volume measurements, 2.9% in the surface measurements, and 6.9% in the height measurements. In addition, the volumes of five ulcer replicas interferometrically ascertained in serial measurements were compared to the volumes of water held by these replicas (60 mm^3, 370 mm^3, 450 mm^3, 1590 mm^3, and 3690 mm^3). The volumes measured by interferometry on average corresponded to 94.4% of the volumes ascertained through the water-holding capacity of the silicone replicas. The maximum standard deviation of the mean value was 1.2% in interferometric volume measurement.

Assessment of Interferometry

Our interferometry system features a combination of the phase shifting and gray code sequence projection method and thus optimizes resolution to 100 μm. Interferometric measurements can be made directly on skin or on skin impressions produced by a replica technique. The surface measured by interferometry cannot be directly compared to the base area of ulcers ascertained through conventional image analyses since the entire ulcer surface is measured (including relief structures in the ulcer base), whereas conventionally a plane at the level of the ulcer edge is obtained. The reproducibility of the measurement results obtained by interferometry was shown in serial measurements. The generally low standard deviation of the mean values ranged from 0.5% to a maximum of 1.6% in volume measurements and depends upon the size of the object. The validity of interferometry was ascertained by comparing the known volumes, surfaces, and heights of given test objects to the mean values obtained by interferometry. Pertaining to the ulcer volumes, the standard deviation of the mean value was only 1.2%. Interferometry provided volume values that were on average 4.6% below the corresponding values obtained through the water-holding capacity. This constant measuring error can be compensated for by a correction factor. The three-dimensional display of the measured objects can be enhanced by the representation of grid lines in different colours and by inserting explanatory text (Figs. 7, 8).

In summary, the introduction of interferometry to dermatology has for the first time provided a method for the noninvasive measurement of the extent and volume of cutaneous alterations above and beneath the surface of the skin. Skin conditions that can be evaluated by this methods include ulcers of various etiology and exophytic tumours. Interferometry therefore

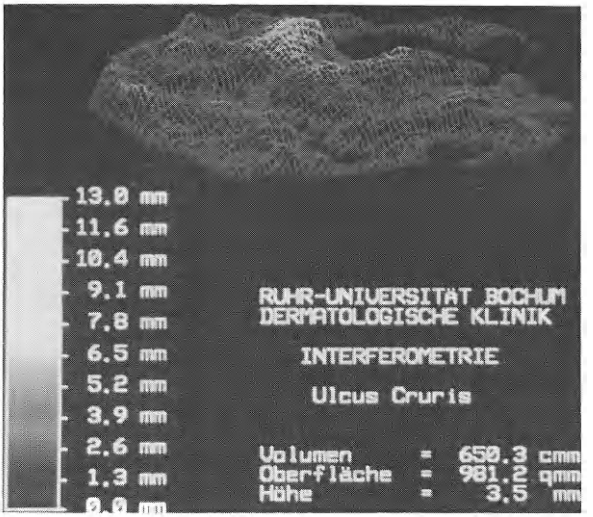

Fig. 7. Three-dimensional display of a venous stasis ulcer by interferometry measurement. Different heights can be distinguished by color representation. Volume, surface, and height of the lesion are presented on the display

Fig. 8. Three-dimensional image of a replica taken from a venous stasis ulcer with nonhomogeneous distribution of heights at the base of the ulcer. The image can freely be rotated for full information on the ulcer from any chosen perspective

plays an important role in monitoring the healing process and therapeutic success in the treatment of ulcerations.

Laser Profilometry

The microtopography of the surface of the skin can undergo significant transformations in the course of repair processes (Rupec 1980). This is illustrated by simple scars or by pathological final states such as keloids.

Unfortunately, subtle structural alterations in the surface of the skin have so far only been accessible to clinical descriptions, whereas evaluations based on measurement techniques have not been possible with adequate accuracy (Mignot et al. 1987). Laser profilometry is a new method of describing and quantifying surface states of the skin with high accuracy (Saur et al. 1991). However, while this technique is considerably more precise than interferometry ($<1\,\mu$m), it is also extremely time consuming. As far as handling is concerned, the new technique clearly poses more problems than interferometry.

Direct scannings of the skin surface fail due to the dynamic character of the skin. Minute movements caused by breathing, pulse etc. prevent a precise measuring process. Therefore, we have to "freeze" the image of the skin surface before we can subject it to a measuring process. This is done by creating silicone replicas of the skin. The "skin impressions" or "prints" obtained in this way precisely reflect the cutaneous macrorelief and microrelief in the micrometer range and can be subjected to measurements.

The UBC 14 laser profilometer (UBM, Ettlingen, Germany) employed in our studies is based on the principle of dynamic focusing (Fig. 9). Laser light targeted at the object is reflected and detected by an array of mirrors and photodiodes, while the system is refocused by a computer. The position of the lens is continuously recorded as a digital value. The data obtained in this way is used to calculate the relief of the surface (Fig. 10).

Software options provided by the system make it possible to ascertain all the characteristic roughness values that have been described and to execute mathematical and statistical procedures including the Fourier transform and autocorrelation functions.

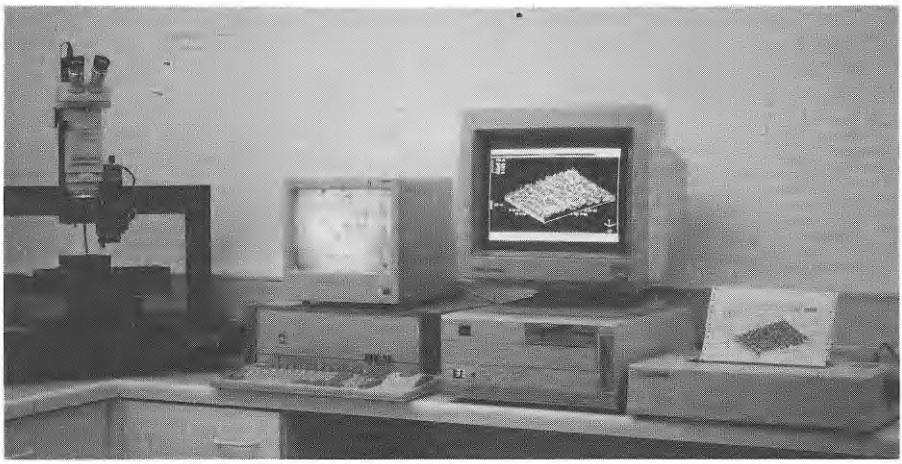

Fig. 9. Laser profilometry unit UBC 14 working place with profilometer, video microscope, computer unit, and printer

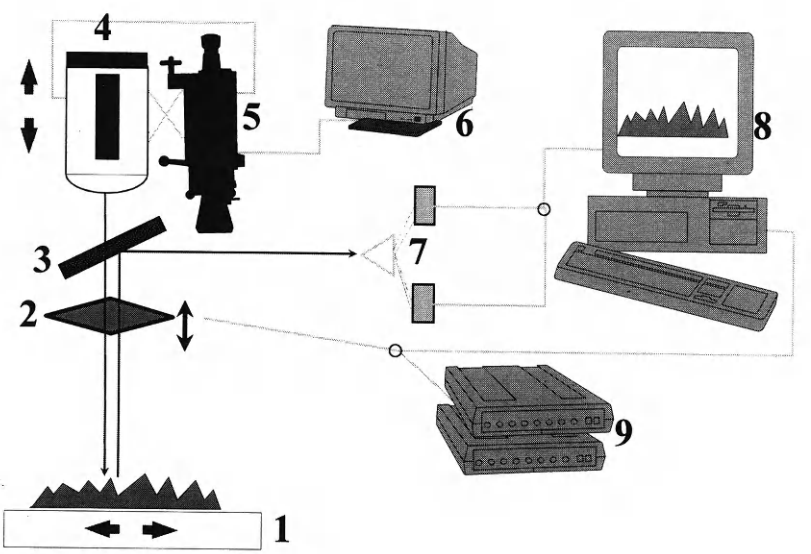

Fig. 10. Technical principal of the laser profilometer. Motor-driven object carrier (*1*), mirror system with lens (*2*) and translucent mirror (*3*); laser unit (*4*), video microscope (*4,5*) connected to a display screen (*5*) for searching for the region of interest on the object; prisma and connected photocells (*7*) are used to control the focus of the system. The surface image is presented on a separate screen (*8*). The system is put into focus by the controller unit (*9*). The position of the lens is continuously recorded as a digital value

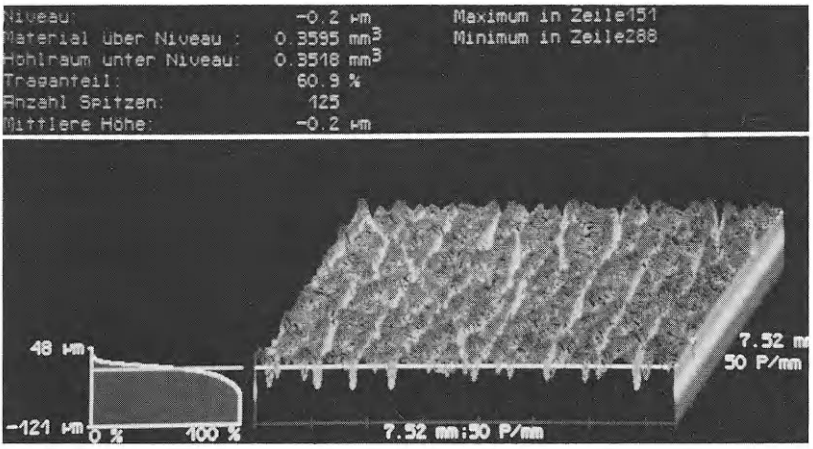

Fig. 11. Laser profilometry image. Volume determination in relation to freely chosen height lines can easily be performed. The architecture of skin lines can be recognized in the image. The volume determination is very useful to evaluate the treatment of scars. We combine the interferometry (large scars) with laser profilometry (small scars)

The extent and volume of cicatricial alterations can be precisely ascertained using this system (Fig. 11). Minor scars that rise up to 2.5 mm over the skin are particularly suited for these measurements. Laser profilometry thus mainly covers the evaluation of minor cicatricial conditions that at present cannot be measured by interferometry. Consequently, the entire range of cicatricial conditions can be evaluated by using a combination of laser profilometry and interferometry equipment. The high degree of accuracy provided by this method can be of particular benefit in the evaluation of medication or techniques for the treatment of scars. Recruiting study participants with minor scars is clearly easier than finding participants with keloids. In addition, the patients' compliance is relatively poor, because the therapy can take a very long time.

Noninvasive Methods for the Evaluation of Microcirculation

Cutaneous perfusion is one of the critical parameters of wound healing. Therefore, perfusion is an important parameter to quantify when observing the healing process and when ascertaining the etiopathogenesis of wound healing defects.

All the examination techniques have to allow for the heterogeneity of the skin's vascular system. The blood flow in the skin supplies the system with nutrients and regulates its temperature and blood pressure (Rupec 1980). A deep cutaneous vascular system is distinguished from a subpapillar vascular system and the capillaries in the papillae. Arteriovenous anastomoses in the corium regulate temperature and blood pressure, while capillaries deliver nutrients to the papillary body and epidermis. The density of capillaries ranges from $29/mm^2$ in the thigh to $155/mm^2$ in the facial region (Oon and Stafford 1987). Thus, measurements must allow for the vertical structure of the vascular system and for its two-dimensional nonhomogeneity so that functional aspects are adequately considered. In the past decades, various methods have been used to investigate cutaneous perfusion. Some of these techniques, however, cause injury to the skin and thus modify microcirculation, while others (e.g., the Xenon Clearance method) are invasive and inappropriate for clinical studies. Definitions of microcirculation, and in particular of the microcirculation in the area of the skin, vary considerably. According to Gaethgens (1981), the term covers blood vessels with a diameter of less than $300\,\mu m$ as well as the blood flowing through them. In the stricter sense of the word, microcirculation is the movement of blood in the terminal vascular system, a term sometimes used as a synonym for microcirculation. More generally, the term designates the nutritive and thermoregulatory vascular system in the skin (Fagrell et al. 1986).

Epiluminescent Microscopy

The microscopic examination of capillaries is the oldest noninvasive method for describing cutaneous microcirculation (Müller 1937). More recently, the measuring set-ups have been optimized (Bollinger et al. 1974). In principle, the skin is made more transparent through the application of oil. The visibility of capillaries is reduced when the stratum corneum is thickened or when layers of eschar or scales are present. Direct capillaroscopy provides information on the individual architecture of healthy and pathological capillaries, on their respective densities, and on the quality of the erythrocyte flow. In the paronychial capillary, the flow velocity of the red blood cells can additionally be ascertained (Bollinger et al. 1974; Bollinger and Fagrell 1990). In the case of progressive systemic scleroderma, the images observed in the paronychial capillaries are almost pathognomonic (Maricq et al. 1989). Ischemic cutaneous necrosis is ultimately caused by the reduced perfusion of the nutritive vascular bed. The vitality of the skin is threatened when the flow of erythrocytes through the papillary capillaries stops (Fagrell 1977). Therefore, capillaroscopy is the method of choice when the risk of cutaneous necrosis has to be predicted. When microscope systems equipped with flexible fiber-optic leads are used, any location on the body surface is easily accessible for examination. Thus, a highly sensitive method is available, which is particularly suited for the examination of peripheral occlusive arterial disease. Above all, the altered morphology of the capillaries is evaluated, which allows conclusions concerning the prognosis for chronic arterial occlusive disease (Fagrell 1972). An important parameter in the noninvasive study of wound healing is the functional capillary density that indicates the number of visible capillary loops per square millimeter. This parameter gives direct evidence on the nutritive vascular bed in the papillary layer. Measurement of the erythrocyte flow velocity – an instructive parameter in the paronychial capillary – is not informative and usually impossible in wounds. The capillary loops must be fully visible for epiluminescence microscopy. This requirement is only met at a few locations, e.g., at the perionychium, while anywhere else only the capillary apex can be observed. Measurements of the erythrocyte flow are useless when the capillaries are pathologically altered, as in the case of telangiectases. For these reasons, the method has not been generally adopted in wound healing studies and is ruled out in our studies because of our specific experience with it.

Transcutaneous Oxygen Tension Measurement

Transcutaneous oxygen tension measurement was first used in 1978 with patients suffering from peripheral occlusive arterial disease to quantify the oxygen supply to the skin (Tønnesen 1978). Studies relating to implant incorporation are also available (Keller et al. 1978). The measurements are

based on polarography. A pO_2 signal is created by the reduction of oxygen at a cathode, which is usually heated to a defined temperature and placed directly on the skin in the absence of air. Transcutaneous oxygen tension measurement registers oxygen diffusing from the subpapillary plexus to the surface of the skin. In this way, the oxygen supply to the upper 100 μm of skin can be assessed (Weindorf and Schultz-Ehrenburg 1988). While various studies have shown a significant reduction in the partial pressures of oxygen with peripheral occlusive arterial disease, a correlation between the clinical stage of peripheral occlusive arterial disease and the $tcpO_2$ value cannot be established (Hiller and Hornstein 1990). $tcpO_2$ measurement and capillaroscopy can be combined by using transparent oxygen electrodes. In this way, the position of the cathode relative to the adjacent capillaries, the current oxygen tension, and the functional capillary density can simultaneously be ascertained (Huch et al. 1983).

We use this method preferably in studies involving medication that influences the blood flow. However, if exclusively used, the method only provides limited information. We therefore always use it in conjunction with flow measurements.

Laser Doppler Flowmetry

The principle of laser Doppler flowmetry is based on the fact that monochromatic light is subjected to the Doppler effect when reflected by red blood cells moving in the microvascular network. A light beam is sent to and from the tissue through a pair of flexible optical fibers. The light source is usually a low-power HeNe laser (1–5 mW). The reflected light subjected to the Doppler effect is received by a photodetector or a photomultiplier converting its respective frequencies into an electric signal. In this way, a signal linearly correlating with the tissue perfusion at the time of measurement can be continuously registered. Stern (1975) pioneered this method in the field of skin perfusion, while Holloway and Watkins (1977) used it for further studies of the blood flow in the skin and compared the method to the Xenon Clearance technique. Nilsson et al. (1980a,b) developed a multichannel system with an improved signal-to-noise ratio to overcome the interfering signals encountered in flowmetry. A signal processor whose signals linearly reflect the concentration and the flow rate of erythrocytes was introduced in 1984 (Nilsson 1984). Since then, laser Doppler flowmetry has been used in a growing number of applications. It is now an established method used not only for blood flow measurements in the skin, but also for perfusion monitoring in other types of tissue, such as bones, muscles, peripheral nerves, and kidneys (Shepard and Öberg 1990). The signal produced by laser Doppler flowmetry is essentially determined by the flow of erythrocytes in the subpapillary plexus (Jakobsson 1992). Wound healing poses a problem that must also be taken into account in transcutaneous

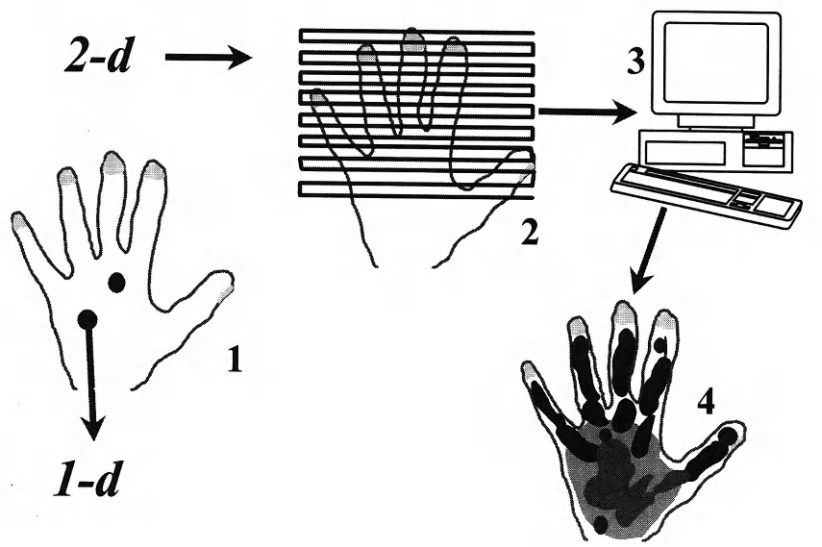

Fig. 12. Laser Doppler. The one-dimensional laser Doppler permits only selective information on blood flow (*1*). An entire survey on perfusion becomes possible by laser Doppler imaging (*2*). The perfusion situation within an entire region of the body can be made visible (*3,4*)

oxygen measurement. Since in both cases spot measurements are made, it is never clear whether the measuring probe has been positioned in a representative region. On the other hand, the method allows continous measurements over long periods of time, which is a considerable benefit. Laser Doppler flowmetry has gained particular importance in combination with two-dimensional methods for the quantification of microcirculation (Fig. 12).

Laser Doppler Scanning

The laser Doppler measuring instruments currently in use allow the evaluation of perfusion at a given point in time in a precisely defined single location. Since the blood flow in the skin varies greatly in different regions (Tenland et al. 1983; Shepard et al. 1987), the method is appropriate for measuring responses to given stimuli over time, while it is less suited for measuring the distribution of blood flow intensities over a given area. An additional disadvantage of this method is the fact that the measuring head of typical laser Doppler instruments must have direct tissue contact for recording, which may cause pain and infections and distort the measuring results. These limitations have been overcome in a newly developed laser Doppler imaging system (Fig. 13) providing valid and precise measurements

Fig. 13. Laser Doppler imaging system (LDI). This Swedish system is commercially available

of cutaneous flux (Wardell 1992). By an array of mirrors and two stepner motors, a HeNE laser (632.5-nm wavelength) is guided over an area of up to 12 × 12 cm in wide curves. The distance between the scanner head and the surface of the skin is 15–20 cm. The light signal is partially reflected by erythrocytes moving in the capillary bed and detected by a photodetector in the scanner head (shift detection). Subsequently, the signals are translated into a two-dimensional perfusion image. The flux values can be individually quantified in each of the 4096 measuring points. The values are represented by false-color coding in six colours ranging from blue and green to yellow and red, reflecting increases in the blood flow. Areas in which no blood flow is observed are rendered gray in wound healing studies. The granulation tissue at the wound edge exhibits increased perfusion (Figs. 14–16). Since laser Doppler scanning shows less variance than one-dimensional laser Doppler flowmetry, this method is better suited for studies over time (Quinn et al. 1991).

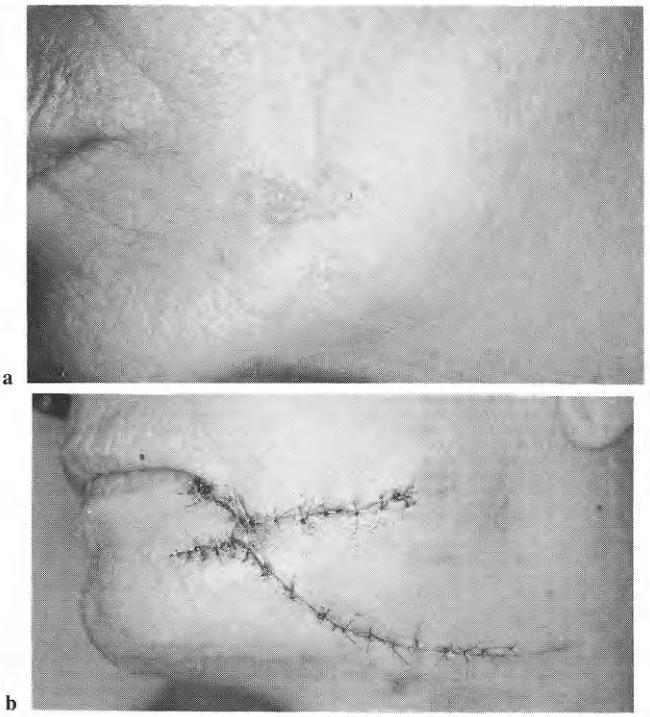

Fig. 14. **a** Basal cell carcinoma on the left mandibular region, preoperative finding. The tumor is much larger than its clinical appearance. The tumor was removed by Mohs surgery. **b** Postoperative result; random pattern flap

Thermography

The various ways in which the human body gives off heat to its environment include heat conduction, heat convection, heat radiation, and evaporation. The skin, and in particular the thermoregulatory vascular system regulating heat exchange via arteriovenous shunts, plays a critical role in the thermoregulation of the body. Thus, measurements of the skin temperature appear to be a suitable method for assessing vascular perfusion in the skin. Thermography measures and records the temperature distribution on the surface of the skin (Stüttgen et al. 1980; Fig. 17). The principle is based on the fact that locally increased metabolic activities or vascular alterations cause a measurable rise or decrease in temperature. The various methods for measuring temperature include contact thermography by means of a detector and contactless thermography based on infrared measurements. Microwave thermography not only registers the temperature on the surface of the skin, but also the temperature of tissue portions up to 10 cm under the surface. Thermography is thus to be preferred for assessments of the total perfusion in areas such as acra (Shwakett et al. 1991). Evaporation cold-generated in

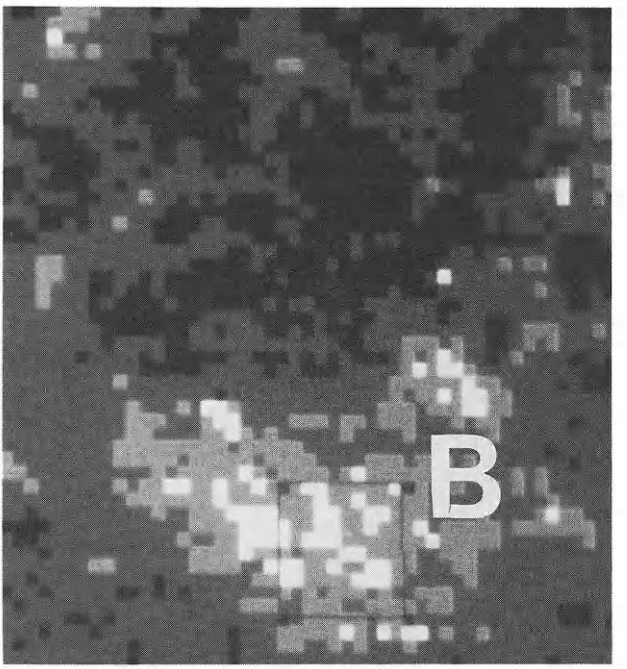

Fig. 15. Laser Doppler imaging of basal cell carcinoma, preoperative finding. Relatively high perfusion in the area of the tumor (*bright colors*) as a result of reactive inflammatory hyperperfusion. It might be that the bright spots around the tumor correspond to parts of the basal cell carcinoma

the presence of wound exudate is an important interference factor that must be taken into account. Measurements of the blood flow in secreting wounds may not be obtained with a sufficient degree of validity due to this factor. We do not use this method in wound healing studies because of its susceptibility to disturbances.

Image Analysis

The term "image analysis" has become a vogue word in medical research. Anything and everything is "analyzed" using computers and specific software. Beginning with radiographs and nuclear magnetic resonance images, these activities have rashly been extended to highly complex fields such as sonography, in which images may contain a large number of artefacts. Even benevolent observers sometimes wonder why certain image analyses presented to them have actually been produced. Nevertheless, image analysis

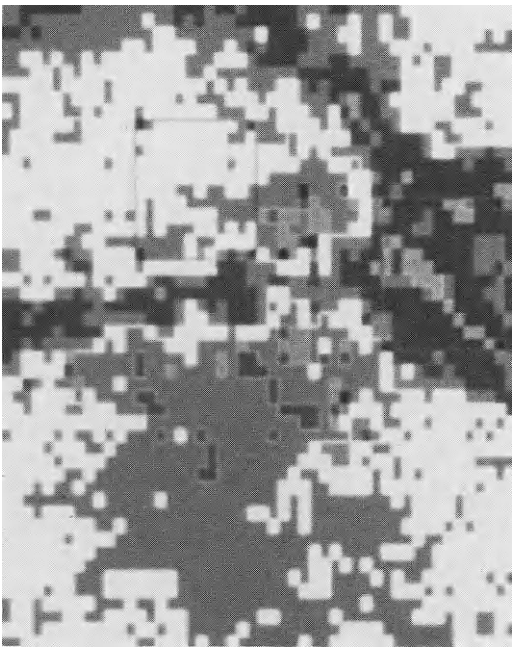

Fig. 16. Laser Doppler imaging on day 8 postoperatively. Hyperperfusion at the cutting edge indicated by *red color*. The center of the flap has gained connection to the surrounding, which means good prognosis for the healing process

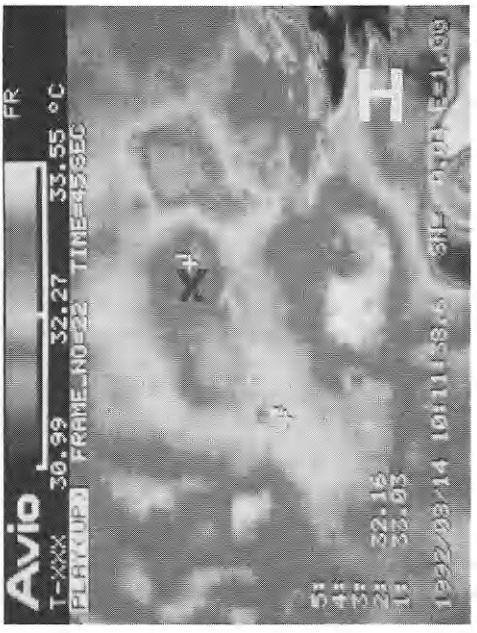

Fig. 17. Thermography image of the face. Color representation of different temperature zones. (The image was taken from a patient also presented in the chapter by Stücker et al., pp. 127 ff; please compare.) This image is comparable to the results we obtained with the laser Doppler imager. *H*, hair; *X*, flap

can provide excellent results in wound healing research. This specifically applies to first order wound healing characteristics, which can be described in mathematical terms. However, some fundamental terms that are frequently confused have to be understood before any discussion of image analysis.

Image Processing

Image processing (IP) is a technology in which the computer input and output consists of images. A variety of image-processing programs offering a multitude of processing functions (filters) is commercially available. If, for example, a digitized image was underexposed when it was initially taken, we can easily change its colors and brightness in the computer system and thus obtain a "usable" version. In scientific research, we can restrict the coloring of an image to a limited set of colors so that a specific area of interest stands out more clearly. Many image analysis studies in wound healing research have made use of this method.

Pictorial Pattern Recognition

Pictorial pattern recognition (PPR) is a technology which describes entire images or detects and sorts specific picture elements for the categorized output in a tabular format. Image data must therefore be stored in a digital format, i.e., the image is transformed into numbers describing its content. This is the underlying cause for the problem mentioned above. Unless the way in which each picture element differs from the next is clearly defined, image analysis must not be used. However, uncritical observers are often led to believe that PPR results in "hard" data that correctly reflects the parameter to be observed. Therefore, it has to be stressed that factors such as the specific illumination applied or the camera distance from the observation object cause problems even when first order wound healing characteristics are to be analyzed. The qualitative description of wound surfaces through image analysis will be described in more detail in another paper by our research team presented in this book.

Pictorial Structure Recognition

Pictorial structure recognition (PSR) is the most widespread and certainly the most useful technique. It measures the surface area, circumference, and the largest and smallest diameter of a wound. Even large image analysis systems provide these parameters. For evaluation purposes, we prefer a combination of the parameters given above and copy the outline of a wound

to film. It is always difficult to obtain a standardized image of the wound, because any changes in illumination, image dimensions, and camera angle have a major effect on the final result. The problem is overcome by the use of special cameras such as the Acmel camera and by including a high-precision color indicator and ruler in every shot. Recently, our research team has used a 35-mm camera (Canon EOS 100) equipped with a macro lens. The built-in electronics of this camera makes it possible to maintain a standardized distance to the photographed objects.

Transparencies can nowadays easily be stored on, and retrieved from, CD-ROMs (photo CDs). The images are then available in a quality highly superior to that of video footage and can easily be further processed as required. Still, it is essential that a series of pictures is taken of a wound from different angles, even when advanced cameras of this type are used. Given the rapidly developing capabilities of modern computer technology and, in particular, of computer graphics, image analysis may well become one of the most important methods of wound healing research in the future.

Outlook

It may be anticipated that as prices continue to drop in the microelectronics industry, the performance of its products will be further enhanced. The capabilities offered by the technologies described above will increase accordingly. Nevertheless, it is certainly too early to recommend the widespread use of bioengineering technologies in routine diagnostics. In our clinic, we use these methods to overcome challenging problems such as the assessment of microcirculation prior to the replacement of a vascularized cutaneous flap or in monitoring the progress of a therapy-resistant leg ulcer. In any case, we think it is essential for physicians to consider the technologies available today.

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Computerized Wound Analysis – A New Method for Assessment of Healing

E. Bengtsson, N. Engström, L. Hellgren, and J. Vincent

In view of the different wound categories and the complexity of wound healing appropriate wound classification is essential for clinically judging healing. Earlier, we introduced, in Scandinavia, a "black-yellow-red" wound classification system, which is based on the clinical stages that occur throughout the healing process [1, 2]. The principal stages are: (1) a *black* necrotic stage with dry and/or moist black necroses; (2) a *yellow* necrotic and/or fibrin covered stage with a dry/moist slough; (3) a *red* granulating stage with healthy granulation tissue.

This classification model is adapted to the gradual shift of the ulcer from the necrotic phase, characterized by the presence of black, yellow necroses, pus, clots or fibrin to the healing stage, in which these tissues are successively replaced by healthy (red) granulations. These macromorphological aspects of healing, in terms of color categories that follow the ulcer condition, were further advanced by Pharmacia/Marion, in Europe and in the US, in the evaluation of various wound dressings [3]. Although this system was suggested primarily for leg ulcers, it encompasses the majority of open necrotic wounds regardless of the cause. In a recent extensive monograph, this three-color classification approach has been adopted as a useful guide both when selecting the various wound management materials and in encouraging medical/nursing staff to assess ulcers in the same clear-cut descriptive terms [4].

Among the currently available evaluation methods, noninvasive approaches, including assessment of the physical appearance of the ulcer, are generally preferred. However, in the clinic, such assessments are largely subjective, mainly because of a lack of reliable tools. The methods availabe may, at best, be considered as semiquantitative, none of them recording ulcer macromorphology directly. In the absence of such important qualitative data (changes in macromorphological appearance), much of the clinically relevant information is lost. Therefore, we have developed a method, based on the three-color classification model, which allows both objective

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evaluation and management of wound healing. This method, *computerized wound analysis* (CWA), is rapid and simple to handle in the clinic, yet sophisticated regarding the analysis [5, 6].

As input, the CWA method uses close-up color wound photographs taken at regular intervals. An Acmel camera with constant focus and lighting ensures a simple photographic routine which is easy to use even for clinical personnel without previous experience in photography. In every wound photograph a reference millimeter-gray scale is included, attached close to the ulcer edge. The shape of the scale and the colors (as seen in the photograph) enables the photographer to check the criteria obtained from an acceptable photograph. Since Polaroid color film is used, direct control of the photos or retake is possible within an interval of 2 min.

The resulting wound pictures are sent to the CWA Institute for analysis. Each photograph from a series of wounds, taken during the whole treatment period, is carefully identified (patient, date and treatment code) and digitized via a charge-coupled device (CCD) video camera. Our computer technology offers a convenient way to analyze a picture by scanning it point by point, recreating the true color and area of the original wound. This makes possible objective measurement of the wound area and the relative proportions of black, yellow necroses/fibrin and red granulations (all being integral parts of the CWA concept). The color classifier in the computer presents the wound in the form of points (pixels), each arranged as a three color value. The color of every pixel is thus expressed as a composition of three intensity values (0–255) representing red, green and blue. The method is implemented using an IMTEC Epsilon image processing system (IMTEC, Uppsala), which offers very short retrieval times and an accurate wound classification (Fig. 1). Briefly, the method includes the following advantages:

1. Photographic documentation of ulcers is done by simple, reliable and quick procedures.
2. Analyses are practicable from a great number of wound photographs.
3. Every point of the ulcer, via the photographic medium, is objectively measured by a computerized scanning process having reference to changes in true color and area.
4. Collected data are specified in accordance with strictly standardized methods of measurement.

In order to assure maximum reliability of the CWA method several validity studies have been carried out. A representative selection of ulcers was assessed by a number of clinicians using different methods (visual estimates or wound tracings). The results from these evaluations were then compared with the CWA data and statistically analyzed. There is no doubt that the CWA method is far more accurate and reliable than conventional "manual" methods [7].

In conclusion it is evident that many of the methods used to assess wound healing parameters are basically unreliable. We are convinced that the advantages of new therapeutic modalities for ulcer management emer-

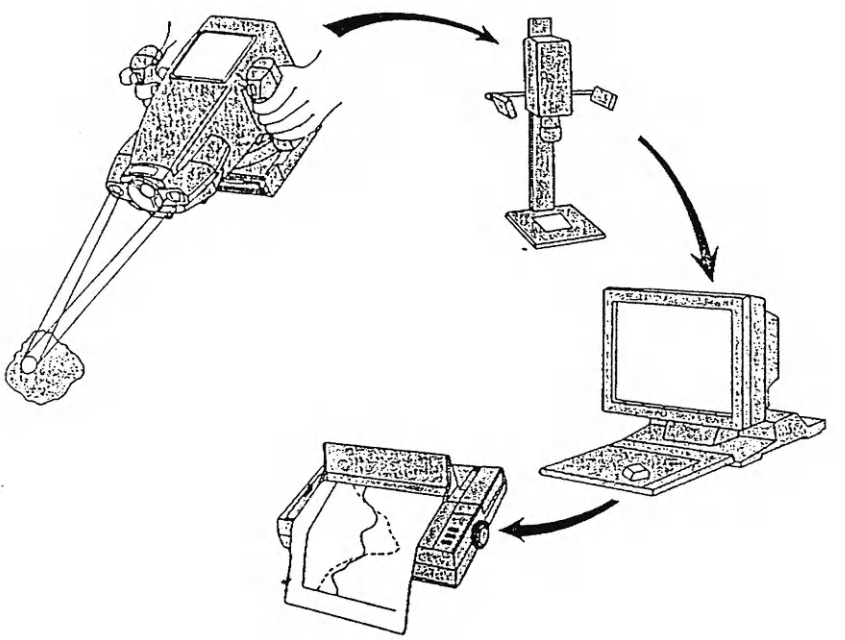

Fig. 1. Flow diagram for computerized wound analysis

ging in the future will be convincingly demonstrated by CWA. This unique tool ensures effective evaluation of the multiple measurements needed for statistical interpretation of wound healing.

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A Color Image Analysis System (CD-CWA) for the Quantification of Wound Healing in Multicenter Trials

S. el Gammal[1], M. Brand[2], C. el Gammal[1], K. Hoffmann[1], and P. Altmeyer[1]

Why Is Quantification of Wound Healing So Difficult?

Selection of Parameters

Clinical trials in wound healing are confronted with difficulties in the selection, definition, and assessment of parameters. Presently, there are no clear-cut, internationally accepted clinical research guidelines to quantify wound healing [1].

Measuring the maximum diameter of the wound neglects wound retraction, which occurs mainly in one direction, leading to spindle shape of a primarily round wound during healing. Most frequently, the wound area is evaluated in order to assess wound healing. However, this ignores wound depth and the quality of the wound surface. A wound can only epithelialize when the granulation tissue has reached the height of the surrounding epidermis.

Therefore, the wound volume has been postulated as the optimal parameter to quantify wound healing. Problems arise from the fact that the volume depends to a great extent on the positioning of the patient, especially when assessing pressure sores. In venous leg ulcers, the curvature of the leg makes surface evaluations much more reproducible than volume measurements.

Apart from these morphometric parameters, the quality of the wound surface is of interest. A necrotic/fibrinous wound surface delays healing. In pharmacological wound healing models [2, 3], it has been demonstrated that the healing time decreases with a pharmacologically induced acceleration of debridement. The three parameters necrotic surface, granulation tissue, and epithelialization interact directly; they can be expressed as a percentage of the total original wound surface. A prerequisite for this qualitative assessment is the measurement of the wound area. Several recent studies [4, 5]

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have used a colorimetric black–yellow–red (BYR) classification scheme [6], which considers only the wound surface and ignores epithelialization. A gradual shift of the ulcer surface from the necrotic phase, characterized by the presence of black elements, of pus, necrosis and fibrin clots, which are yellow, finally to healthy red granulation tissue is assumed.

Classification of the wound surface into the mentioned categories often poses problems, e.g., thin blood crusts overlying granulation tissue can simulate necrosis; hyperergic vasculitis, which can initiate necrosis, may resemble granulation tissue, and the thickness of necrosis is very difficult to judge.

Patient Follow-up

Heterogeneity of wounds, of treatment regimens (in mulicenter trials), of compliance (especially if long follow-up is required), and the variety of underlying diseases complicate the collection of reproducible and comparable data. Epidemiological data on patients with chronic wounds may illustrate some of the problems.

In a community survey [7] with 477 venous leg ulcer patients, the ulcers had been present for a median of 3 months with a range from 1 week to 63 years and with a range of ulcer size from 0.1 to 117 cm^2. There was a very wide range of healing times. Recurrence rates were reported to be very high. In an epidemiological survey, 49% of patients with venous leg ulcers were reported to have a reulceration within 3 months after hospital discharge [8]. Ulcer size, duration of ulceration, age, and deep vein involvement are prognostic factors for the healing time in venous leg ulcers [9].

Longitudinal follow-up of nursing home residents with pressure ulcers showed a healing time of 1 year for the majority of the lesions [10]. The long healing time is a major problem in wound healing trials, as many patients die during follow-up or are referred to other units or nursing homes. According to our experience, even trials with a short observation period are difficult to manage.

Present Approaches to Quantify Wound Healing

Clinical Assessment

The simplest and most widely performed evaluation of wound healing is clinical follow-up at specified intervals. The wound surface and its borders, its size, and shape have been used to evaluate wound healing clinically. The great disadvantage of this approach is its subjectivity and wide interobserver variability.

Planimetry

Planimetry involves direct wound tracing with a transparent sheet placed on the wound in order to delineate the wound edge. Later, series of sheets may be superimposed, making it possible to objectively assess the healing rate. When cut out, the delineated sheets may then serve as basis for a planimetric evaluation using gravimetry. Although foil planimetry may seem acceptable, it causes discomfort to the patient and enhances the risk of wound contamination.

Photographic Assessment

Photography avoids contact with the lesion and thereby prevents contamination and tissue damage. It can be performed quickly and without discomfort to the patient. In enables evaluation independently of the patient and comparison of the wounds at different time points and stages of healing. Several investigators can judge the pictures independently of each other. This provides much better reproducibility and objectivity of the assessments. Brown et al. [11] have used this procedure to study wound healing in patients with donor sites.

Photographs were shot at daily intervals with a Nikon FE2 camera, a Nikon macro lens, and a ring flash at a distance of 46 cm and were evaluated by two observers in a blinded manner.

Polaroid systems have the advantage of instant prints and therefore provide significantly fewer logistical problems with respect to processing, storing, and identification of the photographs in a multicenter setting. Their greatest disadvantage is the low resolution and a low depth of field in comparison to 35-mm slides taken with a closed aperture. In our experience, this is also true for specially designed camera systems such as the Acmel camera [12]. Moreover, with this camera, the missing viewer poses an additional problem.

We also examined the Medical Nikkor 35-mm camera. Here, the aperture is linked to the distance (the greater the distance, the wider the aperture), thus leading to a limited depth of field in the distances most frequently used when photographing chronic wounds. The lack of a motor for the film transport is a further obstacle, as it is desirable to take at least ten photographs per session.

Photographic evaluation is often accompanied by geometrical perspective distortion. Palmer et al. [13] have shown that surface assessment of a predesigned irregular shape on a plane model is quite reliable as long as the camera is within 10° of the right angle to the object. This optic geometrical error can be corrected using transparent graph paper or using a reference scale.

Photographs can be used to evaluate the quality of the wound surface. For this purpose, comparable lighting conditions are crucial.

Casting

Wound volume can be assessed by casting; dental impression materials [14] and silicon replicas [15, 16] have been used for this purpose. The volume can be measured by immersing the cast in a measuring bath (Archimede's principle). The surface topography of the cast can be analysed using stylus, laser [15], or structured light [17] profilometry. Unfortunately, accurate casts of superficial wounds on convex or concave surfaces are difficult to obtain and the interaction of the cast with the wound secretion present unsolved problems.

Photogrammetry

When stereo photo pairs of a wound are available, its volume can be evaluated by stereophotogrammetry [18, 19]. This method has been developed and further refined from map landscapes in geography [20]. Mechanical precision equipment and complex time-consuming computer evaluations considerably limit this method. In contrast to cast techniques, the advantages of photographic wound assessment, as mentioned above, are available as well.

Image Analysis

In recent years, computerized wound analysis has been propagated [18]. Most CWA systems consist of a video camera, a frame grabber, a computer, and image analysis software [21, 22]. The resolution of standard video technology is quite low and varies between 512 × 512 and 1024 × 768 pixels per image. Still video systems (Canon) have 470000 pixels per image. Vincent et al. [23] have shown that computerized wound analysis reduces both intra- and interobserver variablity and that it is reliable in comparison to clinical assessment.

In an ideal situation, wounds of approximately the same size are studied by the image analysis system. A fixed camera-to-wound distance can lead to resolution problems if the size of the wounds varies considerably. If the preadjusted image area is 20 × 20 cm, a small wound of 1 × 1 cm would only represent 0.25% of the total image surface. If the image area has 262000 pixels, the region of interest would only represent 655 pixels. This leads to significant limitations, especially in a multicenter setting.

A New CD Color Wound Analysis System (CD-CWA) Suitable for Multicenter Trials

Ideally, wound analysis should assess the wound surface quality and wound area simultaneously. We designed a color wound analysis system which uses high-resolution images for precise wound quantification. This system has been validated in a red-black-yellow convex wound model so far and wound healing was studied in 100 patients at baseline with seven follow-up sessions. The concepts and features of this system are described below.

Photographic Documentation

Photographic films with fine granularity are particularly suitable for documenting wounds of greatly varying sizes because this analog medium has a high detail resolution. The following requirements have to be met: (a) optical lenses of good quality should be used to reduce geometrical errors and poorly focused picture edges; (b) the camera should be easy to handle; (c) image acquisition and storage should be fast; and (d) the wound region illumination should be uniform. In a multicenter study, fixed automatic camera setting (i.e., photographic distance) without manual adjustments is desirable.

We studied chronic decubitus and venous leg ulcers with baseline areas of between 2 × 2 and 14 × 14 cm. To attain sufficient resolution in the region of interest without changing the optical magnification we use 35-mm slides, because this film material affords an excellent resolution.

Our camera system consists of a Canon 35-mm body (EOS100), a ML 3 ring flash, and a 50-mm macro lens. This camera is suitable for photographic documentation in a multicenter setting. To limit the time of the photo session, the camera is equipped with an automatic film drive. Misfunction is minimized by an automatic adjustment to film sensitivity and shutter blockage when the film is not inserted correctly, already exposed, or rewound. A camera back was used, which fades in the date for identification of the photographs.

We preselect a distance of 40 cm for all photographs by fixing the distance ring. The infrared distance meter emits an acoustic signal when this distance is met. A fixed aperture of 16, a shutter speed of 1/125 s, and an automatic TTI flash exposure is used. We suggest using 35-mm slides, because film development is particularly well standardized. A medium film sensitivity (21 DIN) was selected in order to assure rapid recharging of the flash.

In comparison to other cameras and video systems, this camera set-up is easy to use in a multicenter setting and provides pictures with a good depth of field (aperture of 16 and TTL control of the flash).

The position of the patient and the investigator are recorded at baseline. This position should be reproduced as exactly as possible during all follow-up visits. The reference scale is fixed vertically (as seen by the investigator) next to the left border of the lesion. When taking vertical photographs, the left border of the reference scale appears parallel and close to the left border of the viewfinder. The infrared distance meter is activated by gently pressing the shutter. When moving the camera up and down along the vertical axis of the wound surface, an acoustic signal is heard as soon as the selected 40-cm distance is reached. Ulcers which circumvent the leg surface are documented by tilting the camera at different angles.

Reference Scale

A reference scale is used to calibrate distances and to correct for color variation due to exposure and/or film emulsion in each image (Fig. 1). The

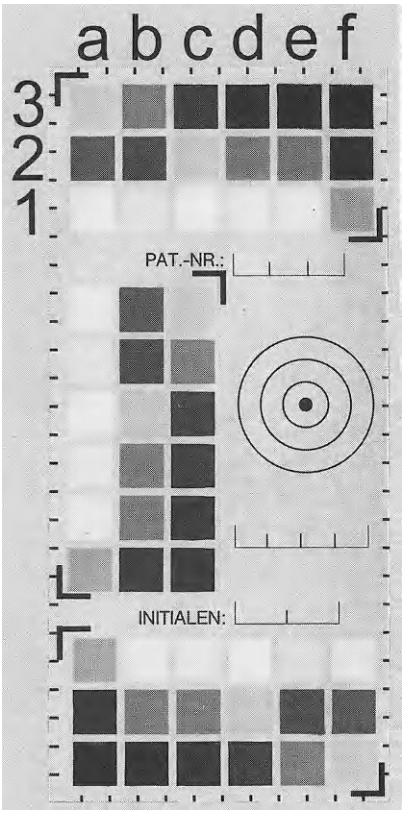

Fig. 1. Reference card used for color correction and distance calibration

Table 1. Lab values of the color block on the reference card of Fig. 1

	Color	L*	a*	b*	L′	a′	b′	Used pigments
1a	Light blue	94.89	−2.83	− 0.04	93.02	−2.14	0.27	8020003 (blue) 0020001 (white)
1b	Light green	93.57	−7.97	− 0.26	92.12	−7.74	−0.40	8020012 (green) 8020001 (white)
1c	Light yellow	95.65	−3.61	12.39	94.5	−3.78	11.78	8020011 (yellow) 8020001 (white)
1d	Light red	91.58	9.26	5.15	90.43	9.02	5.65	8020026 (orange) 8020001 (white)
1e	Light magenta	92.42	5.49	0.19	91.03	5.95	0.69	8020015 (red) 8020001 (white)
1f	Light brown	77.31	−0.14	16.14	78.02	−0.39	13.92	8020011 (yellow) 8020002 (black) 8020026 (orange) 8020001 (white)
2a	Intensive blue	60.09	−22.70	−30.99	59.68	−10.86	−32.00	8020003 (blue) 8020012 (green) 8020001 (white)
		73.38						
2b	Intensive green		−27.84	2.67	56.00	−37.6	−5.3	8020012 (green) 8020001 (white)
		88.09						
2c	Intensive yellow		−2.68	89.32	88.08	−10.8	77.73	8020011 (yellow) 8020001 (white)
		52.11						
2e	Intensive red		53.29	43.22	54.0	53.3	38.09	8020026 (orange) 8020015 (red) 8020001 (white)
		49.78						
2f	Intensive magenta		46.54	5.96	52.69	48.27	6.65	8020015 (red) 8020030 (bluered) 8020001 (white)
		29.18						
	Intensive brown		6.81	8.11	35.97	1.88	7.91	8020026 (orange) 8020015 (red) 8020002 (black) 8020001 (white)
3a	Pure black	34.09	5.07	4.37	32.64	−2.56	2.05	8020002 (black)
3b	1. Lightness	36.08	−2.68	5.79	36.08	0.02	−0.66	8020002 (black)
3c	2. Lightness	43.00	−7.15	−7.71	43.92	1.03	−1.81	8020001 (white)
3d	3. Lightness	42.68	−0.29	−2.55	45.89	−1.42	−2.91	
3e	4. Lightness	68.98	−0.85	−3.67	70.59	−1.43	−2.84	
3f	5. Lightness	86.89	−0.54	−1.14	86.64	−0.39	−0.12	

* Photometer; ′ colorimeter.

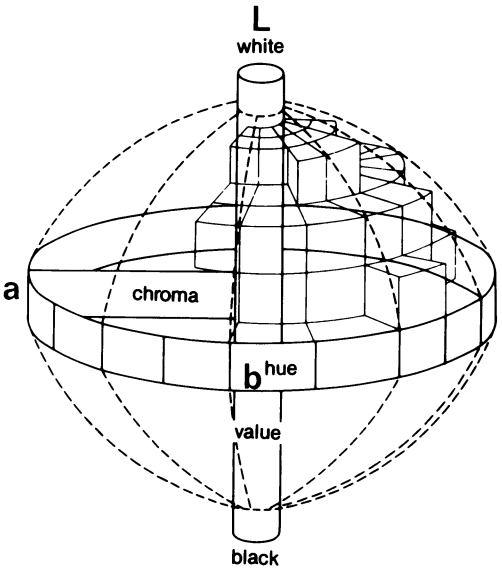

Fig. 2. The **Lab** color representation system forms a three-dimensional ovaloid, where all possible colors are included. **L**, lightness factor; **a**, red–green axis; **b**, blue–yellow axis. By moving in a radial direction, the chroma (saturation) of the color changes; by moving circularly, its hue changes

patient number and initials can also be documented on the card. We designed a self-adhesive paper card (12 × 5 cm) showing concentric circles with 1-, 2-, and 3-cm diameter for distance calibration. If these circles turn out to be elliptic on the photographs, there is significant distortion and perspective error in the images. Often reflections are seen on the images, rendering color evaluation difficult. We therefore included three identical reference color blocks on the reference card at 0°, 90°, and 180°. This redundancy ensures that a complete set of all colors can be used for reference.

Every block has a total of 18 color reference rectangles (12 colours and six shades of gray) with a matte finish. Every rectangle was evaluated using a spectral photometer optronic color flash with diffuse 8° illumination geometry and a Minolta tristimulus colorimeter CR200 (Table 1). Colors are expressed as Lab values within a three-dimensional color ovaloid (Fig. 2). The L value describes the color lightness (intensity) and varies between 0 (black) and 100 (white) on the vertical axis. The a value describes the red–green and the b value the blue–yellow color component. Radial movement in the three-dimensional ovaloid changes the chroma value (color saturation), and circular movement influences the hue of the color (Fig. 2). For every instrument, inter- and intravariability of the identical color rectangle was less than 0.1% during repeated measurements. The manufacturer guarantees high color reproducibility ($\Delta E < 1$ with $\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}$).

Multicenter Monitoring

All exposed films are sent to the same processing laboratory. In multicenter trials, recorded delivery of a larger number of films is advisable. Intense monitoring is a prerequisite and can reduce missing data due to logistic and quality-related problems to 2%–4%.

CD-ROM Transfer

The best 24 × 36-mm slides are selected from each photosession and matched. The following criteria must be met:

1. Identification must be unambiguous, based on the reference scale, patient number, study code, and patient initials.
2. The photo is taken at 40-cm distance (1:6 scale) with sufficient lighting.
3. The entire wound surface is seen.
4. At least one color reference block is in the vicinity of the wound.

The selected slides are digitised on "write once" Kodak photo compact discs (CD). These particular CDs are made of polycarbonate material covered by a layer of organic dye. A recording laser beam changes the reflectivity of the dye. To improve the reflection properties for the playback laser beam, the CD is gold mirrored beneath the dye layer [24]. Each picture is a two-dimensional array of picture elements (pixels) which is saved as a single file on the CD. Every picture is accessible in five different resolutions (192 × 128 to 3072 × 2048 pixels, 16 million colors). At high resolution, the size of a single image file ranges up to 6 MBytes.

Color Image Analysis Software

In most computer applications, pictures are treated as two-dimensional arrays of pixels. In black-and-white pictures, every pixel is visualized as a gray spot. Its gray level is represented in the array as an integer value between black (e.g., 0) and white (e.g., 255). To improve the differentiation of small gray level differences for the eye, some applications map every gray level to a specific color [25].

In true color images, every pixel has a separate Red, Green, and Blue intensity level. All available colors are included in a three-dimensional cube, where the three axes correspond to red, green, and blue intensities (Fig. 3). The number of supported intensity levels varies in different applications. Most color graphic cards support 255 (8 bits) levels per channel (24 bits per pixel). Standard RGB colour monitors are used to visualize these images.

This RGB colour coding is quite abstract and does not correspond to the categories of human color perception. We prefer to use the HSI (hue,

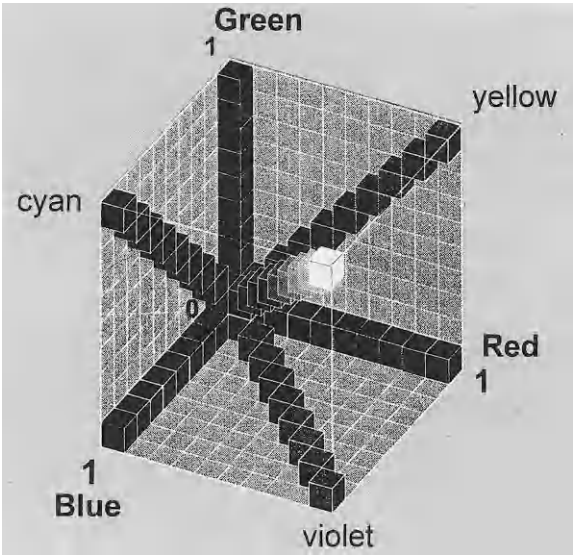

Fig. 3. The **RGB** color coding system can be represented as a cube. Every color is described by three cartesian coordinates (R,G,B), which describe its red, green, and blue intensity. Their intensity varies between 0 and 1. The corners of the cube are red (1,0,0), green (0,1,0), blue (0,0,1), yellow (1,1,0), cyan (0,1,1), violet (1,0,1), black (0,0,0), and white (1,1,1). Shades of gray are located on the black–white diagonal

saturation, intensity) color coding system, because it is more intuitive than the RGB or Lab system. The HSI system is just a different description of the same three-dimensional color ovaloid we had encountered to illustrate the Lab system (Fig. 2). The horizontal cartesian *a* and *b* components of the Lab system have been replaced by a polar coordinate description (Hue and Saturation). The description of the vertical component of the ovaloid is identical in both systems. The *L* value of the Lab system corresponds to the I value of the HSI colour coding system.

