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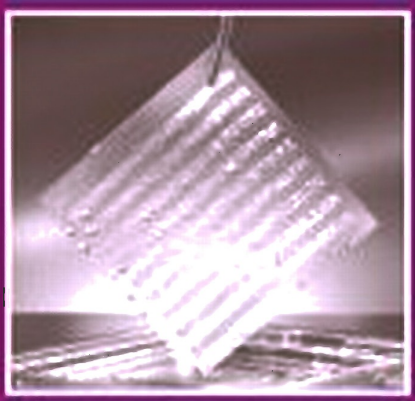

Biomaterials for treating skin loss

Edited by Denis Orgill and Carlos Blanco

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Biomaterials for treating skin loss

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Dennis Orgill
and Carlos Blanco

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Part I

Introduction

1

Introduction: development of skin substitutes

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Abstract: The treatment of large body surface area burns has been the primary motivating factor for the development of skin substitutes. Biological solutions to reconstruction of both the epidermis and dermis have been designed. The need for biomaterials to treat skin loss in reconstructive surgery and chronic wounds has also been developed.

Key words: skin substitutes, biomaterials, skin loss.

1.1 Historical development

Wounds, defined as a disruption in the integument, have long plagued mankind because, if left untreated, they can result in sepsis and death. During the American Civil War, amputation allowed for prevention of death by halting localized infection from spreading. Aseptic techniques and antibiotics were major advances in the 20th century that contributed to increased survival following large wounds and thermal injury. Advances in burn care in the 1960s and 1970s, including early excision and grafting, fluid resuscitation, mechanical ventilation, topical antimicrobials and skin grafting techniques allowed burn victims with large total body surface area burns to survive. The development of the powered dermatome provided uniform thickness sheet skin grafts that were efficient for closing large wounds, but created partial thickness wounds that also needed to heal. As technology improved and larger burns were treated, the available donor sites were reduced. Innovations including widely meshed skin grafts and micro-grafting helped close the wound but had a poor aesthetic outcome. The inability to achieve autologous skin coverage of large burns effectively was a major incentive for the National Institutes of Health to invest in skin substitute development in the 1970s and 1980s. Investment in these projects produced a better understanding of the requirements of skin substitutes and also led to the commercialization of several technologies that are currently used today to treat burn patients and have been extended, in some cases, to their use in reconstructive surgery and the treatment of wounds (Table 1.1).[1,2]

Table 1.1 Many of the early concepts of current skin substitutes were developed at MIT during the late 1970s and 1980s

Investigator	Rheinwald, Green[4–6]	Bell[7,8]	Yannas, Burke[9,10]
Concept	Cultured epithelium	Bilayer cultured graft	Biodegradable template
Clinical application	Large burns	Chronic wounds	Burns and reconstruction
Product	Epicell	Apligraf	Integra Regeneration Template
Company	Genzyme Biosurgery	Organogenesis	Integra LifeSciences

Despite great advances in burn care, the resulting severe scarring and deformity continues to be one of the greatest challenges facing burn victims and their families. Research in recent years has focused on methods to improve regeneration while limiting scarring. The following monograph will review many of the current technologies available to clinicians, highlight some treatments that are in early development phases and point to areas of potential improvement for the future.

A thorough understanding of the biology of skin and its response to injury is essential for designing skin substitutes (Table 1.2). For centuries man has understood the important functions of skin in providing a barrier to bacteria and moisture loss as well as a strong and elastic integument that drapes over complex surfaces. Historically, physicians have turned first to biological membranes that have these basic properties including cadaver skin, pigskin and amniotic membrane. Xenografts, such as pigskin, showed good temporary coverage but the very high antigenicity resulted in predictable failure over the long term. Human cadaver skin works well as a temporary skin substitute but tends to reject between days 10 and

Table 1.2 Some ideal characteristics of skin substitutes

- Bacterial barrier
- Mechanical strength
- Drapeability
- Elasticity
- Semi-permeable to oxygen and water
- Non-toxic
- Non-inflammatory
- Non-immunogenic
- Long-term function
- Heal in response to injury
- Pigment
- Adnexal glands
- Specialized epidermal structure (e.g. glabrous skin)
- Available off the shelf
- Low cost

14 after application. In addition, the supply can be erratic and there is also a possibility of bacterial or other disease transmission. Clearly better techniques were needed to treat very large wounds more optimally.

1.2 Skin regeneration

An injured epidermis heals by spontaneous regeneration, leading to formation of a new, intact epidermis. In contrast, the injured adult dermis generally does not regenerate spontaneously and heals instead by wound contraction and scarring. A superficial injury in the dermis may show restoration as described in an experimental incisional scar model in humans showing that incisions made at a depth of 0.53 mm or less (approximately the top one-third of the dermis) showed no long-term visible scar.[3] In contrast, deep partial thickness burns, full-thickness burns and full-thickness traumatic wounds heal exclusively with scarring and wound contraction.

In the late 1970s and 1980s three groups at the Massachusetts Institute of Technology (MIT) worked independently on three different methods to treat the skin substitute problem. The results of their research provide the foundation for most skin substitute research done today. Howard Green, working with James Rheinwald pioneered cell culture techniques including culturing of keratinocytes.[4] Prior to their innovations, culturing keratinocytes was difficult. Their contributions included specific culture media and the addition of irradiated fibroblasts as a feeder layer for keratinocytes. From a small biopsy of normal skin, taken shortly after the burn injury, they were able to grow rapidly large quantities of keratinocyte sheets referred to as cultured epithelial autografts (CEAs) which could be grafted onto the burn wound within three weeks. This technique became famous when Gallico and O'Connor applied it to two severely burned children at The Shriners Burns Institute in Boston who were able to survive a massive burn injury.[5] At the time, there was a debate about whether or not dermis was a necessary component for the long term success of the technique. Despite the remarkable achievements of Gallico and O'Connor, others found that using CEAs alone resulted in a very fragile skin. Cuono later showed that applying allograft sheets first, and then removing just the epidermis prior to the application of CEAs, resulted in more stable coverage.[6] This technology formed the basis for the company Advanced Tissue Sciences that was later sold to Genzyme® Tissue Repair (Cambridge, Massachusetts). CEAs are still an important adjunct in treating very large burns. Because the number of large burns is decreasing in the USA, the market size for this technique has not grown significantly in the last several years.

Eugene Bell developed a fibroblast seeded collagen lattice and then covered this with keratinocytes.[7] The collagen lattice contracted significantly *in vitro* after being seeded with keratinocytes. These lattices could then be covered with a keratinocyte layer to perform a 'skin equivalent'. This technique never really caught on in the burn community and now is most often used with allogenic cells

derived from neonatal foreskins. Subsequently, Organogenesis Corporation (Canton, Massachusetts) made these sheets for a successful clinical trail in diabetic foot infections.[8] Today, the resulting product, 'Apligraf', is used mostly to treat chronic wounds. Early thoughts were that some of this material actually 'took' into the wounds. Most clinicians today believe Apligraf works as a very advanced dressing, providing both a barrier and a rich source of growth factors to the wound.

Ioannis V. Yannas and John F. Burke worked together to develop a dermal template composed of bovine collagen and chondroiten-6-sulfate derived from shark cartilage. They believed that dermis was the most difficult part of skin to regenerate and if they could solve the problems of scarring and contraction that the epidermal problem would then be less of an issue over the long term. Using a guinea pig wound contraction model, they defined optimal characteristics of the matrix including average pore size, cross-link density and percentage of glycosaminoglycan. This matrix was covered by a silicone elastomer which mimicked the natural permeability of skin. One of the reasons for the success of this technique was the way burns were treated at the Massachusetts General Hospital and Shriners Burns Institute in Boston. Burke was an advocate of early excision and grafting. As such, after excision of the burn, there was a clean sterile bed to apply the matrix. The matrix was left in place for two to four weeks when at a time of election, the patient was returned to the operating room to have the silicone removed and replaced by a very thin epidermal autograft. The advantage of this technique is that the epidermal autograft left very little scarring at the donor site and could be re-harvested. Also, the patient was effectively physiologically closed after the first procedure, could recover with the matrix in place and then, when stable, have the thin epidermal autograft. The surprising outcome in humans was the long-term results of a supple mobile skin, which patients preferred when compared to the standard meshed autografting techniques.[9,10] This matrix was first commercially made by Marion Laboratories (Kansas City, Missouri) and then transferred to Integra LifeSciences Corporation (Plainsboro, New Jersey). Its indications have subsequently been extended to reconstructive surgery.

1.3 Intellectual property and financial issues

Burn patients provide a relatively small market for skin substitutes. Without intellectual property (IP) protection, it is unlikely that corporations would take on the high costs of developing these products without the anticipation of several years of sales without significant competition. Each of these inventions obtained patents that were crucial in the development of the products. Even with patent protection, the small market size for burns combined with limits on charges for products has made it difficult for corporations to reap significant profits from skin substitutes. As a result, companies have looked at other markets to expand the potential use of the products, for example, acute wounds following trauma or surgical excision and chronic wounds. The cost of current skin substitutes range

from US$0.75–22.00/cm^2 with advanced products used in deep dermal and full-thickness burns costing in the US$5.00–12.00/cm^2 range.[11] The challenge comes obtaining both clinical and cost-effectiveness data to justify their use in a particular health care system. As we currently lack good enough metrics to quantify the value of reduction of scar and wound contraction, it is often difficult to make clear economic arguments about the cost-effectiveness of these products.

1.4 Changing medical practice

The introduction of skin substitutes has required education of physicians providing burn care about the proper use of these products. As most physicians are conservative when applying new techniques, it takes many years for new products to be fully utilized in the surgical community. During this learning process, manufacturers of these products will see a gradual increase in the use of the product. Since the original conception of different ideas for skin substitutes, much has changed in the treatment of burns. Fluid resuscitation, nutritional care, improvements in ventilation and topical antimicrobials have all improved. Many surgeons have begun to use the concept of dermal preservation as a method of maximizing the function of skin. As a result, more mid to deep dermal burns are now treated expectantly and allowed to heal in. This has reduced the demand for excision and grafting for many of our large burn patients. We now have more information on the survivability of large burns with a better understanding by the public of what is involved in burn care for the severely burned victim. There is greater acceptance among both physicians and families not to treat aggressively certain large total body surface area burns which have a high likelihood of mortality. Along with the decreasing numbers of large burns, the combination of the aforementioned factors have resulted in a reduction in demand for skin substitutes for burn victims in the USA.

In the following chapters, we carefully study the science of skin regeneration, examine specific products in detail and also look forward to new ideas and challenges that will allow scientists to develop the next generation of skin replacement technologies.

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2

Skin replacement products and markets

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Abstract: The skin replacement marketplace is becoming an increasingly complex environment as both the number of problems and number of solutions are growing. The future needs for skin replacement, specifically, and wound care, generally, are likely to be significantly greater than they are today owing to demographic and epidemiological trends. The development of technologically advanced skin substitutes and wound management systems has been driven, in large part, by industry. The commercial success of various products has been modest compared to the market size. Future research and development may be impeded by the length of the FDA-approval process which increases the cost of the various products and limits investor enthusiasm.

Key words: cultured epidermal autograph, dermal matrix, diabetic ulcers, fibroblasts, keratinocytes, skin replacements, wound care.

2.1 Introduction

To understand this arena better, we need first to understand the problems that require skin replacement and then to understand the available solutions. Lastly, we will explore where these solutions have fallen short, what opportunities may still exist, and which are the most promising future directions for development.

Skin has great importance to body form and function. It is a complex organ with a myriad functions, many of which are necessary for the survival of the patient. When disease or injury results in loss of skin, replacement of the lost surface area is of critical importance. Wound closure is not only medically important, but significantly reduces the cost of caring for the patient. Through the development of various skin replacement and wound care technologies, the marketplace has spawned companies and products that meet many of the requirements of native skin. As the demographics indicate, the future needs for skin replacement specifically and wound care generally, are likely to be significantly greater than they are today. Therefore, the skin replacement marketplace may well be an attractive one for investment, especially as new technology expands our ability to treat a wide variety of problems effectively.

Loss of skin results from multiple causes but there are several with major implications for patients, physicians, the healthcare system and the marketplace. These several causes have significance because they affect large and growing

portions of the population or they represent massive resource utilization in the care of smaller numbers of seriously ill or injured patients. The most common disease processes that require skin replacement include venous stasis ulcers, diabetic ulcers, pressure ulcers, burns and trauma. The economics of treating these different etiologies of skin loss vary widely owing to the specifics of the patient population being treated. However, while a small diabetic ulcer may seem insignificant compared to a 90% total body surface area burn in terms of cost-to-treat, the burgeoning epidemic of diabetes, obesity and advanced age in the United States is making treatment of these wounds a major cost center for the payors and a major revenue center for companies with skin replacement technologies.

Treatment of wounds in the United States costs the health care system approximately US$20 billion annually. The three largest causes of complex wounds are venous, diabetic and pressure ulcers. These problems alone affect up to three million people and cost the US health care system US$10 billion annually. Total yearly costs for wound care products for these indications approaches US$2.5 billion. This figure has increased by approximately 20% per annum since 1998.

The characteristics of an ideal skin substitute or replacement have been known for more than two decades.[1,2] Not surprisingly, replacement skin, like a replacement kidney or lungs, should perform the functions of native skin as closely as possible. In addition, there are cost and useability issues that are factored into the equation. The ideal skin substitute is:

- inexpensive
- long lasting
- a bacterial barrier
- semi-permeable to water
- elastic
- easy to apply
- painless to the patient
- non-antigenic and non-toxic
- cosmetically acceptable
- durable.

2.2 Indications for skin replacement

The advanced wound care market is enormous and growing. The major etiologies of complex wounds in the United States include venous, diabetic and pressure ulcers. Other high-cost wounds include burns and infections involving the skin and soft tissues. Wound care has become a multimodality specialty with the utilization, in some circumstances, of negative pressure therapy, bioengineered skin substitutes and growth factors, ultrasound or combinations of these. In this section, the major indications for the use of skin substitutes and wound care products will be covered. Special attention will be paid to the epidemiology and cost of treating these conditions.

Around the world, the population is aging. This is secondary to decreasing fertility rates and increasing life expectancy. The number of people over the age of 65 years in developed countries will increase by nearly 440 million to 690 million between 2000 and 2030. In the United States, over the same period of time, the number of people over 65 years of age will double from 35 million to 71 million.[3] The aging of the population will bring a commensurate rise in complex wounds and the need for skin replacement as the incidences of nearly all of the causes of complex wounds increase with age.

Diabetes is a major causative factor in the development of complex wounds. According to Centers of Disease Control 2005 estimates, there are over 20 million people in the United States with diabetes mellitus. This represents approximately 7% of the population. Over 1.5 million people were diagnosed in 2005 with diabetes. As a result of the neuropathic and vascular morbidity attributable to diabetes, over 80 000 diabetics will undergo lower extremity amputation.[4] Worldwide, over 350 million people will suffer from diabetes by 2030.[5]

Diabetic foot ulcers are caused by the complex, degenerative effect of chronically elevated blood glucose concentration on nerves and small blood vessels. The combination of decreased sensation and impaired perfusion make the development of non-healing wounds of the feet more likely. Between 4.4 and 10% of diabetic patients will suffer from a non-healing wound or ulcer at any given time, giving an upper estimate of the prevalence of approximately 2 million patients.[6] Over the course of a diabetic's lifetime, there is a 25% chance of developing a foot ulcer.[7] Approximately half of these patients will require advanced wound care. The association between diabetes and lower extremity amputation is clear and significant. Lower extremity amputations are 15 times more likely in diabetics than in the general population. Diabetic foot ulcers can be thought of as sentinel events predicting future amputation as they are present in 84% of non-traumatic amputations in this patient population.[8] The financial costs are significant as well. Care for patients with a diabetic foot ulcer costs approximately US$14 000 per year and that for patients who require an amputation are between US$20 000 and 60 000.[8,9]

Venous stasis ulcers occur when chronically impaired venous return, often from the lower extremities, results in skin breakdown. Often co-existing conditions such as obesity, diabetes, inactivity, cardiovascular or renal disease cause or exacerbate the development of venous ulcers. It is estimated that there are nearly one million patients in the United States alone who suffer from this condition and that approximately 30% will require advanced wound care.

Development and treatment of pressure ulcers are major health care issues in the United States. Pressure ulcers develop when soft tissue is compressed between a hard surface (mattress, seat, wheelchair, etc) and a bony prominence for a critical amount of time. After a period of time, the tissue becomes ischemic and ultimately necrotic. Critically ill, chronically debilitated, paralyzed and malnourished patients are at especially high risk of developing pressure ulcers. Millions of patients are at risk. Recent Medicare legislation has made prevention of pressure ulcers a

Table 2.1 Patients needing advanced wound care

Wounds requiring advanced care	Number of patients affected
Pressure ulcer	200 000
Venous ulcer	300 000
Diabetic foot ulcer	400 000–1 000 000
Burns	40 000
Amputations	100 000–200 000
Trauma	500 000

priority by refusing payment for ulcers that develop during in-patient hospitalization. Data on prevalence and incidence of pressure ulcers vary widely, depending on the population studied. It is estimated that pressure ulcers will affect between 5 and 9% of hospitalized patients and over 14% of the nursing home or acute care facility population. Stage III and IV ulcers (those that require advanced wound care) represent 20% of all pressure ulcers.

Certain acute wounds such as amputations, burns and those related to trauma, infection or surgical wounds require advanced wound care. Over 500 000 patients require treatment for burns each year in the United States. Over these, 40 000 will be hospitalized and 4000 will succumb to burn injuries.[10] A search of the National Center for Health Statistics database revealed over 3 million traumatic wound admissions each year. It is estimated that 10% of these will require advanced wound care representing 300 000 wounds. Finally, of the roughly 45 million surgical procedures performed each year, approximately 5 million are open procedures and, of these, approximately 5–10% may need advanced wound care. This would add another 500 000 wounds to the total. When taken together, the combination of diabetic, venous and pressure ulcers, combined with amputations and traumatic, surgical and burn wounds, it is estimated that there are over 2 million wounds in the United States alone that will require some type or types of advanced wound care (Table 2.1).

Over time, some of these numbers will change. The projected increases in the number of obese, diabetic and elderly patients in the United States will dramatically increase the number of venous, diabetic and pressure ulcers. Better supplemental restraint systems in automobiles, improved fire and industrial safety practices and more types of minimally invasive surgery will decrease the numbers of traumatic, burn and surgical wounds.

2.3 The products

Skin substitutes are engineered dressings designed to facilitate wound closure by performing as many of the key roles of normal skin as possible. They lack dermal appendages, an intact microvascular network, immune cells or melanocytes. In broad terms, the skin substitutes currently available can be grouped into those

Table 2.2 Bioengineered skin substitute products

Skin substitute	Manufacturer	Origin	Structure
Apligraf	Organogenesis	Human fibroblasts, keratinocytes	bilayer
OrCel	OrCel International	Human fibroblasts, keratinocytes	bilayer
Dermagraft	Advanced Tissue Sciences	Human fibroblasts	dermal
TransCyte	Advanced Tissue Sciences	Human fibroblasts, silicone sheet	dermal
Integra	Integra LifeSciences	Bovine collagen, chondroitin	dermal
Alloderm	Lifecell	Human dermal matrix	dermal
Biobrane	UDL Laboratories	Porcine collagen, nylon	dermal
Oasis	Healthpoint	Porcine small intestinal submucosa	dermal
Epicell	Genzyme Biosurgery	Autologous keratinocytes	epidermal

designed to replace the dermal component, the epidermal component or both, and those with a cellular or acellular dermal matrix (Table 2.2).

Apligraf (Organogenesis, Canton, MA, and Novartis, East Hanover, NJ) is a double-layered bioengineered skin substitute derived from neonatal foreskin fibroblasts and keratinocytes. The dermal component is constructed using the cultured fibroblasts combined with bovine type I collagen. Cultured keratinocytes are then added and allowed, over a period of days, to epithelialize. Apligraf has been FDA-approved for the treatment of diabetic foot ulcers and venous ulcers . It costs US$51/cm^2 and has a 10-day shelf life. It is designed to be applied weekly. While Organogenesis sells other products, Apligraf is its largest revenue generator and is responsible for most of the company's US$55 million 2007 revenue. Organogenesis is currently in late-stage development with its next-generation skin replacement product, called VCTO1, which, like Apligraf, is an allogeneic, bilayered skin substitute.

Dermagraft (Advanced Tissue Sciences, La Jolla, CA) is a cryopreserved, human fibroblast-derived, dermal substitute. It is a product similar to Apligraf in that its dermal component is derived from neonatal fibroblasts. In constrast to Apligraf, however, Dermagraft does not contain either an epidermal component or bovine collagen. The extracellular matrix is produced by the fibroblasts which are grown in a biosynthetic, polyglycolic acid mesh. Dermagraft is FDA-approved for the treatment of neuropathic and diabetic foot ulcers and wounds secondary to epidermolysis bullosa. It costs US$34/cm^2 and has a six-month shelf life. Dermagraft and TransCyte were sold by Smith + Nephew to Advanced BioHealing, in 2006.

TransCyte (Advanced BioHealing) is a product similar, in nature, to Dermagraft.

Neonatal fibroblasts are cultured on a nylon mesh scaffold coated with bovine collagen. The dermal layer (fibroblasts–collagen–nylon lattice) is then coated with silicone to provide antimicrobial, fluid and temperature regulation. It was FDA-approved for the treatment of full and partial thickness burns.

Integra (Integra Lifesciences Corp, Plainsboro, NJ) is an acellular, purely biosynthetic dermal substitute. The Integra Dermal Regeneration Template was the first FDA-approved skin substitute. It is composed of (bovine) Type I collagen and chondroitin-6-sulfate on a silicone backing. The collagen–chondroitin dermal matrix is designed to allow in-growth of cells from the wound bed, while the silicone layer functions as an artificial epidermis, regulating heat and fluid loss and providing some degree of antimicrobial protection. After integration with the wound bed, the silicone backing is removed and the neodermis serves as a recipient bed for a split thickness autograft. Integra is approved for use in both full and partial thickness burn wounds.

Alloderm (Lifecell, Branchberg, NJ) is a dermal matrix derived from cadaveric skin treated with Lifecell's proprietary technology. The epidermis and cellular components of the dermis are removed during this process, leaving a scaffold composed of collagen, elastin, fibronectin, proteoglycans and the three-dimensional lattice-work of the microvasculature (without the actual vessels). The myriad applications reported for Alloderm include those for breast reconstruction, urogynecological, orthopaedic and trauma. 2007 revenue from Alloderm was over US$167 million.

Biobrane (UDL Laboratories, Inc, Rockford, IL) is a completely biosynthetic dressing made from porcine collagen-coated nylon mesh embedded in silicone. It is a temporary dressing used to cover burn wounds. It can be stored at room temperature for three years.

OrCel (OrCel International, New York, NY) is a product that is also similar to Apligraf, in that it is an allogeneic, bilayered substitute comprising cultured human fibroblasts and keratinocytes. The differentiating feature is that the fibroblasts are seeded onto a preformed matrix while the fibroblasts in Apligraf are co-cultured with collagen in solution. While Apligraf has a stratum corneum as a result of exposure to air during the epithelialization process, OrCel does not. OrCel is FDA-approved for use on split-thickness skin graft donor sites and for treatment of mitten hand deformity following epidermolysis bullosa.

Oasis (Healthpoint, Ltd, Fort Worth, TX) is an acellular, dermal substitute made from porcine small intestinal submucosa (SIS). Like other complex, three-dimensional scaffold products on the market, Oasis works by facilitating in-growth of native cells into the matrix. Over time, the body absorbs the matrix. Oasis has a two- year shelf life at room temperature. The cost to treat a small lower extremity ulcer over 12 weeks with multiple applications of the matrix was US$320.

Cultured epidermal autograft (CEA), one of the first bioengineered skin substitutes, was pioneered in the early 1980s by Rhinewald and Green. The promise of this technique was the ability to generate amounts of autograft from a very small

skin sample. The utility in patients with high percentage body surface area burns is obvious. The shortfallings of CEAs, however, have limited their use. These include expense, friability and lack of a dermal layer. CEAs have been relegated to the role of a biological dressing. The other existent options for biological dressings for patients with extensive burns now make use of CEAs impractical.

Epicel (Genzyme Biosurgery, Cambridge, MA) is the best known CEA on the market. Autologous keratinocytes are expanded *ex vivo* in co-culture with murine 3T3 fibroblast feeder cells. After expansion, the neoepidermal layer comprises keratinocyte layers of variable thickness and arranged in sheets. These sheets are placed on a carrier and shipped back to the patient in need. Epicel is expensive (US$21/cm^2) and has a 24-day shelf life in the cooled packing system.

2.4 The marketplace

For obvious reasons, the skin replacement marketplace has been an area of intense interest for both physicians and Wall Street investors for the past decade. As we have shown, the potential market for these products is enormous. Skin substitutes represent a high risk, high reward proposition for all involved – companies, physicians, patients and investors. The financial term for volatility, and therefore risk, is beta. Why is this a high beta sector?

In 2000, Krieger and Shaw investigated the financial characteristics of five publicly traded companies whose primary revenue source came from skin substitutes.[11] The companies and their products were Organogenesis/Apligraf, Advanced Tissue Sciences/TransCyte, Lifecell/Alloderm, Integra Lifesciences/Integra and Genzyme Biosurgery/Epicell. The purpose of their study was to compare Wall Street's attitudes towards these companies to other, similarly sized companies in the biotech space.

The rationale for such a study is that Wall Street tends to assess the risk profile of companies in predictable ways. Although not always accurate or foolproof, the metrics used to evaluate the potential profitability of a company provide observers and investors with some objective tools by which to compare different companies within a particular sector. The metrics used in the Krieger study included the degree to which different independent analysts agreed on revenue projections, the volatility of the stock price and the concordance of buy/sell recommendations by the analysts. The results of the study are not surprising to surgeons with experience using these products. Companies who make skin substitutes have higher stock price volatility and lower concordance among analysts regarding both revenue targets and buy/sell recommendations than do other companies in this sector.

The authors' explanation of these results was that there was an inefficient correlation between reported clinical results and potential market share of a given product. In other words, what companies regard as success has not translated into the magnitude of sales predicted by analysts. This leads to overestimation of revenue projections, which, when unmet, results in stock price volatility. At the

same time, the potential bonanza for the company that gains a significant foothold in this space encourages continued attention and investment by Wall Street.

2.5 Conclusion

Despite scientific advances over the last 40 years in engineering more sophisticated skin substitutes, their failure to achieve a major share of this enormous market has been notable. Certainly cost and the lack of clear and reproducible success associated with these various therapies have been partially responsible. The length of the FDA-approval process for biologically based skin substitutes increases the cost of the various products and limits investor enthusiasm for new research and development.

The market for skin substitutes, theoretically, should continue to expand for the foreseeable future. There is no end in sight to the increasing prevalence of diabetes, obesity and vascular disease – all key contributors to the development of complex wounds. Perhaps because these already daunting numbers are increasing so rapidly, the proposal of expensive, engineered skin substitutes as the solution will continue to meet resistance. None of the products described in this chapter represents the silver bullet for complex or advanced wounds. With a potential patient population in the tens of millions, expensive, imperfect solutions will not be welcomed by payors.

Simultaneously, the rise in negative pressure dressings for complex wound care has also made the choice of expensive bioengineered substitutes a more difficult one. The VAC dressing system (KCI, Inc) is relatively inexpensive, flexible, consistent and easy to use. Through transduction of micromechanical forces, the VAC dressing accelerates wound healing in a myriad situations and anatomical locations. The clinical results from its use in all of the above-mentioned circumstances (except epidermolysis bullosa where this has not been described) have been adequate to excellent.

The key to gaining prominence in the skin replacement marketplace in the upcoming years seems to demand development of less expensive technologies that can be used to treat the millions of patients who will require care for complex wounds. The technology that will win the day in this marketspace is going to be disruptive – inexpensive and just good enough. The answer is probably not in the development of more costly and advanced solutions. Neither the medical community nor the market has responded favorably to this line of attack over the past two decades.

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3

Biomechanics of skin

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Abstract: This chapter first describes the importance of studying the biomechanics of skin. After a brief description of the structure of skin, the chapter gives an overview of some mechanical properties needed in the study of mechanics. It ends with a description of wounded skin and modeling skin using finite element analysis.

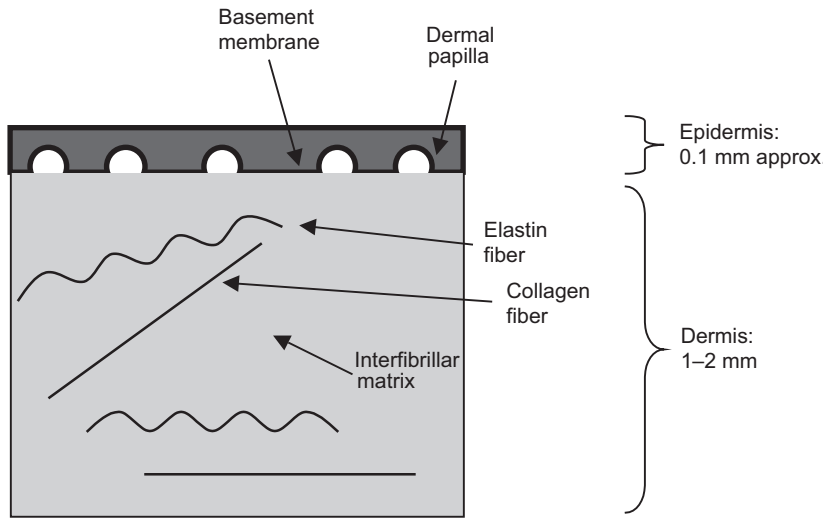
Key words: skin, mechanics, stress, strain.

3.1 Skin biomechanics

Biomechanics is defined as mechanics applied to biology, and mechanics itself is the response of bodies to forces or displacements. For example, when we displace (or try to displace) a body, it may move or it may stretch. It is important to study the mechanics of skin so that we can understand how it will behave under different conditions (for example, we may be interested in seeing how the skin behaves in non-typical environments – deep-sea diving, sky diving) or we may be interested in seeing how the skin responds to various medical interventions, such as micromechanical force therapies (or even non-medical interventions, such as various forms of body modifications including neck rings, used by certain tribes in Thailand, and body piercing).

External conditions applied to a system that we are studying are termed boundary conditions. We can have externally applied forces or we can have externally applied displacements. Forces and displacements are related through the constitutive laws describing the behavior of the material we are studying. Specifying a boundary condition does not mean that the other condition does not exist. It just means that we have chosen that condition to be the independent variable, while the other condition then becomes the dependent variable. For example, when we say that we are applying a force of 3 N to an object, we are letting the displacement be determined through the constitutive law. On the other hand, we may sometimes need to apply a constant displacement, and then the forces will be determined by the constitutive law. Forces and displacements cannot both be independently imposed on a system simultaneously.

Once we understand the way the skin responds to various forces and displacements, we can design our medical device to operate within a range where



3.1 Structure of skin.

the forces or displacements are meaningful but not large enough to cause structural damage.

The skin serves many functions. It serves as a mechanical barrier keeping outside environmental agents (bacteria, toxins, chemicals, UV radiation) separated from the internal parts of the body. It also serves as a protective covering that shields our bodies from minor trauma and abrasions[1] as we perform the day to day activities of moving and handling objects. The skin also helps preserve the temperature of our bodies by preventing heat loss in cooler environments and by alternately mediating heat loss through sweating and increased blood flow.

3.2 Structure of skin

The skin is made up of two layers, epidermis and dermis (Fig. 3.1). The epidermis is 80 μm (or about 0.1 mm) thick, is multicellular and has four sublayers: the stratum basalis, the stratum spinosum, the stratum granulosum and the stratum corneum.[1] New cells are continuously made in the deepest layer of the epidermis, the stratum basalis and move outward toward the surface of the skin.[2] Since the epidermis is not supplied with blood vessels (and therefore only obtains its nutrients from the dermis), cells near the surface die and form the outermost layer of the epithelium or the stratum corneum.

Epidermal cells are strongly attached to one another by tight junctions and desmosomes (desmosomes also help connect these cells to their neighbors below). The lowermost layer of epithelial cells right above the basement membrane is attached to the membrane by hemi-desmosomes. Such junctions provide the

epithelial layer with the mechanical strength needed for it to withstand various stresses placed upon it.

The basement membrane separates the epidermis from the (relatively) much thicker dermis (1– 2 mm in thickness and thus one order of magnitude thicker than the epidermis). The dermis is responsible for the mechanical properties of skin.[1] The dermis is made up of fibers of connective tissue that run in all directions.[3] These fibers are made up of elastin and collagen. Collagen fibers provide strength to the skin, while the elastin fibers provide recoil strength.[3] The tensile strength of the dermis can range from 500 psi to 10 000 psi (3.4 to 68.9 MPa) (depending on the type of species, orientation of the skin specimen tested and the location on the body).[1] The collagen and elastin fibers are both embedded in an interfibrillar matrix of proteoglycans.

3.3 Definition of mechanical properties

Stress is defined as the total force applied per unit area. In units, stress is exactly the same as pressure (Newtons per meter). Or mathematically:

$$\sigma = \frac{force}{area}$$

where σ is the stress.

The strain is defined as the change in length of an object over the original length written as:

$$\varepsilon = \frac{\Delta l}{l}$$

where ε is the strain, Δl is the change in length and l is the original length. Strain has no units since it is the ratio of length to length. Strain is usually presented as a percentage.

The modulus of elasticity or Young's modulus, E, is defined as the ratio of stress to strain. Or:

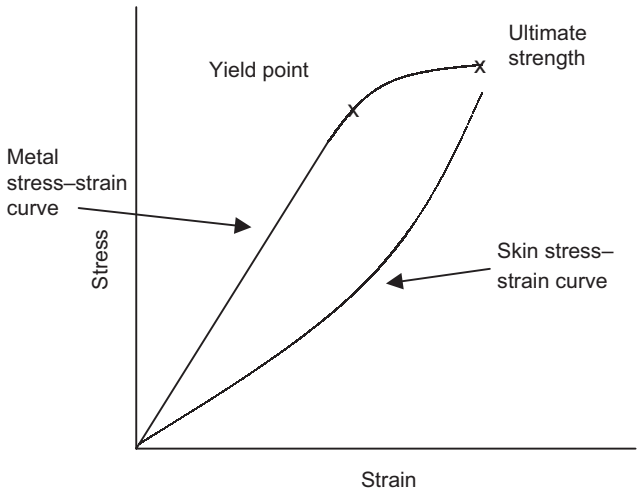
$$E = \frac{\sigma}{\varepsilon}$$

The Poisson's ratio, ν, is the ratio of negative strain in one direction to the strain in an orthogonal direction and is given by:

$$\nu = \frac{-\varepsilon_x}{\varepsilon_y}$$

Creep is the slow deformation of a material under the influence of stress over time where the stress is constant over time, while stress relaxation refers to the change in stress in a material which is subjected to a constant strain.

The stress–strain curve (Fig. 3.2) describes graphically how the material under



3.2 Stress–strain curve for a typical material such as a metal along with that for skin. The Young's moduli for the two are very different. The figure is used to illustrate only the trend in the stresses with increasing strains. Skin shows a positive second derivative – it becomes stiffer as it is stretched.

study deforms under applied stresses. The yield point occurs when the material will no longer deform elastically. In other words, it will not go back to its original length or conformation after the stress is removed. It will have undergone plastic deformation (there will be some deformation in the material even after all stresses or forces have been removed). The tensile strength of the material is the maximum stress the material can withstand and occurs at the point of rupture of the material.

Figure 3.2 also shows the stress–strain curve for skin. As skin is stretched it shows increasing stiffness (it has a positive second derivative). This is because with increased stretch the fibers in skin orient in the same direction, thereby increasing its resistance to the applied stretch.

3.4 Wounded skin contraction

Skin can be damaged under the action of various mechanical, chemical and other stresses. Because the skin serves such an important role in the body, wounded skin needs to be replaced quickly. There are essentially two types of healing responses. One is healing by first intention and the other is healing by second intention.[4] In the former reparative process, after the wounding agent is removed, the edges of the wound bed are in very close proximity. This type of a reparative process involves little or no contraction of the wound bed. However, in the latter type of wound healing, the edges of the wound bed are not in close proximity and thus in order to bring them closer, fibroblasts occur at the edge of the wound.[4] These fibroblasts

then become myofibroblasts and thus have the character of smooth muscle cells in being able to impose contraction forces on the edges of the wound bed in order to close the wound.[1,4] Wound contracture is the primary cause for the wound bed to be reduced to between 5–10% of its size within 6 weeks of the injury. Both types of wound healing involve scar formation (since healing by second intention involved greater loss of and damage to tissues, scar formation is generally greater in this type of wound healing. A scar itself is fibrous tissue that replaces normal skin that was damaged.

Scar tissue contains collagen fibers that are much more densely packed than those in normal skin.[1] This causes scar tissue to be much stiffer than normal skin tissue.[1]

3.5 Modeling skin using finite element methods

In order to understand how skin responds to various forces and stresses placed upon it, we can simulate skin using numerical techniques. One such technique simulates skin by creating a fine network of elements which when put together behave overall like the skin. This is the idea behind finite element methods. One can visualize such techniques by thinking of the three-dimensional structure of skin as being composed of small rectangular elements. These elements are then combined mathematically to yield the response of the system being modeled at each discrete element. For example, by using such techniques we are able to ascertain the stresses within a material under the application of certain boundary conditions (forces, displacements, pressures).

We created one such model of the skin in analyzing how micromechanical force therapies, such as the vacuum assisted closure device, (VAC, Kinetic Concepts, San Antonio, Texas) may work.[5] The skin was modeled as linear, elastic, homogeneous and isotropic. The mechanical properties of the skin were captured by the Young's modulus (measures stiffness) and the Poisson's ratio (measures compressibility).[5] Because wounded skin may have very different mechanical properties from normal skin, and because normal skin itself may have different properties from one person to another (based on age, sex, individual variation, and so on), each of the above mentioned properties were varied. Poisson's ratio was varied between 0.36 and 0.5, while the Young's modulus was varied from 50–100 kPa.

The VAC device applies suction to a wound bed through a sponge packed within a sealed dressing in the wound bed. With the application of the suction the wounded tissue is exposed to sub-atmospheric pressures and is pushed by the external pressure underneath the wound (alternately one can also consider the wound bed to be 'pulled' into the sponge by the sub-atmospheric pressure). The sponge itself collapses into a thin layer at the application of the negative pressure. The wound is thus blocked from rising upwards by the sponge struts and thus the wound rises into the empty space between the sponge struts (roughly hexagonal in

shape). The VAC device properties were captured in our model by varying both strut thickness (values of 0.15, 0.3, 0.5 mm), and strut lumen or pore diameter (0.8, 1.2, 1.6 mm) as well as the sub-atmospheric pressure applied by this device (70, 110 and 150 mmHg below atmospheric pressure).

Our results showed that the average surface strains under the application of the VAC were on the order of 20%.[5] These strains rose as expected with increased negative suction pressure (higher and higher negative values) and with pore size, while they decreased with strut thickness.

3.6 Forces on cells

In our paper discussing the finite element model, we proposed that wound healing was accelerated through the transmission of forces from the external environment onto the cells residing within the wounded tissue. It has previously been shown that cells that are allowed to stretch tend to proliferate whereas those cells that obtain a spherical conformation and are not allowed to stretch become cell cycle arrested and apoptotic.[6,7] Thus, an understanding of the mechanics of skin and its microstructure can help us understand what types of forces may be being transmitted into the cells.

3.7 Conclusion

An understanding of skin mechanics is critical for the design of medical devices that find application in wound healing in the skin. It is also critical in understanding how cells and the extracellular makeup of tissues perceive and transmit those forces. The skin is far from a mechanical scaffold that serves a passive role. It responds actively to forces by transmitting force information on to its cellular components, which then secrete various growth factors, cytokines and so on in response to the mechanical stimuli the tissue perceives from the environment through signal transduction pathways.

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4

The pathophysiologic basis for wound healing and cutaneous regeneration

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Abstract: In this chapter, essential aspects of cutaneous wound repair/scar formation, including the early response and the cellular phase, will be covered. Fundamental differences between wound repair and regeneration, with emphasis on fibroblast and endothelial cell contribution to respective dermal architecture, will be discussed. 'Essential ingredients' for cutaneous regeneration will be explored in detail. Lastly, issues regarding stem cells, progenitors and cellular 'plasticity', as they relate to cutaneous wound healing, will be examined.

Key words: fetal skin, regeneration, scar formation, stem cells, wound healing, wound repair.

4.1 Introduction

Normal wound healing responses in post-natal human skin involve complex and highly coordinated interplay among cells, soluble factors and an extracellular matrix, with the ultimate goal of efficient and effective wound closure. Such responses are likely to have evolved in *Homo sapiens* as a means of minimizing intrusion across the cutaneous barrier by noxious and infectious environmental agents that could threaten survival upon systemic dissemination. A fundamental problem with this phenomenon, however, resides in the fact that this response eventuates in a contractile scar that may impede normal function by limiting motion (contracture) or by replacing essential tissue. Like seemingly more rudimentary organisms, such as salamanders and planaria, which are capable of regenerating complex tissue, the skin of the early human fetus responds to injury via regeneration rather than scarring. Protected from the external environment by normally sterile amniotic fluid, fetal skin can afford to employ the time, inherent plasticity and coordinated differentiation programs necessary for a non-contractile, regenerative process. How fetal skin shifts from a regenerative response *in utero* to a reparative post-natal response and how adult skin may be 're-educated' to procede via a regenerative pathway instead of the wound repair–scar formation pathway, is only now beginning to be explored.

In this chapter, the normal structural and functional complexities of human skin, including biochemical and cellular events, implicit in physiological wound healing

responses are discussed. In so doing, the ingredients of wound repair (that contribute to wound contraction) and scar formation will be emphasized. The essential architecture and cellular components necessary for a regenerative process is explored next. Finally, these ingredients, so defined, are analyzed in the context of available biodegradable matrices that seek to promote cutaneous tissue regeneration.

4.2 Skin microanatomy and physiology

In understanding repair and regeneration, it is essential to be familiar with the anatomic and physiologic functions of normal skin. Histologically, skin is divided into two functionally interdependent layers: epidermis and dermis.[1] Skin is composed of acellular matrix components and a variety of cells, which together, subserve a myriad of protective functions, including mechanical and photoprotection, immunosurveillance, nutrient metabolism and repair.

4.2.1 Epidermis

The majority of the epidermis is composed of mostly keratinocytes (>90%), while the remainder consists of small subpopulations of melanocytes, Langerhans cells, neuroendocrine (Merkel) cells and unmyelinated axons. Architecturally, the undersurface contains downward ridge-like projections (rete ridges) that interdigitate with upward-projecting dermal mesenchymal cones (papillary dermis.) A three-dimensional reconstruction of the undersurface would therefore, resemble a thick-chambered honeycomb.

The epidermis is subdivided into four basic layers (from deep to superficial): basal cell layer, spinous cell layer, granular layer and cornified (horny) layer. Cells forming these layers are bound together by complex membrane-associated plaques called desmosomes. Desmosomes anchor and unite cytoskeletal components of adjacent epidermal cells. Important molecules critical to keratinocyte–keratinocyte bonding (adhesion) includes cadherins, desmogleins, and desmocollins.

The basal cell layer (deepest) is made up of a single 'sheet' of cells that interfaces with the underlying basement membrane, to which they are attached to via membrane-associated plaques (hemidesmosomes.) At the molecular level, structural proteins and integrin (transmembrane receptor protein)–ligand interactions mediate the anchoring of the epidermal layer to the subjacent dermis. In normal skin, most epidermal mitotic activities take place in the basal cell layer and, accordingly, defects in homeostasis of this layer may have critical effects on the maintenance of an intact and viable epidermis. The basal cell layer contains a subpopulation of slow-cycling stem cells that are possibly of critical importance to the regenerative potential of human skin.

Post-mitotic keratinocytes rest on top of the basal cell layer, with the youngest in the spinous layer and oldest in the cornified layer. As keratinocytes 'mature' and

ascend towards the epidermal surface, their shapes become increasingly flattened. In so doing, they synthesize a sturdy, water-repellent keratin protein (keratinization.) The structure of keratin becomes increasingly complex with each ascent. Keratinization is initiated when columnar basal cells differentiate into polyhedral cells to form an overlying spinous layer (5–10 cell layers thick). Further into development, these cells become more squamous (with cytoplasm containing keratohyaline granules), thereby forming the granular layer (1–3 cell layers.) Cells in the granular layer undergo further transformation, losing their nucleus and organelles (via cytoplasmic lysozymes), rendering them technically dead. These dead cells make up the outermost, cornified layer (roughly 10–15 cell layers), and have a turnover rate of about one month.

In areas such as palms and soles where the cornified layer is thick, an additional layer (called clear layer or stratum lucidum) is found. Rich in protein-bound lipids, the clear layer lies between the granular layer and cornified layer.

Depending on the density of disulfide bonds, keratin is classified as soft (as seen in skin) or hard (as seen in hair and nails).[2] The net result of keratinization solidifies cell–cell bonding, producing a barrier that prevents fluid loss and unwanted entry of potentially noxious molecules and organisms.

Keratinocytes also produce several protective immunogenic molecules.[3] These molecules include interleukins (IL-1, IL-6, IL-8), transforming growth factors (TGF-α, TGF-β), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), tumor necrosis growth factor (TNF-α), interferons (IFN-α, IFN-β), and granulocyte-macrophage colony-stimulating factor (GM-CSF or G-CSF.)

Melanocytes, which are largely responsible for skin color, are found randomly dispersed within the basal cell layer.[1] There, keratinocytes outnumber them by a ratio of 10:1. However, repeated exposure to ultraviolet (UV) light increases their population, which in turn, leads to an increased production of melanin (UV-protective pigment.) Melanin is delivered via dendritic processes that insinuate between adjacent keratinocytes, extending to the target keratinocyte. Once pigments are delivered to keratinocyte cytoplasm, they are arranged in an umbrella-like manner, such that the nucleus is shielded from harmful UV rays. Recent data shows that UV light elicits melanization through the activation of the p53 pathway, resulting in the synthesis of melanin stimulating hormone (MSH) by keratinocytes.[4] Interestingly, the p53 pathway also comes into play when constitutive pigmentation fails to prevent keratinocyte mutation (by UV light), thereby inducing apoptosis of defective and potentially premalignant cells.

Langerhans cells (LCs), which are found mostly in the mid-epidermis, participate in cell-mediated immune responses.[5] Also known as epidermal dendritic cells (DCs), this subclass of leukocytes make up about 2–4% of all epidermal cells. Along with self- and non-self antigens, LCs are capable of capturing any haptens that have potential to violate the epidermal surface, rendering them appropriate for initial presentation to naïve T lymphocytes.[6] This phase, termed sensitization, involves migration of antigen-containing LCs from the epidermis

into the dermis, where they subsequently enter the lymphatic spaces and travel to draining lymph nodes. Here, where the audience of naïve T-cells is maximized, they transfer their antigenic information (in an HLA-DR-dependent manner) that transforms the T-cells into ones with antigen-specific memory. Upon re-exposure to antigen, memory T-cells enter the skin, where they release mediators that amplify the immune response, thereby recruiting non-antigen-specific lymphocytes and macrophages, resulting in a delayed hypersensitivity reaction. It is important to realize, however, that skin-homing T-cells normally traffic in and out of skin (on a regular basis) as part of the skin-associated lymphoid tissue (SALT.) This trafficking is mediated by homing interactions between lymphocyte subsets (expressing the skin-homing molecule CLA) and dermal microvascular endothelial cells (expressing e-selectin.) Indeed, it has recently been shown that at any given time, normal human skin harbors approximately 1×10^6 T-cells per cm^2 and an estimated 2×10^{10} T-cells in the entire skin surface, which is nearly twice the number of T-cells in the entire systemic circulation.[7]

Like melanocytes, Merkel cells are found in the basal cell layer.[8] Seldom visible under a light microscope, these cells contain membrane-bound, cytoplasmic neuroendocrine-type granules (identified by electron microscopy.) They occasionally form synaptic junctions with peripheral nerve endings and, in lower vertebrates, may participate in slow-adapting touch perception. However, their function in human skin has not been elucidated and it remains possible that they are vestigial.

4.2.2 Basement membrane

Directly beneath the undersurface of the epidermis lies the basement membrane (BM). Composed mostly of collagen IV, the basement membrane physically 'separates' the epidermis from the dermis. Although seen as a single entity under light microscopy, the BM is of heterogeneous composition when viewed under an electron microscope (and is referred to as the basal lamina.) The basal lamina is complex (in molecular structure) and may be subdivided into the lamina densa (superficial, composed of collagen IV) and the lamina lucida (deep, composed of laminin and other glycoproteins.) Epidermal–dermal anchoring fibrils (made from collagen VII) physically connect the lamina densa to the papillary dermis. Epidermal–dermal communication is permitted through hemidesmosomes (anchoring plaques, contain collagen XVII – also called bullous pemphigoid antigen.)

Compromise of these essential molecular elements may have devastating consequences for dermal–epidermal integrity and homeostasis. Thus, any efforts to regenerate authentic skin must look beyond simple structural integrity to ensure that the essential molecular complexities are faithfully replicated.

4.2.3 Dermis

The dermis is a complex, dynamic microenvironment that harbors a collection of specialized cells within an intricate matrix (formed by soluble and non-soluble molecules.) It is subdivided into the papillary dermis (superficial) and the reticular dermis (deep.) The dermis is responsible for a wide variety of functions, including maintaining the elastic and mechanical integrity of the skin, cutaneous nutrition, immunosurveillance, sensory perception and temperature regulation.

In a normal physiological state, the dermis contains an ensemble of cells that includes (1) fibroblasts, (2) endothelial cells, (3) monocytes/macrophages, (4) dendritic cells (also called dermal dendrocytes), (5) mast cells, (6) lymphocytes, (7) Schwann cells, axons and specialized nerve endings, (8) pericytes and glomus cells and (9) mesenchymal elements with presumed differentiation plasticity (mesenchymal stem cells.) These cells are contained within an acellular matrix, composed mainly of collagen and glycosaminoglycan.

The dermis contains an extensive network (plexus) of blood vessels that provides nourishment for itself and the epidermis. The superficial microvascular plexus defines the boundary between the papillary dermis and the reticular dermis. Endothelial cells, expressing surface markers CD31 (short for cluster of differentiation-31), CD34, and CD144 (vascular epithelium-cadherin or VE-cadherin) line the lumen of the dermal microvessels.[9] Endothelial cells participate in transmural shuttling of macromolecules and are important facilitators of immune cell trafficking.

Dermal microvessels are surrounded by a complement of immune cells, including macrophages, mast cells and dendritic cells. Collectively, these cells collaborate to coordinate intradermal antigen presentation, induction of inflammation and homeostasis in the immediate perivascular microenvironment.[1]

The dermis is supplied with sensory (myelinated up to their terminal branches) and autonomic (unmyelinated) nerves. Found mostly in the papillary dermis (especially near microvessels), autonomic nerves (derived from the sympathetic system) control vascular flow and secretory functions of skin appendages, including sweat glands, but not sebaceous glands (endocrine stimulated.)

Detection of cutaneous sensation is performed by several specialized nerve endings, including free nerve endings, Meissner's corpuscles, Vater–Pacini corpuscles and, in lower vertebrates – Merkel cells (also found in the basal cell layer of the epidermis and the bulge region of hair follicles.) Free nerve endings are involved with temperature, pain and itch (a variant of pain) perceptions. Meissner's corpuscles are structured nerve endings that detect touch; they are found exclusively in the papillary dermis of the ventral aspects of hands and feet. Vater–Pacini corpuscles mediate pressure and vibrational sensation; they are found in the deep dermis and subcutis of palms and soles. Protective roles of these neural components become evident in patients with tissue injury (such as sensory neuropathy.)

Dendritic cells (DCs), which appear in the perivascular region, in close proximity to fibroblasts, make up a diverse subclass of immune cells. These cells are capable of acting as antigen-presenting cells (APCs), acquire phagocytic potential and express an assortment of epitopes, including CD34 (hematopoietic progenitor antigen) and factor XIIIa (activated fibrin stabilizing factor, also expressed by platelets.) Hence, they may play an important role in the early stages of wound healing by virtue of having the ability to cross-link fibrin and fibronectin. With considerable plasticity among subpopulations, these cells can transform their phenotypes from one subtype to another, depending on necessities within the local microenvironment.

As in the epidermis, certain dendritic cells in the dermis are responsible for patrolling against environmental (and possibly endogenous) antigens. Dendritic antigen-presenting macrophages (plentiful in the dermis) subserve a function of Langerhans cells by initiating immune responses directed against locally injected (as well as systemic) antigens. Non-antigen-specific (innate) immune responses are also mediated by macrophages in the dermis; however, these cells tend to be less dendritic and more phagocytic (in dealing with offensive particulates by phagocytosis and subsequent enzymatic degradation within phagolysomes.)

