JOINT HOMEOSTASIS IN TISSUE ENGINEERING FOR CARTILAGE REPAIR

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ORTHOPAEDIE

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JOINT HOMEOSTASIS IN TISSUE ENGINEERING FOR CARTILAGE REPAIR

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Preface

Traumatic joint damage, articular cartilage and the research into methods of restoring the articulation are not new topics of interest. For centuries, clinicians have recognized the importance of cartilage damage and sought ways of learning about the normal form and function of hyaline cartilage as well as the process of degradation and restoration. Unfortunately, only some of the real problems have been solved partially, while a lot of basic knowledge has been available for centuries.

More recently, tissue engineering was introduced as a method of restoring tissue or organ function, and has already been applied to cartilage repair. The possibility of modulating cells and tissues and applying this novel capability towards the restoration of musculoskeletal function provides great promise and opportunity for progress in orthopedics and traumatology. Currently, various strategies have been introduced for tissue engineering in cartilage repair, but so far no solid foundation for full clinical implementation has been established. Therefore, the general aim of this thesis is to investigate influences on cartilage tissue engineering and to identify areas where the outcome can be improved. Given the history of the field of musculoskeletal research, it is important to realize the relativity of individual contributions and the constraints on 'real progress' of scientific knowledge in time. No clearer can this be illustrated than by offering the reader insight in the historically available knowledge. As early as in old Egyptian civilization, descriptions of cartilage as a tissue and the relevance of articular degradation as a disease have been found[30]. Scholars such as Aristoteles, Hippocrates and Leonardo da Vinci already contributed to these investigations, but were also the first to suggest that *'If one way be better than another, that you may be sure is Nature's way'* (Aristoteles, 4± century B.C.)[179] and *'Human ingenuity makes inventions but none as beautiful, simple or to more purpose than Nature'* (Leonardo da Vinci, 15th century)[288]. In the following section, one of the first literature contributions on cartilage biology by the British surgeon William Hunter is represented. This historic hallmark paper demonstrates the value and bright practical insight of (almost) forgotten knowledge, and provides the reader of this thesis with a solid grasp of what is important to orthopedic surgeons who

perform their research in the field of cartilage repair and surgical intervention. Hunter's description of cartilage biology, though not as detailed as our current knowledge, is accurate, relevant and clear. Reading this reproduction of Hunter's original text provides a foundation for the basic problem to be addressed in this thesis and places our experiments in a historical context. The clinical problem colorfully described by Hunter remains largely unsolved, although we have fortunately progressed beyond the point at which our patients *'submit unhappily to extirpation, a doubtful remedy, or wear out a painful though probably short life'*.

Of the structure and diseas of articulating cartilages

William Hunter, surgeon

The fabric of the joints in the human body is the subject so much the more entertaining, as it must strike every one who considers it more attentively, with an idea of fine mechanical composition. Wherever the motion of one bone upon another is requisite, there we find an excellent apparatus for rendering that motion safe and free: We see for instance, the extremity of one bone moulded into an orbicular cavity, to receive the head of another, in order to afford it an extensive play. Both are covered with a smooth elastic crust, to prevent mutual abrasion; connected with strong ligament to prevent dislocation; and enclosed in a bag that contains a proper fluid deposited there, for lubricating the two contiguous surfaces. So much in general, but if curiosity leads us a step farther, to examine the peculiarities of each articulation, we meet a variety of composition calculated to all the varieties of motion requisite in the human body. Is the motion to be free and extensive in one place? There we find the whole apparatus contrived accordingly. Ought it to be more confined in another? Here we find it happily limited. In short, as nature's intentions are various, her workmanship is varied accordingly.

These are obvious reflections, and perhaps, as old as the inspections of dead bodies. But modern anatomists have gone further: They have brought the articulations, as well as other parts of the body, under a narrow inquiry, and entered into the minutest parts of their composition. The bones have been traced, fibre after fibre; but the cartilages, as far as I can learn, have not hitherto been sufficiently explained. After some fruitless events of macerating and boiling the cartilages in, different menstrua, I fell upon the method not only of bringing their fibrous texture to view, but of tracing the direction and arrangement of those fibres. I shall therefore endeavor to give a short account of the structure of articulating cartilages, and make a few observations on their diseases, with a view to advance a rationale explication on their morbid phenomena.

An articulating cartilage is an elastic substance uniformly compact, of a white colour, and somewhat diaphanous, having a smooth polished surface covered with a membrane: harder and more brittle than a ligament, softer and more pliable than a bone. When an articulating cartilage is well prepa-

red, it feels soft, yields to the touch, but restores itself to its former equality of surface when the pressure is taken off. This surface, when viewed through a glass, appears like a piece of velvet. If we endeavor to peel the cartilage off in lamellae, we find it impractible; but, if we use a certain degree of force, it separates from the bone in small parcels; and we never find the edge of the remaining part oblique, but always perpendicular to the subjacent surface of the bone. If we view this edge through a glass, it appears like the edge of velvet; a mass of short and nearly parallel fibres rising from the bone, and terminating at the external surface of the cartilage: and the bone itself is planned out into small circular dimples, where the little bundles of cartilaginous fibres were fixed. Thus, we may compare the texture of a cartilage to the pile of velvet, its fibres rising up from the bone as the silky threads that rise from the woven cloth or basis. In both substances, the short threads sink and bend in waves upon being compressed; but, by the power of elasticy, recover their perpendicular bearing, as soon as they are no longer subjected to a compressing force. If another comparison was necessary, we might instance the flower of any corymbiferous plant, where the flosculi and stamina represent the little bundles of cartilaginous fibres; and the calyx, on which they are planted, bears analogy to the bone. Now these perpendicular fibres make the greatest part of the cartilaginous substance; but without doubt there are likewise transverse fibrils which connect them, and make the whole a solid body, though these last are not easily seen, because being very slender, they are destroyed in preparing the cartilage.

We are told by anatomists that cartilages are covered by a membrane named Perichondrium. If they mean the cartilages of the ribs, larynx, ear, etcetera there, indeed, such a membrane is very conspicuous; but the Perichondrium of the smooth articulating cartilages is so fine, and firmly braced upon the surface, that there is room to doubt whether it has often been demonstrated, or rightly understood. This membrane, however, I have raised in pretty large pieces after macerating; and I find it to be a continuation of that fine, smooth membrane that lines the capsular ligament, folded over the end of the bone from where that ligament is inserted. On the neck of the bone, or between the insertion of the ligament, and border of the cartilage, it is very conspicuous, and may be pulled up with a pair of pincers; but where it covers the cartilage, it coheres to it so closely, that it is not to be traced in the recent subject without great care and delicacy. In this particular, it resembles that membrane which is common to the eye-lids and the fore-part of the eye-ball, and which is loosely connected with the albigulnea, but strongly attached to the cornea. From this description it is plain. That every joint is invested with a membrane, which forms a complete bag, and give a covering to everything within the articulation, in the same manner as the peritoneum invests not only the parieties, but the contents of the abdomen. The blood-vessels are so small, that they do not admit the red globules of the blood; so that they remained in a great measure unknown, till the art of filling the vascular system with a liquid wax brought them to light. Nor even with this method we are able, in adults, to demonstrate the vessels of the true cartilaginous substance; the fat, glands, and ligaments, shall be red with injected vessels, while not one coloured speck appears on the cartilage itself. In very young subjects, after a subtile injection, they are very obvious; and I have found their course to be as

follows; All around the neck of the bone there are a great number of arteries and veins, which ramify into smaller branches, and communicate with one another with frequent anastomoses, like those of the mesentery. This might be called the circulus articuli vasculosus, the vascular border of the joint. The small branches divide into still smaller ones upon the adjoining surface, in their progress towards the centre of the cartilage. We are very seldom able to trace them into its substance, because they terminate abruptly at the edge of the cartilage, like the vessels on the albiguinea ocull when they come to the cornea. The larger vessels, which compose the vascular circle, plunge in by a great number of small holes, and disperse them self into branches between the cartilage and bone. From these again there arises a crop of small short twigs that shoot towards the outer surface; and whether they serve for nourishing only, or if they pour out a dewy fluid, I shall not pretend to determine. However that be, I cannot help observing, that the distribution of the blood vessels to the articulating cartilages is very peculiar, and seems calculated for obviating great inconveniences. Had they run on the outer surface, the pressure and motion of the two cartilages must infallibly have occasioned frequent obstructions, inflammations, etc. which would soon have rendered our motion painful, and at last entirely deprived us of them. But by creeping round the cartilaginous brim, where there is little friction, or under the cartilage, where there is none, they are perfectly well defended from such accidents. It were to be wished we could trace the nerves of cartilages: But, in relation to these organs, here, as in many other parts of the body, we are under a necessity, from the imperfection of our senses, of being satisfied with mere conjecture and thought, from the great insensibility of a cartilage, some have doubted of its being furnished with nerves; yet, as it is generally allowed, that these are a sine qua non in the growth and nourishment of animals, we have no sufficient reason to deny their existence in this particular part. With regard to the manner of their distribution, we may presume, from analogy, that they follow the same course with the blood-vessels. The articulating cartilages are most happily contrived to all purposes of motion in those parts. By their uniform surface, they move upon one another with ease: By their soft, smooth and slippery surface mutual abrasion is prevented: By their flexibility, the continuous surfaces are constantly adapted to each other, and the friction is diffused equally over the whole: By their elasticy, the violence of any shock, which may happen in running, jumping, etc. is broken and gradually spent; which must have been extremely pernicious, if the hard surfaces of bone had been immediately contiguous. As the course of the cartilaginous fibres appears calculated chiefly for this last advantage, to illustrate it, we need only reflect upon the soft undulatory motion of coaches, which mechanics want to procure by springs; or upon the difference betwixt riding a chamber horse and a real one. To conclude, the insensibility of articulating cartilages is wisely contrived, as by this means the necessary motions of the body are performed without pain. If we consult the standard surgical writers from Hippocrates down to the present age, we shall find, that an ulcerating cartilage is found to be a very troublesome disease; that it admits of a cure with more difficulty than a carlous bone; and that, when destroyed, it is never recovered. Hildanus, in considering the diseases, has observed, that when the cartilages of a joint were destroyed, the bones commonly threw out a cementing callus; and thus a bony anchylosis, or immo-

veable continuity, was formed where the moveable joint had been. So far as I had opportunities of examining diseased joints, either after death or amputation, I have found, accordingly to the nature and stage of the disease, the cartilages in some parts redish and lax; or soft and spongy; or raised up in blisters from the bone; or quite eroded, and, perhaps, the extremities of the bone carious; or, lastly, a bony anchylosis formed. But I could never see, nor indeed hear of, the least appearance of an exfoliation from the surface of the cartilage. It appears from maceration, that the transverse fibrils are extremely tender and dissoluble; and that the cohesion of the parts of the straight fibres is stronger than their cohesion with the bone. When a cartilage therefore is inflamed, and soaked in purulent matter, the transverse or connecting fibres will soonest give way, and the cartilage becomes more or less red and soft, etcetera. If the disorder goes on a little longer, the cartilage does not throw off a slough, but separates from the bone, where the force of cohesion is least, and where the disease soon arrives, by reason of the thinnest of the cartilage. When the bone is thus exposed, the matter of the ulcer, or motion of the joint, corrodes or abrades the bony fibres. If the constitution is good, these will shoot forth a callus; which either cements the opposite bones of the articulation, or fills up the cavity of the joint, and for the future prevents motion. But, if unfortunately, the patient labours under a bad habit of body, the malignancy, having got root in the bone, will daily gain ground, the caries will spread, and at last the unhappy person must submit to extirpation, a doubtful remedy, or wear out a painful, though probably short life.

Pages 514-521, Read June 2, 1743

INTRODUCTION

INTRODUCTION

In clinics and emergency departments, patients seek attention on a daily basis because of a recent trauma or joint dysfunction that can be related to articular cartilage damage. Many of these patients are young and active, and unfortunately handicapped to some extent because of their complaints.

Cartilage defects larger than 2 mm in diameter do not heal, and the occurrence of such relevant cartilage defects is high[59,70,112,141,182,185]. As many as 63% of 31.500[77] patients in one study undergoing arthroscopy, and up to 15% of patients with hemarthrosis due to knee trauma have a relevant cartilage defect[128]. Cartilage damage most frequently occurs in the knee, due to for instance trauma, ligamentous instability, malalignment of the extremity, meniscectomy or primary osteochondritis dissecans. The treatment of these cartilage defects forms a considerable challenge for orthopedic surgeons worldwide.

Initial treatment of a cartilage defect consists of symptomatic relief by rest, non-steroidal anti-inflammatory medication and functional mobilization[316]. In most cases, accompanying ligament or meniscal damage may exist and a solitary cartilage lesion is not diagnosed on primary evaluation. Further diagnostic measures such as MRI may be indicated, or even arthroscopic intervention may be necessary. Most, if not all patients with a relevant cartilage defect are seen again in the outpatient clinic weeks to months after trauma and present with complaints of pain, persistent effusion and intermittent locking of the joint. Unfortunately, even as early as

Figure 1.1

These four panels show an arthroscopic view into the knee. (A) Demonstrates a fresh osteochondral defect in the medial femoral condyle, with bleeding from the subchondral bone. (B) There is an accompanying meniscus lesion pulled forward with a probe. Panel (C) and (D) clearly show cartilage degradation both in the defect and on the opposing tibia surface, with signs of synovitis and meniscus fraying present in the same knee after 1 year of follow-up when repeated arthroscopy was indicated due to persistent pain and effusion.
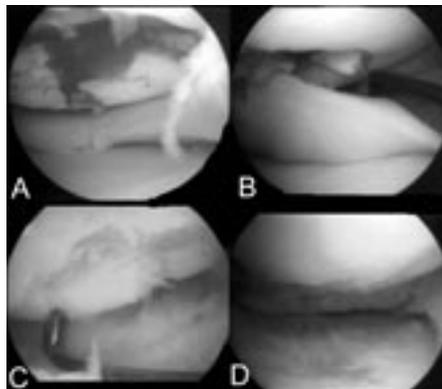
Figure I.2

Panel A shows the knee X-ray of a 16 year old male with considerable subchondral sclerosis (arrows) in the lateral femoral condyle and joint incongruity in the lateral compartment suggesting a cartilage defect. Panel B shows his MRI image where the extensive degree of cartilage damage and bone bruise is clearly visible in the area between the dotted line and the articular surface (arrow).

in 1743 it was known that such cartilage defects do not heal[139]. When left untreated, such defects progress to osteoarthritis[182,184,185]. So far, no successful and lasting treatment method has been developed other than joint replacement surgery. Thus, until the age at which total joint replacement is a suitable treatment, a considerable morbidity arises from our lacking ability to repair a damaged articular surface.

This has sparked an increasing interest in tissue engineering techniques for cartilage repair. Most tissue engineers aim to restore the articular cartilage surface either by repairing the defect so functional demands can be met, or ideally achieving true regeneration of a hyaline articular cartilage, that will fulfill all biological and mechanical requirements for the remaining life span.

The Concept of Joint Homeostasis

An articulating joint has a complex design with many essential components and a multitude of interactions between structures such as synovium, cartilage, menisci, synovial fluid, ligaments and subchondral bone[48,49,139,182]. These anatomical structures are influenced by factors such as motion, loading, alignment, weight, age, hormonal influences and many more. It is evident that this complex environment must be rigorously regulated. Furthermore, metabolic control must be flexible because the external environment of cells is not constant[295]. Studies of a wide range of organisms have shown that there are a number of mechanisms for the control of physiological equilibrium, also referred to as *Homeostasis*. Vogel[313] suggests that 'the crux of a feedback system is the ability to

adjust what it does, depending on conditions outside itself, where those conditions include the result of its own actions. In a strictly mechanical and formal sense, it has self-awareness.' In this thesis, we describe why we feel that the basic scientific concept of homeostasis should be implemented in our thinking when addressing the clinical problem of restoring a damaged articulation. With normal *Joint Homeostasis*, we mean the stable equilibrium of synovium and cartilage matrix void of inflammatory response in a well functioning articulation. When joint homeostasis is disturbed, this equilibrium is changed, and a myriad of intra-articular factors, such as inflammatory, molecular, or cellular components, come into play. Regulatory pathways are typically aimed at restoring equilibrium to normal homeostasis. Well-known examples are blood pressure maintenance, serum glucose regulation, and calcium homeostasis in bone metabolism. Dependant upon the cause and magnitude of a disturbance a permanent alteration may occur. This may again lead to homeostasis but in a different metabolic state. However, in an articulation the initiation of a cascade of change such as in degenerative osteoarthritis will eventually
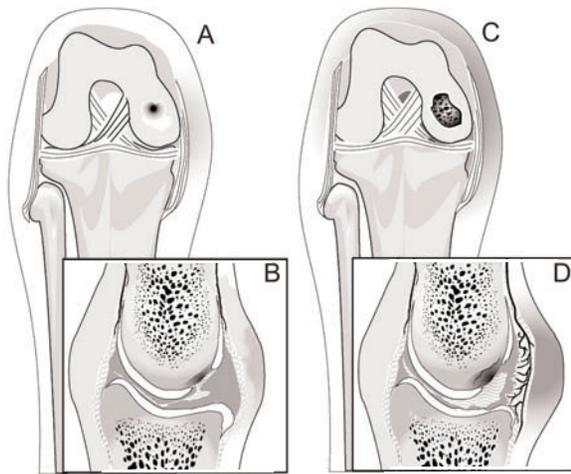
Figure I.3

In normal joint homeostasis, there is a healthy equilibrium between smooth cartilage, subchondral bone, synovial fluid, intact menisci and ligaments. After a cartilage defect as in (A), this equilibrium is disturbed (B). After a persistent defect (C) Joint Homeostasis becomes altered. This environment with matrix degradation, synovial thickening, effusion and subchondral sclerosis (D) constitutes a very different environment in which we attempt tissue engineered cartilage repair.

irreversibly change the joint[48,49]. In this thesis the general biological concept of homeostasis is applied to the intra-articular environment, which stimulated us to investigate some of the factors that may be of influence.

Homeostasis and cartilage defects

The majority of patients have a longer history of complaints before they are treated for their joint damage. For instance, effusion, pain and locking clearly signify that some degree of synovitis and matrix degradation is present. Thus, a disturbed local environment exists that provides a condition quite different from that in the undamaged, healthy joint.

The work presented in this thesis tests the hypothesis that this altered intra-articular environment constitutes a change in joint homeostasis and could provide an explanation for the discrepancy between reproducible basic scientific data and promising *in vivo* examples of cartilage repair, and the variable clinical results reported in current literature. In joints with disturbed homeostasis, the local environment in which we try to achieve cartilage formation is considerably different from those with normal homeostasis in studies using healthy animal knee joints. In light of this hypothesis, the initial *in vitro* findings of Rodrigo and Steadman[269] spark interest. They compared the effect of synovial fluid samples from knees of 25 patients with a fresh or old traumatic chondral defect in a chick limb bud assay. Samples from 11 of 17 acutely injured knees stimulated chondrogenesis, 4 were inhibitory, and 2 showed no effect. Samples from 6 out of 8 chronically injured knees inhibited chondrogenesis; the other two samples stimulated chondrogenesis. They suggested that synovial fluid may contain factors that stimulate cartilage healing in the acute period following traumatic injury, but this effect can become inhibitory if the lesion is allowed to become chronic. We simplify this concept as;

As fish do not thrive in a dirty aquarium, it is our responsibility to clean the tank.

The full extent of altered joint homeostasis cannot be examined effectively in a controlled environment since these conditions only mimic part of the natural organism and not all of the intricate regulatory functions are reproduced. Following the approach suggested by our *Concept of Joint Homeostasis*, not only the damaged cartilage surface needs to be treated, but rather the entire joint will be the target of our attempt at tissue engineered regeneration. This would imply also addressing synovitis, meniscal damage, liga-

ment stability, limb alignment, and normalizing metabolic activity. Establishing true biological joint reconstruction might be of great importance for a large, mostly young, patient population for whom currently no reliable treatment exists.

Homeostasis and loading

Usage-tuned structural change is a valuable accomplishment of nature[313]. Exercise a muscle and it gets larger; put an appendage in a cast for a month or so and the muscles atrophy. Bone responds similarly losing mineral and thus softening if out of use and increasing density in response to repeated loading. Immobilization of a joint or a prolonged period of limited loading changes joint homeostasis. Proteoglycan and collagen metabolism are altered, cartilage becomes thinner and a novel homeostasis is established in a different state. This process is reversible up to a certain point as seen in evaluation of musculoskeletal aspects of space flight as well as in animal experiments[4,65,91]. Similarly, cartilage thickness will vary considerably between joints as well as within a single joint[52,96.] This finding has been related to the amount of load applied to the specific region[52,54]. Many investigators have demonstrated that mechanical factors have an influence on embryogenesis and cartilage metabolism[51,54,55,123,126,162,167,307,311,312]. Up to this point, no detailed *in vitro* data are available on how mechanical stimuli will influence cartilage formation in defect repair and tissue engineering, while a profound effect was shown for example with CPM. We decided to examine periosteal cartilage formation as a model for musculoskeletal response to injury, since periosteum contains mesenchymal stem cells, is frequently used in cartilage repair and plays an important role in fracture healing.

Homeostasis and aging

A lot of degeneration associated with aging may reflect disuse rather than old age itself [313]. However, aging alters homeostasis and regulatory systems. For example, elderly patients have a diminished temperature and white cell count differential in response to inflammation compared to those of infants [129]. Age influences memory, posture and the rate of fracture healing[97,104,190,211]. However, the number of non-united fractures remains similar to that in younger individuals, so some mechanisms remain relatively unchanged. Since age is to date an unalterable essential patient characteristic of which negative influences on the outcome of car-

tilage repair have been suggested, we aim to study the effect of subject age on cartilage formation using an *in vitro* model for cartilage formation[216,219,228].

CONTENTS OF THE THESIS

The central aim of this thesis is to study joint homeostasis and its role in our attempts at repairing damaged articular cartilage. To realistically approach this complex matter we limited our investigation by selecting three aspects of joint homeostasis; metabolic derangement, mechanical stimuli and subject age.

We will attempt to address the following questions and aims:

To study *Homeostasis and metabolic changes due to cartilage defects*
- Do metabolic changes, due to articular damage, influence cartilage formation by tissue engineering?

To investigate *Homeostasis and mechanical factors*
- How do mechanical stimuli affect the chondrogenic capacity of periosteal cells?

To evaluate the effect of age on *Homeostasis and regulation*
- What is the effect of subject age on cartilage formation from periosteum *in vitro*?

Specific aim 1: *Homeostasis* and metabolic changes

After a brief overview on cartilage repair strategies in *Chapter 1*, we will initially address the rationale of our concept. In *Chapter 2*, we describe a validation experiment of outcome tools, which is necessary for the study in *Chapter 3* where we investigate the effect of a disturbed joint homeostasis upon cartilage repair in a large animal model. We modeled a cartilage defect in the medial femoral condyle of the Dutch milk goat since this species has a metabolic rate similar to humans,[22,147,148,213,314] and contrary to *in vitro* studies provides full regulation of joint homeostasis. In the subsequent chapters, we further deepen this approach with *in vitro* studies, where we will focus on the potential importance of single parameter variations: mechanical stimulation by dynamic fluid pressure and subject age.

Specific aim 2: *Homeostasis* and mechanical factors

To study the effect of mechanical factors on the process of cartilage formation in a controlled environment we selected the model of cartilage formation *in vitro* from periosteal explants as developed by O'Driscoll[228] and described in detail in *Appendix A*. This model provides several advantages; the various characteristics of the *in vitro* model have been well examined and form a reliable method to study cartilage formation in a controlled environment. Periosteum provides a source of undifferentiated chondrocyte precursor cells for fracture healing that can also be used for cartilage repair[202,203,207,251,253,272,274,319,320]. In both processes, the quantity of cartilage that can be produced is related to the number of available stem cells[113].

The 'Periosteal Organ Culture Model', has been described for studying the process of cartilage formation in whole periosteal explants suspended in agarose, mimics the events during periosteal chondrogenesis *in vivo*[86,113,196,228,229]. Furthermore, periosteum is used in various tissue engineering cartilage repair strategies, either as a tissue with chondrogenic cells that have the capacity to restore the articulating cartilage and subchondral bone or simply as a cover to retain transplanted chondrocytes in the defect to be repaired. Finally, periosteum plays an important role in fracture healing where it contains the fracture hematoma and provides cells that contribute to the chondral callus.

To apply mechanical stimuli *in vitro* we selected the use of intermittent hydrostatic, or rather dynamic fluid pressure (for details see *Appendix B*). This method has been applied to study cartilage metabolism and bone formation, and provides a reproducible mechanical stimulus with the advantage of excluding directional influences, tissue contact and shear stress as in experiments with direct mechanical loading[48,50,51,54,55,123,126,161,162,167,220,228,307,311,312].

In *Chapter 4*, we discuss the validity of applying a mechanical stimulus in a mixed environment, tissue culture system, which forms the basis for the experimental design in *Chapters 5-7*. *Chapter 5* describes our studies on the effect of mechanical stimulation by applying dynamic fluid pressure (DFP) on periosteal cell proliferation. In *Chapter 6*, we evaluate the specific effects that DFP has on the subsequent phases of cartilage formation; cell differentiation, matrix synthesis and cartilage organization. In *Chapter 7*, we examine the influence of loading characteristics such as magnitude, frequency, timing and duration of the DFP stimulus.
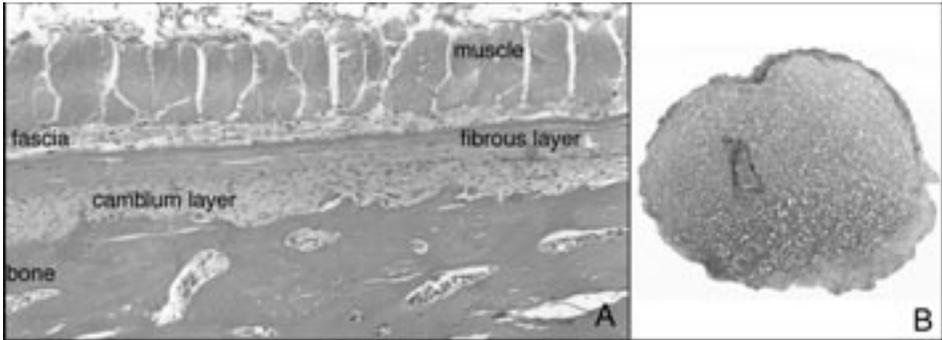
Figure I.4

(A) Cross sectional histological view (H/E, 200x) of rabbit periosteum on bone. The cambium layer is directly adherent to the bone, the covering fibrous layer can clearly be identified in this sample from a two month old rabbit. (B) An example of a periosteal explant in which cartilage has formed in vitro after 42 days in culture.

See CD for full color representation

Specific aim 3: *Homeostasis* and age

The final experiments in *Chapter 8* describe our initial investigations into the relationship between subject age and changes in the chondrogenic process. In closing, *Chapter 9* presents a short summary of the discussions as well as conclusions from the research performed and implications of the data for our ongoing studies and clinical practice.

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Periosteum responds to dynamic fluid pressure by proliferating in vitro
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Journal of Orthopaedic Research 1999; Vol. 17-5: 668-677

Dynamic pressure transmission through agarose gels
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Journal of Orthopaedic Research 2001; Vol. 19-1: 95-103

The enhancement of periosteal chondrogenesis in organ culture by dynamic fluid pressure
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Tissue Engineering 2002; Vol. 4-8: 627-635

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Onder redactie van: Schellekens H., e.a.
Elsevier Gezondheidszorg, Maarssen 2001

Influence of timing, duration and magnitude when stimulating chondrogenesis by dynamic fluid pressure
Saris D.B.F., Mukherjee N., Fitzsimmons J.S., O'Driscoll S.W.
(Submitted for publication)

Joint homeostasis influences cartilage repair; the discrepancy between old and fresh defect
Saris D.B.F., Dhert W.J.A., Verbout A.J.
(Conditionally accepted Journal of Bone and Joint Surgery)

CHAPTER
I

## GENERAL BACKGROUND AND TREATMENT STRATEGIES
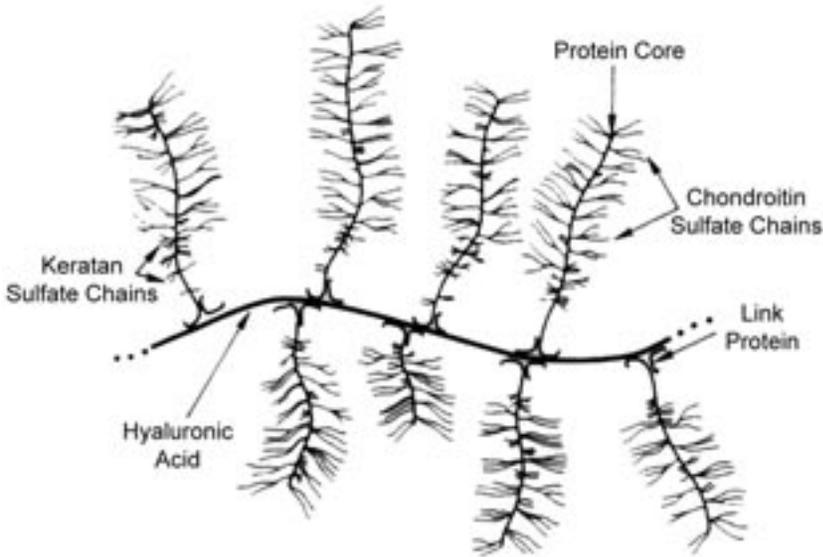
Figure 1.1

This schematic drawing depicts cartilage matrix organization. The central hyaluronic acid core is fixed to the proteoglycans consisting of chondroitin sulfate and keratan sulfate by a link protein. The negative charge in the matrix causes fluid influx. This osmotic swelling gives the matrix its mechanical strength. (Reproduced with permission from Orthopaedic Basic Science; The American Academy of Orthopaedic Surgeons, Edt. Simon Sheldon M.D.)

Normal cartilage function

Healthy hyaline cartilage contributes to a smooth articular surface, and is essential for a proper functioning of the joint[48,50,182,183]. Articular cartilage provides the joint with a low friction surface that, in the normal situation has both an unsurpassed wear resistance and a high compressive stiffness.

This has been efficiently fulfilled by the unique biological and biochemical composition of articular cartilage, as it is essentially composed of a type II collagen sponge supported by water that is held in place by proteoglycans produced by the chondrocytes that are embedded in the matrix. In the normal situation, articular cartilage can perform the required function for a lifetime, although some age related, degenerative, alterations occur. This well designed structure is at risk when trauma occurs since cartilage has a limited capacity for repair. Unfortunately, cartilage defects are not a limit-
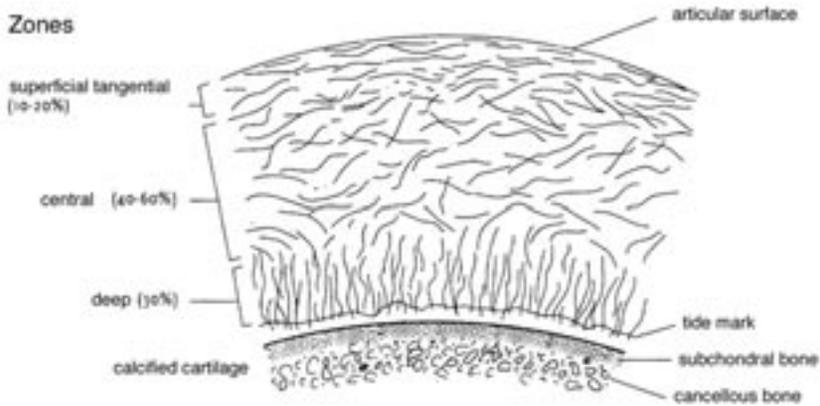
Figure 1.2

Cartilage is organized in four morphologically different zones. From the articular surface to the subchon-dral bone the superficial tangential, the central, the deep, and the calcified cartilage zones are discerned. The superficial tangential zone contains a cell free fibrous layer and in the subsequent tangential layer where chondrocytes are elongated, proteoglycan concentration is low and the collagen fibers are at tan-gential orientation compared to the other layers. In the second, central or transitional zone, chondrocytes are larger in size, rounder and loosely distributed within the collagen network with higher proteoglycan concentration. In the third, deep or radial zone, chondrocytes are largest and a columnar orientation occurs, with collagen fiber orientation radial to the subchondral bone. The fourth zone is called the calci-fied cartilage zone since matrix mineralization is present. On the border between the third and fourth zone the 'tidemark' is discerned. (Reproduced with permission from Orthopaedic Basic Science; The American Academy of Orthopaedic Surgeons, Edt. Simon Sheldon M.D.)

ed problem with an incidence of 2,6 patients per 1000 adults[131]. Defects larger than 2 mm in diameter do not heal, and persistent defects frequent-ly progress to joint degeneration.

Natural healing of cartilage

Spontaneous repair of all musculoskeletal tissue begins with an inflamma-tory response[6,7,60,62,183,185]. Injured cells and platelets release mediators that promote the vascular response to injury. Inflammatory cells help remove necrotic tissue and release mediators that stimulate migration and proliferation of mesenchymal chondrogenic cells. The occurrence of these events during inflammation is critical for initiation of effective tissue repair. In the nonvascular articular cartilage surface this is not adequately

present, therefore many superficial cartilage injuries do not heal. In full thickness defects, cells enter the injury site from the subchondral marrow. These cells provide repair but do not consistently restore the injury with a tissue that has the unique composition, structure, and material properties of normal articular cartilage. The only cell type found in articular cartilage, the highly differentiated chondrocyte, has limited capacity for proliferation or migration because chondrocytes are encased within the tissue. In normal mature cartilage, chondrocytes synthesize sufficient matrix macromolecules to maintain the matrix, and they can increase their rate of matrix synthesis in response to injury or osteoarthritic changes[140]. However, chondrocytes do not synthesize sufficient matrix to repair significant tissue defects[63,64,88], and the matrix macromolecules they synthesize change with increasing age. Another factor that may limit the ability of mature cartilage to repair tissue defects is that the number of chondrocytes declines during aging, thus reducing the capacity of the tissue to repair itself.

Natural healing results in a *repair* that reconstitutes some of the form and function. This is not identical to full restoration of all tissue properties suggested in using the term *regeneration*.

Treatment options for cartilage damage

To restore joint function a large variety of treatment options exists, from conservative measures such as functional adaptation, physiotherapy and medication to surgical intervention ranging from arthroscopic or minimal invasive procedures to whole tissue transplants, joint replacement and more recently tissue engineering. The selected method depends upon presenting complaints and findings at physical examination, diagnostic imaging, patient age, defect characteristics and surgeon preference. In the following section, we discuss the rationale, and results of various strategies[140].