AnalySIS (Soft-Imaging Software GmbH, Münster, Germany) is an open architecture software program under Windows 3.1. It supports virtual image buffers of different sizes, image filtering, automatic paricle detection, and sorting. All functions are available in a macro library and can be combined using the integrated C-code interpreter. Semi-automatic evaluation macros were designed to evaluate decubitus and venous leg ulcers.

Wound Evaluation Steps

Images are loaded as minipictures from the photo CD using Kodak ACESS software. The wound area (region of interest) and the reference card region are cut out separately in every image and saved as TIFF files (Tag Image

File Format Specification Revision 5.0; Aldus Corporation, 411 First Ave South, Seattle WA 98104, USA). The TIFF-image pairs are loaded into the program AnalySIS. The picture of the reference card is necessary to correct geometry, film emulsion, and exposure in the region of interest.

The ulcer outline is detected by AnalySIS using an edge detector. The investigator can accept or modify this outline. Classified wound regions (e.g., fibrinous layer) are automatically detected using HSI-color ranges and the mean color and its standard deviation are evaluated. White reflection spots are excluded from surface color classification and included into total surface area measurement. Finally, the percentage wound surface covered in follow-ups in comparison to the baseline is evaluated for statistical analysis.

We are now developing algorithms which will hopefully be able to detect the classified parts of the wound surface even when total image illumination varies significantly.

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Noninvasive Assessment of the Physical Properties of the Skin

P.G. Agache and P. Humbert

This short presentation of some noninvasive methods used in functional assessment of normal and diseased skin will be discussed from the point of view of the user, namely, the dermatologist.

Mechanical Properties of the Skin

Significant progress has been made in the assessment of skin mechanical behavior during the past decade [1]. This is a very important field if one considers the wide range of diseases in which the mechanical behavior of skin is impaired. It includes inherited disorders such as pseudoxanthoma elasticum, Ehlers-Danlos syndromes, Marfan's syndrome, and osteogenesis imperfecta. Postinflammatory skin sclerosis includes the goitrous ankle and foot skin of postphlebitic syndrome, hypertrophic scars of deep burns, induration of radiodermatitis, scleroderma, morphea. Keloids, and striae distensae. In all of these conditions the mechanism is poorly known and the mechanical trouble is of major concern.

Let us first consider the mechanical tasks of the normal skin. It should resist mechanical injuries, extend upon joint movement or muscle contraction, recoil upon stress discontinuation, withstand frictional forces, and, equally important, adhere to the ground when we run barefoot and allow us to grasp objects without them slipping away.

Skin hardness can be assessed using a durometer. This device consists of a sharp ended rod pressed onto the skin with a given force: the consecutive depression is then assessed. The more specialized tools, called indentometers, are based on the same principle [2] but their use is limited to bony areas. Only prototypes are as yet available. Resistance to mechanical injuries has also been tentatively assessed by an interesting method called by Tosti (from Palerma) ballistometry [3]. It uses a kind of hammer, the rebounds of which over time depend on the skin hardness and its elasticity. This promising technique is not yet commercially available.

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Altmeyer et al. (Eds)

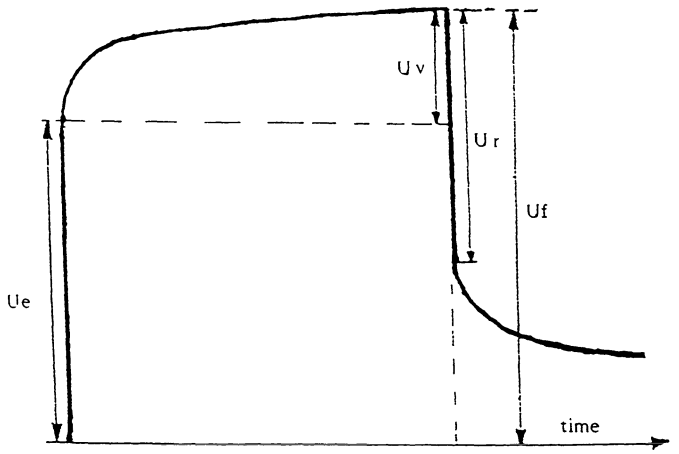

Fig. 1. Skin elongation following application of a constant force (torsional experiment). U_v, asymptotic (viscous) elongation; U_e, immediate (elastic) elongation; U_f, total (final) elongation; U_r; immediate recovery

Most of the progress over the last 10 years has been in the field of skin extensibility assessment. The presently commercially available devices allow experiments using suction (Cutometer, Courage-Khazaka, Köln, Germany and Dermaflex, Cortex Technology, Copenhagen, Denmark), torsion (Dermal Torque Meter, Diastron, Andover, GB) and uniaxial tension (Skin Extensometer, Cutech-Stiefel Laboratories, GB). Skin extension tests can be done in two ways: either by applying a sudden and constant pull and recording the amount of extension vs time, or applying a stepwise increasing pull and recording the amount of extension vs both force and time.

The first type of experiment has been widely used with interesting results. Whatever the device the curve always has the same shape (Fig. 1). An immediate elongation (U_e, e standing for elastic) is followed by a delayed and asymptotic elongation (U_v, v for viscous) or creep, the total elongation being called U_f (f for final). When traction is suppressed, an immediate recovery takes place (U_r) which is always incomplete by this time but will always be completed within a period of time (a couple of minutes to a few hours) depending of the skin tension. This shows that the skin is both elastic (it fully recovers following extension) and viscous (both passive extension and active recoil comprise a delayed phase). The immediate elasticity (i.e., the elasticity in a clinical sense) can be easily obtained by the ratio U_r/U_e. Such a simple experiment has proved useful both in assessing the severity of diseases in which skin extension is reduced or increased and in the follow-up under treatment. However, a healthy symmetrical control area in every patient was needed or, in case of bilateral involvement, the subsequent test needed to be made at exactly the same place as the preceding one.

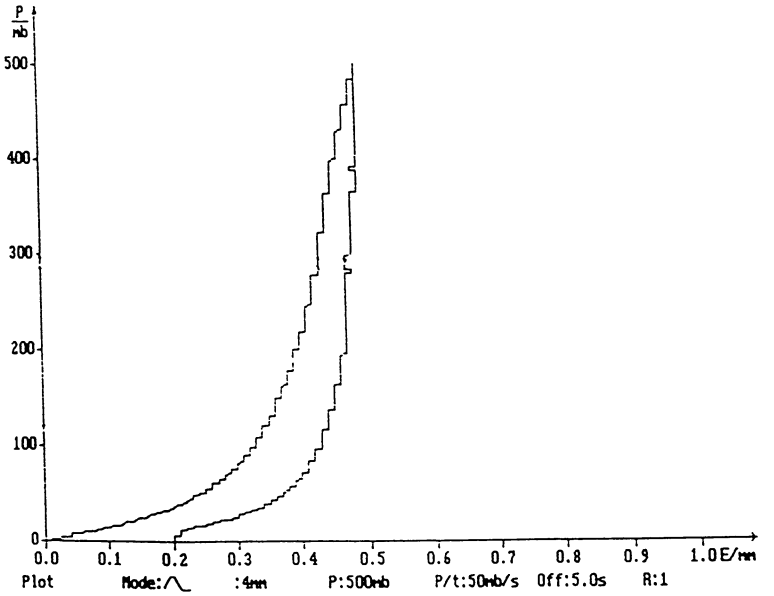

Fig. 2. Force evolved within skin following extension at a constant rate (suction experiment)

When this experiment is repeated after a short time interval, namely, before full recovery is achieved, lower U_e and U_f and greater residual extension ($U_f - U_r$) are obtained, but the difference rapidly and asymptotically tends to zero. Stable U_e and U_f are usually obtained from the fourth run onward. Residual deformation following this series of runs can be used as a viscosity parameter because it is related to the hysteresis loop (see below).

The second type of skin extension test consists of progressively pulling the skin, then going back at the same pace down to zero, while continuously recording the extension (Fig. 2). Upon increasing the pulling force, two types of mechanical behavior are displayed, first a phase of high extensibility, then a phase of low extensibility. The tangent to the curve is an assessment of skin extensibility. When the pulling force is subsequently decreased, the retraction is delayed. The area between the ascending and descending curves is the hysteresis loop. It is related to skin viscosity, more precisely, to the energy spent by the skin viscous component (which depends on the extension rate and magnitude).

With previous assessment of skin thickness at the same site, very important information can be obtained on the skin's intrinsic elastic strength upon extension, namely, the Young's modulus (E) through the formula: $E = \sigma/\varepsilon$, in which ε is the relative elongation (strain) and ε is the stress, i.e., the pulling force related to the skin area submitted to this force. As this area and ε are related to skin thickness (d) and to immediate extension (U_e), respectively, variations of E parallel those of $d \times U_e$. In this way and with

the first type of experiment described above and using a torsional device, we found that, in Ehlers-Danlos syndrome type II, skin hyperextensibility was related only to a decrease in skin thickness, whereas in Marfan's syndrome it was independent of thickness and, accordingly, was an intrinsic property of the skin fibrillar network [4]. However, the $d \times U_e$ product is only indirectly and approximately related to E, as neither the actual relations between U_e and ε, on the one hand, and between force/skin thickness and σ, on the other hand, are known.

Recently, using a suction device in a constant force experiment, we found a way to calculate in vivo and noninvasively the absolute value of E, thanks to a geometrical model of skin deformation that allowed us to obtain the actual value of both stress and strain. We found 26 kPa ($n = 70$) for the median value of E in the volar forearm skin of young adults, with a strongly asymmetrical distribution [5]. This value represents the absolute elastic strength of the skin under normal tension with supine rotated wrist and relaxed fingers. Another value would be obtained in another limb position. With a torsional device E should be replaced by the shear modulus, which can be only very approximately estimated [6].

Assessment of Skin Thickness

Whole skin thickness can be assessed in three ways: (1) using a caliper and measuring a skinfold thickness, (2) by soft X-ray tangential to the skin surface, or (3) by using ultrasound in A or B mode. Caliper assessment always needs to be performed by the same person to be reproducible; however, it overestimates the skin thickness by taking a part of the subcutis fat. X-ray is invasive and no longer used. Ultrasound is considered to be the quickest and most reliable method. As the dermis-subcutis border is deeply serrated (retinacula cutis), B mode, which allows averaging of thickness along a few millimeters, is preferable to A mode, which assesses thickness at a single place [7].

Assessment of Skin Relief

The skin relief pattern is inherited and characteristic of each body site. Anatomically, it occurs mostly within the subpapillary dermis and within the epidermis for the shallower sulci. Its relationship with skin mechanical behavior has been established so that directions of deeper furrows coincide with Langer's lines. These are the direction of lower extensibility, higher tension and higher density of collagen fibers.

The usual way to assess the skin surface relief is profilometry. As skin relief is made of furrows delineating plateaus, the common roughness parameters, such as Ra and Sm, which are based on a mid-plane, are poorly

suited. Assessing the height of both sides of each furrow relative to the next plateaus, and the spacing between furrows, provides more appropriate parameters. Profilometry cannot be done directly on the skin but on a positive replica, usually made of araldite, made out of a negative replica of silicone rubber. Optical scanning is not yet possible, owing to lack of precision. Instead, a diamond cone is placed on the surface and moved by a stepping motor. When parallel scans are made of a skin surface area, the altitudes and positions of all the assessed points can be fed into a computer and result in a 3-D representation of the skin relief over the tested area [8, 9].

Skin relief can also be assessed by image analysis of negative replicas. Altitudes are obtained by computer analysis of the shades induced by oblique illumination [10, 11]. Owing to pattern anisotropy the procedure should be repeated in several directions. The method has the advantage of being much quicker than profilometry, but it is less precise.

When only the relief pattern is to be assessed, without need of depth information, image analysis of transilluminated araldite positive replicas is the best method.

Wrinkle assessment can be made either by profilometry, using a mechanical or an optical sensor, or by image analysis. This requires special parameters.

Profilometric scanning with 3-D reconstruction is the best way to follow up the wound healing rate, because both granulation tissue production (inverse of wound volume variation) and epidermis proliferation (inverse of wound area diminution) can be measured by comparing negative replicas taken at selected intervals [12]. The same method can be used for assessing an increase or decrease in tumor size.

Assessment of Hair Physiology

Hair loss is the most frequent complaint from dermatology patients; accordingly, its reality or severity can be assessed by a trichogram. Usually 40–50 hairs are plucked from three sites (vertex, frontal and parietal areas) and bulbs are examined for growth stage: anagen, telogen, or catagen. Also, dystrophic hairs and bulbs with outer root sheaths are counted. Throughout life, the percentage of telogen hairs is rather stable (Fig. 3), except in the postnatal weeks. Hair loss can be considered as abnormally high if at a site the telogen count is more than 15%. Seasonal variation and aging should be taken into account before interpretation.

Hair density is a useful parameter in assessing several hair diseases: measurement can be done easily by taking a close-up picture of a small area in which the hairs have been cut to approximately 1 mm above the skin surface and then counting the number of hair shafts on the enlarged picture using a grid. In normal subjects the daily loss is about 150 hairs, most of

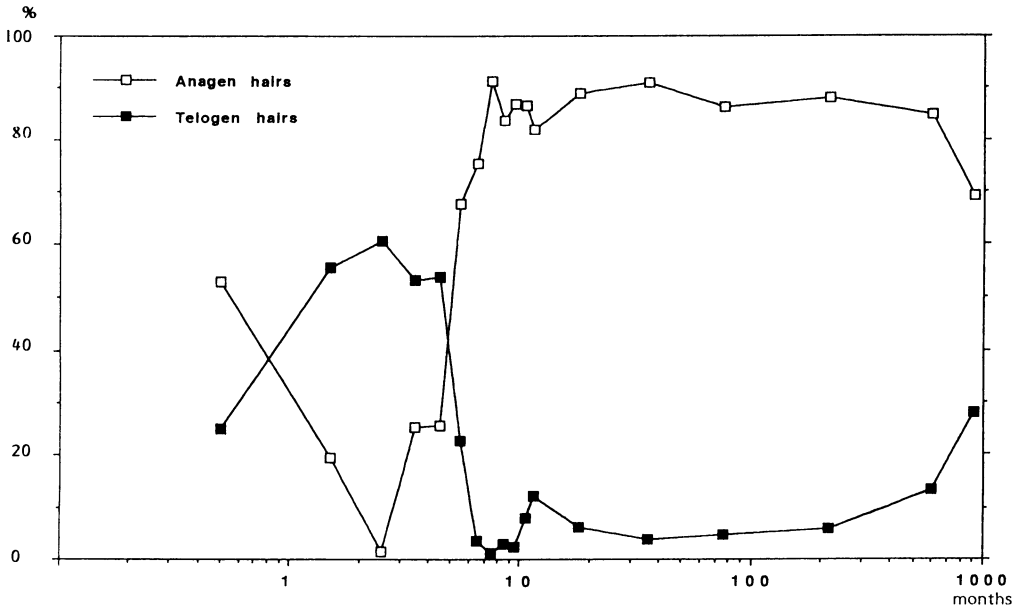

Fig. 3. Percentage of anagen and telogen hairs throughout life (102 normal subjects)

them being vellus hairs. Accordingly, when assessing hair density, terminal and unmedullated hairs should be counted separately, as their cosmetic meaning is different, vellus hairs being almost invisible to the naked eye.

Hair growth rate assessment has little relevance in medicine because while the trichogram allows determination of the proportion of resting (telogen) hairs, no diagnosis is based on an abnormal hair growth rate. Accordingly, such assessment is only useful to demonstrate the efficacy of hair growth promoters. Presently available techniques are based on the measurement of the length of hair grown during a particular time interval and is preferably done by taking close-up pictures of the same area with the hair pressed onto the skull under a piece of transparent tape.

A useful hair parameter would be the assessment of hair life span. Up to now this has not been possible because hairs are periodically cut, so that their length is not relevant to their growth duration. We can only measure hair diameter which is related to the growth rate and usually demonstrates a gaussian distribution.

Conclusion

From these few examples and those which will be discussed in the next chapter, one gets an idea of the impressive progress made in noninvasive

assessment of skin structure and function which has been made over the last two decades. In particular, the assessment of skin static structures (anatomy, histological pictures) has been greatly improved by the use of image analysis and computers. These two parameters, structure and function, are interrelated as function governs structure and structure often predicts function.

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Noninvasive Evaluation of Skin Barrier Function

E. Berardesca

Introduction

One of the more intriguing aspects of skin physiology is the capability of the skin to modulate the exchange of water, ions, and oxygen between the body and the environment and to regulate the penetration of chemicals and topically applied drugs. This complex function of the skin is carried out by the stratum corneum and is referred to the skin barrier function. The organization of the stratum corneum has been efficiently compared by Peter Elias [1] to a wall, in which the bricks are represented by the corneocytes and the mortar by intercellular lipids. The lipids are arranged in lamellar bilayers, are composed of fatty acids, cholesterol, sphingolipids and ceramides and constitute .11% of the stratum corneum weight [2]. There is now good evidence that the barrier function resides within the lowest region of the stratum corneum [3].

Several in vivo bioengineering techniques have been developed over the past decades to measure accurately some of the biophysical parameters related to the integrity and efficiency of the skin barrier. When the barrier is perturbated a change in these functional parameters can be recorded, allowing the investigator to monitor the pathophysiology of skin reactions.

Water Flux

The most important and widely utilized technique to evaluate the skin barrier is the measurement of transepidermal water loss (TEWL) [4]. TEWL represents the amount of water evaporating from the body towards the skin surface and the environment. When the barrier is damaged, the stratum corneum is unable to trap and hold water and therefore TEWL is increased. TEWL can be easily measured using an evaporimeter, which consists of

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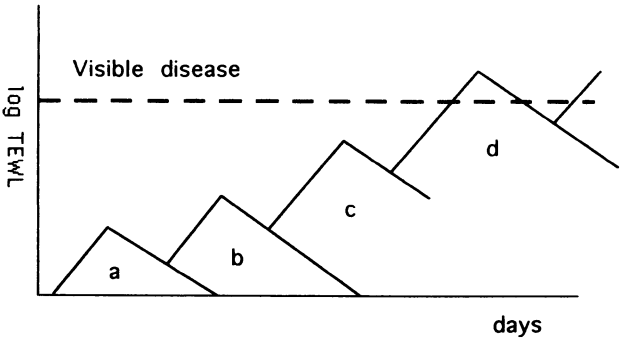

Fig. 1. Chronic irritant dermatitis is the result of several cumulative insults damaging the barrier. This damage can be monitored by transepidermal water loss (*TEWL*) measurement before the appearance of visible disease. (From [6])

two hygrosensors applied to the skin surface, which measure the flux of humidity towards the environment [5].

When the physiology of the barrier is perturbated there is an increased risk of developing contact dermatitis, both allergic or irritant, due to an increased penetration of allergen or irritants through the skin. TEWL can evaluate the state of the barrier and predict the risk of contact dermatitis before the appearance of clinical disease. Figure 1 shows the development of chronic irritant dermatitis, in which the combined action of several factors (which may be constitutional or environmental, e.g., temperature, humidity, or exposure to surfactants) leads to visible disease [6]; by using TEWL it is possible to monitor all these steps before the appearance of visible disease. Indeed, as shown by Pinnagoda [7], there is a linear relationship between the basal state of the barrier, as recorded by TEWL, and the increase of TEWL after exposure to an irritant (in this case sodium lauryl sulfate, SLS); therefore basal measurement of TEWL can be a good indicator of the efficiency of the barrier and predict the proclivity of the subject to develop irritant reactions. A number of studies have been performed to investigate the alterations of barrier function in skin pathology, measuring barrier function before and after the application of irritants. Stress tests like these can be useful in evaluating diseased skin but also in quantifying healthy skin in subjects prone to develop dermatitis. Table 1 shows that the increase of TEWL after open applications of SLS on normal skin in subjects with a history of dermatitis is higher than in subjects reporting no symptoms in their anamnesis [8].

Another way of stressing the barrier in order to better analyze its structure is to remove it by tape stripping: Frodin and Skogh [9] in 1984 studied the relationship between TEWL and stripping, showing the decay of TEWL during recovering of the barrier after stripping. It takes almost 10

Table 1. Correlation between dermatologic history and TEWL increase following open application of 7.5% SLS (from [8])

Dermatologic history	TEWL increase (%)
Hand dermatitis	31
Contact dermatitis	37
Cosmetic irritation	28
Atopy	28
Textile itching	35
Dust itching	29
No symptoms	13

TEWL, transepidermal water loss; SLS

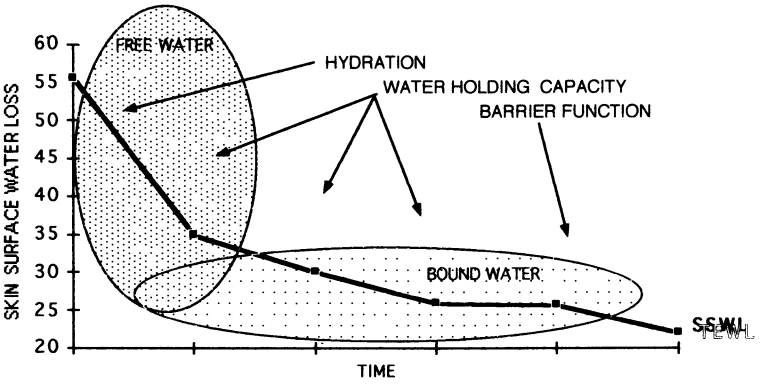

Fig. 2. Plastic occlusion stress test (*POST*) deconvolution curve representing evaporation of water trapped within the stratum corneum (see text for details)

days to rebuild an efficient barrier after removal; formation of a new barrier is not a linear process, as pointed out also by Van der Valk and Maibach [10] in a more recent study in which the role of the outer layers of the stratum corneum in forming the barrier was emphasized. Another in vivo test to measure barrier function and subclinical skin changes is the plastic occlusion stress test (POST), which provides information on skin hydration, barrier function and water holding capacity of the stratum corneum. The POST consists of applying an occlusion to the skin in order to increase its water content; at the removal of occlusion skin surface water loss (SSWL) represents the evaporation of water trapped within the corneum. When all water is evaporated SSWL equals TEWL. A typical POST deconvolution curve is shown in Fig. 2: evaporation lasts about 30 min and the first part of the curve is proportional to skin hydration, whereas the end reflects barrier function. The shape and the slope of the curve are related to the water holding capacity of the corneum. The decay of evaporation is biexponential

with evaporation of free and then bound water. We have used this technique to measure water holding capacity on healthy skin in psoriatics and atopics [11]: barrier and stratum corneum water binding capacity are decreased in atopics compared to the other groups confirming the proclivity of these subjects to develop dermatitis. Interestingly, there are no differences between controls and psoriatics (healthy skin), besides a slight down-regulation of the curve in the evaporation of bound water, which may be due to disturbed keratinization present at a molecular level also in apparently normal skin [11].

pH and Sebum

Another way to study barrier-inducing subclinical changes is the modified alkali resistance test. Short-term application of NaOH increases TEWL up to 15 min causing a perturbation of the barrier [12]. This increase is related to the increase in pH and therefore there is a linear relationship between TEWL and pH, as shown by Wilhelm and coworkers. This difference is further increased after tape stripping which raises the pH under basal conditions [13]. However, it is difficult to say that by measuring skin pH it is possible to evaluate the barrier. pH can easily be measured using pH meters, but the values are very different from site to site and differ during the aging process. According to Braun Falco and Korting we can assume as "normal" a value of 5.4–5.9 for the lower arm of a healthy adult male [14]. The same group recorded increased skin pH and propionibacterial flora after washing the skin with neutral soaps (pH 7.0), stressing the correlation between damage of the barrier and skin irritation with a high skin pH [15]. Different findings, but the same conclusions, that is, high TEWL and pH, low hydration and reduced skin surface lipid content were reported by Thune et al. in a study on the effects of detergents on the skin barrier [16]. In this study the authors measured noninvasively another interesting parameter of skin physiology: the amount of sebum and/or surface lipids of the skin. It is still controversial whether surface lipids have a role in regulating the barrier; most authors in the last decade believe their role is negligible. Nonetheless noninvasive sebum quantification can be performed by either of two techniques: (1) photometric quantification, which measures the amount of sebum on the surface [17] and (2) the use of sebum tape, which also quantifies the follicular distribution pattern [18]. The first method is based upon photometric measurements of light transmission through a transparent plastic tape which is pressed against the skin for 30 s. The sebum tape is polymeric, sebum-sensitive, and contains innumerable air-filled microcavities: an adhesive coating fixes the tape to the skin during the collection period and the sebum excreted from follicles replaces air with lipids which appear in reflected light as black spots. The size of each spot is proportional to the volume of sebum excreted. These changes can be quantified using an image

analysis system which provides data on the number and size of the spots. The two techniques have been compared recently by Serup [19] and a good correlation between the two methods was found; however, due to the occlusive properties of sebum tape this method is less reproducible and seems more specialized for the determination of "oiliness," the last phase of sebum output.

Ions and Gas Flux

The flux of ions through the skin and the movement of gas from the barrier are imporant parameters reflecting the efficiency of barrier function. Ion flux can be measured noninvasively with special electrodes, similar to those placed on skin surface to measure pH. Lo and coworkers [20] used this technique after inducing damage to the barrier by stripping and delipidizing the stratum corneum: physical removal of stratum corneum (stripping) increased ion flux and TEWL whereas delipidization increased only TEWL. These findings suggest that two separate mechanisms control ion flux and water movement through the stratum corneum. Thus there might be two barriers: a water barrier and an electrolyte barrier. Damage to one of these does not necessarily result in damage to the other.

As regards gas diffusion, CO_2 and O_2 are the two most important gases transported through the skin [21]. CO_2 may be produced in the epidermis by the keratinocytes or arrive there from the bloodstream; its emission is higher in areas rich in sweat glands, and the presence of CO_2 on the skin seems to determine the skin alkali resistance [22]. Damage of the barrier results in an increased loss of CO_2. The relationship between CO_2 emission and TEWL and pH has been investigated by Aly et al., who showed that prolonged occlusion of the skin results in increased pH, TEWL and CO_2 emission [23]. Transcutaneous oxygen pressure can be measured using electrodes of the Clark type consisting of a platinum cathode and a silver ring anode provided with a heating element to control the temperature. The stratum corneum offers a great resistance to O_2 flux. Stripping and changes in corneum thickness and/or lipid composition may alter O_2 emission. The transcutaneous oxygen pressure is also related to blood flow and in clinical dermatology can be used to quantify microcirculation and erythema [24]. This technique has been widely used to investigate occlusive disease of the lower limbs, scleroderma and other disorders of microcirculation [25]. Venous ulcers with higher perilesional transcutaneous (tc)pO_2 showed an increased healing response [26]. In terms of barrier function and skin reactivity Prens and coworkers found a good correlation between patch test reactions and increased O_2 loss [27]. Good correlation between severity of skin reactions and $tcpO_2$ was also noted. This technique complements laser Doppler velocimetry (LDV) and is useful in differentiating between reactions $\geq 2+$, when the signal in LDV is frequently saturated.

Conclusions

Bioengineering techniques available today to the dermatologist and the investigator allow noninvasive quantification of skin damage before the appearance of clinical disease. Standardization and widespread use of these techniques and methods should improve our understanding of skin physiology and pathology, allowing repeated monitoring of skin funtion in the course of disease and during treatment.

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A Cellstick Device for Wound Healing Research

J. Viljanto

Introduction

Modern surgery benefits from many new technologies which make operations safer, shorten the period of intensive care and speed up patients' return to work. We can monitor cardiac and respiratory functions, peripheral oxygenation, muscle relaxation, fluid and electrolyte balance, to mention only a few possibilities. We often believe that the usual laboratory values, if normal, are enough to guarantee uncomplicated wound healing (WH), too. We forget the fact that the best possible information on WH is obtainable only from the wound itself. However, preparation of pure granulation tissue from between the wound edges has been difficult, if possible at all. The danger that healthy tissue will also be taken is real, and this has prevented efforts to obtain fine specimens by needle puncture technique. Steel wire mesh cylinders, sponge implants, and direct or indirect means for measurement of tensile strength or bursting pressure of the healing tissue [2, 5, 9, 15], all used in experimental animals [8, 9, 15], are not acceptable in the clinic. Thus, progress has been made in many areas close to surgery, but not in all essential aspects. Continuous monitoring of WH from closed surgical wounds still awaits realization.

This paper describes one solution for your critical trial and comparison with other methods. It offers a more sophisticated model developed from the Cellstick prototype, still composed of two elements, cellulose sponge and silicone rubber.

The original model of the Cellstick device (Cellomeda OY, Turku, Finland), initially named Cellstic – *cell*ulose and Silas*tic* – was first presented in 1975 at the 8th European Rheumatology Congress in Helsinki [12]. Prior to that, numerous animal experiments had been conducted since 1959 to study the suitability of a viscose cellulose sponge, implanted subcutaneously, as a framework for invading inflammatory cells and connective tissue regeneration [7, 8, 10, 15]. The experiences were encouraging, compared with

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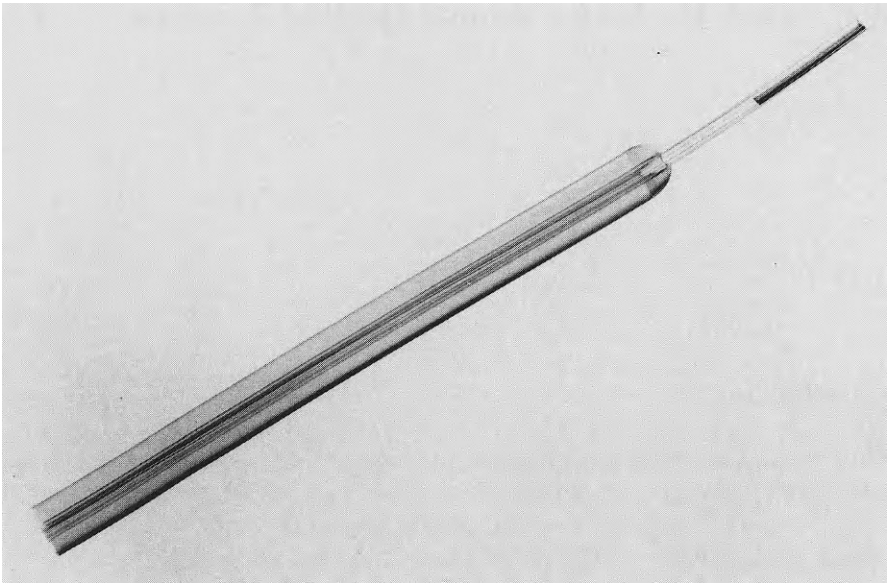

Fig. 1. The Cellstick device reduced to 90% of its natural size

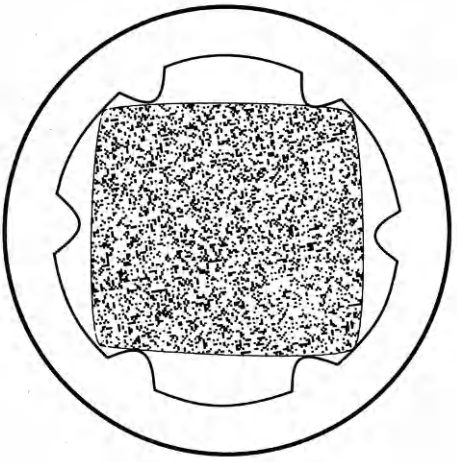

Fig. 2. Diagram representing cross section of the inner silicone rubber tube and cellulose sponge. Diameters of the tube are: outer diameter, 2.50 mm; inner diameter from furrow to furrow, 1.85 mm, from ridge to ridge, 1.58 mm. The sponge is 1.3 × 1.3 × 20 mm in size

those obtained with a polyvinyl alcohol (PVA) sponge [4] and prompted us to try combining a thin wound drain and a cellulose sponge. This was done in order to obtain adequate specimens from inside the wound without disturbing normal wound healing. A silicone rubber tube used as a tiny wound drain was found to meet these requirements better than any

other material available. Due to the thin walls and softness of the tube, however, it had a property of easy kinking, which in turn led to a complete halting of the fluid movement in the tube. To improve the standard of this device, intended for wound healing research in man and to increase the safety of the routine operations, both the cellulose sponge and the tube around it had to be further developed (Figs. 1 and 2).

Structure of the Cellstick Device

Cellulose Sponge

By changing the size of the sodium sulphate crystals, the coagulation temperature, the mixing conditions, and the number of fibers, followed by thorough bleaching and washing, a new high grade medical cellulose sponge was developed. It is characterized by numerous small pores with thin pore walls, numerous interpore connections, and a good overall homogeneity (Fig. 3). It allows reversible compression and expansion without damage of the internal structure, as well as uniform sawing and cutting.

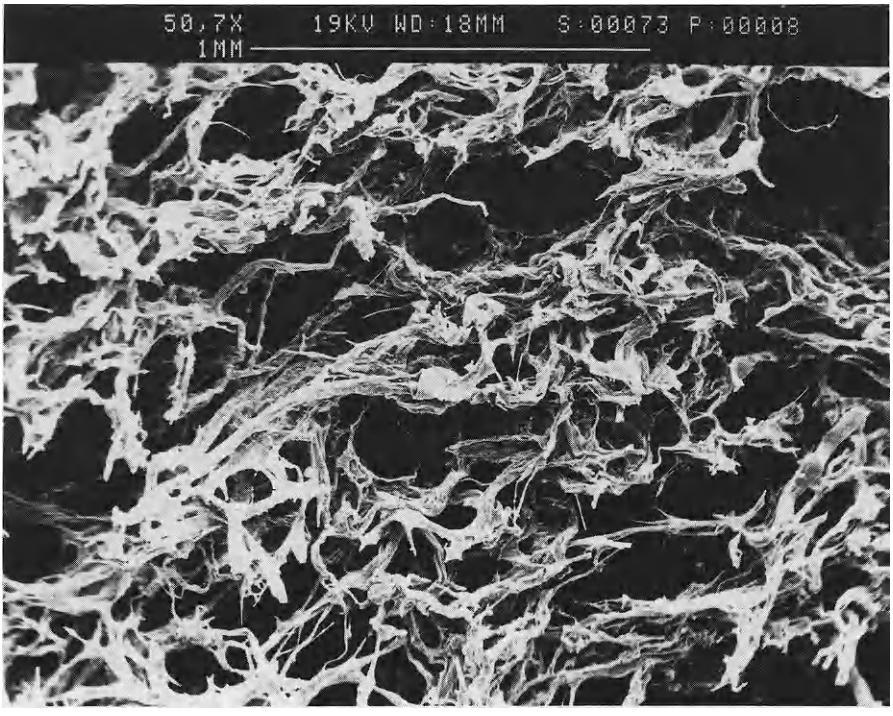

Fig. 3. SEM picture of the Cellstick cellulose sponge. Note 1 mm scale at the upper margin of the picture

The Inner Silicone Rubber Tube

In order to prevent kinking and to increase the free surface area for the attachment of wound cells, the silicone rubber tube was strengthened from inside by six longitudinal ridges. By placing the standard size sponge (1.3 × 1.3 × 20 mm) inside one end of the self-reinforced tube, 2 + 2 opposite furrows remain free, allowing twice as much wound exudate to flow as in the original Cellstic. Furthermore the sponge is more firmly fixed and inward and outward slipping is prevented [14].

Protecting Outer Tube

Experiences gained with the original Cellstic in nearly two thousand patients had taught us to be careful when fixing the silicone rubber tube to the skin with adhesive tape. Nevertheless, occlusion of the tube took place occasionally because of kinking. These test tubes had to be rejected. To be sure that the inner silicone rubber tube will remain open and allow free inward and outward movements of the wound exudate throughout the test period, a protective outer tube with an omega profile was constructed (Fig. 1). The protective tube has an adhesive under surface, causing it to adhere to the underlying skin, and an oblique, elastically bending tip to protect that part of the inner tube just coming out of the closed wound.

Insertion of the Cellstick Device into the Wound

- Choose Cellstick model A for obese adult patients, model B for nonobese adults, and model C for children. The only difference between them is the length of the inner tube.
- Close the incision wound, including fascia.
- Dip the inner end of the device containing the cellulose sponge in a vertical position into the sterile saline solution and allow the sponge to become wet. Lift the device off the solution just before the uppermost part of the sponge has become somewhat wet. Bring the wound edges closer together at a subcutaneous level with a few stitches, if needed.
- Begin to close the skin wound either as a continuous suture or with interrupted stitches.
- Place the inner end of the device through the operation wound subcutaneously, deep enough to allow the bending tip of the protecting tube almost to touch the skin surface.
- Direct the protecting tube medially or laterally upwards against the force of gravity, not along with it.
- Finalize the skin suture.
- Be sure that the inner tube is not constricted.

- Remove the cover slip from the undersurface of the outer tube and allow it to adhere to the skin.
- Dress the wound site and the Cellstick device, but leave the outer end of the tube uncovered or make a small opening through the dressings at the outer tip of the protecting tube to secure free movement of the wound fluid inside the inner tube.
- Record the exact time of Cellstick insertion.

The Cellstick drain works optimally if no other drains are put in the same wound space. It is absolutely prohibited to connect any drain coming out of a wound, including the Cellstick itself, to suction. This would be counter to the basic principle of the device: pressure changes in the wound space, induced by body movements, breathing, coughing etc., force the exudate into the Cellstick tube. Muscle relaxation together with the gravitational force causes a short backflow in the fluid column. Inhibition of the fluid movements, whatever the reason, stops accumulation of wound cells in the sponge and the wound cell specimens obtained with this device no longer represent the time elapsed after surgery. On the other hand, a drain led from the abdominal cavity does not interfere with wound healing tests at subcutaneous level. For additional security the other tube may be fixed, not the inner tube, with a stitch or two to the skin.

The patient is allowed to move and turn around in bed during the postoperative period quite freely. Sitting, standing, and walking are all allowed as usual. However, continuous pressure against the wound site and the Cellstick device caused by the body weight is not recommended. Lying in a supine position after an operation performed on the dorsal aspect of the body should be prohibited.

Removal of the Cellstick Device

After a preselected time interval from a few hours to 24, 48, or 72 h, the Cellstick device is removed simply by drawing it out of the wound and placing it in a sterile lab tube. The wound is covered in the customary manner or left open to heal further. If desired, a microbiological specimen is now taken from the inner tip of the device.

Analytical Alternatives

Histology

The Cellstick device as such, after taking the cellulose sponge from the tube, may be fixed in a 10% formaldehyde solution and processed further according to routine histological techniques. The microtome sections may be stained by any method. The hematoxylin and eosin and van Gieson methods are

Fig. 4. Wound cells in the Cellstick sponge 24 h after surgery. Note the cellulose trabeculae in the center of the picture and fine fibrin threads with blood platelets crossing them

those most often used by us. The cellulose matrix itself does not stain with the usual staining methods, which is an advantage and offers better visualization of the invaded cells and extracellular material.

Electron Microscopy

Transmission [1] and scanning electron microscopic pictures can be made using customary techniques (Fig. 4).

Enzyme Histochemistry

Enzyme histochemical methods have been adapted to sponge ingrown granulation tissue to visualize acid and alkaline phosphatase, adenosine triphosphatase, cytochrome oxidase, aminopeptidase, hexokinase, and isocitrate dehydrogenase [6].

Immunochemistry

Collagen and procollagen types I and III and fibronectin have been identified from the sponge specimens using indirect immunofluorescent methods [3, 13].

Cytology

One of the specific features of the Cellstick device is the possibility to force the cells and extracellular material out of the sponge again by rinsing the inner tube (at a constant speed and constant volume) with isotonic solution (RPMI-1640). The rinsing technique was studied in more detail in order to find optimal conditions for cell detachment. It was found that, instead of a continuous flow, a gentle pulsating rinsing maintains the cellular structure better and removes the cells more completely. Therefore an automatic rinsing pump was constructed to guarantee the uniformity of rinsing conditions. From the wound cell suspension aliquots of 25, 50, 100, or 200 μl, depending on the total number of cells, were taken into Cytospin cuvettes (Shandon Elliot, Shandon Scientific Co. Ltd., London, England) and centrifuged at 1000 rpm for 7 min. After air drying, the glass slides were fixed by immersing them in absolute methanol for 5 min and stained by the May-Grünwald-Giemsa method using an automatic staining apparatus (Shandon Elliot, Shandon Southern Instruments Ltd., Camberley, Surrey,

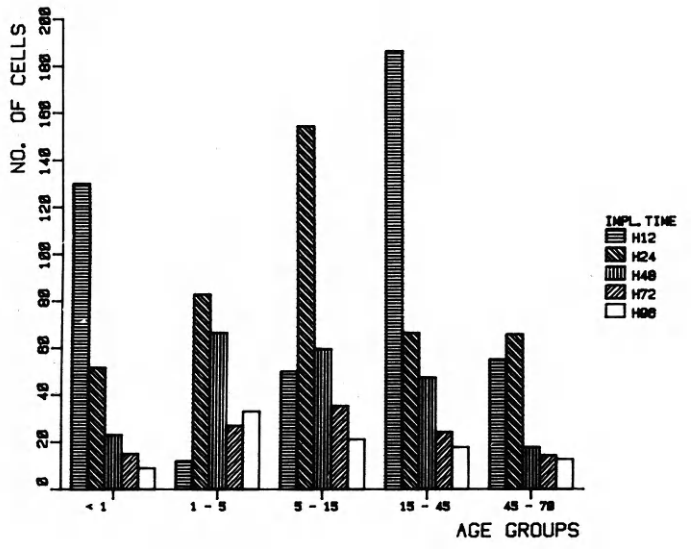

Fig. 5. Relative number of monocytes in the Cellstick device at 12, 24, 48, 72, and 96 h postsurgery in different age groups

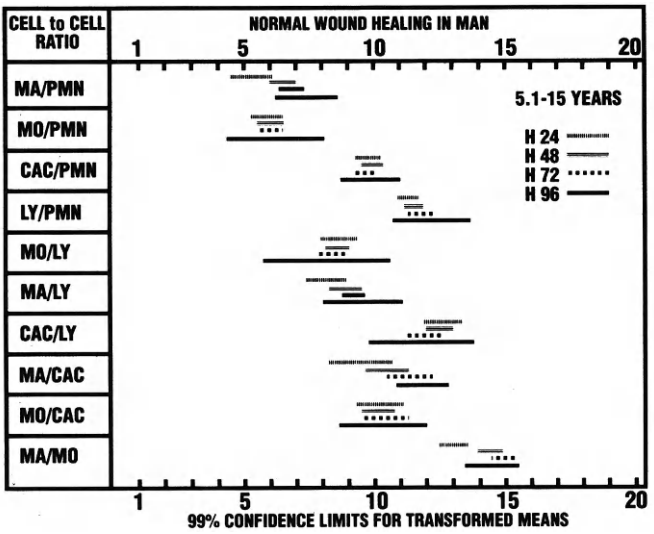

Fig. 6. Cell to cell ratios at 24, 48, 72, and 96 h in children aged from 5.1 to 15 years. *PMN*, polymorphonuclear leukocytes; *LY*, lymphocytes; *MO*, monocytes; *MA*, macrophages; *CAC*, aggregated cells

Table 1. Data for an example of wound healing prognosis produced with the CELLCO software. Age: 15.8 years; diagnosis: colitis ulcerosa; implementation time: 46.4 h

Cell to cell ratio	Reference value	Observed value	Wound healing			Biological healing time	Difference (h)
			rapid	moderate	slow		
MA/PMN	5.24	2.75			*	44.1	−2.3
MA/LY	7.60	5.70			*	45.3	−1.1
MA/MO	14.33	12.72			*	43.2	−3.3
MA/CAC	9.46	5.68			**	45.1	−1.3
MO/PMN	5.03	3.86		⟨ = ⟩		45.2	−1.2
MO/LY	7.18	6.80		⟨ = ⟩		45.8	−0.6
MO/CAC	9.30	6.78			*	46.2	−0.2
LY/PMN	11.69	10.87			*	43.9	−2.5
CAC/PMN	9.54	7.26			*	46.1	−0.3
CAC/LY	12.12	13.84			*	44.3	−2.2

*, ± 1 SD; **, ± 2 SD.

England). The results of the differential count were recorded (Fig. 5) for computerized calculation of the mutual cell-to-cell ratios and their logarithmic transformation. Ten cell-to-cell ratios were selected and their confidence limits in different age groups were determined (Fig. 6), as described earlier in detail [11]. For an individual patient the CELLCO software gives the biological wound healing time in hours. Comparison of this with the

chronological WH time, 24 or 48 h post-surgery, gives the difference in hours indicating either delay, normal healing speed, or enhancement in WH, as compared with the means of healing speed in the same age group (Table 1).

Summary

The Cellstick device is a tool developed for WH research in man. So far it has been used in Finland in 2200 patients in connection with routine surgery without any wound complications or discomfort to the patients. Cooperation with the Finnish study group has recently been introduced in Denmark, Germany, and Austria. Experience accumulated from colleagues during recent years has shown that very careful hands-on teaching is necessary before introducing this technique. I hope this article will substitute to some extent for this training.

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High-Resolution Ultrasound Imaging of Various Types of Wounds

R. Rompel and J. Petres

Introduction

High-resolution ultrasound is used in dermatology for the in vivo evaluation of inflammatory skin diseases and benign and malignant skin tumors [3, 5, 11]. Skin thickness and expansion of normal and pathological structures can be measured [2, 4, 10, 19]. Differences in echo density lead to the differentiation of layers of the normal skin, and structures with deviations in density may be identified and located. Normal skin usually reveals an ultrasonographic image consisting of mainly three layers of different echo reflection (Fig. 1). The initial echo is the result of reflection of the ultrasonic impulse at the border from the water to the stratum corneum and also at the border from the stratum corneum to the stratum malpighii. In 50 MHz ultrasound these two lines can be identified separately [8]. However, using 20 MHz ultrasound scanners only one, thick, echo-dense band is seen as the initial echo, representing mainly the border from the water to the stratum corneum. Below this initial echo a broad band of homogenous medium density is seen. This represents parts of lower epidermal structures and the papillary and reticular layers of the corium. The cause of the echogenicity of the corium has been identified as the regularly stratified collagen [9]. Normal structures can be identified within the corium such as hair follicles and enlarged sweat glands. Benign or malignant tumors lead to a displacement of the normal texture of the corial collagen, revealing a more or less sharply demarcated echolucent area [8, 12, 17]. The transition of the corium to the fatty tissue of the subcutis can easily be determined, which helps to locate the depth of invasion of any pathological processes. Normal subcutis is visualized as an echolucent to echo-free area. Within this area, tendons, fasciae, and muscles are depicted as horizontal echo-dense structures.

Since the corium is the only structure of medium echogenicity, it is best suited for evaluating the course of several processes. Inflammatory diseases of the skin have been investigated in several studies showing the course of

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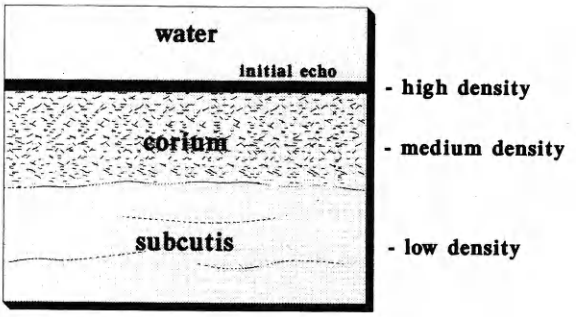

Fig. 1. Schematic ultrasound image of the normal skin

the thickness and density of the corium [6, 15]. Recent studies used high-resolution ultrasound for evaluating wound healing and therapeutic results after cryotherapy of basal cell carcinoma [13, 18].

In the reparative processes of wound healing the corium plays a major role. We investigated the final status of several types of wounds after completion of the healing process. The results are described below.

Materials and Methods

The investigations were carried out in the Department of Dermatology, Municipal Clinics, Kassel, FRG. We used a digital ultrasound imaging system (DUB 20, Taberna Pro Medicum, Lüneburg, FRG), consisting of a high-frequency (20 MHz) ultrasound transducer that conducts the signals to a computer. Using b-scan, the skin is visualized as a two-dimensional color image. Skin and subcutaneous tissue are depicted to a vertical diameter of 5–7 mm with an axial resolution of 80 μm and a lateral resolution of 200 μm.

Patients with various types of healed wounds were included in the study. Studied objects were normal or hypertrophic scars, keloids, healed leg ulcers, radiation dermatitis, free skin transplants and transplant donor sites. For each group six to eight patients were examined. The lesions were compared to healthy skin of the corresponding contralateral location. Three to five images of one lesion were made. Corresponding regions of interest were evaluated using densitometry, the mean values of which were determined: differences were checked for statistical significance using t test analysis.

Results

Normal and hypertrophic scars appeared to be of significantly lower echogenicity than healthy skin of the corresponding region (Fig. 2). The

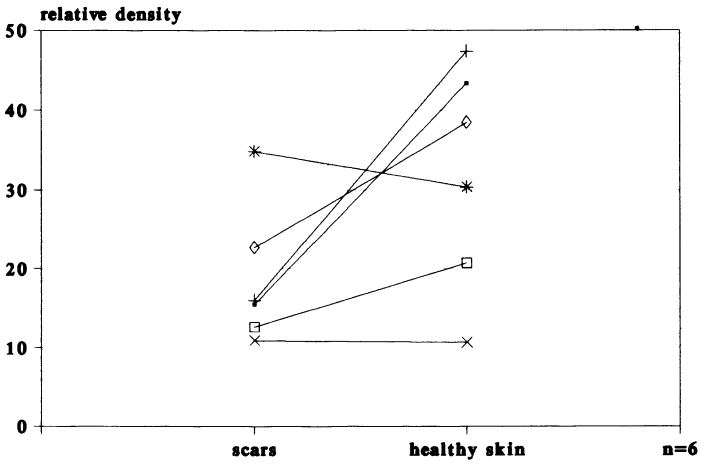

Fig. 2. Significant decrease in the relative density of scars as compared to normal skin ($p < 0.05$)

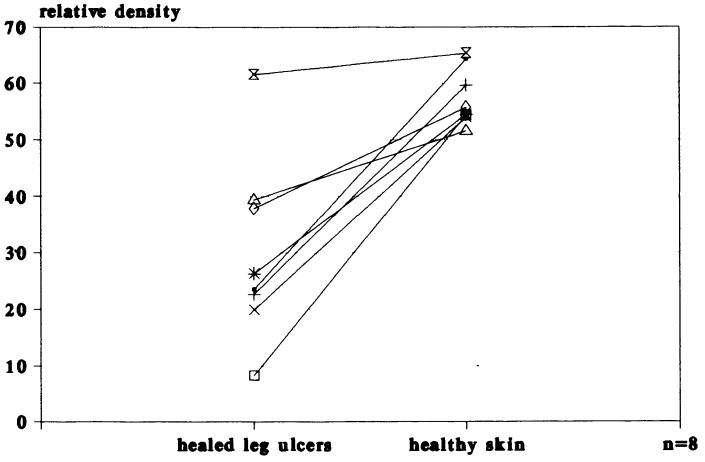

Fig. 3. Significant decrease in the relative density of healed leg ulcers as compared to normal skin ($p < 0.001$)

visible structure of the scar was usually thicker than the corium of the normal skin corresponding to the clinical picture. There was a tendency of increasing echogenicity the older the scar. However, hypertrophic scars remained relatively echolucent even after long duration. Similarly, healed leg ulcers showed a lower echo density (Fig. 3).

Keloids showed a significantly decreased density as compared to normal skin (Fig. 4). The density was even lower than in normal and hypertrophic scars. In addition, the initial echo was markedly reduced in keloids (Fig. 5).