The dermis is thought to harbor a subpopulation of mesenchymal cells with a potential for self-renewal and differentiation plasticity.[10] Recent evidence implicates dermal stem cells having capacity to differentiate along neurogenic and myogenic lines.[10] A similar subpopulation (adipocyte stem cells) has been identified in the subcutaneous fat (subcutis).[11] The contribution of these subpopulations to wound repair and regeneration is yet to be elucidated. Moreover, it remains possible that circulating hematopoietic cells (with stem cell capacities) may seed wounds and play an important role in the healing process (further discussion to follow.)[12]

Dermal extracellular matrix (ECM) is mainly composed of collagen and elastic fibers, embedded within ground substance.[1] All three components are synthesized by fibroblasts. These components are distributed heterogenerously throughout the dermis. Of the three, collagen (the main constituent of leather) is by far the most abundant. Ranging from 2 μm to 15 μm in diameter, collagen fibers are either found in a finely woven meshwork (as seen in papillary dermis) or as thick, dense bundles (as seen in reticular dermis.) The papillary dermis (smaller in size) is dominated by collagen III, whereas the reticular dermis (larger in size) is dominated by collagen I. Overall, the ratio of collagen I to collagen III is 4:1. However, it is variably increased after wound repair. Ranging from 1 μm to 3 μm in diameter, elastic fibers are found intertwined among collagen fibers. Like collagen, elastic fibers tend to be smaller in the papillary dermis and thicker in the reticular dermis.

Spaces not occupied by collagen or elastic fibers are filled by an amorphous material called ground substance – composed mainly of glycosaminoglycan. Also known as acid mucopolysaccharides, glycosaminoglycans (GAGs) are found in sulfated and unsulfated forms. Of the two, unsulfated forms, such as hyaluronic

acid (HA), are more common. These molecules are covalently linked to peptide chains to form high-molecular-weight complexes called proteoglycans. Along with collagen, GAGs are commonly utilized to make bioactive scaffolds used to induce regeneration.[13]

4.2.4 Appendages

Skin appendages (such as hair follicles) are derived from invaginated epidermal tissue and are often found projecting deep into the dermis.[8] In certain regions, such as the scalp, these invaginations actually project into the subcutis (subcutaneous tissue.)

Skin appendages include the pilosebaceous apparatus, eccrine sweat glands and apocrine glands. The pilosebaceous apparatus is composed of a hair follicle, sebaceous gland and associated arrector pili muscle. The lower most portion of a mature hair follicle (bulb) contains mitotically active germinative cells (which produce the hair shaft in a manner somewhat analogous to epidermal keratinization).[14] Eccrine sweat glands main function is thermoregulation, which is accomplished through excreting sweat. Apocrine glands which are found only in the axilla and anogenital regions, produce scents (known in some vertebrate species to function as pheromones.)

A critical issue in wound healing and skin regeneration involves the presence of cells capable of self-renewal and differentiation plasticity, or so-called tissue stem cells. For a number of years, epidermal stem cells were believed to reside exclusively at the tip of rete ridges within the basal cell layer. Recently, an additional epithelial stem cell niche has been identified in the bulge region of the hair follicle, near the insertion site of the arrector pili muscle.[15] There has been active interest in these populations, with respect to their contributions to re-epithelialization, as well as to their potential to differentiate along adnexal lines. The former is a prerequisite for wound repair and the latter is a fundamental component of authentic skin regeneration.

Hair follicle (replete with its stem cell niche) is not a trivial structure in *Homo sapiens*. It is one of few human tissues that retains the ability to involute cyclically, from a mature and structurally complex anagen (growing) follicle, to a rudimentary telogen (quiescent) follicle, and then regenerate back into the anagen form.[16,17] Teleologically and evolutionarily in lower vertebrates, this characteristic may represent the residue of a survival necessity, favoring shorter hair for efficient locomotion or adaptation to temperature variations (as follicular cycling ultimately determines hair length.) However, its persistence in humans provides a unique opportunity to probe the mysteries of how complex structures regenerate in the adult.

4.2.5 Subcutaneous tissue

Lying subjacently deep with respect to the dermis, the subcutaneous tissue has dual

functions. It serves as an energy-absorbing pad that minimizes mechanical injury to skin. It also serves as an energy reservoir (packaged in the form of lipids) which can be easily accessed, and catabolized on demand.

4.3 Wound repair and scar formation

In normal skin, the dermis and epidermis exist in a steady-state equilibrium (with many complex and interdependent components), forming a protective barrier against the external environment. When the protective barrier is broken, the structural integrity is compromised, the underlying vasculature is disrupted and the physiological process of wound healing is immediately set in motion. The classic model of wound healing involves dividing the process into four sequential phases: (1) hemostasis, (2) inflammatory, (3) proliferative and (4) remodeling. Although each phase is distinct, there is considerable overlapping between them.

In brief, upon injury to the skin, platelets aggregate at the injury site to degranulate, thereby initiating the clotting cascade. Within minutes, a fibrin clot is formed and hemostasis is achieved. Within 24 hours, neutrophils arrive at the wound site, ushering in the inflammatory phase (which lasts 3–4 days.)[18] Neutrophils scavenge for foreign materials, bacteria and damaged tissue for phagocytosis. Macrophages appear thereafter (in day 1–2) to continue the task of phagocytosis and in addition, secrete cytokines. Once non-essential debris has been removed, fibroblasts (either mature or perhaps a precursor form) and endothelial cells (or progenitors) are recruited into the wound microenvironment, signaling the beginning of the proliferative phase. During the proliferative phase (occurring from day 4 to day 14), fibroblasts propagate and proliferate, depositing new matrix materials (mainly collagen.) Concurrently, vascular endothelial cells (or precursors) assemble themselves to form microvascular structures. During the remodeling phase (which begins after week 2, and lasts between weeks and months), excess matrix materials are removed, collagen fibers are cross-linked (strengthening the matrix) and contraction of the ECM (by myofibroblasts) occurs.

Alternatively, cutaneous wound healing may be described in terms of an ‘early phase’ and a ‘cellular phase’. This would render less overlapping of the many elements involved. In the early phase, hemostasis is accomplished and cytokines are released, initiating the inflammation process. In the cellular phase, an inflammatory response is mounted, the dermis is repopulated, wound coverage is achieved (re-epithelialization) and a scar is formed.

4.3.1 Early phase

The early phase of wound healing begins immediately following injury. Damage to dermal structures, especially blood microvessels, triggers a cascade of molecular and cellular events, resulting in a hemostatic plug covering the wound site.[18–21]

In addition, blood and lymphatic vessels undergo a brief period of vasoconstriction to minimize blood loss.

In the process of forming the fibrin clot, platelets aggregate along the injured endothelium, where they degranulate, releasing a host of molecules that become involved in the coagulation cascade. Consequently, fibrinogen is converted to fibrin, which collectively assembles at the wound site, forming a fibrin clot that provides hemostasis and early wound covering. The fibrin clot also serves as a rudimentary matrix (or scaffold), providing support for subsequent epithelial migration (re-epithelialization) and cellular infiltration. Aside from fibrin, the hemostatic plug also contains important ECM ligands, including fibronectin (collagen- and fibrin-binding protein), vitronectin (anchoring glycoprotein) and thrombospondin (glycoprotein involved in platelet aggregation.)

Activated platelets also initiate inflammation by releasing chemotactic agents/growth factors, including platelet-derived growth factors (PDGF) and transforming growth factor-beta (TGF-β.) PDGF initiates the chemotaxis of neutrophils, macrophages, fibroblasts and endothelial cells. TGF-β promotes macrophage infiltration (into the wound site), as well as macrophage production of additional cytokines, including fibroblast growth factor (FGF), interleukin-1 (IL-1, acute inflammatory response) tumor necrosis factor-alpha (TNF-α, acute inflammatory response), and even more PDGF. TGF-β also enhances the chemotaxis of fibroblasts and endothelial cells.

Once hemostasis is accomplished, local endothelial cells release prostaglandins and leukotrienes, leading to an influx of cellular elements from the systemic circulation.[22] Prostaglandins cause vasodilatation and platelet disassembly. Leukotrienes increase vascular permeability, chemotaxis, and leukocytic adhesion.

The initiation of inflammation is also done by dermal mast cells (residing in the perivascular region.) Mast cells also degranulate upon adequate mechanical stimuli, liberating histamine and other vasoactive amines, causing blood vessels to become more 'leaky' and permeable to cells. They also release inflammatory cytokines, particularly TNF-α and TGF-β.[18,23]

4.3.2 Cellular phase

As mentioned, during the cellular phase, different cell types work in unison to mount an inflammatory response, synthesize granulation tissue, re-establish the epithelial layer and reduce wound volume (contraction), thereby restoring a rudimentary degree of structural integrity to the region. For simplicity, the cellular phase may be subdivided into the following components:

1 macrophages and related inflammatory components (within 1–2 days)
2 epithelial–mesenchymal interaction: re-epithelialization (phenotype change within hours, migration begins on day 1–2)

3 fibroblasts and myofibroblasts: progressive alignment, collagen production and matrix contraction (between day 4 and day 14)
4 endothelial cells and angiogenesis (begins on day 4)
5 dermal matrix: elements of fabrication (begins on day 4, lasting 2 weeks) and alteration (begins after week 2, lasting weeks–months).

Macrophages and related inflammatory components

After hemostasis is achieved, immune cells (in response to released chemotatic factors) infiltrate the wound site and mount an inflammatory response. This occurs within hours after injury, as neutrophils (in the blood) adhere to activated vascular endothelial cells and rapidly enter the injured area. They are followed by monocytes, which within 1–2 days, are activated to become macrophages. Together, neutrophils and macrophages remove foreign matter, bacteria, non-functioning host cells, damaged matrix components and other non-essential materials. Macrophages play a critical role in the cellular phase, releasing PDGF and TGF-β to promote the migration, proliferation and differentiation of fibroblasts and endothelial cells. Inhibiting macrophage function may lead to a blunted inflammatory response and a delay in wound healing.[24,25]

Although lymphocytes play a major role immunosurveillance, their contribution to the early stages of wound healing is not considered to be significant.[18,20] T-cells are attracted to IL-1, but do not appear until three days after injury (when the inflammatory response is nearing an end.) Nevertheless, lymphocytes remain an essential part of the immune system, functioning in cell-mediated immunity (T-cells) and humoral immunity (B-cells.)

Recently, a unique subpopulation of (mononuclear) leukocytes has been identified to be a major participant in wound healing, especially in excessive healing and fibrosis.[26] Known as 'circulating fibrocytes,' 'peripheral blood fibrocytes' or simply 'fibrocytes', they make up less than 1% of the circulating leukocyte population. These cells are distinctly different from the mature resident fibroblasts – also called fibrocytes.[27] When discovered, they were described as hematopoietic cells with fibroblast-like (spindle-shape) morphology. Fibrocytes express a variety of surface markers, including CD34 (hematopoietic progenitor antigen) and CD45 (common leukocytic antigen), which they maintain until later stages of maturation.

Fibrocytes assist in the coordination of both inflammatory and reparative responses (including wound contraction.)[27–30] Recruited to the wound site early, they have the ability to take on an antigen-presenting role (expressing CD11b, also expressed by monocytes/macrophages) or produce a variety of signaling molecules, including IL-1. They influence epithelial migration and proliferation (via PDGF-α), recruit and activate vascular endothelial cells (via VEGF and TNF-α, respectively), and promote fibroblast differentiation into myofibroblasts (via TGF-β.) Furthermore, they have the unusual ability to secrete collagen I, as well as

the matrix metalloproteinase-9 (MMP-9, type IV and V collagenase.) Their presence is thought to be in abundance in keloids and hypertrophic scars.[31] Although not yet fully elucidated, there is some evidence to suggest that fibrocytes may be a precursor to fibroblasts and myofibroblasts (further discussion follows.)[27,28, 32,33]

Epithelial-mesenchymal interaction: re-epithelialization

Within hours of skin injury, the process of re-epithelialization begins.[20,34] In the presence of epidermal growth factor (EGF, secreted by platelets) and TGF-β (produced by macrophages, platelets, and keratinocytes),[14] epidermal cells undergo phenotype changes that result in the abandonment of (stabilizing) desmosome (cell–cell connection) and hemidesmosome (cell–BM connection) expression, in favor of peripheral cytoplasmic actin filament expression, thereby permitting cell detachment and subsequent migration (which occurs within the first few days.)

The process of re-epithelialization requires epidermal cells (at the wound edge) to migrate centrally, until the epidermal surface is completely restored.[20] Through the expression of integrin (transmembrane receptor protein), epidermal cells are able to interact with a variety of ECM ligand proteins, including fibronectin (binding) and vitronectin (anchoring), such that desiccated eschar are undermined and dissected out (from viable tissue) during migration. Epidermal migration is facilitated by the production of zymogens and enzymes which assist in the removal of fibrin clots (via plasmin) and damaged stroma (via collagenase); both plasmin and collagenase are activated by tissue plasminogen activator (tPA), also produced by epidermal cells.

Recent evidence indicates that re-epithelialization involves the participation of follicular stem cells (residing in the bulge area of hair follicles) through the *Wnt* (signaling) pathway.[35] This would, therefore, explain why re-epithelialization is impaired in deep wounds, where adnexal epithelium is partially or fully destroyed.[36,37]

Proliferation of keratinocytes takes place just behind the advancing front, and continues until all layers of the epidermis are restored. In the early stages of re-epithelialization, this process occurs mainly due to the influence of EGF and tissue growth factor-alpha (TGF-α, secreted by macrophages and epidermal cells.)[18,20] However, during the middle and later stages, the interplay between keratinocytes and fibroblasts gradually shifts away from inflammation, in favor of granulation tissue synthesis and basement membrane formation.[38] Such interaction relies on the production of epithelial mitogens, mainly PDGF and keratinocyte growth factor (KGF), by nearby fibroblasts. Although associated with platelets, PDGF is also secreted by other mesenchymal cells (including fibroblasts), and in event of an injury, epidermal expression of PDGF and KGF (also called fibroblast growth factor-7, FGF-7) receptors are upregulated.

It should be noted that new epidermal tissue is considerably different from uninjured epidermal tissue. This is primarily due to the inability of the re-epithelialization process to form rete ridges (in a proper relationship to papillary dermis) and to regenerate adnexal epithelium. Accordingly, while the new epidermis seals the surface of new dermal tissue, it does not represent a regenerative response to injury.

Fibroblasts and myofibroblasts: progressive alignment, collagen production, and matrix contraction

Physiological response to skin injury occurs (1) in a manner that favors rapid tissue replacement (wound repair/scar formation) and (2) without regard to exact restoration of morphology and functionality (regeneration.) In revolutionary terms, such a mechanism is advantageous to survival because it reduces duration of wound exposure, thereby minimizing opportunities for bacterial invasion. Consequently however, the architectural intricacies of dermis, such as vascular plexus and associated appendages, are inadequately restored.

The period during which the highest rate of cellular proliferation occurs is usually between day 4 and 14. This interval is characterized by a dramatic increase in fibroblast population, which is accompanied by rapid accumulation of collagen deposits in the extracellular space and the resultant formation of granulation tissue.[18,20–22] This process is dictated by levels of TGF-β (mainly TGF-β_1)[39] and PDGF found in the wound microenvironment. TGF-β and PDGF are potent stimulators of fibroblast migration and proliferation.

The reconstitution of dermis proceeds in a manner that is conducive to rapid tissue replacement and effective wound contraction. This approach requires fibroblasts to take up residence in stratified planes/axes, parallel to the epidermal surface (a remarkable contrast to normal skin, in which these same cells are aligned along seemingly random axes.)[13] Consequently, collagen fibers are laid down in a similar configuration, resulting in a fabricated ECM that can be contracted to reduce wound volume. Contraction of the ECM is performed by a differentiated form of fibroblasts, called myofibroblasts.[40,41] Identification of these cells involves the surface marker alpha-smooth muscle actin (α-SMA.) Two weeks after injury, about a half of the fibroblast population in the wound expresses this marker. Wound contraction typically begins around day 5 and continues for about 2 weeks, provided that there are no complicating pathological processes (such as infection or systemic disease.) It is important to note that in surgical wounds that heal by primary intention (wound edges brought together by sutures or the like), wound contraction is not typically observed.

At a molecular level, contraction is mediated by a set of cellular and extracellular proteins. Cellular proteins involved are (1) the transmembrane receptor protein integrin (particularly α_2, α_5, and $\alpha_1\beta_1$ subcomponents),[42,43] (2) the collagen cross-linking enzyme lysyl oxidase[44] and (quite possibly) (3) the transmembrane adhesion

protein OB-cadherin (which is expressed by myofibroblasts, but not fibroblasts.)[40] Aside from collagen, other important ligand proteins include fibronectin and vitronectin. Extracellular ligands fibronectin, vitronectin, and collagen, respectively, which are specific to integrin receptors, are involved in myofibroblasts adhering to, moving through, and contracting the ECM. Although distinctly different, the myofibroblast-ECM interaction (that leads to wound contraction) does bear some resemblance to the actin–myosin interaction (which leads to muscle contraction, but not relaxation.)

Endothelial cells and angiogenesis

As new stroma or granulation tissue forms (around day 4), new blood vessels are also formed to provide oxygen and nutritional support for the new tissue. Referred to as angiogenesis, this process is stimulated by vascular endothelial growth factor (VEGF), bFGF (basic fibroblast growth factor), and TGF-β.[14] These growth factors are released into the wound microenvironment by macrophages, epidermal cells, fibroblasts and endothelial cells, in response to hypoxia and high lactate levels.

The release of VEGF stimulates vascular endothelial cells to proliferate, leading to tubular sprouting.[22] As tubules transform into capillaries, angiogenesis is further driven by nitric oxide (NO), which is produced by local endothelial cells (via NO synthase.) Nitric oxide is a potent vasodilator which protects tissues from hypoxia and ischemia (although tissue hypoxia by itself also stimulates angiogenesis.)

The process of wound repair does not allow for proper reconstruction of dermal vascular structures. Formation of new microvessels proceeds in a manner that conforms to the overall framework of existing collagen matrix, which is dictated by fibroblast orientation. Histologically, vascular structures found in repaired dermis do not resemble those found in uninjured dermis.[13] In normal dermis, networks (plexus) of microvessels are typically found to be in parallel alignments to the epidermal surface; these microvessels have perpendicular offshoots of capillary loops that course within the confines of the mesenchymal cones (of the papillary dermis.) In contrast, microvessels found in repaired dermis are in perpendicular alignment to the epidermal surface; these microvessels have ill-defined plexus and capillary loops. As discussed above, these differences (between scar tissue and normal skin) are due to the intrinsic nature of the reparative response favoring a more rapid, contractile process over a slower, regenerative process.

Dermal matrix: elements of fabrication and alteration

In summary, the reconstruction of the acellular dermis requires fibroblast synthesis of collagen fibers, elastic fibers and ground substance, as well as the presence of ECM-remodeling enzymes. As alluded to earlier, under the effect of growth factors, particularly TGF-β_1, fibroblasts secrete collagen into the surrounding extracellular space (to form granulation tissue.) Intracellular assembly of collagen

molecules begins at the endoplasmic reticulum, with the hydroxylation of proline and lysine.[18,20] After modification and glycosylation, the triple-helical collagen molecules are transported to the cell membrane for release. Collagen fibers, found in granulation tissue, are of type I and type III. However, remodeling of the ECM ultimately results in scar tissue that contains mostly collagen I. Despite attaining greater amounts of collagen cross-linking over time, the maximum tensile strength of scar tissue (achieved during the third month) is no more than 70–80% that of normal skin.[44–45]

About two weeks after injury, when collagen deposits are in abundance, remodeling of the ECM begins. During this time, excess collagen fibers are removed and the remaining collagen fibers are reorganized, adding stability to the ECM and providing a more suitable microenvironment for cellular function (including wound contraction.)[18,20] This process may last between weeks and months, but occasionally can last for years, until equilibrium is fully achieved.[45]

Initial collagen degradation is performed by collagenase (produced by fibroblasts, macrophages and neutrophils.)[18,20] After partial degradation, collagen fragments undergo further breakdown, done by proteolytic enzymes, such as matrix metalloproteinases (MMPs.) These enzymes are secreted by fibroblasts, macrophages, endothelial cells and epidermal cells. Elevation in MMP levels may lead to excess collagen breakdown, resulting in the development of chronic wounds.[46,47]

Relative to collagen, elastic fibers play a much smaller role during wound repair. They are secreted in smaller amounts and at a considerably slower rate.[48] In wound beds, they provide additional sites for endothelial cell attachment and thus may serve as conduits for angiogenesis.[49] Moreover, it is thought that they exert mechanical strain onto attached endothelial cells or precursors, thereby inducing angiogenic growth factor production (further discussion follows.)

Ground substance, as aforementioned, consists mostly of glycosaminoglycans. Both sulfated and unsulfated forms are utilized during wound repair. However, scar tissues typically contain unsulfated versions, particularly hyaluronic acid.

4.4 Pathologic wound healing

Responses to injury may be: (1) physiological, (2) deficient, (3) excessive and (4) regenerative. Physiological response to injury leads to wound repair and scar formation, but abnormal responses can either lead to insufficient healing or excessive healing.

In deficient healing, inadequate deposition of matrix components and/or re-epithelialization occurs, leading to prolonged and incomplete healing.[18,50] Lack of restorative ability and maintenance of structural integrity often leads to chronic wounds. Clinical manifestations include generalized subcutaneous tissue loss (decubitus ulcer), failure to re-epithelialize (venous ulcer) and a necrosis–infection combination (diabetic ulcer.) Excessive infiltration of neutrophils is a significant

biological marker of deficient wound healing. Collagenase (such as MMP-8) and elastase, secreted by neutrophils, are responsible for destroying connective tissue and growth factors, respectively. Excessive use of exogenous corticosteroids, malnutrition, radiation, infection and systemic disease are some causes of deficient wound healing.

Typically seen as hypertrophic scars or keloids, excessive healing occurs when collagen deposits in the dermis (and subcutis) far exceed the amount seen in a typical scar.[51] This is often preceded by an amplified inflammatory response with the resultant overproduction of growth factors. Occasionally, excessive healing can lead to scar contracture – pathological shortening of (completely re-epithelialized and adequately healed) scar tissue (not to be confused with the physiologic process of wound contraction, although the two may be related).

The clinical distinction between hypertrophic scars and keloids is that hypertrophic scars remain within the confines of the wound, whereas keloids extend beyond wound boundaries.[52] Relative to typical scars, fibroblast and myofibroblast expression of TGF-β receptors are upregulated in keloids[53] and thus collagen levels are found to be 2–3 times higher.[18] Interestingly, compared to typical scars, hypertrophic scars contain a higher subpopulation of myofibroblasts, yet keloids appear to contain a significantly smaller myofibroblast subpopulation.[54] Histologically, it is not uncommon to find hypertrophic scars containing areas of keloidal scarring, thus raising the possibility of a biological interrelationship. Recent studies suggest that the development of hypertrophic scars and keloids are associated with an increased presence of fibrocytes, especially in burn patients.[31,55,56]

4.5 Comparison between fetal and post-natal skin

Presently, induced skin regeneration in adult mammalian wounds is partial rather than complete. Secrets for constructing a microenvironment, such that injured adult skin can be induced to regenerate fully, may lie in an in-depth understanding of how fetal skin heals after injury. In general, human fetal skin wounded before the third trimester has the capacity to heal without forming scars.[57–60] Hence, the third trimester is thought to be the transition period, between regenerative fetal healing and post-natal wound repair/scar formation. The ability of the early fetal skin to regenerate an 'exact' copy of damaged/lost tissue is attributed to several factors not seen in the adult counterpart.

4.5.1 Fetal environment and wound microenvironment

Fetal skin is bathed in an environment consisting of warm, sterile amniotic fluid that is enriched with growth factors, hyaluronic acid and other ECM molecules.[58] Nonetheless, such a sterile environment is deemed unnecessary for a regenerative process to occur.[57] An example of this may be illustrated through the ability of a marsupial fetus to heal without scars, while inside the mother's non-sterile pouch.[61]

Despite access to lower oxygenation, the rate of cellular proliferation is much faster in early fetal skin (when compared to adult skin.) The mechanism behind such efficient wound healing, in the presence of hypoxia, is not fully understood. It appears that the lack of inflammatory cells in the fetal wound microenvironment, the degree of fetal fibroblast differentiation, selective expression of cytokine isoforms (primarily TGF-β_3), and altered proteolytic enzymes are key factors that enable the early fetus to proceed towards a more regenerative pathway.[59,60]

Fetal inflammatory response

Unlike the inflammatory response associated with post-natal wounds, the early fetal immune system 'fails' to mount a true inflammatory response following injury.[58–60] Fetal platelets fail to aggregate and degranulate. Likewise, the few fetal neutrophils and fetal monocytes/macrophages present are immature, lacking phagocytic and chemotactic potential.

Growth factors found in early fetal wounds, either have different isoforms (from those typically seen in adult wounds), or are at a different level of concentration. Most strikingly, early fetal wounds contain high levels of the TGF-β_3 isoform. TGF-β_3 downregulates (pro-fibrotic) TGF-β_1 and TGF-β_2 levels, thereby shifting the healing response away from a scarring pathway and towards more regenerative pathway.[58] Differences in levels of PDGF, FGF and VEGF are also observed between early fetal wounds and post-natal wounds.[59,60] Although (pro-fibrotic) PDGFs and FGFs are present in early fetal wounds, they dissipate within 24 hours of injury. Elevated levels of VEGF in early fetal wounds are thought to promote a more rapid angiogenic process.

Fetal fibroblasts and fetal wound matrix

Fetal fibroblasts have innate properties (not seen in adult fibroblasts) that enable them to repair wounds without scarring. Furthermore, the wound microenvironment, in which they function, contains elements (such as high levels of hyaluronic acid) that favor regeneration over repair.

Hyaluronic acid (HA), as aforementioned, is the main component of ground substance and thus is a major component of ECM. HA is also a ligand for HA-receptors, which are upregulated in fetal fibroblasts. HA-receptor transduction is associated with cellular migration, adhesion and proliferation. Thus, in this context, in which levels of HA remain elevated for three weeks in early fetal wounds (versus low levels for less than a week in adult wounds), fetal fibroblasts migrate, proliferate and deposit collagen faster than their adult counterparts.[59] Moreover, fetal fibroblasts have the unique ability to proliferate concurrently and synthesize collagen (not seen in adult fibroblasts.) Owing to the inhibitory effects of TGF-β_3 on collagen I production, collagen deposits in early fetal wounds are predominantly of type III.[58]

Homeobox gene expression

Mechanistic differences between early fetal wounds and adult wounds are ultimately regulated by a set of genes.[59,60] Collectively called homeobox genes, these transcription factors are thought to regulate morphogenesis during development. Eight of these genes (HOX-A4, HOX-A5, HOX-A7, HOX-B13, MSX-1, MSX-2, MOX-1, PRX-2) are known to be expressed by normal fetal cells. Two of these genes (PRX-2 and HOX-B13) are associated with scarless repair of early fetal wounds. Following injury to fetal skin, fetal fibroblast expression of PRX-2 is upregulated, coupled with HOX-B13 downregulation, thereby insinuating an activation–deactivation mechanism (such that PRX-2 promotes regeneration and HOX-B13 promotes scarring.)

4.6 Wound repair versus regeneration: fundamental differences

Within the realm of wound healing, there is a subtle distinction between 'repair' and 'regeneration'. Theoretically, all tissues are capable of repairing injuries.[62] An injury may be defined as an interruption of continuity of the morphology and/or functionality of a given tissue. Repair, more specifically, refers to the physiologic adaptation of an organ after injury, in an effort to re-establish continuity without regard to exact replacement of lost/damaged tissue.[41] True tissue regeneration refers to the replacement of lost/damaged tissue with an 'exact' copy, such that both morphology and functionality are completely restored. Unlike certain organisms (such as newts), the organs of post-natal mammals do not regenerate spontaneously. In some instances, such as skin, 'partial regeneration' may be induced via use of exogenous agents, particularly biological matrices.

Currently, experimentally induced, regenerated skin tissue resembles normal skin in form and function, yet it still lacks the presence of appendages.[41] Nevertheless, the technology has immense value in clinical medicine and will be discussed below. Table 4.1 summarizes some key similarities and differences between wound repair/scar formation and complete regeneration.

4.6.1 'Essential ingredients' for skin regeneration

As alluded to earlier, adult mammalian skin cannot regenerate spontaneously. As a result, much of our understanding of how regeneration occurs has come from models of scarless fetal healing.[41,57–60] Using knowledge from those studies, inferences can be made about the 'ingredients' necessary to promote tissue regeneration in adult wounds. That is, for an adult wound to proceed towards a regenerative pathway, its microenvironment (including exogenous materials) must meet a set of criteria specific to regeneration.

Unlike regeneration, repair is considered the 'default' healing response, with a

Table 4.1 Fundamental similarities and differences between scar and regenerated skin

	Repair/scar formation	Regeneration
Intact epidermis	Yes	Yes
Adnexa	Absent	Present
Rete ridges	Absent	Present
Basement membrane	Present	Present
Fibroblast orientation	Parallel to epidermis	Quasi-random
Myofibroblast presence (contractile fibroblast)	More pronounced	Less pronounced
Collagen organization	Parallel	Quasi-random
Collagen I to collagen III ratio	> 4:1	1:4
Vascular orientation	Perpendicular to epidermis; ill-defined plexus and capillary loops	Parallel to epidermis; well-defined plexus and capillary loops
Relative tensile strength	70–80%	100%
Function	Suboptimal	Normal

Table 4.2 Potential 'essential ingredients' for regeneration

	Required for regeneration
Sterile environment	No
Inhibition of platelet activation	Yes
Inhibition of inflammatory response	Yes
Inhibition of growth factors TGF-β_3, PDGF and FGF	Yes
Inhibit fibroblast differentiation to myofibroblast	Yes
Inhibition of wound contraction	Yes
Source of epidermal and mesenchymal stem cells	Yes
HOX B13 and PRX-2 gene expression	Yes
Surgical conversion to full-thickness wound	Yes
Dermal regeneration template (pore size 20–125 μm)	Yes

smaller set of criteria (which minimally overlap with the regeneration set.) It is important to note that although the conditions required for regeneration and repair are diametrically opposed, they are not necessarily mutually exclusive. However, fundamental differences between repair and regeneration (such as spatial orientation of cellular and acellular elements, as well as wound contraction) allow the inclusion of conditions (that oppose repair) in a list of 'essential ingredients' needed to induce regeneration. Table 4.2 contains a set of potential requirements necessary for the healing response to proceed towards the regenerative pathway.

4.6.2 Fibroblasts and matrix assembly

Histologically, truly regenerated skin is indistinguishable from normal skin (see Figs 4.1 and 4.2 and Table 4.1.) To summarize, fibroblasts in uninjured dermis are orientated in quasi-random axes. Thus, collagen fibers and other cell products are 'randomly' dispersed in the dermis. In contrast, fibroblasts in scar tissue are assembled in parallel planes, with respect to the epidermal surface.[13] Consequently, collagen deposits are arranged in the same manner, thereby permitting effective ECM contraction.

4.6.3 Vascular differences

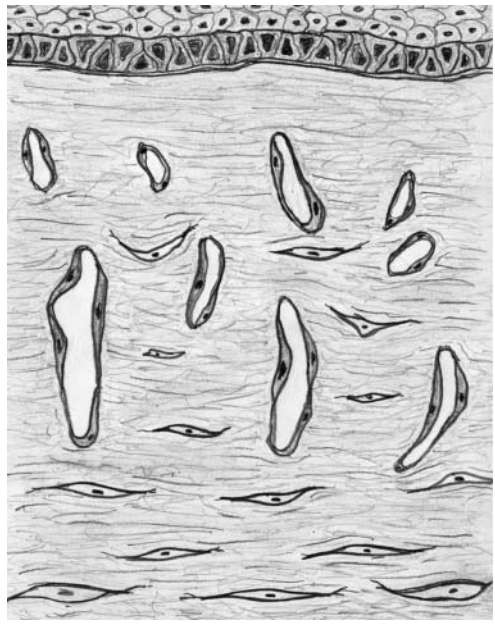
Vascular structures, formed through the process of regeneration, should have structural features that are indistinguishable from those found in normal skin (as aforementioned.) In reference to a horizontal epidermal surface, microvessels in normal/regenerated dermis are organized in a horizontally aligned plexus, with vertical offshoots that form capillary loops (within mesenchymal cones of papillary dermis.) In a remarkable contrast, repaired dermis contains microvessels that typically course along parallel axes/planes, perpendicular to the epidermal surface. These vessels tend to form an ill-defined plexus and give rise to ill-defined capillary loops.

4.6.4 Dermal regeneration template (DRT)

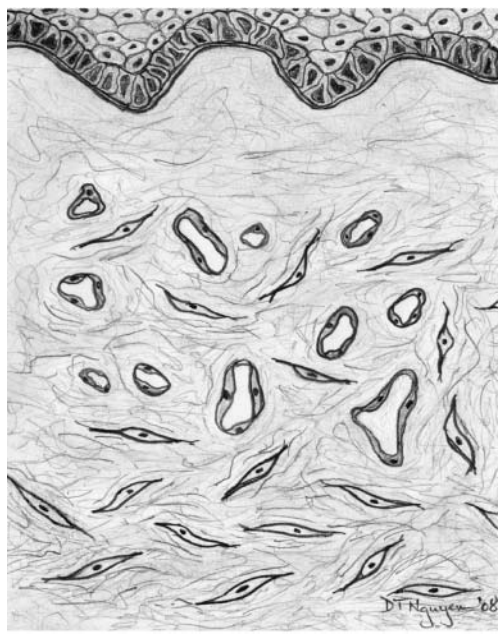
Although complete regeneration of adult skin has not been achieved experimentally, partial regeneration can be attained (by blocking contraction) using a biologically active scaffold.[41] Termed dermal regeneration template (DRT), these sponge-like scaffolds are typically made from collagen-GAG copolymers and thus are biodegradable during the regenerative process. DRTs can also accommodate the seeding of fibroblasts and/or keratinocytes.

The use of DRTs in the treatment of skin loss requires a two-step procedure. That is, after a wound is surgically debrided and prepared, a DRT is grafted into the defined region (first step). At a later date, when the wound is adequately filled with granulation tissue, a split-thickness (autologous) skin graft is gently placed over the new tissue to provide wound coverage (second step.) Alternatively, a return to the surgical suite may be avoided if a composite graft is used; a composite graft comprises a DRT and a thin silicone layer (which serves the role of the epidermis and is subsequently peeled off.)

When compared to untreated wounds, wounds treated with DRTs show about 20% reduction in wound contraction (and an approximate two-week delay in wound closure.)[37] DRT-treated tissues exhibit non-scarring, regenerative characteristics and a lower myofibroblast subpopulation (in comparison to untreated tissues.)[13] In addition to having the functional properties of unseeded DRTs,



4.1 Schematic of representation of fibroblast and endothelial distribution of wound repair/scar formation.



4.2 Schematic of representation of fibroblast and endothelial distribution in wound regeneration.

seeded DRTs have been shown to arrest (and possibly reverse) contraction altogether, allowing for a greater degree of regeneration.[37] Although they lack appendages, adequately healed DRT-treated tissues 'look and feel' similar to normal skin.

Spatial, mechanical and biological considerations for scaffolds

For cutaneous wounds to regenerate, the geometric properties of both the defect and the material (used to patch the defect) are of paramount importance.[41] The defect should have unambiguous, physically contained anatomical boundaries that prevent loss of exudates, as well as entry of bacteria and extraneous tissues. A full-thickness (no dermis remaining) wound would satisfy this criteria, whereas a partial-thickness wound (some dermis remaining) would not.

In general, mechanical properties of DRTs should resemble those of normal skin.[23,41] DRTs must maintain the elasticity, pliability and durability seen in normal skin. At the same time, they must rapidly and uniformly adhere to the underlying tissue. The scaffold pore size (20–125 μm) must not restrict cellular infiltration.[37] Scaffold fibers must allow for cellular attachment. DRTs should be non-toxic and non-antigenic, such that a minimal immune response is elicited. They should also be biodegradable, but stable enough to withstand host enzymes until regeneration has been irreversibly initiated.

Trophic and growth factors

Trophic factors and pharmacological agents, such as cortisone, prednisone and aspirin have not been shown to aid regeneration.[41] Likewise, the external application of growth factors (including PDGF, bFGF, and TGF-β) has not been shown to facilitate regeneration. On the contrary, these pro-fibrotic molecules may inhibit regeneration (as mentioned earlier.)

4.7 Issues of stem cells and cellular plasticity

Virtually all tissues in the human body, in theory, have the ability to regenerate. This ability relies on a group of multipotent adult stem cells that have the capacity to be self-renewing and to give rise to different cell types.[10,15] Stem cells give rise to progenitor cells, which are cells that are not self-renewing, but can generate several types of cells.

The extent of stem cell involvement in cutaneous wound healing is complex and not fully understood. As alluded to earlier, epidermis and dermis are reconstituted by mitotically active stem cells that reside in regions within the skin (called niches.)[10] These regions include the apex of rete ridges,[35] the bulge of hair follicles[17] and the papillary dermis.[66] Moreover, bone marrow may also contain stem cells that play a major role in cutaneous wound healing.[12]

Up until a decade ago, the classic paradigm of wound healing, involving stem cells restricted to organ-specific lineages, has never been seriously challenged.[64,65] Since then, the notion of adult stem cells having 'plasticity' or the ability to differentiate into non-lineage cells has emerged as an alternative explanation. To be more specific, hematopoietic progenitor cells (which give rise to mature hematopoietic cells) may have the ability to 'de-differentiate' back into hematopoietic stem cells and/or 'transdifferentiate' into non-lineage cells, such as fibroblasts.

4.7.1 Basal stem cells (BSCs) and hair follicular stem cells (HFSCs)

It is thought that the epidermis contains two (or perhaps three) groups of self-renewing cells that have the capacity to generate new cells to replenish the epidermal cell population.[10,17] One group (BSCs) resides at the apex of rete ridges (of basal cell layer.) These cells are responsible for generating cells residing outside the hair follicles, sometimes referred to as the interfollicular epidermis (IFE.)

The other group (or perhaps two groups) is responsible for generating hair follicle cells and sebocytes (sebaceous gland cells.)[35] Known as hair follicular stem cells (HFSCs) or bulge stem cells, these cells reside at the hair bulge (region of the outer root sheath.)[15,17] They appear to be multipotential and may have the capacity to generate all subtypes of epidermal cells. Although their regenerative potential is important, HFSCs are non-essential to epidermal homeostasis, as illustrated by the absence of appendages in scar tissue.

4.7.2 Skin-derived precursors (SKPs): dermal stem cells

Recently, a dermal-derived, multipotential stem cell population has been described.[63,66] Known as skin-derived precursors (SKPs), these cells are thought to reside in the papillary dermis and the dermal sheath of hair follicles.[66] In mice, SKPs are typically isolated from normal skin, but in humans neonatal foreskin has been proven to be a superior source.[63]

Interestingly, skin-derived precursors can be manipulated to differentiate along both mesodermal and neural cell lines *in vitro*.[63] SKPs exhibit features similar to embryonic neurocrest cells[66] and can also give rise to neurons, glial cells (Schwann cells), smooth muscle cells and adipocytes.[67] In the presence of fibroblast growth factor and epidermal growth factor, SKPs express nestin (intermediate filament, a neural stem cell marker), fibronectin and vimentin (intermediate filament), as well as embryonic-like transcription factors. The presence of TGF-β seems to promote only proliferation and not differentiation or progency.[67]

4.7.3 Bone marrow-derived stem cells and progenitors

Recent studies indicate that in rare circumstances, such as extensive cutaneous injury, self-renewing subpopulations in the bone marrow are induced to participate in the healing process, whereby they give rise to collagen-secreting cells that seem to play a role during wound repair. These two self-renewal subpopulations are (1) bone marrow-derived mesenchymal stem cells and (2) hematopoietic stem cells.[12,68,70–86,99,100] Bone marrow (BM) also harbors a progenitor subpopulation (endothelial progenitor cells) which, in the same type of setting, are mobilized to aid in the reconstruction of blood vessels.[69,92–98,101–103,107] Moreover, it is thought that, extensive injury to skin also promotes the early trafficking of a unique subclass of leukocytes (circulating fibrocytes) to the injured region, where they perform various functions related to wound healing.[26–32,55,56,88]

Bone marrow-derived mesenchymal stem cells (BM-derived MSCs)

The stroma of bone marrow is home to a group of multipotent stem cells, commonly known as BM-derived MSCs (or simply MSCs.)[10,78,80] MSCs are responsible for generating progenitors that give rise mainly to stromal cells, hepatocytes, adipocytes, myocytes, chrondrocytes and osteocytes. Although MSCs do not generate hematopoietic progenitors, they do give rise to stromal cells that support the development of HSCs. Several non-specific markers are used to identify MSCs, including adhesion markers CD44, CD29 and CD90. In addition, they must not express hematopoietic markers CD34 and CD45.

Despite limited clinical application of MSCs, their potential use in regenerative medicine has created much interest.[81,82] MSCs may have the ability to suppress T-cell activation and proliferation and thus could become a potential treatment for allograft rejection, graft-versus-host disease and autoimmune conditions.[81,83,84] Other potential uses include treatments for myocardial infarction[85] and spinal cord injury.[86]

Hematopoietic stem cells (HSCs)

The other group of multipotent stem cells (found in the BM) is HSCs. Unlike MSCs, clinical applications of HSCs have been met with some success.[87] They are used routinely in the treatment of leukemia, lymphoma, inherited blood disorders (aplastic anemia, β-thalassemia, SCID) and cancer chemotherapy rescue. Although relatively easy to culture, it is not yet known if using cultured HSCs to treat skin injury is efficacious.

HSCs play a critical role in maintaining the homeostasis of the circulating system.[87] Accordingly, all hematopoietic subpopulations are replenished by progenitors that originate from HSCs. Markers used to identify human HSCs are CD34, CD59, Thy-1 and c-kit (low.) Mice HSCs require a different set of markers: Sca-1, Thy-1, CD38, c-kit and CD34 (low.)

HSCs are thought to have some degree of 'plasticity' and under unique conditions (as aforementioned), may be influenced to take on a role not commonly attributed to them. In the setting of cutaneous injury, HSCs and BM-derived MSCs are induced to participate in the healing response, whereby they generate a subpopulation of fibroblasts (BM-derived fibroblasts), capable of collagen production.[12,70–85] Mechanisms by which HSCs and BM-derived MSCs give rise to BM-derived fibroblasts are 'differentiation' and 'transdifferentiation', respectively, with the latter requiring lineage conversion.

Although BM-derived fibroblasts may have collagen-producing capabilities, their contribution to ECM reconstruction remains to be fully described. While both subpopulations of fibroblast (dermal and BM-derived) are capable of transcribing for collagen I, it is thought that only BM-derived fibroblasts can transcribe for collagen III (an important distinguishing factor for regenerative studies.)[79] In addition to skin, HSCs also participate in the injury responses of other organs, including liver, heart and skeletal muscle.[10]

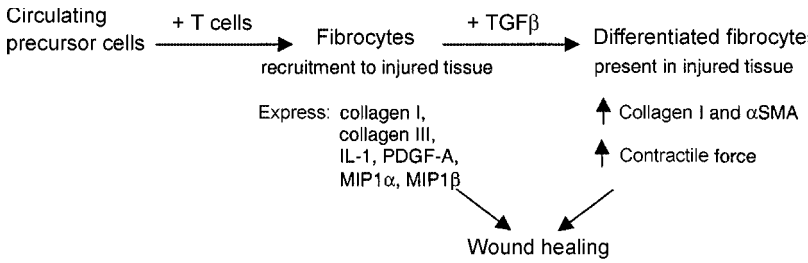
Circulating fibrocytes

The process by which HSCs generate progenitors capable of 'transdifferentiating' into progenitors of BM-derived fibroblasts is not well-understood and remains controversial. As alluded to earlier, there is some evidence to suggest that circulating fibrocytes may be an intermediate between HSCs and BM-derived fibroblasts.[27–29,32] Fibrocytes may have plasticity potential and in the context of variable hybrid phenotypes and associated functions, it is conceivable that they are indeed the transitional cell of interest.

While much focus on fibrocytes refers to cutaneous wound healing, the strongest evidence in support of fibrocytes as an intermediate between HSCs and BM-derived fibroblasts is found in studies of pulmonary fibrosis.[32,88–91] Figure 4.3 summarizes the many elements of fibrocytes and a possible transdifferentiation pathway.[28] Some observations linking fibrocytes as a transitional form include (1) early preference for CD34 and CD45 expression, (2) early APC function, (3) production of migration and proliferation cytokines, (4) sequential downregulation of CD34 and CD45 in response to TGF-β_1, coupled with upregulation of collagen I and α-SMA and (5) late production of collagen and collagnase.[89]

Endothelial progenitor cells (EPCs)

Although their contribution to cutaneous wound healing is not clearly defined, BM-derived progenitor cells with angiogenic properties have been described.[69,92–97] Known as endothelial progenitor cells (EPCs), these cells are known to express CD34, CD31 (also known as platelet-endothelial cell adhesion molecule one or PECAM-1), CD144 and VEGF receptors. Furthermore, they express the transcription factor GATA-2 (associated with hematopoiesis.) They are reportedly



4.3 Proposed differentiation pathway of circulating fibrocytes (Abe *et al.*, 2001,[28] reproduced with permission)

capable of generating new endothelial cells, especially in ischemic tissues.[94] EPCs are thought to participate in both angiogenesis and neovascularization.[12,69,94–100] They are capable of attaching themselves to injured (mature) endothelial cells[101–102] and ECM fibers.[49] They promote tubular spouting via secretion of angiogenic growth factors (such as VEGF.)[101–103] EPC production of angiogenic growth factors is thought to be induced by the mechanical strain exerted on them by resident endothelial cells[102–104] and ECM fibers.[49,105,106] Although uncertain, it appears that BM-derived endothelial cells are not incorporated into the long-term architecture of the dermal vascular network.[12,107] Hence, dermal blood vessels in scars are lined by resident endothelial cells.

4.8 Historic developments and future trends

Efforts towards achieving effective cutaneous regeneration had their inception in the 1970s. In 1971, J.F. Burke (of Massachusetts General Hospital) collaborated with I.V. Yannas (of Massachusetts Institute of Technology) to design a scaffold, intended for use on patients with severe burn injuries, especially those without sufficient skin surface (<50% viable skin) for autologous skin grafts. In 1975, they succeeded in synthesizing a biodegradable scaffold composed of collagen-GAG copolymers. These DRTs were grafted onto wounded guinea pig skin, where diminished wound contraction, coupled with partial regeneration was observed.[108,109] The efficacy of the scaffold is related to fiber composition, fiber size, pore size and the spatial orientation of individual fibers (facilitating cell attachments along more-physiological axes.)

In 1980, clinical trials (with positive results) were conducted on burn patients.[109,110] In 1989, Yannas and Salzman developed a method of selectively 'melting' the quaternary collagen structure, without affecting tertiary structure.[111] Known as 'collagen banding', this process improved DRTs by adding an anti-platelet clotting property. In the 1990s, Yannas and Orgill (of Brigham and Women's Hospital) developed composite grafts (DRT with a removable silicone membrane), thereby reducing a two-step grafting process to one and, in addition, allowing for seeding of cultured autologous keratinocytes.[112] After extensive clinical trials, the Food

and Drug Administration (FDA) approved DRTs (commercially known as Integra™) for the treatment of life-threatening burn injuries (in 1996) and the reconstruction of scar contractures (in 2002.)

During the 1970s, and through the early 1980s, there were several independent groups (in addition to the Yannas–Burke collaboration) involved in researching and developing regeneration templates and skin substitutes. One group was led by Harvard biologists Howard Green and J.G. Rheinwald. Their focus concentrated on the development of neoepidermis (single layer) from dissociated keratinocytes[113] and cultured fibroblasts.[114] Their work resulted in cultured epidermal autograft (CEA), also called Epicel.™ Although CEA met with some success, its application relies on the structural support of the underlying dermis and BM. While the epidermis regenerates spontaneously, it cannot do so without an underlying buttress to support the migrating epidermal cells.[37] Without anchoring, neoepidermis avulsion inevitably occurs within weeks of its application. Another group, lead by MIT biologist Eugene Bell, focused on culturing neonatal fibroblasts and keratinocytes in a medium that eventually becomes a 'solid' mass of collagen, which can then be applied onto wounds.[115,116] These cells were derived from neonatal foreskin and thus had low antigenic properties. Later known as Apligraf,™ this product has met with some success and was approved by the FDA in 2001 for clinical use.

Although great strides have been made in the field of cutaneous regenerative medicine and tissue engineering, the technology itself is still in its infancy. Research efforts are currently focused on developing biodegradable grafts, involving the relevant regenerative templates that incorporate skin cells into scaffolds (made from natural or synthetic fibers.)[23,117–119] Although these approaches have produced promising results, clinical use has been limited primarily to burn patients. The reasons for their limited use include economic considerations and comparable results for autologous skin grafts. Owing to their low cost, availability and proven success, autologous skin grafts (particularly split-thickness skin grafts) are still the mainstay in the treatment of chronic wounds, traumatic wounds, pressure wounds and burn injuries.

With the advent of stem cell technology and gene therapy, it is not unreasonable to anticipate that new approaches (to the treatment of skin loss) will appear in the near future. Specifically, using the technology of biodegradable DRTs, with the infusion of relevant stem cells (with more embryonic or regenerative potential), may hold significant promise. Recently, it was reported that adult mouse fibroblasts can be 're-programmed' to become cells with embryonic stem cell characteristics (*in vitro.*)[120] By introducing four genes (c-Myc, Oct4, Klf4 and Sox2) into adult fibroblasts, via a retrovirus, these altered cells exhibit embryonic stem cell phenotypes, including the presence of all three germ layers. Although it is premature to envision such technology being applied to a clinical setting, guarded optimism is warranted for its evolution towards a stem cell-seeded, cost-effective dermal substitute that promotes efficient cutaneous regeneration.

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5

Skin grafts

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Abstract:: The ideal replacement for missing skin is skin itself. To date, there remains no permanent off-the-shelf replacement that provides the physical and physiologic functions of human skin. This chapter will discuss the biology of skin, the history of skin graft use, the immunology of skin and, finally, the techniques of autograft, allograft and xenograft usage.

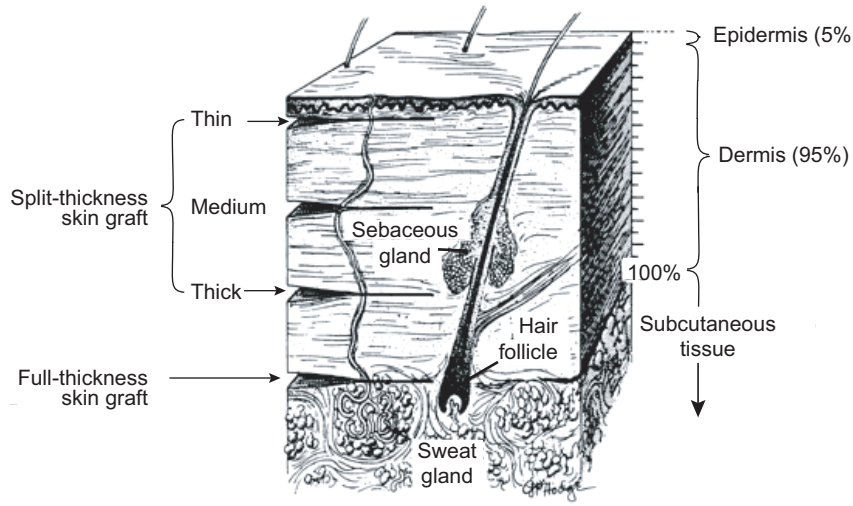
Key words: autologous skin graft, allograft, xenograft, full thickness skin graft, split thickness skin graft.

5.1 Definitions

Several terms are pertinent to this discussion and require definition. A *graft* is tissue separated from its donor bed and relies on nutrient delivery from the recipient tissues. An *autograft* (or autologous graft) refers to tissue transplanted from one location to another in the same individual. *Isograft* refers to tissue transplanted between genetically identical twins. An *allograft* (termed *homograft* in older texts) is tissue transplanted between unrelated individuals of the same species. A *xenograft* (termed *heterograft* in older texts) is tissue transplanted between individuals of different species. *Split thickness skin graft* refers to skin grafts that contain all of the epidermis and a variable amount of the dermis; whereas *full thickness skin grafts* are grafts that contain epidermis and all the dermis and associated dermal appendages.

5.2 Skin anatomy and physiology

The skin performs numerous important roles. It provides a protective layer against mechanical, chemical and microbiologic insults and it performs crucial roles in thermoregulation and ultraviolet radiation protection. In addition the skin is a primary sensory organ and is important in vitamin D metabolism. The skin consists of two distinct layers: the epidermis and the dermis (Fig. 5.1). The outermost layer, the epidermis, is derived embryologically from ectoderm. Similar to other ectodermally derived structures, the epidermis is capable of regeneration. Wounds involving only the epidermis heal by regeneration of epidermal cells not only from



5.1 The anatomy of skin (from Place *et al.*, 1997).

the wound's periphery but also from skin adnexal structures, including hair follicles, sebaceous glands, and sweat glands. Given the ability of the epidermis to regenerate, pure epidermal wounds heal without scarring (Paletta *et al*., 2006).

