Cartilage Wound Healing and Integration

Koen Bos

Paranimfen: K.L. Bos
A.J.B.W. Brouwers

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LIST OF ABBREVIATIONS

ACI	autologous chondrocyte implantation
ACL	anterior cruciate ligament
BMP	bone morphogenetic protein
BSA	bovine serum albumin
DMB	Dimethylmethylene blue
DMEM	Dulbecco's Modified Eagle's Medium
DNA	desoxyribonucleïnezuur
ECM	extracellular matrix
FCS	fetal calf serum
FGF-2	fibroblast growth factor type 2
GAG	Glycosaminoglycan
H&E	Haematoxylin & Eosin
ICRS	International Cartilage Repair Society
IGF-I	insulin-like growth factor type I
IGF-II	insulin-like growth factor type II
IgG	immunoglobulin
IL	interleukin
MRI	magnetic resonance imaging
OA	osteoarthritis
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
SD	standard deviation
TGFβ1	transforming growth factor β1
TGFβ3	transforming growth factor β3
TNFα	tumor necrosis factor α

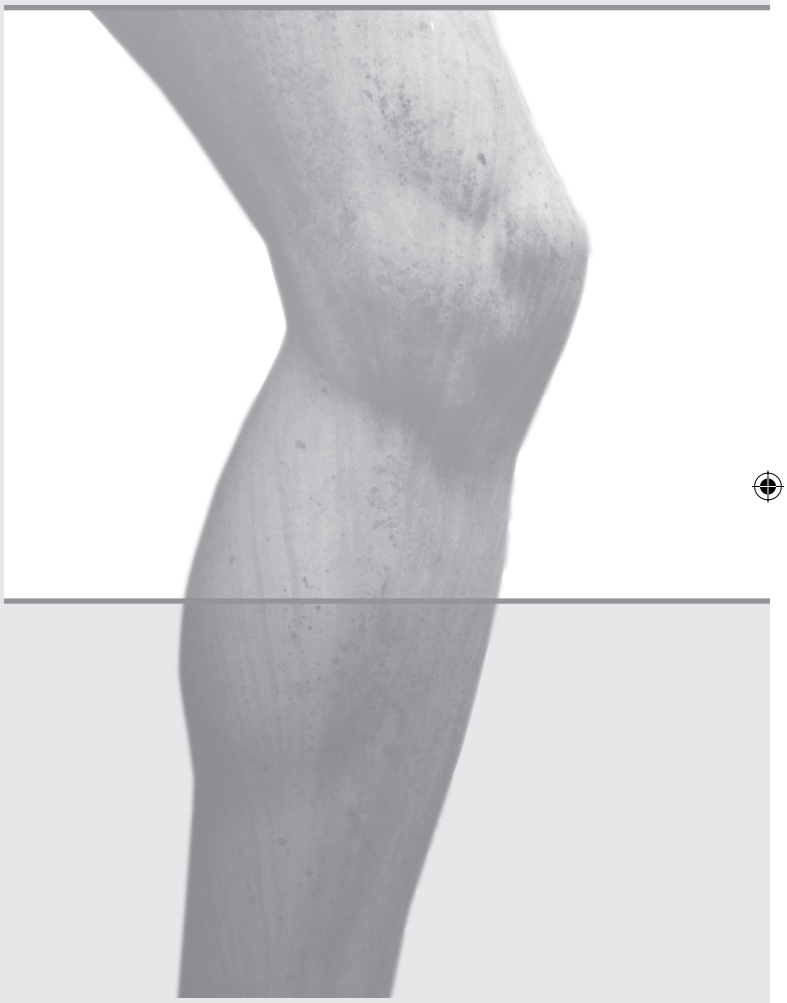
"It should be clear that cartilage does not yield its secrets easily and that inducing cartilage to heal is not simple. The tissue is difficult to work with, injuries to joint surface - whether traumatic or degenerative - are unforgiving, and the progression to osteoarthritis is sometimes so slow that we delude ourselves into thinking we are doing better than we are. It is important, however, to keep trying."

Henry J Mankin, Boston, USA. Editorial NEJM, 1994.

Aan mijn ouders,
Ellen, Maud en Jilles

Chapter 1

General Introduction



GENERAL INTRODUCTION

Articular cartilage is a highly organized avascular tissue composed of chondrocytes embedded within an extracellular matrix of collagens, proteoglycans and noncollagenous proteins. It makes normal, painless, low friction movement of synovial joints possible. Synovial joints are composed of several tissues including joint capsule, ligaments, synovium, hyaline cartilage, menisci and subchondral bone[17]. Hyaline cartilage covers the subchondral bone and forms the articulating surface of synovial joints. It functions as a mechanical shock-absorber and distributes the applied load over the subchondral bone[39].

The regeneration capacity of articular cartilage following injury is considered to be limited.

Partial-thickness articular cartilage defects, limited to the cartilage itself, are not repaired and full thickness defects are repaired with fibrocartilage[81], which has inferior biological and biomechanical properties compared to hyaline cartilage[52]. Complete repair of partial-thickness cartilage injury has only been reported in one study[65]. Superficial lacerations created in situ in fetal lambs were not detectable 28 days later, suggesting that such cartilage wounds may heal completely.