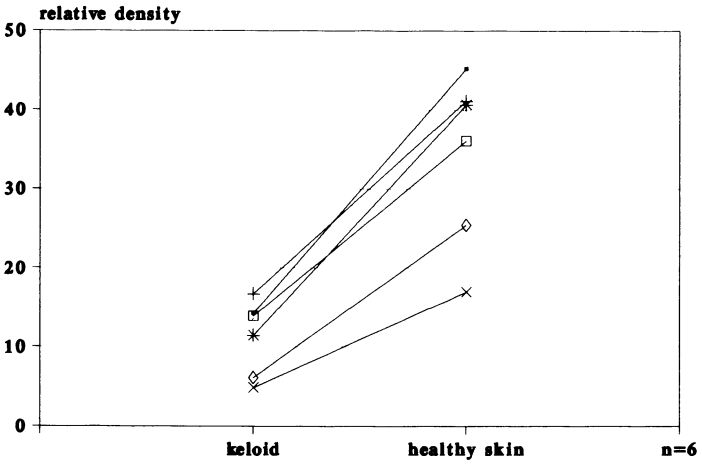

Fig. 4. Significant decrease in the relative density of keloids as compared to normal skin ($p < 0.001$)

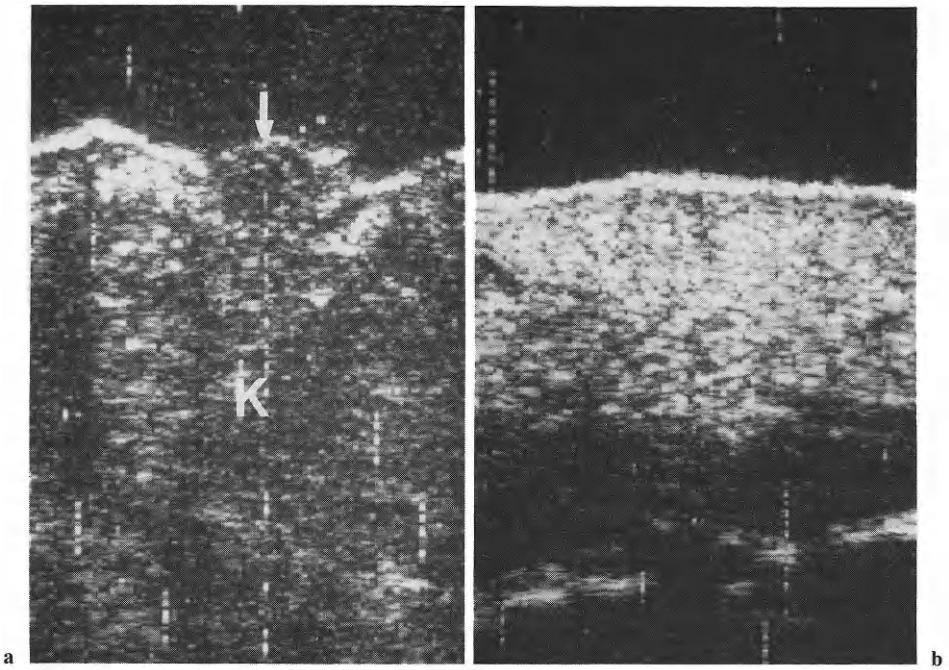

Fig. 5a,b. a Ultrasound image of a keloid. Decreased density within the keloid tissue (*K*) and reduced initial echo (*arrow*) **b** Healthy skin of the corresponding location

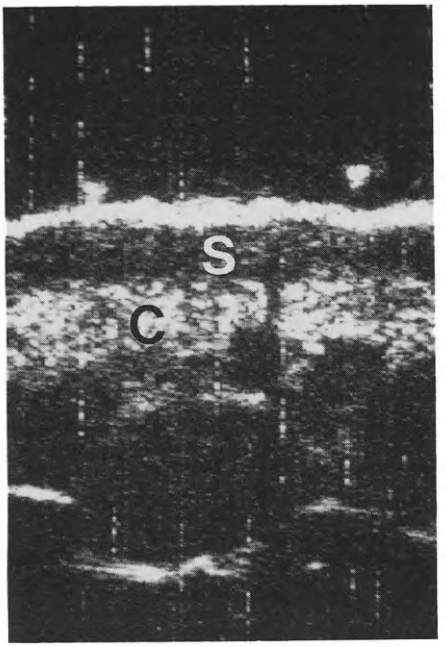

Fig. 6. Split-thickness graft donor site. Echolucent band of the upper corium representing superficial scarification (*S*); normal lower corium (*C*)

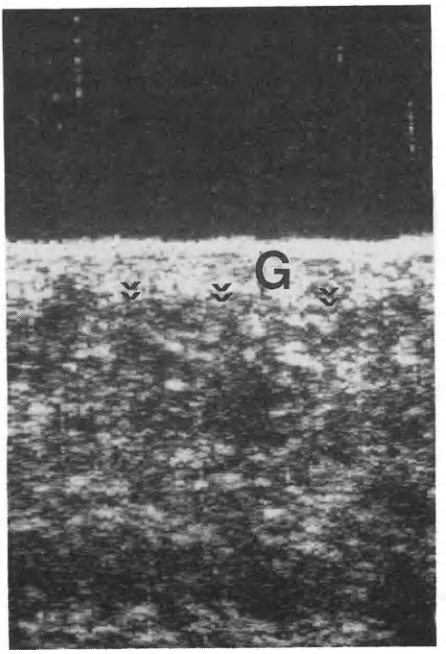

Fig. 7. Split-thickness skin graft 6 months after surgery The graft (*G*) extends down to the lower granulation tissue of decreased density (*arrowheads*)

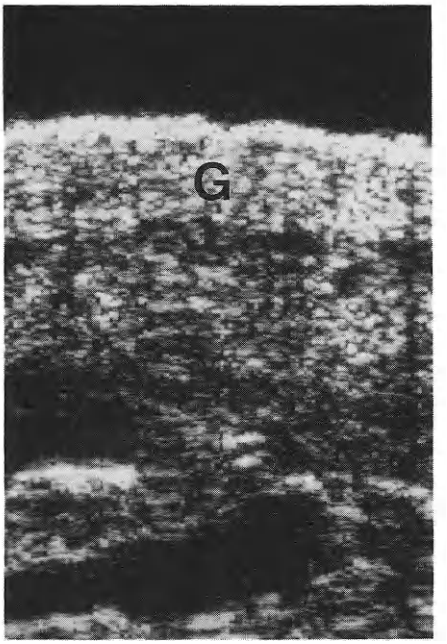

Fig. 8. Full-thickness skin graft 5 months after surgery. The graft (*G*) (thicker than in Fig. 7) extends down to the lower granulation tissue of decreased density

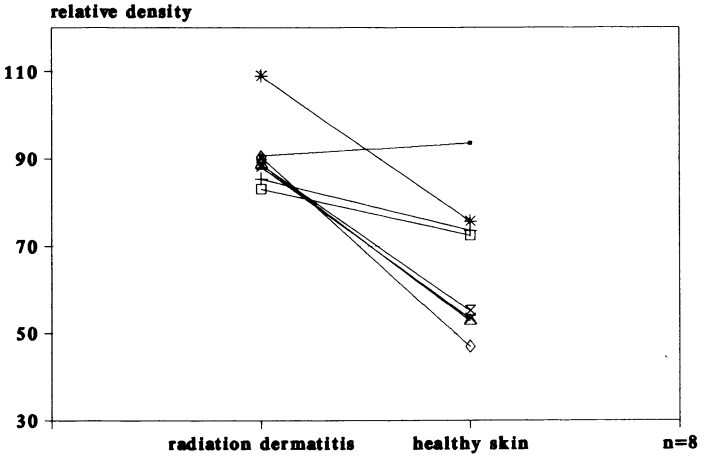

Fig. 9. Significant increase in the relative density of chronic radiation dermatitis skin as compared to healthy skin of the corresponding location ($p < 0.001$)

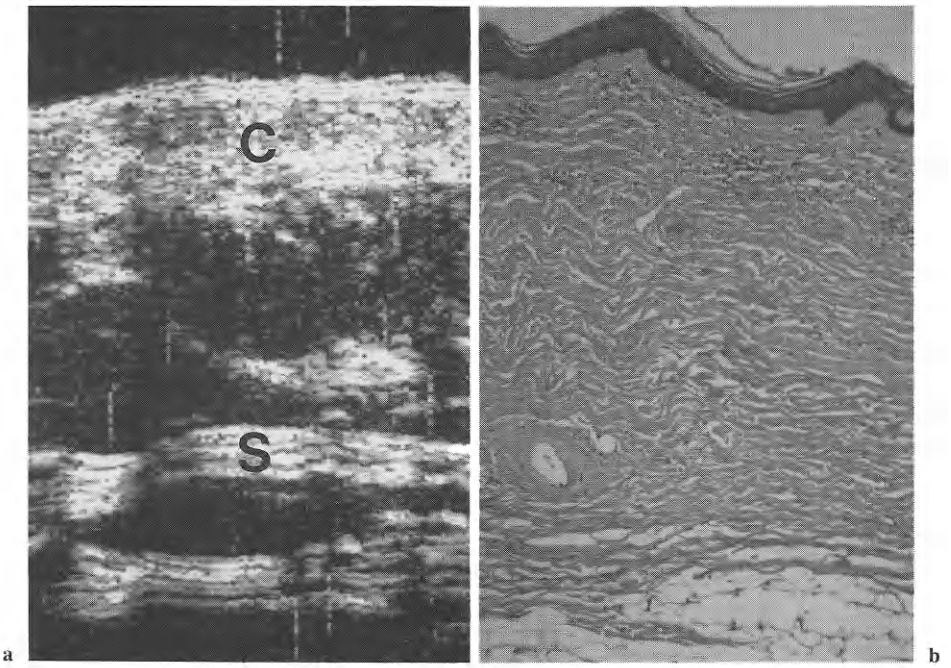

Fig. 10. a Ultrasound image of chronic radiation dermatitis: increased echogenicity of the corium (*C*) and the structures of the subcutis (*S*). **b** Corresponding histology: hyalinization of the dense collagen tissue

Donor sites of split-thickness skin grafts revealed a lowered echogenicity of the upper corium, while the lower corium appeared quite normal (Fig. 6). There was no marked difference in thickness of the corium in transplant donor sites. Free skin grafts, no matter if full-thickness or split-thickness, could easily be demarcated in ultrasound during the first 4–5 months after transplantation (Figs. 7, 8). Older skin grafts showed a homogenous corial texture.

The ultrasonographic appearance of the corium in radiation dermatitis (late stage) stood in contrast to that of other types of wounds. A highly significant increase in density of the corium was found (Fig. 9). Also, the lower corium and even the structures of the subcutis were of markedly higher echogenicity (Fig. 10).

Discussion

High-resolution sonography is useful for the in vivo evaluation of inflammatory skin diseases and benign and malignant skin tumors. Recent experimental studies have shown its efficacy in measuring wound healing [13,

18]. In particular, processes within the corium may easily be determined and measured [16]. This is due to the medium density of the corium as compared to the initial high density echo and, on the other side, the subcutis which is of low density. The main histological correlate of the echogenicity within the corium is collagen [7, 9]. Most structures within the corium, either physiological or pathological, may be identified by their lower density, which is explained by the replacement of the local texture of the corial collagen [7, 16].

Different types of wounds are due to differences in traumatization and scarification of skin structures. Our investigation was intended as a horizontal study on various types of wounds after completion of the wound healing process. The results show that the site of regenerative scar tissue may be located by high-resolution ultrasound. Normal scars appear to be of lower density than normal skin, as reported by previous investigators [13, 16]. Healed leg ulcers were of similarly lower density.

Keloids revealed an even higher degree of echo transparency than normal scars. The phenomenon of lowered echogenicity is probably due to irregularities in collagen structure within the scar tissue. Similarly, it is clinically evident that scars and keloids are thicker than the normal corium. A special finding in the case of keloids is the reduced density of the initial echo. This can be explained by the very thin epithelial layer of the keloid.

Healed split-thickness donor sites showed very characteristic ultrasonographic features. As a result of superficial scarification a small band of lowered density could be identified within the upper corium while the lower corium appeared quite normal. Free skin grafts, even months after the operation, could be identified as a structure with a normal initial echo and a small fragment of normal corium which was demarcated from the lower structures. This lower tissue of decreased density represents granulation tissue. The thickness of the grafts correlated to the thickness of the split-thickness and full-thickness skin grafts.

The ultrasonographic picture of chronic radiation dermatitis stood in contrast to those of all the other wounds we examined. Chronic radiation dermatitis revealed a significantly higher density of the corium and even of the structures of the subcutis. The histological substrate of this increased density may be identified as the dense fibrotic tissue with its high degree of hyalinization. This hyalinization is located within the corium and even around the structures of the subcutis [1, 14]. Blood vessels surrounded by hyalinized collagen make up the main part of the highly echogenic structures within the subcutis.

Some of our investigated objects showed a time-dependent ultrasonographic appearance. As previously reported, there was a time dependence of scars [7, 13]. The appearance of healed leg ulcers, split-thickness donor sites, and skin grafts were also dependent on time. With the reorganization of the normal structure of the collagen, an increasing echogenicity is noted; however, keloids and chronic radiation dermatitis seemed to reach a stage with no further change in echogenicity.

We conclude that different types of wounds show some characteristic morphological features which are the result of the different quality and location of both the trauma and the scar.

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A 20 MHz Ultrasound Examination of Lipodermatosclerosis

J. Welzel[1], W. Schmeller[1], and A. Plettenberg[2]

Introduction

Lipodermatosclerosis (LDS) is a common finding in patients with chronic venous insufficiency (CVI). Clinically the skin of the lower legs is thickened and indurated. Computed tomography, nuclear magnetic resonance tomography, laser Doppler flux and transcutaneous oxygen measurement show pronounced changes in morphological and functional parameters [4–8]. We used high-resolution ultrasound to determine the degree of LDS and to study the acoustic behavior of skin and subcutaneous fat.

Patients and Methods

The study included 23 CVI patients (mean age 67.5 years, 33 legs) and 11 healthy controls (mean age 57.8 years, 21 legs). Regions of healthy skin, moderate and severe LDS were determined by palpation. For comparison the skin of the healthy subjects was examined at similar locations. A total of 480 ultrasound measurements was done with determination of thickness and echogenicity of the echolucent band, the dermis and, if possible, of the subcutaneous fat. Echogeneity depends on the height of a-scans in a region of interest (without defined dimension). Examinations were performed with a 20 MHz b-mode ultrasound scanner (Dermascan C, Cortex Technology, Denmark). For comparison with histological findings 14 corresponding skin biopsies of seven CVI patients were taken from regions of severe LDS.

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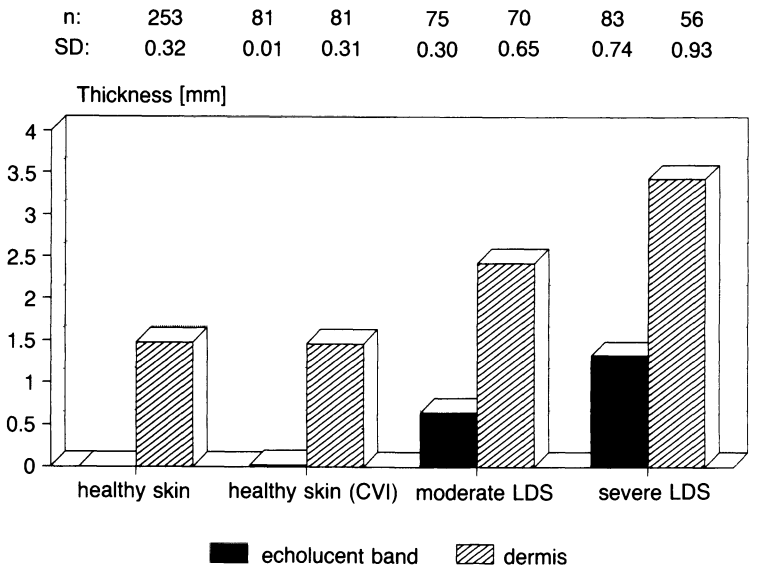

Fig. 1. Ultrasound measurement of thickness. *CVI*, chronic venous insufficiency; *LDS*, lipodermatosclerosis

Results

The thickness of the dermis increased from 1.48 mm in healthy subjects and 1.46 mm in healthy skin of CVI patients up to 2.42 mm in patients with moderate LDS and 3.43 mm in patients with severe LDS (average values). An echolucent band appeared in the upper dermis of patients with moderate LDS. The mean thickness was 0.64 mm and increased up to 1.32 mm in patients with severe LDS (Fig. 1).

The echogenicity of healthy skin, with mean values of 48 and 46 in healthy subjects and CVI patients, respectively, decreased with the amount of LDS: it was 25 in patients with moderate LDS and 15 in patients with severe LDS.

The echolucent band showed a decrease in echogenicity from 13 (moderate LDS) down to eight (severe LDS) (Figs. 2–5). Even in the healthy appearing skin of CVI patients the upper dermis showed a small subepidermal region with lower echogenicity.

The difference of thickness and echogenicity between healthy skin and severe LDS was statistically significant ($p < 0.01$, Student's t test).

In histological sections the echolucent band corresponded either to glomerular blood vessels (five biopsies), edema (four biopsies) or siderophages in a band-like formation (five biopsies) in the upper dermis.

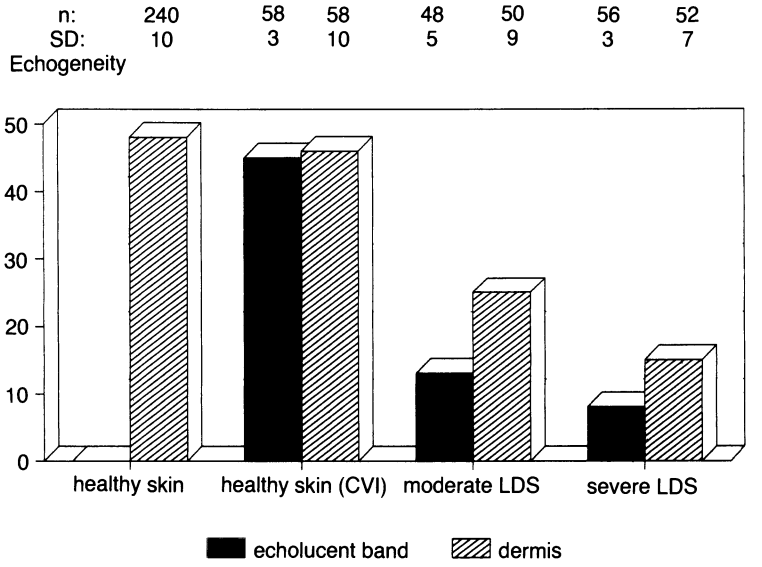

Fig. 2. Ultrasound measurement of echogenicity. *CVI*, chronic venous insufficiency; *LDS*, lipodermatosclerosis

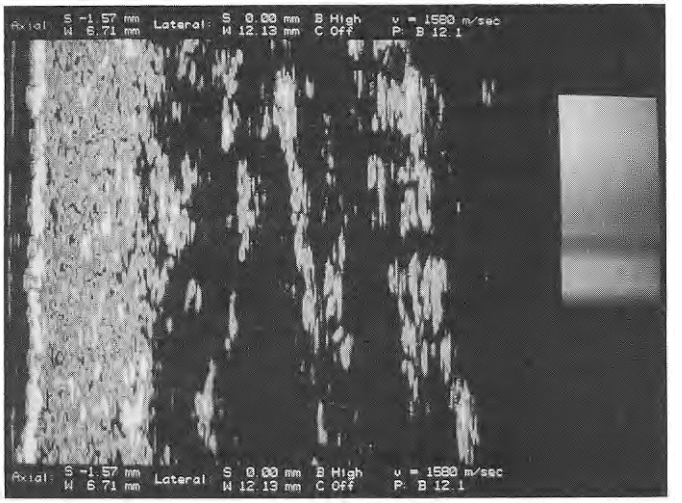

Fig. 3. Ultrasound of healthy skin. *Upper level* (left), entry echo, echo-rich, well-defined dermis; *lower level* (right), echo-poor subcutaneous fat with bundles of connective tissue, muscle fascia

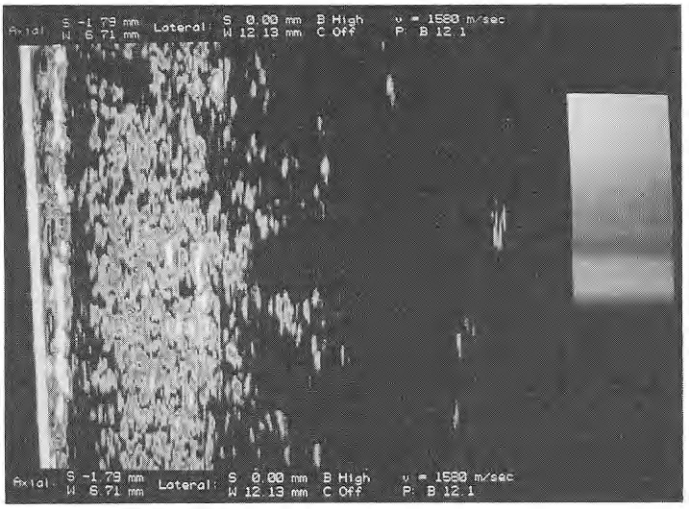

Fig. 4. Moderate lipodermatosclerosis. Subepidermal echo-poor band, dermis less dense, subcutaneous fat

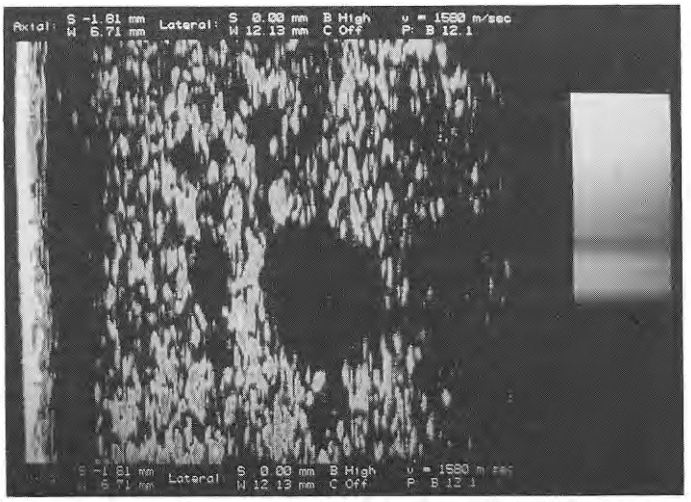

Fig. 5. Severe lipodermatosclerosis. Subepidermal echolucent band, echo-poor dermis with a dilated blood vessel, poorly defined border to the subcutis

Discussion

As expected, in regions of LDS the dermis was thickened; this was confirmed by exact measurements using 20 MHz sonography. Other noninvasive methods for determination of skin thickness such as computed tomography,

nuclear magnetic resonance tomography and xeroradiography result in similar values for LDS [8]. The advantage of ultrasound is that there are no side effects and a higher degree of resolution is obtained.

The general decrease of echogenicity in LDS patients' skin is similar to the ultrasound changes seen in morphea [3]. In both diseases the homogenization of the collagen bundles is sonographically represented by a region of echo-poor dermis.

Histology shows different changes in the upper dermis whereas sonographically the echolucent band is a constant finding. This can be explained by the physical interaction between ultrasound waves and tissue. Edema leads to low reflection; tumors and infiltrates cause scattering, refraction and absorption of the echo signal [1]. The uniform result of these phenomena is characterized by low echogenicity. Therefore, an echolucent region in ultrasound represents different microscopic structures.

The echolucent band was noticed adjacent to venous ulcers before and has been described as an "ulcer band" [6]. It was physically explained by stasis [7]. In the present study, the echolucent band was also observed in legs without ulcer or edema, depending on the degree of sclerosis.

In summary, the characteristic changes measured by 20 MHz sonography allow quantification of LDS and offer a possibility for simple and noninvasive follow-up examinations. High-resolution ultrasound is a good tool to study the process of wound healing and might be helpful in evaluating the prognosis in patients with venous leg ulcers.

Conclusions

Ultrasound examination in LDS shows: (1) an echolucent band in the upper dermis; (2) a pronounced thickening of the dermis; (3) a general decrease of echogenicity; (4) a poorly defined border to the subcutaneous fat; and (5) a replacement of fat by fibrotic tissue.

The echolucent band – a constant and characteristic finding in sonography of LDS – represents different histological structures.

High-resolution ultrasound is a valid and noninvasive method for localization and quantification of LDS.

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Physiological Changes in Aging Skin

A. Lalova, K. Nikolova, and M. Gantcheva

Introduction

The physiological changes that take place in aging skin include impairment of the barrier function, decreased turnover of the epidermis, impairment of its structure, and a reduction in the vascular network, particularly around hair bulbs and sweat glands [1]. A number of environmental factors influence the rate of skin aging, for example, exposure to sun and to chemical agents [2]. As the skin ages, its physiology undergoes significant modification. The skin continues to carry out the functions of protection, absorption, secretion, excretion, thermoregulation, pigmentogenesis, accumulation, sensory perception, and immunity, all of which are essential to survival of the human body; however, these functions are performed less efficiently with aging [1, 3, 4].

Material and Methods

The following four parameters were evaluated. (a) Transcutaneous oxygen pressure ($TcPO_2$) was measured at the following sites: presternally (below the fourth right rib; control measurement), symmetrically on the inner side of both thighs 10 cm above the knees, on both legs 10 cm below the knees above the tibial bones, and on the dorsal aspect of both feet above the second cuneiform bones. The measurements were performed using the Oxymeter (Hellige), with a polar graphitic electrode of the Clark type, applied to the previously cleaned skin. (b) Sebum secretion was measured electromechanically (Sebometer, FRG) over 2 weeks on the forehead, cleaned previously with saline solution. (c) Water-binding capacity was established indirectly by measuring the pH of the skin at the same sites and using the same equipment. (d) Skin temperature was measured by con-

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tact thermometry (Temp-60, Soviet Union) at symmetrical points of the forehead, front of the neck, forearms, and chest. A total of 40 subjects was examined, none showing clinical evidence of skin pathology. Those in group 1 were aged 16–30 years, and those in group 2 were aged 31–80 years. For the measurement of $TcPO_2$ there were 20 subjects in each group; the other parameters were assessed in 10 subjects in each group, the same persons in each case.

Results and Discussion

The measured values for $TcPO_2$ showed no significant differences between group 1 (60–85 mmHg) and group 2 (50–85 mmHg) at any of the sites. The value of 50 mmHg was measured on the dorsal site of the foot. The rate of $TcPO_2$ decreased as the electrode was moved distally but was not influenced by age.

The quantity of oxygen measured at the surface reflects the balance between local metabolism and total blood perfusion. It may be altered by inflammation, which affects the barrier function of the stratum corneum and the oxygen consumption [5]. Pericapillary changes in tissue, especially edema, markedly inhibit oxygen diffusion through the skin [6]. On the other hand, the various compensatory mechanisms of the skin microcirculation system should be borne in mind, which in our opinion could account to a certain extent for the lack of age-related differences in $TcPO_2$.

Mean sebum secretion was 93–120 U in group 1 and 41–59 U in group 2. This difference in sebum secretion demonstrates the decrease in sebum production in the elderly. Although the number of sebaceous glands remains unchanged with aging, sebum production is reduced in elderly subjects [7]. This decreased sebum production, however, does not seem to be of clinical relevance [1]. According to Sauder et al. [8] this is due to a subclinical decrease in androgen production.

Mean water-binding capacity was 91–100 U in group 1 and 72–86 U in group 2. This decrease in elderly subjects is probably related to the lack of natural moisturizing factor in aging skin, which accounts for the dryness, wrinkle formation, pruritus, and impaired regeneration process. Acording to Stüttgen et al. [6], the environmentally induced changes in skin pH lead to increased transepidermal water loss.

Mean skin temperature in group 1 and group 2, respectively, at the various areas of the body was as follows: forehead, 35.5°C; neck, 35.2°C and 34°C; forearms, 37°C and 34.4°C; chest 34.6°C and 32.6°C. These differences are obviously related to the arteriolar circulation [9], but without any clear correlation between the circulating blood volume in the dermal capillaries and heat radiation of the skin.

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Diagnosis and Treatment of Venous Diseases and Leg Ulcer

N. Weindorf

Introduction

The first step in assessing patients with chronic venous insufficiency with or without ulceration is a full clinical history and examination. It is important to look for arterial occlusive disease or complaints of orthopedic or neurological character. A sign for a former pelvic vein thrombosis is given in the case of varicose veins in the mons pubis region.

Diagnosis

Investigation of the venous system can mainly be done with the aid of noninvasive techniques, especially Doppler ultrasound and photoplethysmography.

Using continuous wave (CW) Doppler studies with a directional device the anatomical site and level of venous insufficiency can easily be determined. Of great importance are the sapheno-femoral junction and the sapheno-popliteal junction, the function of which can be tested by Valsalva maneuver and calf decompression, respectively. In this way the investigator gets essential information on the superficial venous system. In some cases there exist atypical refluxes which are not always easily recognized, e.g., the examiner finds a Valsalva positive reflux phenomenon along the vein distally but not proximally [1]. Such a "paradoxical reflux" can be transmitted, for example, by side branches, communicating veins or by the deep venous system. An example is shown in Fig. 1. These atypical refluxes are of significance especially for the treatment of varicose veins, because it should be the aim of therapy to leave the sufficient venous segment intact and to eliminate only the insufficient distal venous segment. Thus, in case of a reflux in the long saphenous vein with intact sapheno-femoral junction but

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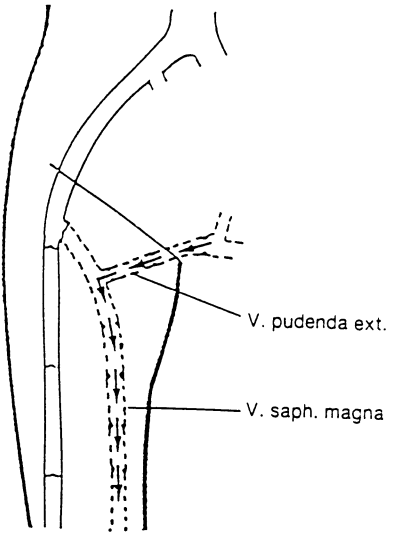

Fig. 1. Reflux in the long saphenous vein transmitted by the external pudendal vein. (From [1])

reflux transmitted by insufficient perforating veins, elimination of the junction must be regarded as unnecessary.

Using the Doppler device an investigation of the deep venous system can also easily be done. With the aid of several test maneuvers, such as Valsalva, thigh compression and decompression and calf compression and decompression the whole deep venous system from the foot to the groin can be examined for pathological refluxes. If the investigator can also use a range-gated, pulsed Doppler, differentiation between superficial and deep vein signals is much easier.

An arterial cause of leg ulcer may be excluded by measuring the ankle pressure. However, the ankle pressure measurement may not be reliable in diabetics. In these cases segmental oscillography or digital plethysmography at the toes can be done.

A very reliable but expensive method is duplex scanning, which will supply information about venous function and anatomical structure. Using this method detailed information can be achieved not only about superficial and deep veins, but also about the perforating veins.

The investigation of the patient should be completed by a method which allows quantitative measurement of the impaired venous function. Various diagnostic tests are available for this assessment. Peripheral venous pressure measurement is the most exact method but involves puncture of a peripheral vein. Noninvasive alternatives are strain gauge plethysmography and air plethysmography, foot volumetry and photoplethysmography. The latter is most widely used because of its simple handling and portability [3]. Diag-

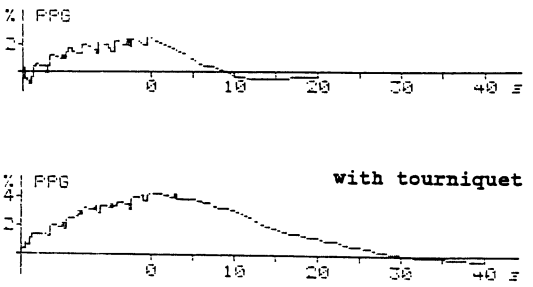

Fig. 2. Photoplethysmography (*PPG*) curves of a patient with treatable chronic venous insufficiency. Venous refilling time ($t0_0$) 12 s, with tourniquet 30 s, i.e., normalized

nostic tests should be performed with and without a narrow tourniquet below the knee. A shortened venous refilling time, i.e., less than 25 s, is caused by calf pump insufficiency. If the venous refilling time can be normalized, as is shown in Fig. 2, operative therapy or sclerotherapy is indicated. In this way the test will give information about the degree to which chronic venous insufficiency can be improved.

Treatment

In the case of an improvable chronic venous insufficiency surgical therapy should be strived for. This includes mainly crossectomy, (partial) stripping of the long or short saphenous veins and ligation of incompetent perforating veins. Alternatively, compression sclerotherapy after the Doppler controlled method of Tournay may be done. The latter method needs a sclerosing plan starting with the highest point of insufficiency and leading to the smaller varices. In a Doppler controlled follow-up study, Tourbier and Schultz-Ehrenburg evaluated the 3 year (and 5 year) results of sclerotherapy of the long and short saphenous veins [2]. Restricting themselves to the Doppler sonographic criterion of absence of refluxes, they found a 3 year cure rate of 52% and 38%, respectively. However, when they formed subgroups of patients without and with deep venous refluxes in the femoral vein (concerning sclerotherapy of the long saphenous vein) or in the popliteal vein (concerning sclerotheapy of the short saphenous vein), the cure rate of patients without deep venous refluxes was 67% and 73%, respectively.

Patients with non improvable chronic venous insufficiency usually need compression therapy all their life. In the acute phase short stretch bandages should be preferred. Normally two bandages are applied, the first in a clockwise direction, the second counterclockwise. After the edema has disappeared medical stockings can be used. The resting pressure at the level of the ankle should be at least 20–30 mmHg. There should exist a pressure gradient from the ankle to the knee.

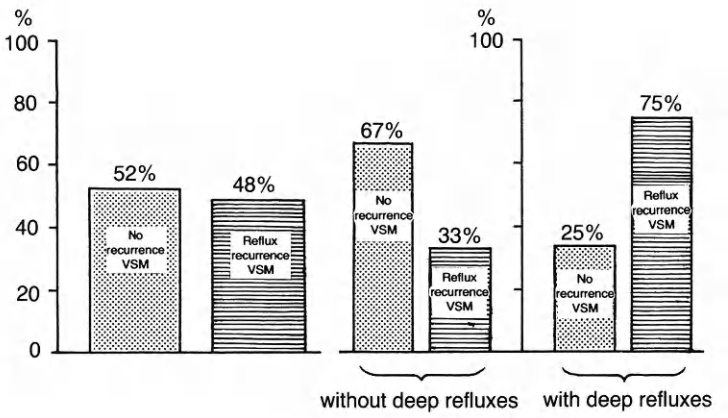

Fig. 3a,b. Reevaluation of the 3 year results of sclerotherapy of the long saphenous vein (*VSM*): **a** all patients; **b** subgroups according to the initial findings of the deep venous reflux diagnosis. (From [1])

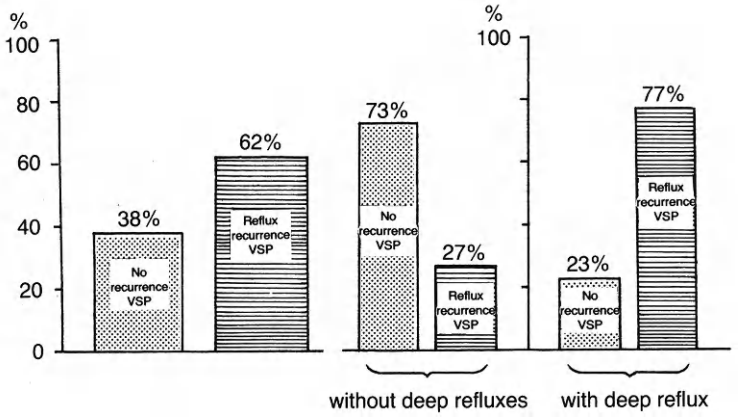

Fig. 4a,b. Reevaluation of the 3 year (and 5 year) results of sclerotheraphy of the short saphenous vein (*VSP*): **a** all patients; **b** subgroups according to the initial findings of the deep venous reflux diagnosis. (From [1])

The success of the compression therapy depends to a great extent on the mobility of the patient. In patients with partial lack of mobility intermittent compression with a pneumatic cuff can be useful.

In patients with leg ulcers a suitable dressing is needed. The choice of the dressing depends on the phase of the ulcer. In ulcers with granulation but without epithelization, skin transplantation using the mesh graft technique may shorten the healing time considerably.

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VIII. Surgery and Physical Therapy of Wounds

Wound Healing in Oncologic Dermatosurgery

R. Rompel and J. Petres

Introduction

The major aim of oncologic dermatosurgery is curative treatment of the malignant tumor; this is best done by total elimination of the neoplastic tissue. Depending on the location and the size of the tumor the resulting defect may cause severe functional impairment for the patient. Tumors of the head and neck often affect important organs such as the nose, ears, lips or the orbital region [9, 11, 18]. Total tumor clearance leads to varying degrees of destruction of those structures. Therefore, the second step in oncologic dermatosurgery is optimal reconstruction, taking both functional and aesthetic aspects into consideration.

All dermatosurgical procedures strive for an optimal wound healing process, which is of major importance for the final result. There are several factors that influence the wound healing process. They may be subdivided into general, preoperative, intraoperative, and postoperative factors. Each surgeon has an individual concept based on his or her own experience and skill. In the following, we present a review of our main principles and of our experiences in obtaining optimal wound healing in oncologic dermatosurgery.

General Aspects

The decision whether to perform surgery depends on the general condition of the patient. If, for example, there are substantial internal contraindications another method of treatment will be chosen. The advantage of surgical treatment of elderly patients is a short duration of therapy as compared to alternative methods such as radiotherapy or cryosurgery [17, 21].

Mainly, the extent of the planned surgery is decisive in choosing the modality of anaesthesia. In general we prefer local anaesthetics without

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additional epinephrine. A reduction of intraoperative hemorrhage improving the visibility of the region being operated on may seem advantageous. However, there is always the danger of postoperative bleeding of vessels at the time when the effect of epinephrine is over. Therefore it is preferable to use local anaesthesia without epinephrine so that all blood vessels that should be ligated are well visible during the operation [13].

Minor operations are performed under local anaesthesia. Histographically controlled surgery, as it is used for example in the treatment of basal cell carcinomas and squamous cell carcinomas, often requires several operations until tumor clearance is achieved. That is why local anaesthesia in those cases is always preferable to general anaesthesia. The advantage of local anaesthesia is that the patient is conscious during the operation. This is of great significance in operations of critical areas (facial nerves, nerves of the extremities) since only the conscious patient can play an active role in monitoring his or her functions [13]. A disadvantage lies in the fact that the injection of local anaesthesia creates an edema of the wound area so that exact adaptation of the wound margins may be difficult. Excision of malignant tumors which exhibit a risk of spreading tumor cells should be performed under marginal wall anaesthesia. The anaesthetic is administered by circular fan-shaped injections around the focus avoiding an intraoperative spread of the tumor cells [13, 15]. Large areas of the extremities may be managed using conduction anaesthesia. Extended surgical procedures require general anaesthesia.

The age of the patient is an important factor in the choice of the reconstructive procedure since the tension of the skin is reduced with increasing age. The skin may be moved more easily and skin folds are useful so that the reconstruction gets simpler in the elderly patient. However, special skin regions may have a reduced blood supply. This should be considered when positioning local flap plasties.

Preoperative Aspects

Prior to operation the patient must be informed and prepared. A confidential talk with the physician should calm the patient and relieve worry and anxiety. The area of the operation must be shaved and cleaned with local antiseptics.

The surgeon should make preoperative considerations on the extent of the surgery and the choice of the anaesthesia. In particular, the horizontal and vertical safety margins for the tumor excision should be fixed preoperatively [3, 4]. Histographically controlled surgery should be used in facial regions and/or in the case of indistinct tumor margins [1, 5, 6]. In case of very large defects that will not be closed by any flap plasties a wound conditioning is necessary [20]. This is a prerequisite for future skin grafting [10, 14]. When reconstruction will be performed with local or

regional flap plasties they should be carefully planned in the preoperative period.

Intraoperative Aspects

The choice of the correct surgical instruments frequently determines the course of healing and the aesthetic result. Coarse forceps, large needles, unwieldly needle holders and scissors traumatize the wound edges causing microthrombi and superficial necroses which lead to wide scars [13]. Small round-bodied needles and atraumatic suture material are preferred.

The surgeon should master the incision and suture techniques. It is important that the incision is made as vertical as possible to the surface of the skin. The choice of the suture techniques will be made individually, for instance, interrupted sutures, matress sutures, continuous sutures and continuous intracutaneous sutures. Proper horizontal and vertical adaptation of the wound margins is decisive for the final result. Resorbable subcutaneous sutures are mainly responsible for holding the wound edges together and must primarily lead to a good adaptation. Final corrections in adapting the wound margins are made by the skin sutures. If the wound margins are free of tension a continuous intracutaneous suture may be used for best aesthetic outcome. However, this technique requires some experience because the vertical aligning of the wound margins is more difficult than with interrupted sutures.

The thickness of the suture material depends on the localization. Fine sutures (6-0 to 7-0) are used in the face while operations on the trunk and extremities require 3-0 to 5-0 and on the scalp 1-0 to 3-0.

Concerning the reconstruction in oncologic dermatosurgery the cosmetic result may sometimes be better if a simpler technique is chosen. Very extensive reconstructive procedures may seem very spectacular to the surgeon; however, the amount of tissue traumatization and scarification is much higher.

Simple reconstructive techniques are: (1) primary wound closure after elliptical excision and (2) VY-shaped wound closure. To achieve tension-free adaptation the surrounding skin area must be undermined subcutaneously by means of dissecting scissors. Using this technique the skin stretches and primary closure of the wound, previously impossible, is achieved [2].

Large defects require local or regional flap plasties for reconstruction. The vascular supply of the flap must be considered when planning the operation to avoid flap necroses. Most skin flaps are random pattern flaps. This means that the supply of the skin flap is mainly via the dermal-subdermal plexus [7]. The planning of the random pattern flap is independent of direct vessel supply and so the flap can be adapted to local anatomical conditions.

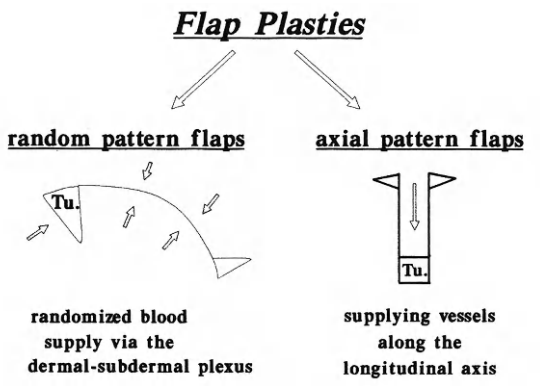

Fig. 1. Local flaps subdivided into random pattern flaps and axial pattern flaps. *Arrows* indicate blood supply. *Tu*, tumor

If the site allows it, the flap should be oriented in its longitudinal axis to supplying blood vessels. This is what is called an axial pattern flap (Fig. 1). The vessels arise from the base of the flap. These vessel-supplied flaps heal better and can be planned with a smaller base which implies that they are more mobile than wide-based flaps [12]. Most of the following examples of local and regional flap plasties have a random pattern blood supply. If width and length of the flap are in an adequate relation wound healing will be excellent. In special areas the amount of the axial pattern blood supply can be increased if anatomical conditions are considered. The relaxed skin tension lines, which are of major importance for proper adaptation of the wound margins [2, 19], may stand in contrast to blood supply if axial pattern flaps are used. Usually, reconstruction will reveal the better result when incision lines are configured along the relaxed skin tension lines and the length-width relations of the flaps are adjusted. However, in each individual condition the surgeon must weigh these factors when planning the flap.

Examples for basic local flap plasties are advancement flaps, rotation flaps, transposition flaps and island flaps. These techniques may be combined when dealing with relatively large defects especially in centrofacial regions such as the nose and lips [16]. If possible in any way, defect reconstruction should be made by using local flaps since the quality of the transplanted skin area is very similar in structure and function as that of the excision site (Fig. 2) [13]. Favorable cosmetic results are achieved when reconstruction involves the skin of the same aesthetic region. In case of relatively large defects in special areas, regional or distant pedicled flaps are used [12]. The pedicled flap is brought into the surgical defect from another site. After healing of the top of the flap the pedicle is returned to the donor site during another session. If a defect of the donor site is remaining it can be covered by a free skin graft. Pedicled flaps are often used in reconstruc-

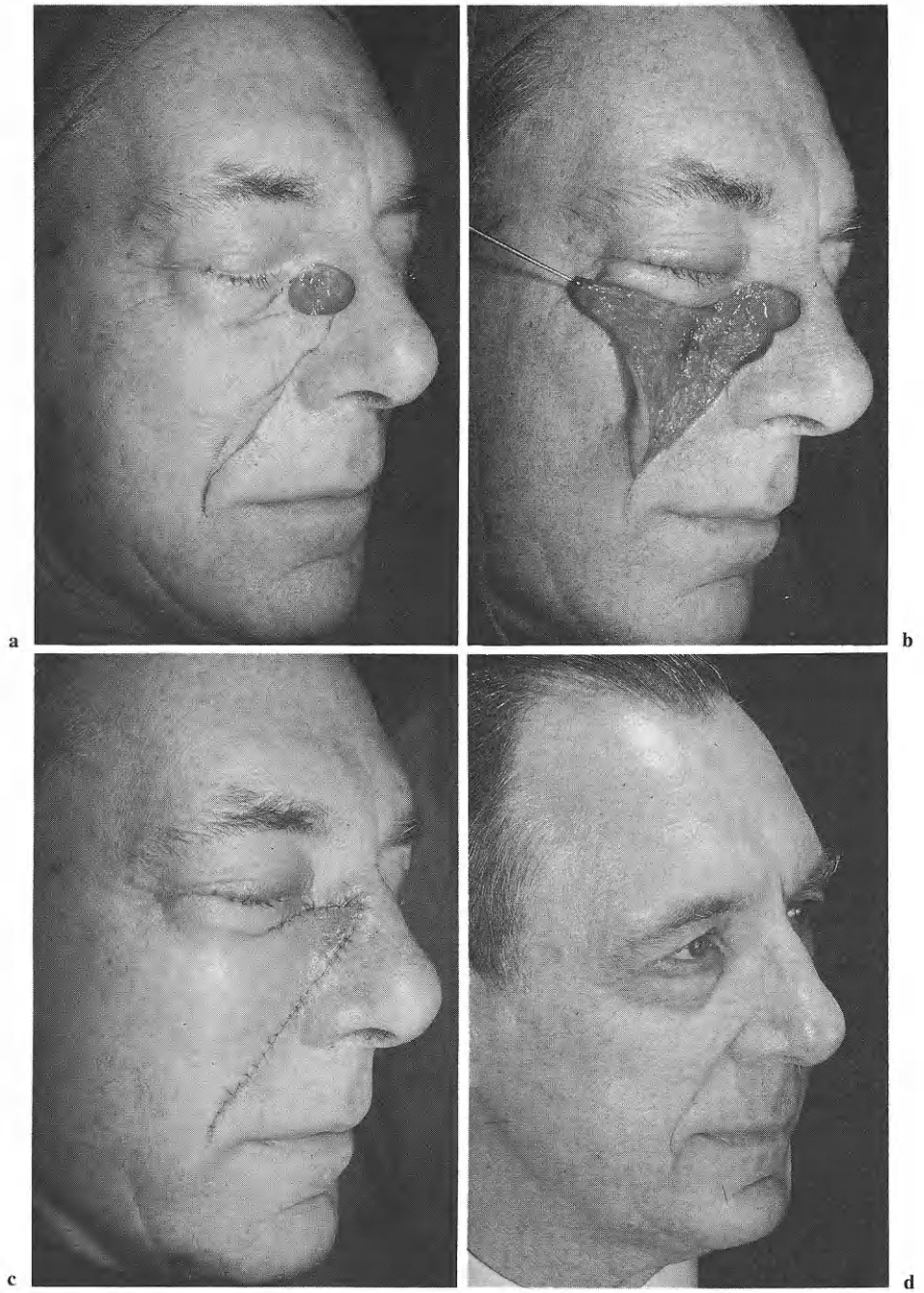

Fig. 2a–d. Advancement flap: **a** defect after histographic surgery of a basal cell carcinoma; **b** mobilization of the flap; **c** end of the operation; **d** 3 years after

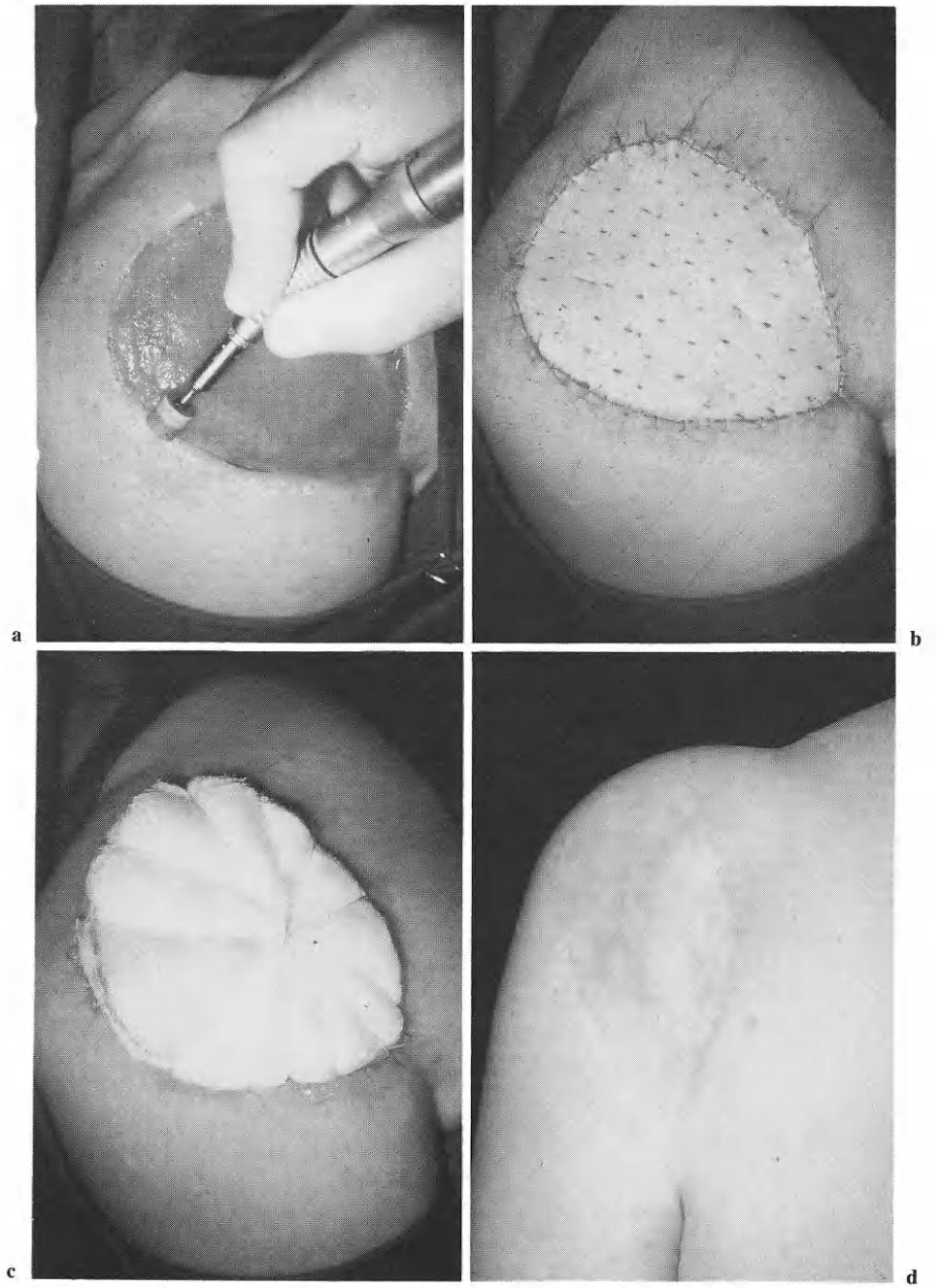

Fig. 3a–d. Split-thickness skin graft: **a** defect after wide local excision of a malignant melanoma and after the wound conditioning, adjusting the wound by dermabrasion; **b** split-thickness skin graft sutured with resorbable material; **c** bandaging technique; **d** 5 years after the operation. (Photographic documentation: C. van Velzen)

tion of large defects of the nose by means of a median or mediolateral frontal pedicled flap [8, 12].

Free grafts are recommended if there is insufficient material available for local grafting. They are often used after wide local excision of malignant melanoma. A prerequisite for optimal wound healing and cosmetic outcome is wound conditioning, which is best done using polyurethane dressings that induce the formation of granulation tissue. The ideal status after wound conditioning is a firm and well vascularized wound. Adaptation of the skin graft is optimized by abrading and adjusting the granulation tissue to the level of the surrounding skin area (Fig. 3a) [10]. The advantage of full-thickness skin grafts is a minimal likelihood of shrinkage and the ability of the graft to withstand pressure. If the wound bed has been properly prepared full-thickness grafts heal well in craniofacial regions. A preferable donor site is the supraclavicular region. Very large defects require covering by split-thickness skin grafts or mesh grafts. The advantage of these grafts is a substantially smaller risk of necrosis than occurs with full-thickness grafts. Split-thickness skin grafts should be avoided in cervicofacial regions because of the tendency to shrink. They are often used in regions of the trunk and extremities after wide local excision of malignant melanoma (Fig. 3 a–d). The preferable donor site is the thigh. Both full- and split-thickness skin grafts require an optimal bandaging technique [10, 13]. Light pressure should be applied to establish a direct contact between the graft and its nourishing ground. The dressing should protect the graft against horizontal displacement (Fig. 3c). In regions of the extremities immobilization by means of a plaster cast is recommended.

Postoperative Aspects

Postoperative wound treatment should assure continuation of proper wound healing. As mentioned above, any wound needs immobilization. Antiphlogistic and antiinflammatory drugs may be prophylactically used to minimize postoperative edema. The wound dressing depends on the type of wound. In temporary wound covering, which is used, e.g., in histographic controlled surgery, sterile and inert dressing material must be used. Primary wound closures and VY-plasties may be managed either by dry or moist wound treatment. In case of local flaps we prefer the moist wound dressing using a combination of fatty ointment and paraffin gauze. This moist wound treatment supports the random pattern blood supply of the wound margins. The dressing should be renewed daily and in case of local irritation the wound covering material should be changed.

Depending on the localization the sutures should be removed, e.g., after 5–7 days in the face, and after 12–14 days on the trunk and extremities. If the suture material is left too long on the wound, infection and foreign body granuloma may occur.

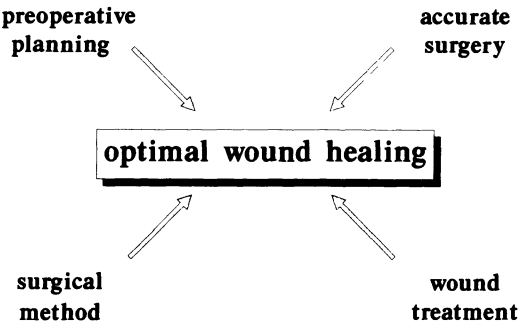

Fig. 4. Influence of several factors on the wound healing process in oncological dermatosurgery

Conclusions

Based on our experience, it is not only the surgical method that is important when optimal wound healing is pursued in oncologic dermatosurgery. Rather a combination of careful preoperative planning followed by accurate operative procedure, considering the above mentioned principles, and individualized postoperative local wound treatment assures a good functional and aesthetic postoperative result (Fig. 4). Advanced reconstructive techniques should be performed by a skilled and experienced surgeon. The broad spectrum of operative techniques shows the necessity for careful choice of the correct reconstructive procedures following radical tumor surgery with respect to localization and size of the tumor.

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Treatment of Keloids and Hypertrophic Scars with Cryosurgery and Silicon Gel Sheeting

H. Tilkorn, K. Ernst, A. Osterhaus, A. Schubert, and V. Schwipper

Introduction

Patients often judge the quality of surgical treatment and the surgeon by the appearance or invisibility of the scar. A protruding, disfiguring, itching and painful scar is a distressing problem for patient and doctor. Moreover, the doctor often is inclined to give into the patient's desire to surgically correct the wrong scar at the wrong time, which worsens the problem.

Since Alibert (1906) [1–3] first described keloids, there has been a seemingly endless number of publications on the etiology and treatment of "problematic scars." Investigators often do not differentiate carefully enough between keloids and hypertrophy. Since there is no histological difference between these two types of scars, one has to depend on the clinical and anamnestic assessment.

Differentiation

The *hypertrophic scar* starts as a reddish-blue, itching, noticeable proliferation of the connective tissue which *does not or only very slightly spread over the lip of the scar*. During the following months the hypertrophy turns pale, the itching disappears, a slow spontaneous involution goes on, and the scar looks flat and tender (Fig. 1).