Conservative measures

Non-pharmacological treatment

A meta-analysis of 10 trials comparing patient education and medication demonstrated a significant reduction in pain scores but no improvement in functionality over the medication group[297]. Sufficient indication exists that physiotherapy, adaptation of life style and similar methods provide

considerable pain relief, but no reliable data are available on the long-term prognosis or the modification of progress.

Pharmacological treatment

Non-steroidal anti-inflammatory *medication* is used to decrease pain and reduce synovitis. Improvement of pain and function has proven to be better than in placebo groups, but no lasting effect after two years has been shown[95,316]. Gastrointestinal side effects are considerable, renal function and blood coagulation is affected. To address these side effects and retain a similar level of pain relief, COX II inhibitors were introduced[2,31]. COX II inhibitors have been shown to have a similar therapeutic result as NSAID with gastrointestinal side effects comparable to the placebo group[130,172].

Food supplements such as chondroitin sulfate and glucosamine are available and in use in the United States more than elsewhere. Some indications of a beneficial effect are available[188]. However, the idea of dietary supplementation of these cartilage matrix proteins is not supported by information on long-term efficacy or level of pain relief [23,142,151,188].

Topical treatment with NSAID or Capsaisin is applied in patients with inadequate pain relief from systemic medication or in those who cannot tolerate such therapy. NSAID application was effective in 65% vs. 30% in the control group[93]. Capsaisin modulates the neurotransmitter substance P and thereby attenuates transmission of pain stimuli[321,322]. The agent is well tolerated and provides better relief than placebo.

Intra-articular injections are used in osteoarthritis when signs of effusion or synovitis are present. Corticosteroids have been shown to provide significant pain relief and reduction of effusion for 4-6 weeks. The duration of a possible long-term effect is blurred by confounding factors, such as surgical intervention or parallel treatments, in most studies. Because of a negative effect on cartilage metabolism and acceleration of cartilage damage, it is suggested that these injections should not be the only therapeutic strategy. Hyaluronic acid is a polysaccharide present in normal synovial fluid that provides lubrication and shock absorption. It also plays an important role in embryonic joint formation where it regulates cartilage cavitation[1,14,19,249]. In osteoarthritis, there is a reduction in hyaluronic acid level. Randomized controlled trials have shown pain relief superior to

placebo, comparable to corticosteroids, but lasting longer[133]. Up to 20% of patients experience a flare up of complaints shortly after injection. The exact placement and cost effectiveness of this therapy within a treatment algorithm remains to be determined[82,83]. In current treatment of cartilage defects injection therapy is not used, since these patients are young and not arthritic. In future investigations intra-articular substances could be tested to see whether joint homeostasis may be normalized prior to treatment of a cartilage defect by tissue engineering.

Surgical intervention

As early as 1861 Fergusson reported resection of the joint as treatment for painful dysfunction. Verneuil in 1863 performed the first interposition of fascia, popularized by Campbell between 1920-1930.

Total knee replacement is one of the most successful surgical reconstructive procedures[17,68,110,292]. After attempts since 1940, Gunston was the first to report on modern unconstrained total knee arthroplasty in 1971. A well-implanted, modern joint prosthesis replaces the degenerated osteoarthritic surface and will function properly for 15 to 20 years in a relatively sedentary older patient population. Between 88% and 93% well functioning implants are described at 15 year follow-up[115,205,247,260,292]. Unavoidably at some point increasing symptoms will occur and signs of prosthetic loosening can be detected. With increased loading and more strenuous activities, the longevity of the joint replacement has been suggested to decrease[90]. Revision surgery can be a technical challenge, the outcome of which is considerably inferior to that of primary surgery. Therefore, it is generally accepted that total joint replacement is not a realistic treatment option for young patients.

Lavage and debridement are performed to overcome mechanical impediment by removing loose or unstable cartilage and to limit the rate of degeneration. This indication is currently being scrutinized. Since the introduction of modern arthroscopy in the 1960's, this limited invasive procedure has become widely accepted. At 6,5 years follow-up 68-77% of patients felt there condition had improved or were even cured and that this would be a lasting effect[12]. On the contrary, Moseley[200] demonstrated a similar relief of sham arthroscopy compared to debridement, although the sham group was better two weeks after 'surgery'. Interestingly, the

Figure 1.3

Arthroscopic view of a cartilage defect treated by perforation of the subchondral bone after cleaning of the defect. Drilling or microfracturing will create bleeding into the joint from the marrow cavity, resulting in fibrocartilage filling of the defect. This is considered a safe minimally invasive treatment method that provides relieve from symptoms during a limited period.
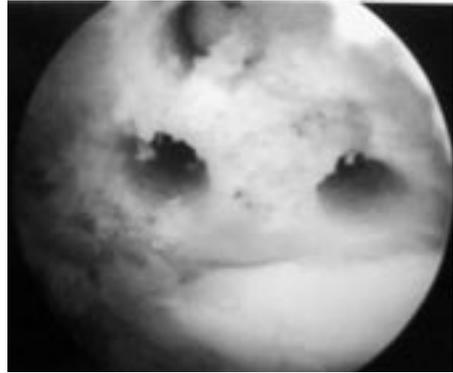
sham group scored similar at one-year follow-up. Overall literature suggests that the operation is palliative not curative and symptomatic relief is achieved for a limited period[176,193].

Perforation of the subchondral bone opens the marrow cavity and provides a source of undifferentiated stem cells that have osteochondrogenic potential. Abrasion arthroplasty[152,154], drilling described by Pridie in 1959[254] and micro fracturing suggested by Steadman[293,294] are similar methods. Under the stimulus of movement and regulated load bearing, bone marrow cells in the clot, on the surface of the exposed bone, will undergo metaplasia to fibrocartilage[149,294]. The techniques used vary considerably which creates difficulty in comparing outcome of such procedures[48]. Beneficial outcome is reported between 45 and even more than 90% of patients, with most techniques. There is a uniform decline in results with duration of follow up. In animal experiments, the repair tissue after subchondral perforation more closely resembles hyaline cartilage than that in large superficial defects[39,143,259,293]. A functional repair of the articulation was seen for up to two years. After 6 months, 100% of patients felt improved while 70% were convinced the effect would be lasting. Most authors agree that these methods provide relief for 3-5 years. These arthroscopic techniques are currently accepted as initial treatment of choice for cartilage defects.

Osteotomy provides relief of symptoms and the possible prevention of (fur-

ther) joint degeneration[74,317]. The goal of such surgical intervention is to decrease the load on the most severely damaged cartilage, to bring regions of the joint that remain intact into opposition with regions that lack articular cartilage, or to correct misalignment or incongruence as in dysplasia[71,116,138,317]. The most frequently performed osteotomies correct valgus and varus deformities of the knee and hip, and correct the anterocranial dysplasia of the acetabulum. A decrease in pain and functional restoration of the joint is seen in relation with local widening of the joint space and a decrease of stress on the damaged region. Negative predictive factors are older age; obesity; ligament instability; over- or under-correction and severe degeneration. Of valgus producing tibia osteotomies, 73-86% good results for a period of 6-10 years have been reported. Even patients deemed optimal candidates for osteotomy given these parameters show deterioration with time at long-term clinical follow-up. Osteotomy is generally indicated in patients too young for arthroplasty with angular deformities of more than 5° and limited walking distance with pain at rest.

Figure 1.4

In osteochondral transplantation cylindrical osteochondral transplants can be used to fill the cartilage defect in the weight bearing area. The osteochondral tissue can be either autologous using smaller plugs from a less loaded area that may be implanted either arthroscopically or by arthrotomy as in this operative view. Alternatively, allograft material can be chosen that allows some degree of matching for location and geometry. (Provided to us by Kln J.H. Postma; Orthopaedic Surgeon, Central Military Hospital. Utrecht, The Netherlands)

Osteochondral techniques involve transplanting single (large, arthrotomy) or multiple (small, arthroscopy) autologous plugs from less loaded areas or using donor tissue[5,21,33,34,80]. A wide range of reasonable to excellent results has been described[11,16,21,69,107,114,127]. Autografts can only be harvested from a limited area and insufficient proof exists that this procedure does not induce arthritic deformation, as matrix integrity and joint home-

ostasis are disturbed. Allograft osteochondral transplants provide the advantage of matching size and geometry of the full defect. Animal experiments have shown good repair, prolonged viability and incorporation. Clinical results in focal post-traumatic defects were described as good to excellent in 27 of 31 patients with a follow-up of two to ten years[191] and successful in 75% at 5 years, 64% at 10 years 63% at 14 years[69,121]. Allograft related factors such as storage influences and disease transmission remain to be addressed.

Tissue Engineering

Autologous cell or tissue transplantation aims to provide a durable regenerate the surface rather than only instigate short term repair[24]. In procedures such as *perichondrial and periosteal grafting*, the defect is cleaned and extended into the subchondral region[10,37,72,73,86,89,99,136]. Periosteum from the proximal tibia or perichondrium from a rib is placed in the bottom of the defect with the cambium layer facing outward towards the joint[163,220,223,229,231,279,308,320]. The mesenchymal stem cells instigate a chondrogenic process that restores both the cartilage surface and the subchondral bone[72,73,105,106]. Using perichondrium, Homminga described good initial results in 85% of patients[136,137]. Bouwmeester[37,38] reevaluated this group at ten years and noted a marked decrease (38% had good results). No longer could a difference be found when comparing the perichondrial-grafted patients to a matched group that was treated by arthroscopic drilling. The addition of Indometacin (a non-steroidal anti inflammatory agent) during 2 days post operatively prevented the calcifications of the grafts even as long as up to 5-7 years post surgery. Some information on the clinical outcome of periosteal grafts is available. Engkvist described the use of periosteum in resurfacing arthritic joints with good symptomatic relief[100,103]. Angermann described 14 consecutive periosteal transplantations evaluated 1 year postoperatively, 9 knees were pain-free, but with 6-9 years follow-up, only 2 knees were pain-free. Six knees had developed arthrosis. Hyaline-like cartilage was documented in 1 patient and assessed as possible in 1 more, but in 10 patients the tissue formed in the defects was not hyaline cartilage[13]. Fifty-seven consecutive patients with isolated full-thickness patella defects were treated by autologous periosteal transplantation to the cartilage defect. When treated with continuous passive motion (CPM), after a mean follow-up of 51 months 29 patients (76%) were graded as excellent or good, 7 patients (19%) were
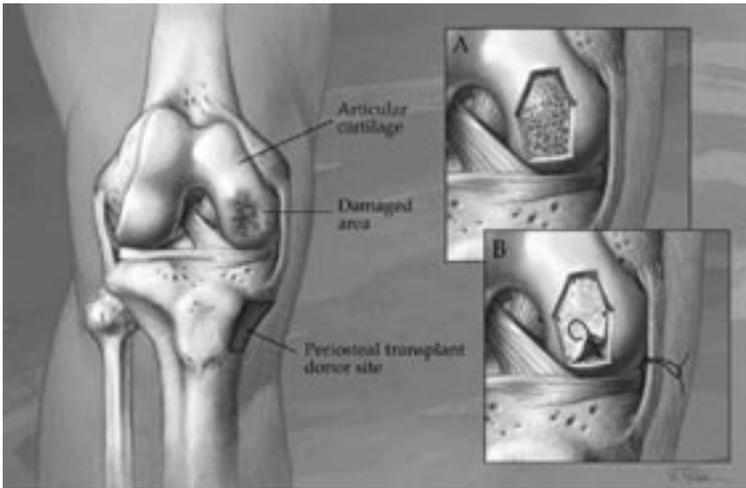
Figure 1.5

Surgical drawing of the technique for periosteal transplantation as described by O'Driscoll. Periosteum is harvested from the proximal tibia and sutured into the bottom of the defect with resorbable sutures through bone tunnels, with the cambium layer facing outward into the joint. (Reproduced with permission from S.W. O'Driscoll, Mayo Clinic Rochester MN, USA)

graded as fair, and 2 patients (5%) were graded as poor. With active motion, but without CPM, 10 patients (53%) were graded as excellent or good, 6 patients (32%) were graded as fair, and 3 patients (15%) were graded as poor. The authors conclude that results are good if CPM is used postoperatively, but results using active motion postoperatively are not acceptable, especially not in patients with chondromalacia of the patella[178]. These data are consistent with the findings of O'Driscoll and Salter on the effect of CPM stimulating periosteal chondrogenesis in rabbits[220,229,230]. The indicative clinical data of 35 defects in 20 knees since 1985 presented by O'Driscoll in 2002 suggest 60% improvement at 1-8 years, 40% not better and non were worse. There were 12 reoperations overall and the author summarized the results as 'disappointing for sure'.

Autologous chondrocyte implantation is the most commercially involved strategy. Chondrocytes are harvested from a less weight bearing area of the knee. Cells are culture expanded during 3-4 weeks and reimplanted
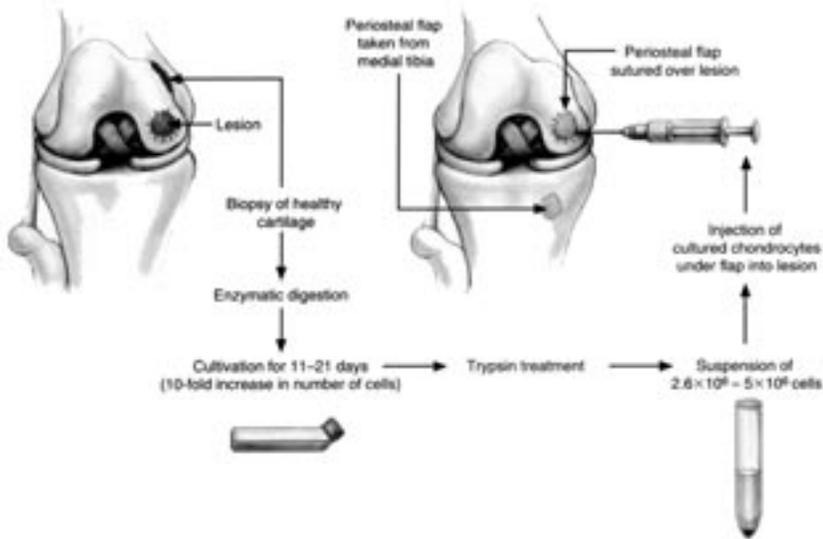
Figure 1.6

Autologous chondrocyte transplantation as described in the clinical application by Brittberg et al. Chondrocytes are harvested arthroscopically and expanded in culture. After 3-4 weeks second stage surgery (arthrotomy) is performed. A periosteal flap from the proximal medial tibia is sutured into the defect with the cambium layer facing inward and the cells are injected under the flap. (Reproduced with permission from the principal author M. Brittberg)

under a periosteal covering sutured into the surrounding cartilage on top of the transplanted cells. Both small and large animal models were used and results are similar: short to mid term follow-up experiments evaluating the percentage of defect filling, quality of cartilage and incorporation into surrounding defect show 70-85 % good results[20,34,43,117,118,194,248]. Results decrease somewhat with longer follow-up. Peterson (ICRS 2002) described 1067 defects since 1991 (MFC 387, LFC 160, pat 253) with more than 200 patients at 7,5 year average follow-up (2-11 yrs) 78% were good to excellent in the total group while 90% were good to excellent in the MFC group. Poor results were seen in 22% if the patients were good after 2 years they were still good at 11 years. After the introduction by Brittberg and colleagues[43], a staggering amount of replications of experimental work on cartilage defect repair using chondrocytes with or without matrices has been published with varying results.

The clinical results as demonstrated by Brittberg and co-workers in fol-

low-up of up to 9 years in 102 patients are encouraging[42,43,248]. Furthermore, these authors showed that there was a correlation between the quality of the biopsy material and the clinical results. There are, however, several problems, which limit the efficacy of this technique: The cells may not survive and multiply in culture. The cartilage cells in culture may undergo dedifferentiation to fibroblasts. The fate of the implanted cells is uncertain. The periosteal covering provides growth factors and contains cells that can contribute to the repair, but also undergo calcification and hypertrophy.

Commercial interest is considerable as can be deducted from patient registries kept by industry, which document over 4500 patients treated, and from the large number of biotechnology companies that have launched a cartilage repair product. At this time, more than 16 companies both in Europe and the United States are offering one or more options for cartilage repair. First generation products comprise cultured cells to be implanted under a periosteal flap or collagen sheath. Second generation products aim at implantation in a (mechanically) solid matrix thereby enabling minimal invasive or even arthroscopic implantation.

Only some initial prospective scientific data are available. These publications indicate a limited beneficial effect over drilling, as did perichondrial grafts[38]. At the 2002 ICRS it was reported that clinical results may in some cases not even be better. However, these clinical results were never as good as expected from animal experiments, with dedifferentiation, side-wall debonding and periosteal hypertrophy and calcification being the main drawbacks. Unfortunately, a realistic outcome of clinical cartilage repair can only be judged after 5-10 years or more, and this long-term requirement is in conflict with the short time commercial interests of companies involved. Long-term randomized, prospective trials have not been started. Obviously such comparisons and subsequent analysis of cost effectiveness would be necessary to progress towards solid implementation of tissue engineering as a clinical treatment strategy.

GOAL:

The purpose of this study is to determine the validity of histological scoring systems used for scoring cartilage repair.

HYPOTHESIS:

The simple Pineda score is more reliable than the O'Driscoll score, which provides more information.

RATIONALE:

Many cartilage repair scores have been introduced, modified and applied. Some authors do not describe the rationale for modifications; compare their outcome to other scores and to the original score without further scrutiny. In the original descriptions of two of the most frequently applied cartilage repair scoring systems, no information on inter- and intra-observer variability was provided, nor is this available from subsequent literature. Other 'standard scores' were shown not to be as reliable as needed. Validation of these scores is essential for the study proposed in chapter 3.

METHODS:

Histological sections of cartilage defects with a varying quality of repair were evaluated. Three observers applied two scoring systems at two points in time. Using the graphic calculation methods described by Bland and Altman, and applying the criteria formulated by Landis and Koch the intra-observer and inter-observer variability as well as the correlation between the scoring systems were determined.

CHAPTER
2

THE CORRELATION AND REPRODUCIBILITY OF
HISTOLOGICAL SCORING SYSTEMS IN CARTILAGE REPAIR

INTRODUCTION

In the last few decades a rapidly increasing number of investigators have studied the process of cartilage healing and communicated to each other on repair and attempts at regeneration. This stream of publications has increased since the seventies and grew near exponentially in the eighties and nineties, leading to over one hundred and fifty scientific publications on articular cartilage repair during the past twelve months.

In these investigations, different methods of regeneration were developed, tested and reported. The difficulty with evaluating and comparing these results is finding a good (semi-) quantitative system for grading the healing process of cartilage defects. Such a system must be a reliable indicator of the pathophysiological condition of the tissue under investigation; furthermore, it should have good reproducibility within and between observers and preferably correlate with similar scoring systems to allow comparisons between investigations.

As research activities increased, multiple histological scoring systems in cartilage repair research were introduced, modified and used to evaluate results. Two of these are since then more frequently reported, the system of Pineda[250] (table 2.1) and the score by O'Driscoll[220,221] (table 2.2). Although these scores have been widely used during the past decade, there is so far no publication of their reliability and reproducibility to be found in literature. Such an evaluation is however essential for progress and quality of research in this field.

After wide implementation and general acceptance, the Histologic/ Histochemical Grading System (HHGS) for osteo-arthritis monitoring, developed by Mankin,[187] was evaluated for the inter- and intra-observer variability. In these investigations Ostergaard[240,241] and Van der Sluijs[306] demonstrated that, though acceptable, the intra- and inter-observer reliability of this scoring system was not as good as expected.

The purpose of this chapter is to demonstrate the validity of histological scores selected for use in a subsequent *in vivo* experiment further described in *Chapter 3*. For this purpose two frequently used histological cartilage repair scoring systems were compared for their inter- and intra-observer variability as well as their correlation. This comprises important knowledge relevant for cartilage repair investigations since a good qualification of the scoring systems enables researchers to make more reliable comparisons between different publications and different methods in cartilage tis-

sue engineering. Neither O'Driscoll, nor Pineda included such validation in their original description of the scoring system, nor were they subsequently compared in literature.

Characteristics	score
Filling of defect	
125%	1
100%	0
75%	1
50%	2
25%	3
0%	4
Reconstruction of osteochondral junction	
Yes	0
Almost	1
Not close	2
Matrix staining	
Normal	0
Reduced staining	1
Significantly reduced staining	2
Faint staining	3
No stain	4
Cell morphology	
Normal	0
Most hyaline and fibrocartilage	1
Mostly fibrocartilage	2
Some fibrocartilage, but mostly nonchondrocytic cells	3
Nonchondrocytic cells only	4

Table 2.1
Cartilage repair score by Pineda and co-workers

Characteristics	score
Nature of predominant tissue	
Cellular morphology	
Hyaline articular cartilage	4
Incompletely differentiated mesenchyme	2
Fibrous tissue or bone	0
Safranin-O staining of the matrix	
Normal or nearly normal	3
Moderate	2
Slight	1
None	0
Structural characteristics	
Surface regularity	
Smooth and intact	3
Superficial horizontal lamination	2
Fissures 25 to 100 percent of the thickness	1
Severe disruption, including fibrillation	0
Structural integrity	
Normal	2
Slight disruption, including cysts	1
Severe disintegration	0
Thickness	
100 percent of normal adjacent cartilage	2
50-100 percent of normal cartilage	1
0-50 percent of normal cartilage	0
Bonding to the adjacent cartilage	
Bonded at both ends of graft	2
Bonded at one end, or partially at both ends	1
Not bonded	0
Freedom from cellular changes of degeneration	
Hypocellularity	
Normal cellularity	3
Slight hypocellularity	2
Moderate hypocellularity	1
Severe hypocellularity	0
Chondrocyte clustering	
No clusters	2
<25 percent of the cells	1
25-100 percent of the cells	0
Freedom from degenerative changes in adjacent cartilage	
Normal cellularity, no clusters, normal staining	3
Normal cellularity, mild clusters, moderate staining	2
Mild or moderate hypocellularity, slight staining	1
Severe hypocellularity, poor or no staining	0

Table 2.2
Cartilage repair score by O'Driscoll and co-workers

MATERIALS & METHODS

Cartilage samples:

Four hundred and fifty six observations of articular cartilage were made. These samples (n=38) were obtained from the *in vivo* goat study described in *Chapter 3*. The samples represented the full range from normal to severely disrupted articular cartilage, with varying degrees of repair after periosteal transplantation.

Histological processing:

Immediately after harvesting samples were fixed in 4% buffered formalin and decalcified. The cartilage samples were embedded in paraffin and from the center of each sample 3μm thick sections were cut. Three slides from each cartilage sample were stained with Haematoxilin-Eosin, Alcian Blue or Safranin O-Fast Green.

Examination:

All samples were examined under direct light-microscopy by each of the observers. For each of the grading scales, three observers examined all samples twice. Between observations there was an interval of at least one week. The group of observers consisted of three orthopedic researchers with different levels of experience in cartilage research, ranging from inexperienced to experienced. The observers were asked to examine and grade the samples according to the two different grading systems. Before examining the experimental cartilage samples, the observers were allowed to become familiar, by self-education, with each of the grading-systems using sections of relevant tissue not included in the current investigation. All samples were presented to the observers in a blinded and random order.

Statistical analysis:

The statistical methods that were used to calculate the intra- and inter-observer reproducibility were based on the graphic techniques and calculations as described by Bland and Altman[32]. The intra-observer reliability was calculated from the difference between the two scores from that observer as compared to the mean of that section. For the inter-observer statistics we determined the difference between observer scores for a given section versus the mean for that tissue sample. Correlation between the two scores was determined from linear regression analysis of the average

scores for each cartilage sample. Correlation and Kappa values are inter-preted according to the guidelines described by Landis and Koch[171]: <0.00 as poor, 0.00-0.20 as slight, 0.21-0.40 as fair, 0.41-0.60 as moder-ate, 0.61-0.80 as substantial and 0.81-1.00 as almost perfect.

RESULTS

O'Driscoll cartilage repair score

The intra-observer variability was very low (figure 2.1 A). The average difference between the first and the second observation was very small, 0.05 (range: -0.08 to 0.18). The standard deviation of the observations was 0.93 (range 0.85 to 1.06) within a 24 point score. Kappa values were
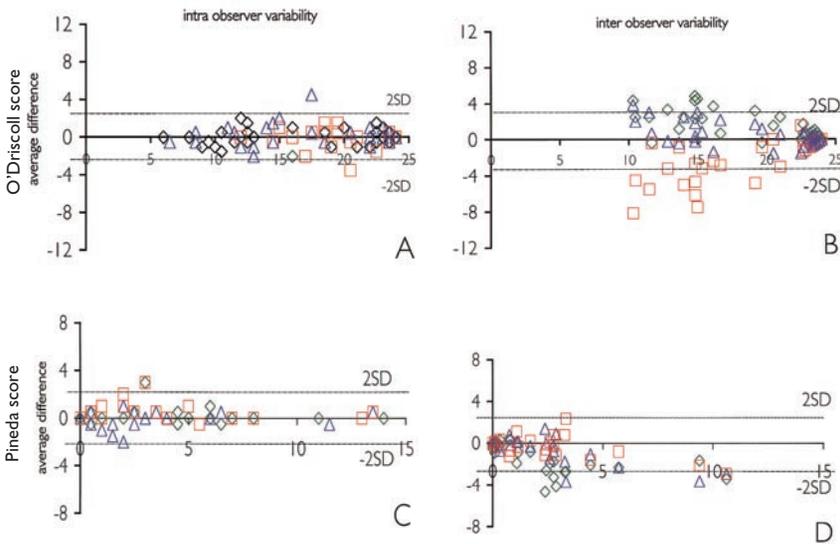

Figure 2.1 A-D

Intra- and inter-observer variability (columns) for both the O'Driscoll system (top row, A&B) and the Pineda score (bottom row, C&D) for all three observers. The difference between the first and second observation for each observer is plotted against the average score for that sample (A&C). Similarly the difference between observations and the average for all observers is plotted against the mean score for a given sample (B&D). Each observer is represented by a different symbol, the dotted line represent the plus or minus two standard deviation level.

high for all three observers; 0.92 for observer A, 0.78 for observer B and 0.87 for observer C. This results in an overall kappa value for the O'Driscoll score of 0.87, which is qualified as almost perfect. The overall kappa value for the inter-observer reliability scored even higher (figure 2.1 B) with 0.92, an average difference of 0.001 and a standard deviation of 2.25. All results are summarized in table 2.3.

O'Driscoll score						
	Intra-observer			Inter-observer		
Observer	r^2	Average Diff	SD	r^2	Average Diff	SD
A	0,92	0,04	0,86	0,98	1,39	1,54
B	0,78	-0,08	0,85	0,89	-1,89	2,36
C	0,87	0,18	1,06	0,96	0,50	1,22
Overall	0,87	0,05	0,93	0,92	0,00	2,25

Pineda score						
	Intra-observer			Inter-observer		
Observer	r^2	Average Diff	SD	r^2	Average Diff	SD
A	0,91	-0,38	0,55	0,88	0,91	0,73
B	0,86	0,75	3,17	0,82	1,21	1,36
C	0,80	0,37	0,53	0,94	0,76	0,98
Overall	0,86	0,86	1,38	0,89	0,82	0,96

Table 2.3
Overall summary of the results from the intra- and inter- observer variability analysis, as well as the correlation between observers and scores.

Pineda cartilage repair score
This system had a small overall average difference (0.86) for the intra-observer reliability (range -0.38 to 0.75) (figure 2.1 C). The standard deviation (SD) within the 14 point score was somewhat higher at 1.38 (range 0.53-3.17) as compared to that in the O'Driscoll score. This SD is

somewhat confounded by the fact that one of the less experienced observers had a SD of 3.17, whereas the other two observers revealed a SD of 0.53 and 0.55 respectively. Again, results show high kappa values; 0.91 for observer A, 0.86 for observer B and 0.80 for observer C, leading to an overall kappa value of 0.86. For the overall inter-observer reliability the kappa value was high, 0.89, average difference 0.82 and SD=0.96 (figure 2.1 D).
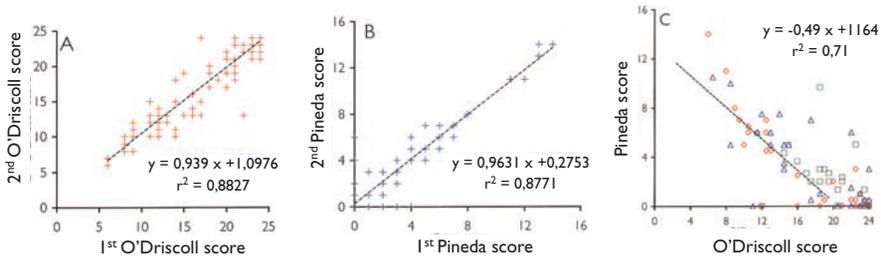

Figure 2.2 A-C

These graphs depict the result of regression analysis determining the correlation between the first and the second observation of each score (A&B) as well as between the mean O'Driscoll score and the mean Pineda score for each sample showed a favorable inversely proportional correlation (C). Kappa values and correlation presentation are provided in each graph. According to Landis and Koch such values are to be considered as representing a substantial correlation.

Correlation between scores

We compared the results of the O'Driscoll system with the results using that of Pineda to determine the amount of correlation between them. Regression analysis determining correlation between the mean of observations with each of two scoring systems showed a considerable correlation. The correlation in all three comparisons is depicted in figure 2.2 A-C. According to Landis and Koch, such values are considered a 'substantial' correlation.

DISCUSSION

Based upon our current observations, the system by O'Driscoll proved to be a reliable scoring system for grading cartilage repair as it was shown to have both very high intra- and inter-observer reproducibility (kappa values of 0.87 and 0.92 respectively). For the system by Pineda, we found comparable values for both items (0.86 and 0.89 respectively). According to the guidelines described by Landis and Koch[171] all these results can be classified as almost perfect. For both systems, the average difference between observations as well as the standard deviation within the score range per sample was low. The high standard deviation of observer B on the Pineda system may be attributed to the relative lack of experience of this observer with this system. Those results did not lead to significant loss of discerning capability.

We may conclude that both these systems for scoring cartilage repair are reliable semi-quantitative systems for grading the healing process of cartilage defects. We had originally hypothesized that the system by Pineda would show better reproducibility because of simplicity. This hypothesis was shown to be incorrect. The system by O'Driscoll proved to be as reliable even though it is more complex. This similarity can in part be explained by the larger number of parameters included in the overall score. When the observers assess one or two parameters differently, using the O'Driscoll system, this could be compensated by a multitude of other parameters. The Pineda system, with only four parameters, does not allow for such possibilities.

To assess the validity of comparison between the two systems, we looked at the correlation between them; we found they were inversely proportionally 'substantially' correlated ($r^2=0.71$), according to Landis and Koch. All observers judged the more extensive O'Driscoll system to be a bit more complex, because of the larger number of parameters to assess. We initially felt that investigators should perhaps gain more experience to use it as efficiently as compared to the simpler Pineda system. However, the statistical analysis showed almost perfect reliability, even with an inexperienced observer, and there was no difference when compared with the Pineda system. Finally the O'Driscoll system does provide considerably more information about the exact quality of the cartilage repair due to the larger number of included parameters, and allows comparisons on subitems. This may be of interest when comparing the results of different tis-

sue engineering strategies.

Both the cartilage repair scoring systems as first presented by O'Driscoll and the score developed by Pineda are reliable semi-quantitative cartilage scoring systems with good correlation. There is no apparent need to apply modifications or to develop new systems given their successful application during many years. These systems will increase reliability and to some extent give us the opportunity for comparisons between studies, which is of benefit for better understanding of cartilage repair publications and the impact of their results.

GOAL:

To study the influence of metabolic alterations in joint homeostasis on the outcome of cartilage repair by comparing untreated, early treated and late treated defects.

HYPOTHESIS:

The presence of a persistent articular defect causes cartilage degeneration in the rest of the joint. The subsequent alteration of joint homeostasis will have a detrimental effect on cartilage formation by tissue engineering. Early intervention may prevent cartilage degeneration so homeostasis is retained and thereby improve outcome.

RATIONALE:

Upon reviewing the literature, it becomes apparent that results from *in vitro* and *in vivo* experiments are considerably better than clinical results. Typically, fresh defects in an otherwise healthy articulation are studied. Most patients present some time after trauma with prolonged complaints of pain, effusion and even previous surgical intervention. In these joints, homeostasis is disturbed to an environment less conducive for successful repair.

METHODS:

A large animal model in the Dutch Milk Goat was developed to compare the results of natural healing of a cartilage defect with the outcome of early treatment in the healthy joint and late treatment in the presence of disturbed joint homeostasis. Proteoglycan synthesis, release and retention were measured to quantify metabolism. To test our hypothesis the histological cartilage repair score was related to these metabolic parameters of homeostasis.

CHAPTER
3

JOINT HOMEOSTASIS INFLUENCES CARTILAGE REPAIR

INTRODUCTION

Various authors have described the incidence of relevant cartilage lesions, the lack of adequate healing and subsequent indications for surgical intervention[43,128,185,256]. Durable restoration of damaged articular cartilage is a valuable but as yet unachieved goal[44,48,49,186,192,194,215,228,232,268]. The need for cartilage repair and use of tissue engineering strategies for restoration of an articular cartilage defect have been well established. Different strategies for tissue engineered cartilage repair were conceived and established by extensive basic scientific, *in vitro* investigations, *in vivo* animal experimental and even initial patient outcome studies[37,43,49,194,208,209,215,248]. However upon reviewing the literature, as described in the treatment strategy section of *Chapter 1*, an obvious discrepancy exists between the favorable outcome demonstrated in preclinical research and clinical practice, where these results have not yet been reproduced. Only a few examples of reliable long-term clinical results are available and the initial patient results that sparked enthusiasm in the field are significantly less favorable at long-term follow-up[37,38].

Most if not all good results published, originate from an optimally controlled laboratory environment or animal experimental work with normal articulations where a fresh cartilage defect was treated by tissue engineering. *In vivo* studies with only a periosteal/perichondrial flap or with cultured chondrocytes under a periosteal or collagen cover demonstrate restoration of the articular surface, with histological and biochemical analyses indicating a regeneration tissue in the defects with hyaline cartilage resemblance. In the clinical setting more disappointing results are seen. There is a tendency for incomplete bonding between the tissue-engineered construct and the wall of the original defect. Surface restoration is only partial. The periosteal flap may undergo hypertrophy and calcification of the outer surface[248]. Eventually the new matrix will show loss of normal metabolic activity.

We hypothesize that a delay in treatment of an old defect, which permits it to initiate cartilage degeneration and cause changes in joint homeostasis, will have a negative effect on the process of cartilage repair. To test this hypothesis we used a large animal model in the goat, and compared cartilage formation between an untreated defect and after periosteal transplantation in a defect treated early with normal homeostasis or a defect treated late when homeostasis was disturbed.