The primary cell of the epidermis, the keratinocyte, forms five layers, or strata, representing its progressive differentiation, a process known as cornification. The five strata from deep to superficial are the stratum basale (i.e. the basement membrane), stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum. The stratum corneum is a layer of non-viable cells that provides a protective layer from the environment. Tight intercellular connections within the epidermis form the basis of its physical integrity and lamellar granules, containing sterols, polar lipids and hydrolytic enzymes, released into the intercellular space give the epidermis its impermeable quality (Landmann, 1986; Paletta *et al*., 2006). Three other cell types populate the epidermis: the melanocyte, the Langerhans cell and the Merkel cell. Melanocytes reside in the basal layer of the epidermis (the melanocyte to keratinocyte ratio is 1:10) (Cochran, 1970). Melanocytes produce pigment melanin containing vesicles known as melanosomes, which are in turn phagocytosed by nearby keratinocytes. The primary role of melanin is to protect the skin from the harmful effects of sunlight (Boissy, 1988). The Langerhans cell resides in the middle layers of the epidermis and is important in immune surveillance and allograft rejection (Choi and Sauder, 1986). Merkel cells reside in the epidermis of the palms and soles, nail beds, and oral and genital epithelium and serve as mechanoreceptors in a close relationship with neurons.

In comparison to the epidermis, the dermis is relatively non-cellular, composed primarily of collagen, elastic fibers and ground substance. The dermis contains all of the nerves, vessels, lymphatics and most of the glandular elements of the skin. The dermis is between 15 to 40 times thicker than the epidermis; however, given that it is less cellular, the dermis consumes less energy than the epidermis. Its embryologic origins are mesodermal and like most mesodermal derivatives, the dermis is incapable of true regeneration in postnatal life. In contrast to the epidermis, the dermis heals through a process known as scarring.

Structurally, the dermis is a complex network of cellular and acellular components. Collagen is the major acellular structural component of the dermis, constituting approximately 70% of the skin's dry weight. Type I and type III collagens represent 80% and 15% of the total dermal collagen, respectively. Elastic fibers comprise approximately 2% of the dry weight of the skin. They play a crucial role in wound contraction after a loss of skin integrity. Finally, glycosaminoglycans (GAGs) are the third major acellular component of the dermis. The principal GAGs in the dermis are heparin and heparan sulfate. Major functions of GAGs include the binding of water and cationic molecules, serving as cofactors for multiple enzyme pathways and participating in cell adhesion and basement membrane formation (Cuono, 1988). In addition, two important dermal GAGs, chondroitin sulfate and dermatan sulfate have been implicated in endothelial proliferation during wound healing (Faham *et al*., 1996).

The major cell type of the dermis is the fibroblast. It is responsible for both the synthesis and the degradation of dermal proteins. In addition, the dermis contains a small number of hematopoietic derived cells including macrophages, mast cells, lymphocytes and eosinophils. Other cell types found in the dermis included those associated with the vascular, lymphatic and nervous systems, and the epidermal appendages.

The mature dermis can be divided into two main layers: the superficial papillary layer and the deeper reticular layer. The papillary dermis contains a disorganized collection of collagen bundles, elastic fibers, fibroblasts and ground substance. A highly developed microcirculation in the papillary dermis provides a blood supply for the overlying epidermis (the epidermis has no inherent blood supply). The papillary dermis and the basal layer of the epidermis are intimately related at a region known as the basement membrane zone (BMZ). Here at the dermal–epidermal junction, projections of the dermis, known as dermal papillae, interdigitate with in-pockets of epidermis known as rete ridges. In contrast, the reticular dermis is less cellular. Within the dermis is a combination of mucopolysaccharides, chondroitin sulfate and hyaluronic acid, which forms the ground substance and takes on a gel-like consistency; with age it is gradually replaced by fibrous tissue.

An understanding of the biology and physiology of skin is paramount to

understanding skin grafting. The skin forms a continuous protective layer over the entire surface of the body. The character of skin is variable between individuals and between different body regions of the same individual based on age and sun exposure. During the first decade of life, skin is thin but progressively thickens until the middle of the fourth decade. By middle age, the dermis undergoes gradual thinning and a decrease in elasticity and sebaceous gland content. Within the same individual, skin varies by body region. The skin of the eyelid, post-auricular and supraclavicular areas, and medial thigh is thin in comparison to the much thicker skin of the back, palms of the hands and soles of the feet.

5.3 Autologous skin grafts

5.3.1 History of skin grafts

The history of skin transplantation mirrors the evolution in the understanding of skin anatomy, physiology, the biology of wound healing and the immunology of transplant rejection. The earliest reports of skin grafting date to the 3rd century BC; the *Sushruta Samhita* sanskrit texts document skin transplantation in ancient India. Ancient texts describe Koomas caste members (the potter and tile-making guild) performing nasal reconstruction after mutilation as punishment for crimes (Bhishagratna, 1963). Subsequent reports of skin transplantation did not emerge until the 15th century. Brancas, and later Tagliacozzi, report the utilization of skin grafts for nasal reconstruction of facial battle wounds and tissue infection caused by syphilis. A seminal work by Tagilacozzi in 1597 in *De curtorum chirugia per isitionem*, established his role as the pioneer of modern plastic surgery (Herman, 2002).

Prior to the 1800s reports of skin grafting in Western medicine were anecdotal and not widely accepted. The Italian Guiseppe Baronio famously described successful autograft transplantation of sheep skin in 1804. Notably, Baronio's experiments with transferring skin from a mare to a cow failed – hinting at the immunologic barriers in xeno-transplantation to be elucidated in the future (Davis, 1941). In 1817, British surgeon Astley Cooper successfully removed skin from an amputated thumb and covered the base of the remaining stump with the full thickness skin graft. In 1823, Bunger revived ancient Indian methods of nasal reconstruction by repairing nasal defects with full-thickness skin grafts (Patterson, 1977, Chick, 1988). Swiss surgeon Jacques Reverdin is credited with performing the first epidermal allograft ('pinch grafts') and the first split-thickness skin graft, in 1869. His work demonstrated that skin transfer from a donor site to an open wound in the same individual not only survived but hastened healing. Two years later, Ollier furthered Reverdin's work by demonstrating a better skin graft outcome with faster wound healing and less scarring by using grafts composed of epidermis and dermis (Ollier, 1872).

As an extension of Reverdin's work, Girdner published the first report of skin grafting using human cadaveric skin (Girdner, 1881). He employed allograft skin,

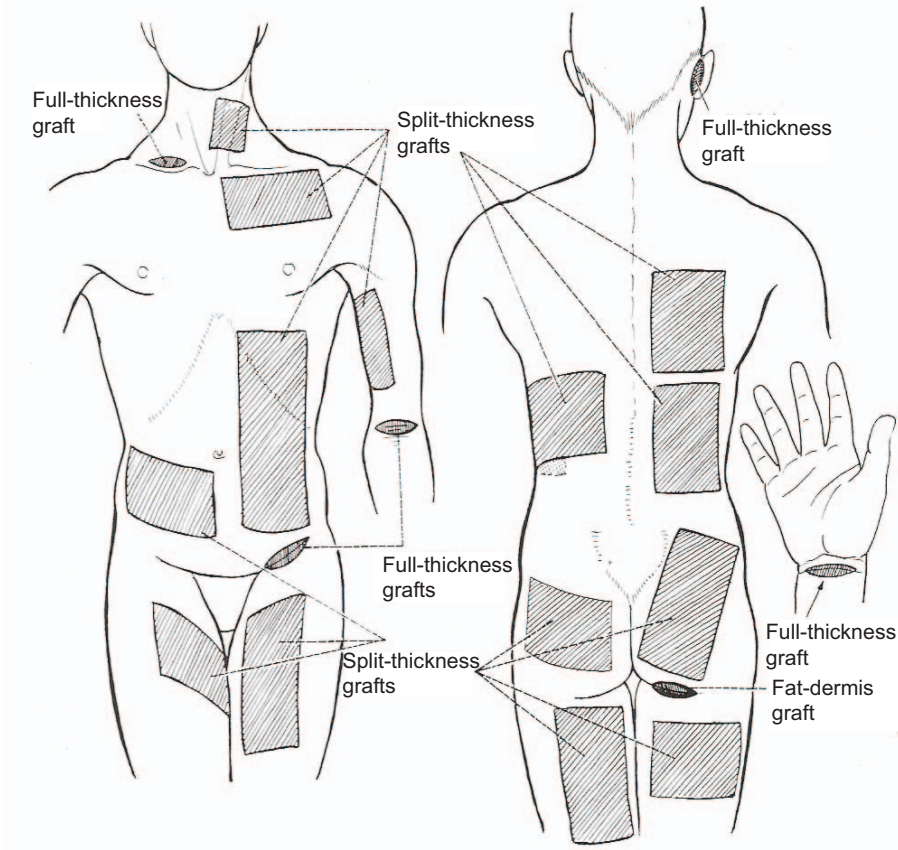
taken from a suicide victim, to treat a patient with a severe burn from a lightning injury and reported an immediate take of 75%. German surgeon Carl Thiersch first recognized the importance of preparing the recipient bed. In 1874, he described the removal of granulation tissue from the wound before graft application, which dramatically improved the success of engraftment (Thiersch, 1874). At the turn of the 19th century, skin graft use was not widely accepted owing to unreliable success, difficulty with harvesting and the belief that skin grafting creates two wounds from one (i.e. the donor site and the wound needing coverage). An important advancement occurred with the advent of meshing the harvested skin by Swedish surgeon, Otto Lanz, enabling the combined grafting of the donor site and the wound (Paletta *et al*., 2006). However, it was not until Saint Louis plastic surgeon James Barrett Brown and oral surgeon Vilray Papin Blair described their skin graft techniques did reproducible results from skin grafting gain wide acceptance. Brown and Blair distinguished between full-thickness, intermediate-thickness and epidermal grafts and, importantly, they showed the reliable healing of donor sites if a portion of the dermis was removed, a common misconception (Blair and Brown, 1929; Brown and McDowell, 1949).

Once the principles of successful skin grafting were disseminated, the primary challenge of skin grafting was the harvest. The first device designed for skin harvesting was developed by Humby in 1936. Prior to this grafts were generally harvested with a razor or long blade in a freehand fashion producing grafts of variable thickness. The Humby knife is a guarded razor which prevented surgeons from harvesting grafts too thick; however, it still did not allow for fine control of graft thickness (McDowell, 1977). During the era of World War II when the demand for a quick and consistent method was required by the army, Earl Padgett, an American surgeon from Kansas, in collaboration with an engineer named George Hood, developed the first dermatome to allow skin graft harvest of precise thickness in 1939 (Padgett, 1939).

The history of skin graft use is intimately associated with the treatment of burn injury. The first reported use of skin grafts to cover burn wounds was introduced by Pollock in 1871. He courageously donated small portions of his own skin in combination with the burn victim's skin to cover a large burn, establishing one of the most important modern functions of skin grafts (Freshwater and Krizek, 1978; Pollock, 1871). The use of skin grafts has revolutionized the care and, importantly, the morbidity and mortality of burn patients. World War II provided impetus for the use of refrigerated skin as a temporary dressing (Webster, 1944), the foundation of the first skin bank in the United States (Trier and Sell, 1968) and the discovery of a cryo-preservative agent that permitted freezing of tissue and subsequent thawing in a viable state (Polge *et al*., 1949).

5.3.2 Principles and techniques of autologous skin grafts

Autologous skin grafts are broadly categorized based on their thickness as full or



5.2 Possible skin graft donor sites (from Rudolph and Balantyne, 1990).

partial-thickness. Regardless of graft type, all require a vascularized wound bed. Full thickness skin grafts have several advantages, including a better cosmetic result, with less associated contraction, in comparison to thinner grafts, but require a healthier, more vascularized bed. The size of a full-thickness skin graft is limited if primary closure of the graft donor site is desired. Common full-thickness skin graft donor sites include post-auricular, pre-auricular, supra-clavicular, anticubital fossa, inguinal crease and volar wrist crease skin (Fig. 5.2). In cases of elective reconstruction, larger full thickness grafts can be obtained by tissue expansion of the planned donor site. This typically cannot be done for treatment of an acute wound given that several weeks are typically required for adequate expansion. In this situation, the full-thickness skin graft donor site may need to be closed with a split-thickness graft from a third site.

Full-thickness grafts are most commonly used on the face and the hands. It is important to consider consistency, thickness, color and texture with resurfacing defects on the face. For example, eyelid skin, which is thin with few glandular

elements, is best replaced with contralateral eyelid skin; whereas the skin of the nose is thick and high in glandular elements, and it is better covered with thicker skin of the nasolabial fold, supraclavicular, or anterior auricular area. Common donor sites in hand surgery include the hypothenar, wrist crease and elbow crease areas. Full-thickness grafting is preferable for most wounds in children, given that the grafts will grow with the growing child and reduce the risk of scar contracture (Baran and Horton, 1972). Defects on the face are frequently closed by local flaps or full-thickness skin grafts. When skin grafts are used on the face, they should be harvested from the 'blush' zone where color match is the best. The blush zone comprises harvest sites above the shoulders, specifically the neck and supraclavicular area (Paletta *et al*., 2006; Valencia *et al*., 2000).

Autologous split-thickness skin grafting is the most commonly practiced form of tissue transplantation in plastic surgery. Split-thickness skin grafts can be of different thicknesses depending on the level of harvest through the dermis. Advantages include a large area of available donor sites and better engraftment rates, given that the metabolic activity of partial thickness grafts is less than their full-thickness counterparts. However, split-thickness grafts are more likely to result in wound contraction, hypertrophic scarring, pigment irregularities and are more susceptible to trauma (Rudolph and Klein, 1973).

Split thickness skin grafts can be taken from any area of the body, including the scalp (Fig. 5.2). In general, epithelial cells within epidermal appendages, such as hair follicles and sweat glands, regenerate a split thickness skin graft donor site within 7–21 days. Despite their ability to heal, donor sites frequently demonstrate scarring and discoloration. Therefore, when possible, split graft harvest sites should be hidden by current clothing styles, including most commonly, the thigh, trunk and buttocks. In addition, the thickness of the donor skin is important in choosing a harvest site. Skin is typically thin in infants and the elderly. Men typically have thicker skin than women regardless of anatomic site. Skin is usually thicker on the trunk and thighs and thinnest on the eyelids and postauricular areas.

Skin grafts can be applied as sheet (or unmeshed) grafts, or they can be meshed at ratios ranging from 1:1 to 4:1. Meshing allows the egress of serum and blood from wounds, thereby minimizing the risk that hematomas or seromas will form that could compromise graft survival. In addition, meshed grafts can be expanded or stretched to cover larger surface areas. When grafts are meshed at ratios of 3:1 or higher, allograft skin or another biologic dressing can be applied over them to prevent the interstices from becoming desiccated before they close (Herndon and Parks, 1986). Because of the lack of dermis in the interstices, widely expanded meshed skin grafts always scar and contract, require longer healing times and result in permanent unattractive mesh marks.

Sheet grafts should be used on the face, the neck, the hands and, whenever possible, on the forearms and the legs. In these exposed areas, the superior cosmetic and functional results obtainable with sheet grafts make such grafts more desirable. Because sheet grafts have no interstices, they must be closely monitored

and, periodically, underlying fluid collections must be expressed by rolling the graft with a cotton-tipped applicator. Any blebs of blood or serous fluid that form beneath the graft should be incised with a No. 11 scalpel and drained expeditiously. Use of fibrin sealant at the time of grafting may decrease the incidence of seroma or hematoma formation under sheet grafts (Gibran *et al*., 2007; Mittermayr *et al*., 2006; Greenhalgh *et al*., 1999; Saltz *et al*., 1989).

Skin grafts require a vascular bed and will seldom engraft on exposed bone, cartilage or tendon without the presence of periosteum, perichondrium or paratenon, respectively. In addition, close contact between the skin graft and its recipient bed is crucial for revascularization; thus, hematomas and seromas under the skin graft or sheer stress will compromise its survival. It is crucial to ensure the wound to be grafted has a vascularized bed free of infection or malignant disease and hemostasis has been achieved (Gingrass *et al*., 1975). Finally, the thicker the graft, the more well-vascularized the bed must be to support engraftment.

5.3.3 Process of successful engraftment

The success of skin grafting or 'skin graft take', depends on the ability of the graft to receive nutrients and, subsequently, the ingrowth of vascular elements from the recipient bed. First and foremost, wound beds need to be adequately vascularized and free from debris and infection. This process of successful skin graft acceptance occurs in three stages. The first stage, lasting over approximately 24–48 hours, depends on *plasmatic imbibition* (Converse *et al*., 1969). Plasma leaks from recipient venules into the space between the graft and the host bed (Kikuchi and Omori, 1970). Fibrinogen, within the extravasated plasma, settles and forms a glue-like substance that anchors the graft to the bed. Nutrient absorption into the graft occurs by passive capillary action from the recipient bed. Once in contact with the recipient site, the graft becomes edematous and increases in size up to 30% (Converse *et al*., 1957). The energy demands of the graft fall as metabolism occurs via anaerobic respiration (Hira and Tajima, 1992).

The second stage requires the cut ends of the recipient and donor end capillaries to align and form microscopic anastomoses – a process known as *inosculation* first coined by Thiersch in 1874 (Thiersch, 1874). This process begins immediately after graft placement, and vascularization occurs by four days (Davis and Traut, 1925). Most reports indicate that vascularization becomes normal by four weeks (Converse and Rapaport, 1956; Haller and Billingham, 1967; Rolle *et al*., 1959). In contrast, flow within allograft skin improves until day 6 then halts by day 9 owing to rejection (Scothorne and Mc, 1953; Kamrin, 1961; Egdahl *et al*., 1957).

The final phase is marked by revascularization of the graft after capillary alignment. Multiple theories exist to explain this process. One prominent theory proposed by many German surgeons in the late 1800s describes how the original graft vasculature degenerates. Subsequently, host endothelial cells and capillary buds invade the graft via the acellular graft basal lamina, which provides a conduit

for the new vascular tree (Paletta *et al*., 2006; Smahel, 1967; Converse and Ballantyne, 1962).

Once the graft becomes vascularized, the graft continues to mature and contract over the course of one year. An ungrafted wound will undergo contraction. Wound contraction is mediated by a cell-type known as the myofibroblast (Guber and Rudolph, 1978; Rudolph *et al*., 1977). Prior to wound grafting, multiple fibroblasts within the wound will differentiate into myofibroblasts, which will contract the wound regardless of the presence of a skin graft. This process is termed *primary contraction*. Skin grafts will also cause contraction, termed *secondary contraction*. The thicker the skin graft the less contraction that is observed, perhaps because the dermal elements inhibit myofibroblast differentiation (Rudolph, 1976; Corps, 1969). The probability of secondary contraction is important to consider when grafting wounds over areas of functional or cosmetic importance, such as over a joint, in a hand webspace, or in the eyelids. In these sites, full-thickness or thick split-thickness skin grafts should be used whenever possible. During the graft maturation process, the epidermis undergoes a tremendous degree of hyperplasia – up to eight times thicker at 2 weeks than the original graft. This occurrence, manifested clinically as crusting or scaling, allows the re-epithelialization of meshed graft interstices and adjacent ungrafted areas (Gillman *et al*., 1953).

The regeneration of skin appendages in grafted skin is variable. Nerve fibers appear to degenerate within the graft during the first month after transplantation. New host nerve fibers invade the skin graft from the base and the periphery after 40 days. At 2 to 3 months, nerve fibrils appear to re-populate the skin graft and innervate end organs, including hair follicles, sweat glands and sensory endings (Ponten, 1960). Sensation is often not normal and for up to a year after grafting the graft may be hypersensitive. Pain sensation usually returns first, then touch, temperature and tactile discrimination return later (Paletta *et al*., 2006). Sensation is usually better in full-thickness skin grafts compared to split-thickness, although sensation depends not only on the thickness of the graft, but also on the condition and depth of the recipient wound (Weiss-Becker *et al*., 1998). Similarly, sweat gland function depends on neural innervation, so it typically does not return prior to three months (Ponten, 1960). Sweat gland function is superior the thicker the graft. Once sweat glands are innervated, they behave like skin of the host bed not the donor site. For example, grafts to the palm will sweat in response to emotional stimuli, regardless of donor site (Paletta *et al*., 2006). Finally, hair follicles are frequently destroyed during split-thickness skin graft harvest, so subsequent hair growth is unusual. In full-thickness skin grafts, hair growth usually resumes and maintains the characteristics of its donor site. Caution should be used when hair-bearing full-thickness scalp skin is transplanted to traditionally non-hair-bearing tissues.

5.3.4 Graft and donor site dressings

Once the graft is secured in place, a dressing may be applied to protect it from

shearing, as well as to accelerate closure of meshed graft interstices. Numerous options for graft dressings exist, including various topical agents in combination with a graft immobilization strategy. The use of a non-adherent dressing such as Conformant 2 (Smith & Nephew) along with an outer antimicrobial wet dressing allows the overlying dressings to be periodically removed without dislodging the graft from the wound bed. Bolsters consisting of cotton and greasy gauze are employed to help grafts conform to concave wound surfaces and splinting of extremities may be necessary for safe graft immobilization, especially over joints. The Vacuum Assisted Closure system (Kinetic Concepts Inc., San Antonio, Texas) is another option for graft fixation. Alternatively, an Unna boot can be placed on both the upper and the lower extremity to immobilize the graft and provide vascular support, allowing mobilization of the extremity in the immediate postoperative period. Sheet grafts can be either left open to the air to allow continuous monitoring and rolling (depending on the patient) or wrapped with dry dressings, which can be removed if necessary to allow interval inspection and removal of underlying blebs.

There are also various options for donor-site dressings. The ideal donor-site dressing would not only minimize pain and infection but also be cost effective. Greasy gauze and Acticoat (Smith & Nephew, Largo, Florida) are often employed for this purpose. Typically, these dressings are left in place until the donor site re-epithelializes, at which time the dressing is easily separated from the healed wound. Op-Site (Smith & Nephew, Largo, Florida), a transparent polyvinyl adherent film, is also commonly used.

With Op-Site, the underlying wound is easily examined without removal of the dressing. however, intermittent drainage of the wound fluid that accumulates is necessary. Op-Site does not work well over joint surfaces and concave or convex areas (e.g. the back). Silver sulfadiazine in a diaper is an excellent covering for buttock donor sites in children; dressing changes can be done with each diaper change.

5.4 Principles of allogeneic skin grafts

As opposed to autografts, allograft skin is harvested from a cadaver. In the following sections, unique issues related to allograft skin are discussed, including the history of its use, relevant immunology and techniques for application.

5.4.1 History of allogeneic transplantation

The history of plastic surgery is intimately intertwined with that of transplantation. Crucial contributions to the understanding of graft survival began in the early 20th century (Herman, 2002). The limited survival of allogeneic and xenogeneic skin grafts in humans was investigated by Schone and Lexer in the early 20th century. They demonstrated that both types of grafts did not survive for more than 3 weeks

after transplantation. During the early stages of World War II, Scottish plastic surgeon Tom Gibson collaborated with British zoologist Peter Medawar to investigate why skin taken from one human being will not form a permanent graft on the skin of another person. In 1943, Medawar and Gibson discovered that the rejection of skin graft was mediated by an antigen–antibody reaction of the host immune system (Gibson and Medawar, 1943). Medawar was eventually awarded the Nobel Prize in Medicine & Physiology in 1960 for his continued research in transplant immunology. The latter half of the 20th century saw a growing use of human cadaveric allograft skin as a biological dressing in burn patients (Brown *et al*., 1953), particularly after allograft skin was demonstrated to stimulate neo-vascularization (O'Donaghue and Zarem, 1971). In 1955, Harrison reported the first successful kidney transplant between identical twins (Harrison *et al*., 1956) and the same transplant group reported a successful cadaveric renal transplant in 1963 (Merrill *et al*., 1963). Subsequent advances in immunologic typing of tissues and immunosuppression have allowed solid organ allograft transplantation to achieve long term graft function and widespread clinical practice. Research in transplant immunology is now focusing on induction of tolerance in allogeneic and xenogeneic tissues in the absence of long-term immunosuppression.

5.4.2 Immunology of allogeneic transplantation

The rejection of allogeneic tissue transplants occurs via cellular and humoral immunologic responses. These responses are generated when the host defense system detects foreign antigens expressed on the donor cell surface. The antigen-presenting molecules are termed major histocompatibility complex (MHC) antigens or human leukocyte antigens (HLA) in humans. The HLAs provide a molecular fingerprint based on six closely linked genes providing more than 100 serologically identifiable combinations (Auffray and Strominger, 1986). Each individual has two MHC regions: a paternally and a maternally inherited collection of HLA genes, or haplotype. HLA genes are divided into two classes: class I HLA genes referred to as HLA-A, -B and -C, and class II genes referred to as HLA-DR, -DQ, and -DP. In humans, class I antigens are expressed on all nucleated cells and platelets, whereas class II antigens are only expressed on hematopoietic cells, such as B lymphocytes, activated T lymphocytes, and monocytes and tissue fixed antigen presenting cells (APCs), such as macrophages and dentritic cells. Matching of HLA-A, -B, and -DR is the most important factor determining long term renal allograft survival (Lee and Butler, 1997).

The process of allogeneic rejection begins with the presentation of a foreign alloantigen on the surface of APCs in conjunction with class II MHC molecules. Identification of this alloantigen pairing by immature T cells results in their maturation and amplification. The dissemination of immunomodulatory cytokines, including interleukin-1 and -2, results in sensitization and activation of T helper/inducer, CD4+ cell subpopulations, and the clonal expansion of both cellular (T

cells) and humoral (B cell) responses. The cellular response results in allograft infiltration and destruction by natural killer cells, cytotoxic T cells and macrophages (Hayry, 1984). The humoral response results in cell lysis through both complement-dependent cell death and antibody-mediated cytotoxicity (Lee and Butler, 1997). Given the coordinated immune response to foreign antigen, allogeneic transplantation without immunosuppression is only possible in transplants between identical twins (Murray *et al*., 1958; Burke *et al*., 1974).

5.4.3 Tolerance

Tolerance refers to the state of immunologic acceptance or unresponsiveness of the recipient to the donor allograft or xenograft (Billingham *et al*., 1955) in the absence of prolonged immunosuppression. Methods of attaining tolerance in adult humans vary. One method employs the use of total body irradiation to remove mature recipient T cells followed by donor bone marrow transplantation, prior to organ transplantation. This induces a state of stable chimerism in which the recipient is tolerant of both donor and recipient tissues. Further discussion of tolerance is beyond the scope of this chapter

5.4.4 Allogeneic skin grafting techniques

Skin allografts were the first allograft organ transplants successfully achieved in humans. However, skin is strongly antigenic and subject to almost inevitable rejection in the absence of long-term immunosuppression. Current uses of allograft skin among burn and plastic surgeons are threefold: the temporary coverage of freshly excised wounds, use as an overlay on widely expanded autografts, and its use to improve recipient bed quality and vascularity for a period of time prior to autograft application (Blome-Eberwein *et al*., 2002; Moerman *et al*., 2002). In addition to burns, allograft is used in cases of skin loss caused by trauma (full thickness wounds with bone and/or tendon exposed), surgery (after the debridement of extensive, necrotizing soft tissue infections), donor sites for autologous skin grafts, reconstruction of critical facial defects, as temporary coverage (after laser-resurfacing and dermabrasion), and in the cases of disease (as temporary coverage for toxic epidermal necrolysis, congenital bullous epidermolysis, diabetic ulcers, venous ulcers, pressure and trophic ulcers).

Viable allograft is often regarded as the gold standard in temporary skin coverage, possessing many of the qualities of the ideal wound dressing. The characteristics of ideal large wound dressings include lack of antigenicity and toxicity, reduced water permeability, heat retention, barrier to microorganisms, pain reduction, firm adherence, protection of underlying tissue and resistance to trauma, low cost and long shelf life with simple storage requirements (Pruitt and Levine, 1984; Burd and Chiu, 2005) (Table 5.1). The extreme antigenicity of skin inevitably results in the rejection of allograft.

Table 5.1 Properties of allogeneic skin which make it the present gold standard for temporary wound coverage (from Burd and Chiu, 2005)

1. The intact stratum corneum provides a barrier function which:
   a. limits desiccation of the wound
   b. limits evaporative water loss
   c. reduces bacterial contamination
   d. protects underlying viable tissue
   e. limits exudative tissue fluid loss.
2. The biomechanical properties of the dermal component allows:
   a. effective draping of the wound
   b. permits movements of joints
3. The biochemical properties of the skin cause:
   a. in the partial-thickness burn
      i. reduced pain
      ii. enhanced healing
      iii. decreased scar
   b. in full-thickness excised wounds
      i. promotes angiogenesis on the wound bed which aids subsequent autograft take.

The use of allograft skin is beneficial in the treatment of large burns with or without concurrent autografts. Allograft skin is used as a temporary dressing while awaiting the healing of autograft donor sites between sessions of harvesting (Herndon and Parks, 1986). In addition, allograft skin can be employed as a biologic dressing pending definitive surgical coverage of deep full-thickness burns or spontaneous healing of partial-thickness skin loss. Early excision and grafting with allograft skin avoids mortality and morbidity caused by disseminated burn wound sepsis, by reconstituting the protective barrier of skin against micro-organisms. Allograft skin also reduces the pain of an exposed wound, decreases evaporative water loss, improves re-epithelialization and leads to improved cosmesis (Seah, 1992). The technique of covering burn wounds with widely meshed autograft and then allograft, known as the 'sandwich technique', may improve healing (Burke *et al*., 1974; Burke *et al*., 1975; Burke *et al*., 1976; Alexander *et al*., 1981). In addition, the immunocompromised state of critically ill burn patients may delay rejection for several weeks. Some authors employ a variation of the sandwich technique by using cultured keratinocyte spray as well as meshed autograft with allograft applied over both. The allograft acts as a temporary dressing without vascularization, by providing a moist protective environment for re-epithelialization underneath (Burd and Chiu, 2005).

Cuono and colleagues demonstrated an important potential use of allograft tissue and an insight into the immunogenicity of allogeneic skin transplants. In an attempt to provide durable wound coverage, they employed cryopreserved, acellular allograft dermis as a substrate for cultured autologous keratinocytes in two patients with extensive burns (Cuono *et al*., 1987). In both patients, burn wounds were excised and covered with allograft skin. Once keratinocyte grafts were ready

for use, the engrafted allogeneic skin was de-epithelialized by derm-abrasion instruments. Thus, durable skin replacement was achieved using allograft dermis under autologous keratinocyte grafts. The authors postulate that allograft rejection is mediated by donor MHC class II expressing Langerhans cells in the allograft epidermis, which are absent in the dermis. As a follow-up to these observations, Wu and colleagues demonstrated in an *in vivo* model of immune rejection where allograft dermis does not generate rejection whereas allograft epidermis does (Wu *et al*., 1995). This report added credence to the observation that allograft dermis is immunologically inert or, at least, expresses minimal immunoreactivity.

5.4.5 Limitations of allogeneic skin graft use

Major problems with skin allograft are cost, limited supply (given that harvesting and banking services are not uniformly available) and infectious disease transmission (Kealey, 1997). In the absence of immunosuppression, the rejection process leads to allograft destruction or sloughing, usually within 10–14 days. This rejection process is mediated by T cells activated by donor epidermal Langerhans cells which migrate from the allograft to the regional lymph nodes of the recipient. In addition, donor dermal dendritic cells play a role in rejection (Larsen *et al*., 1994; Richters *et al*., 2005).

Concern about infectious disease transmission from the cadaveric donor to the recipient is not without validity. The primary cause for concern arises from an incident in 1986, in which human immunodeficiency virus (HIV) seroconversion followed the use of allograft skin for a burned patient at the Burn Unit of Queen Mary's University Hospital in London (Clarke, 1987). This unit has subsequently abandoned the use of human allograft skin, and as a substitute they have described widely meshed autograft skin overlaid with meshed allograft from parents for coverage in large-area burns in children (Phipps and Clarke, 1991). Recently, Burtonboy and colleagues demonstrated HIV by PCR in fresh and cryopreserved skin from HIV-1 positive donors, verifying the hypothesis that HIV infection can be transmitted by skin graft (Gala *et al*., 1997). Although the prevalence of infectious contamination of donor skin is unknown, Pianigiani and colleagues, in Sienna Italy, recently reported up to 16% of skin allograft specimens (among 461 specimens screened) are discarded based on a current serologic and molecular biologic screening regimen for HIV, hepatitis B and C virus, human T-cell lymphotropic virus, cytomegalovirus and *Treponema pallidum*. The most common seropositive result among their cohort was for hepatitis B viral infection among 14.8% (Pianigiani *et al*., 2006).

Allograft skin is currently used after employing either of two storage methods: refrigeration or cryopreservation. Allograft is rarely used fresh because the brief period of sampling and use is insufficient for complete donor screening for eligibility of tissues. Skin harvested from cadavers is usually either treated with solutions containing high concentrations of glycerol (up to 87% with refrigeration

storage between +4 and +8 °C) or by cryopreservation, comprising storage in special solutions (for example, 15% glycerol) and dry storage at –80 to –196 ºC (van Baare *et al*., 1998b; van Baare *et al*., 1998a; Cameron *et al*., 2000). The main difference between the two methods is that refrigerated, glycerolized tissue is not viable but maintains its structure and mechanical properties, whereas, cryopreserved tissue consists of viable cells which can migrate into the wound bed. Cryopreservation can enhance the safety of allografts beyond fresh specimens, but cannot replace extensive donor screening (Pirnay *et al*., 1997); whereas refrigerative, glycerol treatment appears to inactivate extracellular and intracellular HIV (van Baare *et al*., 1998a; van Baare *et al*., 1994; de Backere, 1994). Theoretically, the risk of infection by allograft skin transplant is greater than for other forms of organ or tissue transplantation given that one donor may be used to treat a large number of recipients. In addition, recipients may have a depressed immune system for pathologic (e.g. due to burns, cancer or severe trauma) or physiologic reasons (e.g. extremes of age) which may predispose them to infectious agent transmission. Thus, widespread use of allograft has been hampered, in part, by concerns over disease transmission.

5.5 Principles of skin xenografts

5.5.1 Xenogeneic transplantation

Several issues unique to xenogeneic transplantation, not seen among allografts, are observed. First the transplanted tissues may not function properly in the new xenogeneic environment (Auchincloss and Sachs, 1998). Second, the presence of preformed antibiodies to xenogeneic tissue in the recipient in the absence of prior exposure is common and leads to hyperacute rejection (Hammer *et al*., 1998). This precludes xenogeneic transplantation of tissue into humans from most species except non-human primates. However, the use of successful xenogeneic skin grafts as temporary wound coverage continues despite these issues.

5.5.2 Xenogeneic skin grafts

Xenografts have been used as temporary wound covering with wide success. Since the 1960s, the primary donors are pigs in the United States, by virtue of their relative affordability and similar histologic structure compared to humans (Bromberg *et al*., 1965). Porcine skin grafts are dressings rather than grafts, as they do not become vascularized (Chiu and Burd, 2005). Although the animal skin may become extremely adherent and indeed occasionally incorporated into the healed wound, xenografts are not true grafts or transplants (Sokolic *et al*., 1960; Chang *et al*., 1973; Aronoff *et al*., 1976). No capillary ingrowth or vessel to vessel connections occur after porcine xenograft placement (Pruitt, 1997; Lee, 1972). Passive plasmatic nutrition maintains hydration and cellular viability for a period of time,

but dessication and avascular necrosis inevitably occurs. Similarly, no immunologic response develops to the xenograft tissue and no antidonor antibodies develop in the recipient after usage (Bromberg *et al*., 1965; Sokolic *et al*., 1960; Rappaport *et al*., 1970; Miller *et al*., 1961). The porcine skin is sloughed rather than rejected, by the growth of complete epithelium beneath it (Chiu and Burd, 2005). Similar to allograft skin, porcine skin can be either used fresh and is potentially viable (refrigerated up to 30 days) (Rappaport *et al*., 1970), or treated but rendered non-viable, by lyophilization (freeze-drying) or chemical dehydration with glycerol.

There are multiple uses of porcine skin. Given that porcine skin does not incorporate into wound beds it can be used as a temporary biologic dressing over wounds expected to heal without skin autografts, including partial thickness burns (Still *et al*., 1996), split thickness skin graft donor sites (Chang *et al*., 1973; Aronoff *et al*., 1976), or exfoliative conditions (such as toxic epidermal necrolysis) (Heimbach *et al*., 1987; Honari *et al*., 2001; Imahara *et al*., 2006). When used over open wounds, the porcine skin must be inspected daily to assure adherence. Similar to allograft 'sandwich technique', porcine xenograft can be placed over widely meshed autograft in the treatment of extensive burns, thereby reducing dessication over the wound interstices and subsequent scarring (Burd and Chiu, 2005).

There are multiple reports in the literature attesting to the benefits of porcine skin in the treatment of extensive wounds, including a decrease in healing rates for partial thickness burns and granulating wounds (Chang *et al*., 1973; Pruitt, 1997), a reduction in pain when placed over burns (Pruitt and Levine, 1984, Aronoff *et al*., 1976; Lee, 1972; Rappaport *et al*., 1970) and a decline in heat, fluid, protein and electrolyte loss (Elliott and Hoehn, 1973; Pruitt and Levine, 1984). Furthermore, porcine skin provides a physical protective layer for a re-epithelializing wound (Zawacki, 1974; Pruitt and Levine, 1984) and it decreases bacterial overgrowth (Sokolic *et al*., 1960; Burd and Chiu, 2005; Pruitt and Levine, 1984). As mentioned before, data from our own institution demonstrates the effective use of porcine xenograft as a temporary biologic dressing in the treatment of toxic epidermal necrolysis, when employed to protect the dermis until re-epithelialization occurs (Imahara *et al*., 2006). The major disadvantages affecting the widespread use of porcine skin are bacterial infection, cost and the theoretical risk of zonoses such as meningitis, brucellosis, hydatidosis, cysticercosis and influenza (Pirnay *et al*., 1997). However, these risks may be abated if the pigs are raised in a clean laboratory environment.

5.6 Future trends

The continual demand for techniques and products to aid in the rapid and durable closure of extensive burns and wounds, while re-establishing normal skin architecture remains a challenge. Important barriers to advances in this area of plastic surgery remain an evolving understanding of the allogeneic transplant immunology,

limitations of autologous tissue engraftment and a growing understanding of the complex biologic and biochemical properties of skin tissue. Successful, long-term allogeneic skin transplantation will evolve as the ability to induce stable immunogenic tolerance and mosaicism expands. Important advancements in the improvement of skin engraftment will include the employment of fibrin sealants. Future research will aim to create skin substitutes, probably using cultured epidermis which under appropriate circumstances may provide a wound cover that is as durable and cosmetically acceptable as conventional skin grafts. Current skin substitutes appear to fall short of reconstituting the normal skin architecture and long-term durability.

5.7 Summary

The history of wound closure with skin grafts parallels the history of transplant immunology and the evolution of the profession of plastic surgery. Despite an increase in the knowledge and understanding of skin engraftment and immunology, the durable coverage of extensive and complex burns and wounds remains a challenge. Given that optimal closure of full-thickness wounds requires the replacement of both the epidermal and dermal component of skin, no material to date has been developed that will restore the skin in its entirety. However, nearly three millennia after the writing of the *Sushruta samhita*, skin grafts remain the ideal replacement for skin lost from injury. Extensive wounds with limited donor sites often may be temporarily closed with biological dressings such as allograft and xenograft.

5.8 References

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6

Understanding the cellular basis of skin growth

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Abstract: Skin is a complex body organ with several tissue layers and many more cell types. A good knowledge on the exact composition, structure and development of the skin is important in understanding its function and eventually in engineering artificial skin.

Key words: dermis, epidermis, hair follicles, keratinocytes, skin.

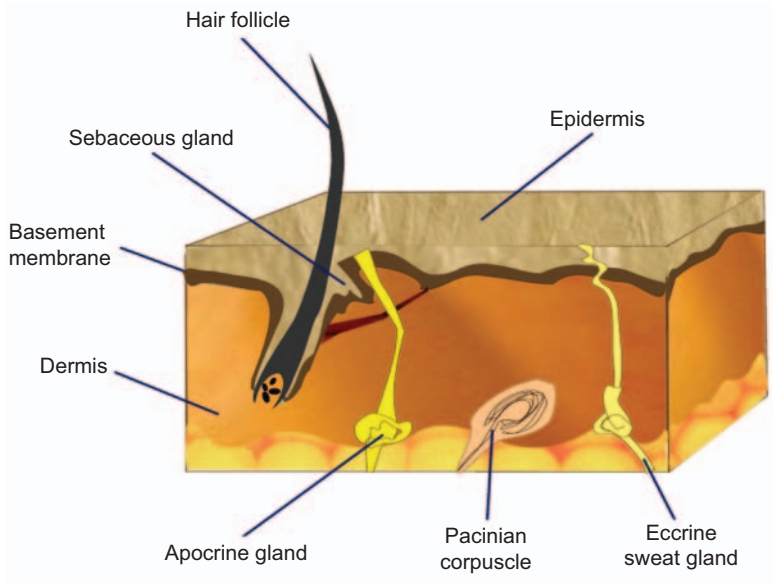
6.1 Introduction

Skin is the body's largest organ and makes up approximately 16% of a human's total body weight. It performs several necessary and important functions and its loss presents a serious medical challenge. Often, physicians can replace lost skin by grafting in undamaged skin from another area of the body. However, sometimes artificial skin constructs can be utilized for large skin defects or to minimize donor site morbidity. To create better skin substitutes, it is important to learn more about the structure and functions of skin. This chapter aims to describe skin's function and structure as well as the different cell types that make up the skin.

Mammalian skin has evolved to fulfill a number of complex and diverse functions. It protects against outside materials and some types of radiation, regulates heat loss and mediates sensation. The skin is relatively impermeable, which prevents internal fluids from escaping. The skin is also the site for synthesis of vitamin D (an important regulator of calcium and phosphate metabolism), as well as a site for biochemical interconversions (e.g. androgenic steroids) and excretion of ingested toxins. Lastly, the skin's aesthetic attributes affect social interaction.

6.2 Structure of the skin

The skin consists of a stratified, cellular epidermis and an underlying dermis of connective tissue (Fig. 6.1). Below the dermis is a fatty layer, usually designated as 'subcutaneous'. In most mammals (e.g. mice) this fatty layer is separated from the rest of the body by a flat sheet of striated muscle (Rook *et al.*, 1986). Dermal flexibility and elasticity united with epidermal strength and impermeability allows for the skin to serve as a protective barrier.

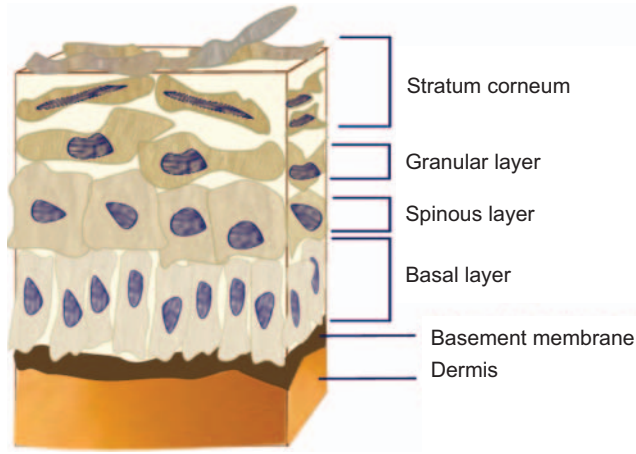


6.1 The structure of human skin. The epidermis and the dermis together constitute the skin. In this drawing, a dark, wavy line (representing the basal layer of the epidermis) separates these two components. The hypodermis, or subcutaneous tissue, lies directly below the dermis. The structural complexity of the skin and its myriad cell types are evident. Note the presence of various epidermal appendages (such as hair follicles and glands), nerves and sensory receptors, blood vessels and subcutaneous adipose.

6.2.1 The epidermis

The epidermis contains a variety of cells. Most of the cells are keratinocytes, which are formed by division of stem cells in the basal layer. As the cells move upwards, they differentiate while synthesizing a group of insoluble proteins and creating keratin, which will collect within the cells. Pigment-forming cells, or melanocytes, exist in the basal layer of the epidermis and in the matrices of hair follicles. The epidermis also contains mechanoreceptors known as Merkel cells and Langerhans cells and lymphocytes that engage in immunologic protection (Fawcett, 1986).

Histologically, the epidermis is composed of four keratinocyte strata: the basal layer, the spinous layer, the granular layer, and the stratum corneum (Fig. 6.2). Only basal layer keratinocytes have the capacity to proliferate. The innermost basal layer is the only mitotically active layer and is tightly connected to the dermis through the basement membrane. The basement membrane is a barrier against the dermal–epidermal exchange of cells and large molecules and is made of a highly organized matrix of fibrous proteins including type IV collagen, laminin, fibronectin and heparan sulfate proteoglycan. Integrin-mediated adherens junctions and



6.2 The various strata of the epidermis.

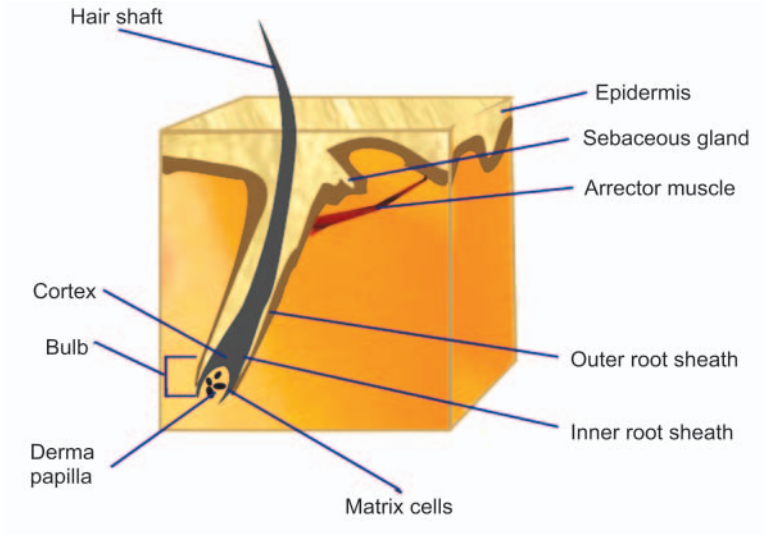
specialized hemidesmosomal junctions anchor basal keratinocytes to the basement membrane. When a basal cell undergoes terminal differentiation, it leaves the basal layer and enters the spinous layer. Related structures also hold adjacent keratinocytes together to form a continuous epithelial sheet. The three remaining suprabasal layers of epidermis are progressively more differentiated. Spinous cells are metabolically very active and they increase in size. In the granular layer, keratinocytes loose metabolic activity and exclude cytoplasmic organelles. By the time they reach the outermost stratum corneum, cells form only highly cornified, dead squames. With these features, the epidermis provides an effective barrier to microorganisms, a semipermeable barrier to water and oxygen and a weak mechanical barrier.

6.2.2 The dermis

The dermis, composed of fibrous proteins, such as elastin, collagens and additional extracellular matrix proteins, provides skin with mechanical flexibility. It is populated by fibroblasts, lymphocytes and mast cells. The dermis has a rich blood supply owing to its many capillary vessels. The dermis also contains sensory innervations. Several types of epidermally derived glands reside in the dermis and account for secretory and excretory functions.

6.2.3 Hair follicles

Hair follicles (Fig. 6.3) are comprised of pockets of epithelium, which are continuous with the superficial epidermis and extend deep into the dermis. A hair follicle forms a bulb around the specialized dermal cells, the dermal papillae. The relatively undifferentiated matrix cells are derived from the embryonic basal layer

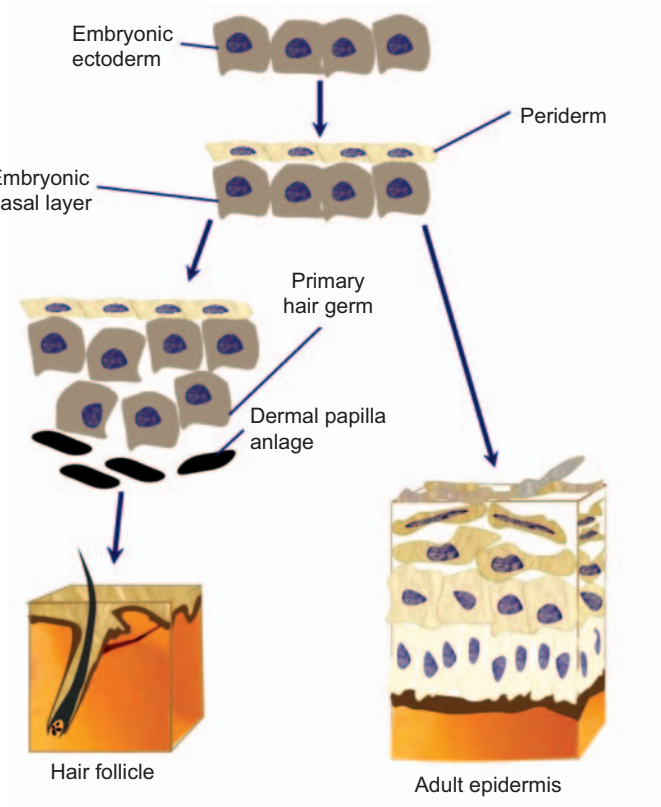


6.3 The structure of a hair follicle.

of developing skin and give rise to concentric rings of differentiated cell types, including the medulla (the cortex and the cuticle of the cortex). The hair is surrounded by inner and outer root sheaths. The inner root sheath cells extend to the dermal papillae, where they are mitotically active and produce cells that migrate upward. The outer root sheath cells are continuous with the basal epidermal cells and do not come into direct contact with the dermal papillae. They are contiguous with the basal epidermal layer but are distinct and self-propagating.

6.3 Skin development and growth

Skin consists of the epidermis and the dermis. During embryonic development, the epidermis derives from a single layer of cells called the embryonic ectoderm (Fig. 6.4). This early embryonic ectoderm has a morphology and biochemistry similar to those of simple epithelial tissues (Dale *et al.,* 1985) and also is a basis for the neuroectoderm. Midway through mammalian development, an embryonic epidermis forms consisting of an inner and outer layer. The inner layer is the embryonic basal layer, which, in reponse to different stimuli, is the precursor of the epidermis and its appendages. The outer layer, termed periderm and unique to primates, still possesses properties of simple epithelial cells. The periderm is later shed from the skin's surface as the true epidermis keratinizes beneath it (Rook *et al.,* 1986). In all mammals, the first signs of epidermal differentiation appear when the epidermis stratifies to form a third layer. This happens at around 13 days of mouse embryogenesis and around 8–11 weeks during human embryonic development (Rook *et al.,* 1986). From here on, the development of the epidermis, sebaceous glands and hair follicles begin to take separate paths. The primary hair



6.4 Development of the epithelial components of skin.

germs appear as invaginations of the embryonic basal layer directly above aggregations of specialized mesenchymal cells, the anlage of dermal papillae. From the primary hair germ the sebaceous gland, the hair follicle and the apocrine gland will develop. The embryonic basal layer stratifies to form the epidermis where dermal fibroblasts, rather than dermal papilla cells, exert their influence.

Embryogenetically, the dermis comes from mesoderm which is brought into contact with the inner surface of the epidermis during gastrulation. The mesoderm is essential for inducing differentiation of the epidermal structures, as is classically demonstrated in the feather germ of birds. In fact, the dermis remains instrumental later during development in maintaining adult epidermis and this epithelial–mesenchymal interaction is known to be dependent upon the interplay of many factors synthesized by both the epidermis and the dermis.

At roughly the same time that a multi-layered epidermis forms, primitive hair germs start developing. The first indication of a hair follicle is a crowding of nuclei (termed primitive hair germ) in the basal layer of the epidermis. This process is believed to be initiated by the specialized mesenchymal cell

aggregations underneath, called the anlage of dermal papillae (Davidson and Hardy, 1952). In the absence of dermal papilla signaling, the embryonic basal cells stay as epidermal cells. The committed hair germ cells then begin to grow obliquely downward into the dermis and the advancing extremity becomes bulbous, gradually enveloping the mesodermal papilla. At this stage, two to three epithelial swellings appear on the posterior wall of the follicle and they eventually become the sebaceous gland, the apocrine gland and the arrector muscle attachment site (Rook *et al.,* 1986).

6.4 Experimental models for predicting cellular interactions

Based on the classic transplant experiments where researchers mix-and-matched epidermis and dermis from different parts of the body, it has been hypothesized that epidermal growth and differentiation is controlled by epithelial–mesenchymal interactions. However, little is known about the exact mechanisms of this interaction owing to the number of variables involved, such as the different dermal cell types and the superimposed influences of systemic factors in the blood circulation.

6.4.1 The *in vitro* model

In vitro model systems have been developed to mimic epidermal–dermal interactions and to study regulation of epidermal cell proliferation and differentiation (Sawyer and Fallow, 1983). These models have shown that coculture on postmitotic mouse or human dermal fibroblasts are required to support human keratinocyte growth at clonal densities in serum-containing medium. Under conventional (submerged) culture conditions, keratinocyte proliferation is the predominant event while terminal differentiation and tissue organization are reduced or atypical compared to the *in vivo* situation (Holbrook and Hennings, 1983). However, using organotypic culture systems where keratinocytes are cultured at an air–liquid interface on fibroblast-embedding collagen gels, improvement of tissue architecture and induction of terminal differentiation markers have been achieved (Kopan *et al.,* 1987).

With the help of such an elaborated *in vitro* cultural system that can closely imitate the *in vivo* situation, it is possible to examine the functions of individual factors during epithelial–mesenchymal interactions and a number of extracellular regulators controlling the balance between epidermal growth and terminal differentiation have been identified. These factors can be categorically assigned to two groups based on their mitogenic activity on keratinocytes, positive growth regulators and negative growth regulators. Stimulators of keratinocyte growth include EGF, TGF-α, low concentration of retinoic acid (Kopan and Fuchs,

1989) and two interleukins, IL-6 (Grossman *et al.,* 1989) and IL-1a (Kupper *et al.,* 1989).