In the beginning there is no difference between a keloid and hypertrophy. As the distinguishing characteristic, the true *keloid* [4] *proliferates over the border of the scar by sending out claw-like ramifications*. Every new operation and injury is followed by an increase in size. A keloid can finally have a monstrous extension (Fig. 2). Spontaneous involution of a keloid has been observed in very rare cases only. According to many publications, growth over the confines of the original wound seems to be the most

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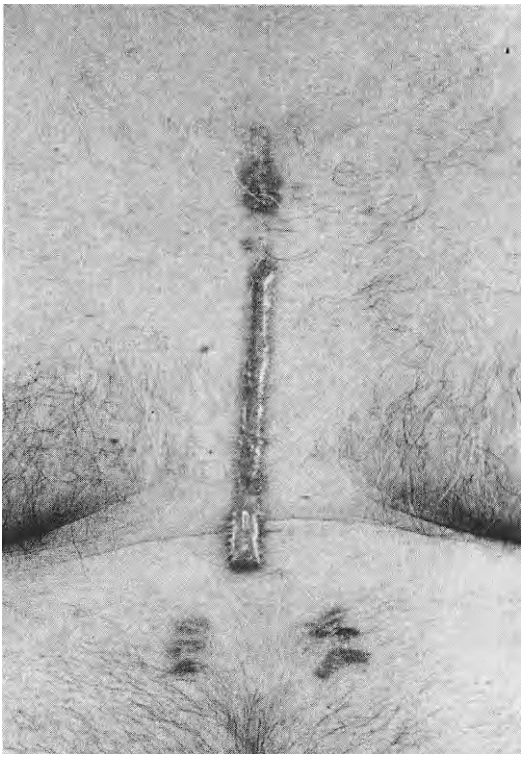


Fig. 1. Hypertrophic scar (sternal region) which does not spread over the border of the scar

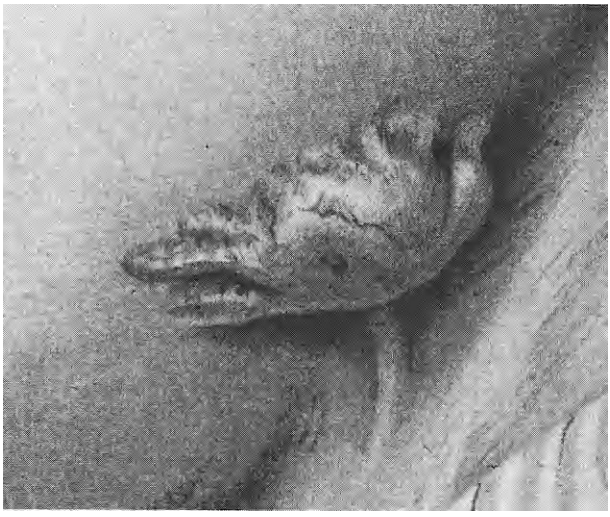


Fig. 2. A true keloid which proliferates over the border of the scar with claw-like ramifications

important criterion in diagnosing a true keloid. Careful differentiation between a hypertrophic scar and a keloid is of decisive importance for the success of treatment [5].

Treatment

For all problematic scars it is essential that treatment begins as early as possible because a fresh keloid or hypertrophy reacts more successfully. However, in the case of a hypertrophic scar, it is no problem to wait and see because of the tendency of the scar to spontaneously regress. This process can be accelerated by different therapeutic techniques [6]:

While surgical correction [7] of an old hypertrophic scar can be useful and sufficient, for a true keloid it is not recommended as the only choice of treatment. In fact, surgery alone is a malpractice! Here an additional therapy must be included such as: (1) *cryosurgery* [8–12], with liquid nitrogen sprayed on the protruding scar, (2) *silicon gel sheeting* [13–19] in combination with *cortisone* [20] injection and/or ointment. These are the most effective, additional, therapeutic measures.

Pressure [5, 14] treatment, which has proven to be very effective for large burn scars can be an inconvenience for the patients over the long term, depending on size, location of the scar, and the time of the year. *X-ray treatment* [21, 23–25] is very efficient, but in our opinion, and following FDA recommendations, it is prohibited or obsolete for treating hypertrophic scars because of the late occurring side effects such as disfiguring radio dermatitis and even radiotherapy-induced cancer. It should only be considered in those cases of real keloid when all other therapies have failed. *Tissue expander*, *laser* and *oral therapies* have not been very promising up to now.

Our Experience

Our experience includes more than 350 patients with keloids and hypertrophic scars who have been treated cryosurgically since 1980 and, since September 1991, 134 patients who have been treated with silicon gel sheeting. Many of these patients had more than one protruding scar [8, 13].

The failure or recurrence rate for the cryo-treated patients is about 15%, including those who withdrew from treatment because it was too long lasting or painful and difficult. Real failures of this therapy occur only in 10% of patients. Of the patients treated with silicon gel sheeting, not a single one has discontinued treatment. On the contrary, compliance is extremely good, as the patients very quickly experience relief of pain and itching and see an improvement of the scar (Table 1).

Table 1. Our clinical experience and results of treatment

	Results	
	Cryosurgery	Silicon gel
Time:	12 years	14 months
Patients	$n = 350$	$n = 134$
Keloids	90% improved 10% failure rate	$n = 12$: 6 good, 5 improved, 1 worse
Hypertrophic scars	−/−	$n = 122$: all improved

Clinical Cases

Two patients with true keloids treated with cryosurgery and three patients treated with silicon gel sheeting are discussed below. Of the latter group, two patients had keloids and one had a full-thickness skin graft.

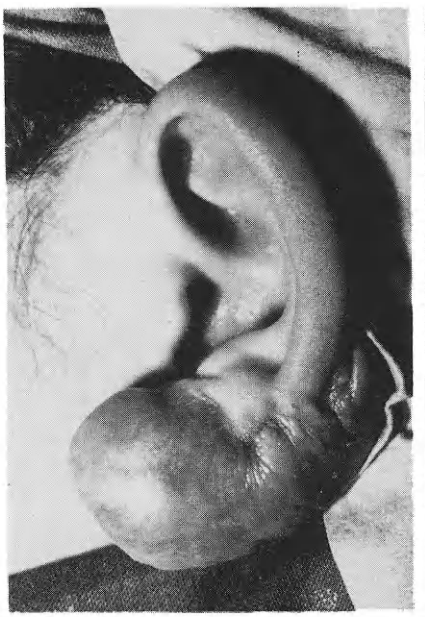
Cryosurgery

Case 1. A boy, born in 1969, was treated in his first year of life for a bilateral hernia. Between 1970 and 1981 a voluminous keloid developed in both inguinal regions as a result of five unsuccessful surgical scar corrections. Each operation made the original scar worse. From March 1984 to September 1987 this boy was treated in our hospital for about 2 weeks as an inpatient; an internal mass reduction of the tremendous sized keloids was performed surgically. Immediately after normal wound healing, cryosurgery was added for about 3 years in monthly intervals as outpatient treatment. A combined therapy consisting of operation + cryosurgery + cortisone (Volon-A) injections with a dermojet was used.

After surgical removal of the keloid, the scar was treated with liquid nitrogen spray 69 times and with Volon-A cortisone injections 26 times. Up to now there has been no recurrence for 4 years.

Case 2. A now 16 year old girl developed typical ear lobe keloids 4 years after she started wearing earrings. She had undergone several surgical removals of the keloids with the result of the tumor growing bigger after each operation. When she was first examined she had hard, not painful, pale keloids of $2 \times 2 \times 3$ cm on each ear lobe.

The first step of treatment was to cut the keloid with the scalpel up to a small layer of fibrotic tissue. The old keloid was converted into a new or fresh one. Some days later cryosurgery was applied to the open wound and a dermojet was used to inject cortisone occasionally. The entire treatment



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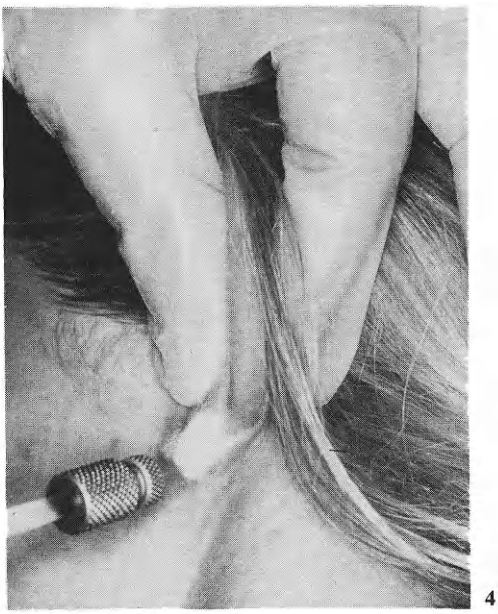

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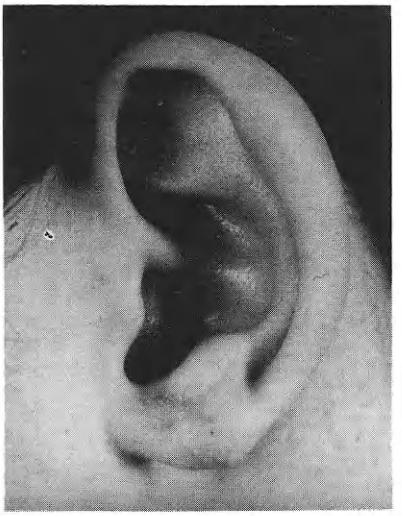

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Fig. 3. Ear lobe keloid
Fig. 4. Ear lobe keloid removed and treated cryosurgically
Fig. 5. Ear lobe keloid treated cryosurgically: result after more than 2 years

lasted 1 year. She had to attend the hospital once a month. She is without recurrence for more than 2 years now (Figs. 3–5).

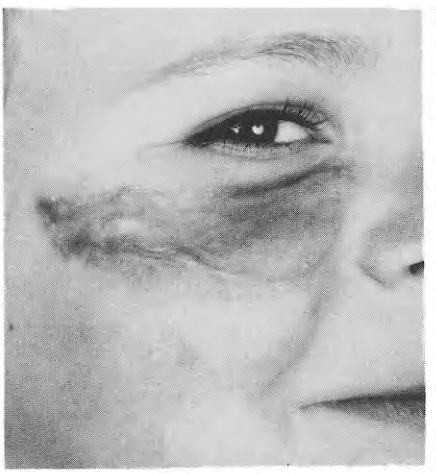
Silicon Gel Sheeting (Silastic Gel Sheeting)

In September 1991 silicon gel sheeting was introduced as treatment of hypertrophic scars and also for some true keloids. Difficulties with the

cryosurgery include painful and long-term treatment. By contrast, there was the simple and successful handling of the silicon gel sheet. We are now using Silastic gel sheet (Dow Corning).

In the first year of our experience 134 patients were treated. Of the 12 patients with keloids, six have had good results, five no change, one worsening of the final scar. Of the 122 patients with hypertrophic scars (hypertrophic scars following burns, operations, skin grafts, donor sites, acne scars or dermabrasion), all have improved under silicon therapy.

Due to these encouraging experiences and based on the publications of Perkins et al. 1982 [13] and Quinn 1985, 1986, 1987 [14–16] the treatment was changed as follows: Keloids first are treated by internal scar mass reduction and intralesional cortisone injection. A few days after surgery silicon gel sheeting is applied for 3–8 months, depending on the response. The silicon gel sheeting is directly applied to the hypertrophic scar, when the



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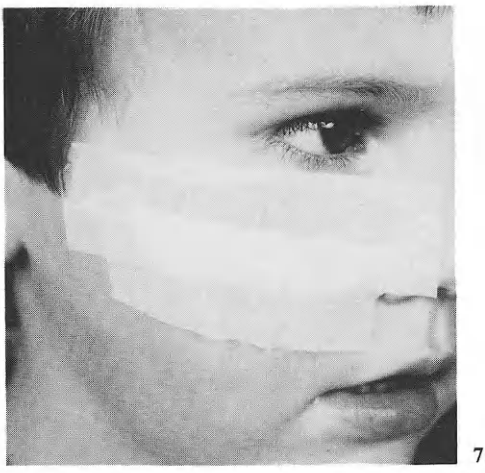

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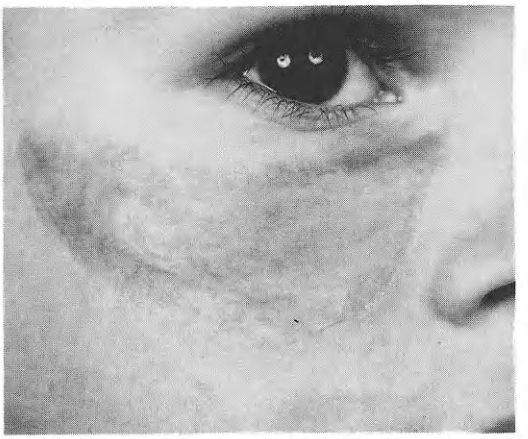

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Fig. 6. Full thickness skin graft 3 weeks after operation
Fig. 7. Silastic gel sheeting fixed with an adhesive plaster
Fig. 8. Result after 2 months of treatment with Silastic gel sheeting

scar is still active, that means red, itching, or painful. An old and not active hypertrophic scar is corrected surgically and silicon gel sheet is applied as soon as the wound is epithelialized or the stitches are removed. The silicon gel sheet should be used 8–12 h daily for 2–3 months. So far, pressure treatment, in addition to the silicon gel sheet, has not been necessary.

Case 1. A 26 year old male patient had a typical true keloid on the sternum after removal of a nevus and two keloid operations. The keloid was cut off

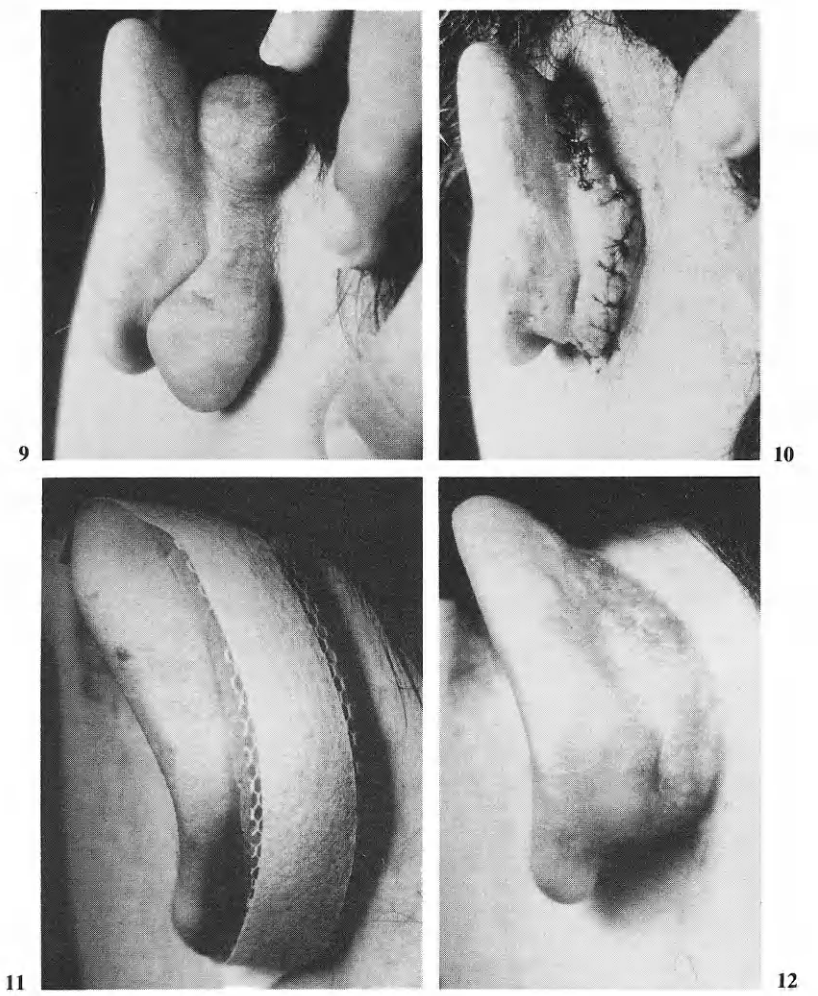

Fig. 9. Real keloid after otoplasty
Fig. 10. Correction of the retroauricular keloid by internal mass reduction and intralesional cortisone injection
Fig. 11. Silastic gel sheeting fixed with adhesive plaster
Fig. 12. Encouraging result after 6 months of treatment with Silastic gel sheeting

and cortisone was injected into the wound. After 8 days, silicon gel sheet treatment was started. This patient used the silicon gel sheet for 8 months and has now, 14 months after the beginning of silicon treatment, had no recurrence of the keloid.

Case 2. In a 4 year old boy a giant nevus was removed and the defect on the cheek was covered with a full-thickness skin graft. To improve the result the parents and the boy were trained to use the silicon gel sheet for 2 months. The result was a much quicker softening of the edges of the graft. The boy tolerated the overnight treatment without any problems. Instead, he asked for it when the parents forgot because he was pleased with the improvement of the scar (Figs. 6–8).

Case 3. A 16 year old boy had a tremendous keloid behind the ear after otoplasty and two unsuccessful scar corrections. An internal scar mass reduction with intralesional cortisone injection was performed and 10 days after surgery the application of silicon gel sheeting was started. The patient is in no way disturbed by the silicon treatment, since he uses the gel sheet overnight and can fix the dressing himself easily with an adhesive plaster (Figs. 9–12).

Conclusion

Silicon gel sheeting is now the first step in treatment of hypertrophic scars and keloids. If we do not see an improvement, we start with surgery or with cryosurgery. We will control our results for 2 more years to be sure that these promising experiences give us a reliable and long-lasting result.

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Surgery of Venous Leg Ulcers

W. Vanscheidt

Introduction

A broad variety of surgical procedures have accompanied the history of phlebology. Basically one can distinguish three different aims of surgical procedures for venous leg ulceration: (1) interventions on the ulcer (excision, grafting procedures), (2) surgery to correct the underlying venous disease and thereby the venous hypertension (bypass operations, crossectomy, stripping, perforator ligation and dissection, ambulatory phlebectomy) and (3) surgery with the fascia as the target organ.

No single type of venous surgery is applicable to all cases of venous leg ulceration. Moreover, to regard surgical therapy as the only possibility would be a serious fault. It is just one method among many including compression therapy and other supportive measures.

Preoperative Diagnostics

The anatomical site and level of any superficial venous incompetence or obstruction can be estimated by continuous wave (CW) Doppler studies. A combination of a functional and an anatomical test is essential to obtain a complete picture of the venous abnormality. We prefer the CW Doppler examination in combination with photoplethysmography. Any vein which is supposed to be ligated or removed should be evaluated by an occlusion test. Prolongation of the venous refilling time by such a maneuver is a convincing argument for surgery. At least one imaging procedure is recommended prior to venous surgery. Nowadays preoperative phlebography is still the method of choice. If it is available, duplex sonography can favorably replace phlebography.

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Choice of the Surgical Procedure

As a rule technically simpler procedures should be carried out before more complicated operations are attempted. Full correction of insufficiency of superficial veins and/or perforating veins should always be carried out prior to considering surgery of deep venous insufficiency.

Correction of Venous Hypertension

Crossectomy

It is accepted worldwide that proper surgical treatment of the orifice of the great saphenous vein in the groin is obligatory in case of a primary varicosis with insufficiency starting at the orifice. Crossectomy is senseless in cases of incomplete stem varicosis with competent orifice valves. Therefore a skillful preoperative Doppler sonographic examination is perhaps more important for the success of the operation than surgical expertise.

Crossectomy of the Great Saphenous Vein

A 4–5 cm skin cut is made on the skin fold in the groin. Cutting more distally must be avoided as problems in reaching all side branches during crossectomy can arise. The adipose tissue and its superficial fascia are cut down to the vein. The connective tissue adherent to the great saphenous vein is cut through, the anterior surface of the great saphenous vein is prepared down to the orifice, and its side branches are demonstrated. Ligation of the long saphenous vein cannot be done before its orifice has been identified. After its identification the great saphenous vein is ligated

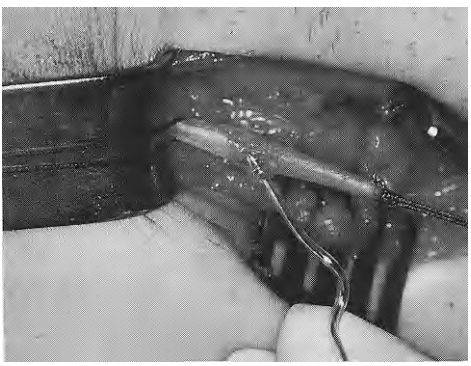


Fig. 1. Distal ligation of the long saphenous vein and insertion of the stripper

distally, which helps to avoid bleeding from the periphery if the vein is injured (Fig. 1). Then the side branches are first ligated close to the orifice. This can be done by individual ligations, but we prefer to do this with one ligation which saves time and has proved to be as safe as individual ligations. The peripheral segments of the side branches are clamped or ligated and the veins cut. The ligated vein stump is prepared and the anterior surface of the femoral vein exposed.

We advise not to inspect the lateral walls of the femoral vein for directly draining side branches for two reasons: (1) higher risk of postoperative lymphedema and (2) postoperative femoral vein insufficiency because of the removal of the fibrous tissue adherent to the femoral vein. Hemodynamically it seems rather improbable that deeply draining side branches of the femoral vein can be the reason for recurrences.

Complications of Crossectomy

Minor complications are postoperative hematomas in the groin and wound infections. Depending on the thoroughness and experience of the surgeon they are usually seldom. Rare but hazardous complications are lesions of the femoral artery and the femoral vein. This risk is higher during surgery for recurring varicosities in the groin. Mistaking the femoral artery for the great saphenous vein and stripping it has been described in the literature. The same is true for the femoral vein. Lesions of the femoral vein by clamps are surely more frequent than published. To avoid them, it is wise to keep in mind the rule: In case of bleeding in the groin the first step is to stop the bleeding by compression of the bleeding vessel with compresses. The next step is to prepare the aspirator and visualize the bleeding point, which is usually quite small; clamping it bears the risk of enlarging the lesion. The best approach is to try a tobacco pouch suture with a thin needle, which enables stitching within the vein wall. Before knotting the suture inject 5000 IU heparin in the bleeding site of the vein to prevent thrombosis. Minor lesions of the femoral vein can also be stopped by turning the saphenous stump for 2 min.

The best help to avoid these complications is excellent knowledge of the anatomy in the groin and its enormous variety and never to ligate before the anatomy has been established.

Crossectomy of the Short Saphenous Vein

Even though the term crossectomy is not correct for ligation of the short saphenous vein, it has been introduced into clinical routine and is now generally accepted.

The site of the orifice of the short saphenous vein varies considerably and is sometimes difficult to locate by Doppler sonography. Therefore preoperative phlebography is essential before this procedure with careful localization of the height of the orifice.

The skin cut is done above the orifice, the adipose tissue bluntly taken apart by wound hooks and the fascia cut longitudinally. The next step is to cut the skin behind and above the lateral ankle. The stripper is introduced into the distal saphenous vein and moved towards the knee. The short saphenous vein is more easily identifiable if the stripper is within the vein.

The vein is then prepared and carefully hooked by an elastic loop. One prepares the vein downwards to the popliteal vein. Because of the small space even minor bleedings can be hazardous and should be avoided. Regularly one encounters the femoropopliteal vein coming from the proximal side and overseeing it guarantees a recurrence. Close to the popliteal vein the ligation is done. It is still a matter of discussion if one has to ligate dilated gastrocnemic veins if they drain into the orifice of the short saphenous vein. Some surgeons do so, others are hesitant to ligate deep veins. Fortunately this situation is seldom.

After ligation careful closure of the fascia with resorbable sutures is essential to avoid postoperative complaints and cysts.

Stripping

Every concerned phlebologist still has to fight against association of varicose surgery, or even phlebology as a whole, with the term stripping. For doctors and patients removal of the long or short saphenous vein is most impressive even though this part of the operation is pathophysiologically often of minor importance. Since most patients have aesthetic interests, removal of the varicosities satisfies this aspect to a high degree.

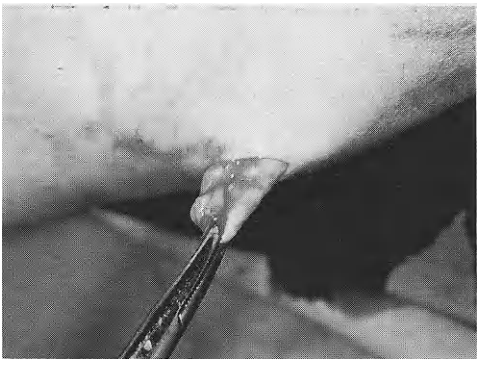


Fig. 2. Phlebectomy of side varicosities

Since autologous vein grafts are best done with segments of the long saphenous vein, segments of about 25 cm should be left in place. Therefore we prefere a retrograde stripping of the long and short saphenous vein. Another advantage of this procedure is that the saphenous nerve is usually not injured. The stripping procedure can be repeated with tubular side branches whereas curved varicosities do not easily permit it. In these cases it is cosmetically much more satisfying to continue with a phlebectomy (Fig. 2). As a rule we perform longitudinal skin cuts and do not parallel them to the relaxed tension lines with the exception of the anterior knee region. These scars remain less visible.

Surgery of Perforator Veins

The perforator veins nowadays are known not to be strictly situated where Frank Cockett and others described them to be. So a careful preoperative investigation is essential. Furthermore it is necessary to demonstrate their functional importance by a plethysmographic occlusion test.

Surgical Technique

The surgical approach to insufficient perforator veins may be subfascially or epifascially. The subfascial ligation of perforator veins is the safest method to prevent recurrences. Its main disadvantages are that it is time consuming and bears an increased risk of wound infections. Simple perforator dissection with a clamp gives comparable results and is technically very easy.

Endoscopic Perforator Dissection

A special endoscope is introduced into the subfascial space by a skin cut near to Linton's line. The crossing perforator veins are prepared, coagulated and cut through (Fig. 3).

Surgery of Deep Chronic Venous Insufficiency

Several procedures have been suggested among which bypass operations are the most frequently performed:

1. For the Palma operation the contralateral long saphenous vein is dissected distally and anastomosed along the pubic area with a segment of the femoral vein distal to the venous obstruction.

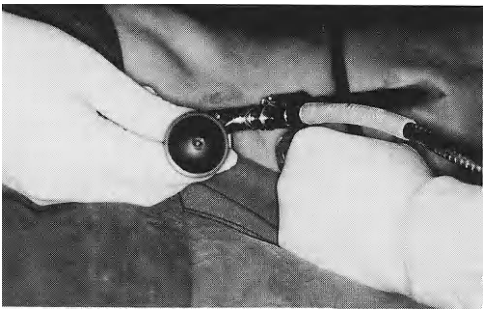


Fig. 3. Endoscopic perforator discission

2. The Warren-Husni operation uses the homolateral long saphenous vein as anastomosis with the proximal popliteal vein. Psatakis instead tried to reconstruct the inefficient muscle pump of the postphlebitic lower limb by prolongation of the tendon of the m. rectus internus [10].
3. Valvuloplasties (Kistner) and interposition of healthy venous segments of the axillary veins are technically even more difficult.

All these procedures have in common that they are difficult and have not been evaluated concerning their prospective results. Therefore they should be restricted to specialized centers which perform randomized controls.

Surgery on the Fascia of the Lower Limbs

Chronic venous insufficiency can lead to dermatoliposclerosis with affliction of cutaneous and subcutaneous structures and subfascial structures. The pathophysiological role of the lower limb fascia has been neglected so far.

The basic therapy of leg ulcers in dermatoliposclerotic lower limbs still remains skillful compression therapy and, if possible, elimination of pathological reflux paths by surgery or sclerosing therapy.

Surgical procedures with the fascia of the lower limbs as the target structure have a long tradition. In 1912 Kondoleon [7] performed a lateral skin incision resecting a broad, 5–7 cm long strip of the fascia and the superjacent dermal tissue. Linton's operation was carried out by a long skin cut with incision of the fascia and dissection of all accessible perforator veins [4, 5]. The essential drawback of this method was the long operation time, the considerable tissue trauma and wound healing problems within dermatoliposclerotic skin.

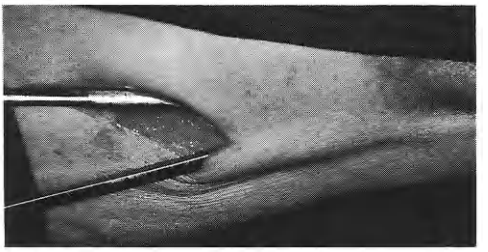


Fig. 4. Paratibial fasciotomy

Paratibial Fasciotomy

The paratibial fasciotomy (Fig. 4) developed by Hach [6] avoids the side effects of previous surgical techniques: The skin cut is made in healthy cutaneous areas so that wound healing disorders are avoided. By means of a strictly paratibial fascia lancing it is possible to preserve subcutaneous lymph vessels and the vasa tibiales posteriores. The new idea behind paratibial fasciotomy is that the fasciotomy split is left open intentionally.

The venous origin of the leg ulcer has to be assessed carefully. Phlebography is performed before operation. Venous refilling time is determined before and after occlusion of reflux paths in a tourniquet test. Approximately 24 h before surgery we take a wound swab to determine the actual bacterial contamination of the ulcers. Then we apply wet compresses with polyvinylpyrrolidone overnight. While introducing anesthesia the patients gets broad-spectrum cephalosporin as perioperative antibiotic prophylaxis.

After skin desinfection and sterile draping of the ulcer, we perform the skin cut 2 cm paratibial on the medial side of the limb. It is important to stay far away from the dermatoliposclerotic area to avoid postoperative wound healing disorders. Then we incise the fascia and digitally mobilize the subfascial space.

The fascia is then taken by a long Metzenbaum scissors and lanced down to the medial ankle. During this procedure it is important not to cut with the scissors but to open the fascia by carefully pushing the scissors downwards and to remain close to the tibia. Hereby lesions of the posterior tibial artery can be avoided. In a "second look" the posterior perforator veins are dissected subfascially. A redon drainage is inserted for 4–5 days. The skin wound is closed and the fascia is left open. A compression bandage is applied immediately.

Postoperatively we place the leg on a Brown splint for about 24 h. Discharge from hospital can be affected as a rule on the seventh postoperative day. Until complete healing of the ulcer, compression therapy is continued. Afterwards we prescribe compression stockings with an integrated truss pad.

Based on our own experience and reports in the literature, the main complications are subfascial haematomas, a superficial skin necrosis above the fasciotomy split, and wound infections. Lesions of the tibial posterior artery were described.

In all cases the immediate pain relief is impressive. This operation can therefore be very helpful in extremely painful cases of ulcerated atrophy blanche. The pain relief seems to persist.

This procedure is still in the experimental phase and explanations as to how it works can only be given hypothetically. Chronic venous insufficiency is not restricted to the cutis and subcutis but involves subfascial structures to a considerable degree. Thickening and induration of the fascia seem to play an important role in venous pathophysiology. By producing a broad paratibial fasciotomy the vascular communication between subfascial and epifascial structures in the dermatoliposclerotic skin is renewed. The immediate increase in transcutaneous oxygen tension after paratibial fasciotomy is remarkable. This operation can be combined with the aforementioned endoscopic perforator discission.

Local Surgery of Leg Ulcers

Ulcer Excision

An internationally accepted way to treat venous leg ulcers is to excise them with their underlying fascia. The recurrence rate is comparatively low. Venous ulcers of the lateral lower limb are, in my personal impression, quickly and successfully treated by this procedure. Recently a new surgical approach consisting of a combination of ulcer excision and muscular flaps was recommended for these ulcers by Hach [6]. For any ulcer excision it is very important to remove ossifications of the subcutaneous tissue (Fig. 5).

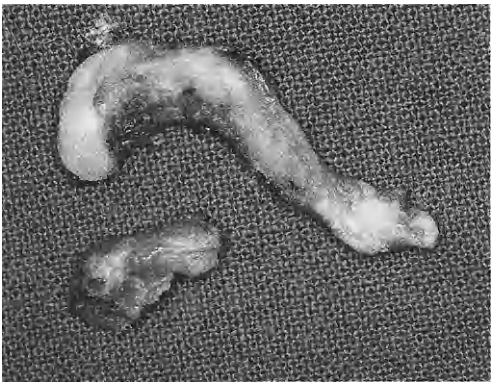


Fig. 5. Ossification in a venous leg ulcer

Skin Grafts

Principally, split skin grafts and mesh grafts (Fig. 6), homologous and heterologeous keratinocytes (Fig. 7), with or without calf skin collagen solutions, and pinch grafts are used. Heterologous keratinocytes act rather as a biologically active wound dressing.

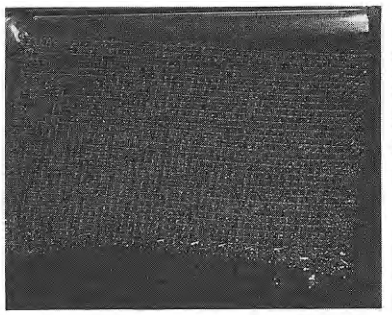
According to recent publications pinch grafts produce comparable results concerning graft acceptance as allogenic keratinocytes. Since the former procedure is much less time and resource consuming it seems to be the procedure of choice (Fig. 8).

Edema removal by compression bandages and elevation of the leg are essential in preparing the ulcer for skin grafting. The ulcer surface should be clean and bacteriological swabs are recommended to prevent graft failure by wound infections. We routinely use polyvinylpyrrolidone wet compresses for 2 days before the day of surgery.

After the operation the patient is immobilized for 10 days and antibiotics are routinely administered as perioperative infection prophylaxis.

The first change of the wound dressing is performed on the seventh postoperative day.

The most impressive improvements in the surgical treatment of venous leg ulcers in recent years are due to better preoperative diagnostics and more detailed knowledge about the pathophysiology of chronic venous insufficiency. In the future the role of the lower limbs fascia seems to be especially worth further evaluation.



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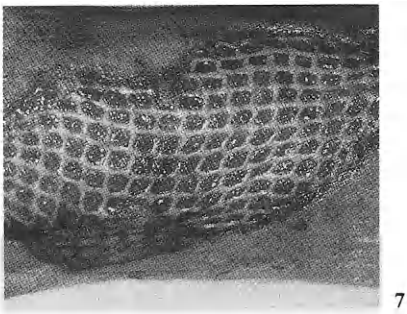

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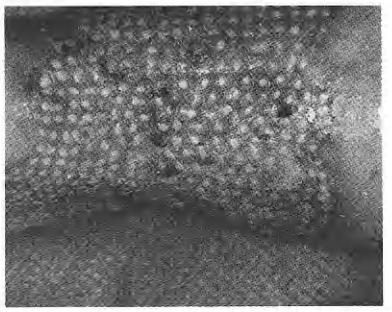

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Fig. 6. Graft of allogenic keratinocytes
Fig. 7. Mesh graft on a venous leg ulcer
Fig. 8. Punch grafts on a venous leg ulcer

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Treatment of Venous Leg Ulcers with a Soft Laser Light

K. Nikolova

Introduction

Laser therapy has been used for many years as a means of stimulating wound healing, but there are rather contradictory data on the effect of soft laser irradiation in the treatment of venous leg ulcers (Iupatov et al. 1990; Timen et al. 1990; Sugrue et al. 1990; Lundeberg and Malm 1991).

Patients and Methods

The pilot study was comprised of 14 outpatients (seven men and seven women), age 54–73 years, with the following diseases: postthrombotic syndrome (PTS) with venous ulcers (n = 12), PTS with ulcerated atrophie blanche (n = 1), and vasculitis livedoides with ulcer (n = 1), The number of leg ulcers was 22. Seven ulcers were treated with a single helium neon beam (He-Ne), 11 with scanner, and four with 3IR + He-Ne (Laser Medical System 301-5, OELT-EOOD, Sofia, beam power of 5 mW with a wavelength of 632.8 nm). Twelve patients received thrice weekly exposures of 10 min and two-twice weekly exposures of 10 min. No other local or systemic treatments were used. The duration of the treatment was 10 weeks. The subjects were interviewed and assessed on admission to the study and thereafter at two weekly intervals regarding subjective symptoms, size and condition of the ulcers (presence of slough, granulation tissue and epithelization). The ulcer area was measured in square millimeters by drawing it on polyethylene foil and transferring it to millimeter ruled paper. The assessment of granulation was made using the following scale: 0, without granulations; 1, single granulation; 2, moderate granulations; 3, abundant granulations; and 4, full epithelization. Slough was assessed using a similar scale: 0, without slough; 1, single area of slough; 2, slight diffuse slough; and 3, dense slough.

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Statistical evaluation of the results was carried out using Student's t test.

Results

Most of the subjective symptoms reported on admission to the study such as pain, paraesthesia, and cramps at night significantly improved or disappeared at the end of the trial. Ulcer size decreased steadily during treatment (maximum at the tenth week by 56.4%, $p < 0.05$) (Fig. 1). Ulcer slough

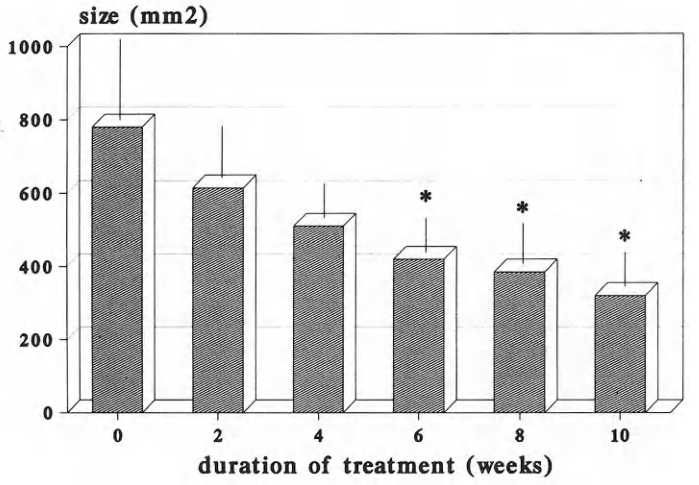

Fig. 1. Changes in ulcer size during laser treatment; $^{*}p < 0.05$ vs control

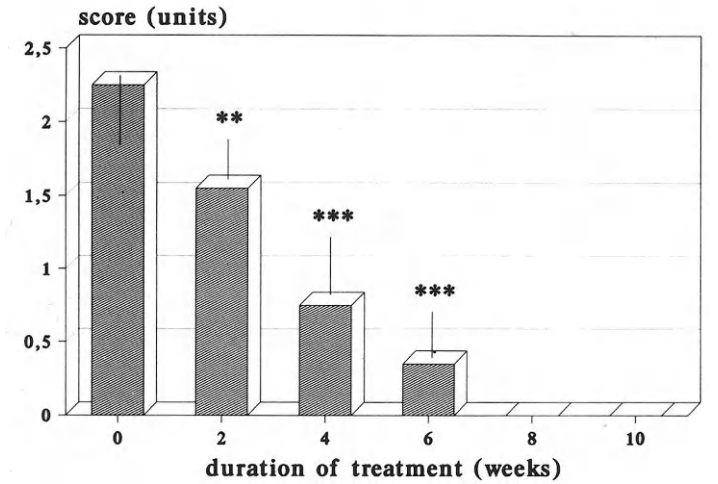

Fig. 2. Changes in ulcer slough during laser treatment; $^{**}p < 0.01$; $^{***}p < 0.001$ vs control

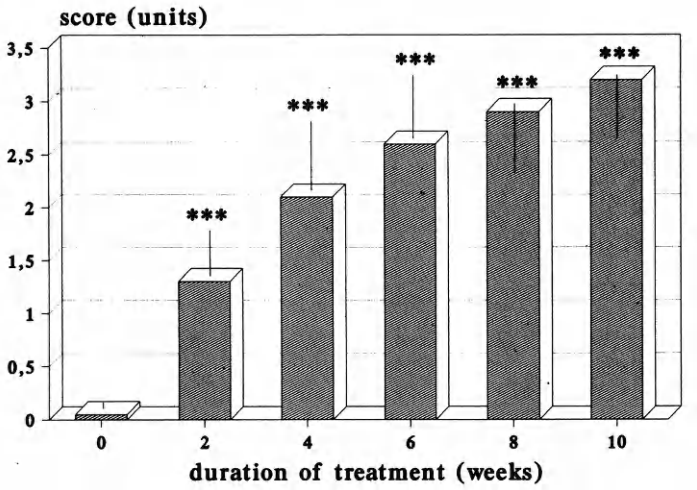

Fig. 3. Changes in ulcer granulations during laser treatment; $^{***}p < 0.001$ vs control

disappeared after the sixth week (Fig. 2), and granulations increased from 0.05 to 3.2 units ($p < 0.001$) at the tenth week (Fig. 3). Global evaluation of the treatment was: epithelized ulcers, 11 ($n = 8$); with improvement (decrease of the ulcer size by one half to one third from the initial one), 8 ($n = 6$). Two patients failed to respond, and the condition of one was aggravated. There were no side effects.

Discussion

Our study has shown considerable improvement of the subjective and objective symptoms of the patients included in the trial. Our results correspond well with these of Sugrue et al. (1990) who performed a similar pilot study on 12 patients with chronic venous ulcers unresponsive to conservative measures. After 12 weeks treatment with infrared laser irradiation the authors reported complete healing of two ulcers, 27% reduction of the size of the remaining ulcers, considerable increase in granulation tissue, and dramatic reduction of pain. Iupatov et al. (1990) and Timen et al. (1990) also reported a very good effect of laser therapy in the treatment of trophic ulcers of the lower extremities in patients with chronic venous insufficiency. However, using low power He-Ne laser Lundeberg and Malm (1991) did not find a significant effect of laser irradiation on venous leg ulcers.

The biological effects of soft laser have been thoroughly described by Mester et al. (1985). The authors reported an improvement in blood circulation of the regenerating tissues and a significant increase in revasculariza-

tion. Amelioration of the microcirculation plays an important role in the healing process in peripheral venous disease by improvement of oxygen and nutrition supply to the tissues. Mester et al. (1985) also reported an increased number of dividing cells and modulation of prostaglandin formation. Other reported effects of soft laser are: increased wound contraction and cellularity of the wound bed (Dyson and Young 1985), and edema reduction and regeneration of the vein and lymph vessels (Lievens 1985). These effects could be a satisfactory explanation of the beneficial action of the laser treatment observed by us.

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IX. Dressings and Agents Promoting Wound Healing

Dressings as a New Concept for Topical Treatment of Ulcers

H. Degreef and M. Flour

Introduction

Many new discoveries have been made in the last decades in dermatology. The therapeutic use of corticosteroids, retinoids, and photochemotherapy has changed dermatology from an art, into a science in which basic research, controlled studies, and new etiopathogenetic insights prevail. With this approach, contentions and dogmas were checked and often found wanting. However, some traditional treatments have become, after some adaptation, important aids in treating skin diseases, e.g., psoralens and vitamin A and its derivatives.

Furthermore, in the last 20 years, better insight has been obtained into the phenomenon of skin regeneration and cicatrization. These new insights have resulted in changes in the treatment of wounds and have brought new materials on the market that made use of this new knowledge of the physiology of wound healing. In the beginning, there were only a few products and these were critically and very skeptically received. The results obtained, however, stimulated the pharmaceutical industry to bring out new and, it was contended, improved products. This recent and rapid evolution has led to much confusion. One could almost speak of a jungle of dressings. My intention here is to sketch a few of the main roads through this jungle but not to examine any one tree. I do not intend to discuss the healing process as such but rather to point out the characteristics of the new synthetic dressings and their use, their indications and contraindications, and their advantages and disadvantages.

According to modern insights, a wound dressing must satisfy the following requirements:

1. Excess exudate and toxic products must be removed without dehydrating the wound itself.
2. A humid environment must be maintained at the wound surface in order to permit optimal division and migration of cells in the wound.

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3. Permitting the exchange of gas is important primarily for superficial wounds. In deep wounds, a slightly acid environment and a hypoxic condition promote the development of granulation tissue.
4. There must be good thermal insulation on the wound surface. The temperature should be as close as possible to body temperature. A decrease of 3°–4°C will reduce the phagocytic activity of macrophages and seriously reduce the effectiveness of a dressing.
5. Protection must be afforded against secondary infection.
6. The dressing may contain no products or byproducts that are toxic for the wound.
7. The dressing must be easy to remove without damage to the granulation tissue or new epithelium.

In the literature, one speaks of "environmental dressings," designed to create a microenvironment in which the healing process of the various kinds of wounds can take place in an ideal way. The new synthetic dressings can be classified in many ways. The division we use here is as follows:

1. Transparent polyurethane film dressings
2. Hydrogels
3. Foam dressings
4. Hydrocolloid dressings
5. Non-adherent dressings
6. Xerogels and xerodressings
7. Alginates
8. Biodressings

Obviously, no one product can satisfy all the above mentioned desiderata. Our intention is thus to point out the specific characteristics of each group of products and, where necessary, to indicate the differences in the materials used.

Transparent Polyurethane Film Dressings

The first group of dressings that were developed consisted of thin sheets of polyurethane (polymeric semipermeable films). These are thin, elastic, transparent sheets or films. Thus, they fit the form of the body or of the treated part very well and no other disturbing dressings are needed. The wound surface remains visible for inspection. Transparent film dressings are permeable to air, oxygen, and water vapor but without being porous. Thus, the patient can wash and even shower without any problem. If the dressing is small, it is even possible to take a bath or go swimming. The dressings also protect against bacterial invasion of the wound.

The wound exudate remains at the wound surface, so the migration of epidermal cells is promoted. Moreover, crust formation, or the formation of

eschar, is prevented. Nerve ends are protected by the dressing as though it were a second skin, which means a rapid reduction of pain.

Indications

As for all the new synthetic dressings, the manufacturers give a long list of indications for their use. Nevertheless, there is a more specific indication area for each group of dressings. We think it is important to stress the following specific indication areas.

Preventive indications for the use of transparent film dressing include prevention of decubitus ulcers at friction sites. The dressings have a high resistance to both shearing and tearing, so the skin under the dressing is not only protected against mechanical loads but also will not be macerated by urine or dry incontinence, etc. In surgery, transparent films are used preventively to protect the area surrounding the incision area.

The primary curative indication for polyurethane film dressings is in treating sterile superficial wounds. Thus, we may consider first degree burns and first degree bedsores, superficial wounds, e.g., friction blisters, and small cuts. In surgery, the primary indication is certainly the covering of donor areas of skin grafts. Furthermore, transparent film dressings can also be used in dermatology for the treatment of laser wounds and dermabrasion areas. Under the dressing, a certain amount of serous fluid is retained. It has been shown that the presence of this serous fluid has a self-sterilizing effect and assists in the healing of the wound.

Contraindications

Necrotic, infected, and very exudative wounds must first be treated lege artis in another way before they can be considered for these polyurethane dressings. When the ulcer or the wound is surrounded by thin, vulnerable skin, it is best not to use this kind of dressing because the removal of the adhering dressing will damage this surrounding skin.

Examples

Opsite (Smith and Nephew) was the first product in a long series of new synthetic dressings that came on the market. Before applying the dressing, the environment is carefully cleaned and degreased, preferably with an alcohol solution, and then dried. The Opsite dressing is then stretched tight over the wound surface after the protective film is removed. The dressing has to extend 5–6 cm beyond the wound and can remain in place for several days, up to a week.

The disadvantages of Opsite include:

1. Opsite is a polyurethane film with a hypoallergenic adhesive based on polyvinylethylether, so the dressing sticks to healthy skin but not to the wound surface. When two parts of the dressing with adhesive come in contact with each other, they stick together, so a certain amount of dexterity is needed to apply the dressing. If the wound is large, it is advisable to have two people put the dressing on. In order to alleviate this problem, other application systems have been developed by the manufacturer. This disadvantage can be converted into an advantage for the application of a hand dressing in the form of a glove, as can be desirable for some wounds and particularly burns. This product is called Opsite Glove (Smith and Nephew), and the special Opsite Glove Applicator is required. In the mean time the manufacturer has adapted the adhesive so that this is no longer a problem. Salves can be applied under the dressing. For burns, a cream based on 1% silver sulfadiazine will often be used.
2. When strongly exudative wounds are treated, the exudate builds up under the dressing. Thus, there is an accumulation of bloody and serous fluid that will escape along the edges of the dressing. To prevent this, the dressing can be changed more frequently. This increases the chances of bacterial infection, and it can damage the new granulation tissue. The fluid can be drawn off with a thick needle and the resultant hole in the film sealed with sticking plaster or a small piece of Opsite. Nevertheless, it must be noted that drawing off fluid increases the chances of infection.

Other Polyurethane Dressings

In order to overcome the disadvantages of Opsite, all sorts of new polyurethane dressings have been developed. Absorbing polyurethane dressings were also developed, but they are no longer used because of the other kinds of dressings now available. For example, *Mitraflex* wound dressing contains an absorptive membrane under the polyurethane film. This layer consists of a microporous polyurethane membrane that absorbs and acts as a reservoir for wound exudate. The dressing can be used when Opsite is indicated, not only in low but also in moderate exudate wounds.

Non-self-adhering polyurethane dressings have also been developed. These not only have an acrylic adhesive but often also have different application systems. Examples of these non-self-adhering are: Tegaderm (3M), Opraflex (Lohmann), and Bioclusive (Johnson and Johnson). The indication area of these non-self-adhering dressings is the same as that of Opsite.

Hydrogels

As we have noted, one of the disadvantages of the polyurethane film dressings is the lack of an absorbing substance. When the exudate formation significantly exceeds the loss by evaporation through the dressing, considerable fluid builds up under the dressing.

In order to overcome this disadvantage, different kinds of products have been developed. Xerodressings are dry materials that are transformed into a gel during the absorption of water and wound exudate. Hydrogels are ready-hydrated products in the form of gel sheets. They are thus moist materials that are still capable of absorbing large amounts of fluid without being water soluble.

In contrast to all the other dressings, a hydrogel is capable of absorbing large amounts of wound fluid while still providing a moist environment. The product does not adhere to the skin, so the surrounding skin is not damaged. The product also has a very clear pain alleviating effect. Hydrogels can be easily used over quite large superficial wounds.

The hydrogel is cut out in the form of the wound, pressed on, and closed with an ordinary dressing. Generally, the dressing must be changed daily.

The major disadvantage of the hydrogels is that they do not offer clear protection against bacterial invasion.

Indications

Like polyurethane films, hydrogels are indicated for sterile superficial wounds. In contrast to polyurethane dressings, strong exudation of the wound is not a contraindication. Because a moist environment is maintained, epithelialization is promoted and the sealing effect greatly decreases pain. Since hydrogels are delivered in sheets, rather extensive wounds constitute no difficulty and they are much easier to use than xerodressings.

Hydrogels are probably the best dressings after dermabrasion since strong exudation generally occurs, but they can also be used after laser treatments. It is not surprising that the first hydrogels were marketed for the treatment of friction blisters as a "second skin" in a first-aid kit. All sterile superficial wounds and sterile burns are also good indication areas for these dressings.

Contraindications

Necrotizing ulcers, certainly when an anaerobic infection is suspected, are contraindicated. Deeply fissured wounds are also contraindications. Third

degree burns are not good indications for these dressings, certainly when necrotic tissue must still be removed.

Examples

Vigilon (Bard) consists of 4% polyethylene oxide and 96% water in a colloidal suspension. In the middle is a thin polyethylene network that gives the gel its sturdiness. In addition, both sides are covered with a thin, transparent polyethylene film. Before the dressing is applied, the film on the side that comes in contact with the wound surface is removed. The dressing is permeable to oxygen, even when the outer sheet is left in place. The dressing can absorb approximately its own weight in exudate. When still more exudate is expected, the outer sheet can also be removed. In this way, more fluid can evaporate, which is then absorbed in a dressing that is placed over the hydrogel.

Cutinova gel foil (BDF) consists of a polyvinyl alcohol hydrogel. The sturdiness of the dressing is provided by a thin polyester network. There is only one protective sheet, so that water evaporation is not so adjustable.

Geliperm (Geistlich) has a somewhat different composition and consists of an insoluble cross-linked polyacrylamide agarose polymer and 96% water.

Foam Dressings

These can be divided into silicon foam dressings, e.g., Silastic foam dressing (Dow Corning), and polyurethane foam dressings. The indication area for *silicon foam dressings* consists of deep, irregular wounds. The foam dressing is shaped in situ to form an exact cast of the cavity of the wound. This dressing forms a kind of sponge that can take up in its alveolar structure excess exudate, cell debris, bacteria, etc. The dressing can be removed from the wound, cleaned, soaked in an antiseptic solution, and replaced.

Polyurethane foam dressings are much easier to use and also have their specific indication area. The dressings consist of a soft, hydrophilic, polyurethane foam in which exudate, necrotic cells, cell debris, pus, and bacteria can be absorbed into the alveolar structure and removed when the dressing is changed. The outer side is hydrophobic, so the moisture necessary for good wound healing is retained.

The foam dressing is cut out in the shape of the ulcer with a border of 2–3 cm and then pressed on well. The hole is closed off with an ordinary dressing. Changing daily or every 2 days is necessary.

Indications

Chronic, necrotic, and still quite strongly exudating wounds, for example, vascular ulcers and decubitus ulcers, constitute the primary indications for these dressings.

Foam dressings are also very suitable for the treatment of not yet completely clean second and third degree burns. Once the wound surface has been cleaned, a skin graft can be applied. Thus, the dressings can be considered temporary in such cases.

Contraindications

Too dry or too atonic wounds are not well suited for foam dressings because it is impossible to have good contact with the wound surface. Also, in the case of serious superinfection of the wound, such dressings cannot be used. Suitable anti-infection treatment must be used first.

Examples

Cutinova Plus (BDF), Epigard (Parke-Davis), Syspurderm (Hartmann), Lyofoam, and Lyofoam C (Ultra Laboratories) are some examples of foam dressings. Cutinova Plus has a protective, hydrophobic top layer made of Cutinova gel foil. Epigard is a sheet of polytetrafluorethylene. Lyofoam C also has another upper layer of activated charcoal to combat odors.

Hydrocolloid Dressings

These consist of a thick, adhesive, absorbent hydrocolloid mass and a thin, water-resistant film on the outside. The hydrophilic particles are contained in a hydrophobic polymer matrix. The chemical composition and structure varies somewhat from product to product, but the biological and therapeutic effects are very similar. Various polymers are contained in hydrocolloid dressings, and gelatin, pectin, sodium carboxymethyl cellulose, and karaya gum are now available. The adhesive matrices are usually either polyisobutylene or elastomeric substances such as styrene isoprene copolymers or ethylene vinylacetate. In some hydrocolloid dressings the matrix is cross-linked, which results in a honeycomb structure within which the hydrocolloid particles are held. This gives the dressing material greater cohesion, as little or no free gel forms as it hydrates. In the non-cross-linked types, hydration leads to more free gel formation. It remains a matter of some debate as to the significance of this property in terms of wound healing, although it seems likely that different types of wounds are suited to different

types of hydrocolloid. A more exudating wound is a better indication for the honeycomb type, whereas a rather dry wound would dictate the use of the non-cross-linked type of hydrocolloid. The function of the outermost membrane is the same for virtually all the products. The outermost material of Biofilm, however, is somewhat permeable for gasses and water vapor but also for bacteria.

The dressing is cut according to the form of the ulcer, but a 2–3 cm margin is necessary on all sides to obtain good adhesion to the skin.

Before applying the dressing, the protective membrane must be removed. The dressing must be changed when it begins to leak, which will be after 5–7 days with good indications.

Common features of hydrocolloid dressings are:

1. The hydrophilic particles form the gel-forming substances that will form a semisolid gel (actually a suspension) after contact with wound fluid or water vapor from the wound base. A moist microclimate is thus created that is necessary for the migration of epithelial cells.
2. These dressings are impermeable for oxygen and water vapor. For a very long time, the advantages and disadvantages were discussed of oxygen-rich and oxygen-poor environments on the wound surface. Present knowledge indicates that an oxygen-poor environment promotes neovascularization in cases of full-thickness injury.
3. The sealing outermost layer protects against bacterial infection.
4. Generally, the dressing can be changed without damaging the newly formed granulation tissue because the hydrocolloid mass above the wound surface is softened. Nevertheless, it remains advisable to remove the dressing very carefully when changing it because the newly formed epidermis, or thin, vulnerable surrounding epidermis, can be damaged.
5. There is better debridement of necrotic tissue of the ulcer, probably promoted by macrophages and proteolytic enzymes that are released from granulocytes, which can better reach the wound surface when it is covered with an occlusive dressing.
6. Generally, there is also a clear reduction of pain.
7. The dressing can be left in place for several, generally 5–7 days. Before it begins to leak at the edges, it must be changed. When it turns out that the dressing has to be changed too often, such as every day or every 2 days, then it is advisable to opt for another kind of treatment because this indicates a weeping ulcer. To a certain extent, this can be dealt with by strewing extra hydrocolloid granules on the ulcer base in addition to the dressing. Indeed, these hydrocolloid granules are dry (xerocolloidal) and, like xerogels, actively absorb wound exudate and so have a debriding effect.