MATERIALS AND METHODS

Experimental design:

Twenty one adolescent, female Dutch Milk Goats (average age 6.2 months, 5.9 - 6.5, 22.8 ± 2.1 kg) were acquired from a commercial vendor and kept in group housing for a minimum of 2 weeks before surgery. All experiments were approved and monitored by the institutional animal experimental ethics committee. In all animals a defect was made in the medial femoral condyle[148] (figure 3.1 A), the contra lateral knee was left untreated to serve as control. Subsequently animals were randomized into one of three groups:

- **No treatment**: Natural healing was studied in the 'No treatment' group where the defect was left untouched. Unrestricted weight bearing and motion were stimulated; animals were terminated after 10 weeks of follow-up.
- **Early treatment**: Cartilage repair in a knee with normal homeostasis was studied in the 'early treatment' group, where the fresh defect was immediately transplanted (figure 3.1 B). Animals were terminated after 10 weeks of follow-up.
- **Late treatment**: The defect in the 'late treatment' group was left untouched for a period of 10 weeks, allowing free weight bearing. After this period the cartilage scar was resected (figure 3.1 C) and transplantation was performed identically to the previous group, but in an environment of disturbed homeostasis. Animals were terminated 10 weeks after transplantation.

Surgical technique:

All animals received premedication consisting of Detomidinehydrochloride (10 mg/kg, Pfizer). Subsequent induction of anesthesia was achieved with Thiopental (50 mg/kg, Rhône Mérieux). Throughout the surgical procedures anesthesia was maintained on a Magill system with O_2-NO_2 1:2 and Halothane 1-1,5% (Zeneca). Postoperative pain medication (Buprenorfinehydrochloride 0,15 mg daily, Schering-Plough) and antibiotic prophylaxis (Amoxicillin 15 mg / kg, Pfizer) were given for a period of 5 days. After shaving, standard aseptic measures and draping, a medial Para patellar exposure was used. After retracting the patella laterally, the medial femoral condyle could be exposed. A standardized full thickness cartilage defect of 0.8 x 0.5 cm was made in the medial femoral condyle using as
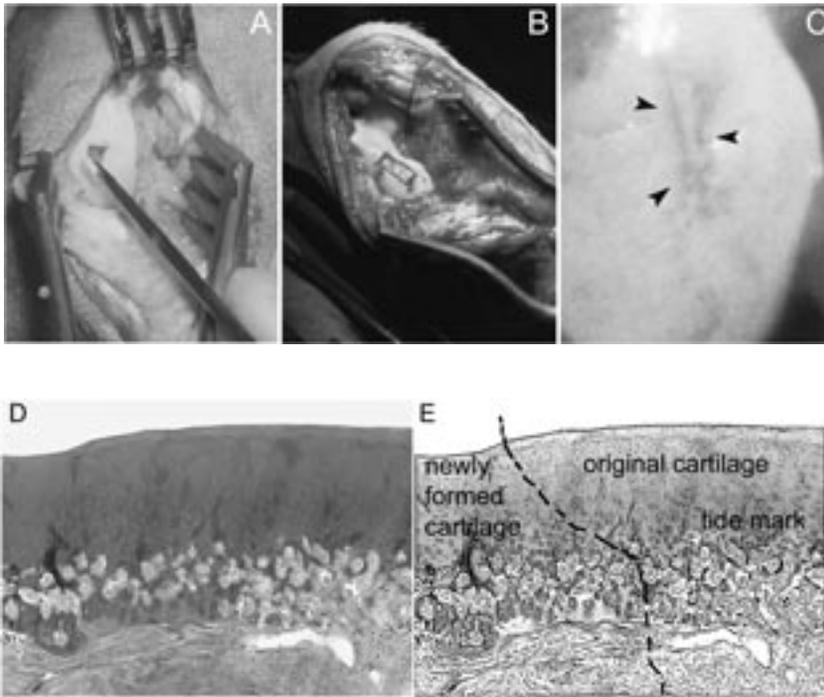
Figure 3.1

Examples of surgical technique, sutured defect, macroscopic and histological results in the goat defect model. Panel A: represents and operative view of making the standardized defect in the medial femoral condyle. Panel B: shows the defect treated with a periosteal graft obtained form the proximal tibia, sutured into the bottom of the defect, with the cambium layer facing into the joint. Panel C: demonstrates the macroscopic aspect of the natural 'healing' process after 10 weeks of unlimited weight bearing and motion, of a defect left untreated. Panel D: The best repair result seen after periosteal transplantation in the 'Early treatment' group. Panel E: schematic representation of the histological section in D to show the margin of the cartilage defect and near perfect restoration of cartilage surface subchondral bone and the tidemark.

See CD for full color representation

sharp tissue elevator (figure 3.1 A). The periosteal tissue was harvested from the proximal, medial tibia using a standardized technique[45,218,227]. From the graft thus acquired, a 2 mm by 10 mm strip was sent for histological analysis to confirm adequacy of the periosteal cambium layer in the graft. The tissue graft was sutured into the bottom of the defect with the cambium layer facing outward into the joint using non-traumatic 3.0

Characteristic	Value
Range of motion	
Full	2
< 20 decrease	I
> 20 decerease	0
Intra articular fibrosis	
None	2
Minor	I
Major	0
Restoration of contour	
Complete	2
Partial	I
None	0
Cartilage erosion	
None	2
Graft	I
Graft + cartilage	0
Appearance	
Translucent	2
Opaque	I
Discolored / irregular	0

Table 3.1

The macroscopic articular evaluation parameters as described by O'Driscoll

resorbable Vicryl (Ethicon-Johnson & Johnson) sutures, through predrilled bone tunnels (figure 3.1 B).

Imaging studies:

Preoperatively, immediately post operatively and at 5-week intervals during the study as well as upon completion of follow-up, AP and lateral knee radiographs (15 msec / 25 kV) were obtained to visualize the growth plate and generally detect postoperative complications as well as developing subchondral sclerosis.

Macroscopic outcome evaluation:

To quantify intra-articular adhesion, stiffening and general appearance of the joint, all knees were scored using the macroscopic outcome parameters as described by O'Driscoll[220,221,229] (table 3.1). The *in vivo* parame-

ters as applied after completion of follow up contain five categories within which an individual value of 0-2 points can be awarded.

Biochemical assays:

For biochemical analysis, cartilage explants were obtained from the weight bearing articular surface surrounding the defect (figure 3.2) in a standardized fashion[168]. These explants were cultured individually in DMEM (D-MEM, Gibco 074-01600; 0.81 mM SO_4^{2-}; 24 mM $NaHCO_3$) supplemented with ascorbic acid (0.85 mmol/l), glutamine (2 mmol/l), penicillin (100 IU/ml), streptomycin sulfate (100 IU/ml), and 10% heat inactivated, pooled female goat serum, on standard ninety-six well plates (200μl culture medium/well, 37°C, 5% CO_2 in air) according to previously published procedures[168].

DNA content was determined as a measure for cartilage cellularity. Samples were digested in papain (2h; 65°C). DNA was stained with fluorescent dye (Hoechst 33528) and measured using calf thymus DNA as a reference[166].

Proteoglycan content was determined by measuring the total amount of glycosaminoglycans (GAG). GAGs in the papain digest were precipitated and stained with Alcian blue for subsequent photometrical analysis. The GAG content was expressed in mg GAG normalized to the wet weight of the cartilage explants (mg/g). Proteoglycan synthesis rate was measured by evaluation of sulfate incorporation after 1 hour of pre-incubation. 148 kBq $NA_2{}^{35}SO_4^{2-}$ (Dupont, NEX-041-H, carrier free) in 10 μl DMEM was added to 200 μl incubation medium containing the explants. After 4 hours of labeling, the samples were digested in papain. Glycosaminoglycans were precipitated by addition of cetylpyridinium chloride (CPC). The $^{35}SO_4^{2-}$ radioactivity was measured by liquid scintillation counting. The incorporation rate was calculated from the specific activity of the medium and normalized to the cartilage wet weight. Values are expressed in nmol of sulfate incorporated per hour per gram of cartilage wet weight (nmol/h.g). Alcian blue precipitation and scintillation counting determined Proteoglycan release of both the total amount and the newly synthesized PG's. Cartilage samples were labeled with 370 kBq/200μl of ^{35}S-sulfate as described above. After 4 hours of labeling, the samples were rinsed 3 times in fresh culture medium (37°C) and incubated for a subsequent 3 day period in the absence of label. The GAGs in the culture medium were precipitated with Alcian blue. The total amount of GAG released

was determined by spectrophotometrical quantification of blue staining. The percentage release was calculated from the total amount of GAG released and the initial GAG content of the tissues. The amount of newly formed GAG released was determined by scintillation counting of SO_4^{2-} and is expressed in nmol/g of wet weight during 3 days[168].

Figure 3.2

Schematic representation of the harvest locations of cartilage samples for biochemical and histological analysis (Courtesy of reumatology research laboratory at the UMC Utrecht). Centrally (H) lie the medial or lateral femoral defect with the opposing tibia cartilage that was harvested for histological analysis and scoring to analyze the effect of cartilage damage and the outcome of repair. The surrounding tissue was harvested in standardized sequence and samples distributed evenly into groups for biochemical (b) and histological analysis to determine the onset of early cartilage degeneration.
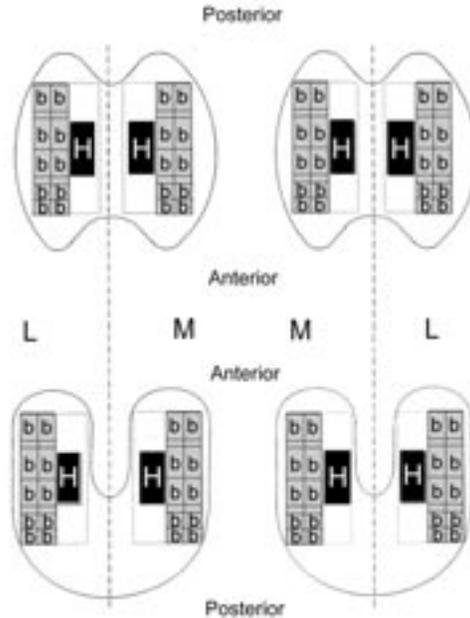
Histological samples:

Upon completion of the 10 or 20 week follow-up period, all animals were terminated by an overdose of Thiopental. Synovial fluid, synovial lining tissue and cartilage samples were gathered according to a standard explantation layout as depicted in figure 3.2. From the center of each cartilage sample, multiple 3 μm sections were cut and stained with either; Haematoxilin-Eosin, Alcian Blue or Safranin O-Fast Green. Generalized cartilage degeneration, filling of the defects and appearance of synovial tissue were scored using the previously described criteria by O'Driscoll (see earlier table 2.2) that were validated[201] for their use in *Chapter 2*.

Statistical analysis:

The minimally required sample size was calculated to be n=6, Seven animals were included in each of the three study groups. Repeated measures analysis of variance controlled for the individual animal and location of the tissue within the joint, and post hoc testing using Duncan Multiple Range as well as Student-Newman-Keuls test, and a 2-tailed Wilcoxon test were used to determine potential differences between groups at a confidence level of 95%. Data are represented as means ± 1 standard deviation unless otherwise stated.

RESULTS

General findings:

Two animals died on the day of initial surgery due to anesthesia related, early post-operative complications (one in the 'early treatment' and one in the 'late treatment' group, thus 6 animals remained for both periosteal transplantation groups and 7 in the control group). All other animals recovered quickly and uneventfully from anesthesia and the surgical procedure. Within four hours after surgery, the animals ambulated with intermittent, partial weight bearing on the operated hind leg. From the fifth post-operative day normal weight bearing was seen on all four legs. In some animals a brief period of limping and effusion was noted between day 8 and 14, there was no correlation to the experimental

Figure 3.3

We compared the difference between control knees that were not operated and the experimental knees that were divided in 'No treatment', 'Early treatment' or 'Late treatment' using the criteria in table 3.1. There were considerable but not statistically significant differences with a higher score (better result) in the 'Early treatment' group as compared to the 'No treatment' and 'Late treatment' group. Furthermore, the standard deviation in the 'Early treatment' group was lower than in the two other groups, which may suggest a more reliable process of articular surface repair.

Characteristic	Experimental group					
	No treatment		Early treatment		Late treatment	
Control (C), Experimental (E)	C	E	C	E	C	E
n=	7	7	6	6	6	6
Range of motion						
Full	7	4	6	6	6	4
< 20 decrease	0	3	0	0	0	2
> 20 decerease	0	0	0	0	0	0
Intra articular fibrosis						
None	7	2	5	1	6	1
Minor	0	3	1	5	0	4
Major	0	2	0	0	0	1
Restoration of contour						
Complete	7	0	6	2	6	1
Partial	0	3	0	3	0	3
None	0	4	0	1	0	2
Cartilage erosion						
None	6	0	5	1	4	0
Graft	0	0	1	2	2	3
Graft + cartilage	1	7	0	3	0	3
Appearance						
Translucent	6	0	6	1	5	0
Opaque	1	3	0	4	0	3
Discolored / irregular	0	4	0	1	1	3

Table 3.3

Description of macroscopic findings using O'Driscoll' criteria. Five parameters were scored from 0-2 points for each knee of all animals in the three groups. This table represents the number of animals in a group that were given a corresponding score.

group. No fractures or other complications were noted on radiological examination. All defects caused radiologically visible alteration in the sub-chondral bone, most notably in the 'No treatment' group.

Macroscopic evaluation of explanted knees:

The articular surface contour was disturbed in both the 'No treatment' and the 'Late treatment' group with discoloration of the cartilage surface. An increased frequency of intra-articular fibrosis with decreased range of motion resulted (table and figure 3.3).

Biochemical analysis:

The cartilage defects disturbed joint homeostasis by causing reproducible early osteoarthritic alterations in all groups. There was a differential pattern of significantly increased proteoglycan (PG) synthesis ($p=0.018$, figure 3.4). This was most pronounced in the cartilage of the area surrounding the defect in the medial femoral condyle as well as the opposing tibia surface, but also present in the lateral joint compartment.

Figure 3.4

Proteoglycan synthesis by wet weight as determined with $^{35}SO_4$ uptake studies demonstrates the significant increased synthesis rate measured in the surrounding cartilage after making a standardized defect in the medial femoral condyle. (Sampling location: both femur (F) and tibia (T), either lateral (L) or medial (M). C: control, E: experimental.
$* = p \leq 0.05$, ns $= p > 0.05$)
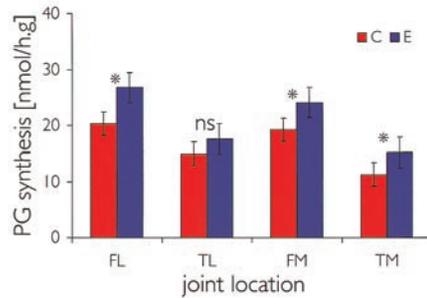
The total amount of PG synthesis increased significantly and to a similar rate in all experimental groups (figure 3.5, table 3.4). The release of glycosaminoglycans (GAG) from the matrix was significantly increased in the cartilage surrounding the 'No treatment' ($p=0.018$) and 'Late treatment' group ($p=0.028$), while no significant loss was found in the 'Early treatment' group (figure 3.6, table 3.4).

There was a significant decrease in total GAG content indicative of a loss of matrix integrity, in both the 'No treatment' group ($p=0.032$) as well as the 'Late treatment' group ($p=0.046$, figure 3.7, table 3.4). We found no significant increase in GAG release nor a decrease in total GAG content in the 'Early treatment' group.

Histological evaluation:

Hematoxillin / eosin staining and microscopic examination revealed that all periosteal flaps contained an adequate cambium layer upon transplantation into the bottom of the defect. Safranin O / Fast greenstaining of control and experimental defects using the O'Driscoll score demonstrated

Group	No treatment	Early treatment	Late treatment
Proteoglycan synthesis contr.	19.3 ± 1.9	10.8 ± 2.5	14.2 ± 0.8
expt.	24.1 ± 2.9	13.3±2.5	18.8 ± 1.0
p value	<0.018	<0.028	<0.015
GAG release contr.	2.0 ± 0.1	1.3 ± 0.4	2.3 ± 0.2
expt.	3.0 ± 0.2	1.8 ± 0.5	3.8 ± 0.2
p value	<0.018	n.s.	<0.028
Total GAG content contr.	35.9 ± 1.1	32.2 ± 2.3	32.2 ± 2.3
expt.	33.1 ± 2.3	30.9 ± 2.2	27.8 ± 1.8
p value	<0.032	n.s.	<0.046

Table 3.4

Biochemical analysis data demonstrate the determined values for each of the biochemical analyses and for the three study groups. Standardized sampling locations were used from both femur and tibia, lateral and medial. Paired data per animal and location within the joint were analyzed, results from the various locations demonstrated considerable statistical similarity. Data represented in this table are from the medial femoral condyle.

Figure 3.5

The proteoglycan synthesis rate in the medial femoral condyle cartilage surrounding the defect for each of the three treatment groups. There is a significant increase for all three groups. A similar effect was found in all medial tibia locations and in the lateral femoral compartment of the 'no treatment' and 'late treatment' group. (No treatment: superficial cartilage defect left untreated, Early treatment: fresh full thickness cartilage defect in normal joint treated with periosteal transplantation, Late treatment: full thickness defect treated after 10 weeks of motion and loading on the previous superficial cartilage defect. C: control, E: experimental, * = p ≤ 0.05)
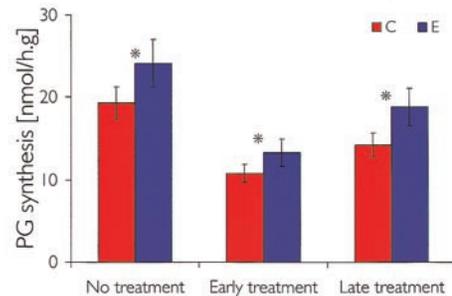
Figure 3.6

In both the 'No treatment' and 'Late treatment' group there is a significant increase in the release of glucoseaminoglycans (GAG) for both newly formed and nascent GAG's from the medial compartment. There was no significant difference in GAG release in the group that was treated immediately. (No treatment: superficial cartilage defect left untreated, Early treatment: fresh full thickness cartilage defect in normal joint treated with periosteal transplantation, Late treatment: full thickness defect treated after 10 weeks of motion and loading on the previous superficial cartilage defect. C: control, E: experimental, * = p ≤ 0.05, ns = p > 0.05)
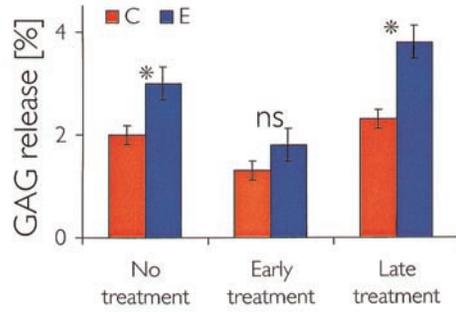

Figure 3.7

We found a significant decrease in total GAG content in the 'No treatment' and the 'Late treatment' group but not in the 'Early treatment' group. This indicates a protective effect on matrix metabolic alterations of early intervention. (No treatment: superficial cartilage defect left untreated, Early treatment: fresh full thickness cartilage defect in normal joint treated with periosteal transplantation, Late treatment: full thickness defect treated after 10 weeks of motion and loading on the previous superficial cartilage defect. C: control, E: experimental, * = p ≤ 0.05, ns = p > 0.05)
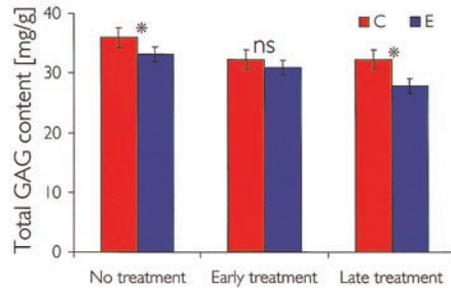
normal values in control cartilage from the intact contra lateral joints which was rated 23.4 ± 0.7 out of 24 points (table 3.5). The defects in the 'No treatment' group did not heal and cartilage repair scores were low,

| Characteristic	Experimental group					
	No treatment		Early treatment		Late treatment	
Control (C), Experimental (E)	C	E	C	E	C	E
n=	7	7	6	6	6	6
Nature of predominant tissue						
Cellular morphology						
Hyaline articular cartilage	7	0	6	3	6	0
Incompletely differentiatied	0	2	0	1	0	2
Fibrous tissue or bone	0	5	0	2	0	4
Safranin O staining						
Normal or near normal	6	1	5	5	6	2
Moderate	1	2	1	1	0	2
Slight	0	2	0	0	0	2
None	0	1	0	0	0	0
Structural characteristics						
Surface regularity						
Smooth and intact	6	0	5	3	4	0
Superficial, horizontal lamination	1	1	2	3	2	3
Fissures, 25-100% of the thickness	0	5	0	0	0	2
Severe disruption or fibrillation	0	0	0	0	0	1
Structural integrity						
Normal	6	1	6	3	6	1
Slight disruption, including cysts	1	3	0	3	0	1
Severe disintegration	0	3	0	0	0	4
Tickness						
100% of normal cartilage	7	2	6	6	6	5
50-100% of normal cartilage	0	3	0	0	0	1
0-50% of normal cartilage	0	0	0	0	0	0
Bonding to the adjacent tissue						
Bonded at both sides and subchondral bone	7	1	6	4	6	4
Bonded partially	0	3	0	1	0	2
Not bonded	0	3	0	1	0	0
Hypocellularity						
None	6	4	6	5	5	3
Slight	1	1	0	1	1	1
Moderate	0	1	0	0	0	2
Severe	0	1	0	0	0	0
Chondrocyte clustering						
None	7	4	6	5	6	4
< 25% of cells	0	1	0	1	0	2
25-100% of cells	0	2	0	0	0	0
Normal cellularity, no clusters, normal staining	6	2	3	1	6	0
Normal cellularity, mild clusters, moderate staining	1	5	0	5	0	6
Mild or moderate hypocellularity, slight staining	0	0	0	0	0	0
Severe hypocellularity, poor or no staining	0	0	0	0	0	0

Table 3.5
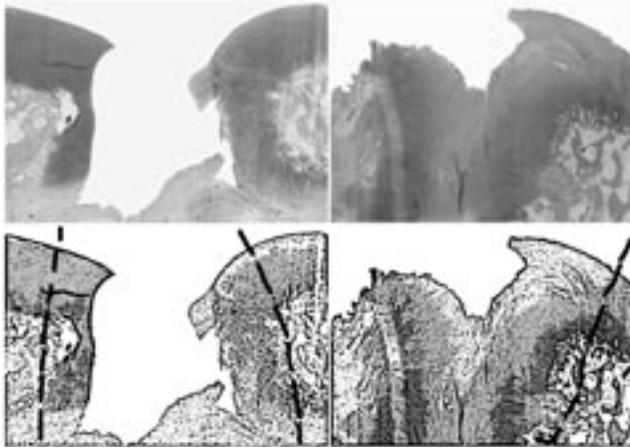
Figure 3.8 A

Top row: histological sections on defects in the 'No treatment' group after 10 weeks of follow-up. (3 μm, Safranin O / Fast green, 200x) Bottom row: schematic representation of the sections depicting the location of the original defect. These two typical samples demonstrate that the cartilage defects that were left untreated either remained unchanged as on the left, or some degree of filling with fibrocartilage or extruding cartilage from the normal cartilage rim occurred as on the right.

See CD for full color representation
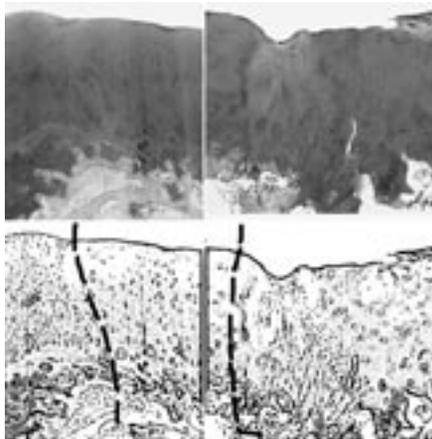
Figure 3.8 B

Top row: histological sections from defects in the 'Early treatment' group (3 μm, Safranin O / Fast green, 200x). Bottom row: schematic representation of the sections depicting original defect. These two typical samples show how the fresh cartilage defect, which was treated early, showed a marked repair of the cartilage surface with a tissue resembling hyaline cartilage. There are some chondrocyte clusters and near normal PG staining throughout the matrix. Some remodeling of the subchondral bone is seen.

*See CD for full color representation*
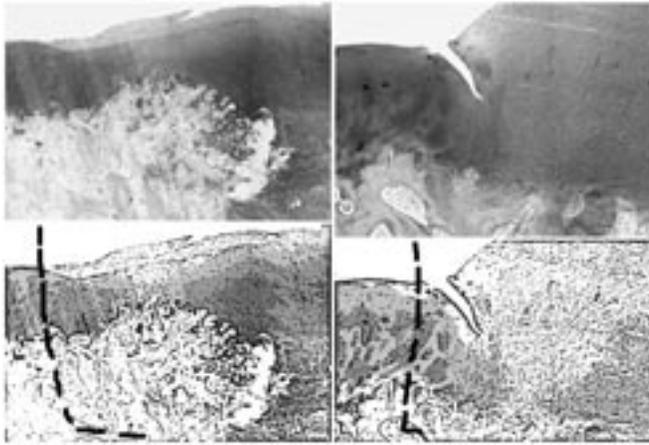
Figure 3.8 C

Top row: histological sections of defects from the 'Late treatment' group (3 μm, Safranin O / Fast green, 200x). Bottom row: schematic representation of the sections depicting the location of the original defect. These two typical samples from the 'Late treatment' group demonstrate an identical full thickness defect as in the previous groups but treated after 10 weeks of motion and loading on the previous superficial cartilage defect. There is irregular fibrocartilage filling and hypertrophy with uneven staining of the matrix. Also, signs of delamination and sidewall fissures were seen more frequently then in the 'Early treatment' group.

See CD for full color representation
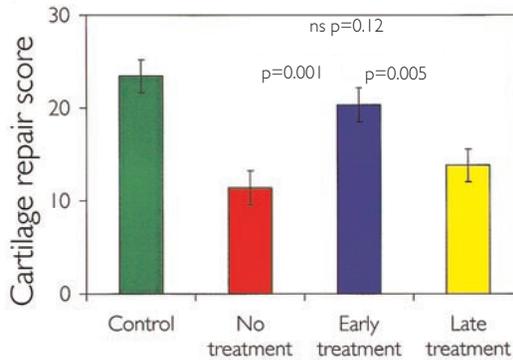
Figure 3.9

This bar chart of the semi-quantitative cartilage repair scores for each sample group demonstrates that tissue engineering outcome is not successful in the 'Late treatment' group, resembling the clinical condition, while the 'Early treatment' group shows much better results, similar to those presented by other authors. (Control: normal cartilage, No treatment: superficial cartilage defect left untreated, Early treatment: fresh full thickness cartilage defect in normal joint treated with periosteal transplantation, Late treatment: full thickness defect treated after 10 weeks of motion and loading on the previous superficial cartilage defect)

averaging 11.4 ± 3.1 (figure 3.8 A). In the 'Early treatment' group, the cartilage repair score was significantly higher (p=0.001) with an average of 20.3 ± 3.1 (figure 3.8 B) as compared to the 'No treatment' group. These sections showed reproducible cartilage formation, with filling of the defect by a cartilaginous tissue, some restoration of the tidemark and smoothing of the articular surface. There was a significant decrease in the cartilage repair score of 13.8 ± 2.0 in the 'Late treatment' group, (p=0.005), where signs of surface irregularity, fibrillation, and loss of interface bonding, with considerable fibrous hypertrophy and more synovitis were present (figure 3.8 C). We found no significant difference (p = 0.12) between the defects treated late and those left untreated (figure 3.9).

DISCUSSION

To test our hypothesis that delay in treatment of a defect, when cartilage degeneration has been initiated and changes in joint homeostasis occur, negatively influences cartilage repair by tissue engineering, we developed a large animal model in the Dutch Milk Goat. The goat model combines favorable joint mechanics, and a cartilage metabolic activity comparable to the human situation. The adolescent animals were chosen since at this age physical growth has ceased while maturation continues, and knee anatomy allows for reproducible surgical technique as well as an adequate amount of material for subsequent analyses. As described in depth as part of our evaluation of the age related effects on chondrogenesis in *Chapter 8*, in older animals the outcome of chondrogenesis declines[232]. We decided to use these relative young adults in which adequate periosteal chondrogenesis was to be expected to include a reliable positive control group in our investigation.

A cartilage defect in the medial femoral condyle resulted in reproducible early degenerative changes with significant histological and biochemical alterations. As an initial sign of matrix degeneration and disturbance of joint homeostasis we found a significant increase in PG synthesis in all groups. There was a decrease in total GAG content, even as early as a few weeks after initial trauma. This was caused by the increased release of matrix components in the 'No treatment' and 'Late treatment' group, but not in the 'Early treatment' group. Interestingly, the signs of early

osteoarthritis were in part reversed in the 'Early treatment' group where the cartilage defect was immediately treated. This creates the suggestion of a protective effect from early treatment. Metabolic alterations in the 'Early treatment' group were considerably less that in the other groups. This may indicate that early restoration of the joint surface may allow us to prevent cartilage degradation and subsequently provide an intra-articular environment more beneficial for cartilage formation.

In the presence of disturbed joint homeostasis, cartilage formation was significantly decreased and the outcome of periosteal grafting became insufficient. The histological findings in our 'No treatment' group are in line with the data from Jackson[148] on natural healing of goat defects and reconfirm that such cartilage defects do not repair spontaneously. Furthermore there was no significant difference between the untreated defects and the defects treated late. The 'early treatment' group showed significant restoration of the joint surface as demonstrated by the O'Driscoll cartilage repair scores. Our normal cartilage, untreated defects and early treated defects have scores that are comparable to data from cartilage repair studies by van Susante[309,310] and those of Driesang and Niederauer[92,210].

We can therefore conclude that a disturbed intra-articular environment negatively influences cartilage formation. These findings have novelty and support our hypothesis that the metabolic aspects of joint homeostasis indeed influence cartilage formation.

These findings are of importance for at least three reasons: primarily since most, if not all, patients currently treated with these methods are known to have a longer existing cartilage defect and thus cartilage degeneration is present in these patients. Our results suggest an explanation for the discrepancy between positive results of experimental repair in healthy joints and the as yet less favorable clinical results. However many questions remain; for instance what can we learn from a comparison between the long-term results of early treatment and late treatment and how do joints respond to pre treatment. The need for the ongoing investigation of these effects is apparent.

Secondly, these findings steer us towards a radical change in our treatment of these patients. We propose that clinical studies should be instigated to compare prompt intervention by tissue engineering with current treatment as described in *Chapter 1*. Immediate diagnosis and treatment after the occurrence of a cartilage defect may offer an opportunity to improve

outcome. Alternatively, we should determine methods of modulating joint homeostasis to create an environment more permissive for chondrogenesis prior to application of a tissue engineering strategy.

Finally, but not less important these data reiterate the relevance of using appropriate animal models. The results of the current investigation suggest that the evaluation of tissue engineering techniques should be done using models of cartilage repair comparable to the clinical situation in our human patients. The sole investigation of fresh defects in healthy joints should not be considered of any predictive value for the success of clinical implementation of the technique studied. An evaluation phase in a model relevant to the clinical setting is imperative.

GOAL:

To establish the validity of applying mechanical stimuli in the form of dynamic fluid pressure to a culture system containing agarose gel, necessary for future *in vitro* experiments.

HYPOTHESIS:

Pressure transmission from air to fluid to agarose gel in which the tissue is embedded is complete and instantaneous.

RATIONALE:

A controlled *in vitro* environment provides an optimal possibility to study the effect of mechanical stimuli on chondrogenesis. To study mechanical modulation of cartilage formation we selected the model for periosteal chondrogenesis *in vitro* extensively validated and published by O'Driscoll and co-workers. As a method of applying mechanical stimulation, we selected the use of cyclic application of hydrostatic pressure, or rather dynamic fluid pressure (DFP). Therefore the need exists to establish whether it is valid to assume that periosteal explants embedded in agarose gel are exposed to the dynamic fluid pressure stimulus in the manner determined by the settings of the machine applying the stimulus.

METHOD:

A materials testing machine was used to apply dynamic pressure variations to an experimental set-up with digital pressure monitoring devices in air, fluid and agarose gel. This set up allows us to apply various loading regimes, wave shapes, gel volumes and viscosities while measuring the resulting pressure at various locations within the system.

CHAPTER
4

DYNAMIC PRESSURE TRANSMISSION IN AGAROSE GELS

INTRODUCTION

Agarose gel is widely used in various fields of biomedical research, particularly in tissue culture systems where it supports the chondrocyte phenotype. These culture systems typically consist of a mixed phase environment of gas, liquid and gel[159,167,228,307,312]. Agarose gel is a necessary component in the periosteal explant model for optimal cartilage differentiation and matrix formation[29,158,228] and may thus be important for investigation in cartilage repair by tissue engineering. The mechanical properties of the media could be important when studying the effect of direct mechanical loading and dynamic fluid pressure on cartilage metabolism and periosteal cartilage formation.

We are interested in studying the effect of dynamic fluid pressure on cartilage formation in periosteum[204,285]. Our experimental set-up applies pressure to the gas phase. Since the tissue pieces are embedded in the agarose gel it is necessary to understand the mechanics of pressure transmission in such a mixed phase environment. Others have studied the properties of agarose gel under direct mechanical loading, and have indicated that it behaves as a poroelastic solid material when seeded with cartilage cells that produce matrix products[55]. This further emphasizes the need for information on the validity of pressure related experiments using these mixed phase culture systems. Currently there is no information on the transmission of pressure applied in the gas phase to the explants that are embedded in the agarose gel.

We tested the hypothesis that agarose gel, as used in our system, does not influence the transmission of pressure to explants cultured in the gel. In other words, the pressure response is virtually instantaneous and complete, irrespective of the applied pressures and frequencies or gel volume and viscosity.

This question is of significance for any conclusions on dynamic pressure stimulation in a mixed phase tissue culture system to be valid, and thus essential for the research on mechanical modulation of cartilage repair proposed in our introduction and described in the subsequent chapters.

MATERIALS & METHODS

Agarose gel was exposed to pressurization. For this we applied dynamic compression of the gas phase above the fluid covering the gel, which is confined in a rigid cylindrical container. Using an air cylinder (AAC advanced automation 4-ms-4-FC,12 Chicago, IL) placed in an MTS (model 810, MTS, Eden Prairie, MN) connected to a pressure chamber containing the gel we could accurately control pressure, frequency and wave shape (figure 4.1). Pressure transducers (msp-300-100-p-3, MSI, Fairfield, NJ) were placed in four locations: the gas and liquid phases as well as centrally and peripherally in the gel.