6.5 Conclusions

Skin is a complex organ comprised of many cell types with their own specific functions interacting with one another. Understanding the functions of the different cells contained within the skin is important in the process of designing bio-engineered skin that has the same properties as the natural organ. Rudimentary artificial skin such as Integra has already been developed and can serve as a template for newer generations of engineered skin. The ultimate artificial skin will be composed of other tissue types described here which contribute to all the functions of native skin.

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7

The regulatory approval process for biomaterials for treating skin loss

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Abstract: The regulatory approval and registration process for biomaterials for treating skin loss can be complicated and is evolving as technology advances. This chapter reviews the approval process in the United States of America with the FDA and the approval process in the European Union. The 510K premarket notification, premarket approval (PMA), design dossier under the Medical Device Directive process, regulations and procedures are delineated. The clinical trial process, regulations and protocol considerations for biomaterials for treating skin loss are discussed. Special considerations and regulations for human and cellular based biomaterials and combination products are summarized. The user fees for various submissions are also discussed. Sources for further review of regulations, guidance, documents and information on the regulatory approval and registration process are provided. Future trends such as the harmonized submission of a summary technical documentation submission that is accepted by all regulatory agencies and other globally harmonized submissions are reviewed.

Key words: regulatory approval, Food and Drug Administration (FDA), Medical Device Directive (MDD), biomaterials for skin loss, combination products, clinical trials, medical devices.

7.1 Introduction

The regulatory approval process for biomaterials for the treatment of skin loss varies greatly depending on the type and source of the biomaterial and more importantly, the indication for use for the product. As technology progresses and more advanced biomaterials evolve, as well as combinations of biomaterials in one product, the regulatory approval process becomes more complicated and detailed. Incorporated in the regulatory approval process is the compilation of clinical data to produce valid scientific evidence of safety and efficacy, and to support the indication for use. At the same time, more countries are recognizing that there need to be new standards to regulate these advanced medical products for the treatment of skin loss.

This chapter discusses the regulatory process for biomaterials for skin loss in the United States of America and the European Union. There are extensive regulations, standards and guidance documents regarding the regulatory approval process.

This chapter will outline the most relevant regulations and provide references to obtain additional information.

7.2 Regulatory requirements

The use of biomaterials for skin loss spans a wide range of materials, from products which are allograft based (cadaver), products of animal origin derived from bovine, porcine, equine and other species, such as shark cartilage, and synthetic materials. The most advanced materials are often a combination of products incorporating drugs as biologics in a delivery device or products that are cell seeded with human tissue (patient or donor tissue) as well as growth factors.

There have been significant advancements using biomaterials and a combination of biomaterials for the treatment of skin loss resulting from burns, trauma, or other disease entities and injuries which have had dramatic results in patient outcomes and quality of life. These advanced biomaterials and combinations of these materials also present challenging regulatory approval pathways. There are requirements and regulations governing advanced biomaterials, combination products and products of human and animal origin. As technology advances, the regulatory processes are changing as well to ensure the safety and effectiveness of these high technology products.

There are several regulatory pathways in the United States (USA) through the Food and Drug Administration (FDA) for approval. The initial step in the approval process is to determine the classification of the product. There is a similar process and classification system for product approval in the European Union as part of the CE Mark certification process under the Medical Device Directive.[1] Classification of a product determines the pathway for product approval in almost every country with medical device regulations. The classification determines the relative risk of the product and this determines the amount of data and testing needed to support the safe use of the product. The classification of the product also determines the regulatory pathway.

It must first be determined whether the product will be classified as a pharmaceutical or a drug, device, or biologic, or a combination of these categories. Regulations regarding human tissue will also be discussed, which will be subject to the regulations for human tissue and cellular based products.

This chapter will concentrate on the regulatory approval and clinical trial process for medical devices and combination products in the USA by the FDA and in the European Union under the Medical Device Directive.

7.2.1 Definition of a medical device

The definition of a medical device is an

> 'instrument, apparatus, implement, machine, implant, *in vitro* reagent, or

other similar or related article, including any component, part or accessory which is:

- recognized in the official National Formulary, or the United States Pharmacopoeia, or any supplement to them,
- intended for use in the diagnosis of disease or other conditions, or in the cure, mitigation, treatment, or prevention of disease, in man or other animals, or
- intended to affect the structure or any function of other body of man or other animals, and

which does not achieve its primary intended purposed through chemical action within or on the body of man or other animals and which is not dependent upon being metabolized for the achievement of any of its primary intended purposes' [21 USC 321(h)].[2]

Devices are classified by FDA as either Class I, II or III in the United States. Class I devices are those for which general controls are sufficient to provide reasonable assurance of safety and effectiveness. General controls include complying with regulations concerning adulteration and misbranding provisions, registration and listing, premarket notification and other general requirements.

Class II devices are those for which general controls alone are insufficient to establish safety and effectiveness. Class II devices must generally meet general controls (including premarket notification requirements for most Class II devices) as well as special controls, if applicable. Special controls include performance standards, postmarket surveillance of the product, patient registries, FDA guidelines, recommendations and other requirements as indicated.

Class III devices are those for which general controls and special controls alone are not sufficient to ensure safety and effectiveness. Class III devices as defined by FDA are those devices used in supporting or sustaining human life; for a use which is of substantial importance in preventing impairment of human health; which present a potential unreasonable risk of illness or injury; or are not substantially equivalent to a legally marketed Class I, Class II device or preamendment Class III device. Class III devices must meet general controls and must be approved by FDA prior to marketing the product.[3]

The classification of a device can be determined by several methods such as those listed in the Code of Federal Regulations,[4] researching the FDA Health Product Code Database,[5] researching a competitive device, contacting the FDA or reviewing the FDA's website. The Food and Drug Administration has an extensive, informative website which covers a wide variety of topics on regulations and is a valuable tool for research on the regulatory approval process, FDA Guidance Documents as well as information available on products cleared via the 510(k) Premarket Notification or Premarket Approval (PMA) process.[6]

7.2.2 Medical device approval process – the Food and Drug Administration

The routes to marketing a medical device product in the United States are a 510(k) Premarket Notification, Premarket Approval (PMA) or Product Development Protocol (PDP). Combination products that are a combination of more than one type of product such as a drug/device, biologic/device, drug/biologic will be reviewed later in this chapter as these products are regulated according to the primary mode of action, but also may be subject to more than one type of review process and regulations.

Food and Drug Administration premarket notification

The premarket notification 510(k) process requires a medical device manufacturer to 'notify' FDA 90 days before they propose to begin marketing a new or certain modified device. This notification submission allows FDA to determine whether a device is substantially equivalent to one or more predicate devices.

By law, a device is substantially equivalent (SE) to another legally marketed device if it has the same intended use and either the same technological characteristics or different technological characteristics but is as safe and effective as the other device and does not raise different questions of safety or effectiveness FDCA 513(I)(1)(A)g.[7] The goal of a 510(k) Premarket Notification submission is to demonstrate substantial equivalence to a device that is already legally marketed. The name comes from section 510(k) of the Food Drug and Cosmetic Act (FDCA).[7] The 510(k) process involves a comparison of one device to another legally marketed device. The device used for comparison purposes is referred to as the 'predicate' device. The FDA recommends a format for submitting 510(k) Premarket Notifications which is outlined in the guidance document *Format for Traditional and Abbreviated 510(k)s*.[8]

Elements of a premarket notification 510K submission

Information to be submitted in a 510(k) premarket notification to FDA includes:

- submitter's name, address, contact person, authorized agent in the USA if the submitter is outside the USA
- name of device
  - classification name
  - common name
  - proprietary name (brand name)
- FDA establishment registration number and address
- classification of device/classification panel
- performance standards

- labeling for the device and predicate device
- device description/substantial equivalence evaluation to the predicate device
- software information (if applicable)
- performance testing – examples: thermal, mechanical and electrical safety, electromagnetic compatibility/electromagnetic interference (if applicable)
- biocompatibility testing
- animal and clinical data (if applicable)
- sterilization information (if applicable)
- 510(k) summary
- indications for use statement
- truthful and accurate certification.

The Food and Drug Administration premarket approval process (PMA)

The Premarket Approval Process (PMA) is required for most Class III devices. Most of the high technology and advanced biomaterials for treating skin loss have been subject to the PMA process. These include many of the products discussed in this book such as INTEGRA Dermal Regeneration Template (Integra LifeSciences, Plainsboro, NJ), DermaGraft (Advanced BioHealing, La Jolla, CA) and Apligraf (Organogenesis, Canton, MA). The FDA considers the following factors in determining the safety and effectiveness of a product: persons for whose use the device is intended; adequate directions for use; probable benefit to health from use weighted against any probable injury or illness from use of the device; reliability of the device; valid scientific evidence and clinically significant results for a significant portion of a target population. The clinical data must support the intended use and provide reasonable assurance of safety and effectiveness.

The conduct of clinical trials under an FDA approved Investigation Device Exemption (IDE) will be reviewed in more detail under the clinical trial requirements.

The PMA is the approval process for most Class III medical devices and is governed by the Center for Devices and Radiological Health or Biological Product where both are required to achieve the intended use, indication or effect [21 CFR 3.2(e)].[9]

7.3 Medical device approval in the European Union

In the European Union, medical devices are regulated under the Medical Device Directive (MDD), Council Directive 93/42/EEC of 14 June 1993.[10] Article 3 of the MDD states 'The device must meet the essential requirements set out in Annex I [of the MDD] which apply to them…'.

7.3.1 CE mark certification

Devices considered to meet the essential requirements must bear the CE mark of

conformity when placed on the European market. In the effort to demonstrate conformity with the essential requirements, the manufacturer must determine the classification of the medical device. The device classification determines the conformity assessment procedures available to the manufacturer.

Advanced biomaterials for the treatment of skin loss may contain collagen or other components derived from material of animal origin. Section 4.5, Rule 17 of Annex IX of the MDD[11] concerning device classification provides that 'All devices manufactured utilizing animal tissues or derivatives rendered non-viable are Class III except where such devices are intended to come into contact with intact skin only.'

According to this rule, the majority of collagen medical devices are considered Class III in the European Union. Article 11 of the MDD provides the conformity assessment procedures. There are two alternative procedures provided for Class III devices. The first procedure, the most commonly used procedure for Class III devices, is detailed in Annex II of the MDD. Under this procedure, the manufacturer must obtain the applicable ISO 13485:2003[12] certification of their Quality System from a Notified Body (chosen by the manufacturer) to the current ISO standard, which is currently ISO 13485:2003. In addition, the manufacturer must submit a design dossier for examination by the Notified Body. The design dossier must demonstrate conformity with the essential requirements and describe the design, manufacture and performance of the product in question. Upon approval from the Notified Body, the manufacturer may apply CE marking to the device.

The second procedure, EC type-examination, is available for companies that do not have an ISO 13485:2003 certified quality system. It involves verification by the Notified Body that samples conform with the essential requirements and product design specifications. Depending on the depth of the manufacturer's quality system, continued monitoring is achieved either by examination and testing of samples from every batch or by periodic inspection and assessment. Because of the requirements for continued testing and inspection by the Notified Body, the first procedure for conformity assessment is preferred for products produced frequently, with a great number of batches.

7.3.2 Medicinal product approval in the European Union

There are currently two systems for medicinal product approval in the European Union: a decentralized approval on the national level and a centralized approval from the European Agency for the Evaluation of Medicinal Products (EMEA).

The decentralized system relies on the principal of mutual recognition of national authorizations. The process involves obtaining approval from the national authority of a member state in the European Union and that approval is then extended to other member states as identified by the applicant. However, if the original national authorization is not recognized by the secondary member state, the dispute is submitted to the EMEA for arbitration.

Council Regulation (EEC) 2309/93[13] creates the centralized community procedure, in which a single marketing authorization application (MAA) is submitted directly to the EMEA. There is a single evaluation and a single market authorization allowing direct access to the European Union. This procedure is compulsory for medicinal products developed by means of one of the following biotechnological processes outlined in the Annex to Regulation 2309/93,[14] Part A.

> 'Part A products are medicinal products developed by means of one of the following biotechnological processes:
>
> - recombinant DNA technology
> - controlled expression of genes coding for biologically active proteins in prokaryotes and eukaryotes including transformed mammalian cells
> - hybridoma and monoclonal antibody methods.'

The centralized procedure may also be used for innovative new products as described in the Annex to Regulation 2309/93, Part B.[15]

There are two scientific committees in the EMEA responsible for overseeing applications and preparing the Agency's opinions. The Committee for Proprietary Medicinal Products (CPMP) is responsible for drugs for human use and the Committee for Veterinary Medicinal Products (CVMP) is responsible for veterinary products.

Four to six months prior to submission in the centralized procedure, applicants are asked to notify the EMEA of their intention to submit an application and provide the intended month of submission. The letter of intent to submit should include a justification of the product's eligibility for evaluation under the centralized procedure. Additional information about the content of the letter of intention to submit and the centralized procedure can be obtained from the EMEA website at www.emea.eu.int.[16]

7.4 Combination products

The Food and Drug Administration (FDA) Safe Medical Devices Act (SMDA) of 1990[17] explicitly recognized the existence of products that 'constitute a combination of a drug, device, or biological product' and provided a mechanism for determining which agency within the FDA would be assigned the administrative responsibility of regulating a particular combination product. Currently, medical products could be reviewed by the Center for Devices and Radiological Health (CDRH), the Center for Drug Evaluation and Research (CDER) or the Center for Biological Evaluation and Research (CBER). The Food and Drug Administration Modernization Act of 1997 (FDAMA)[18] further refined the assignment process by providing a mechanism for requesting that FDA classify a product as a drug, biological product, device, or a combination product, in addition to determining which agency within the FDA would be assigned to regulate the product.

Combination products as defined in further detail in the 21 Code of Federal Regulations (CFR)[19] (Part 3) are composed of two or more different regulatory entities, that is, drug-device, drug-biologic, device-biologic, or drug-device-biologic products. Such products often involve cutting edge, novel technologies that raise unique scientific, technical, policy and regulatory issues. Furthermore, the FDA multi-center aspect of the premarket review and regulation of combination products presents unique challenges in review management. The combination of two distinct components that would normally be regulated under different regulatory authorities introduces additional factors to consider in the assignment of a lead center and the formulation of appropriate regulatory requirements. FDA will receive significantly more combination products for review as technological advances continue to merge with therapeutic products and blur the historical lines of separation between the FDA's medical product centers.

7.4.1 Definition of combination product

A combination product can be defined as:

(1) a product comprising two or more regulated components, i.e. drug/device, biologic/device, drug/biologic, or drug/device/biologic, that are physically, chemically, or otherwise combined or mixed and produced as a single entity;
(2) two or more separate products packaged together in a single package or as a unit and comprising drug and device products, device and biological products, or biological and drug products;
(3) a drug, device, or biological product packaged separately that according to its investigational plan or proposed labeling is intended for use only with an approved individually specified drug, device, or biological product, where both are required to achieve the intended use, indication, or effect and where upon approval of the proposed product the labeling of the approved product would need to be changed, e.g. to reflect a change in intended use, dosage form, strength, route of administration, or significant change in dose; or
(4) any investigational drug, device, or biological product packaged separately that according to its proposed labeling is for use only with another individually specified investigational drug, device, or biological product where both are required to achieve the intended use, indication, or effect.[9]

In accordance with the Federal Food, Drug, and Cosmetic Act section 503(g)(1),[7] the FDA is required to assign review responsibility for combination products based on the product's 'primary mode of action'. The designation of an FDA division does not preclude consultations with other FDA divisions and when such consultation is used, the involvement of more than one FDA center in the premarket review process presents unique challenges in review management. In addition, where the FDA finds it is appropriate, they reserve the option to require separate applications to be approved (by either the lead FDA center or a combina-

tion of FDA centers) for the individual components of a combination product. Requiring the approval of a second FDA center for a product presents additional issues, requirements and costs for the applicant.

The FDA has developed policies and procedures for the review and regulation of combination products and in February 2002 established a Combination Products Program within the Office of the Ombudsman to coordinate such activities. The Office of Combination Products also has assumed the functions of the Combination Products Program begun in 2002 within the FDA Office of the Ombudsman. The Office of Combination Products primary functions are: assigning an FDA Center to have primary jurisdiction for review of combination products; ensuring timely and effective premarket review of combination products by overseeing reviews involving more than one agency center; ensuring consistency and appropriateness of postmarket regulation of combination products; resolving disputes regarding the timeliness of premarket review of combination products; updating agreements; guidance documents or practices specific to the assignment of combination products; submitting annual reports to Congress on the Office of Combination Product activities and impact. In addition to serving as a point of contact for industry and the FDA Centers (CDRH, CBER, CDER) on combination products issues, the Office of Combination Products is developing guidance documents on a variety of policy issues for combination products. The FDA office of Combination Products was established by the Medical Device User Fee and Modernization Act of 2002.[19]

7.4.2 Food and Drug Administration request for designation for a combination product

Currently, combination products need to submit a 'request for designation' (RFD) to the FDA Office of Combination Products. By submitting a RFD, a company may obtain a formal agency determination on which FDA center they should work with in developing a new combination product. A company may submit an RFD for a combination product or for a drug, device, or biological product, when the jurisdiction is unclear or in dispute. The agency will make its jurisdictional determination no later than 60 days after filing the RFD. The RFD process is outlined in 21 CFR Part 3.[9]

7.4.3 Food and Drug Administration marketing applications of combination products

The FDA Safe Medical Device Act (SMDA)[17] required that the primary mode of action of a combination product must determine which FDA center would be responsible for premarket review, but did not address which authorities, including which type of marketing application, should be used to review the combination product, beyond authorizing FDA to use any resources necessary to ensure an

adequate premarket review. The selection of regulatory authorities to be applied to for a combination product is intended to ensure appropriate review and regulation, but may also affect the potential for generic competition and the availability of certain regulatory mechanisms or processes (e.g. a device component of a combination product regulated solely under the new drug application).

The FDA may also require separate applications for the different components of a combination product. The designation of one FDA agency as having primary jurisdiction for the premarket review and regulation of a combination product does not preclude consultations by that component with other agency components or, in appropriate cases, the requirement by the FDA for separate applications. This flexibility is important because the most appropriate regulatory approach for a given combination product may need to be tailored to the associated scientific and policy issues. Some sponsors of product applications have questioned the need for separate marketing applications for the components of a combination product, perhaps based on the perception that the regulatory burden would be less with a single application. On the other hand, some applicants have objected to FDA's decision to require only a single application because separate applications were considered to be advantageous for future development and/or marketing opportunities.

Elements of a premarket approval application submission

There are general requirements and the following is an outline of information needed to support combination product where the principal mode of action is a medical device. This proposed outline incorporates the elements of a Premarket Approval Application (PMA).[20,21]

- Standard required information on the company
  - Name, address, telephone numbers, facsimile numbers, FDA establishment registration number, contact person;
- If the facility is located outside the United States, FDA regulations require an authorized agent in the United States;
- Reports of prior investigations
  - data from preclinical studies; clinical trials performed outside the United States prior to the pivotal clinical trial;
- Description of the product
  - all significant components of the product
  - principal mode of action of each of the product components;
- If collagen is a component of the product
  - type of collagen
  - tissue and species
  - country of origin/sourcing

  - nature and potential for infectivity
  - methods used to inactivate or remove possible transmissible agent
  - viral inactivation study
  - route of administration
  - bovine spongiform encephalopathy (BSE)/transmissible spongiform en encephalopathy (TSE) analysis;

- If cultured cells of human origin are incorporated into the device
  - complete description of the origin of the cells
  - methods of separation from the host tissue
  - manner in which the cells will be handled and/or pooled
  - culturing technique
  - culture media
  - any agents, such as growth factors, used in the culturing
  - assurance that the cells are free of transmissible diseases and viruses must be provided
  - this should include testing of the donor's blood for $HTLV_{1+2}$, HIV_{1+2}, ALT, hepatitis B, hepatitis non A/hepatitis non B, RPR, and CMV IgM at the time of cell donation
  - the test for HIV_{1+2} should be repeated after six months
  - individual cell strains should be tested for infectious agents, including mycoplasma, sterility, HIV_{1+2}, $HTLV_{1+2}$, HSV_{1+2}, and CMV before pooling
  - an *in vitro* viral assay should be repeated on the pooled cells before being placed in a master cell bank
  - final product testing should include sterility, mycoplasma and endotoxin/pyrogenicity tests
  - individual cell lines should be tested to establish the normal human diploid karyotype
  - number of population doublings permitted should be identified
  - quality control procedures used to monitor the cells during the manufacturing process for unusual morphology or growth characteristics must also be described
  - if the cells are to be plated onto a substrate, the methods used to monitor cellular viability and density should be described and the minimum levels of acceptability identified
  - the validation process should also be described as well as the frequency with which it will be performed;

- Biocompatibility testing conducted according to ISO 10993[22]
  - dermal irritation
  - dermal sensitization
  - cytotoxicity
  - acute systemic toxicity
  - hemocompatibility/hemolysis

  - pyrogenicity
  - mutagenicity studies
  - subchronic toxicity
  - chronic toxicity
  - Carcinogenicity studies (if indicated)
  - immunogenic potential
  - reproductive/developmental toxicity (if indicated)
  - absorption studies
  - ADME studies (absorption, distribution, metabolism, excretion) (if indicated)
  - other studies dependent on the biomaterials being evaluated;

- Pre-clinical testing
  - animal species selected should exhibit a biological responsiveness to the test agents
  - multiple animal models are typically used to assess activity of the agents
  - animal studies selected will depend on the type of indication for use and claims being sought
  - biodistribution and pharmacokinetics studies
  - toxicity studies;

- Clinical trial data
  - complete investigational plan
    * intended use for the product
    * objectives of the study
    * number of patients to be enrolled and the number of investigational sites that will participate in the study
    * expected duration of the investigation
    * description of the design of the study (e.g. multi-centered, single-blinded, double-blinded, randomized, etc)
    * inclusion and exclusion criteria which will be used to determine patient eligibility for the study
    * methodology which will be used to assign patients to either the experimental or control groups
  - pre-treatment regime
    * patient pre-screening for eligibility
    * baseline evaluations
    * laboratory testing (hematologic, immunologic, urinary)
    * hypersensitivity screening
  - protocol to be followed
    * treatment regimen for both the experimental and control groups
      ◦ description of both the control and experimental treatments
      ◦ frequency of the treatments
      ◦ other care the patients will receive

- control treatment must be recognized as the current standard of care for this patient population
- description of how uniformity of the control and experimental treatments will be maintained across the investigational sites must be provided

* post-treatment regimen
  - description of the follow-up schedule must be provided
  - frequency of the follow-up visits as well as a description of treatment
    - all laboratory testing
    - all treatments and evaluations
* device effectiveness evaluation
  - study endpoints must be clearly identified
  - rationale for the selection of these endpoints
  - parameters used to evaluate the effectiveness of the dressing in the management of the indicated wound
  - comparison of standard care
* effectiveness evaluation
  - validated evaluation scales
  - histology of the tissue repaired
  - long term follow-up
  - patient and investigator evaluations
  - photographs (if indicated) evaluated by a panel of masked evaluators
  - radiographs, MRIs, and so on evaluated by a panel of masked evaluators
  - patient satisfaction/quality of life scale using validated measurement tool
  - any other treatment effectiveness evaluation evaluated by a panel of masked evaluators
* post-approval requirements
  - post-approval study or post-market surveillance study if indicated to collect additional information about the safety, effectiveness and reliability of the product
  - restriction on the sale and distribution of a product because of a high risk of harm or the need for collateral measures to ensure safe and effective use
  - requirement to add a prominent display of warnings, hazards, and precautions necessary for safe and effective use of labeling and to the advertising of restricted product
  - requirement to include identification codes on the product, in its labeling or on card given to patients with implants, if necessary to protect public health (if indicated)
  - requirement to maintain product tracking records in order to trace patients, if it becomes necessary to protect public health (if indicated)

7.4.4 Regulation of combination products in the European Union

Similar to the approval process in the United States, the first step in determining the European regulatory requirements for a medicinal product–delivery vehicle combination is to determine the product designation. According to the Medical Device Directive (MDD), Council Directive 93/42/EEC, June 14, 1993[23]

> '"medical device" means any instrument, apparatus, appliance, material or other article, whether used alone or in combination, including the software necessary for its proper application intended by the manufacturer to be used for human beings for the purpose of:
>
> - diagnosis, prevention, monitoring, treatment or alleviation of disease,
> - diagnosis, monitoring, treatment, alleviation of or compensation for an injury or handicap,
> - investigation, replacement or modification of the anatomy or of a physiological process,
> - control of conception
>
> And which does not achieve its principal intended action in or on the human body by pharmacological, immunological or metabolic means, but which may be assisted in its function by such means.'

Article 1(3) of the MDD addresses the governance of devices intended to administer medicinal products:

> 'Where a device is intended to administer a medicinal product within the meaning of Article 1 of Directive 65/65/EEC,[24] that device shall be governed by the present Directive.
>
> If, however, such a device is placed on the market in such a way that the device and the medicinal product form a single integral product which is intended exclusively for use in the given combination and which is not reusable, that single product shall be governed by Directive 65/65/EEC (concerning medicinal products). The relevant essential requirements of Annex I to the present directive shall apply as far as safety and performance related device features are concerned.'

Further guidance on the designation of combination products can be found in the European Commission guidelines relating to the demarcation between active implantable medical devices, medical devices and medicinal products (MEDDEV 2.1/3 Rev 5.1 – March 1998).[25] When a medical device is used to deliver a medicinal product, the combination will typically be regulated as a medicinal product.

7.5 The Global Harmonization Task Force (GHTF)

The Global Harmonization Task Force (GHTF) was formed in 1992.[26] This task force consists of the European Union, United States of America, Japan, Australia and Canada. The GHTF was formed in an effort to harmonize regulations globally. As medical devices become more complex and technologically advanced, the regulations governing these products also must change and become more adept at the review and regulation of these devices to assure safety and effectiveness. Review of the quality systems that manufacture these complex products and the postmarket surveillance also become more complex. At the same time, efforts must be made to allow these products to be available for the advancement of patient care and treatment. The GHTF provides a forum for both regulators and representatives from the medical device industry to address complex issues.

The harmonization of the review process includes clinical trial data and equally important quality system requirements. This chapter concentrates on the regulations of the United States of America and the European Union. However, the medical device and pharmaceutical market is highly regulated all over the world. The goal is to provide a unified global regulatory model to harmonize regulations.

7.5.1 Summary technical documentation (STED)

The summary technical documentation (STED) is a harmonized submission format developed by the (GHTF). STED was intended to be a standard, harmonized format accepted by multiple regulatory agencies globally. The details of the format and documentation required are outlined in the GHTF document *Summary Technical Documentation for Demonstrating Conformity to the Essential Principles of Safety and Performance of Medical Devices (STED)*.[27]

The United States Food and Drug Administration initiated a voluntary pilot premarket review program using the STED in June, 2003. FDA's guidance document *A Pilot Program to Evaluate a Proposed Globally Harmonized Alternative for Premarket Procedures details*[28] the documents and format for the STED are detailed in this guidance document. The FDA must be contacted first to determine if the premarket submission can be made in the STED format.

The STED contains documentation that is an expanded version of documents required in a design dossier submission for Class III medical devices under the European Union Medical Device Directive and FDA Premarket Approval application.[3]

7.6 Quality system

The quality system which includes the manufacturing process is integral to the approval process in the USA, the European Union and almost every other country.

In the USA, if the product is a medical device subject to the PMA or a

pharmaceutical or biologic product subject of a new drug application (NDA) or biologic subject to obtaining a biologic license, the product will not receive final approval until the FDA has reviewed a detailed submission on the quality system and manufacturing process. These applications usually require an inspection of the manufacturing facility for compliance with the FDA's quality system regulations (devices) current good manufacturing practices (drugs) or both if the product is a combination product.

In the European Union there is a centralized process of CE mark certification. It is a requirement that the manufacturer and manufacturing facility be certified to the most current standard for quality systems which is ISO 13485:2003.

This chapter concentrates on the approval process in the USA and the European Union; however it is noteworthy to mention that almost every other major country requires compliance and certification of the quality system to ISO 13485:2003.[12] The quality system section of a PMA includes quality management, quality organization, device design, buildings, equipment, purchase and handling of components, production and process controls, packaging and label control, device evaluation, distributions, installation, complaint handling, sourcing and records. The requirements of a quality system under FDA, 21 CFR Part 820[29] with ISO 13485:2003 are similar but to date have not been harmonized.

7.7 Clinical trials

The FDA approval process for high technology medical devices, combination products and pharmaceuticals usually requires clinical trial data. In the European Union clinical data is required for all submissions and there are revisions to the Medical Device Directives which further define the clinical data required to support the CE mark certification of medical devices.[30]

Clinical trials in the USA for advanced biomaterials for the treatment of skin loss which are Class III medical devices will require valid scientific evidence of safety and effectiveness from a clinical trial conducted under an approved investigational device exemption (IDE)[31] or NDA. Clinical trials in the USA and European Union should be conducted under good clinical practice regulations. Good clinical practices (GCPs)[32] are an international ethical and scientific quality standard for designing, conducting, recording and reporting clinical trials that involve the participation of human subjects. Compliance with this standard provides assurance that the rights, safety and well-being of subjects in a clinical trial are protected and consistent with the principles of the Declaration of Helsinki[33] and that the clinical trial data is credible.

7.7.1 Good clinical practices

The Expert Working Group of the International Council on Harmonization of the Technical Requirements for Registration of Pharmaceuticals for Human Use

(ICH) has developed harmonized guidelines on good clinical practices (GCP).[32] The conduct of a clinical trial according to good clinical practices has been adopted by the USA, European Union, Japan, Australia and most other countries.

Good Clinical Practice Guidelines

These guidance documents outline in detail requirements which include:

- Institutional Review Board (IRB)/Independent Ethics Committee (IEC) approval of the clinical study
  - composition of the IRB/IEC member
  - procedures of the IRB/IEC
  - records
- Investigational qualification and agreements
  - medical care of subjects in the clinical trial
  - adequate resources to conduct the clinical trial
  - communication with the IRB/IEC
  - compliance with the protocol
  - compliance with investigational product regulations
  - randomization and unblinding
  - informed consent of clinical trial subjects
  - records and reports
  - progress reports to the IRB/IEC and sponsor
  - safety reporting
  - premature termination or suspension of a clinical trial
  - final reports by the investigator/institution
- Sponsor
  - quality assurance and quality control systems to ensure clinical trials are reported in compliance with the protocol, good clinical practices and any applicable regulatory requirement
  - contract research organizations
  - medical expertise
  - clinical trial design
  - Clinical Trial Management, Data Handling, Record Keeping and Independent Data Monitoring Committee
  - selection of investigators
  - establish trial related duties and functions
  - compensation for subjects and investigators
  - financing
  - notification/submissions to regulatory authorities
  - confirmation of review by IRB/IEC
  - information on investigational products

  - manufacturing, packaging, labeling, coding investigational product
  - supplying and handling of investigational product
  - access to records and clinical trial source documents
  - safety information
  - adverse event reporting
  - monitoring of the clinical trial
  - selection and qualification of monitors
  - monitoring procedures, responsibilities and reporting
  - audit of clinical trial sites and data
  - clinical trial/study reports
- Clinical trial protocol
  - clinical trial objective
  - clinical trial design
  - selection and withdrawal of subjects
  - treatment of subjects
  - assessment of efficacy
  - assessment of safety
  - statistical analysis plan
  - quality control and quality assurances
  - ethics
  - financing and insurance
  - publication policy
  - supplemental information
- Investigational brochure is a computation of the clinical and non-clinical data on the investigational product relative to the study of the product in human subjects
  - introduction to the investigational product and clinical trial
  - physical, chemical and pharmaceutical properties and formulation
  - non-clinical studies
  - effects in humans
  - clinical trial data
  - safety and efficacy
  - publications.

7.7.2 Clinical trial design

A well designed clinical trial on a biomaterial intended for the treatment of skin loss is critically important to evaluate the safety and effectiveness of the product and support the regulatory approval of the product. It is also important to provide objective clinical evidence of the clinical utility of the product and its safe use by clinicians.

In a highly regulated environment, the clinical trial design should include data important to support reimbursement of the product. Reimbursement agencies in

every country usually now require objective clinical evidence obtained from a multicenter clinical trial demonstrating the clinical effectiveness of the product compared to the standard of care.

In the design of the protocol and clinical trial it is important to delineate the objectives of the clinical trial, the primary and secondary outcomes of the clinical trial, the population the product is intended to treat, inclusion, exclusion criteria, surgical technique and outcome measurements. A robust statistical plan must also be prepared. The statistical plan will determine the number of subjects needed to provide statistical evidence to support the primary outcome of the study.

The study design must be established. Regulatory agencies usually request data obtained from a randomized, controlled, blinded evaluation, multicenter clinical trial. While this design is ideal, it may not always be feasible for trials evaluating biomaterials for the treatment of skin loss, especially resulting from thermal injuries. There is also a design that can be used, which is each person serves as their own control. The advantage of this design is that usually, except in areas of minor skin loss or single wound sites, the patient has more than one area to be treated. This design eliminates any bias associated with randominization especially with regard to age, gender, diagnosis or concomitant medical condition. The same person has the investigational and control (standard of care) product used on different sites of skin loss. The investigational product can be directly evaluated compared to a control site in the same patient. The disadvantage is it may be difficult to find patients with two sites suitable for treatment. If one site (investigational or control) heals with significant better results, this may be an issue medically and psychologically for the patient.

Blinding can be an issue in controlled clinical trials of biomaterials for skin loss, as the products, while they are on the patient, can be noticeably different to the clinician and patient. Blind evaluations of range of motion, healing, cosmetic and functional attributes can be evaluated by a clinician who is blind to the treatment when the products have been removed/incorporated into the patient's skin. Clinical outcomes for evaluation of products for skin loss include time of healing, range of motion, scarring and long term follow-up evaluations of healing, functions and cosmetic evaluations.

Clinical trial design for chronic cutaneous ulcers and burn wounds

The FDA has published a guidance document *Chronic Cutaneous Ulcer and Burn Wounds – Developing Products for Treatment.*[34] The guidance addresses important considerations in clinical study design regarding venous stasis ulcers, diabetic foot ulcers, pressure ulcers and burn wounds.

In the initial phase of a clinical trial design, it is important to establish the 'indication for use' for the product. The product's labeled indication for use is based on substantial evidence obtained in preclinical and clinical trials and safety and efficacy data obtained from the results of the clinical trial.

Preclinical data

Animal wound models: the choice of an animal model should be based upon valid scientific evidence. A literature search should be conducted and an appropriate animal model chosen that can address the desired treatment outcome. For example, contraction and re-epithelialization can be evaluated on a full-thickness excisional wound in a pig model for burns or chronic wounds. Multiple animal model studies may be needed to support the use in human clinical trials.

Study population in clinical trials for biomaterials for skin loss

In clinical trials for biomaterials for skin loss, various factors must be taken into consideration. The patient population must be appropriate for the indication for use for the type of wounds/skin loss. The patient population must also be adequate to detect a treatment effect. It is important to include the patient population, especially the age range for which the product will be indicated for use. It will be important to establish the risk analysis for the product to determine if the product can be safely evaluated in high risk patient groups, especially pediatric or pregnant women.

In burn patients, the depth, surface area, location of the burn wound, percent body surface area, type of burn (thermal, electrical, chemical), demographic information (age, gender), concomitant injuries, especially inhalation injuries as well as trauma scores of the overall severity of the illness should be included such as the trauma – injury severity score (TRISS)[35] and the acute physiology and chronic health evaluation (APACHE) Score.[36]

Chronic cutaneous ulcers

The major categories of chronic cutaneous ulcers are diabetic ulcers, venous stasis ulcers and pressure ulcers. These types of chronic ulcers have very different underlying medical etiologies and treatment regimes. Usually products for treatment of these ulcers must be conducted in separate clinical trials specific to the ulcer type.

Standard of care

Standard of care refers to accepted wound care procedures that will be used in the clinical trial. Most burn centers and wound care centers have standard treatment protocols. These standard protocols may vary between treatment centers. It is important for the purposes of the clinical trial that the standard of care and treatment protocols are standardized between investigators and investigational site.[37]

Standard of care for chronic cutaneous ulcers includes the following:

- removal of necrotic or infected tissue
- establishment of adequate blood circulation
- maintenance of a moist wound environment
- management of wound infection
- wound cleansing
- nutritional support, including blood glucose control for subjects with diabetic ulcers
- bowel and bladder care for subjects with pressure ulcers at risk of contamination
- off-loading of pressure
- compression therapy for venous stasis ulcers.

The standard care for serious burns includes careful attention to the following parameters:

- hemodynamic resuscitation
- management of co-morbidities
- timely burn debridement and excision
- wound closure
- management of wound infection
- pain control
- nutritional support
- measures to inhibit excessive scar formation
- rehabilitation, including passive range of motion when burns overlie joints.

Efficacy endpoints

The clinical outcomes for studies evaluating the use of biomaterials in advanced wound care therapies for skin loss depend on what is the primary outcome desired effect of the product. Generally, the use of these products are evaluated by the rate and improvement in wound healing.[37] However, with advanced therapies, the indication for use and primary outcome measurement may be regeneration of tissue – dermal or epithelial, acceleration of wound healing, quality of healing with claims of reduction in scar formation. The biomaterials may also have primary or secondary outcomes in decreasing and/or treatment of wound infection.

Quality of life outcomes are an important element of any clinical trial evaluating biomaterials for skin loss. There are validated tools that measure the quality of life outcomes depending on the outcome measurements of the trial and the type of skin loss being evaluated.

7.8 Humanitarian device exemption

The Humanitarian Device Exemption (HDE) is an application that is similar to a FDA PMA application. The HDE is exempt from the effectiveness requirement and FDA approval of the HDE authorizes the product to be marketed. The

humanitarian device is a 'medical device intended to benefit patients in the treatment or diagnosis of a disease or condition that affects or is manifested in fewer than 4000 individuals in the United States per year'.[38]

The HDE requires that first an application for designation as a humanitarian device be submitted to FDA prior to submitting the HDE application. The Request for Designation (RFD) as a HDE must include a description of the device and the intended disease or condition the device is intended to treat. Documentation must be submitted to support that the device meets the definition and requirements of an HDE. The humanitarian device must meet the principle requirements that there are no comparable devices to treat the disease or condition and that there are less than 4000 people per year affected. There are regulations regarding the cost of an HDE. The amount charged for the product cannot be more than US$250.00. If the cost is more than US$250.00, a report must be submitted by a certified public accountant verifying the amount does not exceed the cost of research, development, fabrication and distribution of the product.

HDE devices require institutional review board (IRB) approval at the medical facility prior to the product being used on patients. Products that have a HDE are subject to the same quality system (good manufacturing practices), amendments, supplements and reports and regulations as are products approved under the FDA PMA regulations.

7.9 Human tissue and cellular based products

Many of the biomaterials for the treatment of skin loss may be or contain human tissue or cellular-based components. The regulatory approval process varies depending on the type of tissue, whether it is subject to tissue regulations, medical device or biologic regulations. Regulations regarding human tissue-based products vary as well among countries.

In the United States and European Union, there are similar regulations regarding the donation, procurement and testing of donors, as well as traceability requirements, notification of serious adverse events and tissue establishment accreditation and inspection[39–41] for products that are made from human and cellular-based tissue.

In Europe, tissue establishments with activities for testing, processing, preservation, storage or distribution of human tissue need to be accredited by a competent authority. In the United States, tissue banks receive accreditation from the American Association of Tissue Banks and must comply with FDA human and cellular-based tissue regulations. Additionally, tissue banks must also have a tissue bank license from certain states within the USA.

In Europe and the USA, tissue establishments are subject to accreditation and inspection for compliance with regulations. There are traceability requirements from the donor to the recipient. There are stringent requirements for all donor tissue[19] including exclusion of deceased donors for:

- presence of previous history of malignant disease, history of disease with etiology, risk of transmission of Creutzfeldt-Jakob Disease
- systemic infection, HIV, Hep B/C, HTLV I/II, history of autoimmune disease
- transplantation with xenografts, recent history of vaccination with life attenuated virus, evidence of risk factors for transmissible diseases
- there are additional requirements for deceased child donors.

The testing requirements for human donor tissue include:

- HIV 1 & 2
- anti-HIV-1, 2
- hepatitis B
- HBsAg
- anti-HBc Ab
- hepatitis C
- anti-HCV-Ab

All human tissue donors must provide consent for procurement of the tissue.

7.10 User fees

The FDA Medical Device User Fee and Administration Act of 2002 and FDA Amendments Act of 2007[42] established among other regulations user fees for submissions. There are user fees associated with submissions such as New Drug Applications, 510(K) premarket notifications, PreMarket Approval applications and other types of submission. The fee is required at the time of the submission. The fee associated with a submission can be ascertained by information on FDA's internet website. There are fees also associated with review of a design dossier by the notified body in the European Union for CE mark certification. Many other countries have user fees for registration of products.

7.11 Future trends in the regulatory process

The treatment of skin loss will continue to advance with combination therapies and products involving biomaterials that incorporate drugs, biologics, growth factors, and are also used in conjunction with other high technology devices. The role of the Global Harmonization Task Force will become more important in standardizing the approval process globally. A standard technical document that is accepted by the USA, European Union, Japan and Australia will be important in the regulatory approval process.

If regulations were harmonized, a sponsor of these advanced products could prepare one technical dossier that contained comprehensive preclinical, clinical, quality system, manufacturing data, risk analysis, postmarket surveillance programs which would be accepted by multiple global regulatory agencies. The

submission, review and approval by one competent authority, such as the FDA, could be accepted by competent authorities in other countries.

Postmarket surveillance and risk management data will become more critical in monitoring the continued safe use of these advanced biomaterials for skin loss and document long term safety and effectiveness profiles. Providing a framework for approval globally of these products will facilitate the availability of these advanced biomaterials for skin loss to improve the quality of life for these patient populations.

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Part II

Epidermal and dermal replacement technologies

8

Alternative delivery of keratinocytes for epidermal replacement

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Abstract: In bridging the gap between the laboratory and the patient it is essential to consider the differences in the environment the cells are exposed to in order optimize their performance and achieve the goal of expedient wound closure. Successful delivery systems for keratinocytes to the wound bed must consider the viability and proliferation potential of the cells with protection to achieve a mature epithelial layer. The delivery systems are described in two groups, direct delivery or with the use of carrier. The clinical use of pre-confluent keratinocytes has been widely reported to be effective in augmenting wound healing.

Key words: Keratinocyte suspension, aerosol delivery, pre-confluent cell delivery, cell carriers.

8.1 Introduction

In bridging the gap between the laboratory and the patient it is essential to consider the differences in the environment the cells are exposed to in order to optimize their performance and achieve the goal of expedient wound closure.[1] The development of the technique of cultured epithelial autograft (CEA) was driven by the need to achieve wound closure in extensive burn injury with limited skin donor sites.[2] CEA was used in the form of confluent cell sheets in place of split thickness skin grafts.[3,4] As reports appeared in the literature it was clear that wound healing was possible with the CEA but problems emerged.[5,6]

The time taken to achieve confluent sheets, often greater than three weeks[7] presents challenges in caring for the patient, including infection control, wound care and pain management. The processes both in the laboratory and operating theatre are costly, labour intensive and time consuming.[8,9]

Subsequent to healing, secondary loss of the CEA healed surface with blistering is associated with secondary delayed healing and poor scar outcome.[10,11] In addition, contracture of the scar is a problem with the fragile surface making scar management more difficult.[12]

It has been demonstrated that the keratinocytes change in character from the time of initial harvest from the dermal epidermal junction as a dissociated cell population,

to the cells developed into a confluent sheet.[13,14] The use of an enzymatic process to remove the cells from the tissue culture flask further influences the adhesion to the wound bed.[15] The surface integrins expressed change from those associated with proliferation and adhesion to those associated with differentiation.[16–19]

The investigation of the keratinocyte characteristics in different states led to the exploration of the clinical use of CEA in a pre-confluent state.[20,21] The history of 'epithelial cell seeding' is outlined by Horch *et al.*[22] and dates back to an initial report in 1895 when the observation was made that cells would attach to the wound better than conventional skin.

Harvesting the cells prior to confluence would clearly save time and reduce the costs associated with the laboratory.[23] Theoretically the cells would be primed for adhesion and proliferation initially, progressing to differentiation as the epidermal layer becomes established.[24,25] The use of pre-confluent keratinocytes was driven by the need to reduce the time taken between skin harvesting and clinically using the cells, and to reduce the complications reported.[20,23] An alternative solution to the issue of time and availability is the use of allograft.[26] The cell delivery issues are the same with both allograft and autograft. The challenge is to deliver the cells to the wound bed in a condition able to achieve expedient wound healing.

In understanding how to use the cells to achieve the optimal scar outcome the relationships between:

- the time taken to culture the cells
- the desire for a definitive skin repair
- the need to achieve rapid wound closure
- to minimize the scar

need to be considered and investigated.

The patient treatment plan must be based on the assessment of the regenerative capacity of the individual, systemically, and of the wound, locally. It is clear that the performance of cells delivered to the wound is intimately related to the wound bed.[27] A clean wound with residual dermis, as in a partial thickness injury, provides the optimal environment for pre-confluent keratinocytes.[28,29] It can be considered that the prepared wound itself is the optimal 'tissue culture' environment for the cells.

In a situation with more tissue loss, epidermal repair may be achieved with pre-confluent cells, but the absence of deeper dermal elements will result in a poor functional outcome and scar.[5,6] Prior repair of the dermal layer needs to be considered. The dermal repair may be achieved in a variety of ways discussed in Chapter 10.

Healing of full thickness wounds have been described using the delivery of keratinocytes in a three-dimensional construct with the cells seeded into a scaffold in the laboratory.[30] It is possible that the time taken in the laboratory may be reduced by seeding cells from the dermal epidermal junction into a dermal scaffold

with immediate placement onto the wound,[31] once again, exploring the concept of the wound as the 'tissue culture' environment. The development and clinical use of three-dimensional tissue engineered skin constructs are discussed in Chapter 10.

8.2 Methods of keratinocyte delivery

A delivery system needs to ensure the even distribution of viable cells capable of adhesion onto the surface of the wound.[32] The use of pre-confluent cells requires that the surface is protected whilst the cells proliferate and migrate across the wound and differentiate to a confluent, mature epidermal layer.

The keratinocytes are harvested from the dermal epidermal junction via a process of enzymatic and physical dissociation.[2] Initially the cells are a mixed population including melanocytes, papillary dermal fibroblasts and Langerhan's giant cells with the keratinocytes.[33] The clinical use of the cells at this stage has been practiced by our group since 1995 to augment wound healing in acute injuries, donor sites[34] and to improve the surface in scar revision.[1,21] The cells are delivered as a suspension in an aerosol using a nozzle fitted to a standard syringe. Cells have been delivered to the wound bed immediately using a carrier dressing to facilitate placement.[35] The cells have also been seeded onto carrier dressings for later transfer to the wound bed as the cells develop into colonies.[36]

In the process described by Green *et al.*[2] the cells are seeded onto a layer of irradiated mouse 3T3 fibroblasts. As the primary colonies expand, the cells are harvested and at this stage the cells may be used as a suspension delivered to the wound directly[23] or using a carrier, or seeded onto a carrier for later use as the colonies become established.[37,38]

The delivery of cells to the wound can be at any stage in the expansion process by direct application or by the use of a carrier. The seeding density is related to the stage of the cells at the time of clinical use.[39] When used immediately with no prior culture, the cell population will contain terminally differentiated keratinocytes and the cell density will be expected to be greater than cells which are cultured and colonies established prior to clinical application.

The need to understand the clinical indication is common to all techniques with a clear understanding of what epidermal cells can achieve in the wound healing spectrum.[32] There is a balance between the expansion ratio and the speed of epithelial repair.[39] Understanding the influence of the wound bed on the speed and area of epithelial repair for a given cell load will indicate the appropriate clinical use.[1]

8.3 Direct application

Direct application of the cells to the wound can be achieved by dripping, spraying or air brushing techniques. The maintenance of viability of the cells in the system is essential.[40]

We commenced the use of cells in suspension in 1995 making 'blisters' of adhesive dressings filled with fluid containing the cells. We then tested a series of airbrushing techniques, using airflow as a vortex to deliver the cells, we found that this was cumbersome with fluid and cell loss.[23] Nozzles clipped on to standard syringes provided a simple solution with little dead space and allowed direct application. The essential elements of the nozzle are the size of the exit aperture, the internal vortex dimensions and the pressure generated manually by the syringe. Viability was maintained using the nozzle on a 5 ml syringe, avoiding the higher pressures generated by the 1 and 2 ml syringes. We routinely use the nozzle to deliver cells at the time of harvesting, ReCell (C3 pty ltd) and as an expanded cell population CellSpray (C3 pty ltd). The cells are delivered in a balanced salt solution or culture medium with no added proteins. Others have demonstrated the effectiveness of spray delivery systems in the delivery of viable functional cells.[41]

Concern about the fluid runoff and cell loss has prompted investigation of the use of fibrin to facilitate cell adherence and expedite wound healing.[22,42] Fibrin application itself has been demonstrated to improve the speed of epithelial repair. It has been reported in animal models and is effective clinically in providing a system which delivers viable cells to the surface which differentiate into a mature epithelial surface.[43] Commercially available Tisseel (Baxter) fibrin[22,42] and autologous fibrin produced from the patient's own serum[43] have been used.

In both cases the protective dressing system is integral to successful epithelial repair.[44,45] The dressings used may be synthetic, such as Surfrasoft (Mediprof) a woven nylon construct, as the primary layer with secondary gauze and fixation. More complex systems are used, for example the use of Biobrane (Smith & Nephew),[35] a bovine collagen and silicone construct, has been reported. It is unclear from the literature which is a superior dressing system. The principles of wound protection, infection control and atraumatic removal, are relevant to all dressing systems.

8.4 Carrier systems

Carrier systems have been:

- used immediately on initial skin cell harvest
- immediately seeded with cells but maintained in the laboratory to allow colonies to establish
- seeded with cells which have undergone prior expansion in the laboratory.

The carrier system avoids enzymatic removal of the cells from the tissue flask in preparation for clinical use, facilitates handling and takes on the role of the primary dressing.

To be clinically useful a carrier needs to:

- facilitate keratinocyte proliferation
- be non-toxic

Table 8.1 Summary of available products used as cell carriers

Product	Manufacturer	Properties of product	Reference
Biobrane	Smith & Nephew	Nylon fabric embedded in silicone film coated with collagen 1	35, 46
Alloderm	LifeCell	Acellular dermal matrix sourced from human tissue may be preseeded with fibroblasts	22, 47, 48
LaserSkin	Fidia Advanced Biopolymers	Benzyl esterified hyaluronic acid with laser drilled holes may be co-seeded with fibroblasts	49, 50, 51
Myskin	CellTran	Silicone coated with plasma polymer film	37, 52
Chitosan	Experimental chitosan is available from a number of sources	Chitosan with lamanin peptide AG73	53, 54
Epigen	Smith & Nephew	Polymeric film of ethylene vinyl acetate and polystyrene	32, 55, 56
Tegaderm	3M	Polyurethane	57, 58
Hydroderm	Innovative Technologies	Polyurethane	38, 59
Tisseel	Baxter	Allograft plasma and bovine thrombin	22, 41, 42

- be non-allogenic
- allow keratinocyte migration onto the wound
- be flexible to allow contouring to the wound surface
- be shed/degraded as the epithelial surface matures.

Many carriers have been reported in the literature, the composition ranges from synthetic polymers to biological fibrin mats, AG73 chitosan, Alloderm, to combinations such as Biobrane with collagen and silicone. Table 8.1 indicates the literature published in the area of pre-confluent cell delivery using carrier systems which are available. Work has been reported using microcarriers of absorbable synthetic material which have not been widely used clinically owing to the associated inflammatory response.[39]

The cell seeding density varies with the timing of seeding and of clinical use. Various clinical cases are reported in acute and chronic wounds and all report enhanced epithelial repair.

8.5 Summary

With the exploration of the clinical indications for the use of cells harvested from

the dermal epidermal junction come reports of novel systems to facilitate rapid, simple, cost effective methods of delivery. From the literature it is not possible to identify any technique as being superior to another in epithelial repair.

Keratinocytes are delivered to the wound as an element within a three-dimensional construct which is beyond the scope of this chapter. The role of a technique with minimal donor sites and the potential to expand the surface area covered to 1:80 and greater, allows exploration of the indications for use. Rapid cover of partial thickness injury with dermal preservation reduces the risk of permanent scarring. The technique also provides the opportunity to resurface scars to improve the contour and colour using a small site matched donor site.

In reviewing the outcomes it is essential to consider the cost, convenience and complications as use of the technology progresses.

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9

Enhancing skin epidermal stability

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Abstract: Long term epidermal instability has been encountered in full thickness cutaneous wounds treated with cultured epidermal autograft. Here we examine how fibrin and hyaluronic acid matrices have been developed to provide a means of delivering keratinocytes to the wound bed and to improve epidermal stability. The physical and chemical properties of fibrin and hyaluronic acid provide an environment suitable for the generation of a stable and organised epidermis. Studies using such carrier matrices have demonstrated that a stable epidermal repair can be achieved clinically when these materials are used in combination with cultured keratinocytes.