   The difference between xerocolloidal granules and xerogels is that the former form a suspension of very large molecules in water while the latter, also constituted from very large molecules, are physically or chem-

ically bonded to each other in water to form the gel structure. There is thus no dispersion, as occurs with xerocolloidal and hydrocolloid dressings.

8. Some hydrocolloid dressings also have a fibrinolytic activity. These dressings, therefore, have not only a permissive passive role in normal wound healing but also promote the removal of fibrin. This could be of interest in the debriding effect on necrotic wounds and eschars. The fibrin cuffs around the small vessels of the skin play an important role in the origin of venous ulcers. This could, in turn, explain the good results of hydrocolloid dressings in the treatment of venous ulcers.

Disadvantages of hydrocolloid gels are:

1. The gel formed has a characteristic, sometimes alarmingly yellow color that looks like pus.
2. The gel, together with the fibers in it, often has a very unpleasant odor.

It has been suggested that a disadvantage of occlusive dressings is the promotion of bacterial infections. In practice, however, infections are rare. The presence of granulocytes and macrophages, which are not always removed because of too infrequent dressing changes, the temperature under the dressing, and the slightly acid pH could explain why no bacterial invasion takes place and why even an inhibition of bacterial growth has been observed. Nevertheless, after the removal of the gel substance and after cleaning of the ulcer, close wound inspection must always be conducted when the dressing is changed.

Indications

The primary curative indications for occlusive hydrocolloid dressings are vascular and particularly venous ulcers, which give off rather little fluid and have an atonic appearance. Stage two decubitus ulcers and second degree burns also are very good indications. In addition, these dressings can be used after dermabrasion, for the treatment of skin graft donor areas, and in the treatment of traumatic wounds.

Hydrocolloid dressings can also be applied preventatively on friction areas, amputation stumps, and pressure sites. In such cases, they can be left in place for 1–2 weeks.

Contraindications

Clear signs of superinfection or too strong exudation are contraindications for some but not all hydrocolloid dressings.

Examples

The hydrophillic hydrocolloid particles in DuoDERM dressings (ConvaTec, a Bristol-Myers Squibb Company; known in some markets as Granuflex and Varihesive) consist of pectin, gelatin, and carboxymethyl cellulose. The hydrophobic matrix in which the particles are embedded consists of polyisobutylene. A polyurethane film-foam laminate forms the outermost layer. When the base of the ulcer is very irregular and deep, or where there is a large amount of exudate, the base can be filled up with hydrocolloid granules (DuoDERM Hydroactive granules) or DuoDERM Paste. Recently a new formulation of the wafer, DuoDERM E (known in some markets as Improved Formula Granuflex and DuoDERM CGF), has been launched worldwide. DuoDERM E consists of the same hydrocolloid particles as DuoDERM, dispersed in a cross linked adhesive matrix which remains cohesive when hydrated. DuoDERM E therefore handles large amounts of exudate in a different way. Other forms of dressings in the DuoDERM range include DuoDERM Extra Thin, and DuoDERM with an adhesive border. The products are available in a range of sizes.

The hydrophillic granules in Comfeel ulcer dressing (Coloplast) are carboxymethyl cellulose. They are imbedded in a styrene-isoprene, copolymer, adhesive, elastic wax, and here too there is an outermost polyurethane film. Aids for deep and more exudative ulcers are Comfeel ulcer paste and Comfeel ulcer powder.

Biofilm (dressing and powder, Biotrol) is composed of sodium carboxymethyl cellulose and karaya gum. This dressing is more elastic, stretchable in various directions, and the top film, made of a nonwoven polyester, is somewhat permeable for gasses and water. The dressing can thus be used for somewhat wetter ulcers, but it does not exclude bacteria.

Other hydrocolloid dressings are Intact (C.R. Bard) and Restore (Hollister).

Non-Adherent Dressings

These are new synthetic dressings to be used where gauze often used to be applied. Vaseline gauze can cause maceration of the epidermis. This certainly is a disadvantage when placed on grafts.

N-terface (Winfield Laboratories, Texas) consists of a high-density plastic woven into a monofilamentous membrane. This dressing is used primarily for the protection of split- or full-thickness skin grafts to keep them from adhering to the covering dressing. When the graft is covered with such a dressing, the dressing can be changed atraumatically. N-terface can also be applied to an ulcer base and xerodressings like Debrisan strewn over. The dehydrating activity of the hydrophillic granules is not reduced by

the dressing, although the changing of the dressing is greatly facilitated, and the hydrophillic granules will not adhere to the wound base.

Other products that can be used for the same purpose are Melolite Contact (rayon manufactured from cellulose, Smith and Nephew) and Adaptic (also a viscous rayon from Johnson and Johnson).

Xerodressings and Xerogels

When a wound or an ulcer gives off too much moisture, it can be desirable to dry it out before proceeding to granulation and epithelialization-promoting dressings. Classically, compresses are used for this, but new products have been developed also for such situations. We mentioned already hydrogel sheets for large superficial, oozing, painful defects, and hydroaphillic particles, when using hydrocolloid dressings. Xerodressings have the common characteristic that they can absorb exudation fluids, cell debris, and bacteria. Antiseptics are added to some of these dehydrating products.

Examples

Debrisan (Pharmacia) beads (dextranomer) consist of dextran, a linear carbohydrate polymer product, cross-linked with epichlorohydrine. This gives the beads a three-dimensional structure. They are very hydrophilic. Substances with a molecular weight less than 5000 penetrate the pores of the beads, while those with a greater molecular weight are sucked up by capillary action in the spaces between the beads. In order to facilitate application, the beads are made into a paste (Debrisan paste), consisting of 60% Debrisan and 40% the very hydrophilic polyethylene glycol (Macrogol 400).

When the beads or the paste are saturated with exudate, which can be seen by the change of color from white to yellow, the granules must be completely removed. Beads that still adhere to the base of the ulcer will crystalize and prevent further wound healing. Generally, this dressing must be changed once or twice a day.

In order to facilitate the changing of the dressing, one can cover the ulcer base with a nonadhering dressing, i.e., Pharmanet, or use Novasorb. The latter consists of a nonadhering rayon sack containing Debrisan paste. Its small size makes it only suitable for small weeping ulcers. If there is a risk that the dehydrating will be too extreme, a moist wound environment can be maintained by applying Pharmaclusive over Debrisan beads or over the Debrisan paste. This is a nonsterile, semipermeable, self-adhering dressing.

Actisorb Plus (Johnson and Johnson) is activated charcoal made by heating cellulose fiber to 900°C. The product is impregnated with 0.15% silver sulfadiazine to prevent bacterial proliferation in the dressing.

Iodosorb (Stuart Pharmaceuticals) is a yellow-brown powder consisting of microgranules of modified starch. It also contains 0.9% iodine.

IntraSite gel (Smith and Nephew) consists of a disposable sack with a gel. This very hydrophilic gel, based on 2% starch, 20% propyleneglycol, and 78% water, will absorb the excess fluid and can be easily removed with water. A dressing can be applied above the gel and medications can be applied on the ulcer base.

Biodressings

In the case of extensive burns or traumatic wounds of the skin, the only solution is often the placement of an autologous skin graft. This skin is not always available. Therefore, interim solutions sometimes have to be sought and uses may be made of homografts or other biological material such as amnion sheets. Perhaps in the not too distant future skin cultures will be available. At present, heterografts can also be used in some cases.

The use of such products is still rather limited in dermatology. Nevertheless, we must mention here that, in cases of very atonic ulcers, such dressings can be applied for a few weeks to the ulcer in order to start granulation. Examples are Collagen-Implant (B. Braun, Melsungen) and E.Z. Derm (Genetic Laboratories). The latter product consists of pig skin that is specially treated to prevent rapid rejection and is also treated with silver sulfadiazine.

Alginates

Alginates are used as xerodressings, as they possess hemostatic and absorbent properties. They are the calcium or sodium salt of alginic acid and are found primarily in certain seaweeds.

Alginates are suitable for the management of most exuding wounds, but should be used with care in deep narrow sinuses or fistulae. In the treatment of burns, donor sites and bleeding wounds, the hemostatic and absorbent properties of alginates are at their most useful (Sorbsan Plus, Kaltostat pads or Sorbsan sheets). They are contraindicated in dry wounds and ulcers. When the exudation is less pronounced, alginates can be used. A thinner pad of alginates bearing a low adherence plastic film may help to conserve moisture and prevent the wound drying out too quickly. In the latter, alginate dressing acts somewhat like a foam dressing rather than as a xerodressing.

Sorbsan (Medical) is an alginate fiber dressing made by processing seaweed. This product can also be used to treat several kinds of strongly secreting lesions.

Conclusion

The classic wound treatments with compresses, salves, creams, etc., under gauze dressings, cotton, and the like have certainly not been consigned to the past. Nevertheless, the new synthetic dressings offer many possibilities. Proper choice of the product, however, is important. Clear insight into the large range of possibilities will certainly facilitate increasing use of these new products.

Further Literature

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The Effect of Allopurinol on Wound Contraction and Granulation Tissue Formation in the Rat

I.L. Naylor and T.C. Teo

Introduction

Various types of aberrant wound healing are encountered in surgical practice. Examples include hypertrophic scarring, keloid formation and a range of conditions collectively called the fibromatoses. All these conditions pose problems clinically, and drug treatments for them have yet to be found. It is to the fibromatoses and their possible drug treatment that this study is addressed.

Although the aetiology of the fibromatoses, an example of which is Dupuytren's Contracture (DC), remains unknown, various pharmacological treatments have been suggested or attempted empirically. The most recent suggestion for such a treatment for DC was made by Murrell et al. (1987) from their serendipitous observations of the ameliorating effects of allopurinol on DC in gout patients. They proposed that allopurinol, a commonly used anti-gout drug which reduces the production of free radicals by competitive inhibition of xanthine oxidase, also reduces the severity of DC because oxygen derived free radicals were implicated in the pathogenesis of DC (Murrell et al. 1990). They further showed that allopurinol and other free radical scavengers modulated fibroblast proliferation in tissue culture (Murrell et al. 1990; Murrell 1992). Unfortunately, such empiricism is required because there is no animal model for any of these fibromatotic states in which drugs can be studied for their potential ability to modify such conditions. A further difficulty is that not only do animals not suffer from any of these conditions, it is also impossible to artificially induce them.

To overcome such problems one practical alternative is to conduct drug trials in experimentally induced conditions in which certain pathological elements of these diseases are present, albeit on a very different time scale. A highly reproducible model which meets some of these criteria is the excisional skin wound. In the repair process the formation of granulation

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tissue, with its contractile properties and its subsequent maturation, is easily measured and is a prominent feature of the fibromatoses.

This chapter describes the effect of allopurinol on excisional wound healing in the rat. The experiments were designed to determine if the drug influences wound contraction and the deposition of collagen.

Materials and Methods

Wound Infliction and Measurement of Wound Contraction Rate

Under general anaesthesia, a standard 15 × 15 mm full-thickness wound was inflicted on the left dorsolateral aspect of adult female hooded Lister rats (control animals, 238 ± 22 g, $n = 32$ and treated 229 ± 16 g, $n = 35$). Each wound was dressed with a transparent semipermeable dressing (Tegaderm) for the first 48 h. After wound excision the animals were randomised into two groups to receive, by gavage, either a daily dose of allopurinol (50 mg/kg) or a vehicle control of 2% methylcellulose (1 ml/kg). The general well being of the animals, their body weights and the wounds were monitored daily. The wound margins of fully conscious animals were traced daily for 7 days onto acetate sheets. The docile nature of the animals facilitated an average of ten tracings to be obtained on each occasion with minimal restraint and hence with little, if any, risk of damage to the healing wounds.

The wound tracings were digitised using a digitiser (Summagraphic Bit Pad Two) and a DIGIT software programme (Department of Opthalmology, University of London) run on a BBC microcomputer which was accurate to an error margin of <0.5%. The daily mean wound area thus derived for each animal was then plotted against time using standard Macintosh microcomputer software (Cricket Graph) producing an exponential curve. The logarithmic transformation of this decline in wound area vs time results in a straight line whose gradient, known as the coefficient of wound contraction (Du Nouy 1919), represents an index of the rate of wound contraction. The sensitivity of using this index to detect the modification of wound contraction by pharmacological agents has been verified in our previous study (Cross and Naylor 1990).

Measurement of Granulation Tissue Volume

At day 7 the animals were killed using intraperitoneally administered pentobarbitone (10 mg/kg) and the whole wound, with margins of about 5 mm, was excised intact and placed fascia side downwards onto a piece of thin card. This was then fixed in 10% formalin before being routinely processed for histological examination. Vertical mid-wound sections (6 μm) were cut and separately stained with haematoxylin and eosin and Masson's

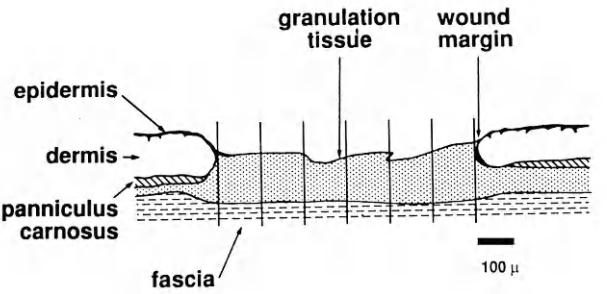

Fig. 1. A representative tracing of a 7 day full-thickness wound as produced by projection of a stained histological slide in a photographic enlarger. The *vertical grid of lines* indicates the points at which the thickness of the granulation tissue was measured

trichrome. All slides were stained at the same time to minimise interslide staining differences.

Four representative sections of the wound from each animal were magnified 10x in a photographic enlarger and a tracing was obtained of each section. Selected dimensions from these tracings were then digitised and the mean mid-wound cross-sectional areas of allopurinol treated and control animals were obtained for comparison. In addition the mean mid-wound thickness of the granulation tissue was also derived from taking the average measurements at seven equidistant points across each wound (Fig. 1). The volume of granulation tissue at 7 days for each group of animals was calculated as the product of the thickness of the mid-wound and the known wound surface area.

Histological Analysis

Sections of mid-wound granulation tissue from the control and allopurinol treated animals were examined using light microscopy and assessed for reepithelialisation, vascularity, inflammatory cell infiltration and fibroblast density. In addition, the overall collagen content of the wounds was also subjectively assessed.

Statistics

The data were analysed on the Macintosh microcomputer utilising the statistical software package StatsView. Dunnett's t test for unpaired samples was used for comparison of body weight changes; Mann-Whitney non-parametric U tests were used for all the other data. A value of $p < 0.05$ was taken to be statistically significant.

Results

Immediately after the wound was produced, its area increased by an average of 15% due to the mobility, elasticity and tension of the surrounding skin. For the first 2 days after wounding there was little change to the size of the wound under the dressings. Once these were removed the wounds entered a phase of rapid contraction between days 2 and 7. All the animals remained healthy and there was no evidence of wound infection. The mean weight loss for the control animals and those treated with allopurinol was not significantly different ($p > 0.05$)

Wound Measurements

In allopurinol treated animals the coefficient of wound contraction (K) was significantly decreased for both study periods taken from either the day of injury (days 0–7; $p < 0.05$) or during the rapid phase of wound contraction (days 2–7; $p < 0.01$) as compared with controls (Table 1).

The mean vertical mid-wound cross-sectional area at 7 days was significantly larger for allopurinol treated animals than for vehicle treated controls ($p < 0.05$; Table 2). Furthermore, as shown in Table 2, the mean

Table 1. The mean coefficient of wound contraction for allopurinol and vehicle control treated animals

Daily oral regimen	n	Days 0–7		Days 2–7	
		k ± s.e.m.	R	k ± s.e.m.	R
Allopurinol (50 mg/kg)	18	−0.0750* ± 0.005	0.88	−0.0590** ± 0.006	0.89
Vehicle control (1 ml/kg)	20	−0.0900 ± 0.006	0.95	−0.0870 ± 0.008	0.91

k, mean coefficient of wound contraction; s.e.m., standard error of mean; R, mean regression coefficient; *$p < 0.05$; **$p < 0.01$.

Table 2. Mean mid-wound area and thickness and volume of granulation tissue from allopurinol and vehicle control treated animals at day 7

Daily oral regimen	n	Mid-wound area (mm^2 ± s.e.m.)	Mid-wound thickness (mm ± s.e.m.)	Volume of granulation tissue (mm^3 ± s.e.m.)
Allopurinol (50 mg/kg)	6	17.5 ± 1.1*	1.64 ± 0.10**	183.7 ± 28.6*
Vehicle control (1 ml/kg)	6	14.2 ± 1.3	1.29 ± 0.10	122.6 ± 19.9

s.e.m., standard error of mean; *$p < 0.05$; **$p < 0.025$.

mid-wound thickness of the granulation tissue was also significantly greater for allopurinol treated animals than for control animals ($p < 0.025$).

The amount of granulation tissue (Table 2) was significantly greater ($p < 0.05$) for animals treated with allopurinol than for controls.

Histological Analysis

Histological examination of the wounds from allopurinol treated and control animals showed that at 7 days the epithelium migrated less than 10% across the wound surface. Thus the closure of the wound during the first week was primarily the result of wound contraction.

The granulation tissue had the everted, typical funnel shaped appearance in cross-section, suggesting that the process started in the subdermal fascial plane (superficial fascia) and the base of the wound and then spread upwards to fill the excised defect.

Within the granulation tissue itself, the new capillaries showed a typical vertical orientation in cross-section, projecting from the wound base towards the surface. Control wounds appeared more vascular, with a greater inflammatory cell infiltrate, than the wounds of allopurinol treated animals. The wounds of allopurinol treated animals had a less cellular appearance and fewer fibroblasts. In contrast, however, these wounds seemed to have a greater amount of collagen–stained bluish green with Masson's trichrome–than the wounds of control animals.

Discussion

A significant decrease in the rate of wound healing was shown in the animals treated with allopurinol as compared with vehicle treated controls. Initially, using the simple technique of deriving the coefficient of wound contraction (Cross and Naylor 1990), it was found that allopurinol significantly inhibited the rate of wound contraction, irrespective of whether the period of measurement was taken from the day of wound infliction (days 0–7) or over the period of most rapid wound contraction (days 2–7). However, measurement of the changes in surface area alone was found to provide useful but limited information on what in wound healing is in essence a complex three-dimensional phenomenon. Consequently, in an attempt to clarify the effect allopurinol has on the wound healing process, further measurements on the granulation tissue were carried out. The mid-wound cross-sectional area and mid-wound thickness of the granulation tissue at 7 days were both found to be significantly greater in the wounds of allopurinol treated animals than in those of controls. Furthermore when the volume of granulation tissue on that day was calculated, this also proved to be significantly greater in allopurinol treated wounds. However, it is accepted that

these values were obtained from histological specimens in which shrinkage must inevitably occur, but since all tissues were processed in an identical manner the comparisons are valid. For the same reason even though this is not a precise measurement of the actual volume of granulation tissue, it nevertheless provides a reasonable indicator of the total wound content.

The combined measurement of the wound surface area contraction rate and the volume of granulation tissue within a single study has not been reported before, yet it may be highly relevant. It certainly provides more information on the effect of a drug on the wound healing process. This is well illustrated by contrasting the effect of steroid administration with that of allopurinol. Steroid treatment has been reported to inhibit the rate of wound contraction (McGrath and Emery 1985) and produces a thin granulation tissue when compared with control wounds (Howes et al. 1950). Our recent study has confirmed these findings (Cross and Naylor 1990). However, allopurinol has been shown in the present study to also inhibit the rate of wound contraction but, in contrast, this was associated with a greater volume of granulation tissue than found in the wounds of control animals. Thus it is quite evident that in order to better characterise the effect of a treatment on the process of wound healing, measurements need to be performed in more than one plane; otherwise the differing influence of a drug may not be detected.

Histological examination of the wounds, albeit subjective, was performed in order to try and correlate the dynamic process of wound contraction with the cellular and connective tissue changes induced by allopurinol. Granulation tissue from the allopurinol treated wounds at 7 days appeared less vascular with fewer inflammatory cell infiltrates and fewer fibroblasts while at the same time there appeared to be more collagen deposition than in control wounds. Although a quantitative assay of the collagen content was not performed, the more extensive bluish green stain of collagen, observed with the Masson's trichrome stain, does correlate with the greater volume of granulation tissue found in allopurinol treated wounds.

It was Carrel (1910) who first suggested that the contractile forces responsible for wound contraction reside within the granulation tissue. With the identification of specialised cells which had contractile ability within granulation tissue (Majno et al. 1971) it seemed that the process of wound contraction could simply be explained on the basis of the contractile activity of these so-called myofibroblasts (Rudolph 1979). However, this appears to be too simplistic a view since it does not explain the role of the connective tissue nor the progressive decrease in the amount of granulation tissue that is observed during the active phase of wound contraction (Abercrombie et al. 1954). Brickley-Parsons et al. (1981) who carried out extensive studies on the biochemical changes in the collagen of the palmar fascia in patients with DC, commented upon the similarities in the connective tissue and cellular changes between the active phase of the disease and that of a healing wound. They postulated that the progressive resorption and re-

placement of the existing collagen with a smaller quantity of abnormally arranged collagen may be the basis for the development of sustainable contraction deformity. It is highly probable that the synergistic interaction of the cellular elements, principally the myofibroblasts, and the dynamic process of collagen deposition, resorption and remodelling is responsible for the wound contraction and the deformity in DC. If the induction of the myofibroblast, clearly shown to be present in DC (Gabbiani and Majno 1972; Tomasek et al. 1987), and its contractile activity provide the stimulus for the subsequent connective tissue events, this could explain the findings in the allopurinol treated wounds: the lesser density of fibroblasts would be associated with a weaker force of granulation tissue contraction which is reflected in a slower rate of wound contraction. The slower process of collagen resorption and remodelling is reflected in the greater volume of collagen, as observed in this study.

What are the mechanisms by which allopurinol inhibits wound contraction? Even though this study does not allow us to draw firm conclusions, several possibilities could be considered. A certain degree of inflammatory response and local tissue hypoxia are present in all healing wounds (Hunt et al. 1967), and indeed such conditions are believed to play a role in stimulating angiogenesis and cellular transformation (Knighton et al. 1981). When there is gross inflammation, ischaemia or infection, this is associated with an enhanced production of a variety of mediators among which the oxygen derived free radicals are potentially highly damaging to tissue (Im et al. 1984; Granger et al. 1986). Recent studies on the breaking strength of intestinal anastomoses have provided evidence to suggest that free radicals are also generated and may have a direct role during normal tissue damage and repair (Hogstrom 1987). In vitro studies by Murrell (1988) have shown that low levels of free radical appear to induce the proliferation of fibroblasts. Allopurinol, by inhibition of free radical formation, could attenuate the inflammatory response and fibroblast-myofibroblast transformation. This could represent a mechanism by which it produced the effects seen in the healing wounds of the rat.

Another possibility is that allopurinol may, by a direct action or acting indirectly via prostanoid mediators (Badalamente et al. 1988) or peptide growth factors (McGrath 1990), inhibit the contractility of the wound myofibroblast while leaving the collagen sythetic activity of the cell intact.

In conclusion the effect of allopurinol on wound healing in the rat may involve the interaction between the inhibition of free radical formation, fibroblast-myofibroblast transformation and contractility and the forces that stimulate the turnover of collagen in granulation tissue. Further systematic studies are indicated to delineate the role of these elements of what is clearly a complex process.

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Effects of Pale Ichthyol in a Model of Impaired Wound Healing

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Introduction

Ichthyol (ichthammol, ammonium ichthosulfonate, ichthyol ammonium, ammonium bituminosulfonate) is a reddish brown viscous fluid with a characteristic odor. It is manufactured form shale oils of high sulfur content. The oily distillate is sulfonated to form sulfonic acids and subsequently neutralized. Sulfonated shale oils are mixtures of a number of compounds. These include saturated and unsaturated hydrocarbons, including heterocyclic sulfur containing hydrocarbons. Dark sulfonated shale oil is a result of treatment of shale oil with sulfuric acid at higher temperature. Pale ichthyol is obtained from gentle sulfonation at lower temperature. In contrast to tar, the sulfonated shale oils are water soluble (Koch et al. 1985).

Due to their antiseptic, antipruritic and antiinflammatory effects, ichthyol containing formulations are used in certain areas of dermatology (Dollery 1991). The treatment of inflammatory skin disorders with ichthyol was first described by Unna (1882). An inhibitory effect of ichthyol on the production and secretion of chemotactic leukotrienes was found by Czarnetzki (1986). Ichthyol inhibited the directed migration of neutrophilic granulocytes which was stimulated by chemotactic agents (Kownatzki et al. 1984, 1986). Ichthyol reduced edema development caused by topical application of croton oil on the ear skin of mice (Diezel and Schulz 1991). An inhibitory effect on esterase activity in the skin was described by Steigleder (1959). An acanthogenic effect of ichthyol was demonstrated in guinea pig skin (Salfeld et al. 1966).

The animal study described here was performed to examine effects of different dilutions of an aqueous solution of pale ichthyol in wound healing.

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An animal model of impaired wound healing was used. The retardation of healing was induced by repeated systemic glucocorticoid treatment.

Material and Methods

Animals

Female mice (NMRI, Hagemann, Extertal, FRG) weighing 25 g were used. Six animals were housed per cage at 22°C with a 12 h light/dark cycle. They were fed with a standard diet (Altromin, Lage/Lippe, FRG).

Drug

The sodium salt of pale bituminosulfonate was from Ichthyol Gesellschaft (Hamburg, FRG). It was applied as an aqueous solution (1%, 2%, 5%, and 10%).

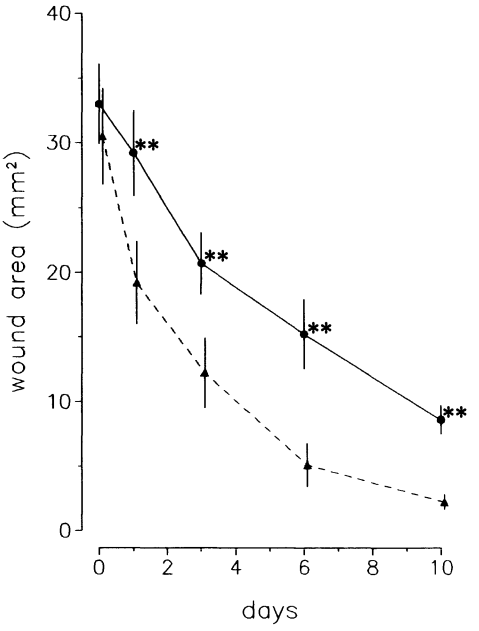

Fig. 1. Wound healing (wound area in mm^2) in female NMRI mice. Normal healing (*filled triangles*) and delayed healing (*filled circles*) in mice treated with dexamethasone-21-phosphate (1 mg/kg body weight per day, subcutaneously). $^*p < 0.05$, $^{**}p < 0.01$ compared to controls with normal wound healing (analysis of variance followed by Newman-Keuls test)

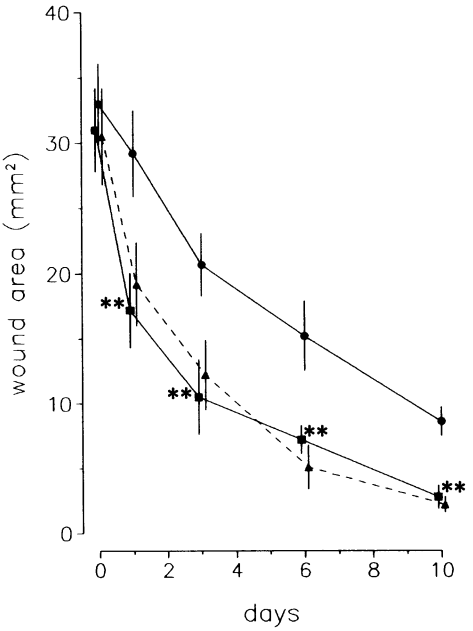

Fig. 2. Effect of daily topical treatment of back skin wounds with an aqueous solution of pale ichthyol (1%; *filled squares*) on the healing rate (wound area in mm^2) of full-thickness skin wounds in female NMRI mice. A delayed healing (*filled circles*) was induced by systemic treatment with dexamethasone-21-phosphate (1 mg/kg body weight per day, subcutaneously); *filled triangles*, normal healing. $^*p < 0.05$, $^{**}p < 0.01$ compared to animals with impaired healing rate which were treated with the vehicle (analysis of variance followed by Newman-Keuls test)

Experiment

Mice were treated with dexamethasone-21-phosphate (Decadron phosphate) over 18 days. The glucocorticoid was applied subcutaneously once daily in a dose of 1 mg/kg body weight. After 1 week of glucocorticoid treatment, the back skin was depilated. After local anesthesia, full-thickness skin wounds were incised with biopsy punches of 6 mm diameter in the back skin. Immediately after wounding, the wounds were treated topically with pale ichthyol. A total of 100 μl of the aqueous solution was applied on the wound surface (open application). Animals of the control groups were treated with the vehicle. The topical wound treatment was repeated once daily. The wound area was measured directly after wounding and after 1, 3, 6 and 10 days. Simultaneously, wound areas were measured in five mice with a normal undelayed healing rate. These animals were not treated with dexamethasone-21-phosphate.

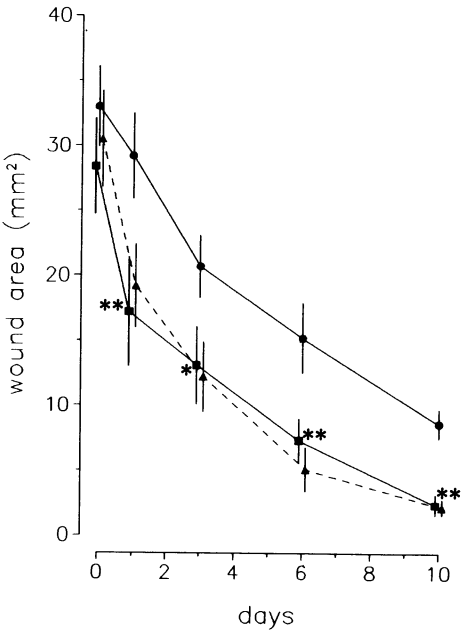

Fig. 3. Effect of daily topical treatment of back skin wounds with an aqueous solution of pale ichthyol (2%; *filled squares*) on the healing rate (wound area in mm^2) of full-thickness skin wounds in female NMRI mice. A delayed healing (*filled circles*) was induced by systemic treatment with dexamethasone-21-phosphate (1 mg/kg body weight per day, subcutaneously); *filled triangles*, normal healing. $^*p < 0.05$, $^{**}p < 0.01$ compared to animals with impaired healing rate which were treated with the vehicle (analysis of variance followed by Newman-Keuls test)

Statistics

The results of the study are presented as mean ± standard deviation of five mice per group. Statistical analysis of the data was performed by one-way analysis of variance. If significant differences were found, the Newman-Keuls test was used to find which pairs, among all possible pairs of means, were different. For statistical calculations, the computer program Pharm/PCS 4.2 was used.

Results and Conclusion

Figure 1 shows the time-dependent decrease of the wound area in untreated and dexamethasone-21-phosphate-treated mice. Dexamethasone-21-phosphate induced a significant delay of the wound healing rate. After 10 days, the wound area was 26% of the initial value, while in mice with not delayed wound healing the wound area was about 7%.

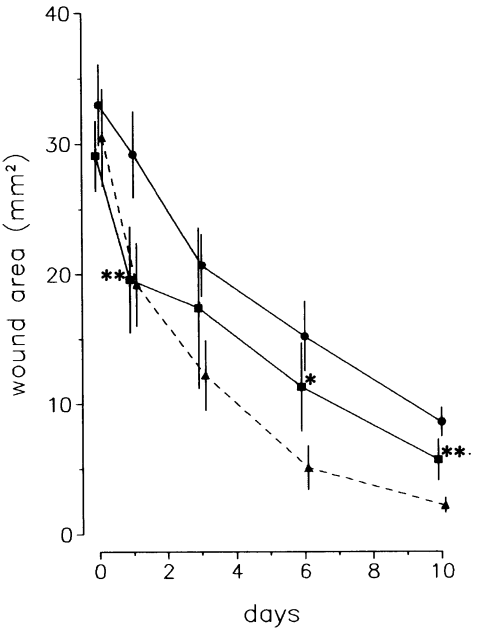

Fig. 4. Effect of daily topical treatment of back skin wounds with an aqueous solution of pale ichthyol (5%, *filled squares*) on the healing rate (wound area in mm^2) of full-thickness skin wounds in female NMRI mice. A delayed healing (*filled circles*) was induced by systemic treatment with dexamethasone-21-phosphate (1 mg/kg body weight per day, subcutaneously); *filled triangles*, normal healing $*p < 0.05$, $**p < 0.01$ compared to animals with impaired healing rate which were treated with the vehicle (analysis of variance followed by Newman-Keuls test)

Light ichthyol sodium containing aqueous solutions of 1% and 2% enhanced the healing rate in mice which were treated with dexamethasone-21-phosphate. The healing rate was in the range of mice not treated with dexamethasone. Compared to dexamethasone-21-phosphate-treated mice, which showed a retardation of wound healing, significant differences were found (Figs. 2, 3). In contrast, 5% or 10% pale ichthyol containing formulations induced no significant acceleration of wound healing (Figs. 4, 5).

Earlier studies showed that the animal model used here is suitable to demonstrate effects on impaired wound healing. The delayed healing of skin wounds was accelerated by occlusive wound treatment and by different agents, while no effect on the normal healing rate was measurable. In contrast to an occlusive wound dressing which enhanced the healing rate, the tetrachlorodecaoxide containing product Oxoferin remained without effect (Kietzmann and Lubach 1989; Heidelck 1991; Itzel-Kietzmann and Kietzmann 1991).

The results described here document a beneficial effect of topically administered aqueous solutions of pale ichthyol on impaired wound healing.

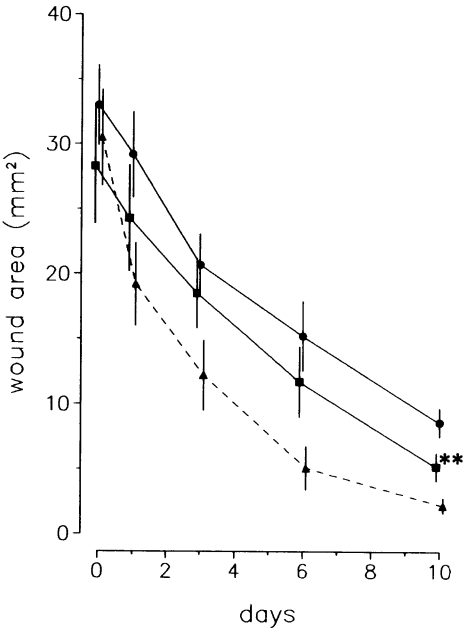

Fig. 5. Effect of daily topical treatment of back skin wounds with an aqueous solution of pale ichthyol (10%, *filled squares*) on the healing rate (wound area in mm^2) of full-thickness skin wounds in female NMRI mice. A delayed healing (*filled circles*) was induced by systemic treatment with dexamethasone-21-phosphate (1 mg/kg body weight per day, subcutaneously); *filled triangles*, normal healing. $^*p < 0.05$, $^{**}p < 0.01$ compared to animals with impaired healing rate which were treated with the vehicle (analysis of variance followed by Newman-Keuls test)

An acceleration of healing was detected for 1% or 2% containing aqueous solutions while higher concentrations produced no significant effects.

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Pretreatment of Photodamaged Forearm Skin with Topical Tretinoin Accelerates Healing of Full-Thickness Wounds

C. el Gammal[1] and A.M. Kligman[2]

Introduction

It has been known for decades that vitamin A and its derivatives may enhance various aspects of wound repair [1, 2]. Epithelialization is stimulated, accompanied by collagen synthesis and angiogenesis [3–5]. In 1968 Ehrlich and Hunt found that systemic as well as topical vitamin A could reverse the retardation of wound healing caused by anti-inflammatory steroids [6, 7]. However the toxicity of systemic vitamin A has restricted its use for this purpose.

Recently, there has been increasing interest in topical tretinoin (all-*trans* retinoic acid) as a wound healing promoter. Several studies in rats, pigs and other animals have demonstrated better repair of partial-thickness and full-thickness wounds after pretreatment with topical tretinoin [2, 8–11]. Corneal wounds in rabbits also respond favorably [12, 13].

Many dermatologic surgeons have adopted the practice of pretreatment with tretinoin before dermabrasion and chemical peels, relying mainly on empirical observations. A few studies in humans were published [14–16] showing clinically accelerated healing after trichloroacetic acid peel, dermabrasion and electroepilation. These were performed after short pretreatment periods of 3–14 days. As more profound changes can be observed after months of treatment [17, 18] we pretreated for 4 months in this study. We chose to study photoaged skin because wound healing is impaired in this abnormal state. Tretinoin has been known to reverse many of the regressive changes in photoaged skin [18].

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Materials and Methods

Eight white elderly men (57–81 years old, phototypes 1–3) were studied after written informed consent was obtained. Each of them had a history of excessive sun exposure. Clinically the forearms showed the stigmata of photoaged skin, i.e., mottled pigmentation and solar lentigos, laxity and wrinkling, teleangiectasias and a rough, scaly skin surface.

On the right forearms 500 mg (2 DELCAP) Retin A cream (Ortho) 0.05% were applied nightly during the first 8 weeks and 500 mg (2 DELCAP) Retin A cream 0.1% during the last 8 weeks. The left forearms served as vehicle-treated controls. We chose Purpose Cream (Ortho; 500 mg applied nightly) because of its similar composition to the Retin A vehicle. Dove soap was used for washing. No other products were applied during the study.

Prior to treatment 4 mm punch biopsies were obtained from the proximal third of both dorsal forearms in all patients. After formalin fixation, 7 um paraffin sections were stained with H&E, Luna for elastic tissue and Hale for glycosaminoglycans. Additionally, 2 μm sections were embedded in soft methacrylate and stained with toluidine blue (JB4 technique). After 16 weeks, 2 mm punch biopsies were taken from the proximal third of both dorsal forearms, down to, but not including, the subcutis. The biopsies were processed as above. The wounds were blotted with sterile gauze to control bleeding and then covered with a dry dressing (Band-Aid, Johnson and Johnson) for 24 h. After that the wounds were allowed to heal without any dressing.

In four patients photographs were taken on days 1, 4, 6, 8 and 11 after wounding. Close-up photographs were taken through a Zeiss OPMI 1 FC operation microscope with a Yashica 108 multiprogram camera at magnifications 19.4 and 30.4 (film: Kodak Ektachrome 64 tungsten). Polarized light photography (cross-polarization) was performed to reveal subsurface microvessels [19]. We used a Minolta X-700 camera with a Tamron SP objective, 90 mm, 1:25 and a Tamron BBAR MC Teleconverter 14 OF. The camera was connected with a single Ultra 1200 white lightning (Paul C. Buff, Inc., Nashville, Tennessee). Polarizing filters were mounted on the camera objective and the flash (orthogonal planes for cross-polarization); photos were taken at a 30° angle with apertures 13.5–19 at a distance of 40.1 cm (Kodak Ektachrome 100 HC). All the photos were coded. The wound margins, wound base and surrounding erythema and edema were graded by two blinded investigators. Stereomicroscopic photos were used to determine the wound areas by means of image analysis (program analySIS, Soft-Imaging Software GmBH, W 4400 Muenster, Germany). The wound margin was outlined with the mouse and the wound area calculated. On day 11 in all eight patients each wound was completely excised by means of a 4 mm punch. The tissue was processed as above after bisection of the specimens to ensure that the evaluations were made on the mid-portion of the wounds.

Results

Clinical Effects of the Treatment

No changes could be observed on the control sides (Purpose-treated). After about 2 weeks of treatment with tretinoin all eight subjects developed moderate irritation (slight erythema and scaling), which persisted throughout the entire treatment phase. After 2 months of treatment, hyperpigmented spots faded in six of the eight subjects; this effect was even greater after 4 months.

Histologic Effects of the Treatment

Stratum Corneum. Before treatment the stratum corneum showed the characteristic basket-weave pattern. After tretinoin, the stratum corneum was markedly thinned and compact without the normal basket-weave pattern (Fig. 7). Purpose cream had no discernible effect.

Epidermis. Before treatment the epidermis was atrophic and flat (four to nine cell layers) with variable numbers of lentiginous downgrowths. The stratum granulosum was thin, with one to two cell layers. Keratinocytes showed varying degrees of atypia, i.e., variation in size, shape and staining properties. There was also loss of polarity.

After tretinoin, the stratum granulosum increased to four layers with large numerous granules (Fig. 1). The epidermal thickness increased to 7–16 layers. The frequency of lentiginous downgrowths decreased. Atypia and disturbed polarity were completely corrected in six of the eight specimens. The epidermis was composed of large, regular keratinocytes. The intercellular spaces were slightly widened. Two subjects responded only moderately with regard to these changes.

No changes could be observed in the control forearms, the specimens being indistinguishable from the ones before treatment.

Dermis. Before treatment, the dermis showed the typical histological changes of chronic photodamage with thickening and clumping of elastic fibers (elastosis), loss of collagen, a narrow grenz zone and increased glycosaminoglycans in the upper dermis.

The tretinoin-treated sides of seven of the eight subjects showed an increased number of dilated capillaries in the upper dermis with an increase in adventitial cells around the superficial blood vessels. Fibroblasts were larger and more numerous (Fig. 1). No striking difference was noted regarding elastosis, collagen and glycosaminoglycans. The control sides did not show any difference to pretreatment (Fig. 1).

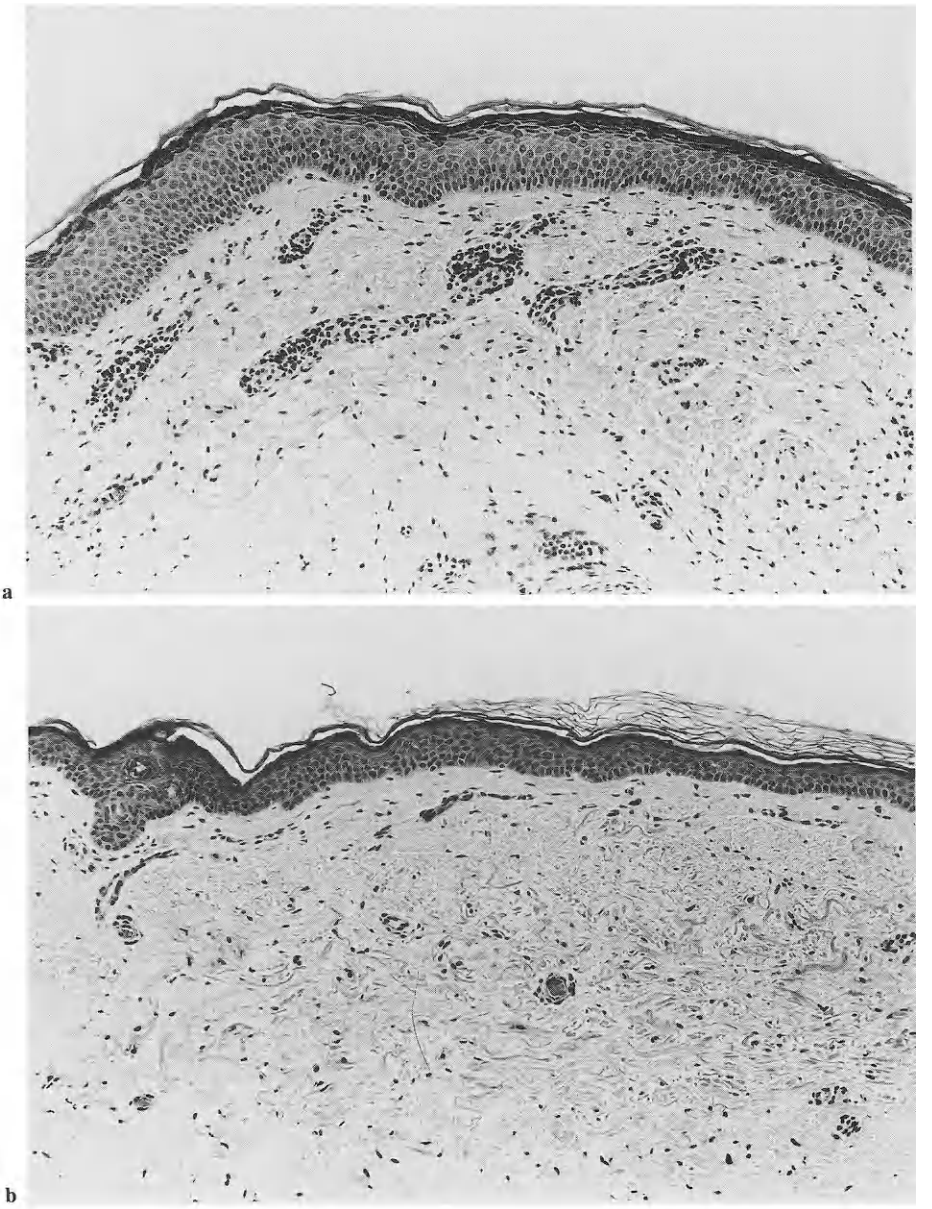

Fig. 1a,b. Subject number 3 after 16 weeks of treatment. **a** Tretinoin-treated skin: compact and very thin stratum corneum; acanthosis and hypergranulosis of the epidermis; widened intercellular spaces. Vessels in the superficial dermis are increased in number, elongated and surrounded by many adventitial cells. **b** Control arm: atrophic epidermis with flattening of the dermoepidermal junction; marked elastosis in the superficial dermis. H&E, ×123

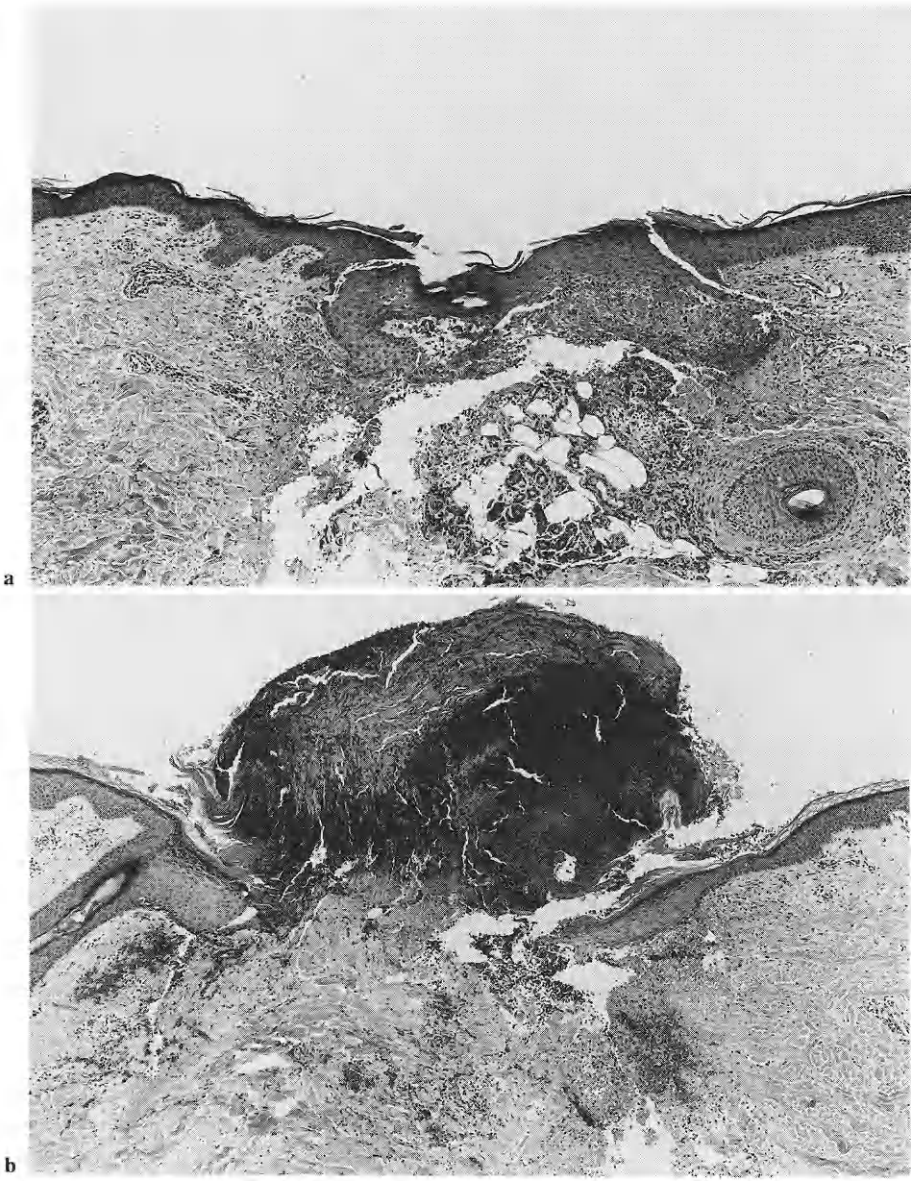

Fig. 2a,b. Subject number 1, excised wound. **a** Tretinoin-treated skin: complete reepithelialization; well developed and highly cellular granulation tissue with numerous vessels. **b** Control arm: large crust with numerous neutrophils; incomplete epithelialization; necrotic areactive tissue beneath the crust in the dermis. H&E, ×49

Histology of Excised Wounds

In all subjects the crust covering the wound was markedly larger on the control arms than with tretinoin. In four subjects more neutrophils were embedded in the eschar on the control side than on the tretinoin-treated side.

All tretinoin specimens showed complete epithelialization. On the control arms, epithelialization was not complete in five of the eight subjects (Fig. 2).

Granulation tissue on the tretinoin-treated arms was much more cellular and showed more microvessels than on the control arms.

Wound Closure

Figure 3 displays the mean areas of the wounds at different time points as evaluated by image analysis. The numeric values are presented in Table 1. The mean wound area on the tretinoin sides was at all times markedly smaller than on the control side: on day 1 the difference between the sides was 32%, on day 4, 37%, on day 6, 50%, on day 8, 47% and on day 11, 50% (Figs. 4–7).

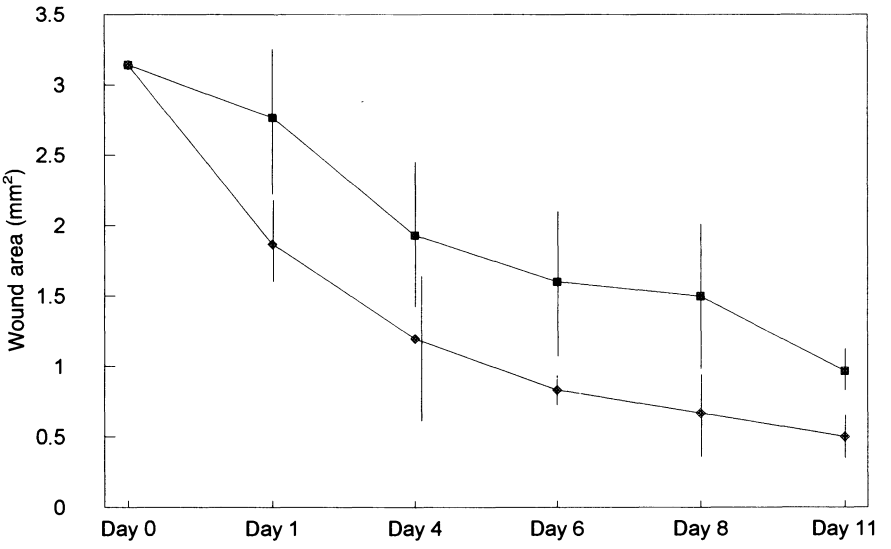

Fig. 3. Assessment (means and standard deviations) of wound healing by image analysis; *filled squares*, control, *filled diamonds*, tretinoin treated

Table 1. Wound healing assessed by planimetry of close-up photographs

Day	Tretinoin (mm^2)	Control (mm^2)
1	1.9 ± 0.3	2.8 ± 0.6
4	1.2 ± 0.5	1.9 ± 0.5
6	0.8 ± 0.1	1.6 ± 0.5
8	0.7 ± 0.3	1.5 ± 0.5
11	0.5 ± 0.2	1.0 ± 0.1

Means and standard deviations ($n = 4$).

Blind Assessments of Close-Up Photographs

On day 1 after wounding, there was beginning epithelialization of the wound margins on the tretinoin sides of all four evaluated subjects. No epithelialization could be detected on any of the control arms. Compared to the round shape of the wounds on the control arms, wounds after tretinoin treatment had elliptical outlines (Fig. 4).

From day 4 on all wounds were covered by a crust (Figs. 5–7). In three of the four subjects the crust was yellow on the tretinoin-treated side and brown-red on the control sides. In one subject the color of the crusts on both sides was yellow.

Blind Assessments of Polarized Light Photographs

After treatment with topical tretinoin the arms of all four subjects displayed moderate erythema. In two there were numerous small vascular spots; the other two showed a more diffuse network of fine dilated vessels. These changes persisted after creation of the wounds.

On the control arms one subject showed slight erythema with diffuse dilated capillaries. No such changes were noted in the others.

Regarding the area directly surrounding the wounds, on day 1 erythema was more pronounced on the tretinoin side of all four subjects than on the control side (Fig. 4). From day 4 on no difference between the sides could be observed.