Figure 4.1

Experimental set-up: stainless steel pressure chamber with rigid gel container for variable volumes and viscosities. Four pressure transducers measure air pressure, fluid pressure, central gel pressure and peripheral gel pressure, with digital data acquisition and analysis. Pressure is applied with a MTS (model 810).
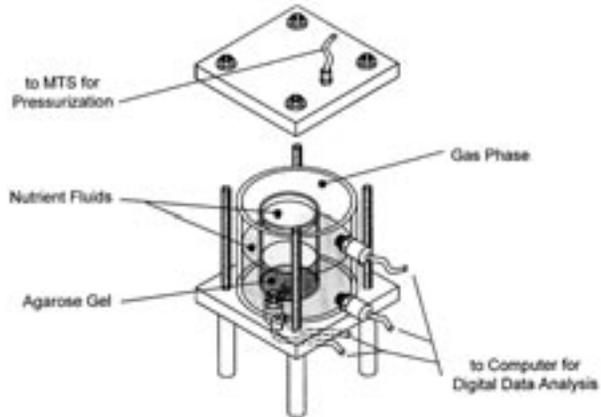
Tested loading regimes:

The pressure response of the gel was tested with single cycle pressurization to 0.15, 0.35 and 0.7 MPa using a 0.1 second pressurization/depressurization slope with a one second plateau (figure 4.2). The independence of gel response to pressure increase was tested by gradually increasing the pressure from 0 to 0.7 MPa at 0.3 Hz (figure 4.3). The effect of frequency variance was studied by pressurizing from 0 to 0.7 MPa at 0.3, 0.6 and 1.2 Hz (figure 4.4). We looked for a possible reverberation echo in the gel after a short (0.1 second, 0.7 MPa, 14 MPa / sec pressure rate) pressure pulse. Finally, the validity of the experimental set-up was determined by

applying the stimuli to the system when the cylinder was closed with a metal lid to exclude indirect pressure transmission as a confounding factor.

Gel parameters:

The influence of gel volume was studied using various gel volumes from 50 ml to 325 ml. Three different gel viscosities were studied by varying the percentage of agarose: *standard*: 50% low melting temperature (Tm) agarose 10 mg/ml (162-0017 Bio-Rad laboratories, Richmond, CA) and 50% double strength Dulbecco's Modified Eagle Medium (12100-046 Gibco BRL, Grand Island, NY); *low*: 25% of low Tm agarose 10 mg/ml and 75% 2xDMEM; *high*: 75% of High Tm agarose 10 mg/ml and 25% 2xDMEM.

Data analysis:

We used a digital data acquisition and analysis system with a sample frequency of 150 Hz to monitor the response. This sample frequency is two orders of magnitude greater then the frequency with which the pressure was applied. All experiments were performed three times with new materials at weekly intervals to confirm reproducibility. We used linear regression between P_{air} and $P_{gel\ center}$ and $P_{gel\ periphery}$ to determine differences. We described the frequency response of the gel by plotting the phase and gain of the system in a Bode plot, when an effect of the gel on pressure transmission was found.

RESULTS

The pressure transmission from gas to liquid in this system was complete and instantaneous; as expected we did not find any difference between the pressure readings from the air to which we applied the pressure and the fluid medium in which we measured the resulting pressure. More importantly we did not find any differences between gas and gel pressures when we varied the applied pressure. Pressurization to 0.15, 0.35 and 0.7 MPa using a 7 MPa / sec pressure rate and a 1 sec plateau did not influence the pressure response in the gel (figure 4.2). Nor did increasing the pressure from 0 to 0.7 MPa at a 0.3 Hz frequency (figure 4.3). We can therefore be confident that pressure variation does not influence the pressure response in this system. The frequency of pressurization did not change the pres-

sure transmission to or within the gel. Increasing the frequency from 0.3 to 0.6 and 1.2 Hz for pressurization between 0 and 0.7 MPa did not influence the pressure response curves in the gel (figure 4.4). There was no pressure differential, or delays among the three phases in this system. The pressurization rate $\Delta P_{pressurization}/\Delta t$ and $\Delta P_{depressurization}/\Delta t$ was equal for all pressure / time combinations. A short pressure burst (0.1 second, 0.7 MPa, 14 MPa / sec) did not cause a reverberation in the gel. Therefore frequency changes did not influence the gel response.

Figure 4.2

To test the response to pressurisation of different magnitude we applied pressure from 0 to 0.15, 0.35 and 0.7 MPa with a pressure rate of 7 MPa / sec. and a 1 sec. plateau. This figure shows applied pressure to the gas and the resulting pressures in the fluid, gel center and gel periphery versus time.
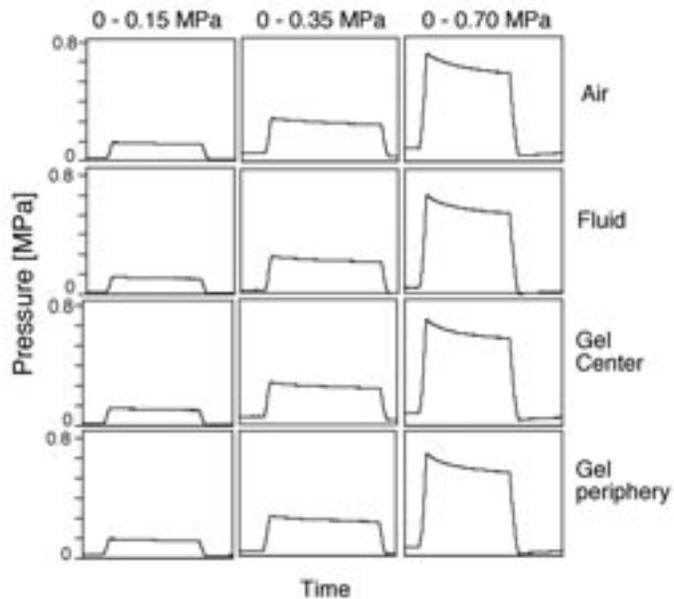
Increasing the gel volume from 50 ml to 325 ml or using low and high viscous gels did not alter the gel pressure response to any of the pressure/frequency regimes. Location of the transducers within the gel was not of influence; central gel pressure and pressure on the interface with the container were equal in all experiments. This indicates that preparation of the gel, filling of the culture wells or placement of the tissue within the gel does not alter the pressure exerted on the tissue.

Figure 4.3
To determine the role of increasing the pressure that is applied to the gas we varied the gas pressure from 0 to 0.7 MPa while cycling at 0.5 Hz. This graph illustrates the pressure curves in all three phases through time. There was no delay or pressure differential among phases.
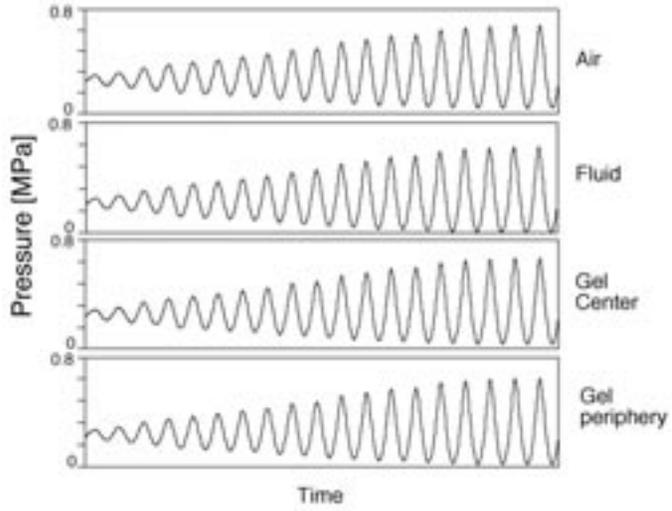

Figure 4.4
To study the influence of the frequency with which pressure is applied we measured the pressure response at 0.3, 0.6, and 1.2 Hz for a 0 to 0.7 MPa pressure. We found no differences among pressure curves.
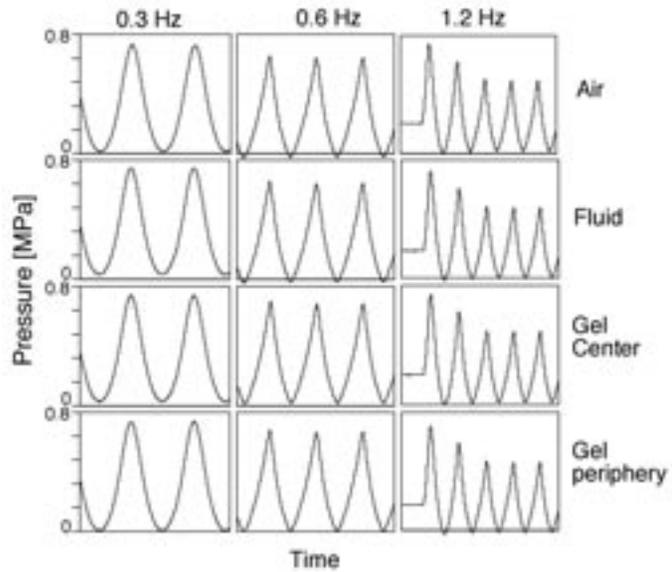
In conclusion we did not find any differences between the pressure applied to the gas and the resulting pressure in the gel for any of the tested parameters. Regression analyses between the applied pressure and the resulting fluid and gel pressures showed an almost perfect linear relation (r^2 = 0.99987, p < 0.0001, f (x) = 0.9982 x - 0.0286). When the lid to the experimental pressure vessel was closed there were no pressure readings in any of the transducers, therefore we can safely assume that the measurements are directly related to the pressures in the air, fluid and gel. No indirect pathway of pressure transmission could be determined. Results from all three repetitions were identical with the same apparatus using new gels each time.

DISCUSSION

We determined it to be necessary to use an *in vitro* model to study the effect of mechanical stimulation on cartilage formation. Since this approach was not previously described, validation of our experimental set-up was essential. Periosteal explants *in vitro* need a culture system that permits suspension in something such as agarose gel for optimal cartilage differentiation and matrix formation[29,195,197,228]. It is of importance for our understanding of cartilage repair to study the influence of mechanical stimuli, which can be reproduced in vitro by applying dynamic fluid, pressure[159,175,223,229,291,312]. The behavior of the air-to-fluid-to-gel transmission of pressure under dynamic pressure changes had to this time remained undescribed but is important for any conclusions on tissue pressure response to be valid.

Other authors have described the role of agarose gel in direct mechanical loading, and when seeded with matrix producing cartilage cells[55,57]. They found it to behave as a poroelastic solid once matrix was produced by the chondrocytes. A limiting factor in such studies is the pressure and frequency range as well as the different volumes and gel viscosities that can be tested. We chose our pressure range between 0 and 0.8 MPa and frequencies of 0 to 1.2 Hz. This includes the range of values used in our culture experiments (typically 13 ±2 kPa at 0.3 Hz). The frequency at which data were sampled and the experimental setup were adequate for measuring differences that would be of influence on conclusion from our *in vitro* experiments. We were able to test the pressure response of agarose gel in a

mixed phase system and found pressure transmission from gas to fluid to gel to be complete and immediate. This was independent of pressure or frequency ranges tested. Neither gel volume, nor viscosity or location within the gel influenced the pressure response within the reasonable range for culture purposes.

We can therefore be confident that a mixed phase environment and our model for dynamic fluid pressure stimulation of periosteum to study cartilage formation *in vitro* are valid. This is an essential prerequisite for the experiments described in *Chapters 5-7*.

GOAL:

To determine the effect of dynamic fluid pressure on periosteal cell proliferation.

HYPOTHESIS:

Dynamic fluid pressure will stimulate periosteal cell proliferation *in vitro*.

RATIONALE:

Mechanical factors are known to be important in normal cartilage development, maintenance, and outcome of repair techniques. Thus, mechanical stimuli can be considered an essential component of joint homeostasis. However the effects on periosteal cartilage formation *in vitro* have not been studied and how cartilage metabolism or repair / regeneration are affected remains to be fully understood. It is essential that we understand the role of mechanical factors in regulating these processes.

METHODS:

Periosteal explants were cultured in the presence or absence of dynamic fluid pressure. The effect on cell proliferation was determined using ^{3}H-thymidine uptake studies, autoradiography, Proliferating Cell Nuclear Antigen immunostaining, cell proliferation blocking assays and total DNA measurements.

CHAPTER
5

PERIOSTEUM RESPONDS TO DYNAMIC FLUID PRESSURE

BY PROLIFERATING *IN VITRO*

INTRODUCTION

The present decade has witnessed an explosion of interest in the field of cartilage repair, as evidenced by the widespread coverage devoted to this topic in scientific publications and the lay press. To better understand the factors and conditions regulating the basic process of cartilage formation requires good *in vitro* and *in vivo* models for studying chondrogenesis, as well as methods for simulating mechanical factors.

Periosteum provides a source of undifferentiated chondrocyte precursor cells for fracture healing that can also be used for cartilage repair. In both processes, the quantity of cartilage that can be produced is related to the number of available stem cells. Optimal cartilage repair or fracture healing requires increasing the quantity as well as the quality of cartilage produced. The quantity of cartilage is believed to be limited by the number of chondrocyte precursors in the cambium layer[113], which can be increased by stimulating cell division in this population.

The periosteal organ culture model, which has been described for studying the process of cartilage formation in whole periosteal explants suspended in agarose, mimics the events during periosteal chondrogenesis *in vivo*[86,113,196,200,228,229]. Prior to expression of the cartilage phenotype in transplanted or cultured periosteum, DNA synthesis and cell proliferation are the first events that occur during periosteal chondrogenesis[196,200,228,229]. Expanding the chondrogenic cell population will increase the quantity of cartilage produced. In periosteal chondrogenesis, the total cell count in the cambium layer of the periosteum, where the chondrocyte precursors are believed to reside, is the rate limiting factor determining chondrogenic potential of periosteum and its diminution with age[113,198,232]. As the first stage of periosteal chondrogenesis is cell proliferation, we need to understand how this can be stimulated.

Klein-Nulend *et al*. have shown that low-level oscillations in hydrostatic pressure (13 kPa at 0.3 Hz) increased periosteal and perichondrial cell density, while Lafeber *et al*., have shown that chondrocytes from normal or osteoarthritic cartilage in culture respond to such a mechanical stimulus by alteration of their metabolic rate and increased proteoglycan synthesis[159,161,167].

As the pressures are dynamic rather than static, a term such as *dynamic fluid pressure* (DFP) might be preferable. Such dynamic fluid pressures have been recorded in synovial fluid during joint motion and gait[223,305].

The effects of experimentally applied dynamic fluid pressure have been studied and found by many investigators to regulate (i.e. stimulate) cellular activity in cartilage and bone[51,54,55,123,124,162,167,307,311,312]. It is recognized that mechanical stimuli are important during the development, homeostasis, repair and degeneration of cartilage[48,50,161,167,220,228].

Biological repair of cartilage is enhanced by continuous passive motion (CPM) of the joint postoperatively[280]. This is true for cartilage regeneration by transplantation of periosteum, which contains undifferentiated mesenchymal stem cells with osteochondrogenic potential[135,150,164,220,221,228,230,273,276,315]. The important role of mechanical factors is illustrated by the finding that continuous passive motion of a joint into which periosteum has been transplanted greatly increases the quantity and quality of the cartilage produced by the periosteum, as compared to that formed in immobilized joints[86,220,221,229,230].

Movement of a joint causes sinusoidal oscillations in the synovial fluid pressure[223]. The beneficial effects of joint motion on repair of damaged cartilage might be explained in part by the contribution of dynamic fluid pressure. At this time there is no published information on the effect of dynamic fluid pressure on periosteal chondrogenesis. However, it is clear that periosteum has a chondrogenic potential, which is influenced by mechanical factors, and motion alters the phenotypic expression of the chondrocyte precursor cells in the periosteum[220]. The major limitation with periosteal transplantation is the age-related decline in periosteal chondrogenic potential which is examined in *Chapter 8*[113,220,232].

The purpose of the present investigation was to test the hypothesis that cell proliferation in whole periosteal explants is stimulated by dynamic fluid pressure (DFP). If correct, this would explain, in part, the previously reported association between the cyclical changes in intra-articular pressure and the increased number of chondrocytes in periosteal grafts or transplants exposed to CPM[223,229]. It might also be useful as a method to expand the stem cell pool (i.e. chondrocyte precursor population) and maintain differentiation and therefore help to reverse the age-related decline in the capacity of periosteum for cartilage repair and improve the pre implantation culture conditions.

METHODS

Harvesting of periosteal explants and tissue cultures:

Four hundred and fifty two periosteal explants were harvested from the proximal medial tibiae of 60, immature (two-month old) male New Zealand White rabbits, as described in *Appendix* A[109,228,229]. The culture conditions were as reported in the periosteal explant model by O'Driscoll *et al* detail of which is provided in *Appendix B*. To act as a positive control for the experiments on blocking the proliferative activity, TGF-ß1 was added to both the agarose gel and the fluid culture medium. This was done in a concentration of 10 ng/ml[228], for the first two days of culture, unless otherwise mentioned. All explants were cultured in standard 48 well flat-bottom plates maintained at 37°C, 100% humidity and 5% CO_2 mixed with 95% air.

Figure 5.1

The dynamic fluid pressure apparatus applies dynamic pressurization to the gas phase of the culture system by movement of a membrane that divides the regulated line air pressure from the culture environment of 5% CO_2 in 95% air. The system is controlled by a computer, which also monitors applied and resulting pressures H. (Piston A is pushed by pressurized air B/C, moves membrane D thereby applying controlled dynamic fluid pressure on the culture plates G in pressure chamber F)
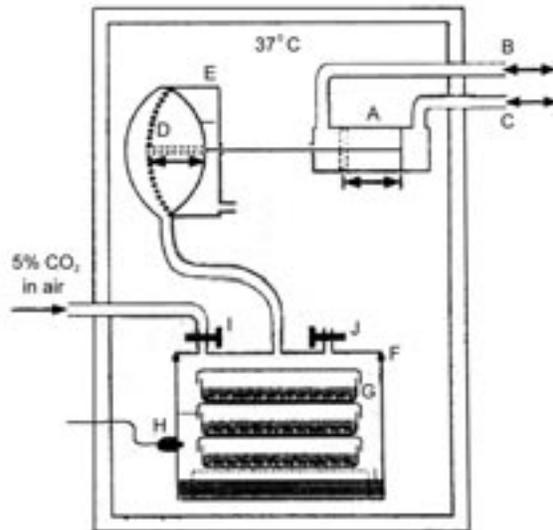
Dynamic Fluid Pressure apparatus:

Dynamic fluid pressure was applied, to periosteal explants based on the validation experiments in *Chapter 4*, using machine number 1 described above, and explained in more detail in *Appendix C* (figure 5.1)[159,167,284,285,312].

A pneumatically driven membrane chamber was used to create dynamic pressurization in the gas phase within the attached pressure chamber, in which the culture plates rest. The pressure oscillated between 0 and 13 ± 2 kPa at a frequency of 0.3 Hz, based on previous publications by other authors[120,123,124,159,167,244,246,278,305,307,312]. The rate of pressure rise produced by the system was 33 kPa / sec.

DNA synthesis measured by ^{3}H-thymidine uptake:

To quantify cell proliferation we evaluated DNA synthesis by measuring ^{3}H-thymidine uptake as described in *Appendix D*[166,298,300].

Autoradiography and Immunostaining:

The location of proliferative activity as determined from DNA synthesis was visualized by ^{3}H-thymidine autoradiography on 40 explants that were cultured for 1, 2, 3, 4, 5, 6, 7 and 14 days as described in *Appendix E*. Histomorphometric analyses of autoradiograms were performed using a previously published counting technique[113]. Photomicrographs of the autoradiographs were taken at 200x magnification. The total number of both labeled and unlabeled cells were recorded using a calibrated rectangular grid laid over the fibrous and cambium layers of the periosteal explants. Counts were performed twice and averaged. The reproducibility of this technique was confirmed previously[113] as well as in this study. The accuracy was validated by the finding that the values for cell density in the cambium layer as well as for thickness of the layers were similar to those previously reported by Gallay[113].

As a second indicator of proliferative activity we performed immunostaining with an antibody to Proliferating Cell Nuclear Antigen on paraffin embedded sections of 8 explants cultured for 1 or 4 days with or without DFP[169,170] (*Appendix E*).

Data Analyses:

All data are presented as means ± 1 standard deviation (SD). All experiments were designed to control for the donor rabbit such that comparisons between experimental and control groups were paired to donor rabbit. We used a sample size for each group of n = 16, to yield an 80% chance of detecting a change in the means ≥ 0.75 standard deviations. This is considered to be between a medium and large effect size[67]. Analyses were performed by repeated measures analysis of variance and

post-hoc testing using Duncan's New Multiple Range Test, with a 95% confidence interval. Scintillation counts have been rounded off to two digits of accuracy.

RESULTS

[3]H-thymidine uptake:

[3]H-thymidine uptake, which was measured on days 1, 3 and 5, reached a peak on day 3 in both the experimental and control groups, consistent with a previous report using this model[197,200]. At each tested time point there was a significant increase in ^{3}H-thymidine uptake (p < 0.001) in the experimental group exposed to dynamic fluid pressure (DFP) of 13 ± 2 kPa at 0.3 Hz, compared to the control group, that was cultured in a similar chamber at atmospheric pressure (figure 5.2). On day 1, there was a 60% increase in ^{3}H-thymidine uptake in the DFP group over controls
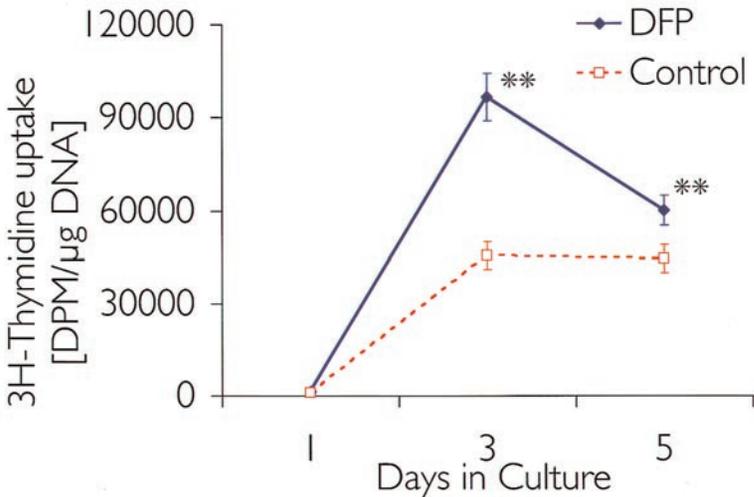

Figure 5.2

*The continuous application of dynamic fluid pressure at 13 ± 2 kPa, 0.3 Hz for 1, 3, or 5 days led to a two-fold increase in ^{3}H-thymidine uptake under DFP compared to controls. The time course for DNA synthesis under the influence of DFP peaks on day 3, which is identical to that in the standard model. Explants at each time point were labeled with ^{3}H-thymidine during the last 24 hours in culture. (Data are presented as means ± SD, n=16, ** = p < 0.001).*

(1500 ± 220 vs. 980 ± 150 DPM/μg DNA). The results on day 3, when DNA synthesis is maximal, showed a 110% increase with DFP (97,000 ± 5,700 vs. 46,000 ± 6,000 DPM/μg DNA). A 40% stimulation of ^{3}H-thymidine uptake with DFP was still present on day 5 (DFP: 60,000 ± 2,700 vs. controls: 45,000 ± 4,900 DPM/μg DNA).

DNA Content:

The DFP-induced increase in ^{3}H-thymidine uptake was reflected by a higher DNA content in the DFP-treated explants when compared to the controls. In the control explants, the total amount of DNA decreases with time, presumably due to the trauma of explantation or adaptation to the culture environment. A similar observation has been described in other systems[270]. There was also a decline in DNA content in the experimental DFP group, but less so than in the controls. The DNA content of the explants exposed to DFP was 60% higher than that of the controls on day 3 (DFP 5,700 ± 720 vs. control 3,700 ± 630 ng/mg wet weight) and 40% higher on day 5 (DFP 5,200 ± 490, control 3,700 ± 390 ng/mg wet weight) (p < 0.01; figure 5.3).
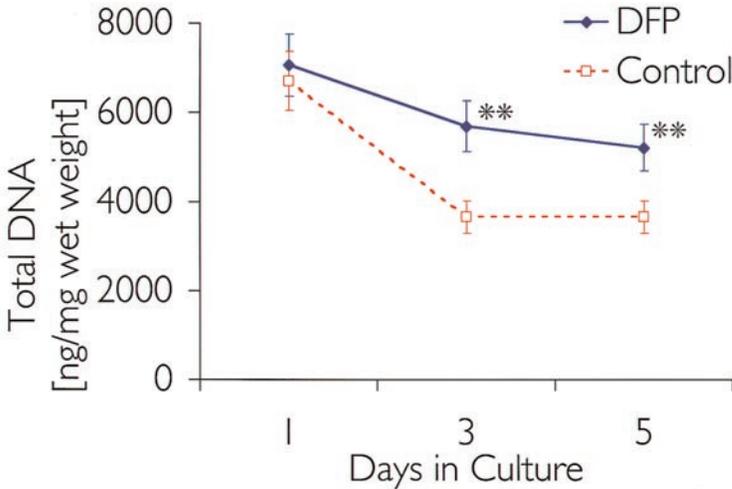

Figure 5.3

*In adapting to the in vitro environment, the periosteal explants experience a decrease in total DNA. This decrease was however, significantly inhibited in the DFP group on days 3 and 5 compared to controls. (Data are presented as means ± SD, n=16, ** p <0.01).*

Blocking of DNA Synthesis:

To confirm that the ^{3}H-thymidine and DNA measurements actually represented stimulation of cell proliferation by DFP, DNA synthesis was blocked with aphidicolin, which inhibits DNA polymerase α[235,237]. DNA polymerase is essential for chromosomal DNA replication and cell division[146,165]. We measured ^{3}H-thymidine uptake in three different groups: a negative control (no DFP, no growth factor), a positive control (10 ng/ml TGF-β1 - known to stimulate periosteal DNA synthesis[197,200], and the experimental DFP group (without TGF-β1). Explants were cultured in the presence of 0, 0.5, 1.0, or 2.5μg per day of aphidicolin until day three when proliferation peaks. The explants were pooled after labeling with ^{3}H-thymidine, and the pooled uptake measured on day 3. The negative


Group	(-) Control				(+) Control				Experimental			
Aphidicolin dose [μg/day]	0	0,5	1	2,5	0	0,5	1	2,5	0	0,5	1	2,5
TGF-β1	None				10 ng/mL day 0-2				None			
DFP	None				None				day 0-3			
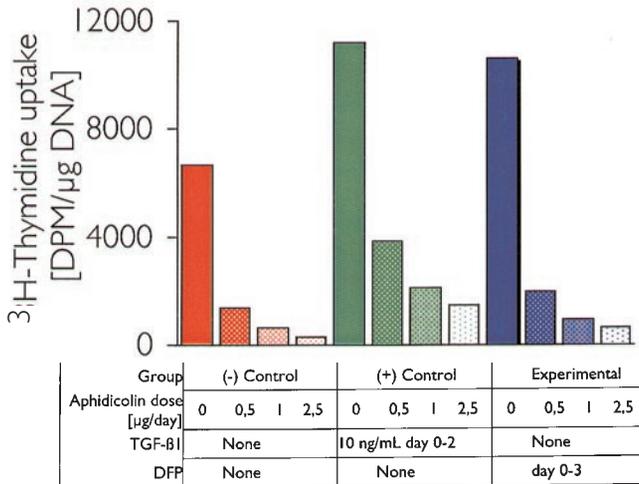
Figure 5.4

[3]H-Thymidine uptake was measured in three groups: a negative control (no added growth factors, no DFP), a positive control (10 ng/ml TGF-β1), and the experimental DFP group. For each group a dose response effect was noted to aphidicolin. Explants were cultured for 3 days (when proliferation peaks) with and without aphidicolin, which blocks DNA polymerase and thus DNA synthesis. The controls showed levels of ^{3}H-thymidine uptake as expected in our standard model; i.e. addition of TGF-β1 increased ^{3}H-thymidine uptake significantly. DFP significantly increased ^{3}H-thymidine uptake. In all groups, the response to aphidicolin was a dose-dependent inhibition of ^{3}H-thymidine, confirming that the ^{3}H-thyumidine measurements truly represent DNA synthesis. Explants were pooled, columns represent values for whole groups, experiments were repeated and reproducibility was confirmed.

Aphidicolin (µg/ml)	Negative control [a]	Positive control [b]	Dynamic fluid pressure [c]
0	820	1.000	1.800
0,5	150	320	580
1	100	220	100
2,5	30	130	210

Table 5.1

Total DNA (ng/mg wet weight) while proliferation was blocked.

[a] Without transforming growth factor β1 (TGF-β1). [b] Treated with TGF-β1. [c] From days 0 to 3; without TGF-β1.

and positive controls showed levels of thymidine uptake as expected in our standard model; i.e., the addition of TGF-β1 increased ^{3}H-thymidine uptake (11,000 vs. 6,700 DPM/mg wet weight) (figure 5.4, table 5.1). In both the positive and negative controls the inhibition of ^{3}H-thymidine uptake with aphidicolin was dose-dependent, ranging from 80% using 0.5μg to 95% using 2.5μg aphidicolin.

These data confirm that indeed ^{3}H-thymidine uptake represents cell proliferation in this system. In the experimental group, DFP increased thymidine uptake and, as expected, this increase was inhibited in the same dose dependent manner by blocking proliferation with aphidicolin (0.5μg: -82%, 1μg: -91%, 2.5μg: -94% inhibition respectively). The aproximately 95% inhibition of ^{3}H-thymidine uptake in all groups indicates that DNA synthesis could be virtually stopped. The total amount of DNA in the DFP group was higher than that in the other groups and this, too, could be blocked with aphidicolin which led to a moderate decrease in total DNA (control group: 820 vs. 150 ng/mg wet weight with 0.5μg of aphidicolin, TGF-β1 group: 1,000 vs. 320 ng/mg wet weight, DFP group: 1,800 vs. 580 ng/mg wet weight)(table 5.1). These data indicate that dynamic fluid pressure does indeed stimulate DNA synthesis and cell proliferation. Alternatively the difference in the total amount of DNA between the TGF-β and the DFP group might be interpreted as an effect on apoptosis, in which DFP helps retain cell viability in this *in vitro* setting, as well as a direct stimulation of periosteal cell proliferation.

^{3}H-thymidine Autoradiography and PCNA Immunostaining:

Periosteum contains two discrete layers: the cambium layer (inner), which is believed to contain the undifferentiated mesenchymal cells, and the fibrous (outer) layer. To identify the location of cells that are stimulated to proliferate we performed autoradiography with ^{3}H-thymidine, incorporated during DNA synthesis and immunostaining with proliferating cell nuclear antigen (PCNA, which appears at the G1/S boundary in the cell cycle during proliferation). The autoradiographic sections (figure 5.5)
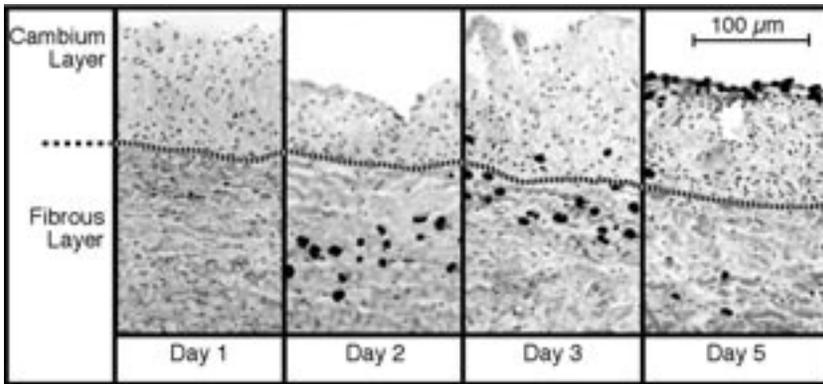

Figure 5.5

Autoradiography with ^{3}H-thymidine shows the onset of proliferation between day 1 and 2 and further increase through time (day 2-5). The black grains indicate the location of labeled cells in the sub-cambial fibrous layer on day 2 which progresses to the cambium layer by day 3, and the outer margins by day 5.

See CD for full color representation

showed that on day 1 a small number of cells were proliferating (fibrous layer: 1% ± 1%, cambium layer: 1% ± 1%). The proportion of labeled cells increased in the fibrous layer on days 2 and 3 (35% ± 8% and 33% ± 7% respectively), but did not rise substantially in the cambium layer (4% ± 6 and 4% ± 5% respectively). The percentage of labeled cells in the fibrous layer peaked at 45% ± 9% after 4 days in culture, at which time the cambium cells were just showing a rise in proliferative activity (16% ± 11%). Proliferation in the cambium layer peaked 48 hours later on day 6, at 18% ± 4% of the total cells being labeled. This difference in timing of the peak activities in the fibrous and cambium layers was statistically significant (p < 0.03) (figure 5.6). This time difference of 24 - 48

Figure 5.6

There was a significant temporospatial difference in proliferative response between the periosteal cambium and fibrous layers. Under the influence of DFP the proliferation rate increased significantly. The peak in fibrous layer response occurs 24-48 hours prior to that in the cambium layer where the chondrocyte precursors reside.
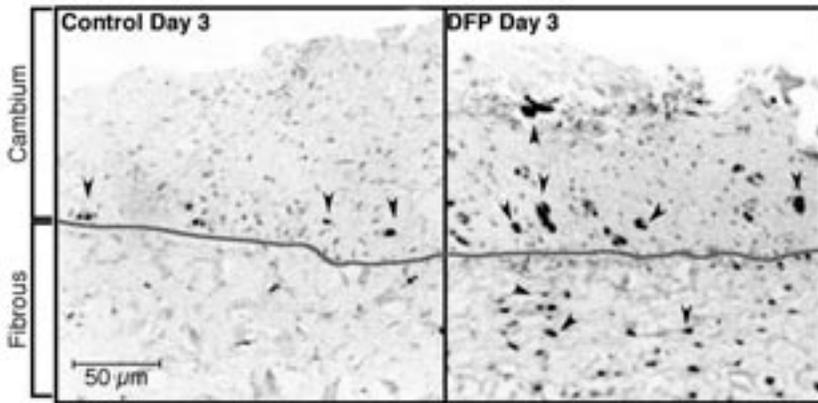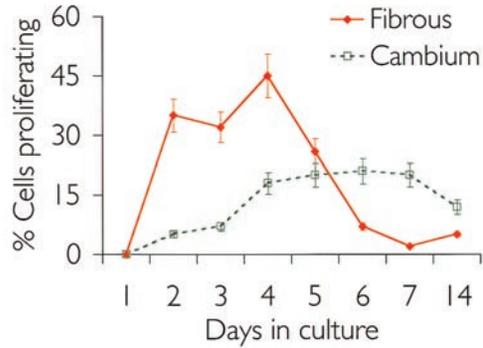

Figure 5.7:

Proliferating cell nuclear antigen (PCNA) immunostaining clearly visualized the difference in proliferative activity between the unstimulated controls and the dynamic fluid pressure (DFP) group. Proliferating cells take up the PCNA stain (arrows) and are localized in the cambium and sub-cambial fibrous layer.

hours would represent 1 - 2 cell cycles. The increased proliferative activity with DFP, as evidenced by ^{3}H -thymidine uptake, was confirmed with greater PCNA immunostaining in the DFP than control explants (figure 5.7). The percentage of PCNA positive (i.e. proliferating) cells in the experimental group (with DFP, no growth factors) on day 4 was 21% ± 3% versus 9% ± 4% in the controls (no DFP, no growth factors) (p < 0.03).