Key words: epidermis, fibrin, hyaluronic acid, laserskin®, keratinocyte.

9.1 Introduction

A variety of approaches have been explored to provide restoration of the epidermis whilst reducing the need to use large amounts of autologous split thickness skin graft. These include the use of allogeneic cadaver skin, cultured epithelial autograft (CEA), sprayed autologous cultured keratinocytes and an array of materials designed to restore the physical and biological function of normal epidermis. The transplantation of cultured epithelial autografts and sub-confluent keratinocytes to repopulate the wound with keratinocytes has achieved some clinical success. However, long term epidermal instability has been encountered and modifications to the existing technique have been sought by combining cell-based treatments with biomaterials. A number of biomaterials have been used to deliver cultured keratinocytes to the wound bed, in order to improve the handling of the transplanted material, the cellular organisation of the repair tissue and its long term stability. This chapter examines how fibrin and hyaluronic acid matrices have been developed to provide a means of delivering keratinocytes to the wound bed whilst providing an environment suitable for the generation of a stable and organised epidermis.

The 'gold standard' epidermal replacement is autologous split thickness skin graft (STSG). However, limitations in donor site availability and the creation of a

secondary wound have led to alternatives being sought in the development of (CEA) and sprayed cultured keratinocytes.

The number of permanent, purely epidermal, biological skin substitutes presently available on the market is limited. Commercially produced autologous keratinocyte sheets are available from a number of companies. The technology is also available from a number of university and hospital laboratories and private research facilities. The sheets are produced either on the surface of culture flasks and then transferred onto a supporting delivery membrane, for example Epicel®, or they are grown on a delivery material throughout the culture process, for example Myskin™ and Laserskin® (Moustafa *et al*., 2007; Ronfard *et al*., 1991).

The use of CEA alone to treat burns has provided inconsistent results, with variable take rates and a tendency to blister (Desai *et al*., 1991; Woodley *et al*., 1988). Sprayed keratinocytes derived from both sub-confluent cultures (Magnusson *et al*., 2007) and from uncultured cells isolated by the digestion of autologous split thickness skin (Gravante *et al*., 2007) have been extensively used clinically. Limited clinical data exist irrefutably proving which mode of keratinocyte delivery provides the most effective wound closure. It is clear that a great deal of work is still required to provide an environment in which keratinocytes or epithelial precursor cells obtained from an autologous biopsy can grow into the highly organised structure which constitutes a stable and fully functional stratified epithelium.

9.2 Fibrin as a repair material

The use of fibrin glue in skin grafts and tissue engineered skin replacements has been reviewed by Currie *et al*. (2001). Fibrin has been used as a haemostatic agent and as a glue for the repair of tissue since the early 20th century (Bergel 1909) although its effectiveness as a glue was only established 60 years later with the introduction of a glue with a high fibrin content (Matras *et al*., 1972).

Commercially available fibrin sealants emulate the biological process of fibrinogen breakdown by thrombin to fibrin monomer and its subsequent polymerisation in the presence of factor XIII and calcium to form fibrin polymer. Numerous commercially available fibrin glues are marketed for use in a wide variety of applications (Currie *et al.*, 2001; Eyrich *et al.*, 2006). Fibrin glues have been used in cutaneous wound repair to improve graft adherence, to deliver keratinocytes in suspension and for their delivery in a matrix or sheet form.

Wound healing progresses by a complex series of cellular and biochemical interactions. These can be modified by providing an environment, in the form of a matrix, in which these interactions are optimised to provide an increased rate of healing with an improved and more stable final epithelial structure. For example, the fibre size and spacing of fibrin fibrils within fibrin matrices has been shown to affect the migration of fibroblasts into the matrix *in vivo* (Pandit *et al.*, 1998). The ability to modify the structure of fibrin in the fibrin matrix, in order to provide

optimal conditions for cell proliferation and differentiation, makes fibrin an attractive candidate as a carrier matrix for the delivery of cells to the wound bed. This attractiveness as a wound repair material is further enhanced as fibrin degrades completely without causing any immunogenic response (Ronfard *et al*., 1991).

The principle ways in which fibrin can be used to deliver cells to the wound bed are as follows:

1 Apply the thrombin and calcium solution to the wound bed and spray the fibrin component containing cells onto the wound. On contact the solutions mix and polymerise. The use of this sprayed technique is covered elsewhere in this book.
2 Mix the thrombin and fibrin fractions with cells and apply to the wound bed as the solution polymerises.
3 Generate fibrin sheets *in vitro* seeded with keratinocytes and apply these as sheets to the wound bed.

This review examines the use of fibrin delivered to the wound as sheet material.

9.2.1 Use of fibrin sheets for restoration of the epidermis

The first reported use of fibrin as a delivery vehicle for keratinocytes was by Hunyadi *et al*. (1987) for the treatment of chronic wounds. In this study keratinocytes were isolated from split thickness skin by trypsin digestion and suspended in the fibrinogen component of Beriplast® fibrin sealant. This was combined with the thrombin and calcium components on the wound bed to form a fibrin matrix containing keratinocytes. This method avoided the use of cultured keratinocytes and although limited data are presented, demonstrated increased epithelialisation. A further study (Hunyadi *et al*., 1988) described the treatment of chronic wounds in 20 patients with fibrin and keratinocytes of which 16 healed well when compared with five patients treated with fibrin alone, where no healing occurred. This idea was further developed by Ronfard *et al*. (1991) who used keratinocytes cultured with irradiated 3T3 mouse fibroblast cells according to the methods of Rheinwald and Green (1975). At the final subculture, cells were seeded into Petri dishes which had been pre-coated with fibrin glue matrix. The fibrin glue matrix was prepared by combining fibrin solution in the presence of aprotinin and thrombin. This mixture was spread evenly over the base of the dish and allowed to polymerise into a homologous fibrin matrix. Proteolytic degradation of the matrix was prevented by the high concentration of aprotinin. Cells were cultured for either 2 days, forming colonies of 10–20 cells or for 10 days after which time they formed a confluent sheet 1–4 cells thick. The fibrin matrices, populated with keratinocytes, were then transferred to the wound. It appears from the data presented in this study that a better take rate occurred when the fibrin matrix was oriented with keratinocytes facing the wound and the fibrin outward. Sub-confluent keratinocytes, rather than

confluent cultures, also appeared to demonstrate improved take. However, as only two patients were treated in this study, and different sites were treated with inconsistent methods, no firm conclusions were drawn. Despite this, when sub-confluent keratinocytes were delivered with the cell sheet down, take rates of 87% were observed suggesting that the technique provided good biological and mechanical support for keratinocytes.

The same group conducted further investigations using an athymic mouse model to assess better the effect that cell culture conditions have on successful wound repair (Ronfard *et al*., 2000). In additional clinical studies it was found that cell spreading was reduced when keratinocytes were cultured on a fibrin matrix compared with tissue culture plastic, although the clonogenic capacity of the fibrin matrix was similar. The fibrin matrix was found to have completely degraded within 11 days following transplantation onto the mouse model. As part of this study, seven patients suffering from burns ranging from 45–95% total body surface area (TBSA) were treated with keratinocytes cultured on fibrin matrices. Histological examinations of punch biopsies demonstrated that structures analogous to rete ridges were present in areas treated with fibrin matrix but were not present in areas treated with cultured epithelium alone, suggesting that the presence of the fibrin matrix may facilitate the formation of dermal/epidermal junctions. These studies demonstrated, above all, that the use of fibrin as a carrier matrix improves the mechanical integrity of CEAs and acts as an ideal carrier material. Additionally, the fibrin matrix was found to retain its size in contrast to CEA which can shrink to one-third of its original area prior to application, therefore allowing greater areas to be treated. Fibrin matrices were also found to be strong and capable of being easily moulded to complex contours.

As a delivery vehicle for keratinocytes, a matrix is required to maintain a population of stem cells or holoclones from which transiently amplifying cells, and ultimately differentiated keratinocytes, can proliferate. A study by Pellegrini *et al*. (1999) aimed to establish whether keratinocytes, cultured on a fibrin matrix, maintained their clonogenic capacity, growth rate and long term proliferative potential. This study demonstrated that keratinocytes delivered on fibrin sheets maintained a clonogenic potential and that cultured autografts containing stem cells can rapidly, and permanently, cover the surface of wounds with epithelium. Conventional light and transmission electron microscopy (TEM) verified that all of the structural components of the dermal–epidermal junction were present, that is the hemidesmosome-anchoring filament complexes, the basal lamina and the anchoring fibrils. In addition, melanocytes were present, as were Langerhans cells which are known to be lost in keratinocyte culture. This suggests repopulation from the underlying wound bed. Clinical studies demonstrated excellent take rate and an epidermis which was stable 2–20 months postoperatively. The authors also examined the cost of using fibrin matrix which was found to be approximately 50% of the cost of CEA owing to the reduced amount of handling during culture and the greater area that could be covered.

Fibrin matrices have also been combined with cultured autologous fibroblasts and autologous keratinocytes to give a co-culture system (Llames *et al*., 2006). Fibroblasts were mixed into a human plasma fibrin matrix from blood donors. The surface of the matrix was seeded 24 hours later with keratinocytes. Of the 20 patients treated, 13 were burns patients. Variable take rates of between 10 and 90% were recorded. Where epithelialisation did occur, epithelial loss, blistering injuries and skin retractions were not observed, suggesting that this was an effective and permanent treatment.

The use of lethally irradiated mouse 3T3 fibroblast feeder layers for keratinocyte culture has, until recently, been viewed as necessary for optimal cell growth. The development of defined culture conditions removes this use of xenogeneic material and any associated risk of cross-species disease transmission. Krasna *et al*. (2005) examined the clonogenic capacity of keratinocytes grown on a fibrin matrix in a defined medium. Colony-forming efficiency was found to be greater on fibrin glue than on tissue culture plastic, as was keratinocyte proliferation. The use of 3T3 free culture conditions were also used to generate a fibrin matrix containing both fibroblasts and keratinocytes (Meana *et al*., 1998). A fibrin matrix was seeded with fibroblasts and allowed to polymerise. Keratinocytes were subsequently seeded onto the surface of this matrix. A stratified epithelium was found to develop in 10–15 days in culture and basal membrane structures also formed. Grafting onto a nude mouse model gave rise to an ordered epithelium resembling human epidermis.

In a further development of the use of fibrin sheets for the establishment of epidermal stability they were combined with the dermal regeneration template Integra® (Mis *et al.*, 2004). This approach was tested *in vitro* and *in vivo* although no clinical results were published. Wounds on athymic mice were initially treated with Integra® which was left in place for three weeks. At the end of this period the Integra® was well integrated into the host tissue and host cells had colonised the matrix. At this stage, following removal of the silicone outer membrane, a fibrin substrate seeded with cultured keratinocytes was grafted onto its surface. In wounds treated with fibrin and CEA, a stratified epithelium resembling human epidermis, which stained positive for the keratinocyte specific marker involucrin, was observed. Better graft take was observed for the fibrin sheet with cultured epithelial cells when compared with CEA alone. In addition, and importantly for scarring, significantly lower wound contraction rates were observed.

An alternative mechanism for keratinocyte delivery has been sought by the utilisation of fibrin micro-carrier beads (Voigt *et al*., 1999). This method allows keratinocytes to be cultured in spinner cultures. This technique provides a high surface area to support cell growth using particles of fibrin (50–500 μm). By culturing and delivering cells on microcarriers the use of enzymatic digestion to form a cell suspension is obviated thus reducing cell damage and improving viability. Keratinocytes delivered in this way were found to produce a closed and keratinized epithelium in a pig model. The use of this technique clinically has been

limited but it would appear to have potential as an improvement on the current delivery systems of CEA and sprayed cell suspensions.

Fibrin matrices have also been used for the delivery of growth factors. For example β-fibroblast growth factor (β-FGF) was used to stimulate angiogenesis in a rabbit ear ulcer model (Pandit *et al.*, 1998). In athymic mice the delivery of epidermal growth factor (EGF) in combination with fibrin matrix and cultured keratinocytes was found to improve generation of the dermal–epidermal junction when compared with keratinocytes and fibrin alone. Little work has been undertaken on the impregnation of cell carrier matrices with growth factors, although these studies would suggest that improved epidermal stability and regeneration could be facilitated by their use.

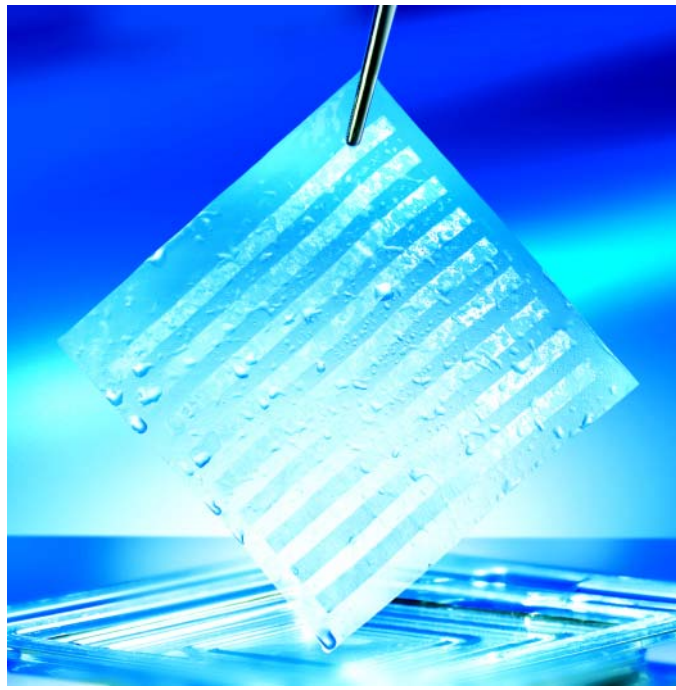
9.3 Hyaluronic acid and Laserskin® as a repair material

Laserskin® is an epidermal membrane delivery system on which keratinocyte cultures can be established and transplanted at sub-confluence (Fig. 9.1 and Fig. 9.2). The sheets are composed of hyaluronic acid (HA) and have regular sized, circular, laser cut, microinterstices 40 μm in diameter that penetrate the 20 μm depth of the material (Zacchi *et al.*, 1998). It can be used as an epidermal replacement in combination with dermal replacements such as Integra® and Hyalograft-3D® (Hyalomatrix®) or can be applied on its own when dermal elements remain.

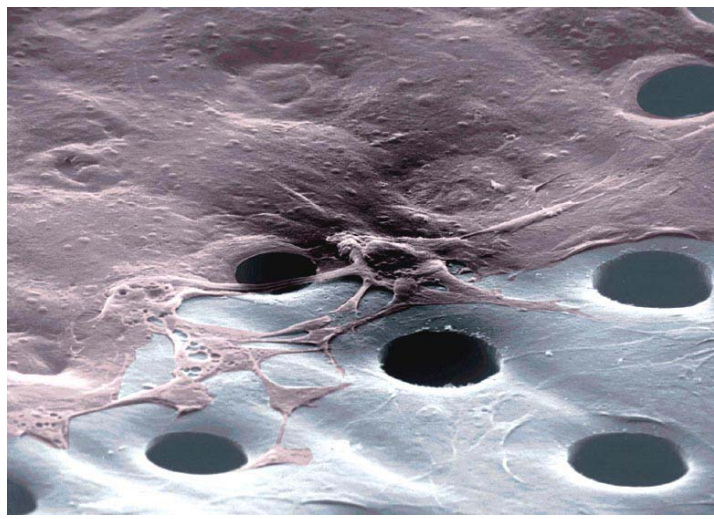
Hyaluronic acid is a high molecular weight glycosamino-glycan (GAG) composed of alternating glucuronic acid and N-acetylglucosamine residues found naturally within the extracellular matrix. These groups are strongly hydrophilic and therefore attract water and form a gel-like matrix. This turgidity provides resistance to compressive forces whereas structural fibrous proteins resist tensional forces.

There are four main groups of GAGs, determined by the linkage between sugar residues and the number and location of sulphate groups. Hyaluronic acid is the simplest of the GAGs and unique among them as it contains no sulphate groups and is not found attached to proteins as a proteoglycan. Unlike other GAGs, which are produced inside cells, hyaluronic acid is expressed from enzyme complexes embedded in the plasma membrane. Hyaluronic acid is produced in large quantities during wound repair, allowing cell migration. During the repair process any excess is degraded by the enzyme hyaluronidase.

HA is a major component of early granulation tissue. Fibroblasts in early wounds produce more hyaluronic acid than fibroblasts in normal skin do. Hyaluronic acid has a stimulatory effect on wound healing and during proliferation and regeneration hyaluronic acid concentrations are elevated, which allow cells to migrate. Prior to mitosis, hyaluronic acid levels are elevated briefly, which allows cells to dissociate. In addition to promoting proliferation, hyaluronic acid inhibits



9.1 Photograph of Laserskin (reproduced with permission of Fidia Advanced Biopolymers, Italy).



9.2 Photomicrograph of cell growth on Laserskin and the topography of the laser cut holes (reproduced with permission of Fidia Advanced Biopolymers, Italy).

cell differentiation. Growth factors may mediate hyaluronic acid-induced cell locomotion. For example, TGF-β1 upregulates hyaluronic acid expression and the receptor RHAMM, hence promoting fibroblast motility (Yang *et al.*, 1993). The stimulatory effect of hyaluronic acid on granulation tissue formation is complex. Hyaluronic acid is known to have pro-angiogenic effects (West *et al.*, 1985) with biodegradants from hyaluronic acid increasing angiogenesis (Anthony *et al.*, 2006).

Hyaluronic acid has a wide range of healthcare applications. This versatility is in part due to the ease with which hyaluronic acid-based biopolymers can be processed. Following esterification, the material can be processed into a number of formulations such as membranes, sponges or microspheres by lyophilising, spray drying or extrusion. HA fibres can be used to produce gauzes, ropes or non-woven materials. It is used to repair cartilage in orthopaedic surgery, in ophthalmology as a replacement for lost vitreous fluid during cataract or lens surgery, in dermatology and plastic surgery for augmentation, to fill facial wrinkles cosmetically, to produce microspheres for drug delivery, in surgery and chronic and acute wound healing, topical gel delivery of drugs and vascular regeneration (Lepidi *et al.*, 2006).

9.3.1 Effect of hyaluronic acid and Laserskin® on epidermal stability

Laserskin® is unique amongst the current epithelial delivery systems and its development addresses a number of the factors that adversely affect the stability and usefulness of epithelial autografts. The physical properties of Laserskin® mean that cells can proliferate and migrate through the material's interstices and structural components formed by the cells can be incorporated into the network. In addition to providing a conduit for cell migration and proliferation, the laser cut holes allow drainage of wound exudate. The supporting nature of the sheet also enhances ease of handling during surgical application and the transparent nature of the membrane means the wound can be observed during dressing changes.

Other advantages of the use of Laserskin® as a delivery system for keratinocytes over the use of fully confluent, layered, differentiated epidermal sheets is that they can be produced in a shorter space of time, saving up to 1 week, which is of particular advantage in the treatment of acute burn injuries (Pianigiani *et al.*, 1999; Chan *et al.*, 2001). Sub-confluent keratinocytes delivered in this way are also in a more proliferative state which may increase the rate of epidermal closure. Growing cells on a biocompatible delivery sheet also overcomes one of the major problems associated with the use of cultured epithelial autografts, which is the use of enzymatic dislodgement, commonly dispase, to remove the sheets from the surface of the tissue culture flasks. Dispase causes damage to the basal cell layer and a loss of laminin 5 laid down by epithelial cells during culture occurs which may adversely affect epidermal stability (Yamato *et al.*, 2001). With Laserskin®,

enzymatic detachment is not required, cell surface proteins are not damaged and therefore take and stability may be enhanced.

Laserskin® has been under development since the early 1990s. However, comparisons with other epithelial autografts, particularly in conjunction with different dermal substitutes have not been conducted (Price *et al*., 2006). Additionally, no studies on the long term stability or persistence of cultured cells delivered on Laserskin® have been carried out. Fibroblast–keratinocyte composite cultures using Laserskin® have been grown *in vitro* at the air–liquid interface and a wide range of target antigens have been examined in order to characterise the cultured skin. There was formation of laminin-5 and collagen type VII, which are the main components of the basal lamina. The fibroblasts seeded in the dermal matrix adhered, proliferated and secreted the main extracellular matrix (ECM) components collagen I, III and IV, fibronectin and laminin I (Zacchi *et al*., 1998).

Differentiation of keratinocytes cultured on Laserskin® has been demonstrated, with specific basal cytokeratins being only expressed in cells growing in the perforations and in the basal layer. Structures resembling hemidesmosomes were also evident in the cells in close contact with the Laserskin® membrane (Andreassi *et al*., 1991). It was suggested by these authors that the cells growing on the Laserskin® were at a point preceding the formation of a true dermal–epidermal junction (DEJ), unlike cell sheets grown on plastic. Initial ultrastructural similarities to a basal layer were noted by Pianigiani *et al*. (1999) in the region close to the Laserskin® substrate although with time these cells became keratinized. They also demonstrated that epidermal cells grown *in vitro* on Laserskin® developed a fully functional stratum corneum providing a physiological barrier, reducing dehydration and thus improving the likelihood of successful take and epidermal stability.

The stability of keratinocyte seeded Laserskin® sheets may be enhanced by alterations to the physical structure. The rate of hyaluronic acid resorption is dependent on both the type of hyaluronic acid and the degree of esterification, with 100% esterified hyaluronic acid having a longer residence in tissues when compared with partially esterified hyaluronic acid. Prolonged action of 100% compared with 75% esterified hyaluronic acid may have promoted more angiogenic effects (Price *et al*., 2006) therefore both the presence and structure of hyaluronic acid are likely to affect the stability of grafted epidermal elements. The hyaluronic acid derivative used to prepare both the microperforated membrane and the non-woven fabric are esters of hyaluronic acid. These esters have a percentage of esterification of between 75 and 100%. By varying the percentage of esterification of the hyaluronic acid esters used, it is possible to control the degradation kinetics of the tissue support and, therefore, the length of time that the device can remain *in situ*. Degradation (to 75%) times of 30 days for the 100% ester and 4 days for the 75% ester were reported by Price *et al*. (2006). Owing to a fast rate of degradation of Laserskin® in the treatment of traumatic soft tissue injuries, an additional split

thickness skin graft was added to improve epidermal integrity/stability (Hollander *et al*., 2001).

The clinical take rate and stability of cultured epidermal cells are greatest when there is a functional dermal element remaining. With a dermal element in place, the take rate of Laserskin® was found to be significantly lower in a porcine model when keratinocyte seeding densities were reduced by half (Myers *et al*., 1997). In a more recent study, keratinocytes were delivered on Laserskin® to full thickness porcine wounds pretreated with Hyalomatrix®. Delays in keratinocyte application reduced take rates, however, this was reduced by repeated pre-treatment with Hyalomatrix® and a maximal take rate of 57.2% was measured (Myers *et al*., 2007). The use of co-cultures of fibroblasts and keratinocytes has not fully been examined although autologous fibroblast feeder layers, instead of murine 3T3 cells have been used in a small number of clinical applications (Lam *et al*., 2001).

Although hyaluronic acid is known to play an important role in wound healing, clinical experience with this polymer is limited. Positive results have, however, been recorded in a number of indications.

Burns

A combination of the dermal Hyalograft-3D® and keratinocyte seeded Laserskin® has been used successfully (Scalise *et al*., 2001) to treat patients with burn injuries, diabetic, vascular and post-traumatic ulcers. No observations were made regarding the stability of the grafts and the timing of long term follow up varied considerably between patients. In another study involving six patients (Travia *et al*., 2003), it was determined that a dermal element is required to heal full thickness burns and that the use of the TissueTech system (dermal and epidermal hyaluronic elements) provides some positive results in terms of overall healing. In the treatment of a full thickness burn (Harris *et al*., 1999), interdigitations resembling rete ridges were visible 24 days after grafting at the DEJ indicating good stability of Laserskin® grafted over Hyaff NW (Hyalograft-3D ®) and after a 7-week period there was no unusual scarring.

Take rates of 60–100% were observed in grafted Laserskin® with a layer of allogeneic fibroblasts on the upper surface onto which a layer of keratinocytes were grown. Low numbers and lack of information about the burn depth meant that little information could be gathered regarding the stability of the sheets. However, they remained clinically intact at 12 weeks post graft (Harris *et al.*, 1999).

Scar revision/nevus

The manufacturer's instructions recommend the use of irradiated 3T3 cells as a feeder layer for keratinocyte culture on Laserskin®. However, Chan *et al*. (2001) raised doubts over the use of 3T3 cells, as washing failed to remove them entirely. They suggest an alternative method of cultivation using autologous or allogeneic

fibroblasts as a feeder layer because graft rejection may possibly be associated with the presence of xenogeneic antigens. The layers of Laserskin® with autologous keratinocytes and either allogeneic or autologous dermal fibroblasts were grafted onto the vascularised neodermis of Integra® (a bilayer artificial skin, Integra® LifeSciences, Plainsboro NJ, consisting of a layer of porous collagen-GAG with a thin outer silicone membrane) immediately after the removal of the silicone membrane. Although there maybe obvious potential problems with the allogeneic approach, for example disease transmission and immunologic effects, a burns patient was successfully treated for contracture release by this method. Two other patients with congenital nevus were treated using the autologous fibroblast approach. There may be an additional benefit of using autologous dermal fibroblasts in that they produce a number of proteins such as collagen and fibronectin that may enhance graft attachment and stability.

Vitiligo

Sheets of Laserskin® without dermal replacements have been used for the treatment of vitiligo (Andreassi *et al*., 1998). The achromatic area was de-epithelised and Laserskin® previously inoculated with autologous keratinocytes and lethally irradiated 3T3 cells was applied. Good repigmentation rates were reported (10 out of 11) with the only failure being associated with sepsis, demonstrating that stability of grafts can be an issue in even relatively minor procedures if wound bed conditions are not optimal. However, the effectiveness is reported as being greater than that of unsupported sheets of CEA.

Ulcers

A small number of studies have been conducted on the use of Laserskin® as a material for chronic wound closure. A large, multicentre, retrospective, uncontrolled study was performed in Italy (Uccioli, 2003) evaluating the TissueTech Autograft System (TTAS) combining autologous fibroblast cultures within a HYAFF based three-dimensional matrix (Hyalograft-3D®) with Laserskin® applied as the epidermal layer. Diabetic lower extremity ulcers, venous ulcers, arterial ulcers, traumatic wounds, pressure ulcers and others were studied in 401 patients from 60 different centres. Complete closure was reached for 70.3% of the ulcers with a mean observation time of 330 days and 63% of those healed within four months. The rate of recurrence was 8.2% (Uccioli, 2003). These figures are comparable to other studies using TTAS (Caravaggi *et al*., 2003) and are slightly higher than studies investigating the effectiveness of allogeneic skin substitutes. As this study was purely descriptive, there were no controls for comparison. However, figures for control groups have been cited in studies investigating other treatments and the healing rates were between 30 and 40%.

Two further studies investigated the use of Laserskin® for treating chronic

wounds. Caravaggi *et al*. (2003) grafted Laserskin® seeded with autologous keratinocytes onto Hyalograft-3D® seeded with fibroblasts on dorsal and plantar diabetic ulcers. For dorsal ulcers there was a significant increase in healed ulcers compared to the control.

Lobmann *et al*. (2003) evaluated use of Laserskin® seeded with autologous keratinocytes applied without a dermal element for the treatment of non-healing diabetic foot lesions. Eleven out of 14 grafts were successful and of the non-responders all had a higher stage arterial occlusive disease. A small tendency for hyperkeratosis was noted although this diminished over time after grafting and long term stability appeared to be promising, with no ulcer recurrences within a 12-month period in responsive wounds. Hyalograft-3D® followed by the application of a Laserskin® autograft has also been reported as a successful treatment for severe scleroderma cutaneous ulcers which had been unresponsive to previous therapeutic attempts with vasoactive drugs, steroids and immunosuppressors (Giuggioli *et al*., 2003).

Aplasia cutis congenita

Laserskin®, in conjunction with dermal Hyalograft-3D® has been used for the reparation of a severe case of Aplasia cutis congenita (ACC) (Donati *et al*., 2001). Full healing was achieved leaving no scarring.

Tattoos

A study by Price *et al*. (2006) involving 23 patients investigated the effect of two different esters of hyaluronic acid (Hyalomatrix®), without cultured fibroblasts, for constructing the dermis followed by application of Laserskin®. Of the two esters, 11p75 (75% esterification) and 11p100 (100% esterification) the overall indication is that the total ester had a beneficial effect on wound healing although no statistical differences were noted for the stability of the epidermal layer. Blistering occurred in both groups but healed readily. Stability is, however, known to be affected by differences in the dermal matrix. Collagen-based matrices (Integra®) have been poor recipients of cultured keratinocytes (Pandya *et al.*, 1998) although there are few reports of the use of keratinocytes grown on hyaluronic acid epidermal matrices in conjunction with collagen dermal elements (Chan *et al*., 2001).

Traumatic soft tissue loss

A combination of Hyalograft-3D® seeded with fibroblasts and Laserskin® seeded with keratinocytes followed by a thin meshed split thickness skin graft was applied to large soft tissue injuries resulting from vehicle accidents (Hollander *et al.*, 2001). A near total take rate of all sheets was reported, as was the formation of a

mechanically stable tissue. Split thickness graft was applied after the Laserskin® had almost completely biodegraded leaving a thin epidermal layer. As no controls were included it is not evident whether a full epidermal layer, resistant to shear forces, would have successfully developed without the STSG.

9.4 Summary

Fibrin- and hyaluronic acid-derived matrices are ideal candidates for the delivery of keratinocytes. The most important role which any wound repair material plays is in providing an environment in which cells of the correct tissue phenotype can proliferate, differentiate and form an organised structure. In order to fulfil an effective functional role, the final repair tissue is required to be as similar as possible in structure and function to the native, undamaged tissue. A successful repair tissue will contain most, or all, of the elements of the original tissue. In skin, the establishment of a functional dermis and a viable basement membrane is key to the formation of an organised and stable epidermis. The organised deposition of proteins such as laminin 5 and collagen VII at the DEJ is known to be vital in basement membrane formation (Breitkreutz *et al*., 2004).

It is well established that epithelium formed as a result of the application of CEA is unstable (Desai *et al*., 1991). This instability is thought to be due in part to the enzymatic treatment required to release the cell sheet from the tissue culture plastic flask prior to clinical application. It could be postulated that enzymatic digestion irreversibly damages the proteins forming the basement membrane such that a subsequent establishment of a stable DEJ does not occur. The delivery of keratinocytes which have been pre-cultured in or on a carrier matrix, such as hyaluronic acid or fibrin, removes the need for enzymatic digestion and cells can be delivered to the wound bed in a proliferative state with their extracellular proteins intact. It could be speculated that keratinocytes delivered to the wound bed in such matrices are better able to form a DEJ than keratinocyte sheets or suspensions following enzymatic treatment. Clinical studies using such carrier matrices have demonstrated their suitability as epidermal repair materials when used in combination with cultured keratinocytes. Certain aspects, such as their ease of handling, make them particularly attractive as repair materials. Few comparative studies exist which directly compare these delivery matrices with other treatments such as CEA, sprayed sub-confluent keratinocytes and sprayed sub-confluent keratinocytes delivered in fibrin glue. As such, it is difficult to elucidate which treatment provides the highest quality and most stable epidermis, and future research should be directed towards establishing which combinations can be used to achieve this. The studies described here of the use of fibrin and hyaluronic acid carrier matrices for delivery of keratinocytes demonstrate that, as a mode of delivery, this approach has huge potential. By further refining the physical and chemical properties of the delivery matrix, such that a healthy and stable epidermis is regenerated following skin loss, further improvements can be achieved.

9.5 Future trends

Tissue engineering approaches to skin wound closure have been in development for over 25 years since the first reports of the use of CEA in the early 1980s. Drawbacks including fragility, delay in availability owing to culture time, high cost, uncertain take rate and poor mechanical stability have led to the development of the current generation of biomaterials specifically designed for skin wound repair. Owing to the nature of full thickness burn injuries these materials are usually required to be seeded with epithelial cells or their precursors. The two materials reviewed here are simple biological molecules and little work has been undertaken substantially to modify their structure in order to improve their function as skin repair materials. The primary goal in the development of epidermal replacement biomaterials should be to understand better how matrices such as fibrin and hyaluronic acid can be further modified to provide a template for organised tissue regeneration. The provision of the optimal environment for the generation of the DEJ may be enhanced by altering the structure and/or the functional groups of the carrier matrix. A material is required which provides an element of wound closure whilst providing the optimal environment for keratinocyte proliferation, differentiation, basement membrane formation and stratification of the epithelium.

Previous experience with Integra®, de-epidermized dermis, Laserskin® and fibrin suggests that modification of naturally occurring matrices is likely to be more successful than totally artificial polymers in providing an effective matrix for tissue regeneration. A combined approach is required with the employment of modern polymer chemistry and fabrication technology to manufacture a material which significantly outperforms other treatments. Other factors are important in the development of such a material. Ease of handling and availability are vital as is the time taken to produce the cell seeded matrix. The cost of the material is also important.

The ultimate goal is to develop an epidermal repair material which is available 'off the shelf'. For this to be achieved, however, the use of allogeneic material is a requirement, as a relatively large number of cells are required for immediate delivery to the wound bed. Allogeneic keratinocytes have been shown to treat partial thickness wounds in paediatric scalds successfully (Rab *et al*., 2005). The provision of pre-made cryopreserved sheets of matrix containing allogeneic cultured keratinocytes or keratinocyte precursor cells offers a potential solution, although the potential for rejection would need to be examined. The seeding of such matrices with allogeneic pluripotent stem cells may provide further advantages such that the transplanted cells differentiate in response to environmental cues on the wound bed into a well organised epidermis. Such a development could be combined with currently available dermal substitutes to offer a viable co-cultured 'off the shelf' bilayered material. Initial studies have indicated that such materials can be manufactured (Dai *et al*., 2005). However,

further refinement is required to produce a clinically effective and commercially viable product.

Further developments would see the inclusion of skin appendages such as hair follicles and sweat glands into skin replacements and it has been shown that allogeneic hair follicle stem cells can result in the regeneration of hair follicles (Reynolds *et al*., 1999). A combination of developments in high-tech materials and a rapidly increasing understanding of cell behaviour and differentiation should make these advances possible.

9.6 References

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10

Human-derived acellular matrices for dermal replacement

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Abstract: AlloDerm, an acellular extracellular matrix (ECM) scaffold derived from cadaveric dermal tissue, was introduced in 1994 principally as a treatment for full-thickness burns. Since then, application of this regenerative tissue matrix (RTM) has been expanded to hernia repair, breast reconstruction and gingival grafting. This technology platform has also been extended to produce products for urogynecologic, orthopaedic, podiatric and laryngologic indications. Through this broad range of applications, over a million grafts have been used clinically with an exceptional satisfaction and safety record. Recognition of the RTM's ability to support the regeneration of cellular and vascularized soft tissue and the transition to a tissue architecture resembling that specific to the treated site has fueled use by clinicians for new and challenging indications.

Key words: extracellular matrix (ECM), regenerative tissue matrix (RTM), acellular.

10.1 Introduction

This chapter will focus on the use of acellular dermal RTM scaffolds for replacement, repair, and regeneration of soft tissue deficits with emphasis on treating skin dysfunction and loss. The overarching rationale for and function of RTMs will be presented in the context of biochemical and mechanical characteristics believed to facilitate a regenerative response rather than a common fibrotic scar repair process. The significance of a properly prepared and intact ECM to support the body's intrinsic regenerative abilities will be demonstrated through *in vitro*, preclinical *in vivo* and clinical analyses. Moreover, the universality and value of this approach will be shown by highlighting its employment in multiple clinical applications targeting various tissue types and by its applicability to various tissues as RTM sources.

10.1.1 The problem

Skin, the largest organ of the body, is a dynamic layered composite comprising the epidermis, dermis and hypodermis (anatomy and function reviewed in Fore, 2006;

Kanitakis, 2002). Each layer has a unique architecture which imparts specific attributes to overall organ function, including general system homeostasis, protection against environmental insult, thermoregulation and the biomechanical stability required for a wide variety of body motion. As a result of this functional importance, wounding initiates a cascade of events with the sole purpose of rapidly regaining integrity.

Wound healing describes a specific and complex biological process initiated by a loss of integrity and is characterized by five overlapping stages: (1) hemostasis, (2) inflammation, (3) cellular migration and proliferation, (4) matrix protein synthesis and wound contraction and (5) tissue remodeling (Monaco and Lawrence, 2003). Regaining hemostatic control is primarily accomplished through platelet activation and aggregation followed by fibrin deposition (Lind, 1995). Platelet activation releases a variety of soluble peptides, including platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), epidermal growth factor (EGF), transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF), all of which direct biological responses to a wound. In addition to growth factors, the wound healing process is regulated by cytokines and chemokines which are first released by polymorphonuclear cells during the inflammatory phase and subsequently by fibroblasts, endothelial cells and keratinocytes as ECM deposition and remodeling occur (Gillitzer and Goebeler, 2001;Werner and Grose, 2003). The coordinated efforts of growth factors, cytokines and chemokines adjust the biochemical and cellular environment of the wound, thereby achieving resolution. Unfortunately, unless the wound depth is less than a critical level (Dunkin *et al*., 2007), the characteristic adult reparative wound healing process results in scar formation with eventual scar tissue contraction. While the ability to heal cutaneous wounds by this reparative process is essential for survival, scar tissue has neither the structural, nor the physiological, attributes of the tissue it has replaced and therefore represents a comparatively dysfunctional tissue relative to native tissue.

Epidermal and superficial dermal wounds typically heal within a couple of weeks without hypertrophic scarring and contracture (Chapman, 2007). However, when the size, depth, or biochemical environment is such that the wound is precluded from healing naturally, medical intervention is necessary. Such wounds requiring delayed closure are associated with increased morbidity and mortality owing to tissue dehydration, reduced thermal regulation and are at increased risk of infection and elevated pain levels (Brown and Barot, 1986). Burns, trauma and chronic ulcerations resulting from co-morbidities such as diabetes are the principal causes of the over 35 million cases per year of significant skin loss ultimately requiring medical treatment (Clark *et al.*, 2007). With nearly 1.2 million hospital visits annually for burns (Sánchez *et al*., 2007) and over four million individuals suffering from chronic wounds resulting from diabetes, venous stasis, or pressure-induced necrosis, it is easy to understand why the overall health care costs for treatment of skin injuries in the USA alone are in excess of $10 billion (Clark *et al.*, 2007). But despite the large incidence and the associated costs of treatment,

advanced wound care treatments have been slow to develop. It is only now, through our growing understanding of the intrinsic ability of human tissue to heal itself via a regenerative pathway, that RTMs have been developed to address the need for more clinically promising wound treatments. RTMs are currently garnering increased clinical acceptance as advanced wound care materials through their ability to support restoration of the structure, function and physiology of damaged tissue previously unattainable through other wound treatments (Harper and McQuillan, 2007).

10.1.2 Solutions

Permanent closure of large full-thickness wounds has historically been accomplished through application of an autologous split thickness skin graft (STSG) from a non-wounded area. The amount of dermis included as part of the graft inversely correlates with the level of scarring and contracture observed in the healed wound (Dunkin *et al*., 2007; Klein and Rudolph, 1972). This response probably results from the presence of myofibroblasts in the wound bed which induce a significantly stronger contractile response than do typical dermal fibroblasts (Germain *et al*., 1994). The effect of dermal thickness on healing characteristics applies to the donor site as well. Thus, a balance must be achieved between providing enough dermis with the graft and leaving enough in the donor site so that both sites can heal and provide the functional and aesthetic outcomes desired by the patient. For very large wounds, the STSG can be meshed to expand graft coverage and minimize the size of the donor site. However, meshed grafts have a tendency to impart a meshed pattern to the treated wound. Nevertheless, despite efforts to minimize additional trauma during the autograft procedure, the limiting factor to this approach has remained the availability of a viable dermis. One potential source of additional dermis has been allograft skin. Such grafts integrate well with the wound bed and minimize dehydration, but are eventually rejected owing to the presence of foreign cellular antigens. Thus, the role of allogeneic skin grafts has been limited to temporary coverings.

The explosion in biotechnology over the past three decades has provided scientists with new tools and the ability to purify or synthesize many biological building blocks. Medical science has thus turned to tissue engineering (TE) to address the limitations of auto- and allograft skin. While TE is customarily defined as the application of engineering and life science principles in the development of biological substitutes to restore, maintain, or improve function, it is not, however, necessarily equivalent to the regenerative medicine concept, which requires restoration of native structure and physiology. By employing combinations of the traditional TE triad of cells, matrix molecules and biochemical factors, a variety of dermal constructs and engineered skin substitutes have been developed and now are commercially available. These devices, their approved uses, benefits and limitations are reviewed elsewhere (Bello *et al.*, 2001; Clark *et al.*, 2007; Simpson,

2006). But because a complete characterization of native dermis is not available and the composition of substitutes is known to affect healing outcomes (Shafritz, *et al.*, 1994), it is uncertain what material components and in turn biological attributes, may be altered or missing in such devices. Thus, while TE products may aid wound closure, they may not provide for fully regenerative outcomes. In contrast, recent advances in processing techniques have permitted native tissues to serve as starting materials for the production of acellular ECM scaffolds. Such materials greatly aid researchers in their understanding of the factors necessary to support regenerative healing while at the same time providing clinician and patient with better wound care options.

In treating cutaneous wounds, RTM use translates to enhanced functional outcomes by providing a thicker graft, thereby reducing wound contracture and increasing patient mobility while maintaining graft take rates (DeClement, Jr. *et al*., 1997; Lattari *et al*., 1997; Sheridan *et al*., 1998). These functional outcomes, the result of biological properties that support fibroblast infiltration, neo-vascularization and epithelializaiton in the absence of inflammation, lead to increases in overall cosmesis (Wainwright *et al*., 1996).

10.1.3 Concept of an acellular dermal matrix

The limitation of allograft skin as a viable treatment for skin loss results from cell-based immunogenicity introduced as part of the graft. Removing all remaining cells in the dermis renders a completely acellular dermal matrix that is essentially immunologically inert and can serve as a general ECM scaffold for soft tissue replacement. Furthermore, by starting with the native ECM, the resulting scaffold retains much, if not all of the native dermal architecture and composition. Thus, acellular dermal ECMs allow for successful engraftment of allogeneic dermis and formation of fully differentiated skin without contracture (Heck *et al*, 1985; Langdon *et al*., 1988). These studies further demonstrate the importance of the dermal matrix in wound healing and its general low antigenicity.

During fetal development, wounds are believed to undergo regenerative healing and the absence of scarring is noted. Thus, the human body has the intrinsic capability to regenerate tissue. However, this capacity is lost during development and replaced by a reparative scarring process (Adzick and Longaker, 1992). Although the adult human heals by this reparative scarring process, in the absence of some underlying dysfunction, the cytokines and pluripotent progenitor cells necessary for regeneration are still available. Seemingly, the tissue in the adult wound environment does not provide the proper chemoattractant and differentiation signals necessary for a regenerative healing pathway. The provisional fibrin scaffold deposited during hemostasis is distinctly different from the native dermal matrix in composition, architecture and mechanical properties, all of which are known to affect cellular responses. Therefore, the key component missing in adult wounds is the native intact ECM scaffold. Such a scaffold, comprised of native

composition and architecture and endowed with the necessary mechanical properties, has the potential to orchestrate a regenerative process resulting in little or no scarring. It is for these reasons that a naturally occurring, structurally preserved ECM template provided the rationale for the development of the acellular dermal matrix as a regenerative tissue matrix.

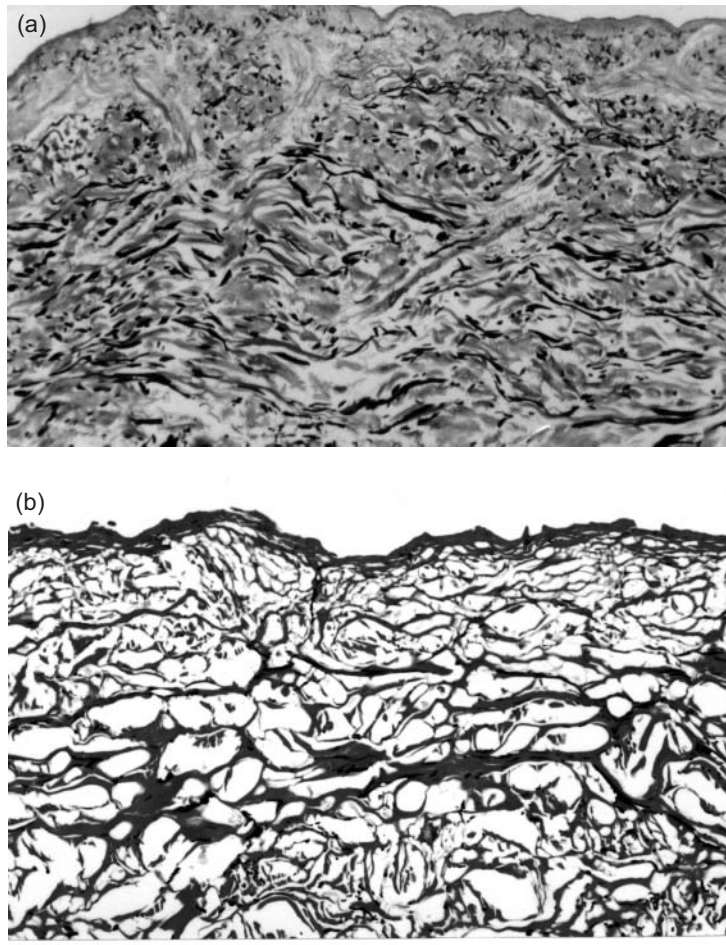
10.2 Processing native tissues

10.2.1 Acellular dermal matrix as an RTM

LifeCell's RTMs AlloDerm, GraftJacket and Repliform are produced from allograft skin procured by banks accredited by the American Association of Tissue Bank (AATB) and in compliance with 21CFR1271. The skin is quarantined until donor medical/social histories and serology testing is complete. Once medical release is obtained, the RTMs are manufactured through a combination of proprietary processes necessary for antigenic epitope removal and stable preservation.

The initial LifeCell processing steps remove the cellular components, eliminating the antigens that would normally promote clinically relevant inflammatory or immunologic responses. The epidermis is next detached by agitation in solutions that modulate ionic strength, pH and divalent cation concentration. These non-enzymatic steps separate the cellular epidermis without proteolytic cleavage of the underlying basement membrane. In this manner, the components of the structure, including laminin and collagen types IV and VII are retained intact. The remaining cellular material of the dermis and dermal vasculature are solubilized by low molecular weight detergents. During these steps, specialized buffers are employed to prevent degradation and promote preservation of the principal ECM components collagen type I, elastin and various glycosaminoglycans.

Once the acellular RTM is prepared, it must be preserved such that it can be delivered to the clinician with retention of its biologic function. The core technologic requirement and the basis for stable preservation is controlling formation and growth of ice crystals during freeze-drying of the matrix. Biocompatible cryo- and lyoprotectants are introduced into the matrix to promote and support amorphous ice formation. Successfully controlling ice formation minimizes damage to the matrix during the freeze-drying process (Fig. 10.1) and LifeCell's drying protocols have been developed based upon the thermodynamic and kinetic stability characteristics of the frozen matrix to prevent damage in the final dried product. LifeCell's RTM materials are processed aseptically without terminal sterilization which can induce detrimental damage to the matrix. This mild and minimal processing results in preserving both the biochemical components and the underlying structural architecture inherent in the ECM that is ultimately necessary for a functional RTM. Rehydration of the RTM yields a material that is flexible and capable of being shaped, cut and sutured depending upon the specific application



10.1 Verhoeff's staining of histological sections demonstrates the preservation methods used to produce AlloDerm RTM maintain structural integrity (a) in contrast with ice-induced matrix damage in poorly preserved tissues (b).

at hand. Despite vigilant screening processes, these RTMs pose certain disease transmission risks associated with allograft transplantation.

10.2.2 Regenerative tissue matrix configurations and universality

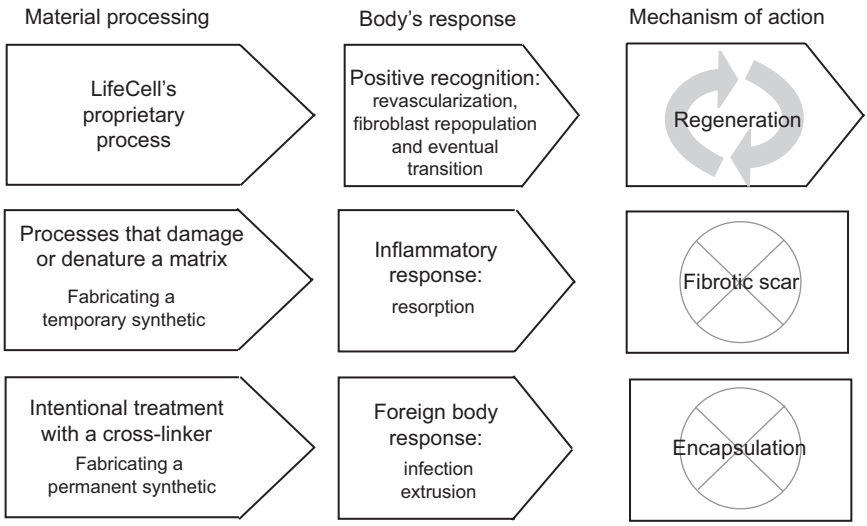
The decellularization and preservation strategy described above can be applied to nearly any tissue type having a significant ECM component, including vascular conduits, nerve segments, tendon, ligament and fascia (Hilbert *et al*., 2004). These

structures have distinct cellular and matrix properties so that individualized methods are necessary to achieve antigenic neutrality and preserve matrix structure. However, once the tissues are prepared, further processing can yield unique formulations and constructs. As an example, the sheet AlloDerm RTM prepared from allograft dermis can be micronized in a cryofracture process that produces particles of intact matrix with an average diameter of 123 μm, allowing for delivery by injection (Sclafani *et al.*, 2002). The same particulate matrix, marketed as Cymetra, can be mixed with allograft demineralized bone to create an easily moldable and injectable putty that retains the cellular ingrowth and osteoinductive properties of the materials which it comprises. Thus, the resulting putty, marketed as AlloCraft DBM, is suitable for the repair of irregular bone defects which have no load-bearing requirement (Qiu *et al*., 2007).

The intact matrix scaffold strategy can also be applied to tissues sourced from non-human species. Owing to its similarities to human skin (Lavker *et al*., 1991; Wollina *et al.*, 1991) and wound healing properties (Rigal *et al*., 1992), much of LifeCell's technology platform and early knowledge base pertaining to a matrix's regenerative potential was developed using porcine skin (Livesey *et al*., 1995). As the uses for products that support regeneration have grown, LifeCell has come full circle with the introduction of the porcine-derived tissue reconstructive matrix device, Strattice. The unique processing in the manufacture of Strattice yields an intact ECM that extends the possible applications and markets for intact matrix products. While Strattice represents the future of soft-tissue reconstruction devices, the foundation of this technology was laid with AlloDerm, the characterization and performance of which will be the focus for the remainder of this chapter.

10.2.3 Processing affects biological responses

Because the methods used to prepare a matrix scaffold from existing tissues have a significant impact on the retention and integrity of matrix components, they ultimately dictate the body's response to the implanted material. These responses are likely to fall into one of three general categories (Fig. 10.2). Processing that causes damage, such as protein denaturation, cleavage or extraction, tends to induce an inflammatory response resulting in a reparative wound healing mechanism characterized by resorption of the matrix, deposition of a provisional matrix, and formation of scar. Cross-linking of matrix components, either intentionally to prevent degradation caused by the presence of hidden antigenic epitopes or unintentionally as the result of poor preservation or incompatible sterilization modalities, can elicit a foreign body response resulting in matrix encapsulation. In contrast to these undesirable outcomes, a properly prepared matrix that maintains the bioinductive, mechanical, constitutional and functional properties of a native intact ECM is likely to support the body's intrinsic regenerative potential. While these responses are most notable in applications where the biomaterial is used as an implant (Valentin *et al*., 2006), they are similar in consequence to those found for



10.2 Schema showing mechanistic pathways of responses to biomaterials.

cutaneous wounds where treatment with damaged or cross-linked matrices leads to fibrotic scar formation.

10.2.4 Regulatory considerations

LifeCell's RTMs, such as AlloDerm and Cymetra, are produced from donated human skin. All tissue is supplied by US AATB-compliant tissue banks following the guidelines set forth by the AATB and US FDA. AlloDerm is considered to be minimally manipulated during processing and is marketed for repair and replacement of integumental tissues. As such, AlloDerm is classified as a banked human tissue by the FDA. Other products based on the matrix template platform, such as AlloCraft and Strattice, have received 510(k) clearance and are regulated by the FDA as medical devices.

10.3 Material characterization

In order to understand better the mechanism by which LifeCell's tissue matrices support a regenerative outcome rather than reparative scar formation, the material has been subjected to a series of biochemical, biomechanical, histological and functional analyses. These assays serve to demonstrate experimentally the inherent properties within an RTM that are believed to be responsible for their clinical success in a broad range of applications.