Discussion

We found a marked acceleration of wound healing by clinical and histologic criteria after pretreating photodamaged forearms for 4 months with topical tretinoin. Already after 1 day, the wound area was on average 32% smaller

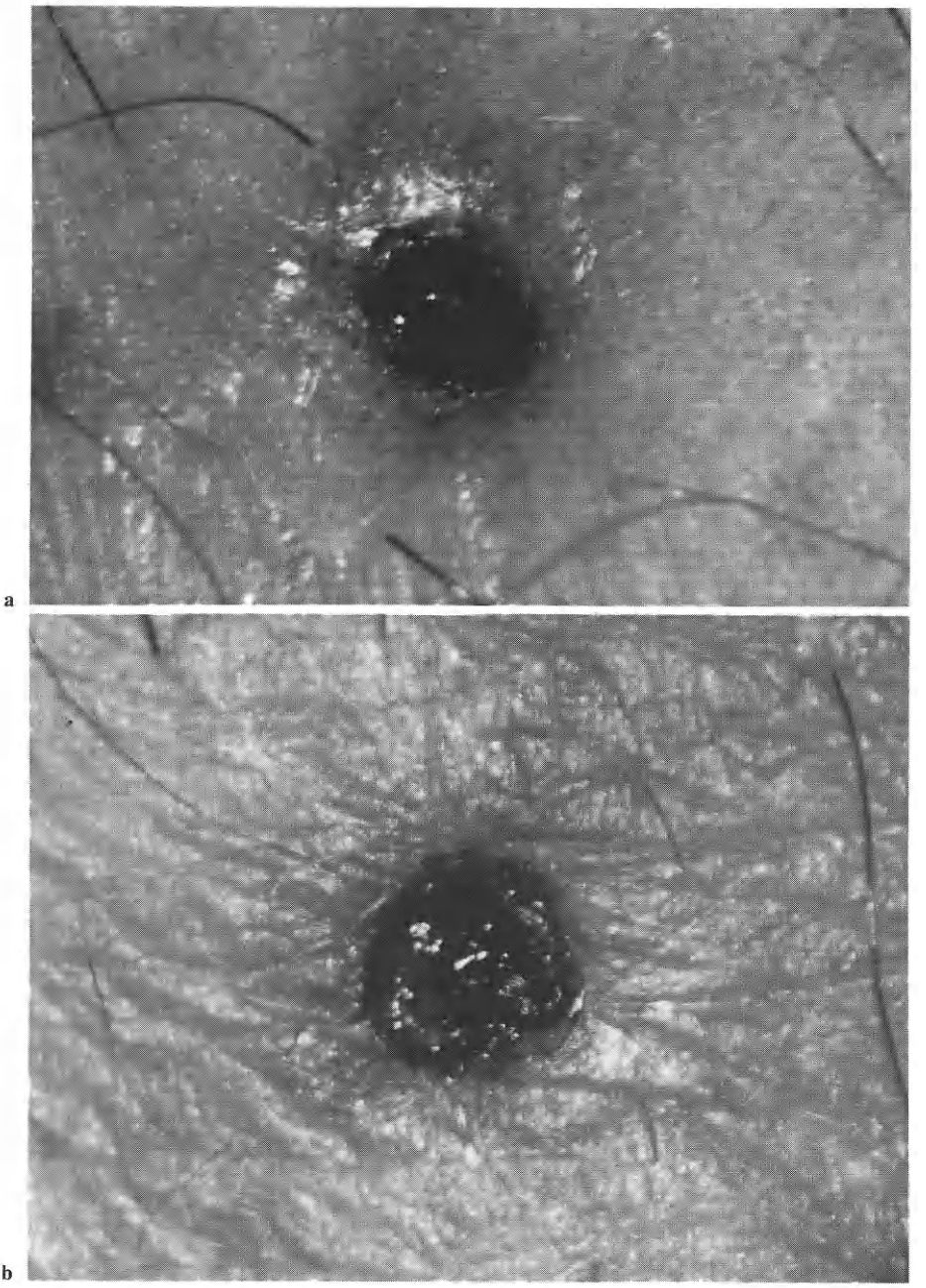

Fig. 4a,b. Subject number 1, day 1 after wounding. **a** Tretinoin-treated side: contracted wound with elliptic shape and beginning epithelialization of the wound margins. The surrounding skin shows numerous small vascular spots. **b** Control side: no wound contraction (round shape), no visible epithelialization of the wound margins. Stereophotographs, original magnification ×30.4

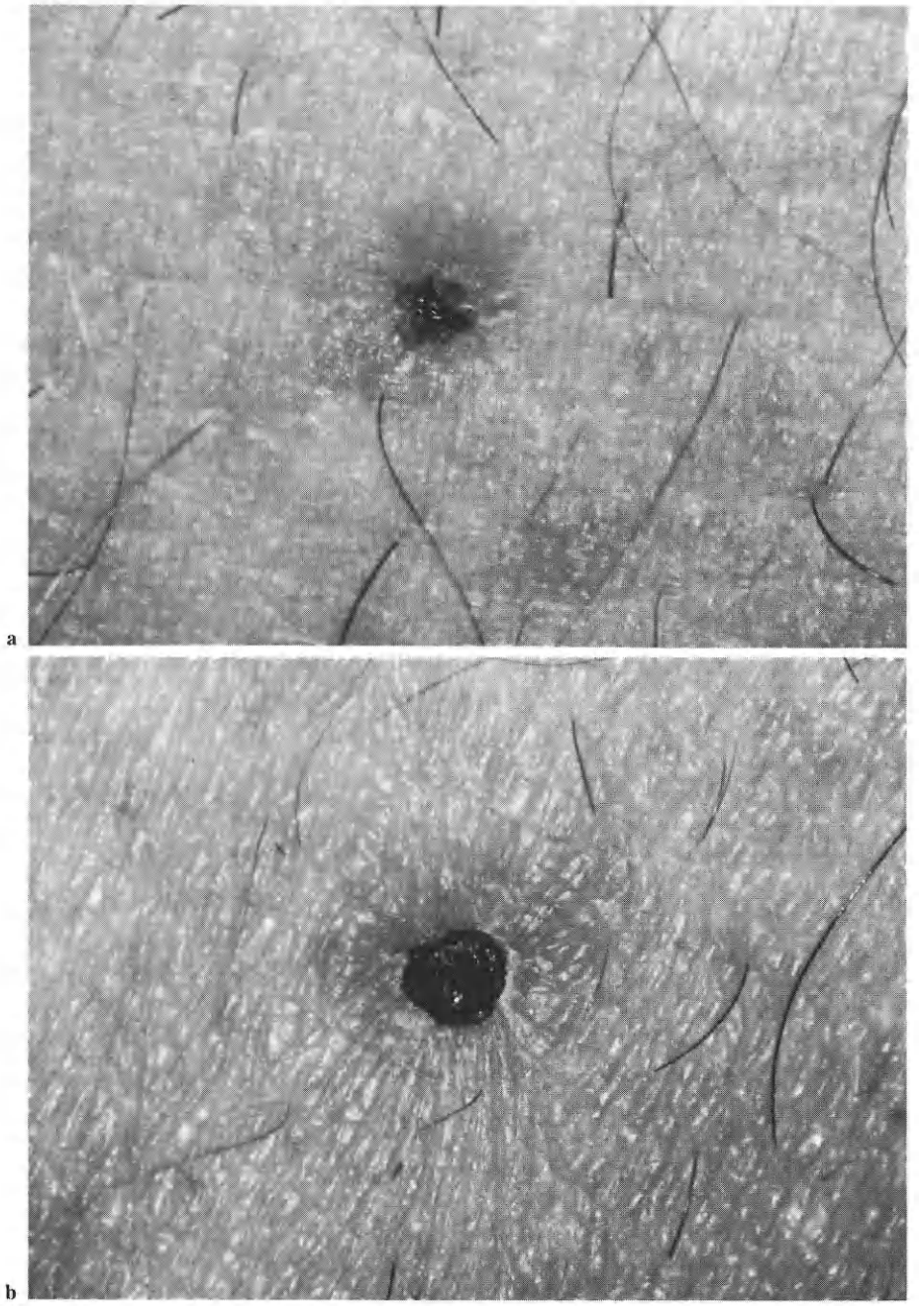

Fig. 5a,b. Subject number 1, day 6 after wounding. **a** Tretinoin-treated side: small yellow crust with dark brown center. **b** Control side: the crust is distinctly larger than the one on the tretinoin-treated side. Stereophotographs, original magnification ×19.4

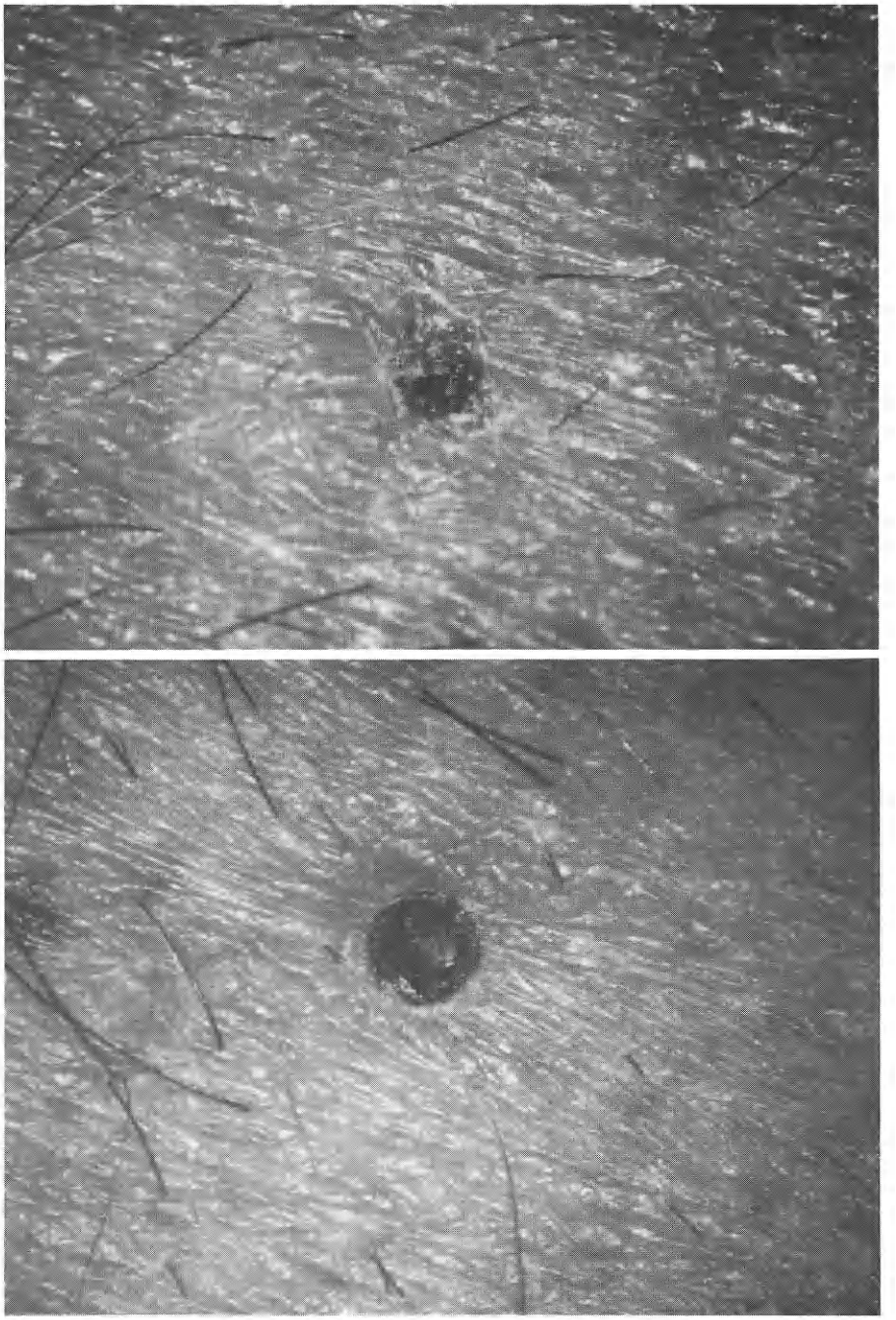

Fig. 6a,b. Subject number 4, day 4 after wounding. **a** Tretinoin-treated side: small crust; moderate irritation of the surrounding skin due to tretinoin-therapy. **b** Control side: slightly indented crust which is much larger than on the opposite side. Stereophotographs, original magnification ×19.4

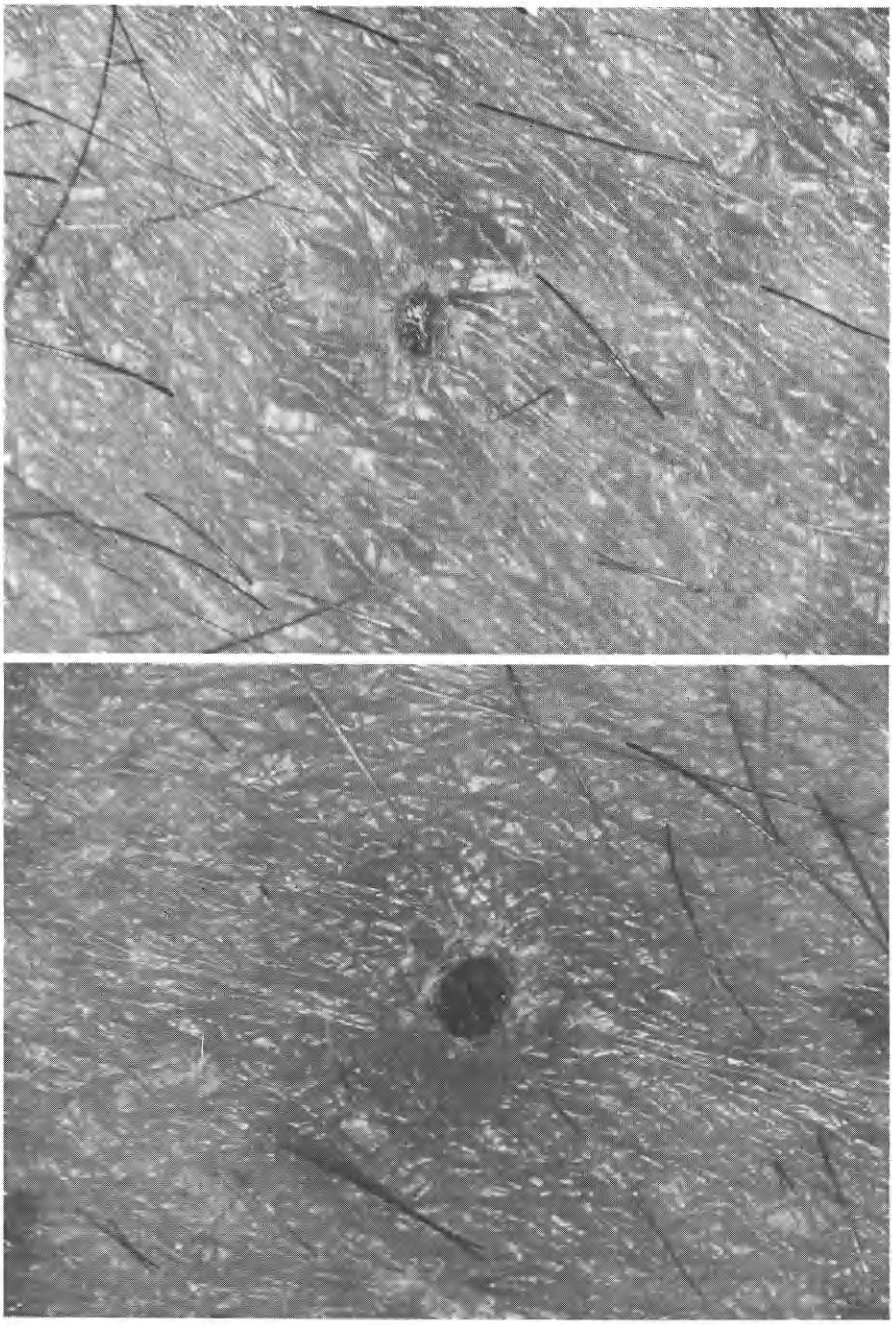

Fig. 7a,b. Subject number 4, day 11 after wounding. **a** Tretinoin-treated side. **b** Control side. Even more pronounced difference between the sides than in Fig. 6a,b. Stereophotographs, original magnification ×19.4

on the tretinoin-treated arm than on the vehicle-treated arm. Better epithelialization and increased wound contraction was seen after tretinoin at all time points, accompanied by smaller crusts. The difference in wound areas increased throughout the follow-up period of 11 days reaching 50%.

It is likely that a combination of the well known effects of tretinoin is responsible for enhanced wound healing. Stimulation of epidermal turnover certainly encourages faster reepithelialization of wounds. Retinoids bind to specific receptors important for the maintenance of cellular differentiation; they are pleioptropic molecules which influence cell division, RNA and protein synthesis, and lysosomal membrane stability [3]. Histologically, the increased cell turnover caused by retinoids is expressed in epidermal acanthosis, hyperplastic keratinocytes, and hypergranulosis, changes that are well documented [17, 18, 20]. These were confirmed by our results.

Another retinoid effect which we were able to observe clearly is the formation of new vessels (angiogenesis) in the superficial dermis. Polarized light photography [19] revealed multiple, minute, dilated vessels which histologically corresponded to an increased number of dilated capillaries and venules. Neovascularization is important for the regulation of blood flow and modulation of solute transport for oxygen and nutrients. Endothelial cells secrete a number of biologically active substances, e.g., prostacyclin, growth factors, and enzymes [21, 22]. Some authors have focused on the influence of retinoids on macrophagic mobilization as a necessary component of wound healing [4, 23]. Finally, retinoids have been shown to increase mucopolysaccharide and fibronectin synthesis, important factors in the formation of granulation tissue [24, 25].

The effect of both topical and systemic administration of vitamin A and its derivatives, especially retinoic acid, on wound healing has been investigated in various animal models. Large methodological differences, however, make it difficult to compare the studies. The studied species included rats [1, 2, 6, 8, 10, 26] rabbits [7], mice [27], pigs [9, 11] and dogs [28]. Wound healing was assessed in different ways, e.g., by measuring the tensile strength of sutured wounds [6, 8, 10, 26], by planimetry of full-thickness open wounds [1, 2, 7, 9, 11, 27], or by measuring the quantity of collagen deposition in subcutaneously implanted polyvinyl sponges [8]. Despite large variations in dosage and methodologies, these studies have consistently demonstrated acceleration of healing when the retinoid was applied *prior to wounding* [2, 9]. Application *after wounding* has yielded conflicting reports. Some investigators witnessed enhanced healing [1, 6, 7, 8, 10, 27], while others reported inflammation and retardation of epithelialization [2, 9, 11]. Tretinoin is apparently too irritating on fresh wounds as are many other agents which are otherwise well tolerated.

It is important to appreciate that wound healing in rats and mice is not entirely comparable to that in humans. In loose-skinned animals wound contraction plays a much greater role than in human skin. Porcine skin more closely resembles human skin. Two studies, one using partial-thickness and

one full-thickness wounds in pigs as a model, both demonstrated delayed healing of wounds with application of topical tretinoin after wounding [9, 11]. Pretreatment of pig skin with tretinoin for 10 days before wounding, however, resulted in improved healing [9].

In human studies *pretreatment* has been the rule. Hevia et al. [15] used planimetry to follow the healing of trichloroacetic acid peels of actinically damaged forearms, hands and faces. They found accelerated healing on tretinoin-pretreated skin. On the forearms, reepithelialization was 50% greater than with the vehicle on the 11th day post-peel. Enhanced wound healing with tretinoin pretreatment has also been demonstrated after dermabrasion of the face [16] and electroepilation of the axilla and groin [14].

This study confirms previous observations that tretinoin partially reverses the structural abnormalities of actinically damaged skin. Epidermal atrophy, atypia, dysplasia, and loss of polarity are completely corrected [17, 18]. Tretinoin also has the ability to correct the regressive structural changes in protected, intrinsically aged skin of the thigh of elderly women [29]. In our model, the capability of tretinoin to enhance wound healing was highlighted because actinically damaged skin heals poorly as might be anticipated from the profound involutional losses in structure and function. In future studies, it may turn out that pretreatment for a shorter period, e.g., 2 months, shows equivalent effects on wound healing.

A variety of clinical applications come to mind in which topical tretinoin might be useful to promote wound healing, i.e., in donor sites for split-thickness grafts, before dermabrasion and chemical peels of photodamaged skin and before cryosurgery of actinic keratoses and malignancies.

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Treatment of Chronic Wounds with Cultured Keratinocytes

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Chronic nonhealing wounds are a major health problem. Previous studies have shown the effectiveness of cultured keratinocytes in healing burns and skin lesions. We used keratinocyte cultures to treat leg ulcers. The grafts were prepared by the Battelle-Institut in Frankfurt according to the method of Green. The epithelium is a layer of two to four noncornified squamous epithelial cells. Its differentiation, which is reflected by the expression of keratin, distinguishes it from native epithelium. Among other things cytokeratins 6, 16, and 8 are expressed; the latter is not found in native epidermis. We could show nerve growth factor (NGF) receptors and vimentin in keratinocytes of the culture, in contrast to the epidermis in vivo. The cultivated epidermis can be put either on a dressing or grafted to cover the wound by means of fibrin glue.

In an uncontrolled study 29 wounds in 28 patients were treated with cultured epidermal sheets. The following skin lesions were covered with cultured keratinocytes: leg ulcers (26 patients), malum pedis (1 patient), necrobiosis lipoidica (1 patient), decubitus (1 patient), and nevus spilus (1 patient). In treating leg ulcers we only chose problematic cases, in which the usual treatment did not show any progress.

In 25 patients we used allografts, in two patients autologous keratinocyte cultures, and in another two patients both treatments.

Good clinical results were obtained in most cases with an onset of epithelization after 7–10 days. In most cases, application of the cultured keratinocytes had to be repeated. Reepithelization notably started from the edge of the wound or from epithelial islands. In only five patients was there no success in treatment.

The advantages of cultured keratinocytes are: (1) the grafting procedure is uncomplicated; (2) no anesthesia is necessary; (3) all patients with painful ulcers described a reduction of their pain; (4) in vitro studies have shown that there is an antibacterial activity of cultured human keratinocytes [1]; (5)

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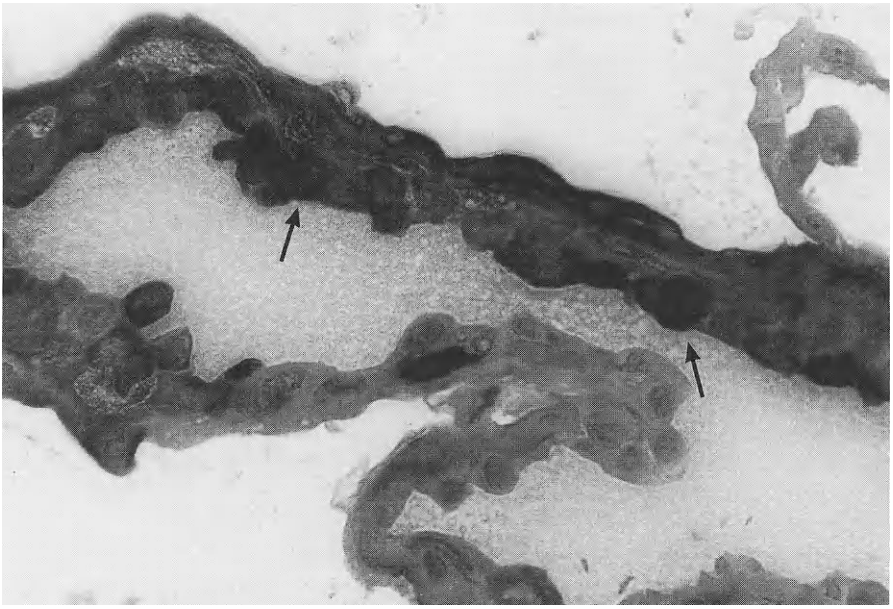

Fig. 1. Keratinocyte sheet expressing cytokeratin 8 in some cells (*arrows*)

the donor region is very small, so that a punch biopsy of 5–6 mm is sufficient; (6) 5 years of follow-up observation of patients at the Harvard Medical School showed that a histologically and immunologically normal epidermis develops without higher risk of neoplasma [2].

Disadvantages of the method are: (1) there is little stability and elasticity; (2) a dermal component is missing; (3) the grafts are vulnerable and at risk of infection, although after 2 weeks the epidermis is cornified and more resistant.

We did not see any difference between autologous and allogenic keratinocytes. We believe that the liberation of growth factors is responsible for the faster reepithelialization of wounds in comparison to conventional wound treatment.

Significant progress has been made in skin replacement options in the past several decades. Despite intensive efforts to develop a skin substitute based on collagen gels and fibroblasts dermal substitute is not possible until now. Therefore, cultured keratinocyte grafts can still be regarded as a good alternative in treating resistant, painful, chronic wounds.

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Influence of Agents Used for Topical Wound Treatment on Phagocyte Stimulation and Fibroblast Growth

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Introduction

Wound healing has always been a fascinating phenomenon for physicians and surgeons. Wounds usually close by forming new granulation tissue, contracting, and re-epithelializing. Before granulation begins, polymorphonuclear leukocytes (PMNs) and monocytes/macrophages (MCs) are chemotactically attracted to sites of injury. Upon contact with various stimuli in the wound including bioactive lipids, complement components, certain cytokines, e.g., tumor necrosis factor-α (TNF-α) [1], granulocyte/monocyte-colony stimulating factor (GM-CSF), interferon-γ (IFN-γ), and interleukin-8, and particles such as microorganisms, phagocytes respond by a respiratory burst consisting of a markedly enhanced uptake of oxygen, activation of the hexose monophosphate shunt, and generation of oxygen intermediates comprising superoxide anion, hydrogen peroxide, hydroxyl radical and singlet oxygen [1, 2]. These compounds are able to kill microorganisms and to destroy tissue resulting in wound decontamination and wound debridement (summarized in [3]).

MCs also release several growth factors important for new tissue formation such as transforming growth factor-a (TGT-α), TGF-β and platelet-derived growth factor (PDGF), also important for fibroblast growth [3]. Fibroblasts produce and organize the major extracellular matrix components including fibronectin and collagen, which are involved in wound healing [3].

To promote healing a number of topical agents are applied therapeutically to wounds such as leg ulcers. Based on the observation that wound healing sometimes ceases despite consistent topical treatment with antiseptics, we investigated the influence of agents used for topical wound treatment on phagocyte stimulation and fibroblast growth. We measured phagocyte stimulation, i.e., the respiratory burst, employing luminol-enhanced chemi-

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luminescence [4] and growth of skin fibroblasts by the incorporation of [^{3}H]thymidine. Our results show that most of the agents we tested suppressed the respiratory burst of phagocytes and inhibited the growth of fibroblasts.

Materials and Methods

Topical Agents Tested

The following products were used: (1) polyvidone iodide complex (Mundipharm, Limburg), 1.0 g dissolved in 10.0 ml Dulbecco's minimum essential medium (DMEM) for chemiluminescence (Boehringer, Mannheim); (2) polyvidone (polyvinyl pyrrolidone, Mundipharm) 1 g per 10 ml DMEM; (3) Lugol's solution containing 3.33 g iodide and 6.67 g sodium iodide per liter of distilled water; (4) gentian violet (methylviolet, Merck, Darmstadt), 5 g/l distilled water. (5) Ethacridine lactate (Riedel-de Haen-AG, Selze, FRG) 1 g/l distilled water; (6) mercurochrome (mercuridide-bromo-fluorescein Merck) 1 g/l distilled water. (7) zinc sulfate (Merck) 5 g/l distilled water; (8) chloramine-T (Merck) 1 g/l distilled water; (9) Oxoferin (tetrachlorodecaoxide, Oxo-Chemie GmbH, Heidelberg). To evaluate the effect on cell cultures, ready-to use solutions were diluted in DMEM as indicated in the results.

Measurement of the Respiratory Burst of Phagocytes

The method of Schopf et al. was used [4]. In brief, mononuclear leukocytes (MNLs) and PMNs were isolated from the peripheral blood of healthy donors. MNLs were separated by density gradient centrifugation (density 1.077 g/ml). MCs in the MNL mixture were identified by esterase staining; their numbers ranged from 8% to 22% in the MNL suspensions. The cell concentration was adjusted to 2×10^5 MCs per assay. It has been shown that the chemiluminescent activity in MNL suspensions is due to MCs and not to lymphocytes [3]. PMNs were isolated from the pellet of the MNL density gradient by sedimentation in 1% dextran solution. Pure PMN suspensions were obtained from the resulting supernatants after lysis of the erythrocytes by 0.98% NH_4Cl solution. The PMN concentration was adjusted to 5×10^5/assay. Phagocytes were stimulated by zymosan particles (0.3 mg/ml). Dilutions of the agents were added to the zymosan-stimulated phagocytes. The $2 \times 10^{-4} M$ luminol-enhanced chemiluminescence was determined in a Berthold LB 9500 luminometer every 10 min for 10 s over 1 h. To compare the effects of the agents, the chemiluminescent activity measured under zymosan stimulation was normalized to 100%. All assays were performed using duplicate samples.

Viability Testing

After isolation of the phagocytes and fibroblasts viability was gauged microscopically by exclusion of 0.4% trypan blue (Merck) and found to exceed 98% in all fresh cell preparations used. Trypan blue exclusion was also used to determine fibroblast viability after culture with the topical agents. To measure viability after phagocyte stimulation, the release of lactate dehydrogenase (LDH) was determined biochemically by measuring the rate of oxidation of reduced nicotinamide adenine dinucleotide at 340 nm (test kit purchased from Boehringer Mannheim). The highest concentrations of the agents that were used with PMNs and MCs were employed.

Cell-Free Luminogenic System

To find out whether the agents, effects on zymosan-stimulated phagocytes were due to quenching of the light generated we employed a final concentration 0.5% H_2O_2 instead of phagocytes.

Fibroblast Cultures

Human fibroblasts were isolated from biopsies of normal skin. The subcutaneous adipose tissue was removed with a scalpel. The epidermis could be peeled off after incubation for 12 h at 4°C with 8 U/ml dispase (Boehringer Mannheim) in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS, Biochrom, Berlin). The dermis was cut in pieces of about 0.5 × 0.5 cm and cultured in 200 ml flasks (Nunc). The fibroblast culture medium consisted of DMEM supplemented with 10% newborn calf serum (Boehringer Mannheim), and 2.5 μg/ml final concentration amphotericin B (Gibco). Fibroblasts were cultured at 37°C, 5% CO_2. After confluence, the fibroblast monolayers were trypsinized (40 U/mg trypsin, 250 μg/100 ml 0.02% EDTA solution). After washing, fibroblasts were subcultured at 10^5 viable cells/ml.

Fibroblast growth was determined for 8 days by seeding 10^4 fibroblasts in 96-well microculture plates (data not shown). Medium was changed every 24 h. To each well 1 μCi [^{3}H]thymidine (specific activity 6.7 μCi/m*M*) was added. After detachment with trypsin/EDTA the fibroblasts were harvested onto glass fiber filters and [^{3}H]thymidine incorporation was counted in a β-counter. Only fibroblasts up to the fifth passage were used.

Dilutions of the agents were added to fibroblast cultures after 24 h. On day 3, 1 μCi [^{3}H]thymidine was added and radioactivity was counted as cpm 24 h later. All samples were run in triplicate. The means of the values of [^{3}H]thymidine uptake of fibroblasts in the absence of agents were normalized to 100% to compare the effects of the agents.

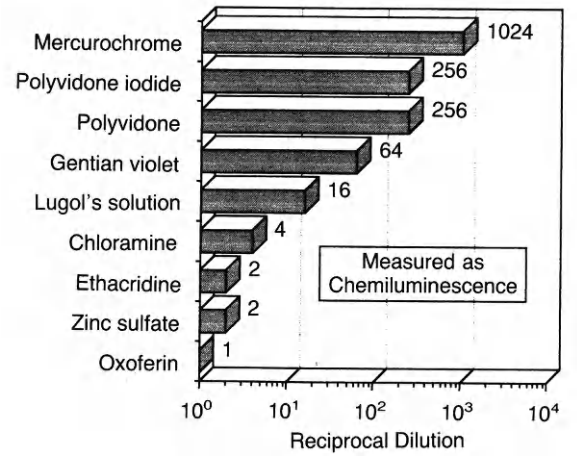

Fig. 1. ID_{50} of agents used for topical wound treatment for the zymosan-induced respiratory burst of 5×10^5 polymorphonuclear leukocytes. Time of incubation: 60 min. Values are the medians three independent measurements. Dilutions were made from ready-to-use solutions for topical use as listed in the methods

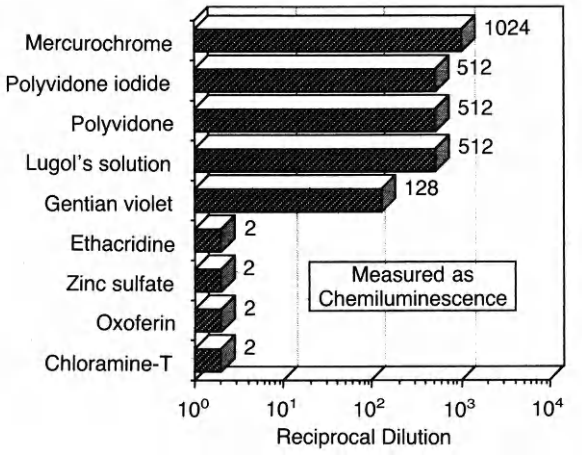

Fig. 2. ID_{50} of agents used for topical wound treatment for the zymosan-induced respiratory burst of 2×10^5 monocytes/macrophages

Results

Effects of Topical Agents on the Respiratory Burst of Phagocytes

At first the effects of the agents on zymosan-stimulated PMNs and MCs were tested. Figures 1 and 2 show the results. The respiratory burst of both PMNs and MCs was markedly inhibited by most of the agents tested.

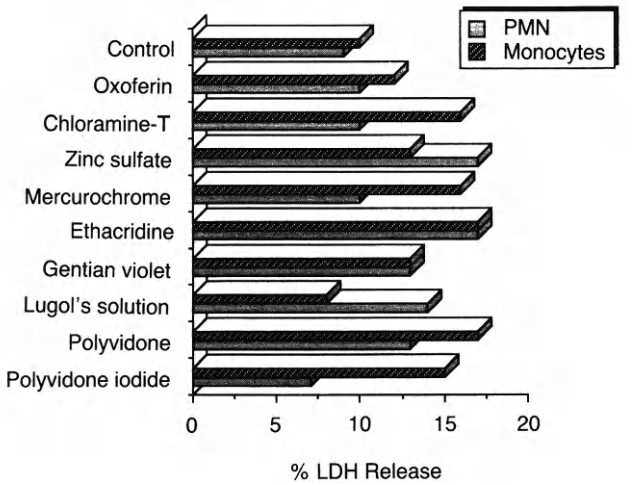

Fig. 3. Viability of 5×10^5 zymosan-stimulated PMN and 2×10^5 MCs incubated for 60 min with the agents used for topical wound treatment

Mercurochrome was the agent with the strongest inhibitory action, whereas tetrachlorodecaoxide (Oxoferin) exerted only marginal effects at the concentrations tested. From these findings it was concluded that agents used for topical wound treatment are able to supress the respiratory burst of both PMNs and MCs.

Viability of Phagocytes Under the Influence of the Topical Agents

The release of LDH was determined under the influence of the agents employed. Figure 3 exhibits the results with zymosan-stimulated PMNs and MCs. The data indicate that the viability of phagocytes remained between 7% and 17%, which was well within the range of the phagocytes incubated with zymosan alone. From these findings it was concluded that the inhibition of the respiratory burst of PMNs and MCs under the influence of the topical agents was not due to a cytotoxic action.

Effects of the Agents on a Cell-Free Luminogenic System

To rule out whether the suppressed chemiluminescent response of the phagocytes under the influence of the topical agents was due to quenching of the light generated during cell activation, the cell-free system outlined in the Methods section was employed. We found that the chemiluminescence generated remained stable under the influence of all the agents tested, indicating that nonspecific quenching of light could not be the cause of

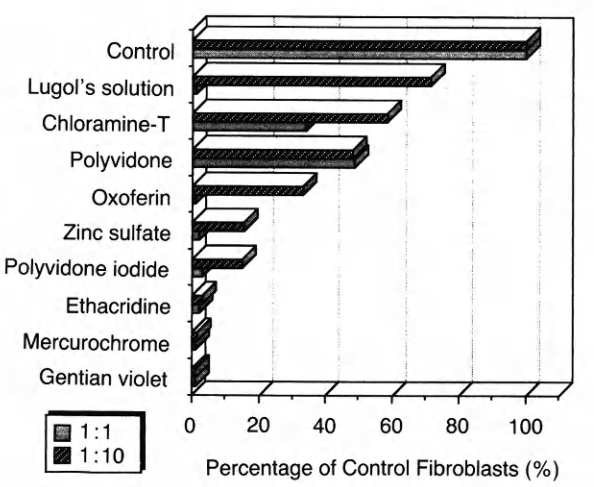

Fig. 4. Influence of agents used for topical wound treatment on the growth of 10^4 human skin fibroblasts. Time of incubation: 4 days. Cell proliferation was measured by [^{3}H]thymidine uptake. The control contained cell culture medium only

suppression of the respiratory burst of phagocytes. It was concluded that the cellular metabolism involved with the activation of phagocytes was suppressed by the inhibitory topical agents.

Influence of Topical Agents on Skin Fibroblast Growth

When skin fibroblasts were incubated together with the agents, it was found that most agents inhibited fibroblast growth. Figure 4 displays the results. Again, mercurochrome was among the agents causing the highest rate of suppression of fibroblast growth. Viability testing ruled out that suppression of fibroblast growth was due to mere cytotoxic action of the agents. From the data it was concluded that the agents used for topical wound treatment inhibited fibroblast growth by suppressing cellular metabolism.

Discussion

Our findings demonstrate that both PMNs and MCs are inhibited by a number of topical agents commonly used to treat wounds. In vivo effects of both PMNs and MCs include wound decontamination by phagocytosis and killing of microorganisms, wound debridement by phagocytosis of tissue debris, and tissue destruction by release of highly reactive oxygen intermediates [1, 3]. MCs, in addition, release growth factors that are responsible

for tissue formation. In particular MCs are able to synthesize and secrete PDGF, TGF-α and TGF-β critical for new tissue formation [6]. As a matter of fact, MCs are among the cells considered to be the most important for wound healing by continually producing growth factors and promoting the transition from inflammation to new tissue formation [3]. The finding that both PMNs and MCs are inhibited by the topical agents tested in vitro may be interpreted with caution in that these cells are also inhibited in vivo in treated wounds such as leg ulcers.

Undoubtedly, antiseptics remove contaminating microbes on wound surfaces. At the same time, however, it should be kept in mind that the physiologic mechanisms of the body to deal with invading bacteria, i.e., phagocytosis, are inhibited by the very same antiseptics. Tetrachlorodecaoxide (TCDO, Oxoferin) may be considered to be an exception among the topical agents tested since this agent has not been shown to exert direct antibacterial activity but rather has enhanced host resistance to bacterial infections [7, 8]. Moreover, TCDO has been shown to improve wound healing when applied topically [9].

The migration and proliferation of fibroblasts results in fibroplasia; the production of extracellular matrix components such as fibronectin and collagen promote connective tissue formation; the production of growth factors provides autocrine stimulation; the dynamic linkage between actin bundles and the extracellular matrix leads to tissue contraction; and, finally, the release of proteases is involved in remodeling of the extracellular matrix [3]. Our findings of suppression of fibroblast growth under the influence of agents commonly used for topical wound treatment are in accordance with those of Lineaweaver et al. [10], who reported that 1% polyvidone iodine, 0.25% acetic acid, 3% hydrogen peroxide, and 0.5% hypochlorite were cytotoxic to human fibroblasts in culture and thus unsuitable for use in wound care. Others have also reported that antiseptics inhibited wound granulation in subcutaneously implanted, viscose, cellulose sponges after treatment with dyes [11]. Older studies have demonstrated that, compared to the addition of antibiotics to fibroblast cultures, the addition of Ringer's solution resulted in far better growth rates than the addition of antibiotics [12].

From the clinical experience that some wounds may fail to heal despite daily continuous application of antiseptics or antibiotics, the reports of the literature [10–12], and from our findings, there seems to be little doubt that many topical agents, in particular antiseptics, do not accelerate but rather suppress wound healing if applied over prolonged time. The question may then arise: for how long is it justified to use these agents if necessary? The answer may be given as follows: if wounds obviously are contaminated by bacteria as indicated by their fetor and if wounds exhibit a marked inflammatory reaction, it may be beneficial to quickly decontaminate wounds and to suppress inflammation by applying topical antiseptics up to 3 days. After that period no further benefit can be expected. Moreover, when applying

topical agents it should be excluded that cellular mechanisms important in would healing are suppressed. Also, if suppression of granulation tissue is desired, such as in pyogenic granuloma, it seems to be justified to apply, e.g., gentian violet, to suppress phagocytes and fibroblast growth.

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Both Wet and Moist Wound Environments Accelerate Cutaneous Healing

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Introduction

Since the early reports on skin wound treatment, showing that skin wounds epithelialize more rapidly under moist conditions than under dry [1, 2], a variety of water and gas permeable dressings have been developed. The principle findings of healing in a moist environment are acceleration of epithelialization and prevention of the progression of wound depth that is observed in a dry environment. In a previous study we have shown that if a skin wound is continuously kept in a liquid environment consisting of saline and cell culture concentrations of penicillin and streptomycin, eschar formation was reduced and the time of epithelialization accelerated as compared to air exposed wounds [3]. In this study we have directly compared three different conditions under which porcine partial excisional wounds can heal: Wet (saline in an occlusive vinyl chamber), moist (hydrocolloid) and dry (air exposure under gauze dressing).

Materials and Methods

Animals and Procedures

All animal procedures were approved by the Harvard Medical Area Standing Committee on Animals. Four female fasted Yorkshire pigs (50 kg) were kept under standardized environmental conditions (20°–23°C, 65% humidity, 12 h light cycle, standard porcine diet). Operations were performed under general inhalational anesthesia (1.0%–1.5% halothane, O_2 and N_2O in a 3:5 ratio). After shaving, surgical prepping and sterile draping, 30 medium, partial-thickness, square excisional wounds (15 × 15,

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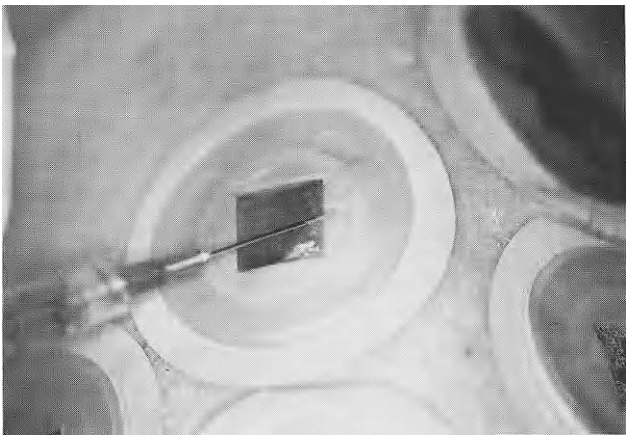

Fig. 1. Vinyl chamber for the treatment of skin wounds in a wet environment

1.2 mm deep) were created on the dorsum of each pig using a Padgett dermatome (Kansas City Assemblage Co., Inc., Kansas City, MO) with a custom made guard.

The wounds were randomly allocated to the treatment modalities: 0.9% saline, moist treatment (hydrocolloid) and air exposure with each group comprising a total of 10 wound sites per pig. Self-enclosed vinyl adhesive chambers (P.A. Medical Corp., Columbia, TN) with a basal opening corresponding to the wound size (15 × 15 mm) were applied to saline treated areas. The chambers were then filled with 1.2 ml normal saline (unbuffered, 0.9% NaCl), containing penicillin G (100 U/mL) and streptomycin (100 μg/mL). (Fig. 1). For the moist treatment group (hydrocolloid), a square patch (5 × 5 cm) of DuoDERM (ConvaTec) was centered on each wound and sealed to the adjacent skin. For the air dried sites, sterile gauze was placed over each wound and adhered to the skin (Fig. 1).

Every 24 h, the saline chambers were exchanged and refilled. Wound fluid volume was measured daily. Standardized photographs of each wound site were taken on days 0, 1, and biopsy days.

Biopsies were taken from day 3 to day 9. Without removing the gauze or hydrocolloid dressing, in order to avoid artifacts, each wound site was biopsied in fourths. The pigs were euthanized with 5 g thiopental sodium (i.v.) after the experiments were completed.

Histology

Biopsies were fixed in 10% neutral buffered formalin, processed for H&E staining, and analyzed under a light microscope. The rate of epithelialization was calculated from eight sections per biopsy across the entire surface aea.

Results

Macroscopic Aspects

In saline treated wounds a clot was seen covering the wounds as early as day 1. This was gradually covered by the regenerating epithelium.

Microscopic Aspects

Healing in the three groups was markedly different. In air-exposed wounds, necrosis of the base of the wound had increased its depth. The regenerating epidermis developed from the wound edges and from the dermal appendages. In moist and saline treated wounds granulation tissue filled out the space of the excised dermis and the epidermis. Epithelial migration was taking place on top of this granulation tissue. In all groups regeneration of the epidermis was observed as early as day 3. Saline treated wounds had all healed on day 6, moist wounds on day 7 and dry wounds on day 8.

Discussion

With the direct comparison of skin wound healing under three different degrees of moisture (wet-moist-dry) we could confirm the results of earlier studies [1, 2, 4–11], which have shown that a moist environmet provides faster healing than a dry environment. Moreover, the new saline filled chamber system provided a faster reepithelialization (day 6 vs day 7) than a conventional hydrocolloid dressing. The chamber system used in our experiments requires a minimum of maintenance but offers a number of advantages:

1. It has been shown to reduce inflammation in a partial-thickness burn model and accelerate reepithelialization [3].
2. It provides a tool for the delivery of, for instance, antibiotics and growth factors, or cell suspensions [14] as well as for monitoring of factors that are secreted or excreted into the wound [12, 13, 15].
3. It provides a useful way to treat contaminated wounds that require the delivery of certain substances in order to facilitate healing.

In this study we have chosen the "ideal" wound for the testing of a hydrocolloid dressing, i.e., the fresh, uncontaminated, partial excisional wound with limited wound exudate.

It is likely that the advantages of the chamber system would be more obvious in contaminated wounds. Particularly in contaminated wounds the possibility of delivery of substances such as antibiotics and the opportunity to replace the fluid provides faster healing of these wounds.

In summary, the study confirms, that the partial-thickness uncontaminated wound is best treated by a dressing that would provide a wet or moist environment.

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Comparative Experimental Study on the Treatment of Open Wounds

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Introduction

Open wounds often involve complex pathophysiological events and, consequently, difficult therapeutic problems [1]. However, our increasing understanding of tissue repair processes has permitted great progress in the treatment of open wounds. To achieve appropriate wound repair it is necessary to consider many parameters [2]. Intrinsic wound factors include:

1. Fibroblasts
2. Collagen deposition
3. Macrophages
4. Bacterial burden
5. pH
6. Angiogenic processes
7. Cellular division, migration and differentiation

Moreover, much importance has been attributed to the quality of tissue repair [3], which includes:

1. Mechanical resistance
2. Good connective tissue organization
3. No pathological scar tissue [4, 5].

The ideal open wound dressing has yet to be developed [6]. The effectiveness of *N*-carboxy-butyl chitosan and of activated synthetic charcoal in tissue repair was investigated and compared with fibrin glue. An experimental model was adopted in conformity with the International Guiding Principles for Biomedical Research Involving Animals.

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Materials and Methods

N-Carboxy-butyl chitosan is obtained from Alaskan king crab chitin and chemically modified so that it is water soluble (chitosan). The average molecular weight is 720 kDa; the degree of acetylation, 0.15; the degree of *N*-carboxy-butylization, 0.27; residual ashes at 600°C, less than 0.1%; and pH of the 1% solution, 6.2 [4].

An activated, fibrous, synthetic charcoal with high sorption capacity for low and high molecular weight physiologically active substances was used. It possesses high strength and elasticity, thermal stability and chemical inertness. It is manufactered at the Academy of Sciences of Kiev (Ukraine) [7].

Human fibrin glue containing fibrinogen and factor VIII (75 mg/ml) was added to a solution containing thrombin and calcium dichloride. The resulting mixture mimics the final steps of the coagulation process [4]. Based on the important experimental and clinical data regarding fibrin glue in tissue repair [8, 9], animals treated with this dressing could be considered as a control group.

In 18 male New Zealand rabbits (body weight 3 ± 0.350 kg) under general anesthesia, an open wound was made by the removal of a 24 cm^2 area from the shaved dorsal region. The dorsal cutis and subcutis were removed down to the surface bundles of furrier muscles in the shape of a rectangle (6×4 cm). The excision was carried out using a sharp lancet; hemostasis was not performed with either an electric lancet or vascular ligatures in order to avoid local necrosis. Animals were divided into three groups with six rabbits per group. The first group was treated weekly with *N*-carboxy-butyl chitosan, the second group with charcoal, and the third with fibrin glue (control group).

Every week, two animals per group underwent a biopsy for morphological analysis. The samples included healthy tissue, lesion margins, granulation tissue, subcutis and furrier muscle. These animals were excluded from calculations of reparative parameters, although they were treated in the same way as the others. For ultrastructural analysis by transmission electron microscopy (TEM) specimens were fixed in 2.5% glutaraldhehyde in 0.1% cacodylate buffer (pH 7.4), postfixed in 1% OsO_4 in cacodylate buffer, dehydrated in increasing ethanol concentrations and embedded in araldite. Semithin sections and ultrathin sections were cut using a Reichert Ultracut E microtome and stained with 2% toluidine blue. Ultrathin sections were counterstained with uranyl acetate and lead citrate and observed with a Zeiss EM 109 electron microscope.

Healing rates (mean and SEM) were calculated for each group. Student's *t* test was used for statistical comparison and a *p* value less than 0.05 was considered statistically significant.

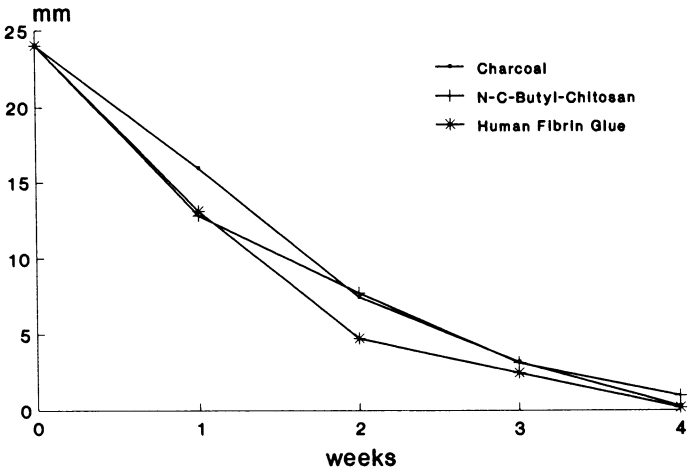

Fig. 1. Wound healing rate

Table 1. Healing rate (mm/week) for each group and animals

	Charcoal	*N*-Carboxy-butyl chitosan	Human fibrin glue
Animals			
1	85.71	81.07	85
2	83.21	82.85	84.64
3	83.93	82.85	84.64
4	84.28	81.43	85
Mean	84.28	82.05	84.82
SEM	0.525	0.468	0.104
p value	$p < 0.05$	$p < 0.005$	

Results

Suppurative complications were not observed. The healing time was shorter in group 2 (charcoal) group than in group 1 (chitosan), as compared with group 3 (fibrin glue) (Fig. 1, Table 1). Statistically significant differences did not exist among groups 2 and 3.

Histology

In *N*-carboxy-butyl chitosan treated animals, at 15 days postsurgery, the pluristratification of the epithelium in the covered area was evident. The epithelium was still immersed and covered by chitosan residues. The stromal

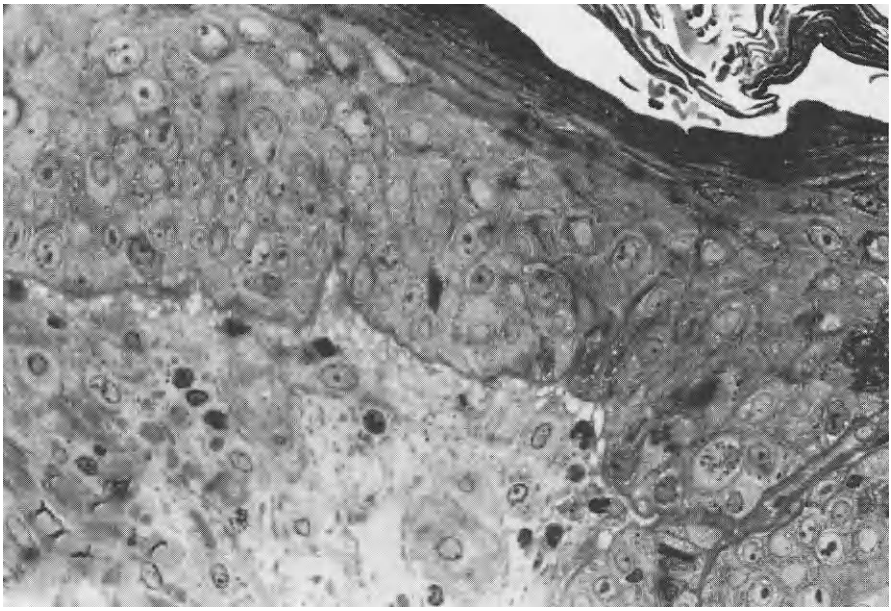

Fig. 2. Histology in *N*-carboxy-butyl chitosan treated animals (×630)

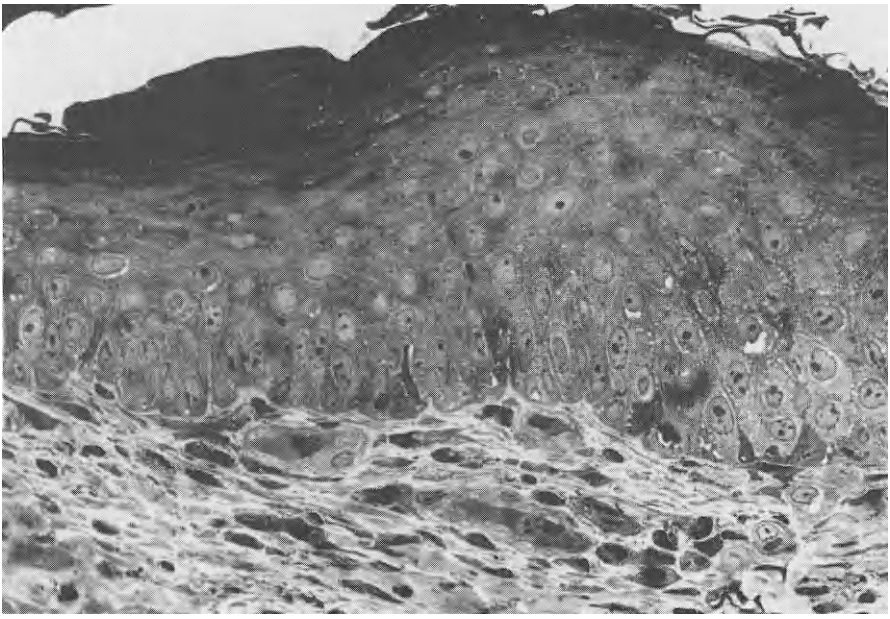

Fig. 3. Histology in fibrin glue treated (control) animals (×630)

tissue was loosely organized, with collagen fibrils showing a rather regular architectural pattern. Elongated mesenchymal cells and microvessels were observed. At 30 days postsurgery (Fig. 2), the dermis was rich in vessels and its histoarchitecture was still regular. The epithelium was multilayered with aspects of final maturation.

In fibrin glue treated (control) animals, at 15 days postsurgery, the front of epithelialization was apparent. The underlying connective tissue showed a less evident architectural order than seen in the presence of chitosan. At 30 days postsurgery (Fig. 3), the epithelial tissue was more multilayered than seen in the presence of *N*-carboxy-butyl chitosan. The connective tissue was more compact, with bundles of collagen fibers which were larger than those in the chitosan treated group. Some inflammatory cells were still present.

In charcoal treated animals, at 15 days postsurgery, the new epithelium had a good degree of maturation; there was no inflammatory reaction in the deep or superficial layers. Pluristratification of the epithelium was confirmed by TEM. At 30 days postsurgery, there were many cells in the dermal tissue with an evident neoangiogenic process.