DISCUSSION

The study presented in this chapter has shown that cell proliferation during periosteal cartilage formation can be stimulated by dynamic fluid pressure (DFP), while the total amount of DNA is better retained in the presence of a mechanical stimulus. The autoradiographs visualized initial proliferative activity in the fibrous layer of the periosteum. Twenty-four - forty-eight hours later similar activity was seen in the cambium layer. Furthermore, they demonstrated the presence of proliferating cells in the cambium layer, which contains the chondrocyte precursors. This was confirmed by PCNA immunophotomicrographs demonstrating an increase under DFP.

The observation that periosteal cells respond to mechanical stimulation by proliferating *in vitro* is important for several reasons. First, the ultimate goal of cartilage repair research is to optimize the quantity, as well as the quality, of cartilage produced. Quantity is limited by the number of chondrogenic cells, which for periosteum corresponds with the number of cells in the cambium layer[113].

As further discussed in *Chapter 8*, the decline in the total cell count in the cambium layer with age is the major cause for age-dependent decrease in chondrogenic potential of the periosteum[220,228,232].

During the early stages of periosteal cartilage formation, proliferation occurs prior to cartilage matrix synthesis, the two stages being discretely separated. These and other data suggest the cell differentiation might need to be preceded by proliferation[25,28,29]. Indeed, in an experiment described in *Chapter 7*, we found suggestion of a permissive relation between periosteal cell proliferation and differentiation. Cell proliferation precedes differentiation so that the stem cell population is at least partially retained, as demonstrated by previous data by Ito[144,145], the proliferative activity increased in the cambium layer cell region where the morphological transition from flat to round cells occur, while it diminished during enlargement of round cells.

Potentially, one of the most important observations from this study is the temporal change in geographical distribution of cell proliferation seen by autoradiography and immunostaining with PCNA. There was a consistent time-dependent pattern, with cell proliferation first in the fibrous layer, followed 24 to 48 hours later by proliferation in the cambium layer. These findings suggest either a paracrine signaling mechanism between

the cells in these two layers of the periosteum, or recruitment / migration of proliferating cells from the fibrous to the cambium layer. We believe it most likely to represent evidence suggesting intercellular signaling between the two layers. Paracrine signaling mechanisms almost certainly exist within the periosteum, and this observation may provide a clue as to how periosteal cells respond to mechanical stimulation. Specifically, periosteum must contain cells with mechanoreceptors, and cell division in the fibrous layer is associated with endogenous expression of chondrogenic cytokines that induce the chondrocyte precursors in the cambium layer to divide and differentiate. The evidence for this claim includes the fact that the extent to which periosteum is induced to form cartilage in a fracture is determined by motion occurring at the fracture site[47,48,286,287]. Periosteal chondrogenesis also is induced by physical intervention, such as when it is separated from the underlying bone by a blood clot[264,267] and by transplantation into a joint[86,229,274]. In each case, mechanical factors are present.

The alternative explanation for this observation could be that proliferating cells from the fibrous layer are recruited or migrate into the subcambial fibrous region or cambium layer, comparable to the lymphocyte recruitment seen in inflammatory response and similar effects in regulation of hematopoesis in bone marrow. These two hypotheses could be investigated by performing *in-situ* hybridization studies early during periosteal chondrogenesis, to look for expression of mitogenic and / or chondrogenic factors in the fibrous layer.

It would be understandable if the cells responsive to mechanical stimulation reside in the fibrous layer of the periosteum. The fibrous layer of the periosteum contains cells dispersed within a meshwork of collagen and elastin fibers. Periosteum contains 2.1% elastin by weight[271], which provides it with mechanical properties including its significant contraction following elevation or stripping. The cambium layer, on the other hand, is densely cellular with little extra cellular matrix. It would therefore be plausible that the cells responsive to mechanical stimuli might reside in the fibrous layer.

The data in this chapter show that after one or two cell cycles of proliferative activity in the fibrous layer, proliferation is induced in the cambium layer, where cartilage formation eventually takes place. Uptake of ^{3}H-thymidine as well as PCNA intensity are more obvious in the group stimulated with dynamic fluid pressure, which is what would be expected in

response to a mechanical stimulus. This result is consistent with published reports showing that mechanical stimulation by cyclical pressure oscillations, such as those seen during CPM of a joint, enhance cell proliferation[223,229].

The mechanism of action of mechanical stimuli on chondrocyte precursor cells is unknown. So far there have been no previous studies into the response of periosteum to mechanical stimulation *in vitro*. However, the observation that these cells are capable of responding to a mechanical stimulus is important. Other authors have shown stimulatory effects of dynamic fluid pressure on cartilage, chick calvariae and bone metabolism *in vitro*[51,159,161,312], positive effects of continuous passive motion[214,229] on periosteal chondrogenesis and cartilage repair *in vivo*, and partial restoration of cartilage in osteoarthritis with joint distraction[305]. Collectively, these indicate that dynamic fluid pressure acts as a mechanical stimulus to influence matrix synthesis and cell proliferation. Mechanical stimuli are of paramount importance for development, maintenance and repair of the musculoskeletal system.

In summary, it can be concluded that dynamic fluid pressure (DFP) stimulates cell proliferation in periosteum *in vitro*. These observations are important for several reasons. First, they show that it is possible to study the effects of mechanical stimuli on periosteum in a controlled *in vitro* environment, and indeed there is an effect. Second, a rate-limiting factor in periosteal chondrogenesis is the number of starting cells, and this can be increased by DFP. Increased proliferation is seen in the cambium layer as well as the fibrous layer. These findings direct us toward seeking a paracrine regulation process or the induction of cell migration from the fibrous to cambium layer. Such paracrine regulation would control an initial response to mechanical stimulation by cells in the fibrous layer, releasing growth factors that induce undifferentiated chondrocyte precursor cells in the cambium layer to divide and differentiate into chondrocytes. Cell proliferation in the early stages of cartilage formation is stimulated by mechanical factors.

These findings provide a possible explanation for the increase in cartilage repair tissue seen in joints subjected to continuous passive motion postoperatively. This increases our understanding of the process of periosteal chondrogenesis, which plays a role in both cartilage repair and fracture healing. It has recently been determined that the physiological explanation for delayed fracture healing with age is a reduction in the amount of carti-

laginous callus produced at the fracture site. As most of this callus is formed by periosteum, the implications for understanding how to increase the number of chondrocyte precursors extends beyond cartilage repair to fracture healing as well. Finally, this work forms the basis for further detailed investigation of the biological response of cartilage repair tissues to mechanical stimulation in which we hope to determine the effect of DFP on differentiation and cartilage formation as well as the role of timing and magnitude of the stimulus.

GOAL:

To evaluate the effect of DFP on cell differentiation, matrix synthesis and cartilage formation.

HYPOTHESIS:

DFP will stimulate cartilage formation by increased synthesis of collagen and proteoglycans resulting in more cartilage.

RATIONALE:

Given the known beneficial effect of loading on musculoskeletal tissues we expect that appropriate mechanical stimuli can be a means of increasing cartilage yield. The stimulatory effect on cell proliferation described in chapter 5 directs us towards studying the effect of DFP on subsequent events in cartilage formation which are proteoglycan, collagen synthesis and matrix organization.

METHODS:

Periosteal explants were cultured in the presence or absence of DFP. The effect on cell differentiation, synthesis of matrix components and cartilage formation was determined using collagen typing, sulfate uptake studies, DNA measurement and cartilage histomorphometry.

CHAPTER
6

DYNAMIC FLUID PRESSURE AFFECTS DIFFERENTIATION, MATRIX SYNTHESIS AND CARTILAGE FORMATION

INTRODUCTION

Motion is as important for joints as joints are for motion. Motion of the joint, such as continuous passive motion (CPM), after transplantation of a periosteal graft has been documented to enhance the chondrogenic response in the grafted tissue[220,230,273,276]. However, the mechanisms by which a mechanical stimulus exerts its effect on cartilage formation are unknown. Based on the observation in *Chapter 5* that periosteal cell proliferation is stimulated by dynamic fluid pressure[285] and that the intra-synovial pressure oscillates during CPM (0.6-10 kPa)[220,223], it was speculated that the cyclical fluid pressures experienced by the graft as a result of CPM stimulate the chondrogenic response of the graft.

The capacity of periosteum to respond to mechanical stimulation by forming cartilage is well supported in many systems. Ritsila, showed that in fracture healing, mechanical factors stimulate chondrogenesis in the periosteum[264,266,268]. Raab has shown that mechanical loading stimulates rapid changes in mRNA levels of TGF-β and IGF-1 genes in periosteum[255]. Both these genes regulate cartilage metabolism[9,26,78,79,87,233,234]. Several studies have shown that free intra-articular periosteal grafts respond to joint motion by producing cartilage[220,272]. Intra-articular pressure measurements during CPM documented sinusoidal oscillations in the range of 0.6 - 10 kPa[222]. Cartilage was the predominant tissue formed by 59% of the CPM-treated grafts vs. 8% of the immobilized-knee grafts. Based on these studies it was hypothesized that low-level fluctuations of fluid pressure (DFP) might be responsible for enhancing periosteal chondrogenesis.

The purpose of the investigation presented in this chapter was twofold. After having established the reliability of DFP in an agarose gel culture system (*Chapter 4*) and our finding that DFP stimulates periosteal cell proliferation (*Chapter 5*) we wanted to determine if DFP enhances all aspects of periosteal cartilage formation. Furthermore, there was the need to standardize the model to permit further studies concerning the mechanisms by which mechanical factors regulate cartilage formation, and allow for extending the culture period.

MATERIALS & METHODS

Culture conditions:

Dynamic Fluid Pressure was applied to periosteal tissue culture using machine 1 described in *Chapter 5 (Appendix C)*. This machine allows us the ability to apply cyclic compression with pressures of 13 kPa at 0.3 Hz in the gas phase above the culture plates in which the periosteal explants were embedded.

Two hundred and fifty six periosteal explants from 21 rabbits were cultured either with or without mechanical stimulation. Periosteal explants were harvested from skeletally immature (two-month old) male New Zealand white rabbits using the methods detailed in *Appendix A*. All culture conditions were as reported in the other chapters (*Appendix B*). Controls used were explants cultured without any growth factors (negative controls) or with 10 ng/ml of TGF-β1, which is known to enhance cartilage formation in the agarose culture system (positive controls). The controls were put in identical pressure chambers as the experimental explants, but were not pressurized. A further set of controls was cultured in a separate incubator in order to assess whether there was an effect of just putting the cultures in the chambers.

Tissue analyses:

Histological evaluation was performed to quantify the progressive appearance of the cartilage phenotype. After 10, 21 and 28 days of culture, the explants were sectioned and stained with Safranin O / Fast green using the methods described in *Appendix E*. The percentage of the explant section that was cartilage was determined by using a published automated histomorphometry technique[223]. Collagen typing (after 28 and 42 days of culture, *Appendix F*) and measurement of ^{35}S-sulphate uptake for proteoglycan synthesis (after 19 days of culture, *Appendix G*) were additionally used to confirm the chondrogenic event.

Data Analyses:

All data are presented as means ± 1 standard deviation (SD). All experiments were designed to control for the donor rabbit such that comparisons between experimental and control groups were paired to donor rabbit. We used a sample size for each group of n = 16, to yield an 80% chance of detecting a change in the means ≥ 0.75 standard deviations.

This is considered to be between a medium and large effect size[67]. Analyses were performed by repeated measures analysis of variance and *post-hoc* testing using Duncan's New Multiple Range Test, with a 95% confidence interval. Scintillation counts have been rounded off to two digits of accuracy.

RESULTS

To determine the feasibility of culturing the periosteal explants long-term in the DFP pressure chambers and to determine if DFP has a stimulating effect on periosteal chondrogenesis, we cultured periosteal explants from 21 two-month-old rabbits in the DFP chambers. The explants in the chambers were treated either with or without DFP at 13 kPa / 0.3 Hz. Histological analysis showed a gradual appearance of the cartilage phenotype over time in the controls and to a much greater extent in the DFP-treated explants. Histomorphometry after 28 days of culture revealed a significant 3-fold increase in cartilage formed under DFP over unstimulated controls ($48 \pm 9\%$ vs. $18 \pm 11\%$ $p < 0.05$; figures 6.1 and 6.2). The content of type II collagen showed a similar trend and was significantly higher in the DFP group versus the controls on day 42 ($43 \pm 8\%$ vs. $10 \pm 5\%$ $p < 0.05$; figures 6.3).

Proteoglycan synthesis, as indicated by the uptake of ^{35}S-sulfate on day 19, was 30% greater in the DFP group versus the controls, but not significantly higher (350 ± 50 DPM/μg total Protein vs. 250 ± 75 DPM/μg total protein; figure 6.4). These data indicated that it is possible to maintain explants in culture under DFP for an extended period of time and that a low magnitude of non-stop dynamic fluid pressure has a stimulating effect on cartilage formation and matrix synthesis during periosteal chondrogenesis. Positive and negative controls grown in separate incubators behaved as expected, with TGF-β1 treated samples growing significantly more cartilage than untreated samples (59 ± 6 vs. $14 \pm 5\%$, $p < 0.05$). There was no significant difference between the negative controls grown in the DFP chambers and those grown in a different incubator.

Figure 6.1

Histological section of periosteal explants after stimulation with either TGF-β1 from day 0-2 (+ control), DFP permanently from day 0-28 and no growth factor or mechanical stimulation (- control). The sections were made from explants harvested after 28 days in culture and were stained with Safranin O (red) and counterstained with Fast Green. The Safranin O targets the extracellular proteoglycans excreted by chondrocytes.

+ Control   DFP   - Control
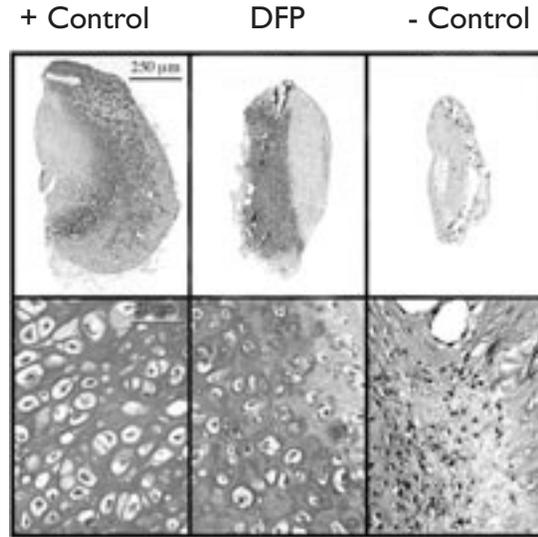
See CD for full color representation

Figure 6.2

Cartilage formation was increased in explants treated by DFP vs. negative control. Quantitative histomorphometry of Safranin O stained sections showed a significant 3-fold stimulation of chondrogenesis by DFP over controls (p<0.05) by day 28 of culture. Note the gradual onset of chondrogenesis over time in both control and DFP treated explants.
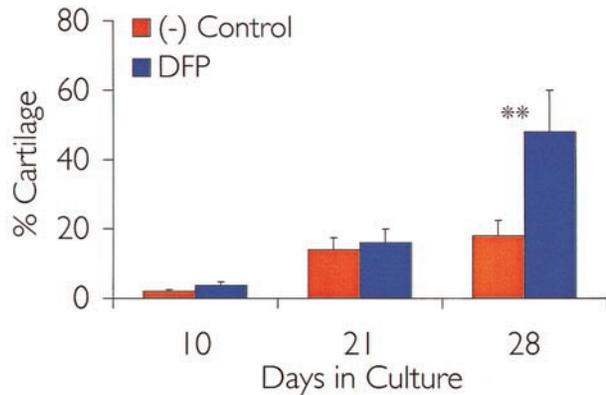
DISCUSSION

In this study, the application of dynamic fluid pressure at 13kPa, 0.3Hz resulted in enhancement of cartilage formation over that seen in unpressurized controls, as demonstrated by increased cartilage yield on histomorphometry, increased ^{35}S-sulphate uptake and increased content of collagen type II protein in the matrix of the periosteal explants.

The observation that the chondrogenic potential of periosteal explants can be stimulated by DFP is important. Since, from the literature[85,108,256,258,261,262], it is known that the maintenance of the chondrocytic phenotype requires ongoing exposure to appropriate mechanical stimulation (too little or too much induces degradation), it is understand-

able, that the induction of chondrogenesis requires appropriate magnitudes of mechanical stimulation.

It would be interesting to speculate what the mechanism of mechanotransduction could be in our system. Several methods have been proposed. Fluid flow alone[58], or fluid flow causing streaming potentials or better transport of food and waste products, could theoretically mediate the effects of unconfined mechanical compression[173,174]. However, the uniform transmission of pressure across the gel in our system would make fluid flow less likely. Other hypotheses include mechanical distortion of cell surface receptor molecules to configurations that permit ligand binding. Mechanical stimulation changes cell shape and organization of stress fibers within the tissue[155,245,246]. Although to a limited extent it was shown that even pure hydrostatic pressure will orient stress fibers and thereby alter cell shape. Several other possible mechanisms of mechanotransduction have been proposed. They involve altered calcium inhibition of cAMP[35,36], prostaglandin E_2, or changes in cellular morphology[242,245], intracellular ion concentration or pH[119], and fixed charge density[51,304,318]. Future studies in well-defined *in vitro* models of cartilage formation will confirm or deny these proposed mechanisms. Thus, it is possible that there is a combined role of mechanical and other factors for the optimum differentiation to the chondrocytic phenotype in any model of cartilage formation.

GOAL:

To determine the influence of modulating DFP characteristics such as magnitude, timing and duration.

HYPOTHESES:

High pressures will be detrimental, while incremental application from low to high will improve cartilage formation. Repetitive application of DFP will elicit an increase in matrix synthesis.

RATIONALE:

The effect and optimal characteristics of a mechanical stimulus will be different for the fragile cells in the cambium layer on day one than for the matrix producing chondrocytes after four weeks in culture. Identifying optimal characteristics can be beneficial for both *in vitro* and clinical application. The specific requirements of magnitude and timing should guide our perioperative mobilization. In tissue engineering, this information may allow us to optimize the *in vitro* conditions and influence dedifferentiation and improve yield.

METHODS:

The role of magnitude was studied by applying low, incremental and high loads of DFP. Duration was examined by testing 30 minutes, 4 hours and 24 hours of exposure per day. Timing was studied by applying DFP either early, late, double (both early and late), continuously or not at all.
The effect on cell proliferation was determined by ^{3}H-thymidine uptake and total DNA measurement. The effect on matrix synthesis was determined through the uptake of ^{35}S-sulfate in proteoglycan synthesis. Chondrogenesis was measured by histomorphometry.

CHAPTER
7

THE ROLE OF DFP PARAMETERS SUCH AS FREQUENCY, MAGNITUDE, TIMING AND DURATION OF EXPOSURE

INTRODUCTION

The experiments described in *Chapter 5* have shown the stimulatory effect of dynamic fluid pressure on periosteal cell proliferation, and identified a temporo-spatial relation between cell proliferation in the periosteal fibrous and cambium layers. In *Chapter 6*, we have shown that DFP influences differentiation, matrix synthesis and increases the amount of cartilage formed. These data suggest that DFP provides a reliable method of simulating mechanical factors in tissue culture. Thus, it would be of interest to better understand the way in which DFP influences cartilage formation *in vitro*. One of the important aspects of the way in which mechanical stimuli affect musculoskeletal tissues lies in the magnitude, frequency and temporal characteristics. It has been suggested that magnitude and frequency have a combined effect where the number of compressions with a certain magnitude per time frame constitutes the load to which the tissue is exposed. Both a low magnitude at high frequency and a high magnitude at a low frequency would amount to the same area (of load) under the curve, and a similar biological effect could result[40,277]. The duration and the time at which tissues are exposed to a certain load are superimposed upon the magnitude / frequency matrix. As exemplified by the few minutes per day of exercise needed by astronaut to minimize muscle wasting and bone resorbtion in space[4,65,301,303]; or the fact that the amount of load experienced until the age of 30 determines the peak bone mass, which predict the risk of osteoporosis in post menopausal women[296,302].

Cartilage formation in periosteal explants has been described as a tri-phasic cascade of events (figure 7.1). As in the classical healing response described in *Chapter 1*, periosteal mesenchymal stem cells respond to external stimuli with DNA synthesis and cell proliferation as the first event to occur. As a sufficient number of cells become available, differentiation is initiated and expression of the cartilage phenotype is seen. Finally chondrocytes begin to synthesize matrix components and a cartilaginous tissue becomes organized[196,228,229]. As illustrated in the introduction to this thesis (figure I.4) the thin periosteum and cambium layer cells constitute a very different structure than that of chondrocytes embedded within an initial matrix seen later in the process of cartilage formation. We hypothesize that the appropriate loading conditions in each phase could also vary considerably.
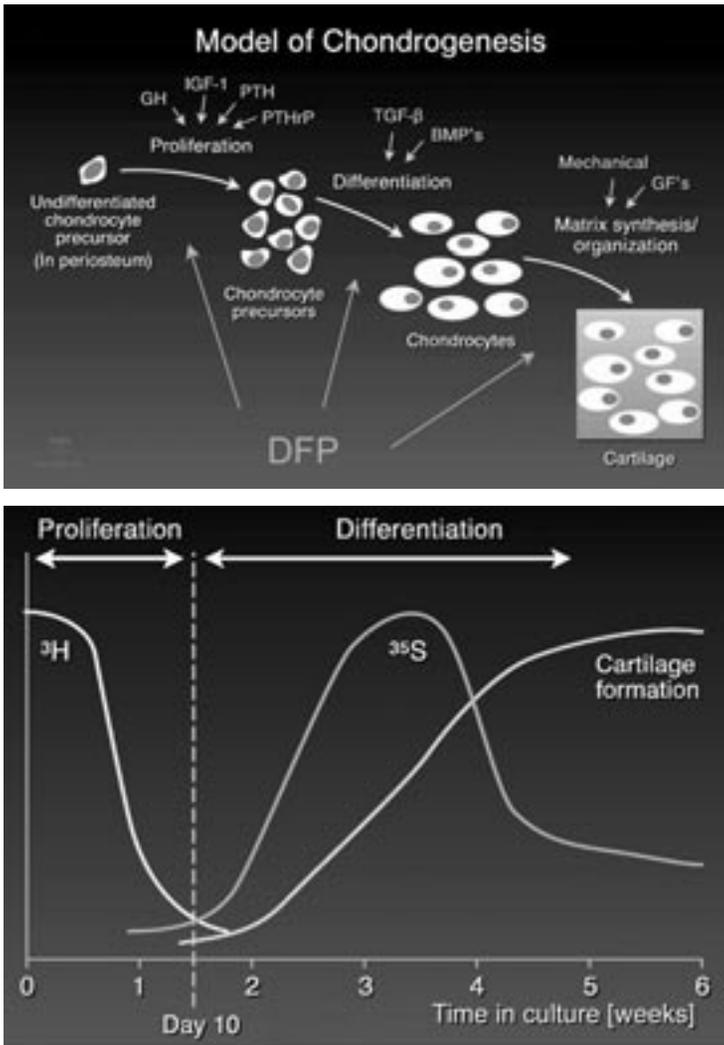
Figure 7.1:

Graphical representation of the consecutive events in periosteal cartilage formation in vitro, as described by Miura et al. Three phases can be discerned: proliferation, differentiation and matrix synthesis with organization following a defined time course. Initially proliferative activity is highest. 3H-thymidine uptake is maximal on day 3 and decreases sharply there after. After proliferative activity subsides, signals of differentiation such as collagen type II gene expression can be found. There is an increase in ^{35}S-sulfate incorporation, which is a measure for proteoglycan synthesis from day 10 until day 24. The third phase is characterized by cartilage formation, which can be seen as early as day 10 and increases until day 42. (Reproduced with permission from the Cartilage & Connective Tissue Research Laboratory at Mayo Clinic, Rochester MN, USA)

Various authors have studied the role of loading characteristics on embryogenesis and metabolism in musculoskeletal tissues[3,8,18,41,51,54,56,66,68,81,84,111,122,155,160,161]. It is generally agreed that there is an interaction between frequency and magnitude as well as between duration and timing of the exposure. These variable parameters form a multi factorial system and create a sheer endless number of combinations to be tested. This poses a considerable challenge for both the statistical design and experimental set up. However, even an initial understanding of factors influencing cartilage formation would provide relevant basic scientific knowledge. Furthermore, the initial step in many tissue engineering approaches includes culture expanding a stem cell population, the outcome of which could be improved if we would be able to apply the appropriate mechanical environment for the tissue / cells at any given time. Finally, the knowledge gained *in vitro* may eventually enable us to adjust peri-operative rehabilitation protocols based upon some initial scientific data indicating the moments on which the magnitude and timing of exposure to mechanical stimulation are most conducive for cartilage formation.

This chapter described a series of experiments designed to acquire an initial feel for the role of modulating some relevant parameters such as; magnitude (low, medium and high loads); timing (early, late, repeated and continuous exposure) and duration (30 minutes, 4 hours, 24 hours).

MATERIALS & METHODS

Experimental design:

The periosteal explant model with DFP as a mechanical stimulus was used to evaluate the effect of modulating magnitude, timing and duration on cell proliferation, matrix synthesis and cartilage formation. A new DFP machine (figure 7.2) was developed, that allows us to apply various loading regimes. This machine (*Appendix C, number 2*) enables the independent selection of experimental settings for 8 tissue culture chambers. Frequency, magnitude, duration and timing of the exposure can be altered during the course of the experiment[284].

Three hundred and four periosteal explants were harvested from 32, immature (two-month old) male New Zealand White rabbits, using the techniques described in *Appendix A*, and divided among experimental

groups. Three different control groups were included. The principal control consisted of cultures placed in a pressure chamber but not exposed to DFP. To the positive controls 10 ng/ml of TGF-β1 was added which has a stimulatory effect on cartilage formation in this model. A further set of control samples cultured in our standard incubator was used in only one experiment to identify possible effects of being in the new DFP machine. All explants were cultured in the standard environment (*Appendix B*) of agarose gel and DMEM with 5% CO_2 in 95% air.
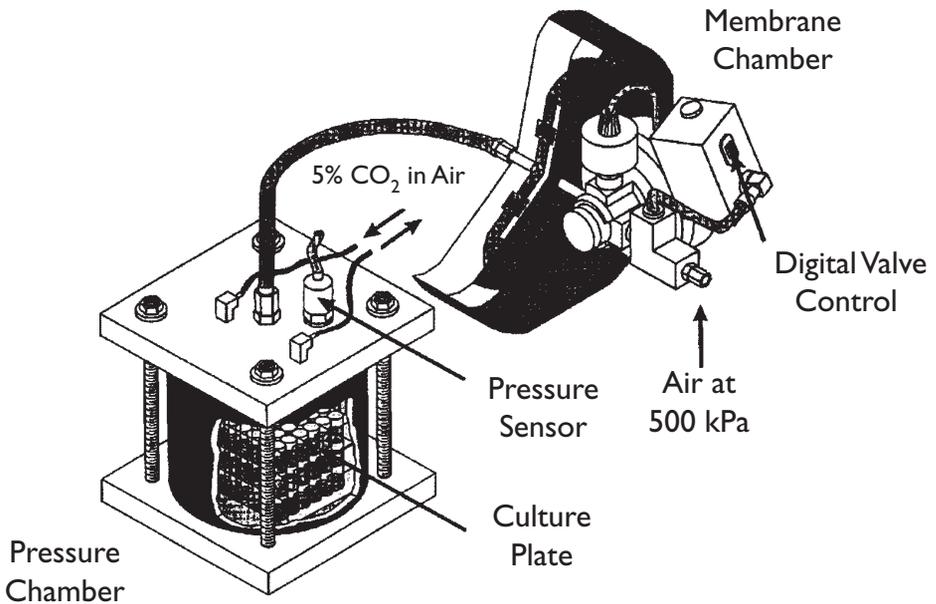
Figure 7.2

Dynamic Fluid Pressure machine number 2 allows individual regulation of parameters such as magnitude and frequency in up to eight culture chambers. (Technical details provided in Appendix C)

Loading regimes:

To study how the duration of exposure to DFP influences cartilage formation, the explants were exposed for 30 minutes, 4 hours or 24 hours per day.

To test the effect due to magnitude of loading three regimes were applied:

- Low (13 kPa, 0.3 Hz for 28 days);

- High (103 kPa, 0.3 Hz for 28 days);
- Incremental (13 kPa, 0.3 Hz for 1 week followed by 54kPa, 0.3 Hz for 1week and 103 kPa, 0.3 Hz for 2 weeks).

To study the effect of timing of DFP on periosteal cell proliferation four variations of exposure were compared to unstimulated controls (group C):

- Early DFP (day 0-3) during the peak of proliferative activity (group E);
- Late DFP (day 18-21) when the proliferation rate is low (group L);
- Combination of early (day 0-3) and late (day 18-21) DFP to identify a possible repetitive effect (group EL);
- Permanent application (day 0-21, group P).

Sample analysis:

Basic analyses were identical to the methods described in previous chapters. Cell proliferation was evaluated by ^{3}H-thymidine uptake. Counts were normalized to tissue wet weight and total DNA which was measured using fluorometry (*Appendix D*)[166]. Proteoglycan synthesis was measured by ^{35}S sulfate uptake (*Appendix G*) and cartilage formation was determined by automated histomorphometry after Safranin O / Fast green staining (*Appendix E*).

In order to study the possible permissive effect of cell proliferation on subsequent differentiation we blocked cell proliferation by adding aphidicolin to the culture medium and performed RT PCR to determine the presence of Collagen type II as a measure of cell differentiation towards the chondrogenic lineage. Aphidicolin was added to block proliferation on day 0-2 when proliferative activity is normally highest. Aphidicolin was also added on day 13 when proliferative activity is low to determine if there was a direct chemical effect on the tissue. Aphidicolin inhibits DNA polymerase α[235,237]. DNA polymerase is essential for chromosomal DNA replication and cell division[146,165].

For RT PCR, each experimental group contained 12 explants from different rabbit donors. After the explants were washed with phosphate buffered saline, they were pooled for the preparation of total RNA with use of Trizol reagent (Gibco BRL) as described by Sanyal[281,283]. Two micrograms of sample was then treated with DNase I before being converted to cDNA with use of Superscript II (Gibco BRL) reverse transcriptase and random primers. Each experiment was repeated at least once, and results were confirmed to be reproducible. For the quantitation of collagen type II gene expression, competitive PCR was carried out

using the rabbit specific primers and ^{32}P dCTP. The amplified PCR products were electrophoresed on agarose gel and were quantitated with a phosphorimager (Molecular Dynamics). The intensities of each of these genes were normalized using Actin as the housekeeping gene.

Statistical analysis:

Eight large periosteal explants (four per leg) could be obtained from each rabbit. To control for error due to biological variation between individual rabbits, only one periosteal explant from each rabbit was assigned to any one group. Therefore, the sample size (n) in these experiments represents not just the number of periosteal explants but also the number of rabbits. Statistical analyses were performed using an ANOVA with Duncan Multiple Range *post hoc* testing to determine significance among groups at $p \leq 0.05$. The number of explants in each group was calculated to give a sample size of n = 12 for all experiments.

RESULTS

The effect of magnitude & duration

Low magnitude DFP stimulates cartilage formation. High magnitudes were detrimental and incremental increase of magnitude from low to high was worse than low level DFP. Histomorphometry after 28 days of culture revealed a higher yield of cartilage in the samples stimulated with low pressures, as compared with the negative controls (figure 7.3, 34 ± 7% versus 14 ± 5%, $p < 0.05$). In contrast, chondrogenesis was completely inhibited using high pressure DFP (103 kPa, 0.3 Hz, 4hrs/day), and incremental DFP at 4 hrs/day resulted in a significantly lower cartilage yield as compared to low DFP, but was not different from the negative controls (10 ± 5% vs. 14 ± 5%; $p > 0.05$). Four hours of stimulation per day produced a better chondrogenic response than only 30 minutes of stimulation per day at low levels of DFP (see table 7.1), and was comparable to the response produced by 24 hours of stimulation per day. The result of continuous DFP in the new machine was similar to that in earlier experiments with the simple machine. At higher pressures, there was no significant difference between cartilage produced by 30 minutes/day and 4 hrs/day stimulation. At higher pressures, longer stimulation times were slightly worse with respect to cartilage yield.

Figure 7.3

The effect of DFP on periosteal chondrogenesis was significant as demonstrated by the clear differences between groups after 42 days in culture: Low pressure DFP (13 kPa, 4hrs/day) stimulated chondrogenesis while incremental pressure rises or high pressure DFP (103 kPa, 4 hrs/day) inhibited chondrogenesis. The letters indicate post-hoc Duncan's multiple range statistical comparisons. Any groups with a letter in common are not statistically different.
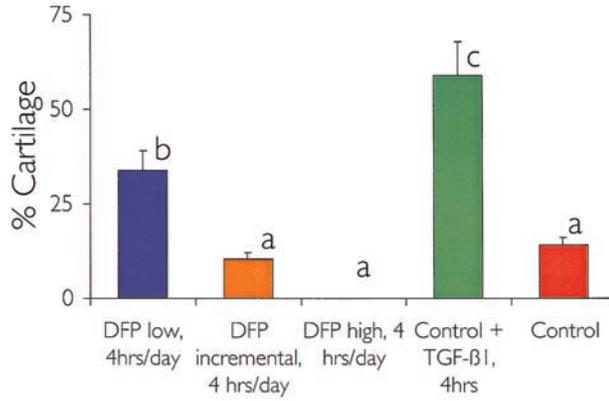
	30 min/day	4 h/day	24 h/day
Low (13 kPa, 0,3 Hz)	12 ± 4 (a,b)	34 ± 6 (d)	48 ± 8 (d)
Incremental	10 ± 4 (a,b)	10 ± 5 (a,b)	
High (103 kPa, 0,3 Hz)	4 ± 3 (a,b)	0 (a)	0 (a)
(-) Control (-DFP,-TGF-β1)			14 ± 5 (a,b,c)
(+) Control (+TGF-β1 day0-2)			59 ± 6 (e)

Table 7.1

Cartilage yields with different DFP protocols. All data are reported as mean ±S.D. Incremental DFP stimulation included low stimulation for week 1, 54 kPa, 0,3 Hz for week 2 and high stimulation for weeks 3 and 4. The letters after the percentage values indicate *post hoc* Duncan's multiple range statistical comparisons. Any two groups with a letter in common are not statistically different.