10.3.1 Structural analyses

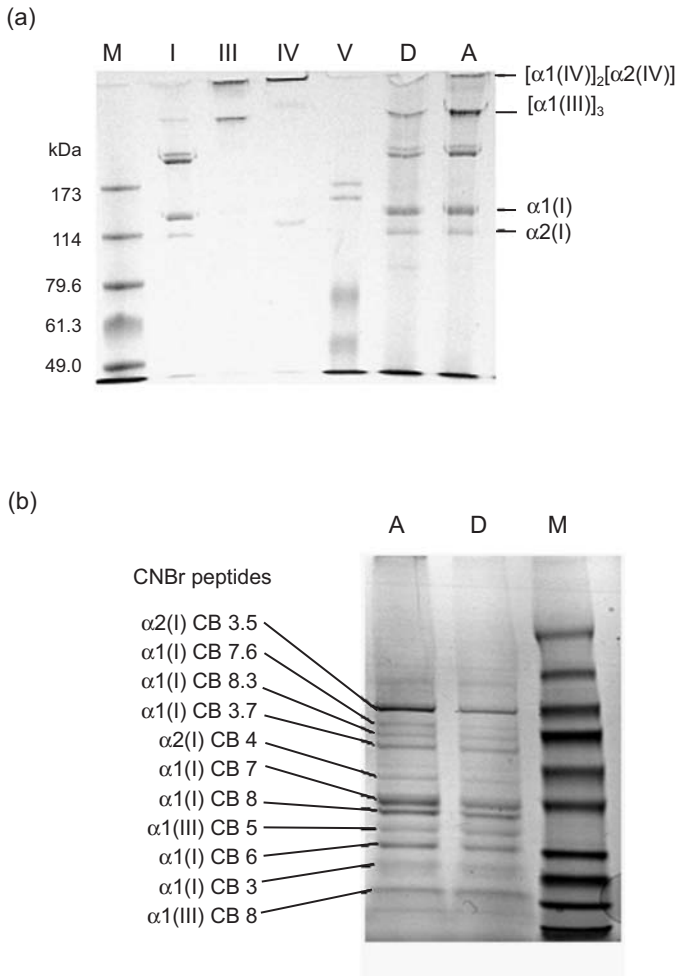
Collagen represents the major component of most ECMs, including that of dermis where collagen type I is the major component. Besides providing the ECM with its primary structural support, it plays a key role in the overall organization of other ECM molecules including other collagens, proteoglycans, laminin, as well as numerous others. Collagen contains specific recognition sequences for the cell-surface integrins that regulate cellular activities such as adhesion, migration, and differentiation. Further, it binds soluble proteins including growth factors which are directly involved in the regulation of cell growth. In order to retain these functions, a processed acellular RTM should possess a matrix composition as close to that of the native ECM as possible.

Collagen accounts for greater than 75% of the dry mass of RTM as determined by hydroxyproline content (Table 10.1). Total hydroxyproline is variably distributed throughout acid soluble, pepsin soluble and pepsin insoluble compartments. This distribution is typical of normal dermal tissue and reflects the relative amounts of newly synthesized, mature and mature cross-linked pools of collagen. This data is consistent with independent analysis of RTM (Derwin *et al.*, 2006) and fresh dermal samples (Poulsen and Cramers, 1982). Soluble collagen prepared following pepsin digestion reveals collagen type I and III to be the predominant forms existing as single chains as well as higher order structures resulting from intra-molecular cross-linked dimeric (β) and trimeric (γ) collagen polypeptides (Fig. 10.3(a)). The molecular complexity of the collagen matrix is further shown by treating pepsin insoluble fractions with cyanogen bromide. The intricate banding pattern observed reveals the expected α1(I), α2(I) and α1(III) peptide fragments characteristic of a mature collagen ECM (Fig. 10.3(b)). Taken together, these data demonstrate that the collagen organization in AlloDerm RTM is typical of a mature dermal ECM complete with the levels of structural complexity provided for by intra- and inter-molecular cross-linking.

In addition to collagen, the RTM retains the important proteoglycans decorin,

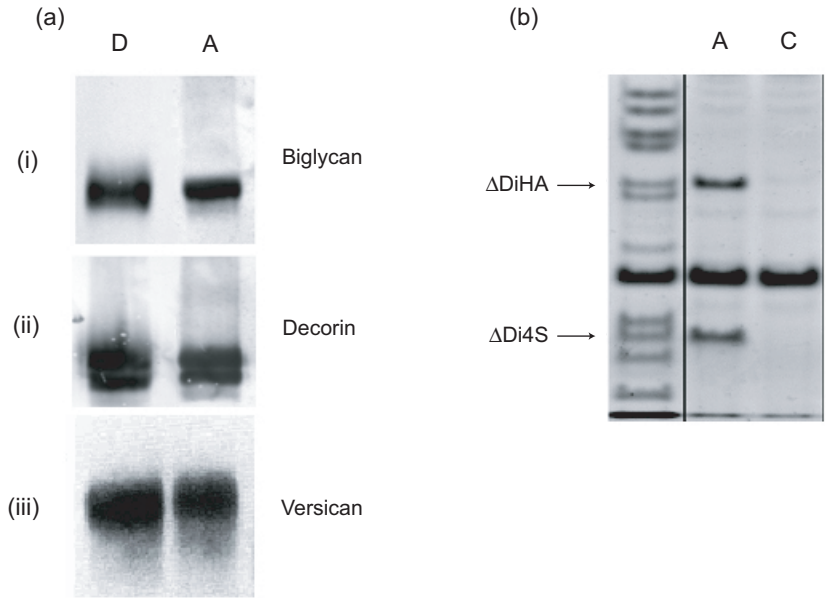
Table 10.1 AlloDerm RTM collagen content. Collagen from AlloDerm was extracted through sequential treatments with 0.5 M acetic acid and pepsin. Hydroxyproline was quantified in each extract and used to estimate total collagen content assuming that the hydroxyproline content of the collagen I and III enriched ECM was 12.5%. Each value represents the mean ± SD (standard deviation) of six different donor lots

	Hydroxyproline (μg/mg dry weight)	Collagen (μg/mg dry weight)
Total collagen	97.5 ± 7.4	780.0 ± 59.5
Acid extract	10.2 ± 6.6	81.7 ± 52.5
Pepsin extract	48.2 ± 17.1	385.3 ± 137.0
Pepsin insoluble	39.1 ± 22.6	313.0 ± 181.2



10.3 Collagen type distribution in AlloDerm RTM. (a) AlloDerm RTM was digested with pepsin and the solublized collagen analyzed following electrophoresis on an 8% polyacrylamide gel. (b) Pepsin insoluble collagen was cleaved using cyanogen bromide and the resulting collagen peptides analyzed following electrophoresis on 4–20% polyacrylamide gradient gel. Dermal samples that were not subjected to the decellularization process were analyzed in parallel and shown for comparative purposes. Lane headings: M = Marker; A = AlloDerm; D = Dermis; I, III, IV, V = purified collagen of the indicated type.

biglycan and versican (Fig. 10.4(a)). In particular, decorin and biglycan have been described as possessing a broad range of functional activities responsible for cell growth regulation, growth factor binding and immunoregulation. Additionally, they both bind collagen and regulate the formation of collagen fibrils. Mutant mice lacking these proteoglycans produce phenotypes with connective tissue disorders



10.4 AlloDerm retains proteoglycan content and glycosylation pattern. (a) Proteoglycans were extracted from AlloDerm RTM (A) and control dermis (D) using 4 M guanidine hydrochloride. Glycosaminoglycan chains were then removed by digestion with chondroitinase ABC. The resulting core proteins were fractionated on a 10% polyacrylamide gel, transferred to nitrocellulose and identified by western blot for decorin, biglycan or versican. (b) AlloDerm RTM was digested with proteinase K and the resulting glycosaminoglycans precipitated with ethanol. Disaccharides were obtained following digestion of the glycosaminoglycans with chondroitinase ABC and hyaluronidase, labeled with 2-aminoacridone and analyzed by fluorophore-assisted carbohydrate eletrophoresis (FACE). Control AlloDerm samples (C) were not treated with chondroitinase ABC or hyaluronidase and show the migration of an irrelevant band not representative of dermal-derived disaccharides.

(Danielson *et al*., 1997; Young *et al*., 2002). In addition to their structural role, these molecules have recently been shown to regulate cellular activities through direct interactions with growth factors such as TGF-β, TNF-α and PDGF, as well as the cell surface receptors EGFR (epidermal growth factor receptor) and ILGFR (insulin-like growth factor receptor) (Csordás *et al*., 2000; Nili *et al*., 2003; Schönherr *et al*., 2005; Tufvesson and Westergren-Thorsson, 2002; Yamaguchi, *et al.*, 1990). In particular, the presence of decorin in ECM scaffolds has been shown to reduce contracture in cutaneous wound healing (Shafritz *et al.*, 1994) presumably by binding and neutralizing TGF-β. Theses findings correlate with analysis of hypertrophic scars showing 75% reductions in decorin content and irregular collagen organization (Sayani *et al*., 2000; Scott *et al*., 1996).

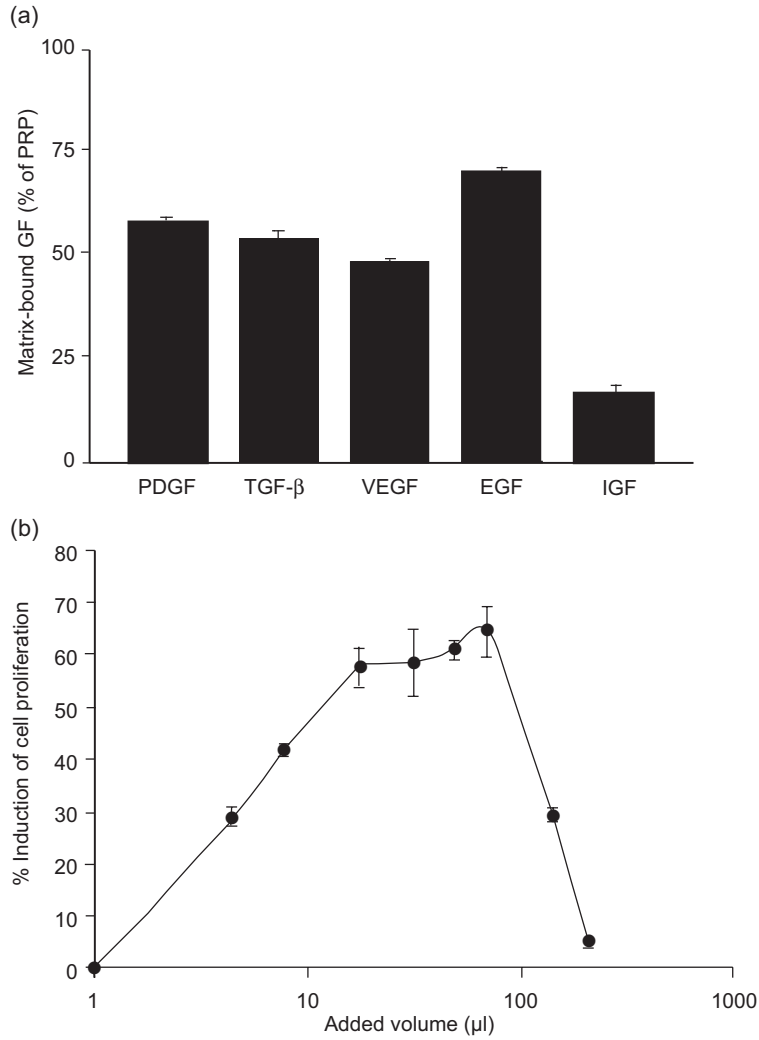
The glycosaminoglycan component of AlloDerm RTM exhibits normal

glycosylation patterns during fluorophore-assisted carbohydrate electrophoresis. Disaccharides isolated from the glycosaminoglycan component consist exclusively of 4-sulfated chondroitin sulfate and hyaluronic acid (Fig. 10.4(b)). Although the relative levels of each glycosaminoglycan tend to vary from donor sample to donor sample, both glycosaminoglycan components are consistently detected in all samples analyzed. Furthermore, no other form of chondroitin sulfate is identified indicating that the glycosaminoglycan component of decorin and biglycan in the acellular dermis is composed solely of 4-sulfated chondroitin sulfate glycosaminoglycan chains.

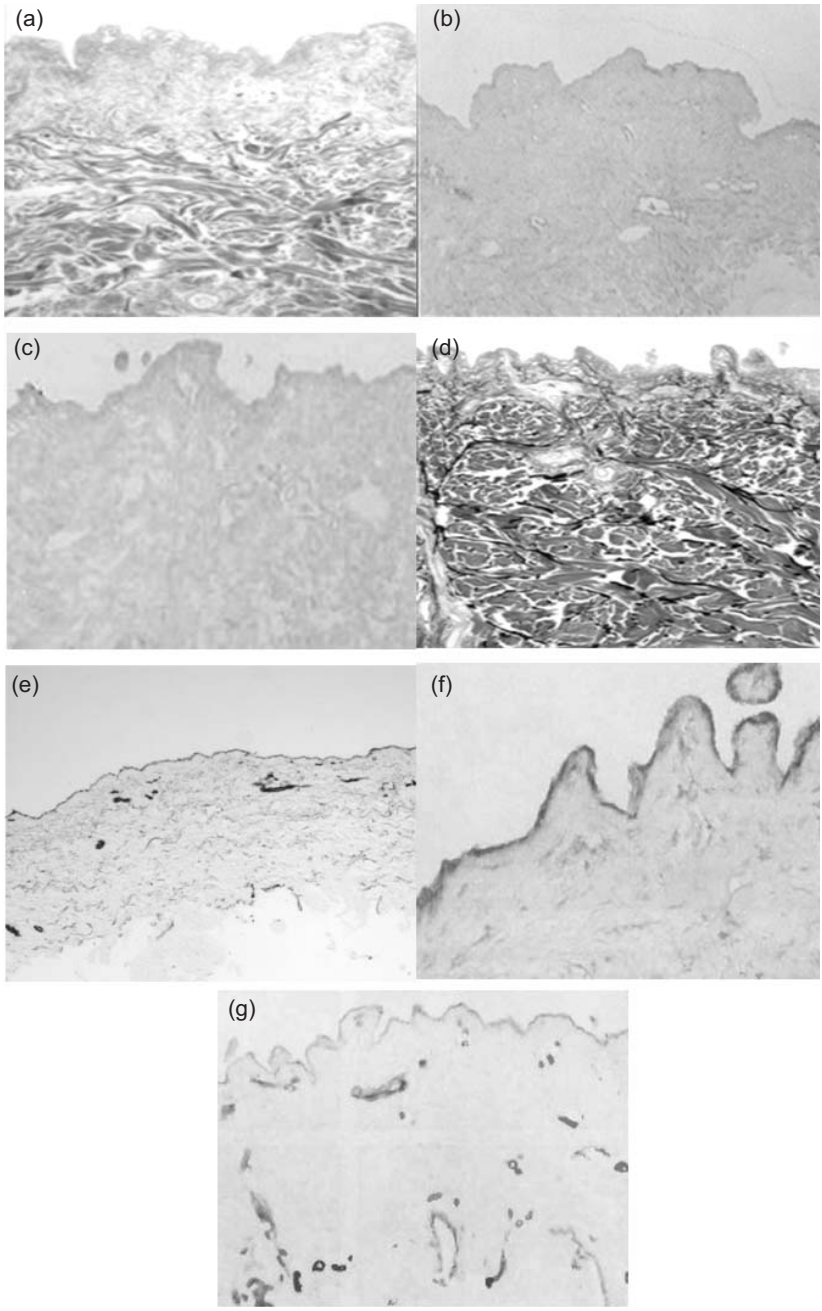
The biochemical analyses described above demonstrate the AlloDerm RTM maintains the expected molecular composition characteristic of dermal ECM. Thus, upon introduction to a wound, the RTM provides the necessary biochemical components needed to support wound healing. This characteristic of the RTM is exemplified by its ability to bind growth factors. Using platelet rich plasma (PRP) as a source of growth factors relevant to wound healing, the RTM matrix has been shown to bind nearly 50% or more of initial PRP content of growth factors TGF-β, PDGF, VEGF and EGF (Fig. 10.5(a)). Moreover, this growth factor matrix combination has been shown to induce fibroblast proliferation in a dose-dependent manner (Fig. 10.5(b)) (Pietramaggiori *et al.*, 2008).

While the biochemical composition and ability to regulate biochemical environment are essential attributes necessary for function, maintenance of the native organizational structure is equally important. Histological analysis demonstrates that the RTM appears structurally intact with collagen fibers arranged in distinct bundles throughout the tissue (Fig. 10.6(a)). Both papillary and reticular dermal layers are easily identified by differences in the collagen staining intensity. The efficacy of the decelluarization process is demonstrated by the absence of hematoxylin stained nuclei or nuclear remnants. The absence of cellular material is supported by the failure to detect major histocompatibility factor (MHC) antigens using immunohistochemical staining (Fig. 10.6(b) and (c). Histologic staining is also useful in identifying additional ECM components that are not characterizable during biochemical analyses. Elastin, which contributes to the resilient nature of the matrix, is distributed widely throughout the matrix but localizes in regions between the bundles of collagen. This elastin network is revealed through Verhoff's staining which indicates it is maintained following processing (Fig. 10.6(d)). Immunostaining with an anti-collagen type IV antibody shows an intact, contiguous basement membrane on the superficial dermal surface at the epidermal–dermal interface (Fig. 10.6(e)). Collagen type VII and laminin are protein constituents of the basement membrane necessary for cellular adhesion and staining demonstrates both are retained in AlloDerm RTM (Fig. 10.6(f) and (g)). In addition to basement membrane components, small blood vessels distributed throughout the matrix are also revealed through staining.

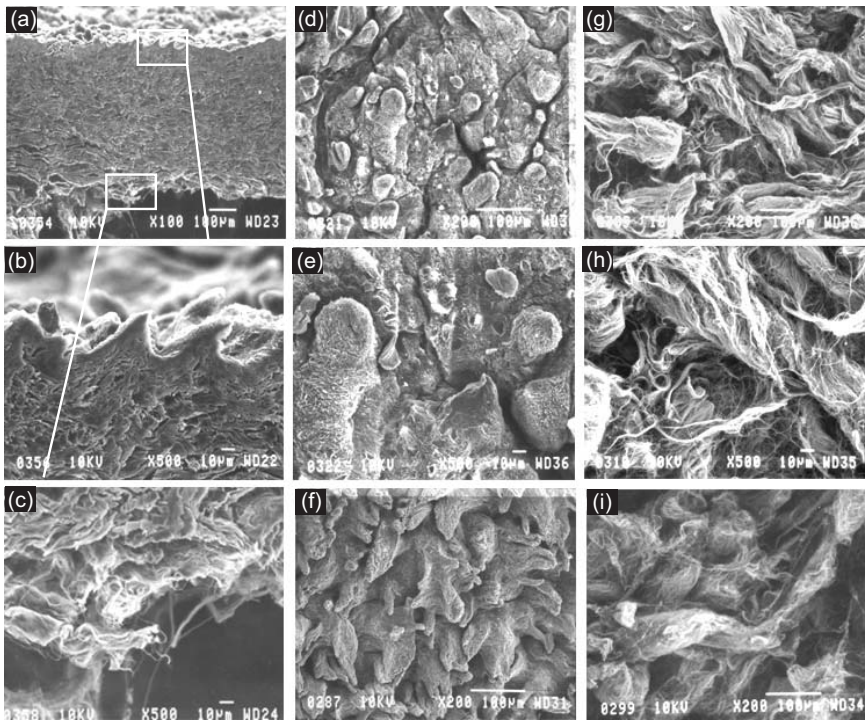
The overall three-dimensional organization of RTM can be visualized at the ultrastructural level using scanning electron microscopy (SEM). Cross-sectional



10.5 Growth factor binding to RTM matrix. (a) The micronized RTM showed the ability to bind variable amounts of the platelet-derived growth factors after incubation with sonicated PRP at 37 °C for 2 hours. PDGF, TGF-β, VEGF and EGF retention were all around 50% of the initial PRP content. IGF binding was significantly lower than all other growth factors evaluated (* $P < 0.5$). (b) PRP was incubated with micronized matrix and freeze dried. Specific volumes of the resulting freeze-dried material were analyzed for proliferative induction potential. A dose-dependent response was observed up to an upper threshold (approximately at 70 μl) above which the induction potential declined. All responses were significant ($P < 0.05$) compared to the matrix alone at 0% induction. Results are shown as mean ± SD (standard deviation). Figure reprinted with permission by Blackwell Publishing from Pietramaggiori *et al.*, 2008).



10.6 Histological analyses of AlloDerm RTM. (a) Hematoxylin and eosin (H&E) staining, (b) immunostaining for MHC I, (c) immunostaining for MHC II, (d) Verhoeff's staining, (e) immunostaining for collagen type IV, (f) immunostaining for collagen type VII, (g) immunostaining for laminin.



10.7 Ultrastructure of AlloDerm RTM by scanning electron microscopy. (a) Full cross-section of RTM showing both the basement membrane surface (top) and reticular surface (bottom). Magnified images show details of the basement membrane surface (b) and reticular surface (c). The basement membrane surface (d and e) retains characteristics of unprocessed dermis (f). Similar structural preservation is observed on the reticular surface of RTM (g and h) compared to unprocessed dermis (i); the surface illustrated represents a section through the central region of the deep dermis as the allograft skin was procured by the use of a dermatome.

views (Fig. 10.7(a)) through the entire depth of the dermal matrix are consistent with the histological staining at the light microscopy level as the overall organization of the collagen network of both the superficial papillary (Fig. 10.7(b)) and deep reticular (Fig. 10.7(c)) layers are easily distinguishable. The superficial basement membrane surface is quite irregular (Fig. 10.7(d) and (e)), and is consistent with the structural preservation of the dermal papillae in de-epidermized dermis (Fig. 10.7(f)). The reticular dermal surface of the processed matrix exhibits a loose network of connective tissue (Fig. 10.7(g) and (h)) indicating that the collagen matrix present in unprocessed dermis has been structurally preserved (Fig. 10.7(i)). These ultrastructural level images, demonstrate the presence of a porous three-dimensional network of collagen fibers comparable to unprocessed matrix.

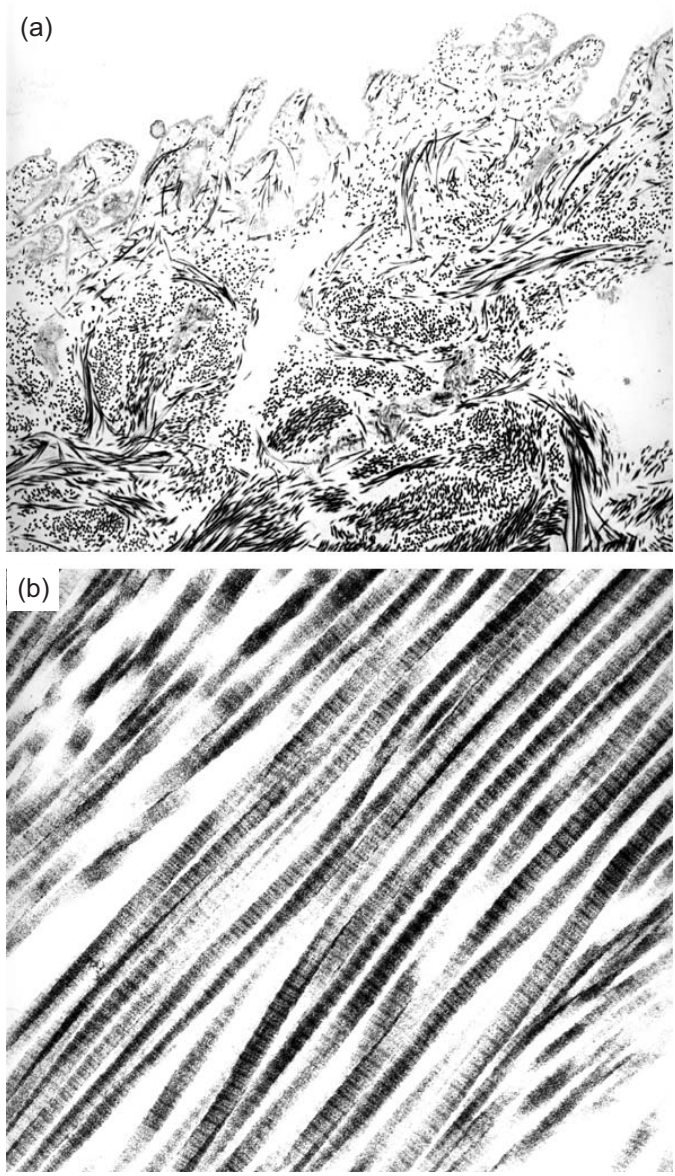
Furthermore, the components identified through biochemical analysis are shown not only to be retained in the RTM, but also to possess their native structural architecture. Taken together, the data suggest that the RTM is likely to retain the cell recognition sequences and structural porosity necessary to allow the migration, adhesion, proliferation and differentiation of host cell populations within the graft.

Molecular detail may also be obtained through the use of transmission electron microscopy (TEM). Such technology allows visualization of individual collagen fibers. As shown in Fig. 10.8(a), the acellular dermal matrix comprises almost exclusively collagen fibers arranged in numerous bundles. These bundles are constructed from the complex fibrillar network reminiscent of that seen following H&E staining (Fig. 10.6(a)). At higher magnification they exhibit their usual repetitive banding pattern characteristic of the arrangement of the individual collagen molecules within each fiber (Fig. 10.8(b)).

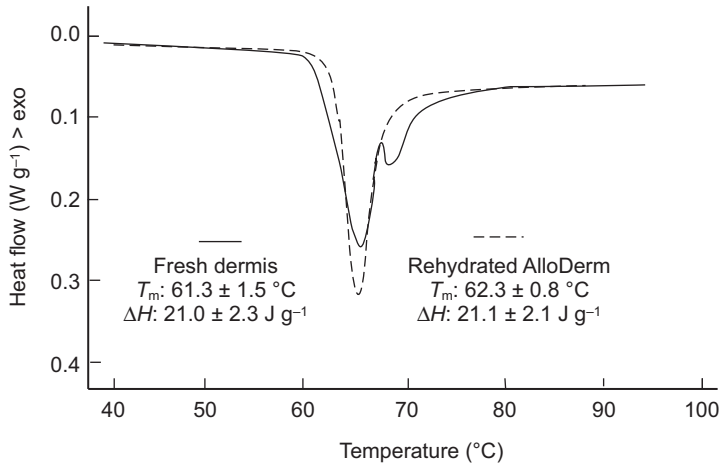
As previously noted, one detrimental consequence of improper processing is cross-linking between individual collagen fibers which can lead to poor functional outcomes. Differential scanning calorimetry (DSC) can be used to measure the thermodynamic properties of the RTM and demonstrate the effect that different preservation techniques can have on the matrix. Collagen melt temperature and the enthalpic change associated with the melt transition are two properties that are greatly affected by various matrix modifications, including cross-linking. DSC analysis of AlloDerm RTM demonstrates peak melting at roughly 63 ºC (Fig. 10.9). This peak temperature is consistent with melting of purified collagen type I (Miles and Ghelashvili, 1999) and is comparable with unprocessed dermis.

10.3.2 Mechanical analyses

The dermis provides the majority of mechanical properties of skin. Just as the biochemical and structural properties of a matrix affect its functionality *in vivo*, so too do its mechanical properties (Badylak, 2007; Clark *et al.*, 2007). Proper processing of native tissue in the production of acellular RTMs is therefore required to preserve the mechanical properties of the native tissues. Because LifeCell's RTMs have been explored for use in loading environments, the mechanical properties of the RTM have been investigated and quantified by a variety of specific metrics. Uniaxial tensile extension on more than 100 donor lots of RTM demonstrates a significant positive correlation between tissue thickness and ultimate load per cm width as tested over a range of 25 to near 500 N cm^{-1} (Fig. 10.10a). Clinically, overall strength is not dependent solely upon the intrinsic strength of the tissue, but also on how strongly the RTM can be fixed to the host. Suture pull analysis has been performed to determine how well the processed RTM retains sutures under tension. Polyblend (FiberWire) sutures in a mattress stitch configuration were pulled through test samples. The tissue–suture composite was fixed in the testing frame by securing the tissue in the lower grip and looping the suture around a metal hook in the upper grip. The composite was extended



10.8 Ultrastructure of AlloDerm RTM by transmission electron microscopy. The bundled nature of the reticular organization is observed by collagen fibers aligned both with and normal to the section plane (a). Preservation of intact collagen fibrils with typical periodicity owing to the staggered parallel arrangement of triple helical collagen molecules is shown at higher magnification (b).



10.9 Differential scanning calorimetric thermograms of AlloDerm RTM (dashed curve) and fresh dermis (solid curve).

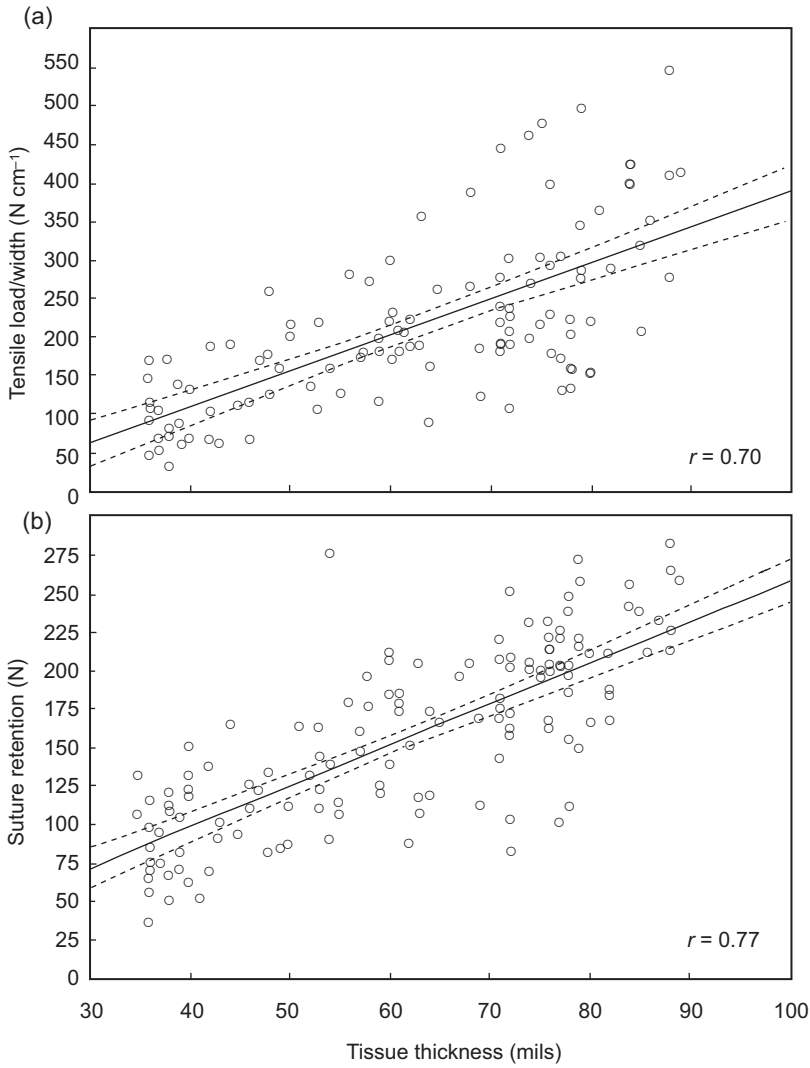
uniaxially until failure. Similar to the ultimate load data, the suture retention strengths demonstrate a relatively broad range of values (~30–300 N) which again correlate positively with tissue thickness (Fig. 10.10b). This data is consistent with published independent studies (Derwin, *et al.*, 2006; Silver *et al.*, 2001) which indicate the mechanical properties of RTM are comparable with that of native skin dermis.

10.4 Functional evaluation

The characterization studies presented above provide evidence that the biochemical, structural and mechanical properties of native dermis are retained in the processed RTM. The matrix template principle suggests that such properties will afford the matrix the functional characteristics required of an RTM. This section will address the preclinical and clinical evidence in support of the matrix template principle by demonstrating the functionality of acellular RTMs.

10.4.1 Preclinical analysis of skin healing

The initial demonstration that preserved acellular dermal ECM scaffold could function as an RTM during wound healing was performed in a full-thickness excisional wound porcine model (Livesey *et al.*, 1995). Test wounds were treated with allograft acellular RTM and a meshed STSG while control wounds received STSG only. Histological assessment from biopsies taken at 16 days postgrafting demonstrate uniform fibroblast infiltration, neovascularization, and an intact stratified epithelium with the absence of an inflammatory response. Take rates of



10.10 Mechanical properties of AlloDerm RTM. (a) 1-cm wide samples were mounted with a 4-cm grip-to-grip gauge length in an Inston mechanical testing frame and pulled to failure at 1.65% min^{-1}. Ultimate load per cm width is presented as a function of matrix thickness. (b) Mattress sutures of #2 FiberWire were threaded with a 5-mm bite through the top section of a 2 cm × 3 cm piece of tissue. The tissue–suture composite was fixed in the testing frame by securing the tissue in the lower grip and looping the suture around a metal hook in the upper grip. The composite was extended uniaxially until failure. A linear correlation with 95% confidence interval is shown for each data set.

the STSG were not statistically different between the two groups, but tissue thickness of the healing wounds above the subcutaneous fat was significantly greater in the dermal RTM treated wound compared to the control group at all time points up to 60 days postgrafting.

These initial results were independently confirmed and extended by assessment of wound contracture and scarring (Reagan *et al*., 1997). Treating full-thickness wounds with RTM and STSG significantly reduced contraction to 30.9% compared to 42.9% for STSG treatment alone. Changes in degree of contraction were noticeable by 6 weeks after grafting and persisted during the 10 week study. Blind cosmetic analysis using a modified Vancouver assessment demonstrated a significant reduction in scar value from nearly 12 to approximately 9.8.

In vivo, cultured keratinocytes have also been shown to support responses similar to STSG (Rennekampff *et al*., 1997). Furthermore, differential cell integrin expression was observed to be a function of both wound healing time and the formation of normal skin architecture. By 21 days after grafting, the laminin binding integrin subunit $\alpha 6$ was exclusively observed on the basal side of basal keratinocytes and the fibronectin binding integrin subunit $\alpha 5$ was not present. These findings are consistent with the integrin expression differences witnessed between normal and healing skin (Cavani *et al*., 1993; Grinnell, 1992; Hertle *et al.*, 1991) .

In vitro studies have assisted in understanding the mechanistic processes believed to support the *in vivo* responses described above. In particular, skin-like organotypic cultures utilizing AlloDerm RTM as a dermal substrate have provided insight into the microenvironmental factors and dynamic cross-talk between epithelial and dermal components necessary for normal epidermal phenotypes and skin morphogenesis (Andriani *et al*., 2003). AlloDerm seeded with fibroblasts accelerated basement membrane (BM) assembly and supported both keratinocyte growth and normalized epidermal architecture. However, AlloDerm cultures lacking dermal fibroblasts exhibited slower assembly than when fibroblasts were present. The role fibroblasts play in regulating matrix metabolism and keratinocyte-produced BM components is likely to explain this result (Smola *et al*., 1998). Cultures using neutralized collagen type I as the dermal substrate failed to support BM assembly even in the presence of dermal fibroblasts, presumably owing to the lack of BM components in the substrate. Thus, normal fibroblast phenotypes and BM components are both necessary to support formation of normal stratified epithelium. This study also demonstrates the potential limitation of *in vitro* experimentation.

Prior studies using AlloDerm employed cell seeding methods that do not support normal infiltration of fibroblasts and therefore failed to mimic the *in vivo* condition (Ng, *et al.*, 2004). Methods that support uniform infiltration produced distinctly different results (Andriani *et al.*, 2003). Similar issues arose when fibroblasts were not included in the culture environments. Integrin expression of keratinocytes cultured on AlloDerm *in vitro* (Rennekampff *et al*., 1996) was

distinctly different from what it was *in vivo* (Rennekampff *et al.*, 1997). Taken together, these results indicate that care must therefore be used when interpreting *in vitro* studies.

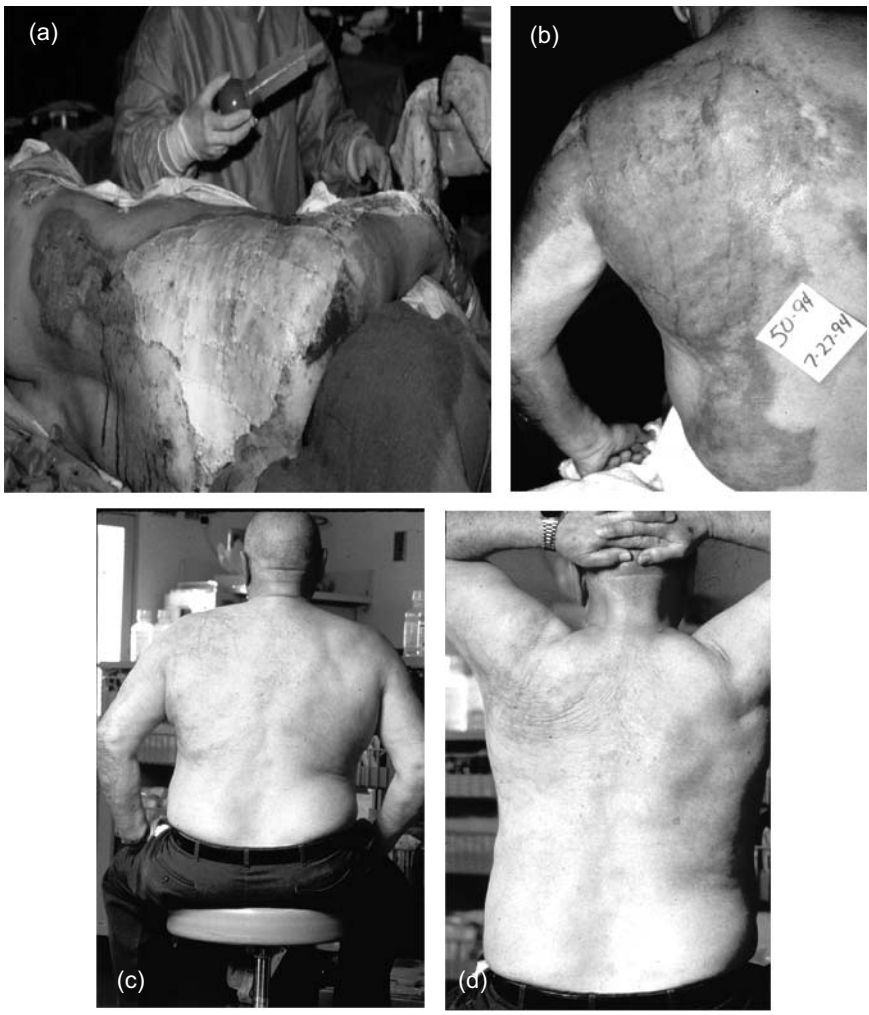
In addition to supporting acute full-thickness wound healing, the RTM is supportive of tissue formation in delayed wound healing models (Pietramaggiori *et al.*, 2008). When placed in genetically diabetic mice, the intact RTM not only supported generation of a dense and vascularized wound tissue, but also demonstrated an elevated fibroblastic cell proliferation noticeable within 9 days of treatment. Cell proliferation declined to levels similar to non-treated controls by 21 days following treatment and the resulting tissue more closely resembled normal dermis complete with epidermal migration from the wound periphery. Although these responses were slightly enhanced by incorporating platelet-rich plasma into the RTM, these results suggest the RTM stimulated enhanced tissue formation in the wound which may ultimately provide accelerated healing when treating chronic wounds.

10.4.2 Clinical studies of skin healing

The wound healing responses demonstrated in model systems have been translated into clinical wound healing practice. In the treatment of full-thickness burns, RTM demonstrated an equivalent graft take rate to STSG alone (Wainwright, 1995). Histological analysis of biopsies taken at 16 days after grafting showed normal collagen structure, fibroblast recellularization, neovascularization and re-epithelialization in the absence of inflammatory cells. However, elasticity and cosmesis of the healed wound with RTM were considered to be superior to STSG alone as judged by both the patient and surgeon. These functional findings were confirmed in a multi-center trial although the qualitative cosmetic assessments of the two treatments were found to be equivalent (Wainwright *et al.*, 1996).

Smaller studies of burn patients have also replicated the ability of the RTM to support immediate thin STSG grafting, although no statistical qualitative differences between test and control groups were found (Gore, 2005; Munster *et al.*, 2001; Sheridan *et al.*, 1998; Sheridan and Choucair, 1997). However, donor sites for thin STSG harvesting exhibited faster healing times, thereby allowing more rapid reharvesting, if necessary (Gore, 2005). Studies of grafting at joint areas, such as hands, feet or shoulders (Fig. 10.11), with RTM and STSG have produced good cosmetic results in these visible areas with limited scarring or contracture (Lattari *et al.*, 1997; Tsai *et al*., 1999). Similar results with respect to graft take and reduced contracture have been shown in a larger study evaluating performance across a wide study demographic exhibiting variations in patient age, burn site, and involved surface area (Callcut *et al*., 2006; Lattari *et al.*, 1997).

Cymetra, a particulate, injectable form, is similar to sheet AlloDerm RTM in that it has been shown to support cell ingrowth and tissue regeneration without producing inflammatory responses and host rejection (Maloney *et al.*, 2004;



10.11 Clinical photos of wound healing – skin grafting. AlloDerm RTM was used to treat a full thickness wound created by scald burn to the patient's back. (a) AlloDerm was sutured into position covering the excised wound. Ultrathin (0.004–0.008"; 0.101–0.203 mm), widely-meshed autologous STSG was applied over the AlloDerm in the same procedure (simultaneous application). (b) Wound healing at 1 month post-treatment showing complete wound closure and 100% take. (c) The healed wound is shown at 1.5 years post-treatment and exhibits good cosmesis and absence of fibrotic scarring. (d) The patient exhibits full range of motion. Photos courtesy of Dr. Robert Voorhees, Fort Wayne, IN.

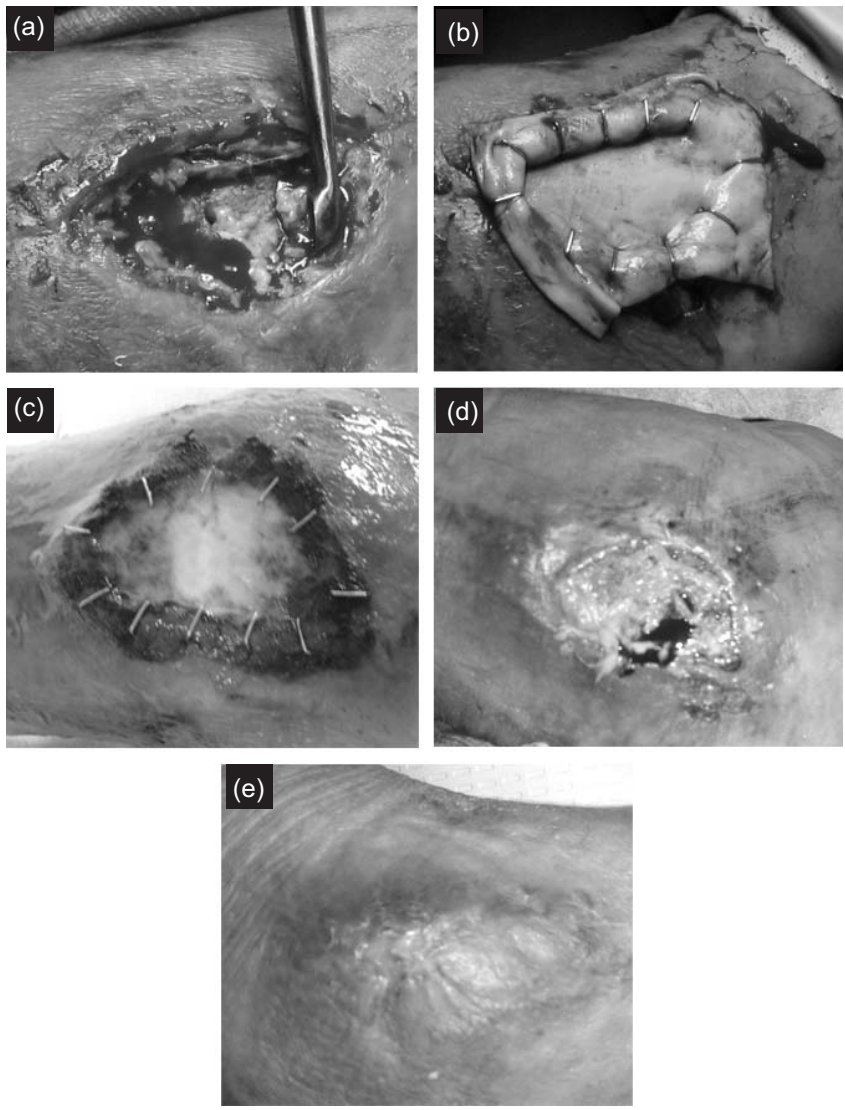
Sclafani *et al*., 2000). These biological properties have translated to successful use in reconstructive surgery and soft tissue augmentation (Homicz & Watson, 2004; Karpenko *et al*., 2003; Maloney *et al.*, 2004).

Standard care for a chronic wound generally follows the paradigm established for acute wounds with only minor alterations (Schultz *et al*., 2003). A chronic wound results when the normal healing process is disrupted and represents an even more challenging clinical problem (Lazarus *et al*., 1994). However, a chronic wound presents a significantly different biochemical and cellular environment, particularly with respect to the presence of necessary growth factors required for healing. Furthermore, the accumulation of excess chronic fluid in the wound may actually retard wound closure (Tarnuzzer & Schultz, 1996; Trengove *et al.*, 2000). These wounds exhibit elevated levels of matrix protease activity (Xue *et al.*, 2006) which adversely affect provisional matrix integrity (Grinnell *et al.*, 1992) and promote an imbalance in matrix metabolism (Trengove *et al*., 1999). All these factors result in the inability of the local environment to facilitate satisfactory healing of the wound.

The medical costs associated with chronic wounds as well as the detrimental effects that chronic wounds have on a patient's quality of life are well established. Most advanced therapeutic treatments, such as recombinant PDGF and skin equivalents, unfortunately require multiple applications to achieve wound resolution. However, in a pilot study of 40 patients, a single application of RTM eliminated the need for skin grafting and demonstrated statistically faster wound closure compared to standard debridement and dressing care protocols (Fig. 10.12) (Brigido *et al.*, 2004). Continued monitoring of patients in this prospective, controlled, randomized trial showed 85% of the patients treated with a single application of RTM had wound closure by 16 weeks compared to 28% in the standard care control group (Brigido, 2006). These results are consistent with a retrospective analysis of deep wound healing in diabetic patients, where 82% of patients' wounds treated with a single application of RTM healed during the 20-week evaluation and represented a mean wound duration of just less than 9 weeks (Martin *et al*., 2005).

10.5 Universality of acellular regenerative tissue matrices for soft tissue replacement

The preclinical and clinical successes described above reflect the ability of an acellular RTM to support tissue regeneration by providing the biochemical and structural environments for wounds that are required to support host recellularization and vascularization. However, these studies demonstrate only the beginning of RTM utility. The clinical need for an ECM material that supports the intrinsic regenerative process has driven the use of RTMs for new indications.



10.12 Clinical photos of wound healing – diabetic ulceration. The wound bed was prepared using full-thickness debridement to remove all necrotic tissue and create a bleeding base (a). The RTM graft was applied using skin staples to affix the graft to the wound margins circumferentially (b). Following application, a moist environment was maintained with a mineral oil-soaked fluff compressive dressing. The dressing was changed and reapplied at 5 (c), 10 (d) and 15 days post-application. After day 15, the wound was covered with a dry sterile dressing. Complete closure of the wound is observed at 4 weeks post-application (e). Figure reprinted from Brigido *et al.*, 2004 with permission from SLACK Incorporated.

10.5.1 Abdominal wall reconstruction

The use of traditional synthetic mesh materials for reconstruction of the abdominal wall are plagued by the occurrence of visceral adhesions, susceptibility to infection, palpable rigidity and discomfort associated with limited mobility (Butler, 2006). Similar to the results seen during skin graft application, AlloDerm embodies an RTM scaffold supportive of biological revitalization while simultaneously providing the mechanical strength necessary for successful closure of an abdominal wall defect. AlloDerm's strength under these conditions has been shown to be comparable to synthetic meshes (Choe *et al.*, 2001). Preclinical hernia repair models demonstrate AlloDerm RTM provides greater strength than surrounding fascia (Silverman *et al.*, 2004), while its integration into the fascia creates a strong repair (Silverman *et al.*, 2004). In addition, AlloDerm RTM was found to support rapid revascularization (Menon *et al.*, 2003) and to reduce the formation of bowel adhesions (Butler and Prieto, 2004). Successful clinical use resulting in a low occurrence of complications (Buinewicz & Rosen, 2004) has been demonstrated in incisional hernia repair (Buinewicz and Rosen, 2004; Butler *et al.*, 2005), abdominal and chest wall reconstruction (Butler *et al.*, 2005; Hirsch, 2004) and transverse rectus abdominis myocutaneous (TRAM) flap reinforcement (Buinewicz and Rosen, 2004). Moreover, because the RTM does not elicit an inflammatory response, it does not impede the body in targeting the sources of potential infection notable in abdominal interventions. Owing to these properties, AlloDerm RTM has been used safely and successfully in abdominal wall reconstruction wherein the surgical field was known to be contaminated (Patton, *et al.*, 2007).

10.5.2 Breast reconstruction

Tissue expansion of the pectoralis muscle together with mastectomy skin flaps is currently one of the most common procedures employed for the reconstruction of the breast. Since many patients lack the necessary tissue to accomplish complete lateral coverage of the implant, a repositioning of the serratus anterior muscle is required. Use of the acellular RTM as a sub-pectoral sling provides an alternative to techniques involving serratus muscle by creating a complete pocket for reconstruction (Breuing and Warren, 2005). Besides the obvious advantage of a reduction in donor site morbidity, this technique also reduces, or sometimes eliminates, the need for tissue expansion and allows immediate reconstruction after mastectomy (Breuing and Warren, 2005; Salzberg, 2006). Although relatively new, applications of AlloDerm RTM for use in breast reconstruction via this procedure are growing (Baxter, 2003; Bindingnavele *et al.*, 2007; Breuing and Colwell, 2007; Breuing and Warren, 2005; Gamboa-Bobadilla, 2006; Glasberg and D'Amico, 2006; Margulies *et al.*, 2005; Salzberg, 2006; Zienowicz and Karacaoglu, 2007). Results have further demonstrated that the RTM provides a viable graft with recellularization and revascularization noticeable up to 6 months (limit of study)

(Baxter, 2003) and thereby is a viable method for reducing morbidity in high-risk patients (Gamboa-Bobadilla, 2006).

10.6 Future trends

After preserving life, the ultimate goal of clinicians is to provide treatment that restores natural structure, function and physiology irrespective of the pathology source, be it acute or chronic, induced or traumatic, local or systemic, genetic or acquired. The growing acceptance of RTMs for use in the repair of soft tissue defects is being driven by the recognition of the overall RTM concept and by clinical need. The studies described herein demonstrate that an intact natural RTM has the ability to support intrinsic regenerative healing and the characterization of these materials is providing insight into the roles that the underlying composition and architecture may play in this process. Continued successful clinical outcomes are likely to support expanded utility and application of the native RTM. Further research on the mechanisms of wound healing and the factors that induce specific functions may support the development of novel functionalized matrices that are designed to elicit more specified healing responses or even to deliver specific molecules that influence stem cell differentiation, direct the synthesis of particular ECM molecules, provide antimicrobial properties, or support accelerated hemostatsis.

By incorporating supporting materials such as bioabsorbable polymers, the mechanical attributes of the ECM might be tailored leading to even broader application of ECM scaffolds. While the RTM supports biological revitalization, the mechanical properties are such that overloading of the structure is likely to occur in environments requiring significant loading, such as is seen in ligament and tendon. Providing an absorbable material with mechanical support while the RTM undergoes biological transition poses the opportunity to engender a completely biological repair in a mechanically challenging environment. Similarly, polymeric materials might be combined with particulate RTM to create unique three-dimensional structures supportive of regenerative responses. Such hybrid devices may find utility in soft tissue orthopaedics and cardiovascular applications.

Devices manufactured in part from acellular RTM are likely to be regulated by the FDA as 510(k) or PMA devices. As such, sterility is expected. Many methods of sterilization have been attempted, but they universally impart some modifications to the matrix structure. These modifications typically translate into reduced biological function and may result in inducing a pathological response. Although sterilization is not a direct advance in acellular RTM *per se*, advanced technologies that better support matrix sterilization with the retention of biological performance and the RTM's ability to elicit a regenerative response would be considered a significant development in the field.

Finally, and most significantly, in the context of cutaneous wound healing, continued characterization of novel RTMs and the manner in which the compris-

ing elements direct the wound healing process will someday allow the creation of a synthetic matrix that is truly equivalent to the native structure. With the ability to provide biocompatible cells already available, successful combination of a native-like matrix with appropriate cell types has the possibility to create a true skin equivalent of varying shape and size.