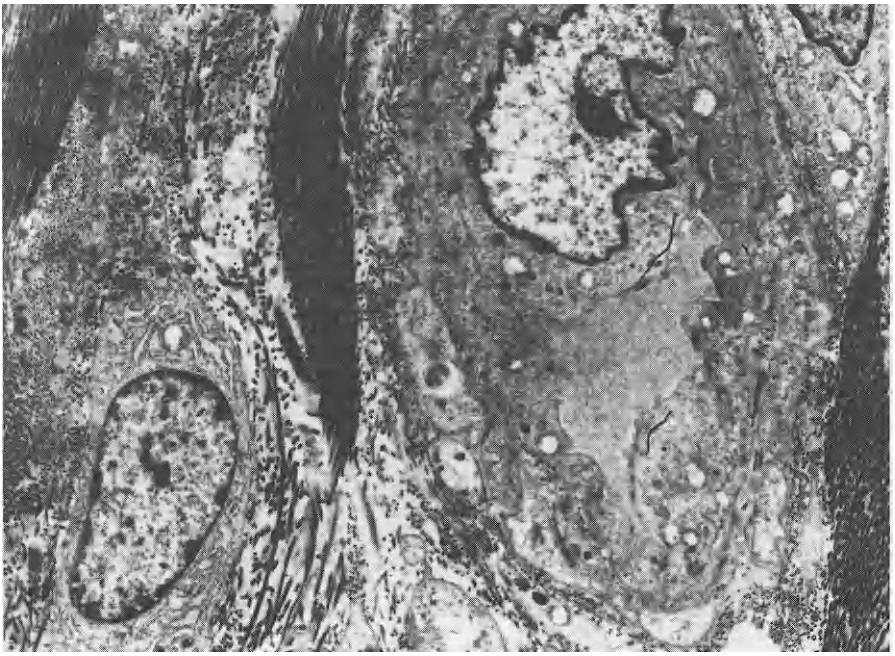

Fig. 4. Transmission electron microscopy in *N*-carboxy-butyl chitosan treated animals (×6000)

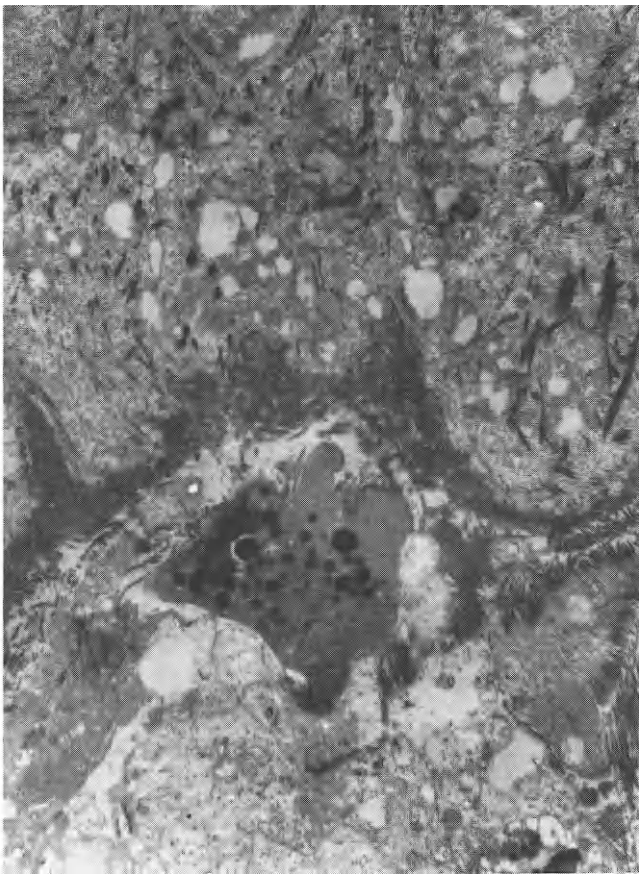

Fig. 5. Transmission electron microscopy in fibrin glue treated (control) animals (×5000)

Transmission Electron Microscopy

In *N*-carboxy-butyl chitosan treated animals, at 30 days postsurgery (Fig. 4), typical microvessels with walls made up of differentiated endothelial cells overlying a basal membrane were seen.

In fibrin glue treated (control) animals, at 30 days postsurgery (Fig. 5), a dermo-epidermal junction was noted. Inflammatory cells were observed at the more superficial level of the dermis.

In charcoal treated animals, at 30 days postsurgery, there was a pluristratified epidermis (Fig. 6) with aspects of hypercellularity at the dermal level (Fig. 7).

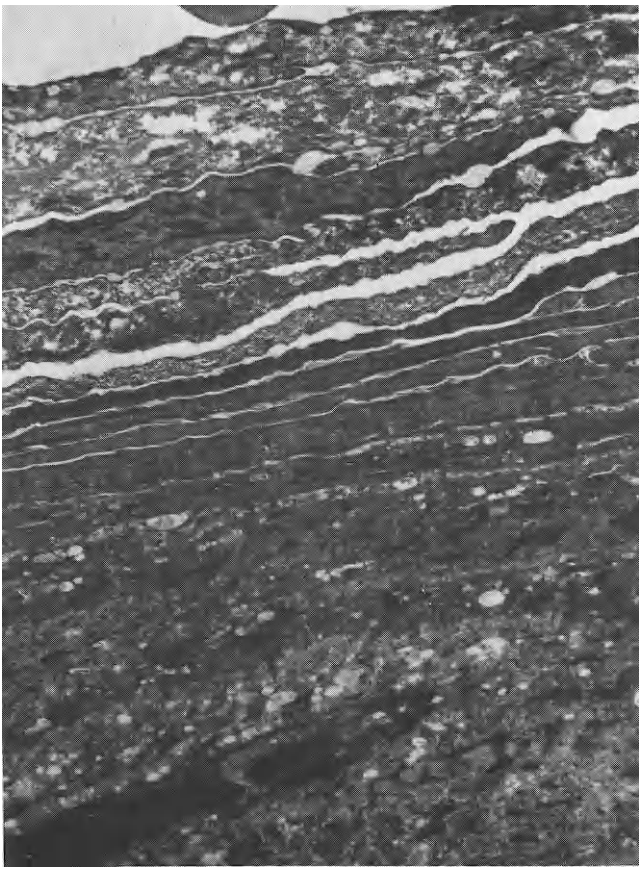

Fig. 6. Pluristratified epidermis in charcoal treated animals (×5000)

Discussion

Open wound dressing and treatment modalities have changed during the last several years. Many dressings are commercially available and they can be classified into biological and synthetic types. It should be noted that while each product has its own advantages, disadvantages, indications and contraindications [2], the ideal dressing should be biocompatible and capable of protecting the wound against injury or infection, shortening the wound healing time, absorbing the wound secretions, and decreasing blood loss and the fibrous reaction.

N-carboxy-butyl chitosan is a biocompatible biological product with hemocoagulative and bacteriostatic properties [12, 13] and structural affinity to glycosaminoglycans (GAGs), which are responsible for reconstruction of an ordered stromal tissue and for new vessel growth. In fact GAGs seems

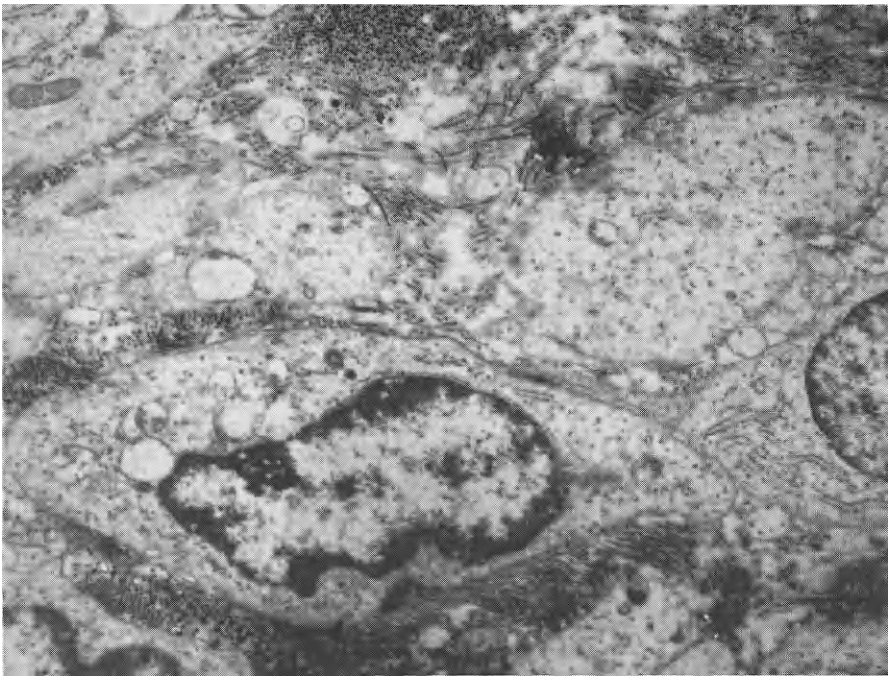

Fig. 7. Aspects of hypercellularity at the dermal level in charcoal treated animals

to adhere to collagen fibers, modulating their caliber. The neoangiogenic capability was confirmed also with studies in rabbit cornea [14].

The high sorption capacity of charcoal is responsible for an indirect bacteriostatic action and also for coagulative and hemostatic activities. Moreover, it has been reported that mast cells degranulate under edematous conditions. Products released by mast cells cause hydration of acid GAGs and deposition of mucinous ground substances; also the secretion of heparin could facilitate collagen deposition [15]. Thus, it is possible that a decrease in edema could stabilize mast cells, also avoiding an excessive neoangiogenic process and formation of keloids or hypertrophic scars.

These processes provide the rationale for chitosan and charcoal use as dressings for treatment of open wounds. Our results showed that chitosan provides results similar to those of fibrin glue, but while the latter seems to facilitate epithelial migration and differentiation, chitosan permits reconstruction, with a more regular histoarchitecture. Charcoal permitted quicker tissue repair than chitosan ($p < 0.05$) while the difference between charcoal and fibrin glue groups was of borderline statistical significance ($p < 0.1$).

The hypercellularity observed in the charcoal treated group at 30 days calls for further study of biocompatibility and carcinogenicity. For these

reasons we have implanted a 2 cm^2 specimen subcutaneously in rats for long-term in vivo evaluation.

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Comparison of Varihesive E with the Conventional Tulle Gras Dressing in Skin Graft Donor Sites Treatment of Infants

W. Wagemann, B. Franke, and H. Krause

Introduction

The conventional open or nonocclusive treatment of split-thickness skin graft donor sites using wide-meshed, lattice tulle gras compresses impregnated with saline, paraffin or ointments requires frequent dressing changes, which are usually painful and often sanguineous. Even today, this complicates donor site treatment in children. Pediatric surgery practice therefore requires a type of wound dressing that: (a) provides a safe, immovable wound cover adhering tightly to healthy skin, even in mobile children, and (b) that can be changed without causing the patient pain.

The Varihesive E hydrocolloid dressing combines with the wound exudate to produce a gel. Thus dressings can be changed without pain and without damage to newly formed granulation tissue or epithelial tissue.

We carried out a prospective, clinical, comparative study to find out if Varihesive E is a suitable alternative to conventional lattice tulle gras ointment dressings in the treatment of split-thickness skin graft donor sites in children.

Materials

Varihesive E

Varihesive E (ConvaTec GmbH, Munich) is a second generation, occlusive hydrocolloid dressing consisting of an outer layer of impermeable polyurethane foam and an inner matrix of a hydrophobic polymer and hydrocolloid particles (pectin, gelatin, sodium carboxymethyl cellulose). It adheres to normal, healthy skin. When the dressing's wound side is applied to healthy skin around the wound, it adheres safely and immovably. There is no need to keep it in place using bandages.

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Conventional Lattice Tulle Ointment Dressing

Wide-meshed lattice tulle compresses were impregnated with a paraffin vaseline mixture and subsequently sterilized. The mixture (20.0 g subliquid paraffin added to 100.0 g yellow soft paraffin) was produced by the departmental pharmacy of the Magdeburg Medical Academy Surgical Clinic.

Methods

A total of 13 children (five girls, eight boys) were included in the study. Split-thickness grafting was necessary in these patients because of extensive soft tissue injuries. The average age of the patient group was 8.6 years (s = 3.27). Patients with a known hypersensitivity to one of the test products, clinically manifest wound infection, known immuno-deficiency, or skin margins of less than 3 cm around the donor site were excluded from the study.

Split-thickness skin was taken from the extensor sides of both thighs using an electric dermatome (Wagner) set to 0.01 cm. The average donor site size in the Varihesive E and tulle gras groups was 30.6 cm^2 and 31.7 cm^2, respectively (Fig. 1).

Immediately after harvesting the skin and arresting local hemorrhage, 15 of the 30 donor sites included in the study were covered with Varihesive E and 15 were covered using a conventional lattice tulle ointment dressing.

In the Varihesive E group, we made sure that each dressing overlapped the wound by at least 2.5 cm on all sides (Fig. 2). The tulle gras dressing was

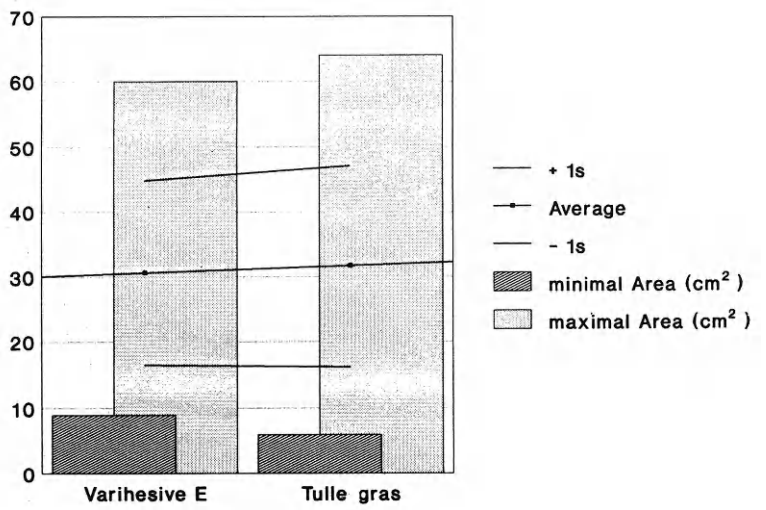

Fig. 1. Surface area of split-thickness skin graft donor sites in cm^2

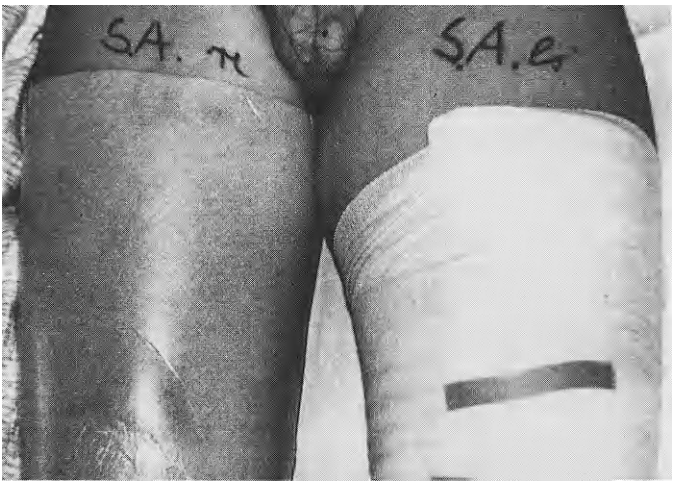

Fig. 2. Dressings in situ; *right*, Varihesive E; *left*, conventional tulle gras dressing

kept in place using bandages. The following parameters were monitored and documented: (1) the healing process and the healing time; (2) the complaints of the children during treatment; and (3) the ease of use of the dressings in clinical practice.

The first dressing change was carried out before the fifth postoperative day only if dictated by circumstances (leakage, detachment, blistering).

Before a new Varihesive E dressing was applied, the viscous exudate adhering to the wound bed was removed by irrigation using Ringer's solution. The conventional lattice tulle ointment dressing remained in situ up to the formation of a closed epithelial layer and was then likewise removed by irrigation using Ringer's solution.

The dimensions of the wound bed were precisely measured during each dressing change to evaluate the extent of reepithelialization. Only fully reepithelialized donor sites were regarded as completely healed.

The sensation of pain during healing or dressing changes was evaluated on a scale ranging from 0 to 10 (0, no pain; 10, intolerable pain).

The study results were recorded in specially designed forms.

The late results of the therapy were evaluated in a final assessment 3 months after complete reepithelialization.

Results

On average, the first dressing change was carried out after 7.9 days in the Varihesive E group and after 9 days in the control group (Fig. 3).

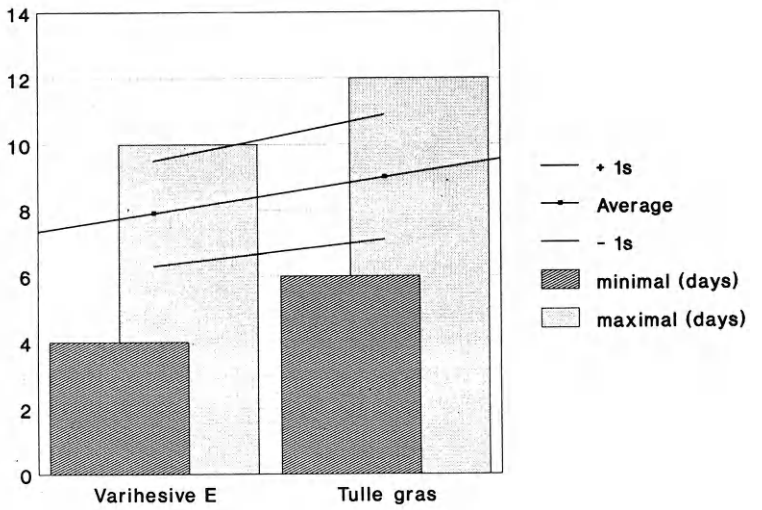

Fig. 3. Time elapsed before first postoperative dressing change

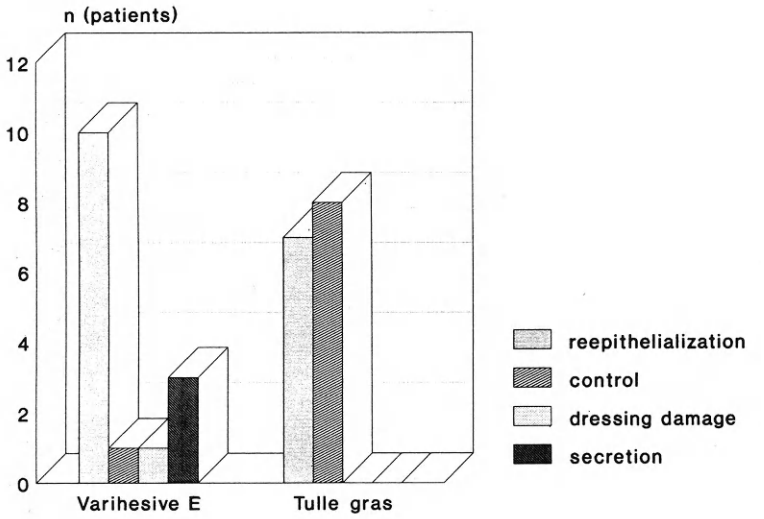

Fig. 4. Reasons for first postoperative dressing change

Two thirds of the wounds treated with Varihesive E were completely reepithelialized at that point, whereas only half of the conventionally treated donor sites were epithelialized at the time of the first dressing change.

Three primary dressings in the Varihesive E group exhibited massive blistering due to heavy wound exudation, and in one case detachment of the dressing dictated the first dressing change (Fig. 4)

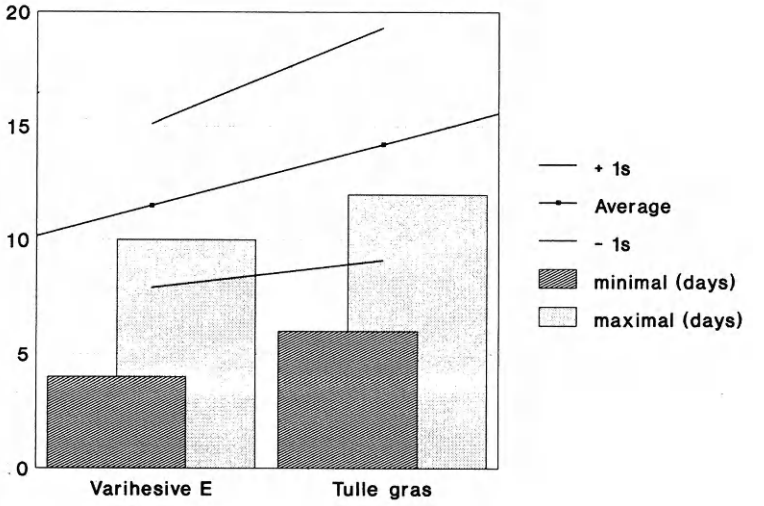

Fig. 5. Time required for complete reepithelialization of split-thickness skin graft donor sites

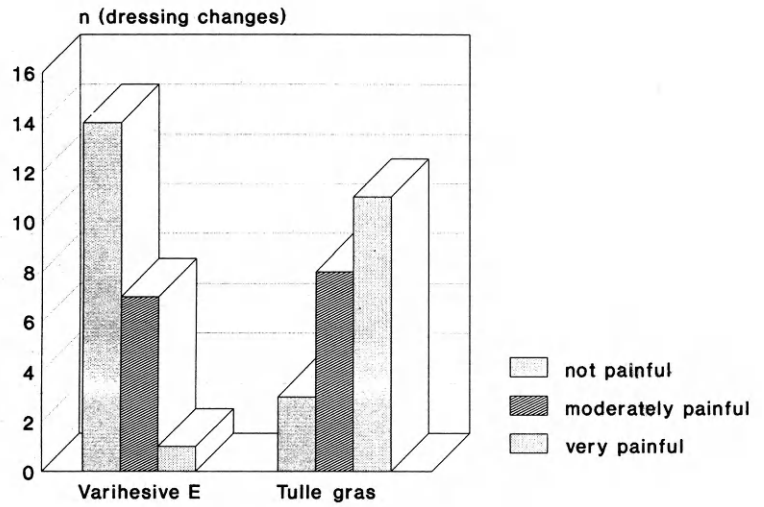

Fig. 6. Pain during dressing change (data for three tulle gras treated patients missing)

Complete reepithelialization of the wounds on average took 11.5 days in the Varihesive E group, as opposed to 14.2 days in the control group (Fig. 5).

The intensity of pain or discomfort felt during dressing changes was ascertained by questioning the children and entering their answers in a scale ranging from 0 to 10 (as described above).

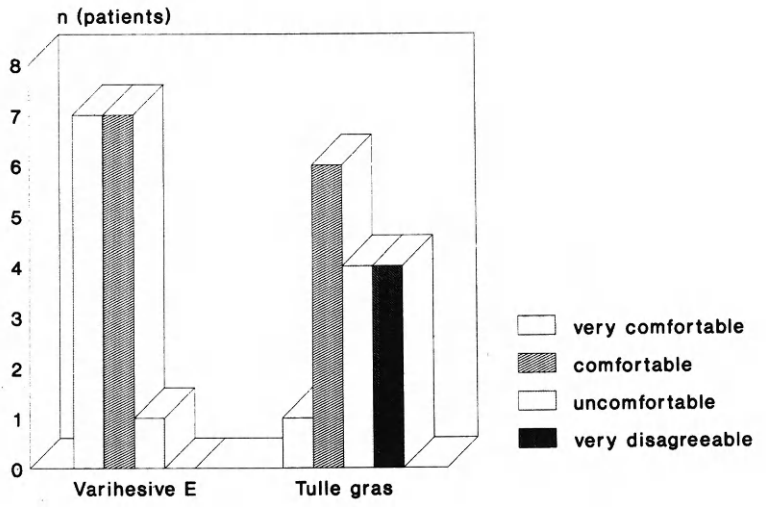

Fig. 7. Subjective assessment of dressing comfort

There was a clear difference between the two methods in this respect. Pertaining to all dressing changes, there were 14 answers in the Varihesive E group describing the process as painless, seven complaining about slight pain and one about intense pain. In contrast, dressing changes were perceived as painless only three times by children treated with the conventional tulle gras dressing, while eight answers reported slight pain and 11 intense pain (Fig. 6).

When asked for a final comfort assessment of Varihesive E, most of the children described the dressing as comfortable or very comfortable (Fig. 7). No patient rated Varihesive E as very disagreeable, while four patients did so pertaining to the conventional lattice tulle ointment dressing.

Late cosmetic results 3 months after complete reepithelialization of the split-thickness skin graft donor sites were evaluated on a skin quality scale ranging from 1 to 10 (1, best rating; 10, worst rating). According to that evaluation, the cosmetic results obtained in both groups were more or less of the same good quality. In the Varihesive E group, eight scars were rated at 1 or 2 and five scars at 3. In the control group, nine scars were rated at 1 or 2, and six were rated at 3. Keloid formation was only observed in one scar in the conventionally treated group.

Discussion

Split-thickness skin graft donor sites are usually aseptic, though often extensive, superficial wounds. Although they generally heal without most of

the complications associated with contaminated skin lesions, the healing process can often be very painful for the patient.

A variety of new dressings for split-thickness skin graft donor sites have been presented in recent years. Most publications focus on the healing process, the shortening of healing time in conjunction with a low infection rate, the ease of application in clinical use, and, last but not least, the cost of treatment [1–8, 10–12, 16].

In most surgical and dermatological institutions, however, superficial wounds are still predominantly treated according to the open or nonocclusive regimen using conventional, sterile, wide-meshed lattice tulle dressings impregnated with saline, paraffin, ointments, antibiotics or antiseptics. This type of dressing is often very painful in the area of the donor sites, a particular problem in pediatric surgery. Dressing changes often require anesthetization, especially with young children.

Literature data on healing time vary between 12 and 21 days to complete reepithelialization.

The first approach competing with the conventional open or nonocclusive wound therapy was published in 1962. After preliminary animal experiments, Winter presented the principle of wound healing under occlusion [15, 16]. Winter's research and more recent prospective clinical studies have shown that occlusive treated wounds heal better than wounds exposed to air [14].

Various authors have described the rapid and favorable healing of wounds treated with Varihesive, a commercially available occlusive dressing (also known under the brand names DuoDERM, Granuflex); according to these reports, the dressing also significantly reduces the painfulness of the wounds [1, 7–9, 11, 13].

Highly exudative wounds such as split-thickness skin graft donor sites reportedly cause practical problems in the clinical use of the dressing. The gel formed by the dressing will often leak, necessitating relatively frequent dressing changes. According to the manufacturer, the gel produced by the second generation hydrocolloid dressing Varihesive E has different physical properties. The same hydrocolloids are embedded in a different polymer, resulting in a stronger bond between the gel and the dressing substrate layer than in Varihesive [8]. Correspondingly, the first dressing change in our prospective clinical study became necessary after 7.9 days (on average). Two thirds of the split-thickness skin graft donor sites were completely epithelialized at that point.

A comparison of the total healing process in the two patient groups reveals that Varihesive E treated donor sites on average healed 3 days faster than conventionally treated donor sites.

The mean reepithelialization time of 7.9 days observed in our Varihesive E group corresponds to the results published in other studies using Varihesive E [2, 10, 11, 13]. Ten children in our Varihesive E group thus required only one dressing each for complete healing. Conventionally treated

wounds, by contrast, required one dressing in five sites, two dressings in eight sites, and three or more dressings in two sites.

Dressing changes with conventional treatment were rated 19 times as "very painful." With Varihesive E, the dressing changes were mostly described as "painless" or "only slightly painful." The children's overall subjective assessment concerning treatment with the occlusive dressing was "very comfortable" or "comfortable."

In no case was it necessary to stop treatment with Varihesive E prior to the complete reepithelialization of the donor site. The obtainable cosmetic results as assessed after 3 months were good in all children.

Conclusions

This prospective clinical comparative study has shown clear advantages of the Varihesive E hydrocolloid dressing over conventional lattice tulle ointment dressings in the treatment of split-thickness skin graft donor sites in children. The wounds required significantly less time for reepithelialization, and pain during treatment and dressing changes was alleviated. The acceptance of the dressing with patients and nursing staff was good.

As far as handling, patient acceptability and the duration of wound healing are concerned, the Varihesive E occlusive dressing is an alternative to conventional tulle gras dressings. We recommend its clinical use with children.

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Hydrocolloid Dressings in the Therapy of Cryolesions

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Introduction

Our understanding of the basic cellular and biochemical processes taking place in wound healing has undergone significant changes in recent years.

For a long time, it was standard medical practice to screen off a wound by a simple dressing and then leave it to heal by itself. In the early 1960s, however, some researchers proposed covering wounds by an external environment promoting the healing process [13, 25]. These considerations were based on the observation that blisters with an intact integument heal more rapidly than opened blisters. Wound healing in a "moist environment" clearly differs from healing under a conventional dry dressing and can be artificially attained by using innovative occlusive dressing materials. Hydrocolloid dressings are a main category of this type of advanced "wet" dressings. They consist of a mixture of hydrophilic polymers of varying structures. The hydrocolloid is dispersed in an elastomer by means of an adhesive. The top of the dressing consists of a semipermeable layer of polyurethane foam, while an adhesive layer faces the surface of the wound. The layer facing the wound absorbs the fluid exudating from the wound and thus forms a colloid gel covering the surface of the wound [6, 12, 22].

The moist environment created under a hydrocolloid dressing apparently accelerates the cellular and biochemical processes taking place in a wound. Fully functional leukocytes releasing proteolytic enzymes and thereby continuously cleansing the wound were found on the surfaces of wounds and in the hydrocolloid itself [1, 9, 26]. In addition, proliferation factors accumulate in the hydrocolloid and influence epithelial and fibrotic processes of wound healing in a variety of ways [7]. Desiccation of the wound surface is prevented, and there is no formation of eschar that would obstruct the epithelialization of the defect. Given these theoretical benefits, hydrocolloid dressing should also exhibit significant clinical advantages over traditional wound dressings.

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Wound Healing and Skin Physiology
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The evaluation of a dressing's effect in a clinical study is often complicated by the fact that wound healing is not only influenced by the dressing itself, but also by other criticial parameters such as chronic venous insufficiency or arterial perfusion disorders in the presence of leg ulcers. The variety of influencing parameters makes it difficult to rank the dressing's role. In order to overcome these problems, we need a method to obtain defined and reproducible defects of the epidermis and corium, in which the effect of external factors is minimized. Cryosurgery is a procedure that comes very close to meeting these requirements. This method has become more and more popular in recent years, since it leads to good cosmetic results in the therapy of epithelial skin tumors. It is particularly suited as a standardized model of wound healing [3, 15, 21, 24].

Digital b-scan sonography allows evaluations of the healing progress in cryolesions on the basis of the echo characteristics presented by the wound bed [14, 15, 17, 24]. In this way, the extent of the postoperative edema and the regeneration processes taking place in a healing wound can be evaluated. Initially, the sonographic image is completely resolved after cryosurgery. The entry echo is completely lost (this echo is an impedance phenomenon that occurs when the beam of sound impinges on the surface of the stratum corneum). The sonographic image of the cryolesion and physiological parameters such as the pH value of the skin can additionally characterize the wound environment.

The following is a report on a clinical, randomized, prospective study in which a hydrocolloid dressing (Varihesive E) was compared to a conventional "dry" paraffin gauze dressing (Oleotüll) combined with sterile compression bandages. The dressings were applied over cryolesions resulting from the cryosurgical therapy of basal cell carcinomas in the facial region and neck.

Wound healing in the two dressing regimes described above is documented on the basis of the clinical progress of healing and by referring to objective parameters.

Materials and Method

A total of 77 patients (40 males, 37 females) were subjected to the cryosurgical treatment of basal cell carcinomas in the facial region or neck. Prior to the procedure, the diagnosed basalioma were histologically confirmed on the basis of 3-mm sample biopsies; 85.6% of the cases exhibited solid basal cell carcinomas. In the remaining cases, we found superficial basilomas (7.9%), sklerodermiformally growing basilomas (5.2%), or adenoid basaliomas (1.3%).

The cryolesions caused by the surgical procedure were treated in a randomized comparative study, in which either a hydrocolloid dressing (Varihesive E) or a paraffin gauze dressing (Oleotüll) combined with dry

compression bandages was applied. A total of 40 patients was included in the hydrocolloid group (18 males, 22 females; mean age, 68.3 years; age range, 52-85 years), and 37 patients were treated with a paraffin gauze dry dressing (22 males, 15 females; mean age, 67.7 years; age range, 52-85 years). The mean diameter of the tumor was 23.5 mm^2 in the hydrocolloid group and 24.1 mm^2 in the dry dressing group.

The inclusion criteria were: cryosurgically treated basiloma in the face or neck; standardized cryolesion (maximum diameter, 40 mm; maximum depth, 4 mm); age, 40–85 years. Exclusion criteria were: ulcerated basiloma; infections in the treatment area; systemic diseases that may affect wound healing, e.g., diabetes mellitus; endogenous or exogenous impairment of the immunological state; intolerance to Varihesive E or Oleotüll. Each patient signed a document in which he or she consented to participating in the study. The Ethical Commission of the Ruhr University, Bochum, examined the study before it was started.

Cryosurgery

Cryosurgical therapy of the basilomas was carried out using the closed contact method. Whilst in the open spray method, liquid nitrogen is directly sprayed onto the skin, the closed contact method involves the direct placement of a metal punch of defined dimensions onto the lesion. The metal punch is then flushed with liquid nitrogen and cooled down to approximately −150°C. The low-temperature flux in the tissue is monitored by a thermoneedle positioned at the base of the tumor (Figs. 1, 2).

After local anesthesia using Scandicain, the metal punch was placed on the skin, and the freezing cycle was started. The cycle was ended when the tumor base had reached a temperature of approximately −30°C or when the

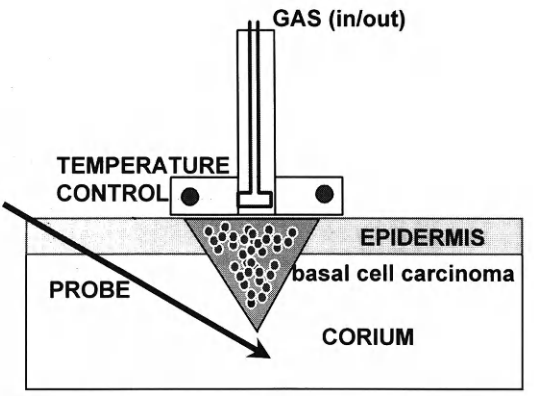

Fig. 1. Functional diagram of a closed cryosystem. The cryoprocess causes a triangular defect

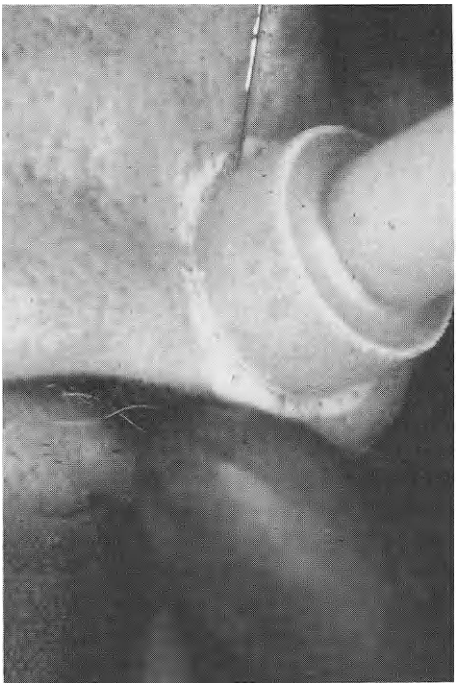

Fig. 2. Clinical realization of cryosurgery at the nose. Cryostamp with incorporated thermosensor. A thermoneedle (probe) is placed in the skin

punch had been cooled to a temperature of −150°C. The size of the punch depended upon the size of the basiloma; the average size of the punch was 17.3 mm in the hydrocolloid group and 18.2 mm in the dry dressing group. The number of freezing cycles depended on the penetration depth of the tumor in the corium, which was sonographically ascertained prior to the procedure. Only two patients in the hydrocolloid group and three patients in the dry dressing group required a second freezing cycle.

Wound Dressing

Each lesion was cleansed with physiological saline and dressed immediately following cryosurgery. The hydrocolloid dressing (Varihesive E) is self-adhesive. The paraffin gauze dressing (Oleotüll) was covered with a sterile compression bandage, which was fixed by Leukosilk adhesive tape. The resulting paraffin gauze dry dressing was changed twice a day, while the hydrocolloid dressing was changed once a day.

The treatment period was limited to a maximum of 21 days. The state of wound healing was examined on the days 0, 1, 4, 7, 10, 14, and 21.

Examinations of Wound Healing

The progress of wound healing was evaluated on the basis of the following parameters: preoperative measurement of the basiloma's penetration depth; clinical evaluation of the wound structure; dimensions of the ulceration (planimetry); corium thickness (sonographic measurement); dimensions of the cryolesion (sonographic measurement); pH value trend in the wound area.

The surface area of the wound was ascertained by planimetry. Initially, the wound area was traced to a transparent plastic film. This area was subsequently scanned into an image analysis system (Microscale TC, Digithurst, United Kingdom) and measured following appropriate calibration of the system.

For the preoperative evaluation of the basalioma's penetration depth and for the postoperative examinations of the healing progress in the cryolesion, 20-MHz sonography was used. The specific ultrasound imaging system employed for this purpose was the DUB 20 scanner (Taberna pro medicum, Lüneburg, Germany). This system allows the examination of skin structures in a depth of approximately 7 mm with an axial resolution of approximately 80 μm and a lateral resolution of approximately 200 μm. A ruler can be superimposed for the precise measurement of a region of interest (ROI) or a structure of interest (STOI) in the μm range.

The pH value in the wound area was measured as a physiological skin parameter on the days listed above. The PH 900 Skin pH Meter (Courage und Khazaka, Cologne, Germany) was used for this purpose.

Statistics

Comparisons between the two treatment groups were made in the course of healing, when there was sufficient homogeneity on day 0. The Wilcoxon test was used for interval/proportional scales, the Mantel Haenszel test was used for ordinal scales, and the chi-squared test was used for nominal scales.

Results

Sonographic Case Study

A solid basalioma on the left zygomatic bone of an 82-year-old male patient was histologically confirmed based on a 3-mm punch biopsy. In sonography, the basiloma appears as a poorly reflecting tumor (T) with nonhomogeneous distribution of internal reflections (*) and dorsal echo enhancement in the corium (arrows; Fig. 3). the impedance difference between

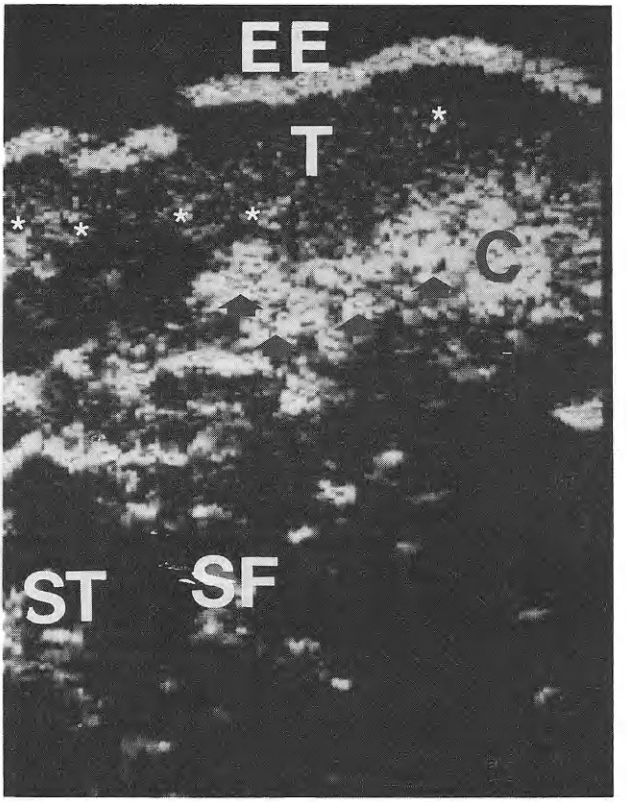

Fig. 3. Sonogram (20 MHz) of a solid basiloma. *EE*, entry echo; *T*, tumor; *stars*, internal echoes; *C*, corium; *arrows*, dorsal echo enhancement; *SF*, subcutaneous fatty tissue; *ST*, subcutaneous trabeculae

the water prepath and the stratum corneum causes an entry echo (EE) linearly covering the tumor. The corium (C) is echo rich.

Subcutaneous fatty tissue (SF) appears as an echo-poor structure. Individual reflections of greater intensity are caused by subcutaneous trabeculae (ST).

On day 1 after cryosurgery, the entry echo (EE) is highly fragmented (Fig. 4). Underneath the entry echo, there is a poorly reflecting area which exhibits only a few internal reflections (*). The corium (C) is echo rich. This sonographic image corresponds to a blister (B) which typically forms after cryosurgery. Deep down, there is a very dense reflection pattern which indicates a slightly consolidated band of corium. The subcutaneous fatty tissue (S) appears as an echo-poor structure.

On day 7 after cryosurgery, the entry echo (EE; Fig. 5) is gradually restituted. Deeper down, the highly reflective margin of the corium has become wider (C). Portions of connective tissue have been newly formed

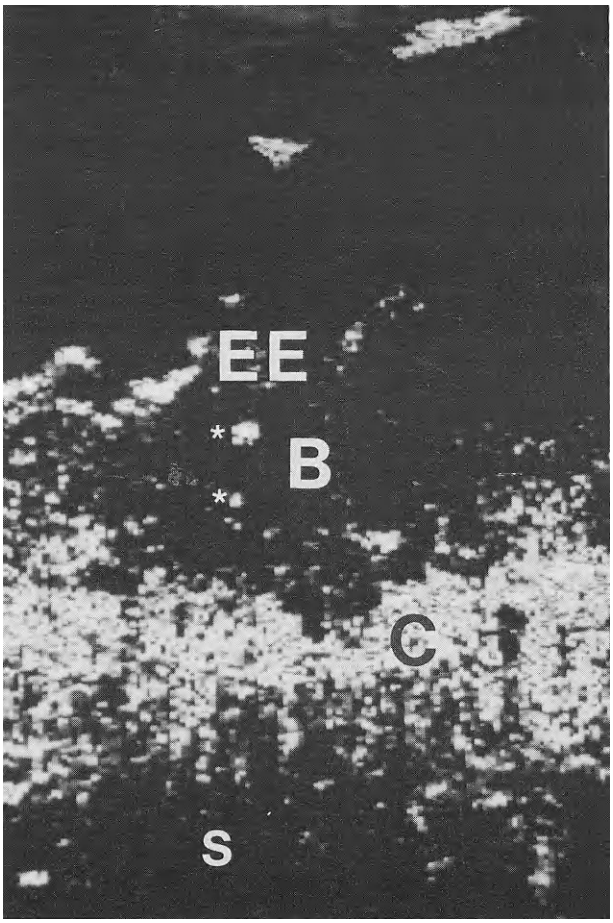

Fig. 4. Sonogram (20 MHz) on day 1 after cryosurgery. *EE*, entry echo; *stars*, internal echoes; *B*, blister; *C*, corium; *S*, subcutaneous fatty tissue

and are advancing from the depth towards the entry echo. There is only a small echo-poor band between restituated corium and entry echo.

On day 21, an epithelialized flat scar is present under clinical inspection. In the sonographic image, the entry echo (EE) has been restituted (Fig. 6). There is only a small poorly reflecting band left between the highly reflective corium (C) and the entry echo (arrows).

Planimetric Measurement of Wound Surfaces

Planimetric measurement initially indicates an increase in the wound surface between day 0 (surgery) and day 1. Subsequently, the surface area begins to

Fig. 5. Sonogram (20 MHz) on day 7 after cryosurgery. *EE*, entry echo; *C*, corium

decrease (Fig. 7). The measured extent of the cryolesions shows that wound healing proceeds faster under a hydrocolloid dressing than under a paraffin gauze dry dressing. Towards the end of the treatment period, the surface area of the wounds is significantly smaller in the hydrocolloid group ($p = 0.0004$).

The mean duration of treatment was 15.8 days in the hydrocolloid group and 20.4 days in the dry dressing group ($p = 0.0001$). Treatment in the dry dressing group on average took 4.6 days longer than in the hydrocolloid group.

Sonographic Measurement

Sonographic measurement indicated that the corium width in the hydrocolloid group was more significantly reduced than in the control group ($p = 0.06$).

In addition, this procedure showed that the sonographic extent of the cryolesion diminishes more quickly in the hydrocolloid group when compared with the rate of reduction in the dry dressing group (Fig. 8). A comparison between the individual patients in the hydrocolloid group clearly showed a significant reduction in the size of the cryolesion ($p = 0.0009$), while the change was not significant in the dry dressing group ($p = 0.251$).

Fig. 6. Sonogram (20 MHz) on day 21 after cryosurgery. *EE*, entry echo; *arrows*, echo-poor band; *C*, corium

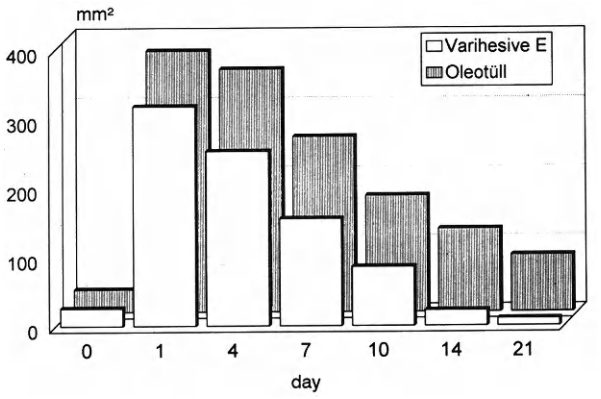

Fig. 7. Results of the planimetric measurement of the wound. The reduction of the wound surface in the hydrocolloid group is superior to the reduction in the dry dressing group

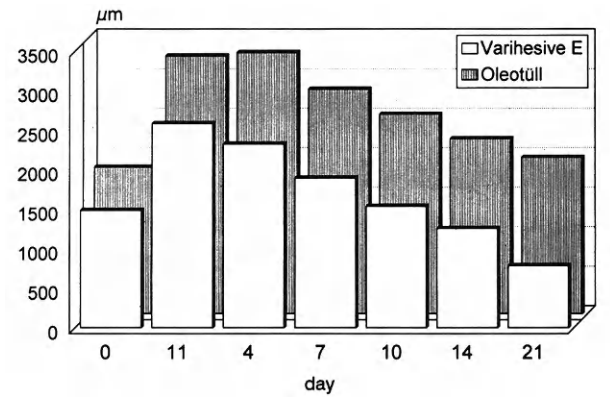

Fig. 8. Sonographically measured depth of the cryolesion. We observed both reduction of the wound surface and reduction in wound depth, as can be measured by high-frequency ultrasound

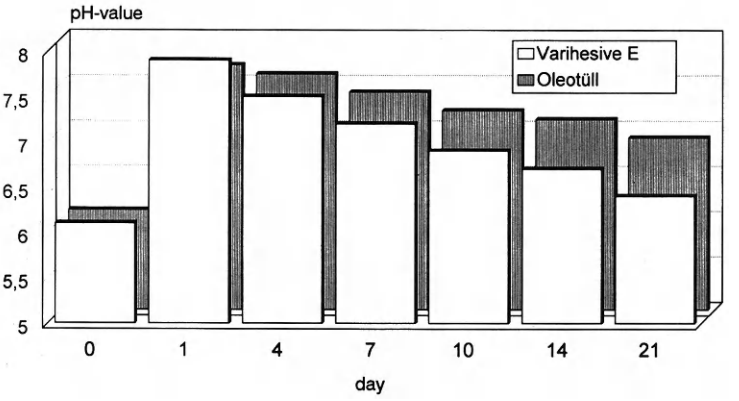

Fig. 9. pH value trend in the wound environment. The pH value decreases in both groups in the course of treatment. This effect is more pronounced in the hydrocolloid group

pH Value Trends

The two groups exhibited very similar pH value trends in the cryolesions, as can be seen in Fig. 9. After an initial increase following cryosurgery, the pH values dropped as treatment progressed. This effect was more pronounced and statistically significant in the hydrocolloid group ($p < 0.0081$).

Infection Rate

In the course of the study, 11 cases of wound infection were confirmed by swabs. While nine of these cases were found in the dry dressing group, only two infections were observed in the hydrocolloid group. The infections were successfully treated by oral administrations of 3 × 500 mg Tetracyclin per day in ten cases or by 2 g Oxacillin per day in one case.

None of the infections caused major clinical symptoms; all of them were detected in swab examinations made at regular intervals.

Discussion

Wound healing invariably aims at the swift and uncomplicated repair of damaged tissue. With respect to lesions of the skin, physicians have always attempted to optimize the healing process through the dressings applied to the wounds. Hydrocolloid dressings are an innovative approach in these therapeutic efforts, since they create a moist environment for wound healing.

Experimental studies have shown that leukocytes remain fully functional in a moist environment and are thus able to perform important tasks in the healing process [1, 8, 9, 11, 19, 22, 26]. Lysosomal enzymes released by neutrophilic granulocytes and macrophages as well as enzymes produced by bacteria are instrumental in the decomposition of necrotic matter. Necrotic matter hinders the formation of granulation tissue and is a major reservoir of pathogens. Layers of eschar that usually form under dry dressings have for a long time been regarded as a physiological wound cover. However, eschar not only contains a large number of pathogens, but is also a major obstacle to the migration of keratinocytes. Hydrocolloid dressings prevent the formation of eschar and allow keratinocytes to advance from the edge of a wound towards the granulation tissue and accelerate the epithelialization of the defect.

Hydrocolloids promote fundamental cellular functions which play a predominant role in wound healing. The rehydration of the wound under a hydrocolloid dressing by itself facilitates the dissolution of necrotic matter and eschar, but the hydrocolloids not only act as a mere reservoir of moisture, but also contain in the wound a large number of factors promoting wound healing. Thus, in addition to cleansing the wound, the dressing strongly promotes granulation and epithelization. Growth factors, which play a critical role in these processes of wound healing, are retained in the gel that forms under a hydrocolloid dressing and can have a favorable effect on dermal and epidermal healing processes [2, 4, 6, 18, 20, 23]. In addition, chemotactically active and mitogenic cytokines are found, which have a substantial influence on cellular activity [7].

The clinical evaluation of the effects described above is usually very difficult, since a direct comparison of wounds is typically precluded by the heterogeneity of the defects. However, cryolesions resulting from the cryosurgical therapy of epithelial skin tumors meet many of the requirements of a standardized wound. The surface area and the depth of a cryolesion can be precisely determined by the dimensions of the metal punch, the freezing temperature, and the number of freezing cycles. The healing process in cryolesions can be tracked by 20-MHz b-scan sonography [16, 21, 24]. Initially, there is only a poor reflection from the defect because of the edematous impregnation of the collagen matrix, which is chiefly responsible for the dermal reflection pattern. The edema causes the dissociation of collagen fascicles, which results in the reduction of interfaces reflecting the ultrasound signal. In the course of wound healing, the edema diminishes and collagen fibers are newly formed.

As the epithelialization of the defect proceeds, the entry echo typical for intact skin is restored; 20-MHz sonography is an important new tool since it not only provides information on the surface of a wound, but also allows evalutions of wound healing in the corium. The method is noninvasive and can be repeated as often as required without causing discomfort to the patient [3, 14–17, 24].

A paraffin gauze dry dressing (Oleotüll) was compared with a hydrocolloid dressing (Varihesive E) in a clinical study on the basis of the wound healing model described above. The study design was similar to a method that had previously been used in an evaluation of wound healing in cryolesions [16]. The advantages theoretically offered by a hydrocolloid dressing were confirmed in clinical practice and on the basis of objective parameters. The mean duration of healing was significantly reduced under a hydrocolloid dressing regime, and the reduction in the surface area of the wounds also proceeded at a significantly higher rate than that recorded in the dry dressing group. These findings are of considerable clinical importance. When earlier closure of a wound is achieved, the integrity of the cutaneous system is restored earlier; the skin sooner resumes its physiological function, and complications caused by a wound are reduced. Sonographic measurements indicated that the reduction in the depth range of a cryolesion likewise was faster under a hydrocolloid dressing than under a dry dressing. Consequently, there is not only a higher rate of epithelialization on the surfaces of wounds, but also an acceleration in the reduction of edemas caused by cryosurgery and initially contributing to the swelling of the lesion. Moreover, the regeneration of connective tissue also takes place at a higher rate.

The pH value in the wound area of a cryolesion continuously diminishes during the course of healing. This value may reflect the increasing activity of leukocytes releasing acidic metabolites or proteases [5], and it could therefore be an important parameter of leukocyte activity. The fact that the pH value dropped faster in the hydrocolloid group may indicate that leukocyte

activity is more intense under a hydrocolloid dressing than under a paraffin gauze dry dressing.

Two cases of wound infection were observed in the hydrocolloid group as opposed to 11 cases in the dry dressing group, i.e., the infection rate was significantly lower in the hydrocolloid group. Under a hydrocolloid dressing, there is a persistent flow of moisture originating in the base of the wound. This flow may be an important factor contributing to the low infection rate, since it results in a continuous cleansing of the wound base. Pathogens and wound debris are absorbed by the dressing, while leukocytes in the wound remain functional and create an antimicrobial environment [10, 18].

The hydrocolloid dressing was shown to be clearly superior to the paraffin gauze dressing in this study. Previous in vitro findings suggesting the superiority of hydrocolloid dressings were objectively confirmed. The most impressive and important study result is the acceleration of wound healing without undesirable side effects. This capacity is the critical requirement determining the quality of each type of wound dressing.

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Treatment of Venous Leg Ulcers Using Occlusive Hydrocolloid Dressings (Varihesive E) Versus Conventional Phase-Adapted Wound Dressing

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Introduction

The topical therapy of venous leg ulcers (ulcus cruris venosum) has to be adapted to the chronology of wound healing. In addition to the elimination of disturbance factors (débridement, local antisepsis), wound healing can be promoted by the application of various topical preparations that stimulate granulation or epithelization. New findings on wound healing mechanisms have initiated a gradual change in topical therapy since the early 1980s. The development of occlusive hydrocolloids and other types of wound dressings has made it possible to replace conventional phase-oriented topical therapy by one type of treatment covering all phases. The performance of various occlusive hydrocolloid dressings has been studied in various types of skin lesions, such as venous leg ulcers [1–7], diabetic ulcerations [8], pressure sores [9–11], excoriations [12], abrasions [13, 14], split-thickness skin graft donor sites [15–17], burns [17–21], and other lesions [20, 22, 23]. Hydrocolloid dressings are easy to use and considerably simplify local therapy.

The present study compares the performance of a hydrocolloid dressing (Varihesive E, ConvaTec) to that of a conventional wound dressing in venous leg ulcer therapy in 22 patients.

Materials and Methods

The efficacy, safety, and ease of use of a hydrocolloid dressing (Varihesive E) and a conventional wound dressing were compared in an open, prospective, randomized study of 22 patients with venous leg ulcers.