The effect of timing on cell proliferation

DNA synthesis as determined from ^{3}H-thymidine uptake, measured on day 3 represents the peak of proliferative activity in this model[197]. After this initial high value there is a sharp decline in proliferative activity

(figure 7.1). To better visually represent the biological effect we selected to represent the data in figure 7.4 as percentage of controls (y-axis) to correct for the overall decline in proliferation rate, while absolute data are provided in this paragraph. As expected, there was a significant increase in ^{3}H-thymidine uptake (p < 0.001), in the experimental groups exposed (groups E, EL, P) to dynamic fluid pressure (DFP) of 13 ± 2 kPa at 0.3 Hz, compared to the control group that was cultured in a similar chamber at atmospheric pressure (group C), and compared to those that had not yet been exposed to the stimulus at that time point (group L). The results on day 3 (figure 7.4, left panel), when DNA synthesis is maximal, showed approximately 80% increase under the influence of DFP (DFP: 127.320 ± 9.700 vs. controls: 69.998 ± 5.300 DPM/µg DNA). In this *in vitro* model the proliferative activity is very low after day 7. However, an iden-
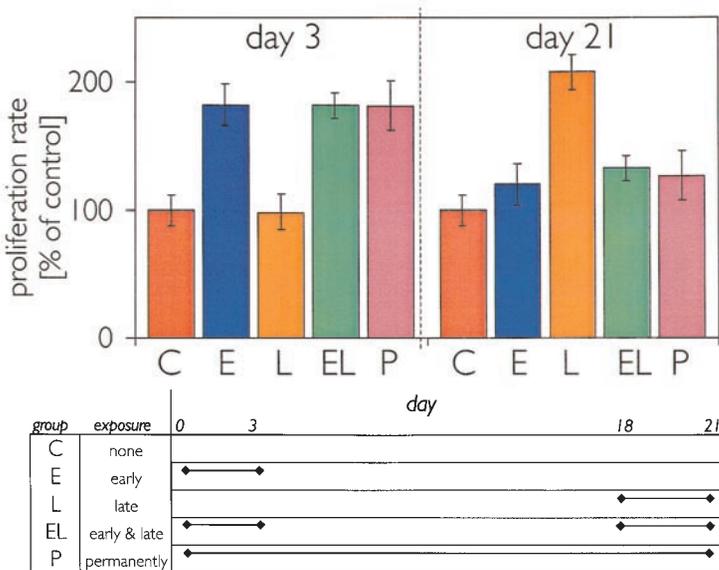
Figure 7.4

Five different loading regimes were applied Group C: control no exposure to DFP, Group E: 'early' exposure from day 0-3, Group L: 'late' exposure from day 18-21, Group EL: 'early and late' exposure from day 0-3 and from 18-21, Group P: 'permanent' exposure from day 0-21. The magnitude and frequency of exposure were 13 ± 2 kPa at 0,3 Hz in each case. Explants were labeled with ^{3}H-thymidine during the last 24 hours in culture. The exposure to DFP led to a significant two-fold increase in ^{3}H-thymidine uptake compared to controls (C). This effect was found after stimulation early (E) as well as late (L) but did not occur twice after exposure early and late (EL), nor was it altered by permanent exposure (P).

tical increase could still be found on day 21 (figure 7.4, right panel, group L: 19.972 ± 3.600 versus group C: 9.634 ± 1.052 DPM/μg DNA, p < 0.003) in the group that had been exposed late. Interestingly, on day 21 there was no increase compared to controls in the other groups that were either stimulated permanently (group P: 12.235 ± 2.871 DPM/μg DNA), both early and late (group EL: 12.972 ± 3.600 DPM/μg DNA) nor was there a persistent increase on day 21 in those explants exposed only at the early point in time (group E,: 11.600 ± 1.720 DPM/μg DNA).

The effect of timing on matrix synthesis:

The effect of DFP on proteoglycan synthesis was determined in two ways. First by permanent application of DFP from day 0-21 while measuring
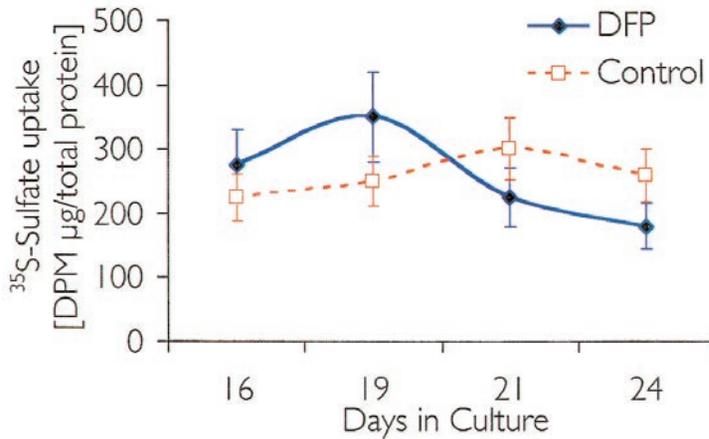
Figure 7.5:

This graph depicts the rate of sulfate incorporation over time, as a measure of matrix synthesis. The effect of timing the stimulus on the rate of sulfate incorporation demonstrates acceleration in the time course of matrix synthesis in the DFP group versus controls. This shift is similar to the significant difference in time of cell proliferation between the fibrous and cambium layer (figure 5.6). There is an increase of 30% in matrix synthesis at day 19 in the DFP group when compared to the controls on day 19. This effect however was not significantly different on statistical analysis.

[35]S-sulfate incorporation from day 16 to 24, secondly in the groups identical to the proliferation study just described. When exposing explants to continuous DFP from day 0-21 we found a gradually increase in proteoglycan synthesis in both control and DFP explants at a somewhat different rate. Evaluation of these data does show a remarkable resemblance in the two curves with a shift in the peak of maximal activity of about 2 days (figure 7.5). The DFP group appears to undergo matrix synthetic activity prior to the onset in the control group. This effect of a time shift clearly resembles the effect found in the autoradiography studies in evaluation of proliferation described in *Chapter 5* where activity in the fibrous layer pre-
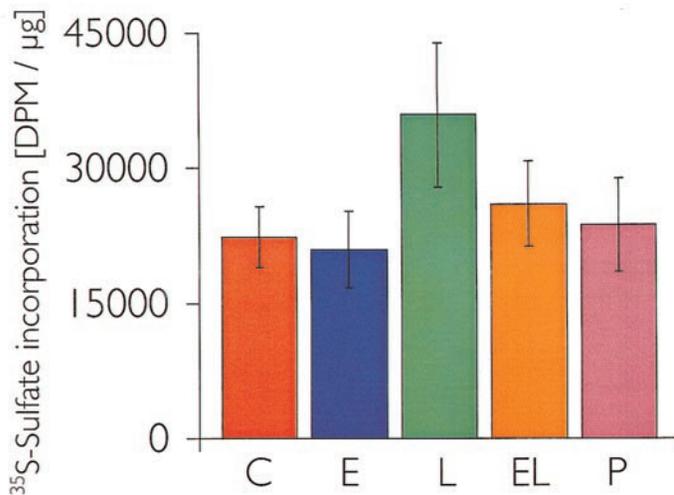
Figure 7.6

Five different loading regimes were applied Group C: control no exposure to DFP, Group E: 'early' exposure from day 0-3, Group L: 'late' exposure from day 18-21, Group EL: 'early and late' exposure from day 0-3 and from 18-21, Group P: 'permanent' exposure from day 0-21. The magnitude and frequency of exposure were 13 ± 2 kPa at 0,3 Hz in each case. Explants were labeled with ^{35}S-Sulfate during the last 24 hours in culture, and harvested on day 24. The exposure to DFP did increase the sulfate incorporation on day 24 in the groups that were exposed early (E), permanently (P) or twice (EL). The highest incorporation rate was measured in group (L) that was exposed during the 3 days before the end of culture (from day 18-21) however, variation between explants was considerable and no significant result was measured.

ceded that in the cambium layer by about 48 hours. However, this differ-ence was not statistically significant due to a large standard deviation in the assay, which was also present in a repeat of this design. Next we used the identical experimental groups (C, E, L, EL, P) as in the evaluation of the proliferation effect above, and measured the ^{35}S-sulfate uptake on day 24 (figure 7.6). The group (L) that had only been stimulated shortly before labeling and analysis had the highest ^{35}S-sulfate incorporation rate, though again SD was too high to demonstrate a significant effect. We found no (statistically) significant difference between the other groups.
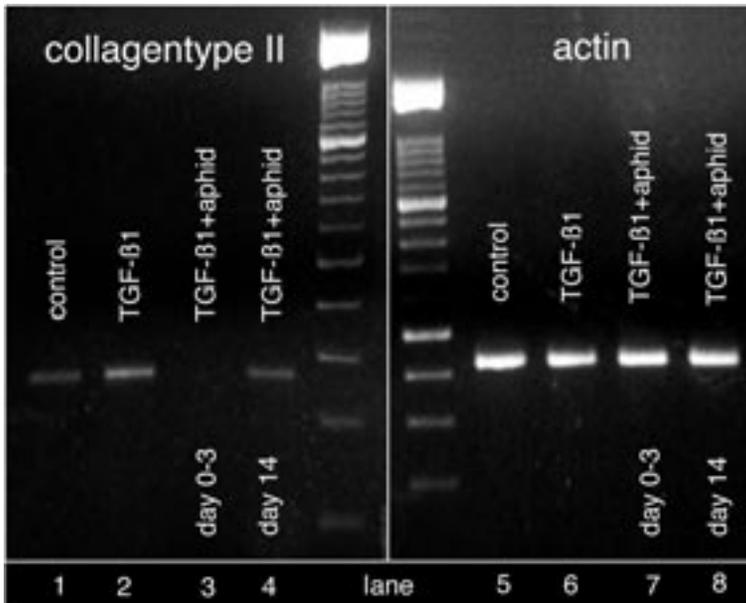
Figure 7.7

The left panel of this gel after RT PCR shows the inhibitive effect of aphidicolin, which blocks cell prolifera-tion, on collagen type II gene expression that can be used to indicate the differentiation state of periosteal cells. There is a down regulation in lane 3 that contains the group where aphidicolin was added during the peak of proliferative activity, but not when it was added after proliferation has subsided (lane 4). Control explants (lane 1&5) were viable and produced collagen type II. As in the standard model the addition of TGF-ß1 stimulated collagen type II gene expression (lane 2).

Proliferation differentiation link:

The gel depicted in figure 7.7 shows the result of the RT PCR experiment studying the effect of blocking proliferation on differentiation. In the positive control group (with TGF-β1) there is a clear increase in collagen type II gene expression when compared to the control explants without additives. However, when up to 95 % of proliferative activity is blocked by adding Aphidicolin from day 0-3 we found only traces of a collagen type II signal with an unchanged Actin (housekeeping gene) message. When the aphidicolin was added on day 14 there was no alteration in the collagen type II expression, confirming the viability of cells and continued matrix synthesis when proliferation had progressed uninfluenced. These data suggest a permissive relationship between proliferation and subsequent differentiation since differentiation will not occur to the same extent if proliferation is blocked.

DISCUSSION

The current study was designed to investigate the effect of variations in application characteristics of the DFP stimulus. The relevance of this approach is supported by the suggestion of homeostatic loading by Dye[94] which hypothesized that the range of load that can be applied across an individual joint in a given period without supraphysiologic overload or structural failure can be termed the envelope of function. This range of homeostatic loading can be graphed with increasing applied loads on the vertical axis and the frequency of loading on the horizontal axis. At least 4 categories of factors together determine the envelope of function for a given joint including anatomic, kinematic, physiologic, and treatment factors. After having found a beneficial effect of DFP on periosteal cartilage formation *in vitro* as described in *Chapters 5* and *6*, it became of interest to examine the boundaries of the 'envelop' of this systems in as far as loading parameters are concerned. Therefore, we executed this set of experiments that study the effect of magnitude, frequency, timing and duration on periosteal cell proliferation, differentiation and cartilage formation *in vitro*.

The response to dynamic fluid pressure was dose-dependent. The results demonstrate that high loads are detrimental to the tissue while a low magnitude increases proteoglycan synthesis, collagen type II content and the

amount of cartilage formed. The incremental increase of load that we studied did not cause significant change. When DFP was applied, 'the timing' has a significant effect on the cell proliferation. Stimulation occurs similarly both early and late in the cascade of cartilage formation, but there was no added effect from permanent or repeated exposure.

The duration was shown to be of influence since 4 hours of exposure was significantly better than only 30 minutes while the response did not increase by using DFP for 24 hours per day. It was thus established that periodic application of low pressure DFP could enhance cartilage formation in periosteal explants. From a practical standpoint, it is advantageous to find that 4 hours of stimulation per day produces comparable stimulation of chondrogenesis in periosteal explants as 24 hrs/day since the eventual clinical application will be more feasible when used for 4 hrs/day than for 24 hrs/day. Also, the use of DFP to improve *in vitro* conditions periodically versus permanently would be more economical since this allows a larger number of cultures to be exposed per volume of 'DFP environment'.

The investigation of effects on matrix synthesis showed changes in proteoglycan synthesis but differences between groups were not significant. Our hypothesis was to find a stimulatory effect on the amount of proteoglycans formed and the sample size for these experiments were calculated accordingly. Due to considerable standard deviation in the assay, and given the sample size we were not able to determine a significant difference between groups. However, we detected a shift in time course rather than an overall increase in matrix synthesis. We therefore assume that DFP influences matrix synthesis *in vitro*, not primarily by increasing the total amount of PG synthesized per cell but rather by accelerating the speed at which the process proceeds which is in line with the temporospatial relation between events in the fibrous layer and the cambium layer demonstrated in *Chapter 5*.

Finally, blocking cell proliferation was shown to prohibit subsequent differentiation as measured by collagen type II gene expression. The finding that blocking of proliferation by Aphidicolin influenced cell differentiation is suggestive of a permissive relationship between these two cellular events. This conclusion is in line with the need for stem cell populations to retain a certain number before differentiation towards a subpopulation. This finding indicates the need to concentrate on methods to stimulate proliferation early in the process of cartilage formation since a differentia-

tion stimulus at that time may be detrimental or decrease the overall yield. However, once the peak of proliferative activity has passed we may need to alter the characteristics of the stimulus aimed at increasing proteoglycan synthesis. In conclusion, the work presented in this chapter supports our hypothesis that characteristics such as magnitude, frequency, timing and duration influence periosteal cartilage formation *in vitro*. We were able to validate the use of the new DFP machine for prolonged maintenance of cartilage cultures under individually variable culture conditions. The matrix of factors studied and the initial results described will guide our ongoing efforts to unravel the multifactorial spectrum of interactions between these parameters. This is necessary to improve our understanding of the mechanisms by which dynamic fluid pressure increases cartilage formation *in vitro*. Orthopedic therapy should be designed to maximize the envelope of function of musculoskeletal systems with the least degree of risk. We feel these experiments will increase our understanding of how mechanical parameters affect cartilage formation, and may eventually provide a scientific basis for improved culture conditions in commercial *in vitro* systems needed for clinical implementation of tissue engineering, as well as suggest guidelines for peri-operative mobilization protocols.

GOAL:

To evaluate the effect of increasing age on periosteal chondrogenesis.

HYPOTHESIS:

There is an age related decline in chondrogenic potential, related to the decline in periosteal stem cellnumber in the cambium layer.

RATIONALE:

Musculoskeletal tissues do not maintain their full functionality through the course of our lifespan. With increasing age a reduced adaptation to loading, a decreased rate in fracture repair and a decline in metabolic rate are found. It has been suggested that there is an age related decline in chondrogenic potential that may be caused by a reduction in stem cell availability while most cellular function remains intact. It is important to study these effects to improve the outcome of tissue engineering in young adults, but also increase our knowledge about the ageing musculoskeletal system in an increasingly older populus.

METHODS:

Periosteal explants from NZ white rabbits of varying ages were cultured using the methods previously described. The effect of age on chondrogenic potential was analyzed by measuring cell proliferation with ^{3}H-thymidine labeling, autoradiography and total DNA measurement. Collagen typing determined the amount of type I vs. type II collagen and PG synthesis was measured by ^{35}S-sulfate uptake to determine matrix maintenance parameters. Finally, we histologically evaluated periosteal morphology and the amount of cartilage formed at the end of culture.

CHAPTER
8

THE CHONDROGENIC POTENTIAL OF PERIOSTEUM

DECREASES WITH AGE

INTRODUCTION

Biological repair of damaged articular cartilage is a realistic and very relevant goal towards which clinicians and researchers are striving based on experimental studies showing the potential for cartilage repair through the use of transplanted chondrocytes[132,134,212], mesenchymal stem cells[46,315], perichondrium[238,239,264,289,290] and periosteum[220,221,230,275,276]. When using *in vitro* models and *in vivo* experiments we try to create reproducible conditions and minimize subject related factors. One of the important and unfortunately limiting factor in the process of cartilage formation is the age of the subject. Unfortunately, (young) adults are precisely the target group for whom 'biological reconstruction' of damaged joints is most needed. Thus, we are compelled to understand the influence of age on periosteal chondrogenesis, if it is to be successfully applied to adult patients.

There are indications that the chondrogenic potential of periosteum is qualitatively and quantitatively inferior in mature and older rabbits than in immature ones[220]. This correlates with the observation that the biological activity of periosteum diminishes with age following the completion of skeletal growth[98,189,220]. Nakahara *et al*. were able to grow cartilage from human rib periosteal cells obtained from patients younger than 20 years old but not those taken from patients older than 22 years of age[206]. Now that periosteum is being used clinically to regenerate cartilage and resurface defects in joint surfaces, it is crucial that we improve our understanding of the effect of aging on the potential for biological regeneration of cartilage using periosteum. Other investigators have found that delayed fracture healing in old rats is due to impaired development of the chondral callus rather than alterations in the process of endochondral ossification.

To investigate the age-dependence of periosteal chondrogenesis, we selected the *in vitro* model of cartilage formation by chondrocyte precursors in periosteum[228]. In this *in vitro* environment cartilage formation has been shown to involve three sequential phases[200]: proliferation, differentiation, and matrix formation as was described in figures 7.1. The goal of this study was to investigate the influence of age on the chondrogenic potential of the periosteum by determining how age affects the three phases cartilage formation *in vitro*.

MATERIALS & METHODS

The relationship between the chondrogenic potential of periosteum and the age of the animal from which it is obtained was determined. Periosteal explants were harvested from rabbits aged 2 weeks to 24 months. A screening study had previously been performed using 2 rabbits at each of following ages: 2 weeks, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 18, 21, and 24 months. After culturing in a standardized cartilage yield assay, these data were analyzed by histology and histomorphometry as well as by collagen typing. As shown in the results section, the histological and biochemical data showed best results in the rabbits at 1.5 to 3 months of age, with linear decreases until twelve months of age, after which little or no chondrogenesis was evident.

For more detailed studies, the age groups were then narrowed down to 4 groups in all subsequent studies: 2, 6, 12, and 24 months (6 rabbits per group, the experiment performed twice). These ages were chosen because chondrogenesis was maximal at approximately two months and minimal by twelve months. Skeletal maturity in New Zealand white rabbits occurs by 6 months[156]. We also included twenty-four month-old rabbits that are well past the age (twelve months) at which chondrogenesis was no longer evident in the initial studies.

Culture and analysis of periosteal explants:

Periosteal explants, 1.5 x 2 mm (small), 2 x 3 mm (large), or 1,5 x 6 mm (strips) were taken from the medial side of the proximal tibia of male New Zealand White rabbits using sharp subperiosteal dissection (*Appendix A*)[229]. The small explants were used for histomorphometry and collagen typing, the larger explants were used for the isotope uptake studies, and the strips were used to assess day 0 periosteal morphology. The larger explants behave similarly to the smaller ones in culture[199]. The explants were cultured in the standard periosteal culture model (*Appendix B*)[228]. TGF-β was added to the medium for the first 2 days of culture.

After the preliminary study across multiple age groups, each age group (2, 6, 12, 24 months) consisted of six rabbits. Explants were divided equally amongst the analysis groups. These were removed from culture at the time points at which each respective outcome normally peaks (tritiated thymidine uptake: day 3, sulfate uptake: day 21, type II collagen and cartilage histomorphometry: 6 weeks)[198].

The experiments were performed twice on different days using an additional 24 rabbits (six rabbits per age group). This permitted testing of repeatability of the experiment and reliability of the data. Seven hundred and thirty six periosteal explants from 82 rabbits were used.

Skeletal maturity:

Skeletal maturity was evaluated by weighing the rabbits, examining the distal femoral and proximal tibia growth plates grossly and histologically at the time of periosteal harvesting. Whole joints including the distal femora and proximal tibiae, were decalcified and sectioned for histological examination, except for the twenty-four month-old rabbits, whose skeletal maturity was certain. This was done using an additional 6 rabbits (2 rabbits per age group). Safranin O and H&E stains were obtained. Thus, a total of 88 rabbits were used in this study.

Histomorphometry:

After six weeks in culture, specimens were fixed in 10% neutral buffered formalin, embedded in paraffin, and a 3 μm thick section cut from the middle of each specimen and stained with Safranin O / Fast Green. This sampling method has been shown to be reproducible and to represent the percentage of cartilage in an explant (*Appendix E*)[113].

Collagen typing:

After six weeks in culture, quantitative collagen typing was performed by measuring the relative amount of type II collagen with respect to type I collagen (*Appendix F*)[231]. This technique permits the analysis of very small samples (1 - 10 micrograms) without initial purification of the collagen[217]. The percentage of type II collagen with respect to type I collagen is determined by measuring the ratio of the $\alpha1(II)CB10$ to the $\alpha1(I)CB7,8$ and $\alpha1(II)CB11$ peaks after gel electrophoresis.

[35]S-sulfate uptake:

[35]S-sulfate incorporation was measured on day 21 to be used as an indicator of glycosaminoglycan synthesis. To measure ^{35}S-sulfate incorporation, the explants were incubated with 5 μCi/ml of ^{35}S-sulfate for 24 hrs. The digested explants were dissolved with 0.5 M NaOH, eluted on PD10 columns, and counted on a scintillation counter[157]. Incorporated radioactivity was normalized to explant wet weight (*Appendix G*).

³H-thymidine incorporation:

Proliferation was evaluated by measuring ^{3}H-thymidine uptake on day 3[27,177]. Explants were labeled for 24 hours with 5 μCi of ^{3}H-thymidine, then digested in 0.05 % proteinase K. DNA was precipitated with 10 % TCA using bovine serum albumin as a carrier, dissolved in 0.25 M NaOH, and ^{3}H-thymidine incorporation was counted on a scintillation counter (*Appendix D*)[166].

Autoradiography:

Autoradiography was performed on tissues that had been grown in culture for one through 14 days. They were labeled for 24 hours prior to harvest with ^{3}H-thymidine, washed with PBS and stored in 10 % neutral buffered formalin (*Appendix E*). To determine the labeling index, explants from two, six, twelve and twenty-four month-old rabbits were cultured for one to 14 days (in daily increments). The method of cell counting involved counting the cells in approximately 30% of the cross-sectional area of each histological section[285].

Periosteal morphology:

Periosteal morphology was measured on day 0 with the periosteum intact on the bone (table 1) and on the large explants (table 2). We found that the measurements were more consistent when the periosteum was left intact on the bone. Just as in the autoradiography method above, each explant was meticulously oriented 'on edge' during the paraffin embedding process such that all of the sections obtained from it provided perfect cross-sections of the cambium and fibrous layers. Using techniques previously described, the cambium layer thickness and total cell count were measured at the time of explantation for each of the four main age groups (2, 6, 12, 24 months). The total cell number in the cambium layer is simply the mathematical product of the cell density and the total volume of tissue. To represent this, we derived a normalized cell number, or cellularity, which is the product of cell density and cambium layer thickness.

Data analyses:

Sixteen small periosteal explants (eight per leg), or four large periosteal explants (two per leg) could be obtained from each rabbit. To control for the individual rabbit influences, only one periosteal explant from each rabbit was assigned to any one group. Therefore, the sample size (n) repre-

sents not just the number of periosteal explants but also the number of rabbits. Each experiment was executed in two identical sub experiments a week apart, by dividing the total number of rabbits into two equal groups. Each group with a sufficient sample size to stand on its own as an individual experiment. These were then combined into one group after confirming that the results were the same in the two sub experiments. The advantage of this design includes not just the opportunity to confirm reproducibility, but also improved management of the randomization and controlling for rabbit with large sample sizes, given that the surgeries have to be performed all in one day. Data were then combined and averaged for analysis. Statistical analyses were performed using an ANOVA with Duncan Multiple Range *post hoc* testing to determine significance among groups at $p \leq 0.05$. The number of explants in each group was calculated to give a sample size of $n = 12$. Data are represented as means ± 1 standard error unless otherwise stated, as the data were averaged from two or more experiments.

RESULTS

Rabbit weights & skeletal maturity

The weights of the rabbits increased steadily until six months of age, at which time they reached a plateau around four kilograms (figure 8.1). Gross and histological examinations at the time of sacrificing the animals for periosteal harvesting, revealed that the growth plates in all of the two month-old rabbits were open, while those in the six and twelve month-old rabbits were closed (figure 8.2). These observations are consistent with published data, which have shown that skeletal maturation occurs by the age of 6 months[10]. Regarding the subsequent studies comparing 2, 6, 12, and 24 month-old rabbits, the exact ages were as follows. The '2-month-old' rabbits were all 2 months and 13 days old; the '6-month-old' rabbits were all 6 months and 18 days old. Rabbits in the '12-month-old' group were 12 months and 10 to 18 days, with an average of 12 months and 15 days. In the '24-month-old' group the ranged from 24 months and 21 days to 25 months and 23 days with an average of 25 months and 10 days.

Figure 8.1

The weights of the rabbits increased steadily until six months of age, at which time they reached a plateau around four kilograms.

Body Weight vs. Age

Age [months]
n=118
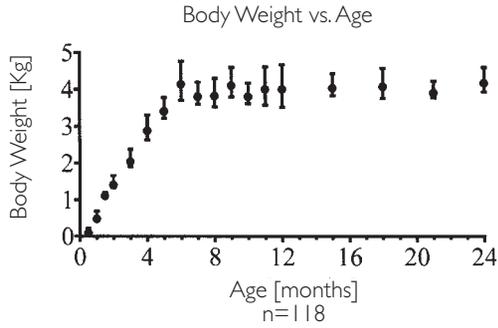
Figure 8.2

The growth plates in all of the two month-old rabbits were open, while those in the six, twelve and twenty-four month-old rabbits were closed, confirming that skeletal maturity had occurred by six months of age in these rabbits.

See CD for full color representation

2 months   6 months   12 months
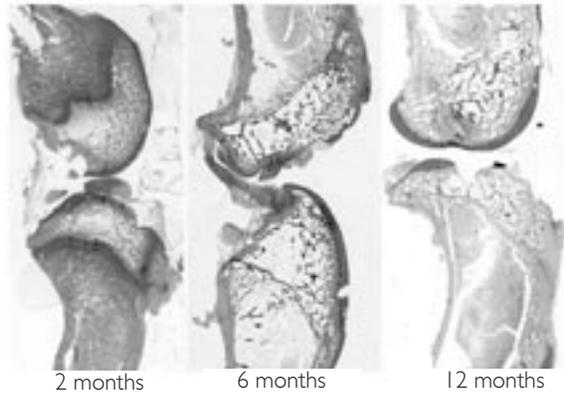
Figure 8.3

The chondrogenic potential of periosteal explants decreased significantly with age (p < 0.0001) as indicated by the decrease in cartilage yield. At each time point, values represent the means and standard errors of eight explants obtained from each of two rabbits.
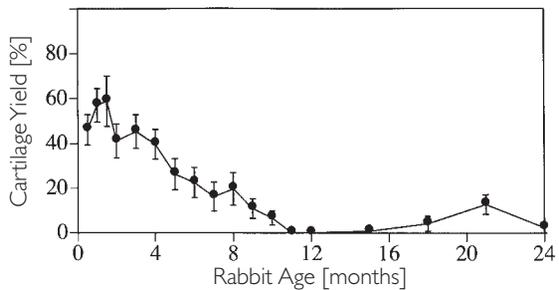
Chondrogenic potential

The horizontal study across age groups from two weeks to two years showed that the chondrogenic potential of periosteum decreased significantly with age ($p < 0.0001$) in a pattern that was inversely related to the weight of the rabbits (figure 8.1 vs. 8.3). Chondrogenesis was maximal in the 1.5 month-old rabbits (cartilage yield = $59 \pm 11\%$), then decreased progressively until twelve months of age. Thereafter, periosteal chondrogenesis was minimal in the fifteen, eighteen, twenty-one and twenty-four month-old rabbits. Only one rabbit in the 15 to 24 month age group had explants that formed any significant amount of cartilage. By the time of skeletal maturity at six months, periosteal chondrogenesis had declined to less than half (cartilage yield = $23 \pm 7\%$) compared to that in the two month-old rabbits.

The experiments involving large numbers of periosteal explants from rabbits aged two, six, twelve and twenty-four months revealed routine pro-
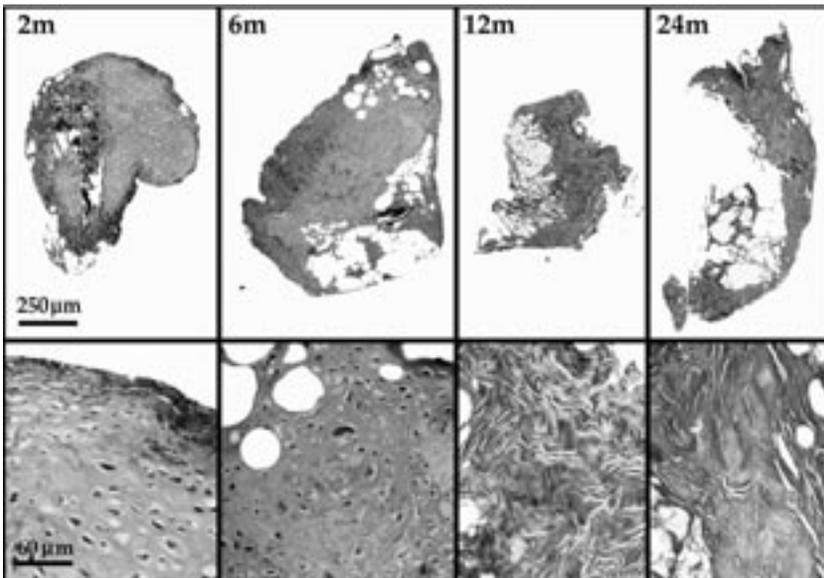

Figure 8.4

Photomicrographs representative of the mean cartilage yield assay results (i.e. % area of cartilage) from the two, six, twelve and twenty-four month-old rabbit explants. Hyaline cartilage, which was routinely seen in the explants from the two and six month-old rabbits, was rarely seen in the twelve or twenty-four month-old groups.

See CD for full color representation

duction of hyaline cartilage in the explants from the two and six-month-old rabbits, but rarely in those from rabbits aged twelve or twenty-four months (figure 8.4). Quantitative histomorphometry demonstrated statistically significant differences in chondrogenic potential among these age groups (figure 8.5 A; $p = 0.0002$). The cartilage yield from the periosteal explants taken from the two month-old rabbits averaged 28 ± 11 % cartilage. This was higher (though not statistically different) than that in the periosteal explants from the six month-old rabbits (13 ± 8 %), the age of skeletal maturity. The quality, and well as the quantity, of cartilage was inferior in the older rabbits (figure 8.4). Chondrogenesis in the periosteal explants from the twelve and twenty-four month-old rabbits was minimal, with cartilage yields of only $0.006\% \pm 0.006\%$ and $0.4 \pm 0.4\%$ respectively. These were significantly lower than those of the 2 and 6 month-old rabbit explants, but not from each other ($p < 0.05$). The cartilage yields from the periosteal explants from the two to six month-old rabbits were significantly higher than those from the twelve and twenty-four month-old rabbits ($p < 0.05$).

Matrix proteins

Indicators of glycosaminoglycan synthesis and type II collagen content correlated with the cartilage yield data, with statistically significant decreases with age of the rabbits from which the periosteal explants were obtained (figure 8.5). The content of type II collagen decreased significantly from 27 ± 8 % in the 2 month-old group to $5\% \pm 2\%$ in the twelve month and $3 \pm 2\%$ in the twenty-four month-old groups ($p < 0.0001$, figure 8.5 B).

[35]S-sulfate incorporation on day twenty-one of culture, decreased similarly with the age of the donor rabbits (figure 8.5 C). Uptake was maximal in the periosteal explants from the two month-old rabbits, then decreased in a pattern similar to the decline in cartilage yield in the six to twenty-four month groups (figure 8.5 C; $p < 0.05$)

Cell proliferation

Cell proliferation, as indicated by the uptake of ^{3}H-thymidine into the periosteal explants decreased significantly with age of the donor rabbits from which the periosteal explants had been obtained ($p < 0.05$, figure 8.5 D). The trend was similar to the decline seen in chondrogenesis, collagen type II content, and ^{35}S-sulfate uptake (figure 8.5 A-C). Figure
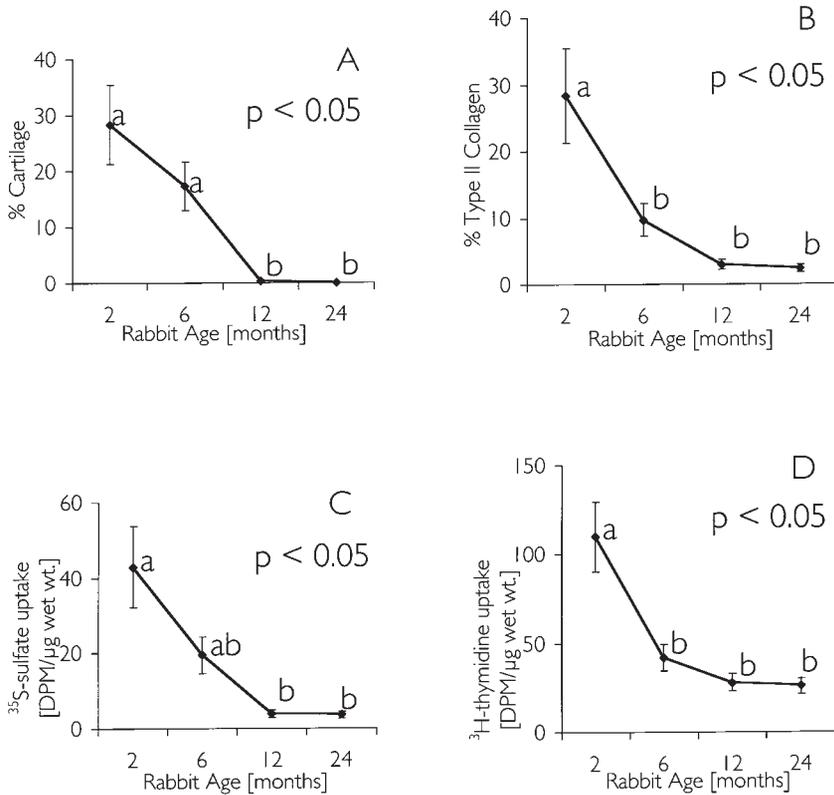
Figure 8.5 A-D

Comparative data from analyzing periosteal explants from four different age groups - two, six, twelve and twenty-four months. Cartilage yield (i.e. % area of cartilage) (A) and type II collagen (with respect to type I collagen) (B) were assayed after six weeks in culture. 35S-sulfate incorporation (C) was measured on day 21 and ^{3}H-thymidine (D) on day 3. The percent cartilage, type II collagen and sulfate contents all decreased in similar patters with age, to minimal levels after completion of growth. The correlation between these parameters of chondrogenesis and cell proliferation, as indicated by ^{3}H-thymidine uptake in D was strong. Letters a, b, and c indicate the result of Duncan's New Multiple Range post-hoc testing. Groups with a letter in common are not statistically different from one another.