10.7 Sources of further information and advice

Further information is available from a variety of sources. For example, the LifeCell company website (www.lifecell.com) provides abstracts from key articles referencing AlloDerm in the areas of abdominal wall reconstruction, plastic reconstruction, including ENT (ear, nose and throat), head and neck, grafting and breast reconstruction. Clinical case studies and monographs can be accessed as well. The locations and dates of scientific conferences of interest to researchers in the area are also posted. The website for the American Association of Tissue Banks (AATB) provides guidance documents, bulletins and abstracts of scientific and clinical conference papers from annual meetings. The AATB site can be accessed via www.aatb.org. The US FDA's Center for Biologics Evaluation and Research (CBER) maintains a website (www.fda.gov/cber) that supplies information about tissue and tissue-based products. Resources include registration, regulatory and safety documents as well as CBER-sponsored meetings and workshops. Additional materials on industry and manufacturing requirements are available. The site also houses a list of approval documents, biological license approvals, products and manufacturers. Information from CBER research programs focusing on the technical and scientific issues relating to safety, potency and efficacy of biologic products is also accessible.

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10.9 Disclosure

The authors are employees and stockholders of LifeCell Corporation, manufacturer of the acellular regenerative tissue matrix products discussed herein.

11

Lyophilized xenogenic products for skin replacement

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Abstract: This chapter describes the need for purified skin substitutes derived from animals. It discusses the difference in products for temporary and permanent skin replacement. Using both porcine and bovine derivatives, skin and tissue replacement has been performed safely, effectively and in most areas of the human body, with great results.

Key words: lyophilized xenogenic skin substitutes, porcine dermis, bovine dermis, porcine small intestine submucosa.

11.1 Introduction

Animal skin has been used for wound coverage for over a thousand years, yet it was in Russia in 1682 that the first documented xenograft was reported: the implantation of a portion of canine skull to repair a combined scalp and skull defect from a Tartar sword injury.[1] In the United States in 1880, a description of a burn wound covered by sheep skin was published. This was followed by reports of the use of skin from a variety of species: frogs, rabbits, dogs, pigs and birds.[1,2] Only in the last 70 years was it determined that xenogenic skin could not replace human skin directly, but rather act more as a biologic dressing. Owing to overwhelming necessity, the short supply of human tissue, and the cost of engineered tissue derivatives, products from animals remain of great importance to the field of epidermal and dermal replacement. Porcine and bovine derivatives of collagen, dermis and small intestine submucosa, have reached the forefront of xenogenic dermal replacement. These grafts are employed in two ways. One as a temporary biologic dressing and the other as a more permanent substitute and scaffold.[3–10]

11.2 Temporary skin substitutes

Temporary dressings are most commonly from domestic swine, where the product is reconstituted dermis and supplied in meshed sheets. Two mainstays are EZ Derm and Mediskin (Brennen Medical, St. Paul, MN), which are both aldehyde cross-linked porcine dermis.[4] The former is stored at room temperature; the latter is stored frozen but does not need reconstitution. Although usually not maintained

Table 11.1 Ideal characteristics of a dermal replacement of xenogenic origin

- Serves as a natural matrix for host granulation, fibroblast proliferation and neovascularization
- Lacks antigencity or immune system stimulatory agents
- Promotes epithelial influx and growth
- Adheres to the wound bed
- Lacks any infectious agents that could cross species
- Fully covers the wound
- Ability to withstand hypoxia
- Flexibility

on a clean wound for more than three to four days, they do provide excellent coverage for second-degree burns, donor sites, and even venous ulcers.[3–5] Reports from a large series of patients have provided favorable results when the dressings of porcine acellular dermal matrix were left for seven to ten days.[11] Porcine dressings provide outstanding analgesia as the wound heals, although because they are of xenogenic origin, they do not vascularize. Bovine dermis is not employed in this fashion, though a bovine tendon collagen derivative, Promogram® (Johnson and Johnson, New Jersey, USA) can be used temporarily. This dressing consists of processed bovine collagen combined with oxidized cellulose.[4]

Initially, these derivatives were used fresh, but with the need for immediate use, technology has allowed them to be lyophilized for preservation and availability. Because of to antigenicity, certain preparation and treatment variations are employed, including radiation and conjugation with aldehydes. Aldehyde cross-linking does not interfere with the structural integrity of the product and, with the addition of silver ions, allows for increased antimicrobial resistance. These preparations are employed for temporary wound coverage.[4–9]

11.3 Permanent skin substitutes

Porcine dermis, bovine dermis and small intestine submucosa are also employed in a more permanent fashion. Optimally, these skin substitutes should act like human dermis (Table 11.1): be adherent to the underlying wound to prevent evaporative loss and protect against infection, and serve as a matrix to stimulate granulation and influx of blood vessels and epithelium. In addition, lack of antigenicity is also desirable, as well as protection against transmission of non-human disease.[4–9]

The more permanent products of porcine dermis and its derivatives come in many forms, conjugated with synthetic and human scaffolds, such as Transcyte™, Permacol™, and Enduragen™ and others, but the latter two will be of focus in this chapter. A previous porcine collagen derivative, Zenoderm™, a product from Ethicon developed and used in the 1980s, is no longer being produced. Permacol™, from Tissue Science Laboratories in the UK, was developed in Britain, and has been available since 1998. Variations include

Enduragen™ marketed by Porex (Newnan, Georgia, USA), Permacol™ Surgical Implant, which is currently being sold in the USA by CR Bard (Murray Hill, New Jersey, USA) in the field of gynecology and urology, Permacol™ Injection UBA (urethral bulking agent) for female stress incontinence and PermaDerm™ Wound Therapy.[11] Because porcine dermal collagen has a structure similar to that of humans, the processing of Permacol involves limiting the amount of cross-linking by conjugating it with isocyanate to make it resistant to human collagenases. Processing the tissue also makes it acellular, limiting the chances of an allergic or immune system reaction. It also allows the ingrowth of fibroblasts and blood vessels, enabling permanent fixture. In the United States, it is a class II medical device (those for which general controls alone are insufficient to assure safety and effectiveness) and has four Food and Drug Administration (FDA) clearances in the United States: a soft tissue patch to reinforce soft tissue where weakness exists and for surgical repair of damaged or ruptured soft tissue membranes, specifically (1) hernias, prolapse, flap reinforcement and pelvic floor reconstruction, (2) plastic and reconstructive surgery of the face and head, (3) where needed for suture and suture anchors, limited to the supraspinatus during rotator cuff repair, and (4) abdominal wall hernias.[12]

Recent studies have indicated favorable results with Permacol™ in each of the uses mentioned above. Even more important is the purported idea that since it is not a synthetic prosthesis, and therefore not a foreign body, removal is not necessitated if infection occurs.[13–15]

Tissue Science Laboratories, UK, has partnered with Zimmer Laboratories, (Warsaw, IN, USA) to produce the Zimmer Collagen Repair Patch™, which is the same product as Permacol, but intended specifically for rotator cuff repair. Recent comparisons to other xenogenic products have been favorable, although there are isolated reports of inflammatory reactions.[16] Permacol, through another permutation, Enduragen™, which is produced by a partner Porex, has been employed in plastic and reconstructive procedures of the head and neck, including lip augmentation, rhinoplasty, and orbital floor repairs.[17]

Bovine dermis has recently come into use, though not as extensively as porcine dermal derivatives. Bovine products are more popular in the form of injectable collagen and come in a variety of forms as well, such as Zyderm™ and Zyplast™. Newer lyophilized bovine dermal matrices have only been on the market for a few years and have not been extensively studied. Nonetheless, these products are similar to the porcine dermal equivalents and show promise. A concern with the bovine derivatives is the transmission of prion disease, namely Creutzfeldt Jakob disease, the human form of bovine spongiform encephalopathy. Although rigorous testing and processing occurs to reduce the risk, the actual effects will not be known as these are new products.[4,5,7]

Primatrix™ (TEI Biosciences, Boston, MA, USA) is a recently FDA approved acellular bovine matrix composed mainly of type II collagen. Using a patented

epithelial basement membrane technology, it is constructed without cross-linking restrictions, unlike Permacol™, and is designed to be replaced by the host's fibroblasts and native collagen. It is indicated for second-degree burns, pressure, diabetic and venous ulcers, donor sites, and Moh's surgical wounds. It is not intended for third degree burns. It is stored dry at room temperature and reconstitutes easily in saline or water. TEI Biosciences does have other forms of Primatrix, for use in orthopedic (Tissuemend™), neurologic (Durepair™), urologic (Xenform™) and, as of October of 2007,[18] Surgimend™ for use in general, plastic and reconstructive procedures. Case reports for the use of Surgimend™ and Primatrix™, and a few studies involving Tissuemend™, do not appear unfavorable at the moment.[19,20]

Currently in the US FDA certification process is Matriderm®, a bovine dermal matrix from a German company, Dr. Suwelack Skin and Health Care. Having been released in Europe within the past few years and not studied in the United States, Matriderm® is a porous, 0.5–2 mm thick membrane of bovine types I, II, and V collagen in combination with elastin obtained from the bovine ligamentum nuchae via hydrolysis. It is irradiated, is not manufactured with any collagen cross-linking chemicals or preservatives and can be reconstituted in saline or Ringer's solution. Human studies on punch biopsies indicate that the scaffold is degraded within six weeks, allowing for a significant influx of fibroblasts and vascular elements.[21] It is indicated for use in a single or two stage procedure. Skin grafts may be laid on the scaffold immediately or in a delayed fashion.[22]

Several European studies have shown that Matriderm® is promising as a dermal substitute. One study, comparing split-thickness skin grafts to split-thickness skin grafts on top of a Matriderm® bed for deep partial and full thickness burns demonstrated no difference in graft survival, but did show increased elasticity with Matriderm® based on the Vancouver burn skin score (VSS).[23] Another study indicated no difference in split thickness skin graft take with Matriderm®, although related less hospital cost and length of stay, in addition to fewer 'skin related problems and decreased itching'.[24] An Austrian group evaluated Matriderm® for use as a dermal substitute in ten patients with severe hand burns and found that in a one stage procedure, there was a 97% graft take, with great pliability, range of motion and lack of hypertrophic scars.[25]

Porcine small intestine submucosa has been under investigation for over a decade. Its commercial popularized forms are Surgisis™ and Oasis™ (Cook Biotech Incorporated, West LaFayette, IN, USA). Derived from jejunum, porcine small intestine submucosa is composed mainly of types I and III collagen. The serosa, tunica muscularis and tunica mucosa are removed, leaving behind the stratum compactum and muscularis mucosa of the tunica mucosa in conjuction with the submucosa. The remaining product still contains glycosaminoglycans, proteoglycans and glycoproteins. In addition, mediators such as transforming growth factor β (TGF-β) and vascular endothelial growth factor (VEGF) are maintained, allowing for more rapid epithelial influx and neovascularization.[26–29]

Oasis™ is one form that is indicated in the treatment of full or partial thickness skin loss, vascular and pressure ulcers and all levels of burn injury. There have been several studies, including randomized control trials attesting to its efficacy in wound healing.[30–32]

Surgisis™ is another form of porcine small intestine submucosa that comes in a variety of forms indicated for a number of applications ranging from hernia repair, soft tissue or body wall defects, urinary slings for incontinence, dural patching and, most recently, an anal fistula plug.[33–39] It has been shown to be effective despite being placed in a contaminated field, although some infectious complications can cause degradation and failure of the patch.[39]

11.4 Conclusions

The xenogenic lyophilized dermal equivalents described have been shown to be useful in the management of dermal replacement. Rigorous testing and extensive research has allowed these animal tissues to be processed to remove unnecessary components and leave behind an acellular frame that allows natural human components to begin the task of rebuilding. Given that the role of the dermis is in maintaining structural integrity, orientation and a blueprint for this process, it is satisfying and humbling to be able to employ this complex structure from other species and for it be so successful.

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Part III

Combined dermal and epidermal replacement

12

Cultured skin substitutes

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Abstract: Biopolymer scaffolds for tissue engineering allow cellular organization into tissue substitutes and can regulate development of connective tissue to minimize scarring. Cultured skin substitutes (CSS), consisting of autologous fibroblasts and keratinocytes on a biopolymer sponge, have been successfully used to treat full-thickness burns. While the current model of CSS has distinct advantages, it is clear that alternative fabrication strategies may improve the quality and function of bioengineered skin. Electrospinning of biopolymers offers advantages in precision of fabrication and versatility of composition compared to freeze-dried sponges. With optimized structures and degradation rates, electrospun biopolymer scaffolds have been shown to increase mechanical strength and stability, reduce wound contraction and promote engraftment of epidermal tissue substitutes. Based on current findings, electrospinning of biopolymers may be expected to contribute to advanced designs for engineered human skin and to reduction in morbidity from skin wounds.

Key words: burns, chronic wounds, cultured skin substitutes, electrospinning of biopolymers, tissue engineering.

12.1 Introduction

Wound closure can be the limiting factor in recovery from several clinical conditions including acute wounds, such as massive burn injuries, and chronic wounds of multiple etiologies. Annually, there are more than 1 million burn injuries in the United States which result in nearly 40 000 hospitalizations and 4000 deaths.[1] Advances in burn care have significantly decreased mortality rates in burn injuries. Sheridan *et al.* reported an approximately 57% decrease in mortality rate in pediatric burn patients with 60% or greater total body surface from 1974 to 1997.[2] Advances in treatment include early excision, improved fluid resuscitation, infection control, nutritional support and aggressive physical therapy.[3–5] Even after very extensive burns, most patients survive the initial resuscitation phase, making wound management critical for recovery. Use of split thickness skin grafts (STSG) has been the prevailing treatment for permanent closure of excised full-thickness burn wounds. Wound closure can be accomplished

using STSG in patients with small wounds but closure is challenging in large burns owing to limited donor sites. Prompt wound coverage in these patients is essential to decrease the probability of infection and sepsis which are major causes of burn mortality.[6]

Conversely, chronic wounds tend to involve relatively small areas of skin but represent a major medical need as they have a high incidence in the general population. The most common chronic wounds are pressure ulcers and leg ulcers.[7] These wounds affect more than 2 million people[8] and are estimated to cost US$1 billion annually.[7] Historically, wound closure has been enhanced with topical agents such as growth factors to stimulate healing and antimicrobial agents to minimize infection.[9] A large percentage of these patients will ultimately require grafting with split- or full-thickness autograft for permanent closure of chronic wounds. However, autograft may not be a feasible option in these patients owing to the underling deficiencies in wound healing which compromise healing in donor sites. A medical need clearly exists for a safe and effective therapy of both acute and chronic wounds, and thus is a major motivation for the design of bioengineered skin substitutes.

12.2 Medical and surgical objectives for cultured skin substitutes (CSS)

Normal human skin performs a wide variety of protective, perceptive and regulatory functions that help the body maintain homeostasis. Ideally, a skin substitute should restore the anatomy and physiology of uninjured skin. However, there are currently no skin substitutes which fully replicate all of the structures and functions of native skin.[10–13] Although skin substitutes cannot restore all the functions of normal human skin, they can provide several advantages over conventional therapy including reduction in donor site area required to close wounds permanently. For example, conventional grafting expands donor skin by about 1:4. In contrast, rapid growth of cells *in vitro* allows coverage of culture surfaces by more than 1000 times the area of the skin biopsy[14,15] and cultured skin substitutes have been shown to heal approximately 60 times the area of the initial skin biopsy.[16] Therefore, skin substitutes containing autologous keratinocytes are ideal candidates for acute injuries where donor sites are limited. By increasing the availability of grafts for wound coverage, autologous skin substitutes can provide several advantages over conventional therapy including reduction in the donor site area required to close wounds permanently, reduction in the number of surgical procedures and hospitalization time and reduction of mortality and morbidity from scarring.[17,18]

The re-establishment of the epidermal barrier to fluid loss and microorganisms[19] is one of the most important objectives for an engineered skin substitute. Ideally, a skin substitute should be readily available, easy to apply, promote complete engraftment without contraction, allow rapid healing forming both dermal and

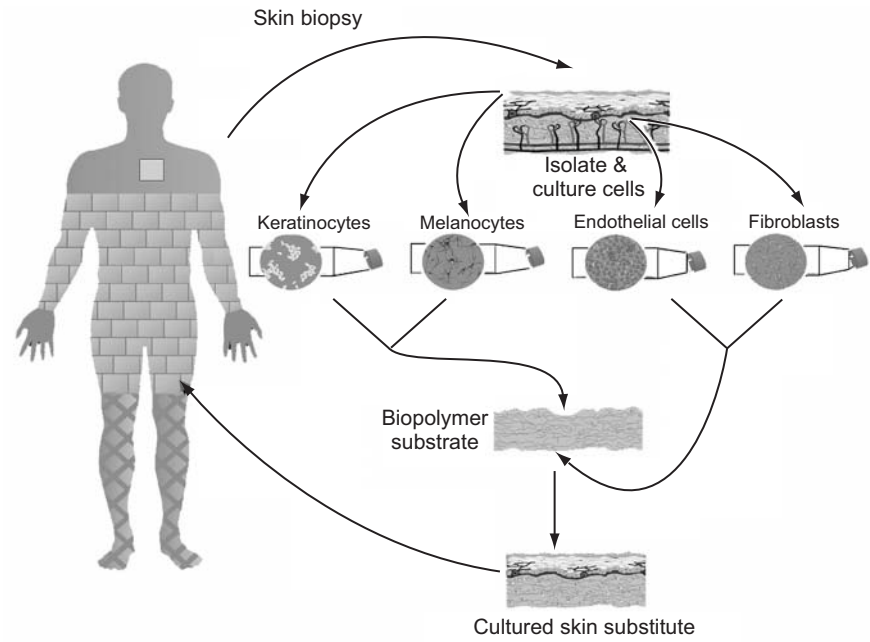
epidermal layers, achieve favorable functional and cosmetic outcome, be free from risk of disease transmission and have minimal immunological reaction, with the ultimate goal of wound closure with the elimination or reduction of a donor site for skin grafts. If these objectives are satisfied, recovery time should hypothetically be reduced and operative procedure reduced.

Delivery of cultured cells to wounds can be facilitated by combination with a biopolymer scaffold. Skin substitutes have been prepared utilizing synthetic polymers such as polylactic/polyglycolic acid (PLGA),[20] polyglycolic acid (PGA),[21] polycaprolactone (PCL)[22] and polystyrene,[23] natural polymers like collagen[24] or composite synthetic–natural polymers such as PCL–collagen.[22] In addition, naturally derived matrices including decellularized extracellular matrix,[25] small intestinal submucosa (SIS)[26] and human fibroblast produced matrix[27] have been studied as a matrix for skin regeneration. Fundamental considerations for biopolymer scaffolds for cell delivery include rate of degradation, immunogenicity/biocompatibility, mass transfer rates (fluid flux) and mechanical properties.

The surgical requirements of skin substitutes are well understood and include rapid adherence, decrease in fluid and electrolyte loss, control of pain, protection against microbial colonization and proliferation, promotion of healing, durability and flexibility, sterility, absence of toxicity, low immunogenicity, cost effectiveness and high availability.[28–30] Clinical complications with engineered skin result predominantly from anatomic and physiologic deficiencies that compromise responses to the wound healing process.

12.3 Design and composition of cultured skin substitutes

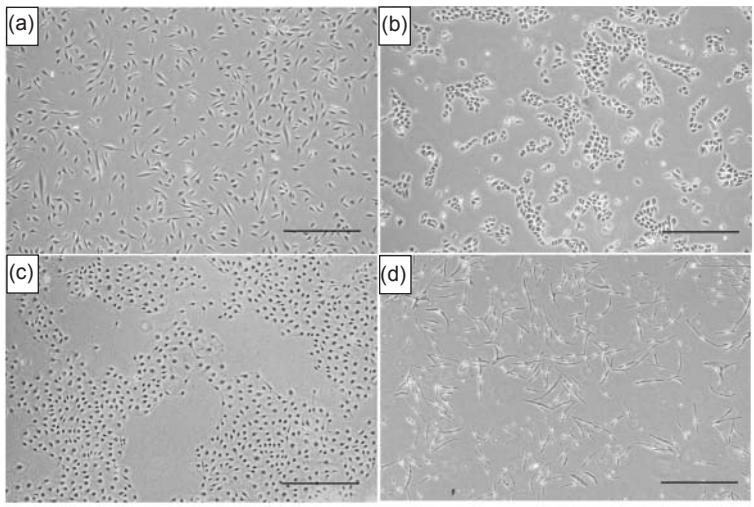
Basic design considerations for replacement skin include control of infection, fluid loss, contracture and scarring, but are only a part of a large set of requirements including rapid adherence and vascularization, mechanical stability and durability and cost-effectiveness. Restoration of skin anatomy includes not only the epidermis and dermis but also skin pigmentation, nerve, vascular plexus and adnexa (glands and follicles). Many skin analogues have been developed[23,31–35] yet none has duplicated *in vitro* all of the structures and functions of native human skin. Cultured skin substitutes are composed of collagen–glycosaminoglycan (GAG) substrates containing autologous fibroblasts and keratinocytes and provide permanent replacement of both dermal and epidermal layers in a single grafting procedure.[36–39] Figure 12.1 shows the general process for the deconstruction of a skin biopsy into its constituent cells and reconstruction of the cultured skin substitute from cells expanded from the initial biopsy and a biopolymer substrate.



12.1 Schematic of the general process for tissue engineering of skin. A small biopsy of split-thickness skin is separated using enzymes and/or mechanical disaggregation to isolate epidermal keratinocytes and dermal fibroblasts. Epidermal melanocytes and dermal microvascular endothelial cells may also be isolated and cultured. Skin cells are grown into large populations and inoculated onto collagen–GAG substrates. Incubation of the skin substitutes at the air–liquid interface promotes epidermal differentiation and morphogenesis of the cells into an analog of human skin that may be transplanted as a graft to excised, full-thickness wounds.

12.3.1 Collection, isolation and expansion of cells from a patient

To fabricate autologous engineered skin substitutes, a split-thickness skin biopsy (~250–300 μm, 0.010–0.012 in thick) from a patient is collected. From this initial biopsy, keratinocytes, fibroblasts, melanocytes and microvascular endothelial cells can be isolated by serial disaggregation using enzymatic and mechanical techniques.[40] Each cell type is placed into culture in a selective growth medium (Fig. 12.2). For current chemical use of CSS, primary cultures of keratinocytes and fibroblasts are grown to sub-confluence to generate stocks of cells that are cryopreserved by controlled rate freezing and subsequently stored in liquid or gas phase nitrogen at –196 ºC. Cells are recovered by rapid thawing and are then inoculated into the intermediate cultures in conventional flasks at a calculated

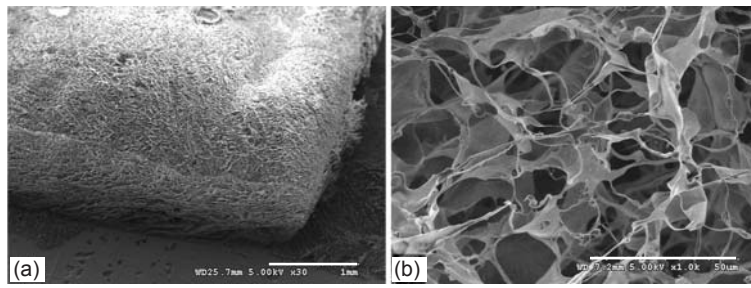


12.2 Photomicrographs of normal human skin cells in selective culture. (a) dermal fibroblasts, (b) epidermal keratinocytes, (c) dermal microvascular endothelial cells, and (d) epidermal melanocytes. Scale bar = 500 μm.

density and grown for 5–6 days. Once cells have reached approximately 80% confluence they are harvested and are then inoculated into the expansion culture in large-scale static culture vessels such as roller bottles or cell factories, or in automated bioreactors to reduce labor requirements,[41–43] to generate the cell populations needed for inoculation onto biopolymer sponges.

12.3.2 Fabrication of biopolymer substrates

Collagen–glycosaminoglycan (GAG) sponges are fabricated from bovine skin collagen and chondroitin-6-sulfate from shark cartilage to generate biopolymer substrates with a controlled thickness and pore diameter. Collagen is solubilized in acetic acid (0.60% wt/vol) and coprecipitated by the addition of chondroitin-6-sulfate at a controlled rate.[44] The mixture is homogenized, cast into a sheet with a given thickness and area and frozen by submersion in a bath of 95% ethanol (EtOH). The reticulated network of collagen is formed as the collagen precipitate is displaced by developing ice crystals.[45,46] Subsequent sublimation of the ice crystals generates a highly porous sponge. The pore size and structure depend on the nucleation and growth rate of ice crystals during the freezing process. Thus the desired pore size and porosity can be controlled by the freezing rate and the concentration of protein in the homogenized solution. The freeze drying process used for the cultured skin substitute model generates collagen sponges that are approximately 200 μm thick with an average pore diameter of 60 μm (Fig. 12.3). The lyophilized sponges are

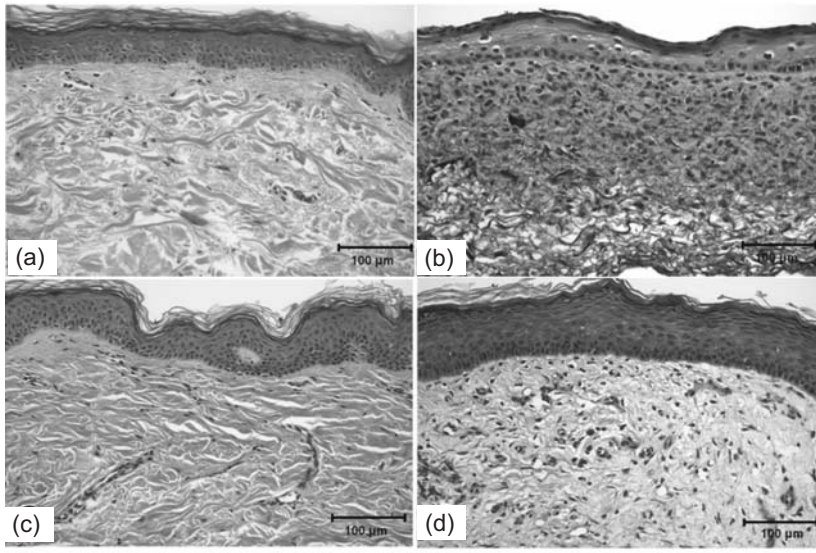


12.3 SEM images of collagen–GAG sponge. Scale bar = 1 mm (a) = 50 μm (b).

dehydrothermally crosslinked at 140 ºC for 24 hours,[47–49] packaged and sterilized by gamma irradiation and stored for later use.

12.3.3 Fabrication of cell–polymer constructs from multiple cell types

As the primary goal of a skin substitute is wound closure, keratinocytes are the essential cellular component of the skin substitute. The importance of a dermal component in skin substitutes, not only for ease of transplantation but also for *in vitro* epidermal development is now well recognized. The role played by fibroblasts has been supported by several investigators who showed that diffusible factors released by fibroblasts regulate epidermal morphogenesis.[50] It has also been widely recognized that for optimal functional and aesthetic outcomes both keratinocytes and fibroblasts are needed.[51,52] Cultured skin substitutes (CSS) consist of a freeze-dried, lyophilized collagen–glycosaminoglycan (GAG) sponge populated with autologous keratinocytes and fibroblasts. Fibroblasts and keratinocytes are serially inoculated into a collagen-based scaffold at high densities[48,53,54] to promote organization of the dermal and epidermal layers. Keratinocytes and fibroblasts have been shown to self-organize when exposed to an air–liquid interface, however the self-organization process increases the time from graft fabrication to patient application.[23] CSS are cultured at the air–liquid interface for 9 to 14 days to provide a liquid to gas transition with nutrient medium contacting the dermal substitute and air contacting the epidermal substitute, resulting in stratification and cornification of the keratinocyte layer.[55–58] In the dermal layer fibroblasts fill the collagen sponge, begin to degrade it and generate a new extracellular matrix. At the dermal–epidermal junction, evidence of basement membrane formation *in vitro* has also been demonstrated.[59] Thus, the blistering encountered with cultured epithelial autografts is not a clinical complication after grafting of CSS because the maturation of the dermal–epidermal junction is accomplished before grafting.



12.4 Histological comparison of native skin and cultured skin prepared for treatment of a pediatric burn patient. (a) Native human skin, (b) cultured skin substitute *in vitro* shown after 6 days incubation. The graft was transplanted to the patient after 10 days of *in vitro* incubation. (c) Healed autograft and (d) healed cultured skin substitute biopsied 3 weeks after grafting to excised full-thickness burn wounds. Scale bars = 100 µm.

12.3.4 Anatomy of cultured skin substitutes compared to split-thickness skin graft

In vitro, CSS resemble split-thickness autograft but exhibit differences in cell density and extracellular matrix structure (Fig. 12.4(a) and (b)). The dermal component of CSS is densely packed with fibroblasts in a porous matrix of thickly reticulated bovine collagen whereas the dermis is predominantly comprised of extracellular matrix with few fibroblasts in native human skin (Fig. 12.4(a) and (b)). After healing, the collagen sponge component of CSS is degraded and new extracellular matrix has been deposited by the fibroblasts yielding a structure that has become more similar in composition to split-thickness skin (Fig. 12.4(c) and (d)).

12.3.5 Cellular differentiation and gene expression in cultured skin substitutes

Two biologic changes result from formation of skin substitutes that contain very high cell densities. First, the proliferation rates of the cells decrease by approximately an order of magnitude from the maximum rate of log-phase, subconfluent

cells in a selective culture. Correspondingly, the nutritional requirements per cell decrease. However, because skin substitutes may contain 10–100 fold more cells per unit area than selective cultures the nutritional requirements of the entire population may increase. Second, the increase of cell density causes an increase in concentration of secreted factors by cells in the tissue substitute. Higher concentrations of secreted factors often confer independence from exogenous growth factors in the culture medium[60,61] and continued addition of mitogens under conditions of high cell densities may result in cytotoxicity. Fibroblasts and keratinocytes are known to secrete a wide variety of cytokines including inflammatory mediators, growth factors, matrix polymers and catabolic enzymes.[51]

The combination of epithelial and mesenchymal cells may allow paracrine mechanisms between cell types to begin to operate. An example is the synthesis of competence factors (e.g. platelet derived growth factor (PDGF), transforming growth factor alpha (TGF-alpha), basic fibroblast growth factor (bFGF)) by the keratinocytes and progression factors (e.g. insulin-like growth factors (IGFs)) by the fibroblasts to support cell proliferation.[62] These and other factors are believed to stimulate the mechanisms of action for healing of skin wounds.[63] The same mechanisms of action have been demonstrated by topical applications of pure growth factors[64] produced by recombinant technology.[65,66] However cellular synthesis and delivery of factors by engineered skin provides a continuous supply and may regulate delivery of factors according to mechanisms endogenous to the wound.

In addition to changes in proliferation rate and nutritional requirements, the formation of skin substitutes from fibroblasts and keratinocytes also results in morphogenesis of the constituent cells into a tissue substitute. Exposure of the skin substitute, containing both fibroblasts and keratinocytes, to the air–liquid interface stimulates differentiation and stratification of the epithelium.[55–57] This culture condition provides a polarized environment with the nutrient medium contacting the dermal layer and air contacting the epidermal layer. Viable keratinocytes in the skin substitute respond to this gradient by orienting the proliferating cells towards the nutrient medium, generating a layer of basal keratinocytes which remain in a less differentiated state. Thus, exposure to the air–liquid interface re-establishes the morphology of a stratified, squamous epithelium.

Skin substitutes aim to recapitulate the properties of native skin, however as they lack many anatomical structures and physiologic functions, it is expected that gene expression in bioengineered skin will not be fully analogous to native human skin. Genes encoding several different extracellular matrix (ECM) proteins, such as fibromodulin, decorin and lumican, are highly expressed in both cultured skin substitutes *in vitro* (CSS) and native human skin (NHS) but at relatively higher levels in CSS.[67] The messages of several matrix metalloproteinases (MMPs), (1, 3, 11, 14, 19) are elevated between 2 and 200 fold over normal tissue, and the tissue inhibitor of metalloproteinase-3 (TIMP3) is decreased three-fold.[67] The expression profile of CSS showed overexpression, relative to normal human skin, of clusters of genes that are known to be overexpressed in hyperproliferative skin disorders

Table 12.1 Clinical limitations and considerations for use of cultured skin substitutes (CSS)

Limitation	Consideration
Mechanical fragility	Special dressings and nursing care
Susceptibility to microbial contamination	Non-cytotoxic topical agents
Decreased rates of engraftment	Increased regrafting
Increased time to heal	Delay of recovery
Very high cost	Resource allocation

and during wound healing. These genes include cytokines, growth factors and receptors, antimicrobial proteins and differentiation markers. The analysis suggests that the keratinocytes in CSS are present in an activated state, similar to wounded human skin. Combined with the expression patterns of MMPs observed in CSS, these findings are consistent with remodeling of the dermal matrix and differentiations of the stratified epidermal layer, processes that are observed during wound healing.

12.4 Clinical considerations

Multiple factors of clinical care can be decisive in whether or not skin repair results from the treatment of wounds by engineered skin substitutes. Anatomic and physiologic deficiencies in the epidermal barrier and vascular plexus within skin substitutes confer practical limitations (Table 12.1) that must be managed to accomplish efficacy of wound closure. Currently available skin substitutes are avascular, slower to heal than skin autograft and may be mechanically fragile. Among the factors that have an impact on the outcome with engineered skin are wound bed preparation, control of microbial contamination, dressings and nursing care and survival of transplanted cells during vascularization of grafts. Therefore multiple factors must be considered prior to using CSS for skin repair.

First, the wound bed must be prepared specifically for CSS. For example, split-thickness autograft can be grafted onto subcutaneous fat with very high efficacy (i.e. 80–90%), but cultured epithelial autografts most frequently fail if grafted over fat. Thus modifications to the wound bed preparation protocol have been made. To stimulate a vascularized wound bed, fresh cadaver allograft may be applied for 7–10 days prior to grafting of CSS.[68] Pre-treatment of excised wounds with Integra® also stimulates a well-vascularized base to which CSS engraft readily.[38] To ensure the highest probability of engraftment and healing, each CSS must pass quality assurance tests and sterility tests prior to grafting. Grafted CSS must then be protected from microbial infection and mechanical shear until the wound is fully healed, which requires specialized dressings and nursing care. Attention to these factors provides an environment which assures the high efficacy of the CSS as a therapy for wound closure.

12.4.1 Release criteria for use of cultured skin substitutes

A definitive requirement for the closure and healing of full-thickness skin wounds is the restoration of the epidermal barrier which protects the body from microbial infection and loss of endogenous fluids. The barrier properties of human skin have been largely attributed to the presence of the stratum corneum (SC) in the upper epidermis.[69–72] In human skin, a water gradient exists across the SC in which hydration levels are lowest at the surface of the skin and highest within more distal layers. Studies have shown that surface electrical capacitance (SEC) can be used as a direct, convenient and inexpensive method of measuring skin surface hydration which is related to barrier function.[73,74]

Within CSS, cells organize by morphogenesis into a tissue analog with expression of tissue-specific phenotypes to partially restore the structure and function of skin. Measurement of SEC on CSS is one of two parameters currently being used for quality assurance in the clinical CSS. SEC measurements, collected over a 2–3 day interval are recorded at four points per graph and averaged. The SEC values of the CSS must decrease during the interval to be eligible for release for surgery, with most CSS approaching SEC values for normal human skin by the end of the interval. In addition, brightfield microscopy of standard histological sections is used as a qualitative assessment. Histologic evaluations consisted of examination of CSS prepared for each surgical procedure. CSS epithelia are scored as either excellent (well organized and stratified epithelium), good (organized and stratified epithelium), fair (multilayered, continuous epithelium) or poor (discontinuous, heterogeneous epithelium). CSS with a histological score between excellent and fair along with SEC measurements which decrease from day 6 to 9 are considered acceptable for transplantation. Poor (non-nucleated epithelium) CSS are not released for transplantation. These analyses are utilized to ensure that only optimal quality CSS are applied to the patient.

12.4.2 Surgical considerations

Clinical complications with engineered skin often result from anatomic and physiologic deficiencies that compromise responses to the wound healing process. Split-thickness autograft contains a vascular plexus and adheres to a debrided wound by coagulum, followed by inosculation of vessels within 2–5 days. In comparison, CSS are avascular and reperfusion results from *de novo* angiogenesis. The additional time required for vascularization may result in cell loss from microbial infection and/or nutrient deprivation. Therefore, nutrients and antimicrobials must be supplied to the CSS while vascularization is taking place.

CSS are delivered to the operating room with a covering of N-Terface, a non-adherent dressing that is stapled to the wound together with the CSS. CSS are placed onto the patient in a patchwork fashion by approximating each CSS on the wound bed until the entire excised wound is covered. For comparison, split-

thickness autograft (AG) is grafted in the same procedure at an adjacent site. To prevent microbial contamination and provide nutrients to the grafted CSS, the CSS and AG comparative site are kept in a moist dressing for 5 days post surgery. Topical antimicrobials in the irrigation solution are used in conjunction with the dressings and must provide effective coverage of a broad spectrum of gram-negative and gram-positive bacteria as well as common fungal contaminants. For use with CSS, topical antimicrobials must also have low cytotoxicity to allow healing to proceed. Previous studies from this laboratory have determined an effective and non-cytotoxic formulation consisting of polymyxin B, neomycin, mupirocin, ciprofloxacin and amphoteracin B.[75] In addition, caution is exercised in order to avoid overlap of topical and parenteral drugs that could possibility lead to resistance to the topical agents and subsequent sepsis.

12.4.3 Nursing considerations

The mechanical fragility of cultured skin substitute is another important source of failure from shear or maceration. These losses may be minimized by development of appropriate handling, securing and dressing of the cultured grafts. For friable grafts such as CSS, the porous, non-adherent N-terface backing material allows surgical application with minimal damage to the graft caused by handling and stapling. This porous dressing allows both the delivery of topical solutions and drainage of wound exudates from grafts during the period of engraftment. To avoid mechanical disturbance, the frequency of dressing changes is low (two to four changes per week) during the first week, and increases to twice daily after a fibro-vascular issue and epidermal barrier develop. With attention to these surgical and nursing factors, closure of excised, full-thickness wounds can be accomplished with reduction of requirements for donor skin autograft.[76]

12.5 Clinical assessment

After treatment of wounds with CSS, the outcome must be measured to determine whether the benefits of a prospective therapy justify any risks associated with the therapy and if risks associated are reduced for the disease being treated. Assessment may range from the level of the individual (e.g. survival),[77] to function (e.g. range of motion, return to work),[78] to tissue integrity (epithelial closure, scar formation),[79–80] to cellular markers (e.g. cell phenotype, synthesis of proteins and nucleic acids).[81]

12.5.1 Quantitative assessment

To evaluate the efficacy of CSS, quantitative measurements of engraftment, closed area to donor area and percent increase in CSS area are collected. A major advantage of CSS compared to that of split-thickness AG is its ability to close a

large area with little donor skin. Using computerized planimetry, measurements of donor biopsy size and area treated with CSS are made and indicate wounds closed with CSS cover on average 61.5 ± 8.4 times the area of the donor biopsy.[16] These values demonstrate the reduction of donor skin harvesting by grafting of CSS in place of AG. In order for CSS to be effective, the grafts must have a high engraftment rate. Percent engraftment at post operative day 14 was 79.5 ± 2.1% for CSS and 97.5 ± 2.0% for AG. These data demonstrate comparable rates of engraftment for CSS and AG and that 1% total body surface area (TBSA) of donor skin can close ~60% TBSA of excised burn. This result defines a new medical benefit for burn patients by autologous CSS with reduction of donor skin harvesting to complete wound closure.

12.5.2 Qualitative assessment

Qualitative outcomes, which rely heavily on the trained eye of the clinician, can be assessed through clinical evaluation integrating multiple properties of the wound. For example, the Vancouver Scale is used to assess burn scar and provides an ordinal score for properties of the skin such as pigmentation, vascularity, pliability and scar height.[82] Such scales assign quantitative values to qualitative measurements and can provide a relative comparison for evaluation. Vancouver scores for CSS have been shown to be statistically lower than AG during the first six months after grafting and not different at one year or after.[16]

12.6 Regulatory issues

Within the United States, the Food and Drug Administration (FDA) regulates the protection of the public from health risks associated with new medical therapies. FDA approval requires that new therapies be safe and effective and that the probable benefits to health outweigh the probable risks of the therapy or of the untreated disease condition.[83] Safety considerations for cultured skin substitutes must take several factors into account including media composition, tissue acquisition, graft fabrication and storage, and sterility testing of the final product.[83] For example, in an effort to reduce the potential risk of exposure to and transmission of bovine spongiform encephalopathy (BSE), the FDA recently proposed a regulation that would prohibit the use of certain cattle materials, including the brain, skull, eyes and spinal cord, in the manufacture of medical devices intended for use in humans.[84] Thus, efforts have been made to develop a defined animal-product free medium for use with cell culture to avoid potential prion infections associated with growth factor extracts such as bovine pituitary extract, a common component of keratinocyte growth medium.[85] In addition, the methods used to propagate primary isolations of keratinocytes for cultured skin substitutes have changed in response to changes in FDA guidelines for medical devices. In 2003, the Department of Health and Human Services defined xenotransplantation as

'any procedure that involves the transplantation, implantation or infusion into a human recipient of either (a) live cells, tissues or organs from a non-human animal source, or (b) human body fluids, cells, tissues or organs that have had *ex vivo* contact with live non-human animal cells, tissues or organs'.[86] Feeder layer cells[15] irraditated to render them non-proliferative for use in co-cultures are considered to be living and thus, within the definition of xenotransplantation. To avoid the potential risks associated with xenotransplantation, alternative methods of improving attachment and proliferation of freshly isolated cells have been used, including using culture vessels coated with extracellular matrix proteins.[85,87] CSS consist of cells attached to a biodegradable polymer and are currently regulated in the United States as class III (significant risk) medical devices, with rigorous requirements for demonstration of safety and efficacy and compliance with good manufacturing practices (GMPs). Thus efforts are made continually to improve laboratory practices to comply with prevailing FDA guidelines.

12.7 Future trends

Much progress has been made by the combined efforts of biologists, chemists, geneticists, bioengineers and materials engineers to develop a permanent substitute for human skin. However, current models of skin fail to replicate all of the structures and functions of normal human skin. Improvements to the design of future models of skin substitutes are likely to include alternative scaffolding materials, addition of cellular components (e.g. melanocytes for pigmentation, endothelial cells for vascularization), biologic regulation of wound healing and genetic modification of transplanted cells.

12.7.1 Improving mechanical strength of cultured skin substitutes

Shear and maceration of cultured skin grafts is an important source of CSS failure. Thus improving the mechanical strength of the CSS prior to grafting may result in increased rates of engraftment. Collagen-GAG scaffolds have been tested for maximum load at failure and have <1% failure loads compared to split-thickness autografts.[88] Improving the mechanical stability and strength of the acellular collagen scaffold is one strategy for increasing the strength and stability of the CSS. Cross-linking collagen scaffolds via chemical methods has been widely utilized to slow degradation rates and optimize mechanical properties. Historically, glutaraldehyde (GA) has been the most widely utilized chemical cross-linking reagent.[89] However, GA cross-linked biomaterials have been reported to exhibit reduced cellular ingrowth *in vitro* and *in vivo*,[90,91] thus alternative reagents have been employed. To overcome reagent toxicity, carbodiimides have been used to cross-link collagen because they are members of the zero-length class of cross-linkers.

Cross-linking collagen-GAG sponges used to fabricate CSS has been shown

greatly to impede scaffold degradation by collagenase and improve ultimate tensile strength (UTS) of acellular collagen sponges.[54] Co-culture of human fibroblasts and keratinocytes on these substrates reveals an apparent cytotoxicty of the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) at high concentrations (≥10 mM EDC) with reduced cell viability and poor cellular organization in CSS fabricated with cross-linked scaffolds. However, intermediate concentrations of EDC can increase collagen sponge stability and strength while providing an environment in which fibroblasts and keratinocytes can attach, proliferate and organize in a manner conducive to dermal and epidermal regeneration. In addition, EDC cross-linked collagen-GAG sponges are cleared from a full-thickness wound in an athymic mouse within 12 weeks, leading to optimal engraftment and stable wound closure.[92] These strategies can be used to produce skin substitutes with increased and more predictable mechanical properties compared to non-modified scaffolds.

12.7.2 Alternative biopolymer scaffolds

Scaffolds for tissue engineering play a critical role in regenerating functional tissues and organs. The current model of skin substitutes utilizes a freeze-dried collagen-GAG sponge populated with dermal fibroblasts and epidermal keratinocytes. Freeze-drying is a labor intensive, costly process that can produce sponges with significant structural heterogeneity [45,93] and is not easily scaled to manufacturing levels. In addition, the collagen sponge is structurally different from the natural extracellular matrix (ECM). Native ECM is fibrillar in structure with micrometer to submicrometer sized fibers, while collagen sponges comprise a reticulated network of collagen with pore walls that are several micrometers thick. To overcome these limitations, alternative scaffold sources and fabrication methods have been investigated.

Naturally derived matrices such as small intestine submucosa[26] and acellular dermal matrices[25] have been utilized as scaffolds for skin tissue engineering. Unfortunately, the inherent variation in natural materials coupled with the inability to alter the structure of the material significantly detracts from their widespread usage. Biopolymer gels have been successfully used both in a laboratory setting[94] and commercially.[21] While these gels have many advantageous properties, such as biocompatibility, direct cellularity and being conducive to ECM deposition, they are weak compared to the native tissue.[27] Electrospun matrices have also been investigated as scaffolds for skin substitutes owing to their ease of manufacture and the ability to select their physical, chemical and mechanical properties.

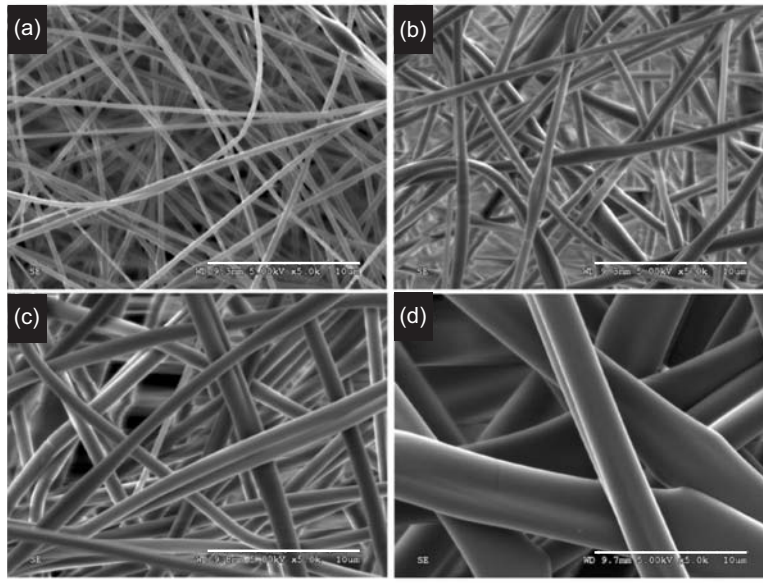
Electrospinning is an inexpensive, scalable process that has been used to fabricate nonwoven fibrous scaffolds from a wide array of materials including collagen.[95–100] In electrospinning, polymer solution is pumped through an aperture (i.e. syringe needle) that is electrically charged. A charge is induced on the liquid droplet at the tip of the needle by the electric potential between the needle and a

grounded collection plate. When the electric field reaches a threshold, the repulsive electric force within the liquid overcomes the surface tension of the solution causing a charged jet of solution to be ejected from the droplet of polymer solution.[96] The ejected polymer jet is accelerated towards the target, which is oppositely charged or grounded. This process generates nonwoven meshes composed of micrometer to nanometric sized fibers. The fiber diameter and morphology of the electrospun scaffold are largely controlled by the concentration and molecular weight of the polymer.[100–103] Many other factors including flow rate, quality of the solvent and surface tension result in variation in fiber diameter and morphology.[96] By altering these factors, a nonwoven mesh with a fibrous structure analogous to native ECM can be generated with narrow tolerances.

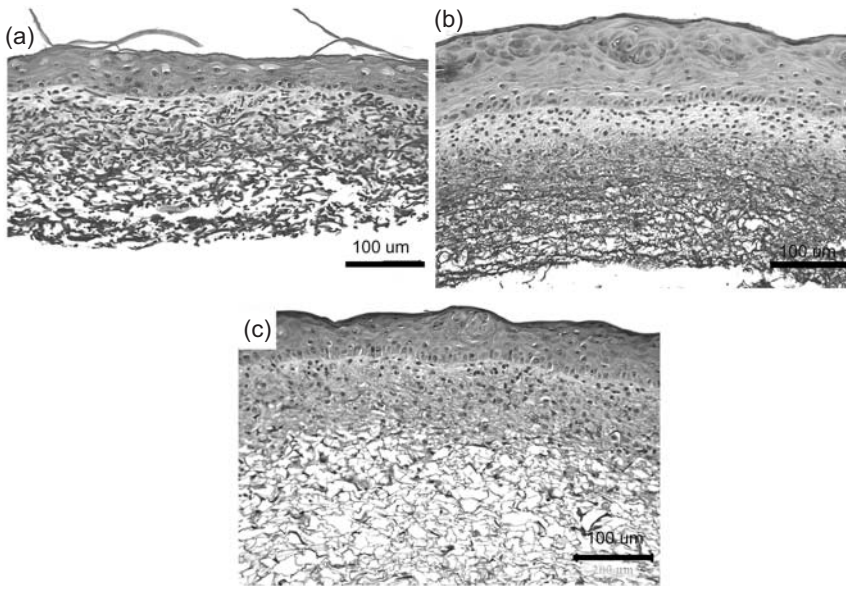
Electrospun polymers, such as polystyrene, have been used to generate skin substitutes *in vitro*.[23] However, the scaffold is not degradable and will remain within the wound. Thus electrospinning of degradable biopolymers such as gelatin or collagen is preferred. Electrospun gelatin can be easily spun into a wide range of fiber diameters (Fig. 12.5) and has been evaluated for its utility in dermal and epidermal tissue regeneration. Electrospun gelatin scaffolds with interfiber distances between 5 and 10 μm appear to yield the most favorable skin substitute *in vitro*, demonstrating high cell viability and optimal cell organization (Fig. 12.6A).[100] Electrospun collagen has also been evaluated as a scaffolding material for skin substitutes in comparison with the standard cultured skin substitute model which utilizes freeze-dried collagen. CSS made with electrospun collagen exhibit the same excellent stratification and a continuous layer of basal keratinocytes seen in the freeze-dried sponge model (Fig. 12.6B and C).[104] When grafted to full thickness wounds in athymic mice, electrospun collagen skin substitutes had high rates of engraftment and appeared to inhibit wound contraction compared to that of wounds grafted with skin substitutes made with freeze-dried collagen sponges.[104] Electrospun biopolymers and polymer blends show great promise for future models of skin substitutes which hope to reconstruct the structure of native human skin more faithfully.

12.7.3 Pigmentation by addition of melanocytes

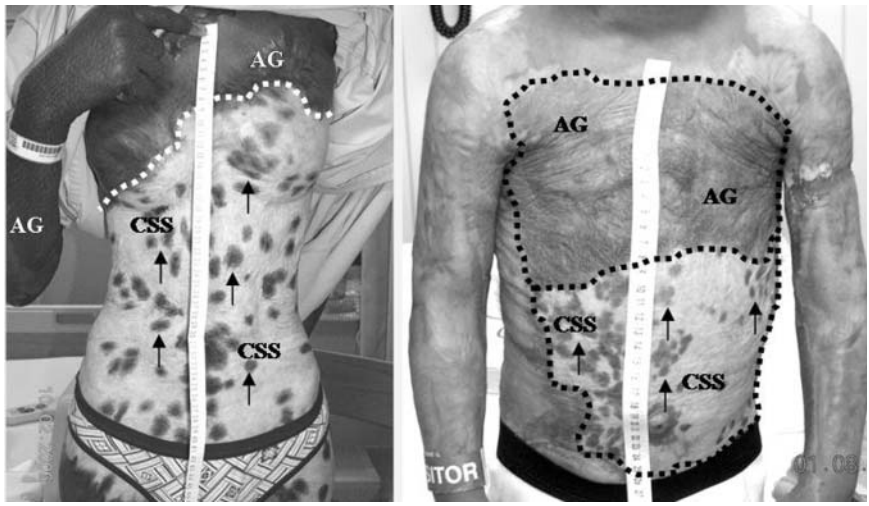
In the current cultured skin substitute model, pigmentation is irregular or absent (Fig. 12.7). In intact skin, pigmentation results from the proper distribution and function of epidermal melanocytes. Pigmentation protects skin from ultraviolet irradiation[105,106] but also shapes a patients body image and personal identity. Melanocytes can sometimes unintentionally exist in cultures of epidermal keratinocytes; referred to as passenger melanocytes, these can result in foci of pigmentation after grafting (Fig. 12.7).[39,58,107,108] In preclinical studies, selective culture of human melanocytes and deliberate addition to CSS showed that uniform pigmentation can be achieved[24] although the intensity of pigment was not regulated. Future models of pigmented skin will benefit from a more thorough understanding of melanocyte function and factors that regulate skin pigmentation.[109,110]



12.5 SEM images of electrospun scaffolds fabricated using (a) 10, (b) 12, (c) 14 and (d) 16 wt./vol.% gelatin in trifluoroethanol. Scale bar = 10 μm.