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Diagnostic Screening

The angiological status was analyzed thoroughly in preliminary clinical examinations. Epifascial and subfascial venous refluxes were diagnosed using Doppler sonography. Arterial closing pressures were ascertained (also using Doppler sonography), and the ankle/arm index was calculated to rule out clinically relevant arterial circulatory disturbances. The severity of the hemodynamic impairment was assessed by direct phlebodynamometric venous pressure measurements (Phlebometron, according to Varady). The following pressure drop intervals (delta p) were defined: Decreases in pressure (Δp) were defined in the following ranges: 50–60 mmHg, normal value; 41–49 mmHg, slight hemodynamic impairment; 20–40 mmHg, moderate hemodynamic impairment; 0–<20 mmHg, severe hemodynamic impairment.

Patients

Exclusion criteria included ulcers of a different genesis, insulin-dependent diabetes mellitus, congestive heart failure, corticosteroid treatment, antibiotic therapy, and other types of treatment that may delay wound healing. Additional exclusion criteria were operative procedures involving the venous system, phlebosclerotherapy during the past 2 months prior to the beginning of the study, pregnancy, and clinically manifest wound infections. The mean age of patients was 63 years (range 35–87), with a slight difference between the two test groups. There was a clear majority of women (82%).

The hemodynamic impairments after direct venous pressure measurement were rated as severe in 16 cases and moderate in two, while in 2 no impairment was measured. The mean decreases in peripheral venous pressure under load was 14 mmHg in the Varihesive E group and 12.6 mmHg in the control group. The results correspond to those generally observed in ulcers with a history of more than 5 years. Doppler sonography invariably showed insufficiencies in the regions of the epifascial and perforating veins and, in most of the cases, around the subfascial veins. Most of the leg ulcers in our patients were located on the left leg, usually around the medial malleolus. Patient histories showed deep vein thromboses to occur predominantly in the left leg, while cases of phlebitis were divided almost evenly between the two legs. Ulcers in either of the legs had been treated conservatively in most of the cases, although a few patients had received surgery.

Local Therapy

Twenty patients each were randomly assigned to treatment with Varihesive E or a conventional wound dressing. In the Varihesive E group initial

surgical wound débridement was followed by monophasic treatment with Varihesive E and compression bandages (elasticity below 100%). The dressings were changed twice a week by the same personnel in the first 6 weeks of the study. In the patients receiving conventional dressing initial surgical débridement was followed by phase-oriented wound dressings according to the normal clinical schedule: *débridement*, enzymatic wound débridement using streptokinase/streptodornase (Varidase Gel); *local antisepsis*, clioquinol (Linolasept Emulsion); *promotion of granulation*, polyurethane foams soaked in NaCl (Epigard); *stimulation and protection of epithelization*, L/W emulsion with pantothenyl alcohol (Bepanthen). In addition, a compression bandage (elasticity below 100%) was applied. The bandage was changed twice a day.

Schedule and Study Parameters

The investigating physician inspected the ulcer twice a week during the first 3 weeks and once a week up to week 6, subjectively judging the acceptability and practical performance of the dressing. In addition, granulation and epithelialization were recorded in terms of a scale, and the ulcer surface was measured by planimetry.

Oxygen partial pressure was measured directly on the ulcer floor using a commercially available transcutaneous oxygen/carbon dioxide monitoring system (Radiometer, Copenhagen, Denmark). Pressure measurements were made at the beginning of the study and during its course at the same location and at 44°C.

We developed a special method of attaching the electrode to the ulcer floor. The procedure causes little discomfort and conforms to hygienic standards. Since the electrode holder depresses the ulcer floor, we initially checked the effect that various pressure intensities have on pO_2 values; the measurements were carried out on intact skin and at the ulcer floor. We thus ascertained mechanical holder effects and were able to exclude artificially induced pressure ischemia.

All the measurements were made by the same investigator at the beginning of the study and on days 7, 10, and 42. These were carried out at the same location in the ulcer, with the patient lying and having acclimatized to room temperature for at least 30 min. The measurements were expected to provide detailed information on the neovascularization of the ulcer floor. We were interested chiefly in the course of values measured during healing rather than focusing on absolute values.

Results

The relative reduction in ulcer surfaces was assessed by planimetric measurement. During the 6 weeks of treatment ulcer surfaces in the Varihesive

Table 1. pO_2 at ulcus floor with Varihesive E and conventional dressing

Patient no.	Day 7 (mmHg)	Day 42 (mmHg)	Difference (%)
Varihesive E			
3	1	19	95
4	1	2	50
5	44	1	−4300
7	5	50	90
9	1	1	0
12	15	25	40
15	1	–[a]	–[a]
16	2	1	−100
18	1	–[a]	–[a]
19	1	20	95
Conventional dressing			
1	4	–[a]	–[a]
2	1	–[a]	–[a]
6	8	1	−700
8	6	0	−100
10	1	1	0
11	1	6	83
13	0	1	100
14	2	0	–[a]
17	0	37	100
20	1	23	96
21	70	85	18
22	13	63	79

[a] Drop out.

E group were reduced by 29.1%, and two out of the ten ulcers healed completely. In the control group, ulcer surface reduction was comparable.

Granulation and epithelization were comparable in the two groups.

The measurement of oxygen partial pressure on the ulcer floor showed widely scattered values. There was no clear difference between the two groups (Table 1). However, the reduction in ulcer surface correlated with a rise in oxygen partial pressure; there are only a few exceptions to this.

Dressing acceptability in terms of itching, burning, skin tension, erythema, and heat sensation tended to favor Varihesive E. Whilst no patient in the Varihesive E group reported pain or a burning sensation during dressing changes, a few patients in the control group complained of considerable pain.

As regards appearance, impermeability to water, and adhesion Varihesive E proved clearly superior to conventional dressing (Table 2). Its appearance was judged as "very good" even after the dressing had been in place for several days. The dressing's excellent impermeability and adhesion allowed patients to shower or bathe while patients wearing conventional dressings had to forego showers and baths.

Table 2. Evaluation of impermeability and adhesion of Varihesive E versus conventional dressing

	Varihesive E (n = 8)	Conventional dressing (n = 10)
Impermeability		
Very good	2	1
Good	6	0
Moderate	0	9
Adhesion		
Very good	5	1
Good	3	0
Moderate	0	9

Discussion

Occlusive hydrocolloid dressings have repeatedly been found to offer significant advantages over conventional dressings. These advantages relate to the dressings' acceptability in the treatment of abrasions [13], burns [18], split-thickness skin graft donor sites [17], and venous leg ulcers [1], particularly as regards the pain involved in dressing changes. No patient in our Varihesive E group complained of pain or burning sensations during dressing changes. Reports in the literature also emphasize that hydrocolloid dressings are more comfortable to the patients than conventional dressings and are thus more readily accepted. This has been observed in the treatment of burns [21], abrasions [13], split-thickness skin graft donor sites [15], and venous leg ulcers [7]. Our findings confirm these observations.

Various wounds reportedly require less time for healing when treated by hydrocolloid dressings, for example, pressure sores [11], abrasions [13], split-thickness skin graft donor sites [16, 17] and burns [18, 20]. Such a statistically significant difference has not yet been established for venous leg ulcers. The planimetric assessments that we have carried out suggest differences in the reduction of ulcer surfaces under the two types of dressing. The same is true for granulation and epithelization. Animal experiments have clearly shown that epithelization can be stimulated under hydrocolloid dressings [24, 25].

The treatment of venous ulcers with hydrocolloid wound dressings promises a number of additional advantages. Varihesive E is clearly superior in its impermeability and adhesive properties evaluated under practical performance aspects. Moreover, Varihese E offers economic benefits. Throughout the treatment period of 6 weeks, the Varihesive E group required only 167 dressing changes compared to 924 in the control group. Conventionally treated hospitalized patients thus require considerably more

nursing care and personnel, outweighing the relatively high costs per dressing change with Varihesive E.

On the basis of this preliminary evaluation the treatment of chronic venous ulcers with Varihesive E hydrocolloid dressings can be regarded as an advantageous alternative to conventional phase-adapted wound dressings, in particular when total treatment cost is considered.

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Weekly Treatment of Psoriatic Plaques with the Hydrocolloid Dressing DuoDERM E in Combination with Triamcinolone Acetonide

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Introduction

It is well established that occlusion of psoriatic plaques has an antipsoriatic potential. In several studies corticosteroids placed under occlusive dressings have been demonstrated to be highly effective in treating psoriasis.

An important improvement of the occlusive treatment approach has been the application of hydrocolloid dressings (HCD). In most patients HCD can remain on the skin for 1 week without causing significant irritation. Plastic occlusives, however, cannot remain on the skin surface for such a period without causing maceration. The semiocclusive Opsite UV 3000 offers an attractive approach as it can remain on the skin surface for a prolonged period and is well tolerated.

The objectives of the present investigation were: (a) to determine whether HCD once a week are effective as a treatment of psoriasis; (b) to determine whether HCD once a week combined with the application of triamcinolone acetonide (TACA) is highly effective in the treatment of psoriasis; and (c) to find out how the efficacy of TACA and HCD in comparison to the efficacy of TACA under Opsite UV 3000.

Materials and Methods

The general plan of the investigation was a four way within-patient comparison. At four plaques the investigator applied once a week: (1) TACA 0.1% under HCD DuoDERM E; (2) TACA 0.1% under Opsite UV 3000; (3) TACA 0.1% lotion only; (4) HCD only. In addition, the unoccluded lesion was treated with TACA 0.1% lotion twice daily by the patient at home.

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Only patients with chronic, stable, plaque psoriasis who had been without any treatment during the 2 weeks preceding the investigation were included. In total 40 patients were included at two centers at the University Hospital Nijmegen and at the Rijn State Hospital in Arnhem. Before treatment and after 1, 2, 3 weeks of treatment, lesions were assessed using a four point scale. We assessed erythema/scaling and induration. Also, side effects and tolerability were recorded.

Results

All 40 patients except one completed the 3 week treatment. Of the 40 patients, 27 judged the DuoDERM E treatment as very comfortable and 23 judged Opsite treatment as very comfortable. No irritation at all was recorded by 29 subjects in DuoDERM treated sites and by 27 in the Opsite treated sites. Moderate to marked irritation was recorded in three patients at sites treated with DuoDERM and at ten sites treated with Opsite. Although irritation tended to be more frequent in Opsite treated sites no statistically significant difference could be shown between DuoDERM and Opsite in this respect. In 28 patients DuoDERM E stayed on the skin during the whole period. In 20 patients Opsite stayed in situ during the whole period of observation. So at the weekly visits the plaque still remained perfectly in situ during the week before.

Opsite and DuoDERM were well tolerated by the great majority of the patients. What about clinical efficiency?

Erythema in DuoDERM treated sites decreased. In the DuoDERM and TACA treated sites the reduction was much more marked. In Opsite and TACA and in TACA only treated sites a tendency of a reduction was recorded. Already after 1 week of treatment, the reduction was statistically significant in both DuoDERM treated sites. Induration in DuoDERM treated plaques reduced, but a pronounced reduction at the combined treatment site were reached. Already after 1 week of treatment the reduction was highly significant in both DuoDERM and DuoDERM and TACA treated sites.

In the Opsite and TACA treated sites the situation was completely different. Although TACA resulted in a reduction reaching significancy after 1 week, TACA and Opsite treatment did not result in a significant reduction after 3 weeks of treatment. We had expected that scaling would decrease in sites treated with occlusive treatment. Again in sites treated with DuoDERM only and in sites treated with the combination a marked reduction of scaling was observed. The reduction induced by TACA was significant after 1 week, but sites treated with TACA and Opsite did not show any improvement.

Conclusions

We can reconfirm that DuoDERM E is, to some extent, effective as a monotherapy in treating psoriasis. In addition, DuoDERM E and TACA is highly effective in the treatment of psoriasis. Comparing DuoDERM E with Opsite UV 3000, we can conclude that both occlusives are well tolerated by patients. However, a major difference was noticed between both occlusives with respect to the antipsoriatic efficacy. It is likely that the partial occlusive capacity of Opsite plays a major role in this respect. It follows that HCD represent an important approach which provides optimal topical drug availability.

Covering Tattoo Dermabrasion Wounds with a Hydrocolloid Dressing (Varihesive E)

J. Ulrich and K.-H. Kühne

Introduction

The art of decorating the human body by painting, stigmatizing or tattooing has been practised for thousands of years. The earliest findings suggesting the practice of tattooing are bone needles from 6000 BC, on which pigments were found and which had apparently been used for tattooing [16].

Schönfeld [23] reports that the irreversible introduction of pigments into the skin was also practiced in classical antiquity, mainly by barbarian peoples such as the Thracians. The tattoos had ornamental function or were regarded as signs of bravery, but they were also supposed to intimidate the enemy in battle.

The discovery of the South Sea Islands led to a revival of tattooing in more recent times. The reintroduction of this form of art is attributed to James Cook and his seamen [18]. The Polynesians taught them how to rub pigments into scratched or burnt skin [18]. The word "tattoo" is derived from the word "tatau" (to draw), which the Polynesians used to describe their art of skin decoration. It is not surprising that the application of ornamental tattoos is still widely used as a signal of belonging to a certain group, in particular among sailors [18, 19].

There is also evidence that even in antiquity physicians removed tattoos in medical procedures, especially motifs regarded as signs of bondage or punishment (slaves, soldiers) [23]. The removal of ornamental tattoos is still a common medical problem. Tattoos are usually acquired in early adulthood, frequently under the influence of alcohol [14, 15, 20]. They can cause great emotional distress in later life.

Various procedures for the removal of frequently psychically distressing tattoos later in life have been described, but none of them has become generally established [1, 8, 11, 20]. While the excision of the tattooed area certainly has favorable cosmetic results, skin grafting is often ruled out

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because of the size of the tattoos. Other methods include CO_2 laser treatment, dermabrasion, skin cauterization or combinations of these methods [1, 8, 20, 22, 24].

Since Schreuss introduced superficial skin abrasion (dermabrasion) in the 1950s, tattoo removal has become one of the main indications for this method, apart from the treatment of lentigines seniles, scars, or keloids [7, 8, 24].

The dressing of dermabrasion wounds is as widely discussed as the removal of the tattoos. Various methods have been published; ointment-impregnated tulle dressings or special wound films are probably the most common dressings in practical use today [6, 21].

In recent years, wound treatment has been revolutionized by the introduction of occlusive mostly hydrocolloid dressings. The use of hydrocolloid dressings is indicated with ulcers of various genesis, split-thickness skin graft donor sites, and dermabrasion wounds [2, 5].

Various studies on the effects of occlusive hydrocolloid dressings have shown the superiority of wet wound treatment. Cells involved in wound healing and different types of growth factors require a moist environment for survival or optimum efficacy [3, 5, 12]. Other effects attributed to occlusive hydrocolloid dressings include stimulation of angiogenesis due to the oxygen occlusion of the wound, increased fibrinolysis and, last but not least, microbiological protection of the wound [3].

The aim of the prospective, randomized, intra-individual, clinical study described in the following was to compare the reepithelialization rate under the influence of a hydrocolloid dressing (Varihesive E) with that under a conventional dressing. Other clinical parameters compared included the painfulness of dressing changes, patient comfort, and wound infection.

Patients and Methods

Approval of the ethical commission of the Magdeburg Medical Academy was obtained before the start of the study. A total of 25 patients wishing tattoo removal participated in the study (10 male patients, 15 female patients, mean age 33.5 years).

After the patients had been checked against the criteria for inclusion or exclusion listed in Table 1, either two tattoos were removed in one session, or one large tattoo was removed in two sessions separated by least 4 weeks. The average tattoo size was $22.4 \pm 9.3\,cm^2$. Two in three tattoos were located on the upper arm or forearm. The majority of tattoos had been applied by nonprofessionals or by the wearers themselves.

After photographic documentation the sites were subjected to preoperative infiltration anesthesia using lidocaine. Dermabrasion was carried out by means of a high-speed skin abrasion instrument (Derma III, Schumann, Düsseldorf, FRG).

Table 1. Criteria for inclusion or exclusion

Inclusion criteria	Exclusion criteria
At least 18 years of age	Completely destroyed corium
Written consent	Antibiotic treatment
Two tattoos comparable in size and location	Clinical or drug-induced immunosuppression
Tattoo area at least 4 cm^2	Known hypersensitivity to skin adhesives
HIV test negative	Pregnancy

Following tattoo removal, the sites were dressed according to the randomization plan, either with the Varihesive E hydrocolloid dressing or with a conventional ointment dressing, Adaptic. In the case of suspected or actual wound infection, swabs for bacteria were taken and the species of pathogen ascertained.

On days 2, 4, 7–11 and 12–18 after the operation, the wound areas were evaluated, and reepithelialization was recorded and photographically documented. The final examination was made at the end of the observation period (day 21), even if reepithelialization of the wound areas was incomplete.

The Wilcoxon matched pair rank test was used for statistical evaluation of numerical data.

Results

All of the 25 patients participating in the study completed the observation period of 21 days. In nine patients, reepithelialization was incomplete by the end of the study: in five patients at the control sites, in two patients at the Varihesive E sites, and in two patients at both the sites.

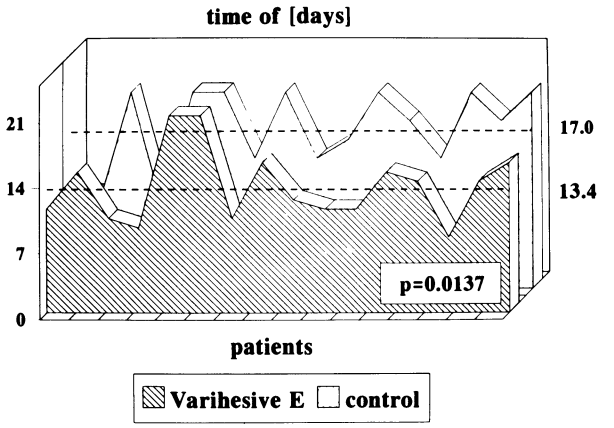

Fig. 1. Reepithelialization times under Varihesive E and control dressing

In 16 patients, the sites were completely reepithelialized by the end of the study. Figure 1 compares the reepithelialization periods. The Varihesive E sites required 13.4 ± 4.0 days for reepithelialization; the control sites took 17.0 ± 4.5 days. This difference of 3.6 days in reepithelialization time in favor of Varihesive E turned out to be statistically significant ($p = 0.0137$).

Another criterion for comparison was the painfulness of dressing changes. The patients rated the sensation of pain on a scale ranging from 0 to 4 ("no pain" to "intolerable pain"). Only one of the 25 patients felt pain during a dressing change at a Varihesive E site, while 15 patients described dressing changes at Adaptic sites as painful or very painful. The difference in the two dressings' pain score was statistically highly significant (Fig. 2).

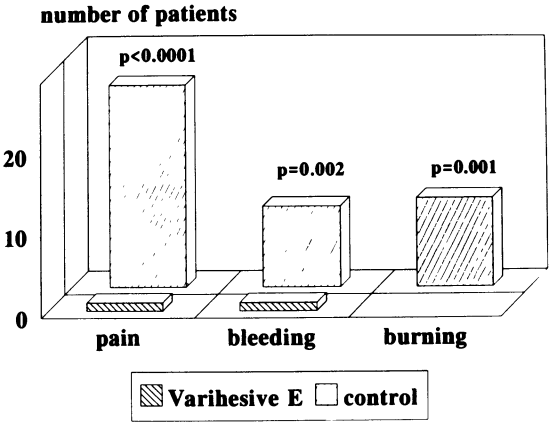

Fig. 2. Comparison of the frequency of pain, hemorrhages and burning under the two wound dressings

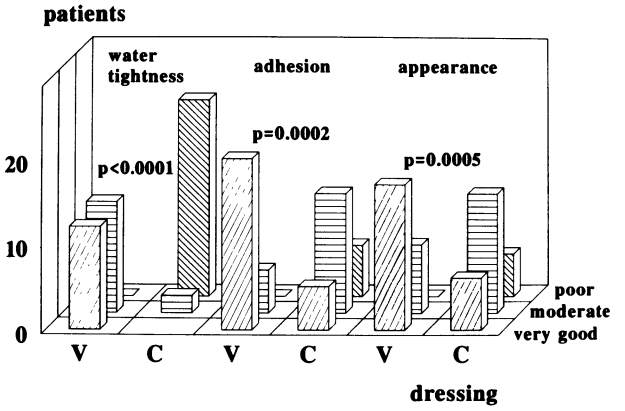

Fig. 3. Patients' ratings of material properties. *V*, Varihesive E; *C*, control (Adaptic)

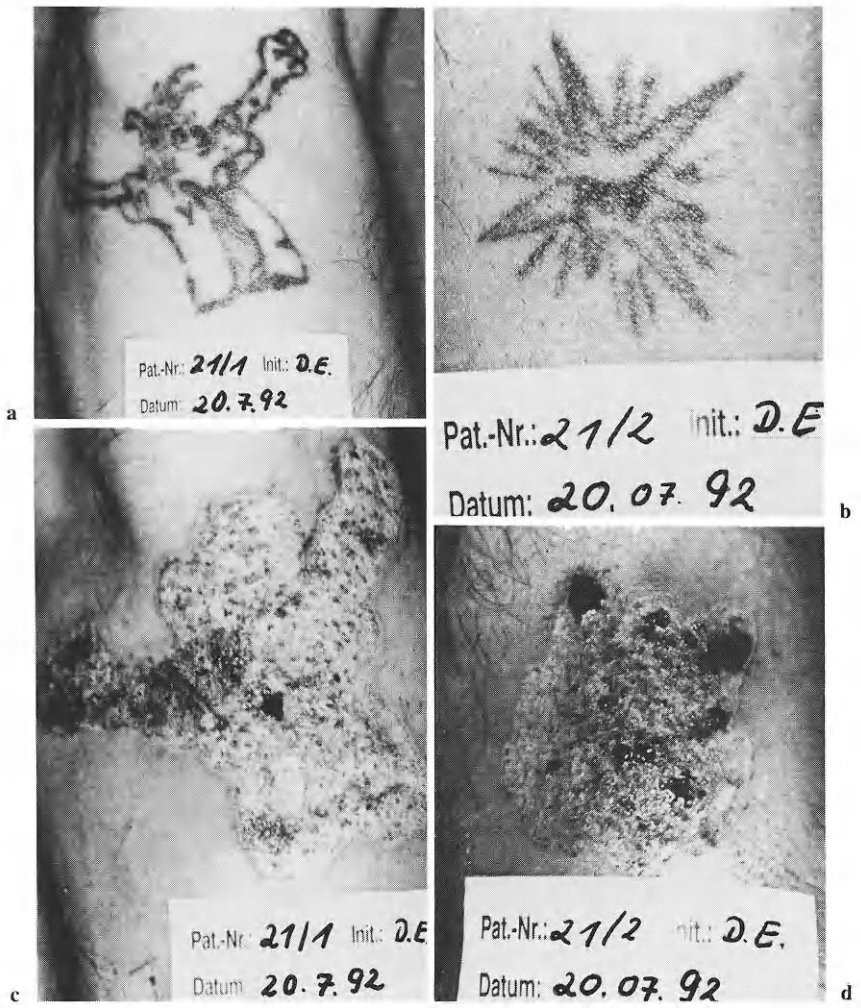

Fig. 4a–k. Clinical progress of wound healing in a 35 year old patient with tattoos on the right and left forearm: **a,b** preoperative findings; **c,d** immediately after dermabrasion; **c** covered with control dressing; **d** covered with Varihesive E; **e,f** findings 2 days after dermabrasion; **e** "dry" wound, punctiform hemorrhages; **f** wound completely covered by wound gel, reepithelialization setting in from the edges; **g,h** findings 9 days after dermabrasion; **g** larger islands of epithelialization and reepithelialization of the edges; **h** reepithelialization complete; **i,k** findings 3 months after dermabrasion; **i** slight cicatrial hypertrophy, **k** excellent cosmetic result

Besides pain, there were frequent hemorrhages from control sites at dressing changes, while no hermorrhages during dressing changes were observed at Varihesive E sites (Fig. 2).

In addition to the painfulness, the sensation of burning during dressing changes was recorded. No patient reported burning during dressing changes

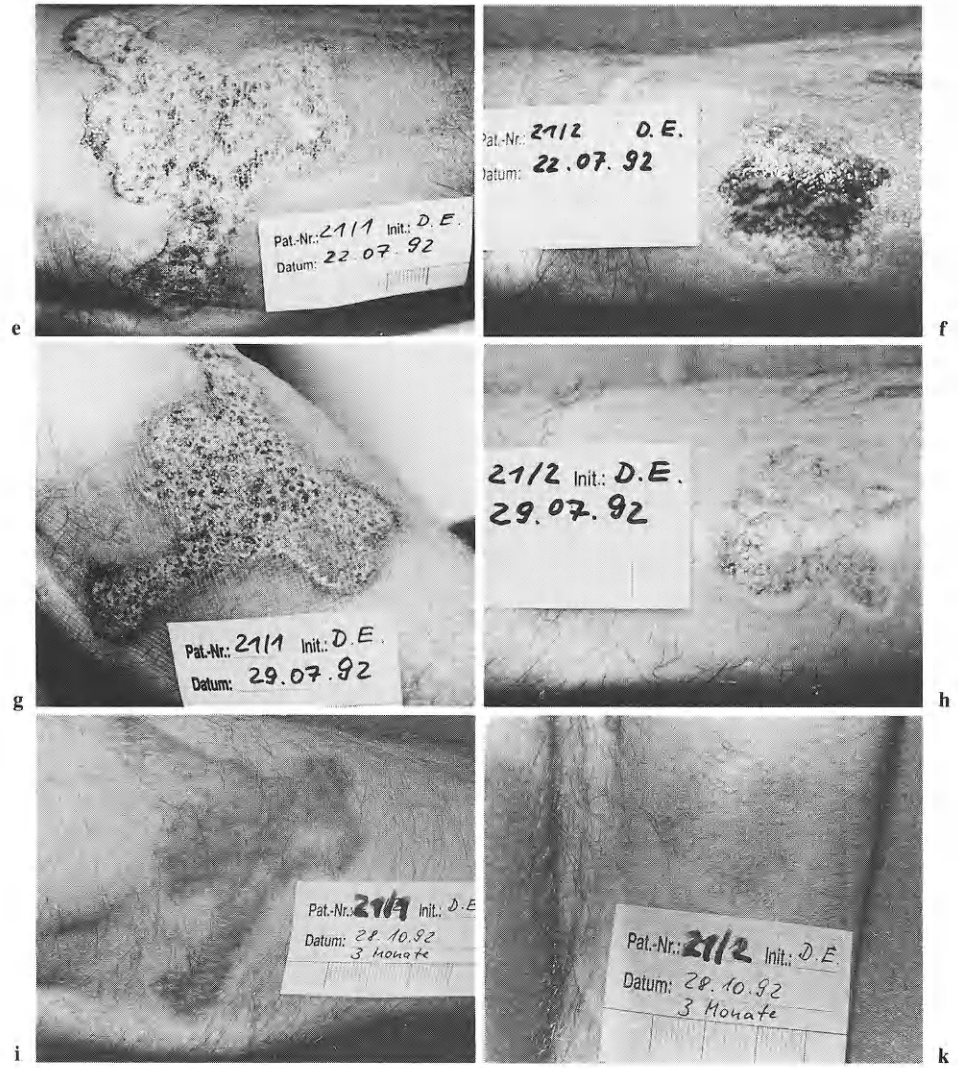

Fig. 4e–k

at Varihesive E sites, while 56% of the patients, in the control group complained of burning (Fig. 2).

There was one single case of wound infection (in the Adaptic group). The pathogen identified was *Staphylococcus aureus*.

The ratings of material properties such as the appearance, water resistance and adhesion of the dressings are shown in Fig. 3. With high statistical significance, the patients rated all the given parameters in favor of Varihesive E.

Figure 4 shows the clinical progress of wound healing in one patient. The difference between the dressings becomes apparent in Fig. 4e,f. The wound shown in Fig. 4e was covered by the control dressing; it appears dry and exhibits punctiform hemorrhages and a negative imprint of the ointment dressing. In contrast, the wound in Fig. 4f exhibits a gel produced by the wound exudate and the hydrocolloid and initial reepithelialization from the wound edges.

Discussion

Superficial wounds such as split-thickness skin graft donor sites or dermabrasion sites are still predominantly treated with special wound films or ointment dressings designed to keep the wounds moist and to prevent the dressings from sticking to the wounds. The wound exudate is mainly drained to the outside, thus making sure that oxygen has access to the wound [5, 21].

The mechanism of action of occlusive hydrocolloid dressings is based on the observation that skin wounds heal faster under intact blisters than under broken blisters. Winter was able to reproduce these findings in animal experiments in the early 1960s [25].

The Varihesive E hydrocolloid dressing meets all the requirements for modern wound dressings. Its inner layer consists of a hydrophobic polyisobutylene matrix combined with hydrophilic particles such as pectin, gelatin, and sodium carboxymethyl cellulose, while its outer layer is a polyurethane film impermeable to air.

The hydrocolloids have passive physical effects (initial absorption of wound exudate, hydration of the wound), and the dressing hermetically seals the wound, thus providing a barrier to exogenous contamination (water, microbes). Apart from these mechanisms, additional effects have been described [3, 5, 12].

The hydrocolloid particles and the wound exudate interact to form a gel covering the wound surface under the dressing. This gel provides a moist wound environment ideally suited for the migration of macrophages and epithelial cells and is actively involved in physiological wound debridment. Moreover, it allows painless dressing changes without damage to the wounds (Fig. 4f).

Many authors regard the oxygen occlusion of the wound and the corresponding decrease in oxygen partial pressure as the main factor driving neoangiogenesis [2, 3, 5, 12].

Macrophages can freely migrate in the moist wound environment under the dressing. The low oxygen partial pressure stimulates them to release a whole series of growth factors [17]. These growth factors cause the stimulation of additional macrophages, mesenchymal cells, and vascular endothelial cells, thus promoting granulation, neovascularization, and ultimately reepithelialization.

The results of this study confirmed the superiority of wet wound healing under occlusive hydrocolloid dressings in the case of superficial dermabrasion wounds. This superiority is primarily manifest in the significantly higher rate of reepithelialization under Varihesive E; there was a variance of almost 4 days between the hydrocolloid dressing and the ointment compress used as a control dressing. Biltz et al. reported a reepithelialization variance of 6 days in favor of Varihesive, when the hydrocolloid dressing or a gauze dressing soaked in saline was used on split-thickness skin graft donor sites [2].

In our study, reepithelialization times were slightly longer than in other studies, in which reepithelialization of split-thickness skin graft donor sites or dermabrasion wounds occurred in 8–15 days on average [2, 15]. The main reason for this may be the large number of amateur tattoos, in which pigments often had been deposited in the deep dermis and sometimes even in the subcutis. Complete removal of the tattoos thus required the dermabrasion of deep layers of the dermis [11].

The hydrocolloid dressing provides the additional advantage of painless and bloodless dressing changes, a benefit which has also been emphasized in other sudies [2]. While patients complained about intense or even intolerable pain during dressing changes at Adaptic sites, Varihesive E dressing changes were virtually painless due to the gel that formed under the dressing. A somewhat unpleasant smell that may result from the use of hydrocolloid dressings is the only factor patients have to get accustomed to. However, this is not a serious problem, provided the patients are thoroughly informed about the dressing and its mechanism of action.

The hydrocolloid dressing should be changed on the first or second day after dermabrasion. Although the hydrophilic particles in the dressing can absorb a considerable quantity of wound exudate, relatively frequent dressing changes are advisable in the highly exudative phase of wound healing. In the case of excessive exudation, wound exudate may leak from the dressing and irritate or macerate surrounding healthy skin. After 4–5 days, the interval between dressing changes may be extended to 5 days.

Wound desiccation and associated eschar formation on the wound were fairly frequent under the control dressing, in which the wound exudate is absorbed by a gauze compress placed over the wound gauze.

Eschar apparently hinders regular reepithelialization and is in some cases more likely to result in hypertrophic scars than wounds sealed with Varihesive E, under which eschar formation cannot occur (Fig. 4i,k) [4, 9]. Follow-ups on this aspect have not yet been completed, so no final statement can be made.

An additional advantage offered by hydrocolloid dressings is the occlusion of the wound from the external environment. This leads to the favorable low oxygen partial pressure mentioned above and prevents microbial wound contamination. None of the wounds treated with the hydrocolloid dressing became infected in our 25 patients. Some authors reporting on studies of the microbiology of venous leg ulcers point out that, although the ulcers were

contaminated with a multitude of bacterial species, healing was not impaired; moreover, even problem bacteria (*Pseudomonas aeruginosa*) were no longer detectable under the dressing after a few days [10, 12, 13].

The water resistance of the hydrocolloid dressing is an advantage to the patients which should not be underestimated. Concerning appearance and adhesion, the hydrocolloid dressing likewise received much better patient ratings than the control dressing. The difference in the ratings was statistically highly significant (Fig. 3). Varihesive E enjoyed great acceptance with physicians and patients due to its significantly higher rate of reepithelialization and its excellent material properties.

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A Prospective Randomized Study of the Efficacy of Hydrogel, Hydrocolloid, and Saline Moistened Dressings on the Management of Pressure Ulcers

G.D. Mulder[1], M. Altman[2], J.E. Seeley[1], and T. Tintle[3]

Introduction

In 1962, a new era began in wound healing when Winter described the effects of a moist environment in the wound healing process [1]. Until this point in time, it was standard practice to keep chronic wounds dry and let them "breathe."

Optimal epithelialization tends to occur in a moist wound environment. It has been shown that epithelialization occurs up to 50% faster in a moist environment [1, 2]. Wound fluid in a moist wound has been shown to contain multiple growth factors which stimulate angiogenesis and epithelialization [3]. Dry wounds deprived of fluids may heal more slowly with less effective repair of skin and blood vessels. A moist wound environment has been shown to cause reepithelialization in 12–15 days while an air exposed wound takes 25–30 days for reepithelialization to occur [3, 4].

Materials and Methods

Study Objective

The objective of this study was to evaluate the safety, efficacy, and functional attributes of Clearsite Hydrogel Dressing (NDM, Dayton, Ohio) and to compare these results with those obtained from standard treatment (wet-to-moist gauze dressing) and DuoDERM hydrocolloid dressing. Clearsite is a hydrogel dressing composed primarily of water and propylene glycol.

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DuoDERM CGF (Bristol Myers-Squibb, ConvaTec, Princeton, N.J.) is a hydrocolloid composed primarily of pectin, gelatin, and carboxymethyl cellulose. DuoDERM CGF is developed from DuoDERM which has been used extensively over the past decade for the treatment of skin ulcers.

Study Design

The study was a prospective, randomized, controlled, three arm, parallel evaluation with treatment groups randomized in a 1:1:1 ratio by a computer generated randomization scheme. A total of 67 patients were enrolled at three independent sites. Each patient was seen weekly for wound measurement, photography, and evaluation of the study ulcer. Saline moistened gauze was changed three times per day while the hydrocolloid and hydrogel were changed biweekly.

Table 1. Distribution of patient enrollment

Investigator site		Clearsite	DuoDERM	Standard	Total
1	Enrolled	8	7	7	22
	Safety	7	5	4	26
	Efficacy	7	7	7	21
2	Enrolled	7	7	6	20
	Safety	5	4	4	13
	Efficacy	5	5	5	15
3	Enrolled	8	9	8	25
	Safety	5	5	3	13
	Efficacy	8	9	7	24
Total	Enrolled	23	23	21	67
	Safety	17	14	11	42
	Efficacy	20	21	19	60

Table 2. Effectiveness of the treatment wound dressings

Measure	Mean
Epithelialization	0.83
Granulation	0.94
Erythema-periwound	1.27
Dressing easy to remove	0.96
Dressing painful to remove	0.44
Wound debrided by dressing	0.67
Tissue damaged by removal	0.50
Pain reduced on application	0.69

Efficacy of each wound dressing was assessed by eight different parameters (Table 1). Quantitative measures of the effectiveness of the treatment wound dressings are listed in Table 2.

Percent reduction of wound area and percent reduction per week were two of the methods used to analyze the data. Per week changes were obtained by dividing an outcome measured by the number of weeks of

Table 3. Wound location by treatment

Location frequency col pct	Treatment			
	Clearsite	DuoDERM	Standard	Total
Ankle	2 8.70	0 0.00	2 10.00	4
Back	1 4.35	1 4.76	1 5.00	3
Buttock	3 13.04	3 14.29	3 15.00	9
Chest	0 0.00	0 0.00	1 5.00	1
Foot	1 4.35	0 0.00	2 10.00	3
Heel	3 13.04	5 23.81	2 10.00	10
Hip	1 4.35	2 9.52	3 15.00	6
Ischium	1 4.35	1 4.76	0 0.00	2
Knee	1 4.35	0 0.00	0 0.00	1
Leg	0 4.35	3 0.00	1 0.00	4
Malleolus	3 13.04	2 9.52	1 5.00	6
Sacrum	3 13.04	0 0.00	3 15.00	6
Shin	1 4.35	0 0.00	0 0.00	1
Stomach	0 0.00	1 4.76	0 0.00	1
Thigh	2 8.70	1 4.76	0 0.00	3
Trochanter	1 4.35	2 9.52	1 5.00	4
Total	23	21	20	64

observation. This, therefore, assumes that healing or lack thereof is linear across the time frame of the study. There is some concern in the literature that percent reduction in wound area overestimates the degree of healing for small wounds [1]. Another limitation is that the measurement does not appear to be valid when the area has a size of zero (i.e., the wound has healed).

Results

Regarding safety, no significant adverse events related to study material were reported during the study. Minor adverse events with probable relationship to material included minor irritation in one patient on DuoDERM, minor sensitivity to DuoDERM in one patient, one case of inflammation with Clearsite, and one case of excoriation possibly related to Clearsite.

Locations of wounds are listed in Table 3. Outcome means and standard deviation by treatment groups with respect to percent reduction and percent reduction per week are listed in Table 4.

There were no statistically significant differences between treatment modalities. All dressings could be considered to perform equally well.

For all measures of healing there were no significant treatment differences. Table 4 summarizes percent reduction in area/ulcer.

Discussion

When no significant differences exist between products, consideration must then include cost of dressing, staff acceptance, patient's tolerance, and reimbursement. The greatest distinguishing features of the Clearsite hydrogel sheet dressing were its transparency, allowing visualization of the wound, nonadhesiveness of its wound contact surface, cooling effect on tissue, ease of application, and comformability to body/skin surfaces.

Table 4. Outcome means and standard deviation by treatment group

	Percent reduction		Percent reduction/week	
	Mean	Standard deviation	Mean	Standard deviation
Cleasite	28.8	52.8	8.0	14.8
DuoDERM	22.4	81.6	3.3	15.1
Standard	21.1	96.2	5.1	14.8

Conclusion

Hydrocolloid, hydrogel sheet and saline moistened gauze dressings were applied to 67 pressure ulcer patients in a randomized fashion. Safety and efficacy were the two major parameters evaluated. Significant differences were not found between the parameters analyzed. All dressings were found to be satisfactory in providing the necessary environment in tissue repair. The hydrogel sheet dressing was distinguished from the other treatments by its transparency, facilitated use, and acceptance by the investigators.

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Pericapillary Fibrin in Venous Ulcers Treated with Hydrocolloid Dressing

G. Mulder, R. Jones, L. Bolton, S. Cederholm-Williams, and M.P. Reich

Introduction

Venous stasis ulcers are chronic or recurrent cutaneous lesions, usually in the lower leg, occurring in 0.5%–1% of the world population [1, 2] and resulting from impaired venous return. As venous return is impeded, by either deep vein thrombosis, faulty venous valve function, or both, edema accumulates in the peripheral leg, reducing capillary flow and exchange.

Capillaries in and near venous stasis ulcers are often surrounded by fibrin cuffs [3–11]. These cuffs are associated with decreased tissue pO_2 and interfere with pericapillary nutrient and waste exchange, contributing to the pathophysiology of venous stasis ulcer tissue deterioration. Lipodermatosclerosis, impaired tissue fibrinolysis, or minor trauma often precipitate tissue damage, which is slow and difficult to repair.

Conservative treatment consists of a primary dressing over the wound and surrounding fragile skin plus 35 mmHg circumferential compression and elevation of the effected limb. It is effective in about 90% of venous stasis ulcer patients [12]. There is growing evidence that hydrocolloid primary dressings foster healing of venous stasis ulcers, but little is known about why this happens.

It has been reported that one hydrocolloid occlusive (i.e., moisture retentive) dressing lyses fibrin clinically [13] in vitro and in vivo [14], and it may be hypothesized that such a dressing reduces the pericapillary fibrin cuffs either by fibrinolysis or by reducing fibrin accumulation in chronic venous stasis ulcers.

We therefore conducted a randomized, prospective, blind, clinical trial comparing fibrin cuffs at the rims of venous stasis ulcers before and after treatment with either a standard occlusive/compressive therapy, Unna's boot, or the same therapy with a moisture-retentive hydrocolloid dressing facing the wound.

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Table 1. Sample characteristics of the two treatment groups[a]

Variable measured	Hydrocolloid dressing plus Unna's boot[b]	Unna's boot
Number of subjects	10 (1)	10
Number males	9 (1)	7
Number females	1	3
Number on left leg	7	6
Number on right leg	3 (1)	4
Number diabetic (controlled)	1	1
Average ulcer age ± s.e.m. (weeks)	113 ± 50	172 ± 57
Number in good health	7	8
Number in poor health	3 (1)	2
Number ambulatory	8 (1)	8
Number with full mobility	7 (1)	7
Number with edema	4 (1)	8
Median posterior tibial Doppler index	1/4	1/4
Median dorsal pedis Doppler index	1/4	1/4

[a] There were no significant differences between groups on any of these parameters.
[b] Dropped patient in parentheses.

Methods

Subjects

Subjects were 20 outpatients with venous stasis ulcers as confirmed by Doppler ultrasound assessment in the popliteal vein. All patients had serum albumin values ≥ 8 mg/dl. Sample characteristics are listed in Table 1. All ulcers occurred on the anterior, medial or lateral leg, malleolus or ankle. The skin around all ulcers was abnormal, with cracking, tenderness, itching, edema or irritation.

Procedure

After obtaining informed consent, initial evaluation, and Doppler measurement (Doppler index), a 6 mm biopsy was taken from the peripheral ulcer rim under local anesthesia. Hemostasis was achieved; then the appropriate dressing was applied according to a randomization schedule. Dressings were either: (1) hydrocolloid dressing (DuoDERM Hydroactive Hydrocolloid Dressing, ConvaTec, Division E.R. Squibb, Princeton, New Jersey) applied to the ulcer surface and covered by an Unna's boot zinc paste bandage (Dome-Paste Bandage, Dome Division, Miles Laboratories, West Haven, Connecticut) and an outside compression bandage applied by a professional

(Coban 3M, Minneapolis, Minnesota) or (2) the Unna's boot plus compression bandage without the hydrocolloid dressing. The dressings remained in place for 1 week (7 ± 2 days), after which the dressing was removed in the clinician's office and a second 6 mm biopsy was taken from the peripheral wound rim approximately 2 cm from the first biopsy site. All patients returned for a follow-up visit at least 1 week later to assure proper healing of the biopsy site.

All wounds were photographed with a centimeter ruler on the adjacent shin before dressing application and after dressing removal for subsequent area determinations using an Optomax System IV image analyzer (Optomax, Inc., Hollis, New Hampshire).

Each biopsy was immediately frozen in liquid nitrogen and maintained at $\leq -40°C$ until staining for 30 min at 37°C with a monoclonal mouse antibody to human fibrin (provided by Dr. Cederholm-Williams) conjugated to fluorescein for blind assessment of fibrin pericapillary cuffs under a fluorescent microscope.

Biopsies were also stained with hematoxylin and eosin for blind evaluation of histopathology, and the frequency of capillaries, to establish that any differences in fibrin cuffs were not due to differences in the numbers of local vessels. Also, these slides were evaluated blind to treatment for inflammatory response on a 0 (none) to 10 (extremely intense) scale, with qualitative assessment of whether the predominant inflammatory cell was the polymorphonuclear neutrophil or the monocyte/macrophage. Additional control stains without the antibody and for keratin were performed to make sure that fluorescence was not due to an artifact of the staining technique or to the presence of keratin in adjacent hair follicles.

Table 2. Rating scale for pericapillary fibrin cuffs

Original rating	Subjective rating	Cuff thickness (corresponding to subjective rating)
0	"None"	No cuffs visible
1	"Some"	Thin cuffs (<5 μm thick) visible on scattered vessels
2	"Medium"	Moderate cuffs (5–10 μm thick) on scattered vessels
3	"Pronounced"	Moderate cuffs on frequent vessels
4	"Dramatic"	Thick cuffs (>10 μm thick) on frequent vessels

Table 3. Frequency of capillaries in the biopsies

Rating	Subjective frequency of capillaries
0	No capillaries visible
1	Few capillaries visible
2	Some capillaries visible
3	Moderate number of capillaries visible

Pericapillary fibrin cuffs and the frequency of capillaries were rated according to the two ordinal scales in Tables 2 and 3, respectively. Separate ratings were obtained for fibrin cuffs around capillaries in surface tissue (top $\sim 100\,\mu m$) and deeper biopsy tissue ($\geq \sim 200\,\mu m$).

Statistical Analysis

Ratings of pericapillary fibrin by the four observers were correlated, then averaged for each biopsy with separate averages being calculated for surface or deep tissue within each biopsy. For a given treatment, the difference between pre- and posttreatment pericapillary fibrin in the two biopsies for a given patient was calculated as pretreatment average rating minus posttreatment average rating for surface or deep tissue. A reduction in fibrin, shown by a positive value, was termed improvement. No change or increase in fibrin cuffs was termed no improvement. By using this procedure, a single value of pericapillary fibrin reduction at each tissue depth was calculated for each patient, yielding a total of 19 observations analyzed at each depth, or one value for each patient at each depth. This averaging procedure avoided the statistical bias toward significant differences which results when large numbers of correlated observations are treated as independent data. The resulting difference scores were analyzed separately for deep and shallow tissue using the chi square test for nonparametric data, with Fisher's exact test adaptation for small numbers of subjects.

Data on the numbers of blood vessels in the biopsies were treated in the same way. Averages and standard errors of the mean were calculated for pericapillary fibrin reduction and for blood vessel frequency for descriptive purposes.

Results

Pericapillary fibrin was reduced from an overall average rating of 2.5 before either treatment to an overall average of 1.7 after 1 week of hydrocolloid treatment as compared to relatively stable fibrin ratings (1.5) before and after treatment with Unna's boot. There were no significant differences in fibrin cuff intensity before treatment. These results are summarized for deep and shallow tissue in Table 4.

Because the data are nonparametric, a Fisher's exact chi squared test was performed to test whether the percent of ulcers improving in fibrin scores was different for the two groups, with the result shown in Fig. 1. Independently for both deep and shallow tissue, 88% of the ulcers treated with the hydrocolloid dressing improved showing reduced pericapillary fibrin after the week of treatment, while only 40% of those treated with Unna's

Table 4. Fisher's Chi-square test for percents of ulcers exhibiting decreased pericapillary fibrin during 1 week of treatment with a hydrocolloid dressing (DuoDERM Hydroactive Dressing) under Unna's Boot versus Unna's Boot alone (Dome-Paste Bandage)

	DuoDERM		Unna's Boot	
	(*n*)	(%)	(*n*)	(%)
Improvement (reduced fibrin)	8	89	4	40
Stable or deterioration (same or increased fibrin)	1	11	6	60
Average ratings of shallow fibrin cuffs				
Before treatment	2.7		1.6	
After treatment	2.0		1.5	
Percent change	−28		−6	
Average rating of deep fibrin cuffs				
Before treatment	2.3		1.5	
After treatment	1.5		1.7	
Percent change	−36		+13	

Chi squared = 0.039. Results were identical for shallow and deep fibrin.

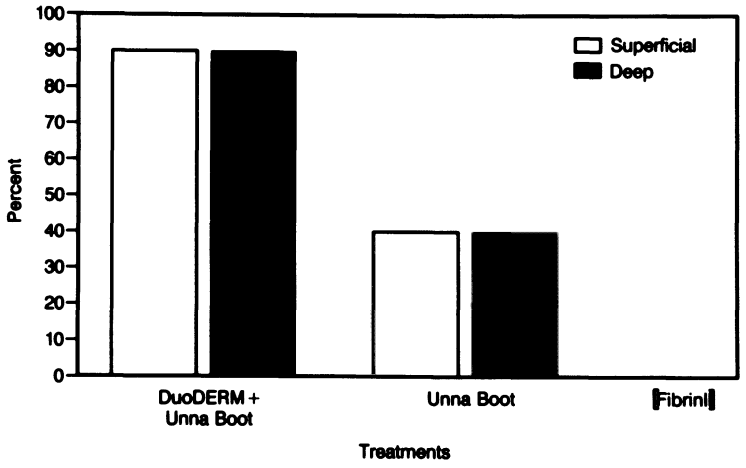

Fig. 1. Percent of ulcers improving in fibrin cuff rating during 1 week of treatment with Unna's boot with or without a hydrocolloid dressing facing the wound

boot improved during the same time. The difference was significant at $\alpha = 0.039$ for both levels of tissue (Table 4).

Figure 2 shows the progressive improvement for a typical ulcer treated with the hydrocolloid dressing, with fibrin ratings of 4 pretreatment (Fig. 2a) and 3 posttreatment (Fig. 2b) vs slight deterioration in an ulcer treated with the Unna's boot progressing from a fibrin rating of 2 (Fig. 2c) to 3 (Fig. 2d).

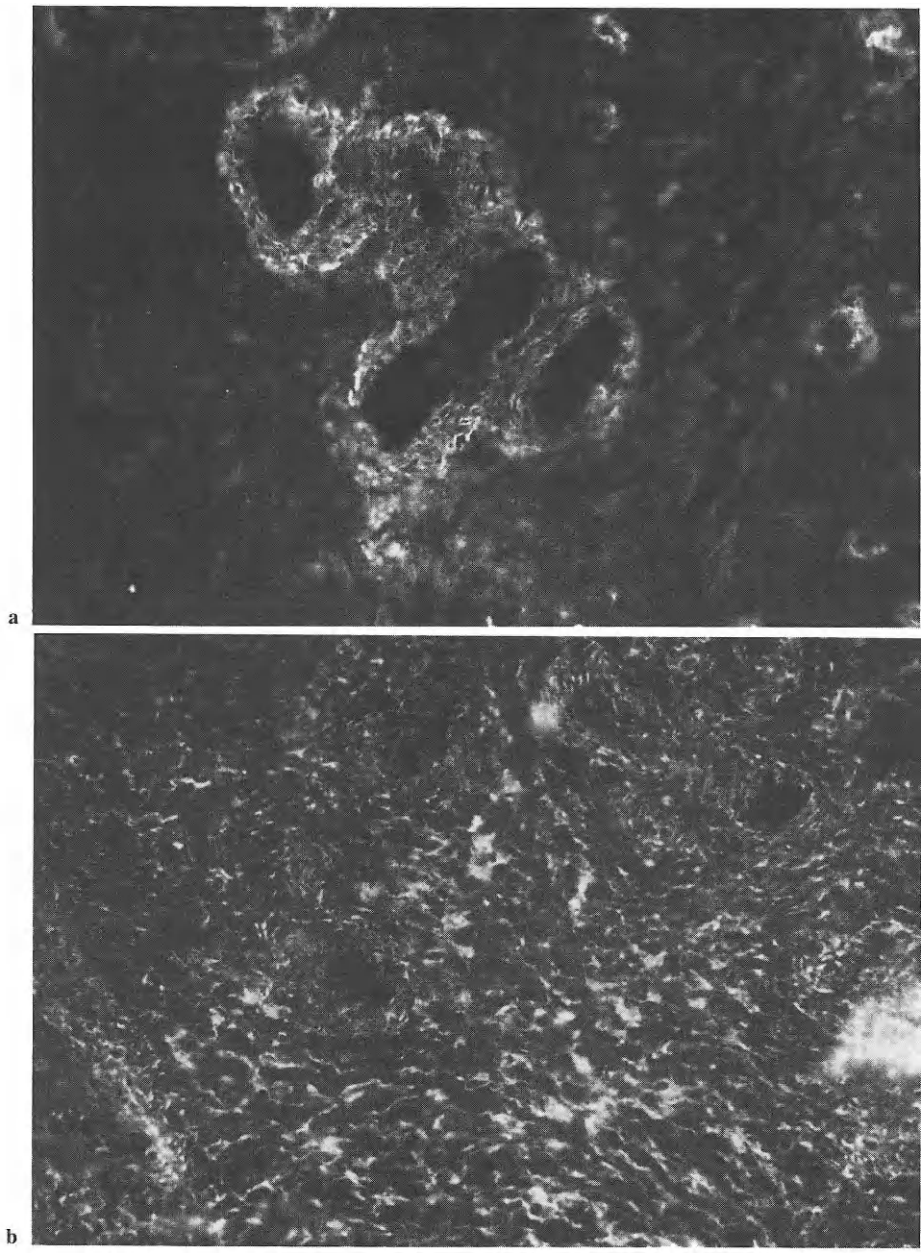

Fig. 2a,b. Fibrin deposits (cuffs) surrounding capillaries in chronic venous ulcers treated for 1 week with Unna's Boot plus a hydrocolloid dressing. **a** Grade I fibrin deposit after treatment with Unna's Boot plus hydrocolloid dressing. **b** Grade IV fibrin deposit after treatment with Unna's Boot alone. All photomicrographs were taken with a 20× objective, at a total magnification of ×220

There were no significant differences in histopathology or inflammation between the two treatment groups either before or after the 1 week treatment period.

Significantly more reduction in pericapillary fibrin cuffs was observed in the group of patients treated for 1 week with the hydrocolloid dressing plus Unna's boot than in the group treated with Unna's boot alone (<0.04 biopsy surface; <0.04 deep tissue). These results are presented in Fig. 1.

All tissue biopsies contained moderate numbers of capillaries, with no significant differences between the numbers of capillaries in the two treatment groups before or after either treatment (Fig. 2).

There were no differences between the two treatment groups on the nature or intensity of the inflammatory response.

No significant difference between treatment groups was observed in wound contraction during this 1 week treatment period.

Discussion

This is the first demonstration that a topical dressing can modify the pathology associated with venous stasis ulcers. The effect may have been mediated by either reduced fibrin accumulation or by fibrinolysis.

Treatment with a hydrocolloid dressing known to lyse fibrin reduced pericapillary fibrin cuffs in 1 week. This effect surpasses that of compression and/or occlusion and is independent of blood vessel numbers during this time period.

The clinical implications of this finding are that conservative treatment with a topical hydrocolloid dressing can reduce pericapillary fibrin deposits beyond the effects seen in response to compression alone. Though this effect surpasses the combined effects of occlusion and compression, these may be necessary accompaniments to optimize the hydrocolloid dressing's effects. The advantages of occlusive dressings for wound healing in general [15–17] and compression bandages [18, 19] for venous stasis ulcers in particular are well documented.

Not all hydrocolloid dressings are fibrinolytic [14]. Therefore, caution should be exercised in using or replicating this clinical finding to select dressings which have scientifically tested fibrinolytic capacity. Clinically, reduction of pericapillary fibrin may signal improved nutrient and waste exchange for tissue surrounding the ulcer [3] and increased chemotaxis to macrophages [20] which regulate wound healing. Consistent, careful techniques generating these local biological effects may yield an 80%–90% clinical success rate for healing venous stasis ulcers with conservative treatment.

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