8.5 D shows that ^{3}H-thymidine in the sixth month group was reduced to 43% of that seen in the two month group, and further reduced in the 12 and 24 month to just 29% of that in the two month rabbits (P <0.05). To determine whether the decline in proliferative activity with age reflect-
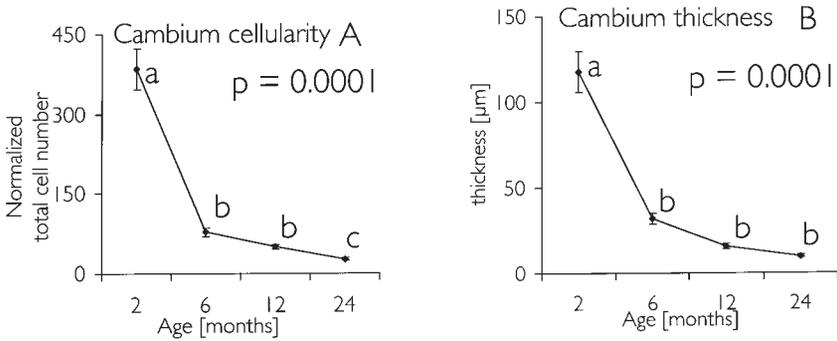
Figure 8.6 A-C

Normalized total cell number, layer thickness and cell density of the periosteal cambium layer versus age. In the cambium layer, there is a marked decrease in cell number and layer thickness with increased age. The total cell number is normalized to a standardized length of periosteum (1 mm). Letters a,b,c, indicate the result of Duncan's New Multiple Range post hoc testing. Groups with a letter in common are not statistically different from one and other.
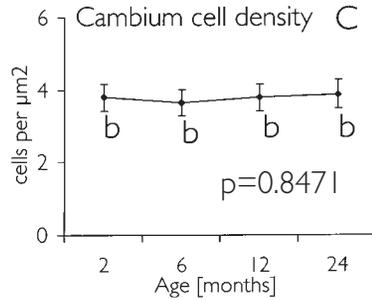
ed a reduction in the number of cells in the periosteum or a decrease in the percentage of cells undergoing proliferation (or both), explants from all age groups were cultured for 1 to 14 days, and labeled with ^{3}H-thymidine for 24 hours before being taken form culture. The autoradiographic sections were obtained and the 'labeling index' was measured. That is, the number of labeled cells was divided by the total number of cells multiplied by 100. Figure 8.8 shows that the peak intensities of proliferation at days 4 - 6 in the cambium layer demonstrated no consistent pattern of change with age.

Periosteal Morphology

Periosteal morphology changed significantly with age, as detailed in tables 8.1 and 8.2 and shown in figures 8.6 & 8.7. Variability was higher in the
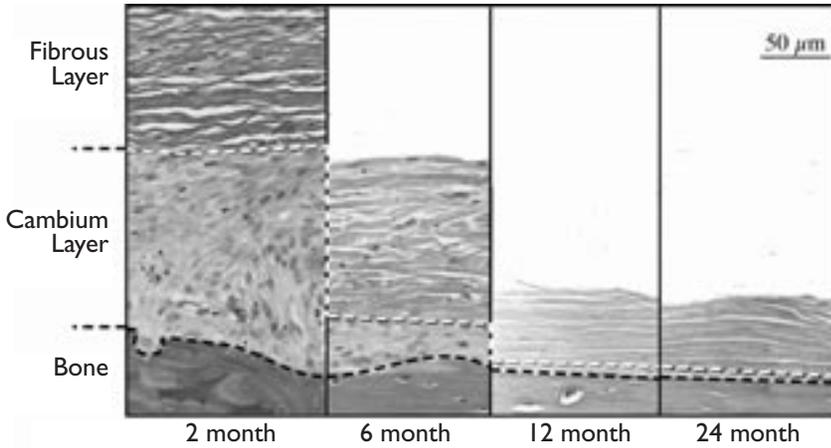
Figure 8.7

Representative photomicrographs of intact periosteum still on the underlying tibial bone, from the same four main age groups used in the other experiments in this chapter, showing how both cambium and fibrous layers become thinner with age. Most notable is the marked reduction in total cell number in the cambium layer, which contains the chondrocyte precursors.

See CD for full color representation

explanted periosteal samples, which showed some evidence of sporadic areas of cambium cell layer losses. Because the measurements were more consistent when the periosteum was left intact on the bone, we believe the data for periosteal morphology to be more reliable in the intact specimens (table 8.1) than in the explants (table 8.2). Most notably, the cambium layer became thinner with age, although changes were seen in the fibrous layer as well. At two months the thickness of the cambium layer of the periosteum, while still intact on the bone, was $118 \pm 43\,\mu$m. This diminished significantly (p < 0.0001) in the six month rabbits to $26 \pm 9.6\,\mu$m and $15 \pm 2.6\,\mu$m at 12 months and $8.3 \pm 3\,\mu$m at 24 months respectively. Cell density was three times as high in the cambium layer as in the fibrous layer, but did not change significantly with age (figure 8.6 C; p > 0.8). The normalized total cell number, or cellularity, in the cambium

Table 8.1:		Rabbit Age [m]					Significant
Periosteum Intact On Bone		2	6	12	24	p-value*	Comparisons**
Cambium Layer	Thickness [µm]	118 ± 43	26 ± 9,6	15 ± 2,6	8,3 ± 3	< 0.0001	2 > 6, 12, 24
	Normalized Cell Number [cells/mm]	393 ± 106	80 ± 27	50 ± 10	29 ± 5	< 0.0001	2 > 6, 12, 24 6 > 12, 24
	Cell Density [cells/cm²]	3,6 ± 0,9	3,4 ± 1,4	3,6 ± 1,1	3,8 ± 1	NS	N/A
Fibrous Layer	Thickness [µm]	172 ± 42	121 ± 27	106 ± 45	68 ± 21	< 0.0001	2 > 6, 12 > 24
	Normalized Cell Number [cells/mm]	200 ± 61	112 ± 28	97 ± 22	61 ± 21	< 0.0001	2 > 6, 12 > 24
	Cell Density [cells/cm²]	1,2 ± 0,3	1 ± 0,3	1 ± 0,4	1 ± 0	NS	N/A

Table 8.2:		Rabbit Age [m]					Significant
Periosteum Intact On Bone		2	6	12	24	p-value*	Comparisons**
Cambium Layer	Thickness [µm]	130 ± 22	37 ± 8,6	21 ± 9	8,1 ± 9	< 0.0001	2 > 6, 12, 24
	Normalized Cell Number [cells/mm]	300 ± 34	72 ± 16	39 ± 11	16 ± 12	< 0.0001	2 > 6, 12, 24 6 > 12, 24
	Cell Density [cells/cm²]	2,4 ± 0,7	2 ± 0,7	2 ± 0,7	1,7 ± 1	NS	N/A
Fibrous Layer	Thickness [µm]	248 ± 36	179 ± 34	162 ± 33	155 ± 35	< 0.0001	2 > 6, 12 > 24
	Normalized Cell Number [cells/mm]	154 ± 12	86 ± 5	67 ± 4	64 ± 4	< 0.0001	2 > 6, 12 > 24
	Cell Density [cells/cm²]	0,6 ± 0,2	0,5 ± 0,2	0,4 ± 0,1	0,4 ± 0	NS	N/A

Table 8.1 & 2

Data shown are means ± 1 standard deviation with n = 12, *Global comparison performed using a repeated measures Analysis of Variance (ANOVA). Pairwise differences identified using Duncan's New Multiple Range *post-hoc* testing, with p ≤ 0,05.
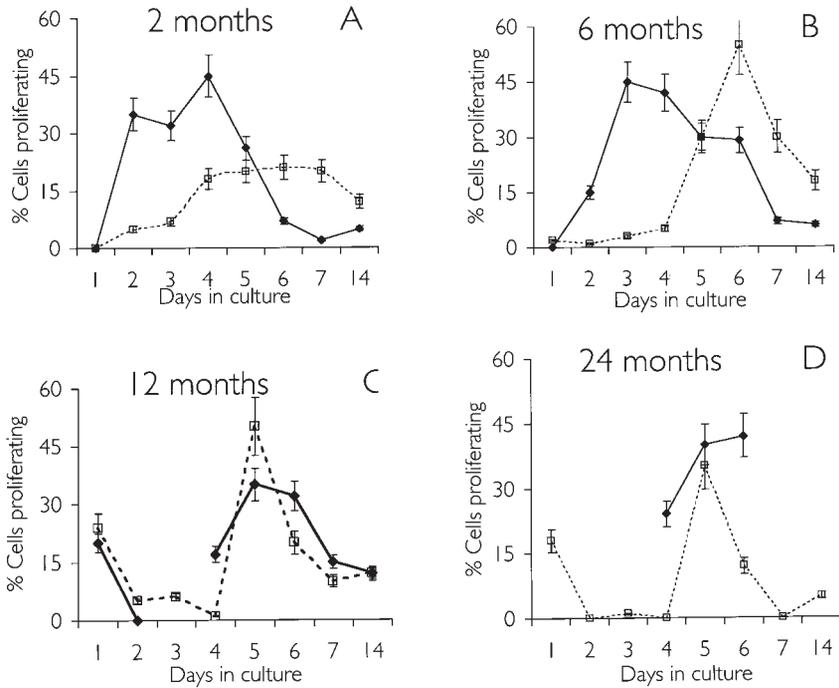
Figure 8.8 A-D

Labeling indices (Solid line, diamond = fibrous layer; dashed line, open square = cambium layer). The labeling index was defined as: (labeled cells / total cells) x 100, as counted on autoradiographs obtained after culturing periosteal explants in the presence of ^{3}H-thymidine. The trends indicate that proliferation in the fibrous layer preceded that in the cambium layer, and that while the labeling indices change over time, there were no obvious age-related differences. In particular, there did not appear to be a reduction in the percentage of proliferating cells in the cambium layer with age.

layer, was calculated as the product of cell density and cambium layer thickness. The normalized total cell number in the cambium layer decreased from 393 ± 106 at two months to 80 ± 27 at six months, 50 ± 10 at twelve months 29 ± 5 cells per mm of length of periosteum (p < 0.0001, figure 8.6). The two and six month groups were significantly different from each other and from the twelve and twenty four month groups. This indicates that the total number of available chondrocyte pre-cursors decreases with age, as these cells are located in the cambium layer[228].

Changes in the fibrous layer are also evident with age, but less dramatically than the cambium layer (figure 8.7). The fibrous layer diminished significantly in thickness and total cell number ($p < 0.0001$). The thickness declined from $172 \pm 42\ \mu$m at two months to $68 \pm 21\ \mu$m at 24 months. As the cell density did not change significantly with age, the decline in total cell number reflected the decline in fibrous layer thickness.

DISCUSSION

The work presented in this chapter has demonstrated that the potential of periosteum for chondrogenesis declines with age. In rabbits, this chondrogenic potential of periosteum begins declining shortly after birth to less than 50% by the time of skeletal maturity at six months. From 6 to 12 months, i.e. an age corresponding to approximately twice that at skeletal maturity, this chondrogenic potential continues to decline, until it reaches a steady state minimal level. Although chondrogenesis was seen in much older rabbits, it was much less frequently.

These data are important for several reasons. First, they establish a baseline for interpretation and planning of future studies related to experimental cartilage formation or repair using periosteum. Second, they offer an opportunity to study and elucidate the mechanisms responsible for the age-dependent decline in chondrogenic potential, not just of periosteum, but also in general. Third, if the mechanisms responsible for this age-dependent decline in chondrogenesis can be elucidated and overcome, it may be possible to apply similar technologies to improve the quality of cartilage repair in younger individuals as well.

The periosteal explants were most chondrogenic prior to skeletal maturity, but continued to demonstrate some chondrogenic potential when taken from rabbits approximately twice the age of skeletal maturity. Nakahara found that isolated human periosteal cells displayed chondrogenic potential when the donors were less than 19 years of age, but not when they were 22 years or older[206]. It would be reasonable to anticipate a reduction in satisfactory outcomes with increasing age in adults. Most patients with damaged cartilage are past skeletal maturity, and the prevalence of this problem rises with increasing age. Experimental studies in lab animals show that ageing is one of the main obstacles to successful cartilage healing[132,180,181,220,280]. Therefore, it will be necessary to understand the age-

related changes in the potential for healing or regenerating cartilage.

The most important observation made in the present study was that the chondrogenic potential was related closely to the total number of cells in the cambium layer of the periosteum, as previously documented for variations in chondrogenic potential with different donor sites[113]. This is the layer in which the chondrocyte precursor cells reside[299]. Tonna and Cronkite speculated that the progressive thinning of periosteum with age might have been due to stretching with radial bone growth, incorporation of periosteal cells into the cortical bone layer, and/or a decrease in proliferative capacity with age[299].

The observation that periosteal chondrogenic potential is determined by the total number of cells in the cambium layer is important, as it offers stimulating proliferation of the chondrocyte precursors or developing chondrocytes as a possible means for enhancing cartilage repair. As stated by Critchlow, cell proliferation is the crucial first step in the development of periosteal cartilage formation[75,76]. In *Chapter 5* we have shown that periosteum responds to mechanical stimulation in the form of dynamic fluid pressure (DFP) by increasing cell proliferation. Subsequently matrix synthesis and cartilage yield were improved (*Chapter 6*). These findings are consistent with reports by Veldhuijzen, in which mechanical factors have been shown to influence chondrocyte proliferation[311].

In summary, these experiments confirm that chondrogenic potential of periosteum decreases significantly with age. This decrease in cartilage formation is associated principally with a decline in the size of the chondrocyte precursor pool. Thus, one possibility for improving cartilage formation by periosteal transplantation in patients beyond skeletal maturity would be to stimulate cell proliferation early during chondrogenesis and/or to increase the final number of chondrocytes by incorporating chondrocyte precursors, mesenchymal stem cells, or chondrocytes in the treatment.

CHAPTER
9

SUMMARY AND IMPLICATIONS OF THE DATA

The novel concept of joint homeostasis forms the central theme in this thesis. Intra-articular homeostasis and factors that influence it should have a more prominent place in our thinking when considering the clinical application of tissue engineering for restoration of articular cartilage defects. The central aim of this thesis is to study joint homeostasis and its role in our attempts at repairing damaged articular cartilage. Our definition of homeostasis encompasses the dynamic mechanism of equilibrium between external influences, such as mechanical stimuli, adaptation to environmental factors and internal factors such as gender, hormonal state, age and joint related parameters such as matrix metabolism, synovial reaction, cartilage integrity, meniscus function and ligament stability. A complex regulatory mechanism is involved in maintaining homeostasis and adapting to the many requirements of a well functioning articulation.

We set out to define some of the many factors that influence cartilage repair by tissue engineering because this remains an unsolved and very relevant clinical dilemma. Since the introduction of tissue engineering as a method of restoring the damaged articular surface, promise has been paramount but general implementation has lagged behind.

Upon reviewing the literature, it became apparent that chondrogenesis and cartilage metabolism are relatively well understood. Many culture systems and animal models enable us to maintain cells in culture and generate cartilage *in vitro* as well as *in vivo*. Although the initial clinical results are encouraging, there is no solid scientific basis for using cartilage tissue engineering in daily practice. Various *in vitro* techniques have shown successful formation of cartilage with considerable resemblance to the hyaline cartilage in a normal joint. Some of these techniques have been implemented in clinical practice and although initial results vary between techniques, outcome reviewed so far is reasonable to good. However, no good long term results are available, the clinical results were never as good as the *in vivo* results and the use of different strategies does not seem to change outcome to any great extent.

To realistically approach this complex matter we limited our investigation by selecting three aspects of joint homeostasis: metabolic derangement of the matrix after cartilage damage, the introduction of mechanical stimuli and role of loading parameters and the way in which subject age influences cartilage formation. We obviously appreciate that this approach can not be comprehensive and that even within the selected aspects, only regions of interest will be examined.

After an initial validation of experimental techniques and outcome measures needed for the proposed investigations we designed an *in vivo* experiment, to determine whether a cartilage defect influences joint homeostasis and how this may influence cartilage repair. Next, we performed various *in vitro* experiments to deepen our understanding of how mechanical factors play a role in regulating chondrogenesis. Finally, we studied the relation between subject age, decreased chondrogenic potential and cellular parameters such as stem cell number and metabolic activity.

SPECIFIC AIM 1: HOMEOSTASIS AND METABOLIC CHANGE

The correlation and reproducibility of histological scoring systems in cartilage repair

Since cartilage histology was to be an important parameter in testing our hypothesis we felt it would be essential to select a scoring system that was internationally accepted and validated. The comprehensive scoring system developed by O'Driscoll, is most frequently applied internationally and seemed ideally suited. Since we found no data on intra- or inter- observer variability, nor was information available on correlation to other scoring systems, we performed a validation of the O'Driscoll score and compared it to the more simple score suggested by Pineda. The data presented in *Chapter 2* demonstrate that both the O'Driscoll score and the Pineda system are reliable and a well reproducible outcome measure for scoring the repair of cartilage defects. These scores can be applied *in vivo* across species as well as in a clinical setting. We chose to use the O'Driscoll score for evaluating cartilage repair in the subsequent *in vivo* experiment because it was shown to be a reliable score, the score allows evaluation of groups of sub-items that may provide additional information about the effect of 'treatment' and this score is frequently used by others, which allows comparison of data.

Joint homeostasis influences cartilage repair

In the *in vivo* experiment described in *Chapter 3*, the defect model in the Dutch milk goat was used to study the outcome of cartilage repair after periosteal transplantation. We reconfirmed that a defect in the medial femoral condyle does not heal and found a reproducible disturbance of proteoglycan metabolism. Proteoglycans are depleted from the matrix, which is a sign of early osteoarthritis and cartilage degeneration. This constitutes an altered intra-articular environment that we refer to as disturbed

joint homeostasis. The outcome of cartilage repair in such an old defect, with disturbed joint homeostasis was significantly inferior to that in a fresh defect in normal joint. When a defect was immediately treated, there was a normalization of matrix metabolic parameters, which suggests a beneficial effect of early treatment on the degree of disturbance in joint homeostasis. The histological scores of cartilage repair were more reliable (higher scores and somewhat smaller SD) in this group than in the group that was treated late. This supports the suggestion of a preventive effect from early intervention. We feel that this finding is of paramount importance since it may provide a way of improving clinical outcome of tissue engineering. It also reiterates the need to critically evaluate the choice of a relevant animal model.

Implications of the data:

If we accept that most patients present with an old defect and therefore altered joint homeostasis, the findings in *Chapter 3* suggest we should now consider changing our treatment strategy by either treating the patients early or by pre-treating the joint in order to normalize homeostasis.

Early diagnosis of relevant cartilage defects becomes essential and therefore rigorous protocols for follow-up and treatment of patients with joint trauma are needed. We should re-think any techniques in which we harvest cartilage from a healthy joint, or even from other locations within a damaged joint, since this will disturb homeostasis even if we were to successfully regenerate cartilage at the location of the traumatic defect. We should try to treat patients as soon as possible, when metabolism is not (yet) permanently disturbed and we may prevent further degeneration by the intervention. It would however be a considerable revolution in our clinical practice to view tissue engineering as a first and not a final resolve. It would be very valuable to quantify joint homeostasis and evaluate the effect of treating patients with systemic drugs or intra-articular techniques in an attempt to normalize homeostasis prior to tissue engineered joint restoration. The result of pre-treatment relies heavily on our capability to determine which patients will progress to osteoarthritis and discern a point of no return. Studies are underway to see whether blood, urine or synovial fluid markers can be identified that indicate patients at risk.

Magnetic resonance imaging (MRI) can identify and localize relevant cartilage lesions. New techniques of MRI spectroscopy by which intra-artic-

ular proteoglycan levels can be mapped provide a non-invasive tool by which we can determine which patient may need treatment and what would be the optimal time to do so.

Various strategies such as non-steroidal anti-inflammatory drugs, COX II inhibitors, intra-articular hyaluronan injection, TNF and Interleukin modulating medication and synovial gene therapy are currently being developed and some initial data suggest these methods could be beneficial in restoring joint homeostasis.

SPECIFIC AIM 2: HOMEOSTASIS AND MECHANICAL FACTORS

Dynamic pressure transmission in agarose gels

In *Chapter 4*, consideration is given to the validation of techniques needed for the *in vitro* research proposed. To study mechanical influences on chondrogenesis we selected a model for cartilage formation in periosteal explants and stimulation by dynamic fluid pressure (DFP) fluctuation. To validate the application of such a mechanical stimulus to tissue explants suspended in agarose gel we studied the transmission of pressure from air, to fluid and through the gel. We demonstrated that there is a complete and instantaneous transmission of pressure applied to the air above the culture medium, and to the explants suspended in the agarose gel. This knowledge is not only relevant for the validity of our subsequent *in vitro* studies, but also provide us with the opportunity to use DFP for stimulation of commercial musculoskeletal tissues culture systems.

Periosteum responds to Dynamic Fluid Pressure (DFP) by proliferating *in vitro*

One of our aims was to investigate mechanical modulation of chondrogenesis. We were able to create and validate a reliable method of sustaining a chondrogenic tissue in culture by the application of DFP for an extended period. The results in *Chapter 5* show that under the influence of DFP, periosteal cell proliferation is increased and cell viability is better retained. Interestingly we found a temporo-spatial difference in the response between the cells in the fibrous layer and the periosteal cambium layer where the chondrocyte precursors are believed to reside. After having found that DFP can be successfully applied to this model for chondrogenesis and that cells respond by proliferating *in vitro* we set out to determine the effect on matrix synthesis and cartilage formation.

The enhancement of periosteal chondrogenesis by DFP

Given the known beneficial effect of loading on musculoskeletal tissues, we expect that appropriate mechanical stimuli can be a means of increasing cartilage yield. Additionally, the stimulatory effect on periosteal cell proliferation described in *Chapter 5* directs us towards studying the effect of DFP on subsequent events in cartilage formation which are proteoglycan synthesis, collagen synthesis and matrix organization. The data presented in *Chapter 6* demonstrate that cartilage formation is stimulated by the introduction of DFP *in vitro*. The amount of cartilage showed a significant two-fold increase over the unstimulated controls. The synthesis of matrix components was monitored by measuring proteoglycan synthesis and by determining the amount of type II collagen, which are both an indicator for chondrogenic differentiation and an essential component for hyaline articular cartilage. Proteoglycan synthesis seemed to undergo an increase and there was a significant two-fold increase in the amount of type II collagen formed when explants were cultured under the influence of DFP. Therefore, we can conclude that DFP enhances cartilage formation in periosteal explants *in vitro* by stimulating cell proliferation and an increase in the synthesis of matrix components, which results in a higher cartilage yield. Given these findings it becomes of interest to study the characteristics of the mechanical stimulus to see how loading parameters may influence the response.

The role of DFP parameters such as frequency, magnitude, timing and duration

In *Chapter 7* we described a set of experiments aimed at studying some variable parameters of a the mechanical stimulus; magnitude, frequency, timing and duration of the exposure. We hypothesize that the response will be different for the fragile cells in the cambium layer on day one than for the metabolically active chondrocytes embedded with their matrix after four weeks in culture.

Our results show that low magnitude DFP stimulates cartilage formation, while high magnitudes were detrimental. An incremental increase of DFP resulted in a significantly lower cartilage yield as compared to low DFP, but was not different from the negative controls. At higher pressures, longer stimulation times were slightly worse with respect to cartilage yield.

The timing of exposure to DFP has a significant effect on the cell proliferation, but we did not find a significant effect on matrix synthesis. Significant stimulation of cell proliferation occurs similarly both early (day 0-3) and late (day 18-21) in the cascade of cartilage formation, but there was not added effect from permanent or repeated exposure.

The duration was shown to be of influence since 4 hours of exposure was significantly better than only 30 minutes while the response did not increase from 24 hours of DFP application.

Finally, blocking cell proliferation was shown to prohibit subsequent differentiation as measured by collagen type II gene expression, which is suggestive of a permissive relationship between these two cellular events.

Implications of the data:

This set of experiments indicate the boundaries of loading parameters to be studied in further detail and establishes the use of DFP in the machine developed for this purpose. The matrix of interactive parameters described in this chapter is currently under more detailed investigation but forms a considerable challenge for statistical design, machine maintenance, investigator patience and the number of tissues to be harvested, cultured and analyzed.

These data suggest the need to use DFP focused on stimulating proliferation early on in the process of cartilage formation, since a differentiation stimulus at that time may be detrimental or decrease the overall yield. However, by day 5-7, once the peak of proliferative activity has passed and differentiation can bee seen, we may need to alter the characteristics of the stimulus aimed at increasing proteoglycan synthesis. Identifying optimal characteristics can be beneficial for both *in vitro* and clinical application. The specific requirements of magnitude and timing should eventually guide our perioperative mobilization. The use of this knowledge in tissue engineering may allow us to optimize the *in vitro* conditions, which may influence cell vitality and dedifferentiation and thus help us to improve both the quality and quantity of cartilage produced. Since the number of stem cells is a confirmed limiting factor in the age related decline in chondrogenic potential, and mechanical stimuli have now been shown to be beneficial, this information can be applied towards improving clinical outcome.

SPECIFIC AIM 3: HOMEOSTASIS AND AGE

The chondrogenic potential of periosteum decreases with age.

Investigating the age related effects on cartilage formation is essential, since ageing as a biological factor is of undisputed importance. Musculoskeletal tissues do not maintain their full functionality through the course of our lifespan. With increasing age a reduced adaptation to loading, a decreased rate in fracture repair and a decline in metabolic rate are found. More specifically, in the field of cartilage repair, it has been shown that young animals have a significantly better capability of repairing cartilage defects than old animals. It is important to study these effects to improve the outcome of tissue engineering in young adults, but also increase our knowledge about the ageing musculoskeletal system in an increasingly older populus.

In *Chapter 8*, we found a clear decline in chondrogenic potential in relationship with increasing age. There was a close correlation between the total amount of cartilage formed and the number of cells in the cambium layer, where the chondrogenic precursors cells are thought to reside. The number of periosteal cells declined with age as the periosteum became more fragile. The percentage of proliferating cells related to the total number of available stem cells was not significantly altered. With the current analysis techniques, we did not see as profound an effect on matrix synthesis and other indicators of cellular activity, when corrected for the number of cells. Therefore, we may cautiously suggest that stem cell numbers are compromised due to increased age, while metabolic activity may be less affected. Our data also suggest a clear need for improving surgical techniques when handling older tissues since they are more fragile.

Implications of the data:

Young individuals have some capacity for cartilage repair, and there is no convincing evidence that young patients with traumatic cartilage damage or osteochondritis dissecans all progress to osteoarthritis. At the onset of adolescence, chondrogenic capacity decreases sharply, related to the number of available stem cells. We should consider these findings carefully in developing our treatment strategy since young patients (open physis) often do not need treatment, and in older patients treatment will be less successful.

The observation that periosteal chondrogenic potential is determined by the total number of cells in the cambium layer is important, as it offers stimulating proliferation of the chondrocyte precursors or developing chondrocytes as a possible means for enhancing cartilage repair. As stated by Critchlow, cell proliferation is the crucial first step in the development of periosteal cartilage formation. Combining these findings with the data from *Chapter 5* steer us towards investigating if and how applying dynamic fluid pressure *in vitro* or similarly, continuous passive motion *in vivo* offers us the opportunity of increasing cell proliferation and subsequent cartilage formation.

The data presented in this thesis suggest the concept of joint homeostasis as an important factor in tissue engineering. Designing, performing and analyzing the experiments have taught us a great deal about metabolic changes, mechanical stimuli and age as relevant factors not only for our studies but for treatment strategies as well.

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APPENDIX:

MATERIALS & METHODS

A: Harvesting of explants
B: Tissue cultures
C: DFP machine
D: Cell proliferation by DNA synthesis
E: Histological evaluation
F: Collagen typing
G: Proteoglycan synthesis

(A) Harvesting of Periosteal Explants in Rabbits

Periosteal explants were taken from the medial side of the proximal tibia. After euthanasia by an overdose of Pentobarbital, Dose: 1 ml per Rabbit (Fort Dodge Animal Health, Iowa) administered through a large ear vein, the hind legs and abdomen were shaven and prepared for sterile harvesting with alcohol and iodine soaks. Placed on their back, both knee joints were exposed through sterile drapes (figure A.1). A medial parapatellar skin incision was made and extended over the tibia. The limited subcuticular tissue as well as the fascia covering the periosteum was removed. The growth plate was identified visually. From a standardized area of 4 mm by 6 mm, 2-5 mm distal to the growth plate, explants are harvested using a sharp subperiosteal elevator[109,229].

Dependent upon the experimental design requirements and analyses techniques to be used, three strips of 1,5 mm by 6 mm, four large explants of 2 mm by 3 mmor eight small explants of 1,5 mm by 2 mm can be retrieved in this way. All periosteal explants were obtained within 10 minutes after death to minimize post-mortem effects on chondrogenic potential[226]. After harvesting all explants were stored for a maximum of 90 minutes in support medium at 4°C until they were plated for subsequent culture using the methods described below.

(B) Tissue Cultures:

The culture conditions were as reported in the periosteal agarose explant model by O'Driscoll[228]. Immediately after surgical harvesting, the periosteal explants were placed in Dulbecco's Modified Eagle Media (DMEM) (430-2100, GIBCO BRL, Grand Island, NY, U.S.A.), with penicillin/streptomycin (50 U/ml/50 μg/ml) (600-5145AE, GIBCO BRL, Grand Island, NY, U.S.A.) and 1 mM proline (p-4655 Sigma, St Louis, MO, U.S.A.) at 4°C for no more than 1.5 hours prior to placement into culture wells. Forty-eight-well flat bottom culture plates (model

3548, COSTAR, Cambridge, MA, U.S.A.) were prepared with previously published techniques[228]. The wells were precoated with high melting temperature (Tm) agarose gel (Bio-Rad Laboratories, Richmond, CA, U.S.A.). The explants were suspended in 0.5 ml of a 1:1 mixture of 1.0% low Tm agarose gel (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and 2x normal concentration DMEM. The final suspension medium contained 0.5% low Tm agarose gel, 1x DMEM, 1mM proline, Pen/Strep (50 U/ml & 50 μg/ml). The agarose gel was allowed to fully congeal at 4°C for 5 minutes. Each 0.5 ml gel layer was covered with 0.5 ml DMEM containing: 10% fetal calf serum (Lot # 37N0840, FCS: GIBCO BRL, Grand Island, NY, U.S.A.), 1 mM proline, Pen/Strep (50 u/ml & 50 μg/ml). In the standard model TGF-β1 (TGF-β1 from porcine platelets; R & D Systems, Minneapolis, MN, U.S.A.) is added to both the agarose gel and the fluid culture medium, in a concentration of 10 ng/ml[228], for the first two days of culture. The medium above the gel layer was replaced every second day. Vitamin C was added daily, in a dose of 25 μg/ml. Vitamin C has a direct role in supporting collagen formation and chondrogenesis *in vitro*[15,263]. Cultures were maintained at 37°C, 100% humidity and 5% CO_2 mixed with 95% air.

Figure A.1

Photograph of a rabbit on the operating table placed on the back, prior to harvesting the periosteum form the proximal, medial tibia. The abdomen and legs are shaven; the skin is disinfected with alcohol and iodine. The two leg bunny drapes are placed over the knee joints for sterile exposure

(C) Dynamic Fluid Pressure Apparatus

Machine 1:

During culture, the tissue could be exposed to mechanical stimulation by application of dynamic fluid pressure (DFP). Plates containing the periosteal explants embedded in agarose were placed in pressure chambers (figure A.4) where the gas phase above the explants could be pressurized. Figure A.2 depicts the set-up of the machine. To apply pressure to the air a piston (A) was moved to and fro by air, controlled by a valve (not shown) that let air at 500 kPa in alternatively on either side (B or C). The piston pushed a membrane (D) to and fro within an steel container (E) which connected to the chamber (F) containing the culture plates (G), thus changing the gas pressure in them. The pressure thus applied was 13 ±2 kPa at 0,3 Hz. The rate of pressure rise was 33 kPa / sec. The chambers contain transducers (H) to monitor the pressure inside (Measurement Specialties Inc, Fairfield, NJ). At the start of each experiment and after media changes the pressure chambers were flushed with 5% CO_2, 95% air mixture and periodically purged manually with an inlet (I) and an outlet (J) valve.

Figure A.2

Dynamic fluid pressure machine number 1, used in the experiments described in chapter 5 and 6. (function of parts A-J explained in the text) (Reproduced with permission from J.P. Velduijzen, ACTA Vrije Universiteit Amsterdam, The Netherlands)
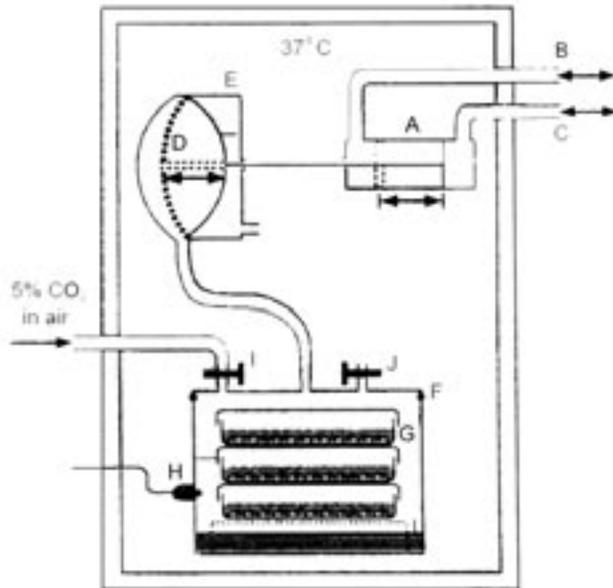
The advantage of this machine is that it can be placed in a standard incubator and is simple to set up and use. The disadvantages are that only three culture plates can be fitted in any chamber, only one pressure magnitude and frequency can be used at a time and the range of pressures that can be generated with it are relatively small (by adjusting the stroke of the piston).