12.6 Histological comparison of skin substitutes prepared using (a) electrospun gelatin, (b) electrospun collagen and (c) freeze-dried collagen scaffolds, shown after 7 days of *in vitro* incubation. Scale bar = 100 μm.



12.7 Photographs of patients who received cultured skin substitutes (CSS) and autograft (AG). Left: After 7 years in an African-American, healed skin from CSS remains pliable, and low in scar formation, except for obvious hypopigmentation with foci of pigment (arrows). Right: At 2 years in this Hispanic patient, CSS also remains hypopigmented. Individual foci of pigment are interpreted as melanocyte populations arising from single melanocytes in CSS. Scales in cm.

12.7.4 Stem cell and gene therapy

Using the tools of molecular biology, genetic modification of cells within skin substitutes can hypothetically be used to overcome limitations in anatomy and physiology resulting in skin substitutes with greater homology to native human skin and improved performance. The gene expression profile of keratinocytes can be altered by the transfer of recombinant genes[111] and these genetically modified cells have been shown to retain their ability to differentiate into a stratified epidermis.[111] Genetic modification can be used ectopically to express cytokines not normally expressed in a particular cell type and to compensate for deficiencies of engineered skin. Alternatively, skin substitutes can be genetically engineered to overexpress growth factors that aid in wound healing to enhance their therapeutic value for wound repair. For example, cultured skin substitutes comprised of keratinocytes genetically modified to overexpress vascular endothelial growth factor, a mitogen for endothelial cells, show enhanced vascularization and improved healing on mice compared to non-modified CSS.[49,112] Genetically modified CSS can also serve as a vehicle for cutaneous gene therapy which could possibly correct genetic diseases such as epidermolysis bullosa (EB). Junctional epidermolysis bullosa (JEB), a severe form of the disease, can result from mutation of genes encoding subunits of laminin-5, a component of anchoring filaments in the

basement membrane zone of skin.[113] Correction of the JEB phenotype can be achieved by gene transfer of LAMB3, which encodes the beta 3 subunit of laminin-5.[114] Organotypic cultures prepared with LAMB3-transduced cells show normal assembly of dermal–epidermal attachment structures indicating a correction of the mutant phenotype.[115] These studies show the feasibility of combining tissue engineering with gene therapy to treat cutaneous diseases.

12.8 Conclusions

Technological advances in the culture of skin cells have permitted the fabrication and testing of engineered skin substitutes. Continued research will be needed to identify more efficient methods of utilizing precious autologous tissue, provide greater amounts of skin substitutes for grafting, and shorten the time required for their preparation. Additional research is aimed at improving the anatomy and physiology of skin substitutes, working toward better homology with native skin autograft. These efforts will lead to enhanced performance of engineered skin graft, greater clinical efficacy and a reduction of morbidity and mortality for patients with burn injuries and other skin wounds.

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13

Use of keratinocytes in combination with a dermal replacement to treat skin loss

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Abstract: The process of induced regeneration of the adult skin is traditionally based on a sequential two-step technique in which an acellular dermal scaffold and a thin skin graft are grafted in the wound bed in two sequential surgical procedures. More recently, it has been shown that autologous keratinocytes can be seeded directly into the dermal scaffolds resulting in contemporary dermal and epidermal regeneration upon grafting. This one-step procedure reduces the morbidity of the patients and the hospital costs, while allowing for improved skin regeneration, compared to the traditional two steps.

Key words: autologous keratinocytes, one-step skin regeneration, cell seeding, neovascularization.

13.1 Simultaneous substitution of dermis and epidermis: from two-step to one-step skin replacement

Skin is an organ in adults that has extensive evidence of induced regeneration (Yannas, 2005a). The process of induced regeneration in adult skin is based on a sequential two-step technique (Murphy *et al*., 1990; Orgill *et al*., 1999). In the first step, after initial excision, full-thickness wounds are grafted with acellular dermal substitutes covered by a silicone sheet which functions as a temporary pseudo-epidermis. During the second step, generally 2–3 weeks later, the silicone layer is replaced by a thin skin graft, which stably closes the wound (Murphy *et al*., 1990; Orgill *et al*., 1999). Despite excellent results in a wide range of patient populations using the two-stage operation, during the waiting period scaffolds are highly susceptible to infection mainly owing to slow neovascularization (Butler *et al*., 1999). In addition, the second step adds morbidity, requiring a significant donor site in many patients who have limited uninjured areas to choose from (Butler *et al*., 1998) and often inducing hypertrophic scarring in children (Compton *et al*., 1998).

Recent evidence has demonstrated that the addition of keratinocytes to the construct significantly improves the performance of acellular scaffolds, allowing

stable wound closure in one surgical step (Orgill, 1983; Orgill *et al.*, 1998; Compton *et al.*, 1998).

13.2 Cell seeding

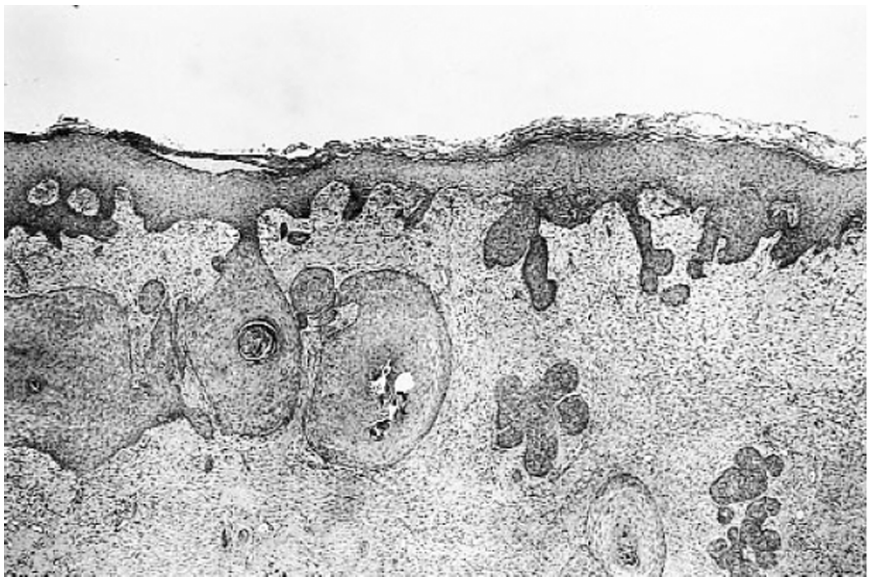
Seeding dermal scaffolds with keratinocytes (Fig. 13.1) leads to simultaneous regeneration of a dermis and an epidermis (Yannas, 2005a). When collagen–GAG scaffolds are impregnated with 50 000 keratinocytes per cm^2, it is possible to achieve epidermal confluence within 19 days from grafting of the cellularized scaffold, with up to a 60-fold surface expansion of the donor epidermis (Butler *et al*., 1998; Orgill *et al*., 1998). Increasing the cell density to 100 000 cells per cm^2 further reduces the time to epithelial confluence to 2 weeks (Butler *et al*., 1998; Orgill *et al*., 1998). Further increases in cell density do not affect the speed of wound closure (Butler *et al*., 1998). Cell suspensions seeded within the scaffold appear also to increase the rate of vascularization (Orgill *et al*., 1998), which reduces the infection risk, probably through the elaboration of growth factors from seeded cells.

Autologous keratinocytes can be seeded by means of centrifugation, a technique described by Butler *et al.* (1998) which induces the formation of thick epithelial cords and islands in the neodermis (Butler *et al*., 1999; Compton *et al*., 1998). Dermal keratinocytes progressively enlarge, coalesce and differentiate to form large horn cysts, and finally reorganize at the graft surface to form a fully differentiated, normally oriented epidermis with rete ridges (Butler *et al*., 1999; Compton *et al*., 1998). Simultaneously, the neodermis is repopulated by endothelial cells, fibroblasts and macrophages, resulting in the formation of blood vessels, production of extracellular matrix and the progressive degradation of the scaffold (Butler *et al*., 1999; Compton *et al*., 1998).

Over time, the stromal cellularity in the neodermis decreases and collagen deposition and remodeling increase to form a neodermal connective tissue matrix beneath the newly formed epidermis (Butler *et al*., 1999; Compton *et al*., 1998; Yannas, 2005a). Complete dissolution of the of the dermal substitute occurs, partly as a result of degradation by an ongoing foreign body giant cell reaction that peaks at 8–12 days post grafting (Compton *et al*., 1998; Yannas, 2005a). Within one month many structural components of normal skin are reconstituted (Butler *et al*., 1999; Compton *et al*., 1998; Orgill *et al*., 1998; Yannas, 2005a).

13.3 Methods of delivery of keratinocytes

The delivery of keratinocytes can be achieved by several different methods. The first methodology described completely is centrifugation of tripsinized epidermis (Orgill *et al*., 1998). Controlled trypsinization splits the dermis from the epidermis to facilitate harvesting of basal keratinocytes as well as fibroblasts and melanocytes and can be completed within 20 minutes, thus making cells available for direct



13.1 Long-term studies in guinea pigs (1 year) show that tissue reorganizes and differs from scar (Butler *et al.,* 1999).

seeding or expansion in culture (Butler *et al*., 1999). While uncultured keratinocytes can be harvested, disaggregated, seeded and grafted within 4 hours (Butler *et al*., 1999), cultured autologous keartinocytes cannot be made available at short notice (Butler *et al*., 1999).

In elective cases, autologous keratinocytes can be expanded in culture to have a more extensive population on the day of grafting. Shorter culture time periods can be used to allow earlier seeded graft production; however, this trade-off significantly reduces the cell expansion capabilities (Butler *et al*., 1999). Although cultured keratinocytes require more time than uncultured ones, they allow selection of proliferating cells and provide the potential to restore bylayer tissues with smaller donor sites (Butler *et al*., 1999). In addition, cultured cell-seeded matrices induced faster (96% on day 14, versus 50% achieved by uncultured matrices) and thicker re-epithelialization when compared to uncultured cell-seeded matrices (Butler *et al*., 1999).

To simplify the surgical manipulation, sheets of cultured, autologous keratinocytes can be also grafted to the undersite of the dermal matrix (Jones *et al*., 2003), showing the natural tendency to migrate upwards and achieve epidermal confluence, but less efficiently than when centrifuged directly into the scaffolds (Jones *et al*., 2003; Orgill *et al*., 1998; Orgill *et al*., 1999).

More recently new methodologies have been proposed to deliver keratinocytes in combination with dermal scaffolds. The introduction of an autologous cell suspension (cell spray) enables the keratinocytes to be delivered to the wound via

an aerosol (Wood *et al*., 2006). This non-cultured cell suspension can be generated using a ReCell autologous cell harvesting device with early evidence of efficacy (Wood *et al*., 2007). Soaking Integra in a cell suspension harvested using the ReCell system appears to have the same self-organizing effect (Wood *et al*., 2007) as cell suspensions centrifuged within a seeded scaffold (Orgill *et al*., 1998).

Seeding dermal scaffolds with autologous keratinocytes is required to accelerate the kinetics of organ regeneration, but is not required to affect the outcome itself (regeneration versus repair) (Yannas, 2005a). In spite of allowing a one-step technique, scaffolds seeded with keratinocytes still induce only partial skin regeneration, mainly owing to the fact that the regenerated tissue still lacks appendages (Yannas, 2005a, b). Hair follicles can be implanted into Integra by micrografting techniques to re-epithelialize the area and allow hair growth (Navsaria *et al*., 2004), but add morbidity and do not guarantee an adequate cosmetic result.

Recently it has been shown that bulge cells, from hair follicles, not only contribute to the epidermis (up to 25% of the cells) during wound healing (Ito *et al*., 2005; Lavker and Sun, 2003), but after isolation, when combined with neonatal dermal cells, regenerate new hair follicles, epidermis and sebaceous glands (Blanpain *et al*., 2004; Morris *et al*., 2004). Since many surface markers of bulge cells have been recently isolated (Mitsiadis *et al*., 2007; Ohyama *et al*., 2006; Rochat *et al*., 2007), including CD200 and FRIZZLED receptor on human bulge cells by laser capture microdissection and microarray analysis for gene expression, the stage is set to isolate bulge cells to seed collagen matrices and regenerate appendages in one step with dermis and epidermis (Cotsarelis, 2006a, b).

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14

Principles of skin regeneration

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Abstract: Partial regeneration of adult organs has been accomplished by induction. This is increasingly an alternative to organ transplantation. Skin was the first adult organ to be induced to regenerate partly in animals and in humans. Skin regeneration was induced following grafting of a highly porous scaffold based on a graft copolymer of type I collagen and chondroitin 6-sulfate (CG scaffold). Peripheral nerves and the conjunctiva have been similarly induced to regenerate. The structural determinants of active CG scaffolds have been determined. The data support a theory of induced regeneration in which contraction of a severe wound in an organ is blocked by the CG scaffold. Contraction blocking appears to require reduction of MFB (myofibroblasts) density in the wound as well as integrin-specific binding of contractile fibroblasts (MFB) on the extensive surface of the CG scaffold. When present in such reduced numbers and oriented quasi-randomly in the plane of the wound, MFB appear to lose their ability to contract wounds. Active CG scaffolds are powerful insoluble regulators of cell function. They appear to modify the adult wound healing to a process that resembles early fetal healing.

Key words: induced organ regeneration, skin regeneration, peripheral nerve regeneration, conjunctiva regeneration, collagen–GAG scaffolds, contraction blocking, fetal healing.

14.1 Introduction

Regeneration is distinct from repair. Although both processes start with an open wound and end with a closed wound, they differ sharply in quality of outcome. Organ regeneration closes a wound by restoring the native structure and function of a failed organ. Repair does not restore the original organ; instead, it simply closes the wound by wound contraction and scar formation. The early mammalian fetus heals injury spontaneously by regeneration while the adult mammal heals injury by repair, a spontaneous repair process, consisting of contraction and scar formation. Spontaneous regeneration occurs without aid from an external agent while induced regeneration requires such aid. This article is focused on induced organ regeneration in adults. Certain highly porous scaffolds, based on analogues of the extracellular matrix, can block wound contraction and induce partial regeneration in skin, peripheral nerves and the conjunctiva. The mechanism of contraction blocking has been largely elucidated. Clinical uses of these scaffolds

in cases of skin loss or peripheral nerve loss are currently being pursued. This chapter outlines the basic principles that are currently known to govern processes of induced organ regeneration, especially skin, in the adult mammal.

14.2 The central problem of skin regeneration

The topic of organ regeneration is best introduced by distinguishing the tissue types in a given organ that regenerate spontaneously from those that do not. This distinction is essential: tissues, rather than the entire organ, which does not regenerate spontaneously, become the focus of the discussion, leading to an enormous simplification of the problem of induced organ regeneration.

Although this chapter focuses on skin regeneration, frequent references will be made to peripheral nerve regeneration. The regenerative behavior of these two organs, skin and peripheral nerves, has been studied much more extensively than that of other organs (with the exception of studies of the liver). Data from these two organ sources are abundantly available; such data are largely missing for other organs. In addition, striking similarities have been identified between the mechanism by which regeneration is induced in skin and nerves. This identification has solidified the conceptual basis of induced regeneration in adults and has suggested methodology for extending the regenerative treatment to other organs as well.

The spontaneous response of organs to injury can be interpreted by referring to the healing response of the 'tissue triad'. Three tissue types, epithelia, basement membrane and stroma, are grouped together in anatomical proximity in most organs. The response of different organs to injury can be readily analyzed, and eventually generalized, by reference to the response of these anatomical structures. The epithelia (epidermis in skin, myelin sheath in nerves) regenerates spontaneously provided the stroma is intact and, while doing so, induce regeneration of the attached basement membrane. The stroma (dermis in skin, endoneurium in nerves) is non-regenerative, that is, it does not regenerate spontaneously in adults. Once the dermis has been induced to regenerate, the epidermis and basement membrane regenerate spontaneously. It follows that regeneration of the stroma is the major problem in studies of induced regeneration of the skin.

14.3 Experimental variables in studies of skin regeneration

Since experimental studies of induced skin regeneration must necessarily focus on regeneration of the dermis following an injury, it follows that the experimental space for the study should be an injury (wound) in which the native dermis is clearly absent, for example removed by excision. Such a wound is the anatomically well-defined full-thickness skin wound, prepared by complete excision of

the dermis and attached epidermis and extending over an area that is sufficiently large to allow detection of the outcome, that is formation either of scar or of regenerated dermis. An incision injures a very small mass of tissue and offers a fleeting opportunity to distinguish between scar and a new dermis. A partial-thickness wound is filled with a layer of residual dermis, making it difficult, though not impossible, to distinguish newly synthesized dermis from the native tissue. In the discussion that follows, the data presented are based exclusively on full-thickness skin wounds, mostly in rodents but also in the swine and the human.

The outcome of an experimental study of induced regeneration can be described in terms of the type of tissue that is synthesized inside the wound. Three processes of wound closure have been recognized: contraction, scar formation and regeneration. Accordingly, the outcome of a wound healing process is simply cast in terms /of the fractional extent to which the original wound area, A_o, has been closed by contraction of skin edges, scar formation or regenerated tissue. Assigning a percentage to the area closed by contraction (C), scar formation (S) and regeneration (R), we arrive at the wound closure rule (Yannas, 2001):

$$C + S + R = 100 \tag{14.1}$$

The quantity C can be determined by monitoring the kinetics of change in the wound area by contraction, optionally using India ink or another label to mark the original wound boundary (identified as the boundary of the injured dermis) and identifying the asymptotic (time-independent) value of the area, A_∞, that remains after contraction of the dermal boundary has ceased. It follows that $A_o - A_\infty$ is the total wound area that has been closed by contraction. This quantity is used to calculate the percent of original area that has been closed by contraction, $C = [(A_o - A_\infty)/A_o] \times 100$.

In studies of repair, for example with full-thickness skin wounds in the adult mammal, where the only outcomes are contraction and scar formation, $R = 0$, and the above equation becomes $C + S = 100$. In such cases the quantity S is then calculated simply as the percent of original area that has closed by scar formation, $S = [A_\infty/A_o] \times 100$.

In cases where it is likely that regeneration has been induced, a distinction between S and R has been traditionally made by histological methods. Conventional histology can be supplemented and extended by quantitative study of collagen fiber orientation, which leads to a clear distinction between scar tissue (planar orientation of fiber axes) and dermis (quasi-random orientation) (Ferdman and Yannas, 1993).

The detailed methodology for measuring C, S and R, which is based on histological tissue sections, has been described elsewhere in detail (Yannas, 2001). There is currently no methodology for quantitative tissue analysis of the three tissue types on the surface of a tissue block that includes the entire wound. Nor is there methodology to study separately the kinetics of each process *in vivo*.

Table 14.1 Healing outcomes of full-thickness skin wounds. Data on configuration of the final state. *C* and *R* values were directly measured; *S* values are estimates[a] (additional data in Yannas, 2001).

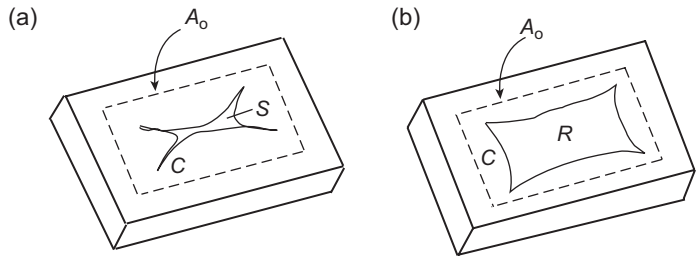
Full-thickness excision of dermis in all cases	Configuration of final state (*C*, % contraction; *S*, % scar; *R*, % regeneration)
General case of organ wound healing	[*C, S, R*]
Ideal early fetal healing of dermis-free wound in skin (perfect regeneration model)	[0, 0, 100]
Spontaneous healing of dermis-free skin wound in Wistar rat dorsum (Kennedy and Cliff, 1979)	[93, 7, 0]
Spontaneous healing of dermis-free skin wound in swine dorsum (Pitman-Moore minipig) (Rudolph, 1979)	[72, 28, 0]
Spontaneous healing of dermis-free skin wound in the adult human forearm (Ramirez *et al.*, 1969)	[37, 63, 0]
Spontaneous healing of dermis-free skin wound in Hartley guinea pig dorsum (Yannas *et al.*, 1989)	[92, 8, 0]
Induced skin regeneration in dermis-free skin wound in Hartley guinea pig dorsum following grafting with DRT[b] seeded with autologous keratinocytes (Yannas *et al.*, 1989; Compton *et al.*, 1998)	[28, 0, 72]

[a]In data from studies of spontaneous healing of full-thickness skin wounds (no experimental manipulation), *C* was determined directly from data on contraction kinetics. A very rough estimate of *S* was then obtained as $S = 100–C$ (note that the difference between two large numbers lacks precision). In some cases it was assumed that regeneration of stroma is not observed in spontaneous healing in the adult mammal ($R = 0$). In studies where regeneration was induced, values of *S* and *R* were estimated from histological data.
[b]Dermis regeneration template, a scaffold described in detail in Yannas *et al.*, 1989.

14.4 Applications of the wound closure rule

To date, only the extent of wound closure by contraction (*C*) has been measured directly in several investigations. Such data have been used to derive rough estimates of the other two quantities, *S* and *R* in a few cases (Yannas, 2001). Examples of such estimates for a few healing models in animals or humans appear in Table 14.1 where values of *C*, *S* and R for the full-thickness skin wound are compactly presented as [*C*, *S*, *R*]. For example, the result of spontaneous healing of a full-thickness skin wound in the dorsal region of the rabbit can be described in the final state by $C = 92 \pm 5\%$, $S = 8 \pm 5\%$ and $R = 0$ (estimated from data by Kennedy and Cliff, 1979). Omitting, for simplicity of presentation, the percentage symbol and error limits from these data yields [92, 8, 0] as the representation of the carefully measured data by Kennedy and Cliff.

A schematic of a rodent full-thickness skin wound that has closed primarily by



14.1 Schematic representation of two tissue blocks that have been excised following closure of a full-thickness skin wound in the guinea pig. (a) Tissue block following wound closure by spontaneous healing. A_0, initial wound area; *S*, fraction of A_0 which has closed by formation of scar tissue; *C*, fraction of A_0 that has closed by contraction. (b) Tissue block following closure by regeneration. *R*, fractional coverage of A_0 by regenerated skin; *C*, fractional coverage of A_0 by contraction.

contraction (and secondarily by scar formation) (Fig. 14.1a) and one that has closed primarily by induced regeneration (and secondarily by contraction) (Fig. 14.1b) illustrate the use of the wound closure rule and pose the methodological problem of separating tissue types from each other prior to determination of the area occupied by each.

Representative estimates of *C*, *S* and *R*, tabulated in Table 14.1, provide insights into the relative importance of these processes in wound closure. In rodents, which have a mobile integument, contraction accounts for almost all of wound closure. In the human, where the skin is tethered to subcutaneous tissues, contraction accounts for little more than one-third of the closure process. The last entry in Table 14.1 illustrates the phenomenon of induced regeneration. For example, spontaneous healing in the guinea pig was described by the outcome [92, 8, 0]; however, following grafting with the keratinocyte-seeded DRT scaffold (dermis regeneration template), the outcome was [28, 0, 72], showing a marked reduction in contraction and a large increase in original area eventually occupied by regenerated skin. The reduction in scar to zero is a rough estimate based on available histological data. We stress here that the entries for *S* in Table 14.1 are rough estimates, useful in identifying large effects, rather than being detailed quantitative data.

14.5 Scar formation may be secondary to wound contraction

A relationship has been shown to exist between fibroblast axis orientation and orientation of collagen fibers synthesized during synthesis of stroma (Birk and Trelstad, 1985). Following synthesis by fibroblasts, collagen fibers are extruded outside the cell with an orientation of the fiber axis that coincides with that of the cell. In a wound that is subject to a tensile field, as in a contracting wound,

fibroblast axes become oriented along the direction of the major contraction vector. Collagen fibers synthesized by these oriented cells should therefore also be oriented along the same major axis. During skin wound healing there is evidence that a mechanical tensile field (a plane stress field) does indeed develop in the plane of the wound (Yannas, 2001). Quantitative measurement of the orientation of collagen fibers in dermal scars by laser light scattering showed that the fibers were persistently oriented in the plane of the wound and along the direction of the major contraction vector of the wound rather than being quasi-randomly oriented, as in physiological dermis (Ferdman and Yannas, 1993).

These considerations suggest the hypothesis that scar formation is the product of collagen fiber synthesis in the presence of the tensile field generated by a wound contraction process. According to this hypothesis, fiber orientation, one of the hallmarks of scarring, should accordingly disappear following cancellation of the tensile mechanical field in the plane of the wound. Blocking of contraction by an appropriate scaffold should cancel such a mechanical field and should block synthesis of oriented fibers (scar). In fact, when scaffolds that block contraction, even to a relatively minor extent, have been used, scar could not be detected in the closed wound (Yannas, 1981; Yannas *et al*., 1981, 1982a,b, 1984, 1989). However, observations of a lack of scar in the closed wound have so far been estimates based on histological data from a very limited number of tissue sections viewed in the microscope and do not support a firm, quantitative conclusion about the absence of macroscopic scar tissue. There is need for data of a much more extensive and quantitative nature to test the hypothesis that scar formation depends on the presence of wound contraction and that abolition of scar depends largely on cancellation of the mechanical field of contraction.

14.6 Experimental studies of partial regeneration of skin

There is accumulating evidence that the healing process of an injured organ in the adult mammal can be modified to yield a partly or wholly regenerated organ. In almost all processes studied to date, the critical 'reactant' supplied by the investigators was a scaffold synthesized as an analog of the extracellular matrix (ECM), occasionally seeded with autologous epithelial cells.

A detailed example of induced skin regeneration, originally referred to as synthesis of an 'artificial skin' (Yannas and Burke, 1980), has been described elsewhere (Yannas *et al*., 1981, 1982a,b, 1984, 1989; Murphy *et al*., 1990; Butler *et al*., 1998, 1999; Orgill and Yannas, 1998; Compton *et al*., 1998). The data describe in substantial detail the structural and functional similarities, as well as differences, between normal skin, scar and regenerated skin in the adult guinea pig and the swine following grafting of dermis-free defects with the keratinocyte-seeded dermis regeneration template (DRT), a scaffold characterized by unusual regenerative activity. DRT is a macromolecular network synthesized as a highly

porous analog of ECM with a highly specific structure that degrades *in vivo* at a controlled rate. Among other characteristics, regenerated skin is mechanically competent, fully vascularized and sensitive to touch as well as heat or cold. The regenerated dermal–epidermal junction, with its extensive formations of rete ridges and capillary loops, leaves no doubt that the newly synthesized tissues are clearly not scar tissue. However, regenerated skin differs from physiological skin in the absence of skin appendages (hair follicles, sweat glands, etc). For this reason, the regeneration of skin accomplished to date is referred to here as partial.

Seeding of the template with keratinocytes leads to *simultaneous* regeneration of a dermis and an epidermis (Yannas *et al*., 1981, 1982a.b, 1984, 1989; Orgill, 1983), while omission of seeded cells leads to *sequential* regeneration of dermis and epidermis. The simultaneous process leads to a clinically desirable result within about 3 weeks but is complicated by the need to prepare the seeded template in the clinical setting. The time required for regeneration can be shortened by culturing keratinocytes prior to seeding inside the scaffold (Butler *et al*., 1999). In spite of these considerations, the sequential process has been used extensively in the clinical setting. In this treatment the template induces regeneration of the dermis and the new dermis is spontaneously epithelialized from the wound margin. However, in clinical studies of large-area wounds with DRT, rather than wait for re-epithelialization from the edges of the wounds (a process with increased risk of infection of the open wound), a thin autoepidermal graft has been preferably applied to the newly synthesized dermis (Burke *et al*., 1981; Heimbach *et al*., 1988; Fang *et al*., 2002).

Although seeding of DRT with autologous keratinocytes was required to accelerate the kinetics of organ regeneration, seeding was not required to effect the eventual outcome itself (i.e. regeneration versus repair). Neither was seeding with fibroblasts required to affect the eventual outcome. Furthermore, studies of skin wounds under the same experimental conditions as above showed that treatment of the wounds with a large variety of growth factors (Greenhalgh *et al*., 1990; Puolakkainen *et al*., 1995), or epidermal cell suspensions or epidermal cell sheets (Billingham and Reynolds, 1952; Carver *et al*., 1993), or with a number of scaffolds based on synthetic polymers (Cooper *et al*., 1991; Hansbrough *et al*., 1993), failed to induce dermis regeneration. These and related observations (for review see Yannas, 2001) motivate study of the mechanism by which DRT induces stroma regeneration. The insight obtained by use of DRT (see below for mechanism) further sharpens the question about alternative methodology to achieve what is currently available by use of an active scaffold.

In spite of the lack of regenerated skin appendages, the cell-free DRT scaffold (clinically used as Integra®) that induces partial skin regeneration has been approved by the US Food and Drug Administration (FDA) for use with patients who have suffered massive loss of skin. Patients treated with this device were those suffering from massive burns (Burke *et al*., 1981; Heimbach *et al*., 1988; Stern *et al*., 1990; Fang *et al*., 2002; Klein *et al*., 2005), patients undergoing plastic or

reconstructive surgery of the skin (Abai *et al*., 2004; Blanco *et al*., 2004), as well as patients with chronic skin wounds (Gottlieb and Furman, 2004). By 2004, over 13 000 patients had been treated worldwide with Integra®.

14.7 Regeneration of adult organs other than skin

In addition to skin, confirmed observations of at least partial regeneration using scaffolds with high biological activity (templates) have been also reported for the following adult organs: peripheral nerves (Chamberlain *et al*., 1998, 2000; Zhang and Yannas, 2005; Yannas *et al*., 2007b) and conjunctiva (Hsu *et al*., 2000). Significant progress in the study of regeneration has been reported independently in studies of bone (Mistry and Mikos, 2005), heart valves (Rabkin-Aikawa *et al*., 2005), articular cartilage (Kinner *et al*., 2005), urological organs (Atala, 2005) and the spinal cord (Verma and Fawcett, 2005). The reader is referred to the relevant publications for further details (see also a compilation in Yannas, 2005a).

14.8 Antagonistic relationship between contraction and regeneration

There are several lines of qualitative evidence suggesting that contraction blocks induced regeneration. Data supporting this view will be reviewed briefly below. An extensive discussion of the data has appeared elsewhere (Yannas, 2001, 2005b).

14.8.1 The early-fetal to late-fetal transition in mammals: emerging dominance of contraction with loss of regenerative activity

A developmental transition, occurring during late mammalian gestation, leads from healing by regeneration without contraction to healing by contraction and scar formation (Soo *et al*., 2002, 2003; Colwell *et al*., 2005).

14.8.2 Amphibian development: gradual replacement of regeneration by contraction

During tadpole development (North American bullfrog), contraction gradually becomes dominant at the expense of regeneration. A small component of scar formation is first observed only after metamorphosis of the tadpole to the adult frog has been completed. At the adult stage, regeneration is abolished and contraction accounts for almost all of closure of the defect (Yannas *et al*., 1996).

14.8.3 Blocking of contraction by use of templates in adult mammals: induction of regeneration of skin, conjunctiva and peripheral nerves.

Skin was the first organ to be induced to regenerate. Wound contraction was blocked for over 25 days, a dramatic delay, when the unseeded DRT was grafted onto full-thickness skin wounds in the guinea pig (Yannas, 1981; Yannas *et al*., 1989). When the cell-seeded scaffold was grafted, contraction was not simply delayed but arrested; the wound closed by simultaneous regeneration of a dermis and an epidermis over most of the wound area (Yannas *et al*., 1981, 1982a,b, 1989).

Contraction was also blocked when DRT was grafted onto a full-thickness wound in the conjunctiva, produced by excision of stroma (analogous in depth of injury to a full-thickness skin wound; Tenon's capsule was also excised) in a rabbit model (Hsu *et al*., 2000). Ungrafted wounds closed by contraction and formation of scar tissue, the latter comprising an aligned array of dense collagen populated by occasional fibroblasts. Grafting with cell-free DRT resulted in regeneration of the conjunctival stroma, followed by spontaneous epithelialization of the stroma (Hsu *et al*., 2000).

Further incidence of contraction blocking coinciding with induced regeneration was observed in studies of a peripheral nerve, the fully transected rat (or mouse) sciatic nerve. This is a standard assay for regeneration studies in the peripheral nervous system. The two nerve stumps, separated by a gap of controlled length, are typically inserted inside a tube fabricated from an experimental material (Lundborg *et al*., 1982). In early studies, it was repeatedly observed that, following transection, the cross-section area of the distal nerve stump eventually was reduced by as much as 50–60% (Holmes and Young, 1942; Sunderland, 1990; Weiss, 1944; Weiss and Taylor, 1944). Later, significant evidence showed that regeneration was marginal, with the incidence of neural scar, when the regenerated nerve connecting the stumps was under substantial radial compression; in contrast, a very high quality of nerve regeneration with negligible incidence of neural scar was observed when compression of the regenerate was cancelled out. Contraction blocking was observed to depend strongly on the use of highly porous collagen–GAG (glycosamino-glycan) scaffolds with a defined structure (Chamberlain *et al*., 2000; Harley *et al*., 2004; Yannas, 2005b).

However, data from studies of impaired healing (e.g. diabetic wounds, wounds in obese animal models) show a different picture; although impaired healing in adults is accompanied by loss of contraction, regeneration is not observed. Experimental study of several models of impaired healing of skin wounds has been based on use of pharmacological agents (e.g. steroids), controlled infection, mechanical splinting, or on animal models of genetically impaired healing, such as the diabetic mouse or the obese mouse. In all of these models contraction was blocked almost completely, yet, regeneration was not induced (see review in

Yannas, 2001). Data from these models clearly shows that blocking of contraction is very probably required but it certainly does not suffice for regeneration.

These observations do not prove the existence of a cause–effect relationship between induced regeneration and contraction blocking. Nevertheless, the indirect evidence linking induction of regeneration and contraction blocking by active scaffolds is extensive and currently forms a useful theoretical framework that best explains the available data.

14.9 Mechanism of regeneration by use of scaffolds

Having highlighted the empirical evidence for an antagonistic relation between contraction and regeneration in adults, we now seek mechanistic pathways that account for such a theoretical relation. In particular, we focus below on cell types that play a dominant role in wound contraction and identify mechanistic pathways for blocking their contractile activity.

14.9.1 The contractile fibroblast (myofibroblast, MFB), is most probably the main cell type associated with wound contraction

The differentiated myofibroblast, referred to here simply as myofibroblast (MFB), has been generally credited with generation of most of the contractile forces in skin wounds (Rudolph *et al*., 1992; Desmoulière and Gabbiani, 1996; Lanning *et al*., 2000; Tomasek *et al*., 2002; Thannickal *et al*., 2003). The specific feature which provides the most useful operational distinction of MFB differentiation is expression of the α-smooth muscle actin phenotype (Tomasek *et al*., 2002). There is considerable evidence that myofibroblast differentiation is regulated by at least one cytokine (transforming growth factor, TGF-β1), the presence of mechanical tension and an extracellular matrix component (the ED-A (extracellular matrix component) splice variant of cellular fibronectin) (Desmoulière *et al*., 2005; Tomasek *et al*., 2002). The concurrent action of these factors appears to result in a feedback loop, in which tension development facilitates TGF-β1 production and activation of α-SMA (smooth muscle actin) expression; in turn, α-SMA expression increases tension development (Hintz *et al*., 2001; Tomasek *et al*., 2002). There is strong evidence that credits TGF-β1 not only with induction of α-SMA expression, but also with modulation of bonding between adhesive receptors (focal adhesions) and extracellular matrix molecules (e.g. ED-A fibronectin) (Hintz *et al*., 2001; Hintz and Gabbiani, 2003; Serini *et al*., 1998; Thannickal *et al*., 2003). An alternative view of the cell type involved in wound contraction has been described (Ehrlich *et al*., 1999). In the discussion below it will be hypothesized that myofibroblasts (MFB) are in fact the dominant cell type required for wound contraction.

14.9.2 Biologically active scaffolds (regeneration templates) appear to block contraction by interfering with the number and organization of myofibroblasts

We start with a simple mechanical model of the macroscopic contractile force that closes wounds in skin, conjunctiva and peripheral nerves. The macroscopic force for contracting a skin wound spontaneously is estimated at about 0.1 N (Yannas, 2005b). An individual dermal fibroblast in culture is capable of developing a force of the order of 1–10 nN (Freyman *et al.*, 2001a,b, 2002). The number of contractile fibroblasts required to develop the macroscopic force that suffices to close the wound is, therefore, at least 10^{-1}N/10 nN = 10^{7} cells, suggesting a factor of this magnitude to scale up the contractile force from cell to organ.

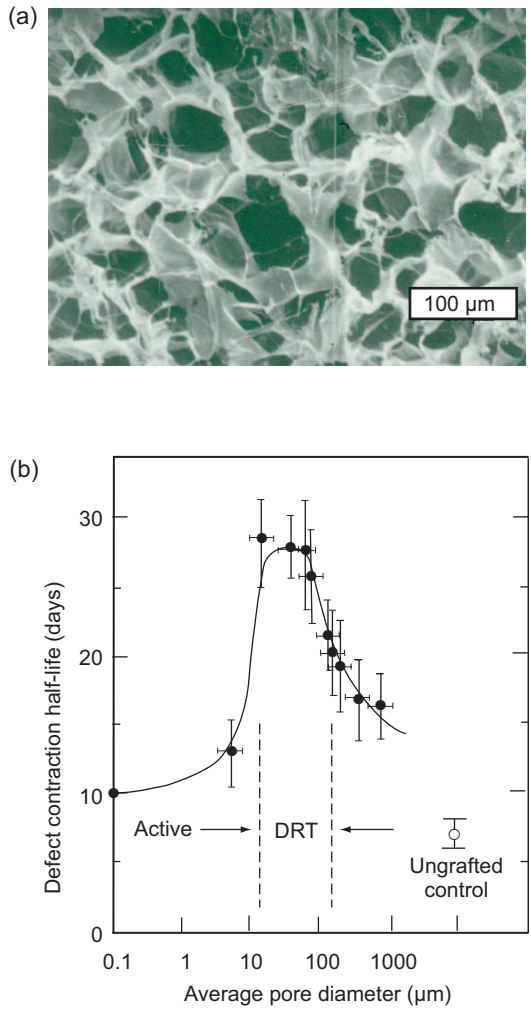
Scaffolds do not block wound contraction by mechanical splinting action. This has become abundantly clear following observations of a series of scaffolds that differed only in pore size but were otherwise identical in structure and in Young's modulus (mechanical stiffness). A homologous series of scaffolds, all with a pore volume fraction of 99.5% and with nearly identical mechanical properties, was studied (see example in Fig. 14.2(a)). Only scaffolds in the pore size range 20–120 μm blocked contraction; scaffolds with a pore size outside this range did not (Fig. 14.2(b); Yannas *et al.*, 1989). If splinting is a viable mechanism for scaffold activity, scaffolds with identical Young's moduli but differing only in pore size should not show such divergent behavior: all scaffolds, irrespective of pore diameter, should block contraction. This is clearly not observed. Other data that indicate that mechanical splinting is precluded as a mechanism of scaffold activity are observations that Young's moduli for these scaffolds are a very low 200 Pa (wet state; Harley, 2006), owing primarily to their very high pore volume fraction of 99.5% (Fig. 14.2(a)). A simple calculation shows that such stiffness values are orders of magnitude lower than necessary to provide the scaffold with any significant mechanical splinting capability inside the wound.

In a simple model of an anatomically well-defined skin wound, contraction results from a plane stress field that is generated by contractile cells with their contractile axes lying in the plane of the wound (Yannas, 2001). The macroscopic force vector, F_c, is considered to be the product of three contributions: the total number of MFB in the wound, N, the fraction of cells bound to the matrix and capable of applying traction, φ, and the average contractile in-plane force vector generated per MFB, expressed as f_i (Yannas, 2005b):

$$F_c = N\varphi f_i \tag{14.2}$$

Two major mechanisms appear to account for reduction of the macroscopic contractile force F_c by scaffolds. The first mechanism depends on reduction of the number of MFB, N, while the second depends on reduction of the effectiveness of forces generated by MFB in the wound.

Persistent observation shows that, in skin wounds that heal primarily by induced



14.2 (a) A scaffold with high biological activity: Dermis regeneration template (DRT). Based on a graft copolymer of type I collagen and chondroitin 6-sulfate. Pore volume fraction 99.5%. Average pore diameter 80 μm. (b) Contraction blocking activity of a homologous series of scaffolds increases with magnitude of vertical coordinate. Sharp differences in contraction blocking activity owing to differences in pore size alone are observed (Yannas *et al.*,1989).

regeneration in the presence of a DRT, MFB comprise only about 10% of the total number of fibroblasts; in contrast, in the absence of a DRT, MFB comprise 50% of total number of fibroblasts (Murphy *et al*., 1990). The observed reduction in the number of MFB probably results, at least in part, from a built-in feature in the DRT structure, the known absence of collagen fiber banding (without loss of triple

helical structure) which prevents platelet aggregation on the surface of collagen fibers. Blocking platelet aggregation probably leads to inhibition of platelet degranulation and concomitant relative depletion of TGF-β1 from the wound environment (Sylvester *et al*., 1989). As mentioned above, TGF-β1 is a known promoter of MFB differentiation. We conclude that DRT, partly at least, blocks one of the normal differentiation process that leads to MFB, thereby depleting the wound of MFB and reducing N in Equation 14.2.

Another mechanism that may account for the observed MFB depletion is based on the finding that TGF-β1 binds avidly, though non-specifically, to the extensive specific surface of DRT (Ellis *et al*., 1997). TGF-β1 binding to the scaffold surface may contribute additionally to the relative unavailability of TGF-β1 and TGF-β2 in the wound fluid and, accordingly, may deplete cells from the cytokine that is specifically required for MFB differentiation.

The second general mechanism for contraction blocking by DRT works by reducing the in-plane vector component of the force per cell (see Equation 14.2), thereby reducing the sum of forces generated by MFB. In the absence of DRT, the wound contracts vigorously; under these conditions, myofibroblasts have been observed to be densely packed with their axes lying primarily in the plane of the wound. In the presence of DRT, however, contraction is arrested. Once having migrated inside the DRT and become bound on the extensive surface of the highly porous scaffold, the long axes of MFB have been observed to be oriented out of the plane of the wound, where they are most effective in contracting the wound edges. MFB have been observed to become almost randomly oriented and relatively ineffective therefore, as a cooperative ensemble, for application of mechanical forces in the plane of the wound. In such a nearly random assembly of force vectors the sum of forces, F_c, must be near zero, leading to near cancellation of wound contraction, as observed. In the context of this second mechanism, scaffolds block MFB function but do not block MFB differentiation (Troxel, 1994; Yannas, 2001).

The structural determinants of scaffold activity have been largely identified (Yannas, 2001). According to the second mechanism described above the contraction blocking activity of a scaffold clearly requires an ability to bind most of the contractile cells in the wound. Accordingly, structural features that control cell-scaffold binding play a major role. For example, fibroblast–DRT binding requires participation of specific ligands, in particular those mediated by the β1 integrins that have been shown (Racine-Samson *et al*., 1997) to control myofibroblast–matrix binding during contraction. Such ligands are richly present on collagen surfaces but not, for example, on synthetic polymers.

Ligand density is another critical feature of scaffold activity; a large concentration of ligands should lead to binding of large numbers of cells on the scaffold, resulting in loss of their ability to scale up contraction forces and leading to blocked contraction. At a very small pore size, cells are prevented from entering inside the scaffold and binding to surface ligands; at very large pore size the specific surface becomes very low (a result simply of decrease in pore size), corresponding to low

levels of the ligand density. Ligand density is accordingly expected to be minimal at large values of pore size.

In a homologous series of scaffolds where scaffold members possess increasingly larger pore size, one should therefore expect that contraction blocking activity should go through a maximum, as observed in the range 20–120 μm (Yannas *et al*., 1989; Fig. 14.2b). Finally, the requirement for an optimal degradation rate in studies of induced skin regeneration (Yannas *et al*., 1989) as well as studies of peripheral nerve regeneration (Harley *et al*., 2004) is explained in terms of the need for the presence of an insoluble scaffold over the entire period (approximately 3 weeks) during which contraction remains active during wound healing. Degradation that occurs too early apparently prevents scaffolds from binding contractile cells, thereby blocking contraction, during the entire contractile period, while persistent scaffold insolubility beyond termination of the contraction process interferes sterically with the synthesis of new tissue. The requirement for a critical scaffold duration period is thereby explained (Yannas, 2001).

14.10 Future trends

Regeneration was first observed about 25 years ago (Yannas *et al*., 1981, 1982a,b). Since then the tempo of publication of studies in this field has steadily increased. Clinical use of the earliest biologically active scaffold (DRT; Integra™) has also increased steadily, especially in recent years.

Several important questions demand answers. Detailed studies of the mechanistic aspects of the activity of regeneration templates, especially transcriptional screening studies of DRT and analysis of concentration changes in wounds treated with DRT, are currently under way and will undoubtedly become more common as the biological properties of templates become better understood. As the clinical use of templates becomes more widespread in skin as well as other organs where there is solid evidence of induced regeneration (peripheral nerves, conjunctiva), questions will be raised about the speed with which regeneration takes place, since this affects a patient's duration of stay in the hospital setting. Other questions relate to the speed with which angiogenesis takes place in the presence of scaffolds. It has been shown, both in animal models and in clinical studies, that angiogenesis proceeds spontaneously from the host's wound tissues into an active scaffold (Murphy *et al*., 1990; Compton *et al*., 1998). However, the kinetics of angiogenesis are likely to be related inversely to the incidence of infection, especially in a clinical setting. This presumptive relationship needs to be studied by working with appropriate modifications in scaffold structure that accelerate angiogenesis.

A greater issue is the development of templates or other means of inducing regeneration in organs other than those in which it has been accomplished. One aspect of organ regeneration that has been inadequately studied so far (Yannas, 2005c; Yannas *et al*., 2007a) is the relation between early fetal regeneration and

induced regeneration in adults. Improved understanding of the relation between these phenomena could revolutionize the field of regenerative medicine, which is rapidly emerging as an alternative to organ transplantation.

14.11 Sources of further information and advice

The most extensive data on induced regeneration are available for skin and peripheral nerves (see review in Yannas, 2001). Relatively simple rules for synthesis of organs by induced regeneration have been formulated (Yannas, 2000, 2001, 2004). Progress in studies by several investigators of the induced regeneration of several other organs have been presented (Yannas, 2005a).

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15

Summary: biomaterials for treating skin loss

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Abstract: Much progress has been made in the development of biomaterials to treat skin loss over the last 30 years. Looking towards the future, we envision a new generation of skin substitutes that will produce a better functional and aesthetic skin that will allow regeneration with minimal scarring.

Key words: biomaterials, skin substitutes, development, market.

15.1 Technological advances

During the time that current skin substitutes were conceived, there have been many technological and biological advances that should be considered as a new generation of skin substitutes is developed (Table 15.1). The focus now must shift from patient survival to quality of life issues such as function, scar reduction and improved aesthetics. For example, despite our best efforts in reconstructive surgery, many facial and hand burn patients have appearances that are grossly different from the norm, making integration back into society very difficult. As we look at the future we should apply the advances made in all fields of science to patients with burns and complex wounds. There have been major innovations in biomaterials that allow us to design lightweight prostheses that can be servo-controlled with direct input from the nervous system. Each year there are significant improvements in microprocessor design and fabrication with recent advances in nanotechnology. Advances in basic biology include composite tissue allografts, genomics, proteinomics, cell signaling and cell culture systems. Advances in surgery include critical care medicine, reconstructive surgery, wound care products and a better understanding of inflammation and scarring.

15.2 Changes in the market for skin substitutes

Burn prevention has had a major impact on reducing the number of large burns in the USA and other developed countries. Smoke detectors are nearly universal in our homes, hot water control mechanisms are common and better occupational

Table 15.1 Current technologies that can be used for advanced design of skin substitutes and skin regeneration.

Technology	Advantages	Disadvantages
Prosthetic materials	Manufacturing reproducibility	Interface with biologic tissues Inability to self-repair Susceptibility to infection
Autogenous tissues	Superior function	Donor site limitations Invasive surgical procedure Scarring
Transplantation	Excellent function	Immunological rejection Opportunistic infections Tumors
Ex-vivo cell culture	Expand from limited donor tissue	Costly Difficult FDA pathway Fragile
Gene therapy	Precise genetic expression	Expensive Poor track record
Embryonic stem cells	Ability to differentiate into many cell lines	Early in development Blood supply Immunologic rejection Ethical issues Difficult regulatory approval
Biodegradable scaffolds	Easily manufactured Proven track record Regenerative properties	Optimal function with cells Not yet optimized

safety measures have drastically reduced the number of severe burns that we treat.[1] As burn specialty care has increased, large burns are now treated in about 100 burn centers in the USA. In developing countries the trends are clearly different. With industrialization, crowding, open fires and poor public safety measures, there are still a large number of burns, particularly in children. Because burn care is poorly organized and technologically behind in these countries, there is little demand for skin substitute products. Nevertheless, the severe scarring and contractures seen following a burn injury continue to motivate surgeons and researchers around the world to find better solutions to these devastating problems.

In contrast, the need for skin substitutes for chronic and complex wounds will undoubtedly increase. Our longer life expectancy along with the aging of the baby boomer generation will cause a large demographic shift to older patients in the USA. The epidemic of obesity sweeping our nation is fueling the expected doubling of diabetics in the next 10 years. Most predict that we will see a rapid increase in the number of diabetic foot infections, venous stasis disease and pressure sores. The increased complexity of surgery and medical care, including radiation therapy, has resulted in other types of complex wounds that are difficult to treat. There is also a steep increase in necrotizing soft tissue infections often

caused by antibiotic-resistant organisms. Already, the increase in both chronic and complex wounds has resulted in the proliferation of wound centers and hyperbaric centers around the nation.[2]

From a health economics standpoint, there is a desperate need for better cost-effective technologies to stimulate healing and regeneration of the integument. To date, very little public attention has aimed at treating wounds because in most cases, they are not life threatening and the discussion of wounds in public is not socially acceptable. As a result, most patients with wounds quietly hope they will heal and rarely organize requests for additional governmental resources to be directed towards these problems. Wound care in the USA is also fragmented by the care provided by practitioners in multiple specialties including surgeons, internists, podiatrists, nurses, therapists and others. As a result, no single professional organization has a unified voice for wound care. For the foreseeable future, most of the investment in improved wound care products will probably originate in industry and is likely to be product focused. It is hoped that academic basic science and clinical researchers can team up with industry to develop and test better products for our patients in the future.

15.3 A more normal skin – regenerative response

Skin differs depending on the site, age and race of a particular patient. It is hoped that in the future, we will have the design capability to match carefully the intricacies of site-specific skin. For example, glabrous skin on the palm or soles has a very different texture and feel than skin on the abdomen or back. Placing a skin graft from the abdomen onto the palm of the hand gives a very unnatural appearance and often causes chafing at the borders. Wood *et al.*[3] reported using a cell spray technique to deliver keratinocyte specific cells to glabrous recipient sites. Melanocytes can then interact with the keratinocytes providing melanin for pigment deposition. It appears that cell spraying techniques may possibly be able to correct pigment deficits.[4,5] The absence of hair follicles, sweat glands and sebaceous glands also significantly decrease the function and appearance of skin. As we understand more about the stem cells involved in each of these organelles, future strategies may be identified that will accurately recapitulate these structures.

The use of composite tissue allografts may make some difficult facial and extremity reconstructions possible with good results. The major problem with these procedures is that skin is highly antigenic and use of allografts requires high doses of immunosupression drugs. Nevertheless, as progress is made in tolerance – better immunomodulatory drugs, or pre-treatment of the allograft – this technically demanding procedure may increase over time.

Reconstruction of the dermis will continue to challenge us for some time. Great progress has been made with the introduction of scaffolds including human cadaver skin, lyophilized human dermis and a variety of collagen-based matrices that have been used with increasing frequency in burns and complex wound cases.

Table 15.2 Additional design characteristics of the next generation artificial skin

- Cell based
- Single staged
- Epidermis
  - Site specific (e.g. glabrous)
  - Melanocyte control
- Dermal matrix
  - Elasticity
  - Strength
- Cost effective
- Low complication rate

The matrix appears to be the critical component in allowing a three-dimensional space for circulating cells from the periphery to migrate into and secrete *de novo* extracellular matrix. The ability to optimize the regenerative capacity of dermis to form an elastic malleable strong integument, in contrast to a stiff, friable firm scar will be critical in improving the design of new skin substitutes.

The next generation of skin substitutes will involve specific cells that are integrated with dermal matrices (Table 15.2). Our ability to mark and study cellular migration within the matrices will allow us to make the best design for this interaction. As we learn more about the cell matrix interactions, we hope that we will be able to predict accurately the functional and cosmetic outcomes of these combinations. These products should be able to be applied in a single stage without technical difficulty. We will also need to understand better reconstruction of the adipose layer. Many severe burns are excised to fascia leaving large contour deformities. Adipose derived stem cells provide a powerful tool that might be useful in reconstructing this layer. Skin substitutes should have resistance to microorganisms to minimize infection and other complications. They will need to be designed in a cost-effective fashion that can be supported by our future health care financing. We are posed with a formidable list of challenges that will require a multidisciplinary focus involving partnership of government funding agencies, industry and academic researchers. The challenge is great, but the rewards of better treatment for our patients will be well worth the effort.

15.4 References

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