Machine 2

After the initial experiments at 13 ± 2 kPa and 0,3 Hz, we decided to develop a new DFP machine. The purpose of this machine was to allow for multiple experiments to be conducted simultaneously, and to enable the use of a variety of pressures between 1 and 500 kPa, and frequencies from 0 to 1,2 Hz for up to 42 days. In this machine (figure A.3) we can run up to 8 chambers at a time, each one independently pressurized permitting different magnitudes at different frequencies. The pressure is generated by directly forcing pressurized air on only one side of a membrane via a computer controlled valve. The valve can be regulated such that any desired pressure waveform (block, sinusoidal, triangular etc) can be applied on the membrane. Springs positioned on the other side of the membrane ensured that the membrane returned to its original position once the air pressure on the side of the valve fell to zero.

Figure A.3
DFP machine number 2: Eight similar pressure culture chambers are individually controlled to allow independent simultaneous experiments with variations in pressure magnitude, frequency, waveshape, timing and duration of exposure to the mechanical stimulus.
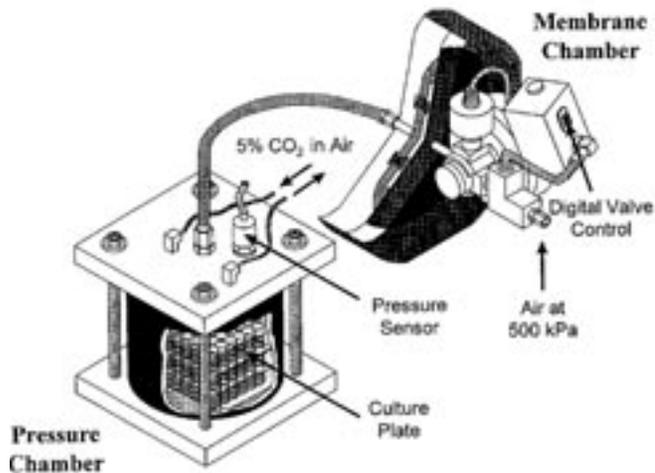
The gas mixture in the chamber was purged periodically (every 4 hours) with a standardized CO_2-air mixture through a computer controlled purge cycle, which opened and closed the inlet and outlet valves in a defined sequence. To generate higher pressures in Machine 2, the computer controlled valve drove pistons, instead of membranes. The high-pressure air would drive the pistons into the cylinders, compressing the gas in the chambers. Once the air pressures fell to zero during the pressure waveform cycle, weights attached to the pistons brought them down to the resting position.

Figure A.4

Photograph of two culture chambers for application of dynamic fluid pressure. Stainless steel cylindrical containers were galvanized to better resist corrosion. Standard 48 well culture plates can be lowered into the chambers on a special tray (bottom left hand corner). The lid of each chamber contains an inlet valve and small purge valves on either side. Pressure transducers in the lid monitor individual chamber pressure.

To generate the highest pressures, an auxiliary piston had to be added, which increased the stroke length of the first piston, thus compressing the gas in the chamber even more. Alarm systems detect and correct situations where the pressures do not meet the user-defined values. Synchronous operation of all the valves controlling all the membranes and pistons was accomplished using the LABVIEW softwarepackage. It requires more maintenance and takes longer to learn how to operate Machine 2. At the very highest pressure settings, e.g. 500 kPa, there were considerable vibrations from the workings of the cylinders and heat generated from the pistons working on the compressed air caused the nutrient media of the cultures to dry up. However, in the experiments in this thesis, those very high pressures

were not used, as it was found that even at 100kPa, there was a negative effect on cartilage formation in the periosteal explants

(D) Cell proliferation / DNA synthesis measurement by thymidine uptake:

For thymidine uptake studies, the explants were labeled for the last 24 hours in culture with 5μCi of [methyl]-^{3}H-thymidine (Amersham, Life Sciences, United Kingdom) in 0.5 ml of media, washed with PBS and digested in 0.1% proteinase K (745 723 Boehringer-Mannheim, Mannheim, Germany). DNA was precipitated with 10% trichloroacetic acid using bovine serum albumin as a carrier, because of the abundance of protein in the explants, dissolved in 0.25 M NaOH, and the radioactivity was measured on a scintillation counter[298,300]. Counts were normalized to wet weight and total DNA which was measured using a Hoeffer DyNA Quant 200 fluorometer and Hoechst H33258 dye[166].

(E) Histological analysis:

All explants were fixed in 10% neutral buffered formalin, and embedded in paraffin. Three micrometer-thick sections for basic histology and 5 μm thick for autoradiography and immunostaining were cut from the middle of each specimen. This sampling method has been shown to be reproducible and to represent the percentage of cartilage in an explant[113].

Histomorphometry:

All sections were stained with Safranin O and counter stained with Fast green. A standard section was always included to correct for variations in the intensity of staining. The samples were always analyzed by a blinded observer using computerized histomorphometry to determine the percentage of the tissue that was cartilage[224,225]. This was performed with a custom-designed software application using the Vidas 2.1 Image Analysis Program from Zeiss (Kontron customized by Carl Zeiss Canada, Don Mills, Ontario, Canada). The analysis of cartilage is based on the uptake of Safranin O stain, with the thresholds and combinations of red, green and blue colors distinguished automatically. With the cartilaginous areas staining red with Safranin O and the non-cartilaginous areas staining blue-green with Fast green, the computer calculates the cartilage yield (i.e. percent area of cartilage) in each explant by measuring the red-stained region of the histological section and by expressing it as a percentage of the whole section's area[224,225].

To determine the location of cell proliferation within the periosteal explants autoradiography was performed by Molecular Histology Labs Inc. (Gaithersburg, MD, U.S.A.). The designated tissues that had been grown in culture for one through fourteen days were labeled for 24 hours prior to harvest with [methyl-^{3}H] thymidine, washed with PBS and stored in 10 % neutral buffered formalin. Each explant was meticulously oriented 'on edge' during the paraffin embedding process such that all of the sections obtained from it provided cross-sections of the cambium and fibrous layers (figure A.5).

To evaluate the percentage of cells undergoing proliferation, a 'labeling index' was determined. The labeling index was defined as the number of labeled cells divided by the total number of cells multiplied by 100. The method of cell counting involved counting the cells in approximately 30% of the cross-sectional area of each histology section.[113,224,225,232,285] Photomicrographs of the autoradiographs were taken at 200x magnification. The total number of both labeled and unlabeled cells was recorded using a calibrated rectangular grid laid over the fibrous and cambium layers of the periosteal explants. Counts were performed twice and averaged. The reproducibility of this technique was confirmed previously[113] as well as in these studies. The accuracy was validated by the finding that the values for cell density in the cambium layer as well as for thickness of the layers were similar to those previously reported by Gallay[113].

Immunostaining:

Immunostaining of explants after 1 to 4 days in culture was performed with an antibody to Proliferating Cell Nuclear Antigen (Dako, Carpentera, CA, U.S.A.) on paraffin embedded sections[169,170] utilizing an avidin-biotin complex method from Biotek Solutions Inc. The primary antibody against PCNA was titered on tonsil and various normal as well as pathological specimens. A positive stain occurs when PCNA antiserum is allowed to bind to a specific antigen within the tissue. The primary antibody is then located by a biotin-conjugated secondary antibody cocktail. The biotinylated secondary antisera is located and conjugated with peroxidase which, after incubation, forms a colored precipitate. The PCNA studies were performed on 8 explants cultured for one or four days with or without DFP.
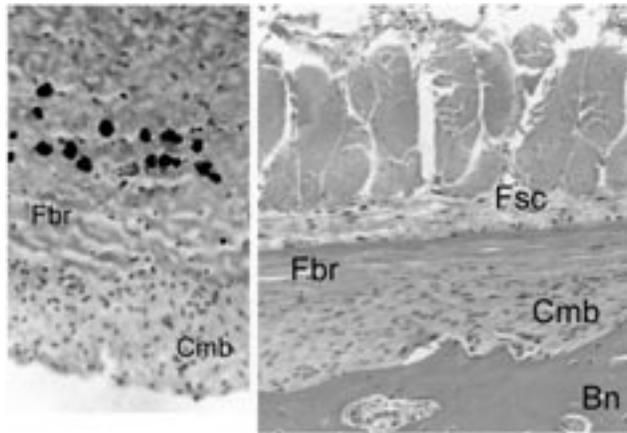
Figure A.5

Light microscopy image of rabbit periosteum mounted 'on edge'. The right panel demonstrates the intact periosteum resting on the bone (Bn) below. The periosteum contains two discrete layers, the cambium layer (Cmb) where the chondrocyte precursor cells are believed to reside, adjacent to the bone covered by the fibrous (Fbr) support layer underlying the fascia (Fsc). The left panel shows an example of periosteal explant mounted 'on edge' for autoradigraphy after ^{3}H-thymidine labeling. The black granules represent cells that have taken up the radiolabeld thymide indicative of DNA synthesis.

(F) Collagen typing

Quantitative collagen typing was performed by determining the relative amount of type II collagen with respect to type I collagen in the tissue samples[231]. This technique has been modified to permit the analysis of very small samples (1 - 10 micrograms) without initial purification of the collagen[217]. All samples were weighed, the individual wet weight is recorded. The collagen peptides were cleaved with 0.5 ml 5% cyanogen bromide (CNBr) in deaerated 88% formic acid. In preparation for electrophoresis, the samples were dissolved in a sample buffer containing 0.063 molar Tris-HCL, pH 8, 3.3 % SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue, at a concentration of 8 micrograms (wet weight) of sample per microliter of sample buffer. One microliter volume of sample was loaded onto 20% gels, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out using a Phast System (Pharmacia LKB, Uppsala, Sweden). A standard derived from articular cartilage was run in the last lane in each gel and analyzed to ensure that the banding pattern for 100% type II collagen was accurately

represented in each gel. The gels were stained with Coomassie blue and scanned on a laser densitometer (Pharmacia LKB). The percentage of type II collagen with respect to type I collagen was determined by measuring the ratio of the $\alpha1(II)CB10$ to the $\alpha1(I)CB7$, 8 and $\alpha1(II)CB11$ peaks in each lane.

(G) PG synthesis ^{35}S -sulfate uptake *in vitro*

^{35}S-sulfate incorporation was measured, as an indicator of glycosaminoglycan synthesis. All explants were incubated with 5 μCi/ml of ^{35}S-sulfate for 24 hrs. After harvesting from culture explants were washed in PBS, weighed and digested overnight in 0.05% proteinase K (Boehringer Mannheim Corp. Indianapolis, IN 6250-0414). The digested explants were then dissolved with 0.5 N NaOH, eluted on PD10 columns (17-0851-01 Pharmacia Biotech, Uppsala, Sweden), and counted on a scintillation counter[157]. Incorporated radioactivity was normalized to explant wet weight and total protein content.

NEDERLANDSE SAMENVATTING

Introductie

Op de polikliniek en de spoedeisende hulp zien wij dagelijks patiënten na een ongeluk of met gewrichtsklachten ten gevolge van een beschadiging van het gewricht kraakbeen. De meeste van deze patiënten zijn jong, actief en worden aanzienlijk gehinderd door deze klachten.

Kraakbeen vormt het gladde oppervlak van het gewricht waardoor in normale omstandigheden vrijwel wrijvingsloos bewegen een leven lang mogelijk is. Kraakbeen bestaat uit een relatief klein aantal cellen in een groot netwerk van collageen vezels met dwarsverbindingen waaraan proteoglycanen zitten die een negatieve elektrische lading hebben. Deze lading trekt water aan waardoor het weefsel opzwelt en zijn glijdende en drukabsorberende functie kan vervullen. Als kraakbeen beschadigd raakt kan het zich niet goed herstellen. Algemeen wordt geaccepteerd dat kraakbeen defecten van meer dan 2 mm in diameter niet spontaan genezen, maar zeker niet alle defecten leiden uiteindelijk tot artrose. De knie is het meest aangedane gewricht, waarbij vaak ook een meniscus beschadiging of kruisband letsel bestaat.

Behandel mogelijkheden

De initiële behandeling bestaat uit pijnstilling, verminderen van de zwelling en rust. In verre weg de meeste gevallen wordt een kraakbeen defect niet direct herkend, en is verder onderzoek met behulp van een MRI scan nodig of wordt de afwijking pas gevonden als een arthroscopie (kijkoperatie) wordt gedaan. Vandaar dat de meeste patiënten met een relevant kraakbeen defect pas worden gezien met klachten van zwelling, slot verschijnselen en pijn, als er al langer gewrichtsschade bestaat. Hierdoor is er 'prikkeling' van het gewricht ontstaan met synovitis (kapsel irritatie) en verlies van matrix componenten (verminderde kraakbeen kwaliteit). Dergelijke onbehandelde gewrichtsbeschadiging lijdt vaak tot artrose. In een poging dit beloop te veranderen worden verscheidene behandelingen toegepast, deze zijn kort samen te vatten als;

- Schoonmaken van het defect en polijsten van de randen waardoor slot klachten en pijn afnemen.
- Het opboren van het onderliggende bot zodat nieuw litteken kraakbeen ingroeit hetgeen 3-5 jaar functioneel redelijk goed resultaat geeft maar daarna weer vergaat.
- Een standsverandering van het been zodat op een ander deel van het gewricht wordt belast dan waar het defect zit. Hierdoor zou de slijtage

vertragen en nemen bij een aanzienlijk deel van de patiënten geduren-
de enkele jaren de klachten af.

- Het vullen van het defect in het dragende deel van de knie door trans-
planteren van kraakbeen pluggen uit een ander deel van de knie of met
donor weefsel. Nadeel is dat elders in de knie een gat ontstaat waarvan
ook kan worden verwacht dat het kraakbeen schade geeft.
- Ten slotte het regenereren van kraakbeen door tissue engineering
waarbij weefsels of cellen worden gebruikt die een delings- en groei-
fase doorlopen (in het lichaam of in een kweeklaboratorium) waarna
zij als kraakbeen cel in het gewricht normaal zouden moeten kunnen
functioneren en zo het defect herstellen. Van deze technieken zijn de
resultaten nog onvoldoende beschreven en niet betrouwbaar genoeg
om als reguliere behandeling te worden bezien.

Helaas zijn wij dus nog niet in staat het gewrichtsvlak betrouwbaar te her-
stellen en is er momenteel geen andere duurzame behandeling beschikbaar
voor de jonge actieve patiënt die nog te jong is voor een kunstgewricht.
Door dit dilemma ontstond interesse in het gebied van 'Tissue
Engineering' ook wel regeneratie geneeskunde genoemd. Het doel is om
beschadigde of verloren weefsels te vervangen door een weefsel dat de
gehele vorm en functie over neemt zodat een duurzaam herstel of liever
'regeneraat' ontstaat dat voor het verdere leven voldoet.

HET CONCEPT VAN GEWRICHT HOMEOSTASE

Een gewricht is een complexe structuur waarin een nauwkeurig samenspel
tussen bot, kraakbeen, meniscus, banden en ligamenten, kapsel en
gewrichtsvocht waardoor beweeglijkheid, stabiliteit, kracht en souplesse
mogelijk wordt. Het regulatie mechanisme dat in deze complexe omge-
ving normaal evenwicht nastreeft noemen we homeostase. Dergelijke
systemen bestaan voor het reguleren van bloeddruk en lichaamstempera-
tuur maar ook voor het zuurstof en suiker gehalte van het bloed en vele
andere biologische processen.
De centrale hypothese van dit proefschrift is dat gewricht homeostase een
zeer relevante invloed heeft op het herstellen van kraakbeen met behulp
van tissue engineering. De beschreven experimenten onderzoeken een
deel van de vele factoren die een rol spelen bij het concept van gewricht

homeostase toegespitst op drie deel gebieden; metabole invloed van defecten, mechanische belasting en leeftijd.

HOMEOSTASE EN GEWRICHT DEFECTEN

Introductie:

Zwelling, pijn, slotklachten en een interval van enkele maanden sinds het initiële trauma dragen bij aan veranderde gewricht homeostase. De biologische condities zijn duidelijk anders dan in een gezond gewricht of in een gecontroleerde laboratorium omgeving waar kraakbeen herstel met tissue engineering gunstige resultaten toont. Wij hebben de veronderstelling dat de gewijzigde lokale condities een belangrijk negatief effect uitoefenen op het proces van kraakbeen vorming door tissue engineering.

Methode en technieken:

Om dit idee te toetsen werd een dierexperiment ontworpen waarbij in 21 geiten een kniedefect werd gemaakt. De dieren werden in drie groepen verdeeld;

1. **geen behandeling:** het natuurlijk beloop van een kraakbeen defect,
2. **vroege behandeling:** het defect werd direct behandeld met een periost (botvlies) transplantaat,
3. **late behandeling:** het defect bleef 10 weken onbehandeld waarna een identieke transplantatie werd gedaan.

Het periost (botvlies) werd los gemaakt van het bovenste deel van de tibia (scheenbeen) en kon met hechtingen in de bodem van het defect worden vast gezet. Periost bevat stamcellen, in de cambiumlaag, die de zowel kraakbeen als bot kunnen maken, en is daarom zeer geschikt voor het bestuderen van kraakbeen groei zowel in een gewricht als ook in gecontroleerde laboratorium omstandigheden. De mate van verstoring van gewricht homeostase na het maken van het defect in het gewricht oppervlak werd gevolgd door de aanmaak, opslag en uitscheiding van kraakbeen eiwitten te meten. Daarnaast werd de hoeveelheid en kwaliteit van het nieuw gevormde kraakbeen bepaald met behulp van een score systeem waarvan eerst de betrouwbaarheid werd getest. Zo werd het mogelijk na te gaan of gewricht homeostase veranderd na het maken van een defect en hoe daardoor kraakbeen groei wordt beïnvloedt, maar ook of kraakbeen groei vervolgens van invloed is op de toestand van de rest van het gewricht.

Resultaten:

In de betrouwbaarheid analyse van *hoofdstuk 2* kon worden aangetoond dat de O'Driscoll kraakbeen herstel score een goed systeem is om kraakbeen herstel te kwantificeren. De vergelijking van uitkomsten tussen diverse onderzoekers is betrouwbaar. De resultaten correleren goed met andere score systemen van vergelijkbare opzet. Het is van groot belang om de betrouwbaarheid van een score systeem te kennen zodat de resultaten van verschillende onderzoekspublicaties in de literatuur kunnen worden vergeleken.

De resultaten van het experiment beschreven in *hoofdstuk 3* herbevestigen dat een defect in het gewrichtvlak niet geneest en aanleiding vormt voor verstoring van kraakbeen metabolisme. Dit geeft een aanzienlijk verandering in de omstandigheden in en rond het gewricht, wat wij aanduiden met verstoorde gewricht homeostase. Het transplantaat in de knie met normale homeostase toonde reproduceerbaar herstel van het gewrichtvlak conform de bevindingen van andere onderzoekers. Het resultaat van kraakbeen herstel in een oud defect bij verstoorde gewricht homeostase was significant slechter dan dat bij een vers defect. Er was geen statistisch verschil tussen laat behandelen en niet behandelen. Daarnaast vonden wij een beschermend effect van het direct behandelen van het defect op de mate van verandering in het kraakbeen metabolisme in de rest van de knie. Derhalve lijkt vroege behandeling een gunstig effect te hebben op het normaliseren van gewrichtshomeostase, terwijl laat behandelen niet verschilde van niets doen.

Implicatie van deze resultaten:

Deze bevindingen zijn van wezenlijk belang aangezien daarmee de mogelijkheid ontstaat om de huidige behaalde klinische resultaten verder te verbeteren. Daarnaast onderstrepen zij het belang van het kiezen van relevante diermodellen om de werkelijk klinische situatie na te bootsen.

Aangezien de meeste patiënten die momenteel worden behandeld een oud defect hebben met verstoorde homeostase tonen deze studies aan dat dat van invloed is op het succes van de behandeling. Daarom moeten wij onze behandel strategie aanpassen door vroeg te gaan behandelen of te proberen eerst de gewrichtshomeostase te normaliseren alvorens tissue engineering toe te passen. Het vroeg herkennen van een relevant kraakbeen defect wordt van groot belang zodat strikte follow-up van ongevalpatiënten en het actief opsporen van verse defecten essentieel kan worden genoemd.

Daarnaast moeten we ons af vragen of het nog wel verantwoord is kraakbeen uit één deel van het gewricht te oogsten om elders in het gewricht te gebruiken. Het zou een revolutionaire wijziging in ons klinische handelen zijn als wij zouden kiezen voor tissue engineering als eerste behandeling en niet als laatste optie.

Hiertoe moeten de huidige mogelijkheden worden gemoderniseerd zodat ook minimaal invasieve behandeling mogelijk wordt, waarbij de mechanische factoren beter worden ingecalculeerd. Ten slotte lijkt het raadzaam om gewricht homeostase te kwantificeren en methoden te ontwikkelen waarmee een verstoorde homeostase kan worden genormaliseerd. Hierbij moeten wij onderscheid proberen te maken tussen patiënten die wel of geen artrose zullen ontwikkelen en ons bewustzijn van een mogelijk 'point of no return' waarna tissue engineering geen nut meer heeft.

HOMEOSTASE EN BELASTING

 "Belasting gerelateerde structurele adaptatie is een waardevolle prestatie van moeder natuur". Dit citaat van Vogel beschrijft het feit dat mechanische factoren leiden tot biologische aanpassing, waarbij een optimaal evenwicht tussen belasting en belastbaarheid het streven is. Mechanische belasting is dus een zeer relevante factor in het concept van gewrichtshomeostase. Voor het steun en beweging apparaat is bekend dat gips immobilisatie of verminderde belasting zoals bij bedlegerigheid of gedurende ruimte vaart aanleiding geeft tot verlies van botmassa en spiervolume. Overbelasting daar en tegen geeft klachten zoals een duim muis, een tennis elleboog of botbreuken bij duursporters.

Vele onderzoekers hebben beschreven dat embryonale groei en kraakbeen metabolisme worden beïnvloed door de mate van belasting. Tot nu toe is echter geen informatie bekend over de invloed van belasting op kraakbeen herstel en tissue engineering. Wij besloten onderzoek te doen naar de rol van mechanische belasting op kraakbeen groei en voor deze studies gebruik te maken van het laboratorium model voor kraakbeen groei uit periost en mechanische belasting door middel van dynamische vloeistof druk.

Periostale kraakbeen groei model:
Om de cellulaire processen in het herstel van kraakbeen te bestuderen is het van belang een gecontroleerd model voor kraakbeen groei ter beschik-

king te hebben. Botvlies (periost) bestaat uit twee weefsel lagen: de stevige fibreuze steunlaag en de dunneren cambium laag die stamcellen bevat met de mogelijkheid om zowel kraakbeen als bot te maken. Gegeven de juiste laboratorium omstandigheden treedt in een periode van 10-42 dagen reproduceerbare kraakbeen groei op en kan dit proces zelfs gedurende een jaar worden vervolgd. Het model van periostale kraakbaan groei is goed gekarakteriseerd en geeft ons de mogelijkheid processen als celdeling, celdifferentiatie en de produktie van matrix onderdelen nauwkeurig te volgen. (Zie voor details Appendix methods and materials A&B) Met dit model is het mogelijk de beïnvloedende factoren te bestuderen en meer te leren over de rol van mechanische belasting en leeftijd als invloeden op gewricht homeostase.

Mechanische belasting door dynamische vloeistof druk:

De zwaartekracht, onze dagelijkse activiteiten maar ook de stabiliteit en belastingsas van het gewricht zijn allemaal factoren die bepalen hoe weefsels mechanisch worden belast en zijn dus van belang in gewricht homeostase. Om te kunnen bestuderen hoe het proces van kraakbeen groei door mechanische stimuli wordt beïnvloed kozen wij voor het systeem van dynamische vloeistof druk (DVD) belasting. Het systeem bestaat uit een gesloten container waarin de kweekplaten met de weefsel stukjes worden geplaatst. De lucht in de container wordt onder druk gezet door een zuiger die heen en weer wordt bewogen op geleide van een computer signaal. De resulterende druk wordt gemeten en via de computer gecontroleerd. (Zie voor details Appendix C).

Eerst werd bepaald (*hoofdstuk 4*) of het valide is om DVD te gebruiken in een kweeksysteem waarin de weefsel stukjes in een gelei onder een vloeistof zitten terwijl de lucht boven de vloeistof wordt gecomprimeerd. Het was daarbij van belang om aan te tonen dat de aangeboden druk ook werkelijk door de weefsel wordt ervaren en te bepalen wat de rol is van wisseling in gelei samenstelling, plaats in de weegschaal en verandering in hoogte en frequentie van de druk. Vervolgens werd in *hoofdstuk 5* gemeten of DVD cel deling beïnvloed en welke respons er in de verschillende cel lagen van het periost kan worden gezien. In de daarop volgende *hoofdstukken 6 & 7* werd gekeken naar de invloed op celdeling, matrix produktie, kraakbeen groei. Ook variabele factoren als de hoogte van de druk, de timing en duur van de blootstelling aan de druk werden gerelateerd aan de verandering van de celeigenschappen tijdens hun groei en uitrijping.

Daarbij was de hypothese dat cellen in verschillende stadia van delingsactiviteit en rijping een verschillende respons op belasting zouden hebben.

Resultaten:

De validiteit van het gekozen model:

Bij het aanbieden van DVD ontstaat een onmiddellijke en volledige geleiding van de aangeboden stimulus naar de weefsels die zich in de gelei bevinden. Deze druk respons blijkt niet gevoelig te zijn voor wisseling in hoogte of frequentie, noch voor wisselende hoeveelheid of samenstelling van de gelei of voor de positie van de weefsels in de kweekschaal. Het is daarom valide om DVD te gebruiken als methode om mechanische belasting op weefsel kweken te simuleren.

Een positief effect op celdeling:

Gebruikmakende van het bovengenoemde kweeksysteem en het model voor periosteal kraakbeen groei konden wij aantonen dat weefsels langdurig onder DVD kunnen worden gekweekt. Dynamische vloeistof druk heeft een duidelijk positie effect op celdelingsactiviteit en helpt een hoger percentage levende cellen in kweek te behouden. Een andere interessante bevinding was het feit dat de eerste reactie van periost cellen op deze mechanische stimulus werd gezien in de fibreuze steun laag en dat de cambium laag cellen waaruit kraakbeen ontstaat pas 24-48 uur later reageren. Dit ondersteunt de hypothese dat specifieke celtypen verschillend reageren op een zelfde stimulus en suggereert een systeem van signalen tussen cellen in diverse weefsel lagen.

Na het aantonen van de validiteit van deze experimentele set-up en het vinden van een positief effect op celdeling werd besloten de invloed op uiteindelijke kraakbeen formatie en de rol van wisselende mechanische parameters te testen.

Stimulatie van kraakbeen groei door dynamische vloeistof druk:

Gegeven het reeds bekende gunstige effect van fysiologische belasting op het steun en bewegingsapparaat mede geholpen door de bevindingen beschreven in *hoofdstuk 5* was het logische om te onderzoeken of niet alleen celdeling maar ook kraakbeen groei werkelijk kon worden verbeterd met DVD. De data in *hoofdstuk 6* tonen dat periostale kraakbeen groei *in vitro* wordt gestimuleerd door de introductie van DVD. Onder DVD ontstond significant meer kraakbeen en was ook het percentage collageen type II, een belangrijke component van goed kraakbeen, twee maal zo hoog als in de controle groep. Wij mogen dus concluderen dat DVD

kraakbeen formatie verbeterd doordat celdelingactiviteit toe neemt, meer collageen en proteoglycanen worden aangemaakt waardoor uiteindelijk een grotere hoeveelheid kraakbeen ontstaat van betere kwaliteit. Gegeven deze bevindingen werd het van belang beter geïnformeerd te zijn over de rol mechanische parameters zoals; timing, grootorde, frequentie en duur van de blootstelling aan DVD die optimaal is.

De rol van belasting parameters:

In *hoofdstuk 7* werd onderzocht hoe parameters als grootorde, timing en duur van de belasting het proces van kraakbeen groei beïnvloeden. De hypothese dat de respons van fragiele stamcellen anders zal zijn dan die van kraakbeen cellen in een matrix werd getest door een spectrum van belasting strategieën toe te passen op verschillende tijdstippen.

Onze resultaten tonen dat een lage dosis DVD kraakbeen groei bevorderd terwijl een hoge dosis juist een negatief effect heeft. Een stapsgewijze toename van DVD gaf een significant slechter resultaat dan lage DVD en was niet verschillend van de ongestimuleerde controles. Timing van DVD heeft een significant effect waarbij zowel vroeg als laat een vergelijkbare stimulatie optreedt, maar geen verbeterd effect gevonden werd na herhaalde of continue toepassing. De tijdsduur van toepassing is relevant want 4 uur DVD was significant beter dan 30 minuten terwijl geen toename ontstond als DVD 24 uur per dag werd toegepast.

Implicatie van deze data:

Deze set van experimenten suggereert een grensgebied waarbinnen de rol van belastingsparameters verder zal moeten worden geëvalueerd. Het bestuderen van deze matrix van interactieve parameters vormt een aanzienlijke uitdaging voor zowel statische analyse als apparatuur en experimentele set-up. Op basis van onze data zou DVD initieel kunnen worden benut om celdeling te stimuleren. Na deze periode van ongeveer 5-7 dagen zou de stimulus langzamerhand moeten worden aangepast aan de veranderende mechanische eigenschappen van de gewijzigde celpopulatie zodat differentiatie en matrix synthese geoptimaliseerd worden. Het identificeren van optimale belasting parameters is van belang voor zowel *in vitro* als klinische applicatie. De belastingscriteria zouden de perioperative mobilisatie protocollen kunnen sturen, terwijl de *in vitro* condities zo kunnen worden gewijzigd dat een hogere celopbrengst met beter kwaliteit wordt verkregen. Het aantal vitale stamcellen is een beperkende factor waardoor de leeftijd van de patiënt van invloed is op de uitkomst van tissue engineering.

Mechanische stimuli blijken een gunstig effect te hebben waardoor deze kennis kan worden gebruikt om de klinische resultaten te verbeteren.

HOMEOSTASE EN LEEFTIJD

Veel van de degeneratieve processen gerelateerd aan ouderdom kunnen mogelijk worden terug gevoerd op onvoldoende gebruik in plaats van op leeftijd zelf. Leeftijd veranderd echter onmiskenbaar de homeostase en controle mechanismen. Ter illustratie bijvoorbeeld het feit dat oude patiënten een minder goede temperatuurscontrole en afweer hebben als reactie op infectie in vergelijking met kinderen. Leeftijd beïnvloed geheugen, houding en de duur van fractuur genezing. Interessant genoeg is het totale aantal niet genezen fracturen bij jonge en oude patiënten nagenoeg gelijk, dus niet alle systemen zijn aangedaan.

Aangezien leeftijd, tot dusver, een onveranderbare grootheid is waarvan nadelige effecten op tissue engineering zijn beschreven vonden wij het van belang deze effecten in verder detail te bestuderen. De leeftijd gerelateerde vermindering in kraakbeen formatie werd bestudeerd, door weefsels van verschillende leeftijden te kweken om daarna kraakbeen hoeveelheid en andere cellulaire processen te evalueren.

Het bestuderen van leeftijdseffecten op de kraakbeen vormende capaciteit is essentieel omdat ouderdom ongetwijfeld een biologisch relevante parameter is. Weefsel van het steun en bewegingsapparaat behouden helaas niet hun volledige functie gedurende ons gehele leven. Met vorderende ouderdom neemt adaptatie aan belasting af, verminderd de snelheid van fractuur genezing en vertragen een aantal metabole processen.

Voor kraakbeen in het bijzonder is aangetoond dat jongeren hun defecten beter genzen dan ouderen. Het is belangrijk deze effecten in detail te bestuderen om zo onze kennis over de mogelijkheid tot het herstellen van kraakbeen in jonge patiënten te verbeteren, maar ook om beter geïnformeerd te zijn over het steun en beweging apparaat op hogere leeftijd in de huidige situatie van een toenemende 'vergrijzing' van de bevolking.

De kraakbeen vormende capaciteit neemt af met stijgende leeftijd:
In de experimenten beschreven in *hoofdstuk 8* vonden wij een duidelijke aan leeftijd gerelateerde afname van de kraakbeen vormende capaciteit. Er was een duidelijke correlatie tussen de hoeveelheid kraakbeen na 42 dagen in kweek en de hoeveelheid stamcellen in de periostale cambiumlaag waar

de kraakbeen voorloper cellen zich bevinden. Het aantal cellen nam af waarbij het periost duidelijk dunner wordt bij stijgende leeftijd. Het percentage delende cellen, gegeven de totale celpopulatie per leeftijd categorie, bleef nagenoeg constant. Met de gebruikte analyse technieken vonden wij geen significante vermindering in de hoeveelheid matrix synthese en andere cellulaire parameters als werd gecorrigeerd voor het aantal cellen. Daarom kunnen we voorzichtig concluderen dat het aantal stamcellen door stijgende leeftijd verminderd terwijl andere cellulaire processen relatief behouden blijven. De resultaten van deze experimenten maken ook duidelijk dat een verbetering van de chirurgische techniek essentieel is in de omgang met oudere weefsels aangezien deze duidelijk meer fragiel zijn.

Implicatie van de data:

Jonge individuen hebben een zekere kraakbeen herstelcapaciteit, en er is geen overtuigend bewijs dat jonge patiënten met een traumatisch kraakbeen defect allemaal artrose krijgen. Na de pubertijd neemt de kraakbeen vormende capaciteit sterk af, het geen is gerelateerd aan het dalend aantal stamcellen. Deze bevindingen moeten in ogenschouw worden genomen bij het bepalen van een behandel plan omdat jonge patiënten (open groeischijf) geen behandeling behoeven terwijl de behandeling bij oudere patiënten minder succesvol zal zijn.

De bevinding dat de kraakbeen vormende capaciteit van het periost is gerelateerd aan het aantal cellen is belangrijk omdat het ons de mogelijkheid geeft celdeling te stimuleren in zowel kraakbeen voorlopercellen als in groeiende chondrocyten om zo kraakbeen herstel te bevorderen. Celdeling is een essentiële eerste stap in de vorming van kraakbeen. Deze constatering gerelateerd aan de resultaten van *hoofdstuk 5* suggereren het nut van het gebruik van dynamische vloeistof druk *in vitro* en als deel van perioperative zorg om zo celdeling te stimuleren en kraakbeen productie te verbeteren.

De data in dit proefschrift suggereren dat het concept van gewricht homeostase een belangrijke rol speelt in tissue engineering. Het opzetten, uitvoeren en analyseren van deze experimenten heeft ons veel geleerd over metabole veranderingen, mechanische stimuli en leeftijd als relevante factoren in wetenschappelijk onderzoek maar zeker ook in het ontwikkelen van klinische behandel strategieën.

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