Articular cartilage lesions are frequently associated with disability and symptoms such as pain, effusion, locking phenomena and disturbed function. Moreover, these lesions are generally thought to progress to early osteoarthritis[16,35,55].

The principal goal for surgical management of the symptomatic chondral and osteochondral defects is to reduce symptoms, improve joint congruence by restoring the joint surface with the best possible tissue, and to prevent additional cartilage deterioration. However, current clinical and experimental treatment methods, for cartilage repair and regeneration, do not result in durable and predictable restoration of the articular surface in damaged joints[14,15].

An important prerequisite for durable repair of cartilage lesions and main topic of this thesis is the integration of wound edges or the integration of repair tissue with the surrounding host cartilage[2]. Failure of repair caused by an impaired integration has been documented by several authors who studied the natural cartilage repair process [40,47,56,81,96], repair following transplantation of periosteal and perichondrial grafts[4,71], osteochondral grafts[1,90], autologous chondrocyte implantation[28], natural[22] and tissue engineered grafts[97].

1.1. ARTICULAR CARTILAGE BIOLOGY

Hyaline articular cartilage consists of low density chondrocytes embedded in a tight extracellular matrix, consisting of two components: tissue fluid and a framework of structural macromolecules that give the tissue its form and stability.

Articular cartilage is an avascular, aneural and alymphatic tissue[52]. Nutrition of chondrocytes occurs by diffusion, which depends on the viscoelastic properties of articular cartilage.

The interaction of tissue fluid and the macromolecular framework give the tissue its mechanical properties. Up to 65% - 80% of the wet weight of articular cartilage consists of water, the remaining tissue contains "structural macromolecules", mainly: collagens and proteoglycans. The extracellular matrix (collagens, proteoglycans, non-collagenous proteins and glycoproteins) makes up 20-40% of the articular cartilage wet weight[53]. Collagens (90-95% type II, also types V, VI, IX, X and XI) contribute more than 50% of dry weight, proteoglycans (mainly aggrecans) contribute 25-35% and noncollagenous proteins and glycoproteins 15-20%. (figs 1.1 and 1.2)

Proteoglycan and noncollagenous proteins bind to the collagenous meshwork and water, attracted by negatively charged glycosaminoglycans, fills the molecular framework.

In adult human cartilage, chondrocytes make up about 1-2% of the tissue volume. Chondrocytes are of mesenchymal origin and are responsible for extracellular matrix production.

Each zone of hyaline cartilage has a characteristic composition and architecture consisting of chondrocytes, collagen, aggrecan, and fluid dynamics that relate directly to that zone's function. Although each zone has different morphologic features, the boundaries between zones cannot be sharply given.

The superficial zone (10-20% of total thickness[63]) consists of a "lamina splendens" layer of tightly packed collagen fibres parallel to the articular surface and a cellular layer of flattened chondrocytes. Horizontal orientation of Type II collagen fibres provide resistance to shear.

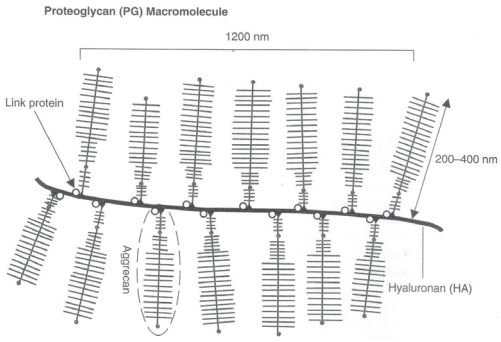


Figure 1.1 Schematic representation of a proteoglycan molecule. In the matrix, aggrecan noncovalently binds to Hyaluronan. Link protein stabelizes this interaction. (reprinted with permission from Mow VC and Hung CT: *Biomechanics of Articular Cartilage*, in Nordin M and Frankel VD (Eds): *Basic Biomechanics of the Musculoskeletal System*, 3rd edition, 2001. Philadelphia, PA, Lippington Williams & Wilkins).

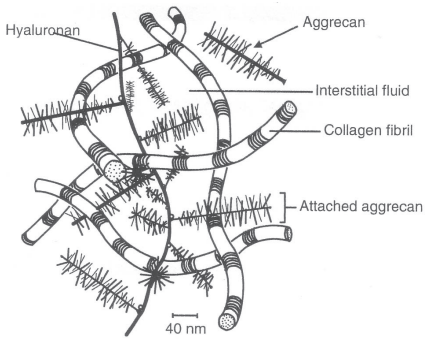


Figure 1.2 Schematic representation of the molecular organization of cartilage. (reprinted with permission from Mow VC and Hung CT: *Biomechanics of Articular Cartilage*, in Nordin M and Frankel VD (Eds): *Basic Biomechanics of the Musculoskeletal System*, 3rd edition, 2001. Philadelphia, PA, Lippington Williams & Wilkins).

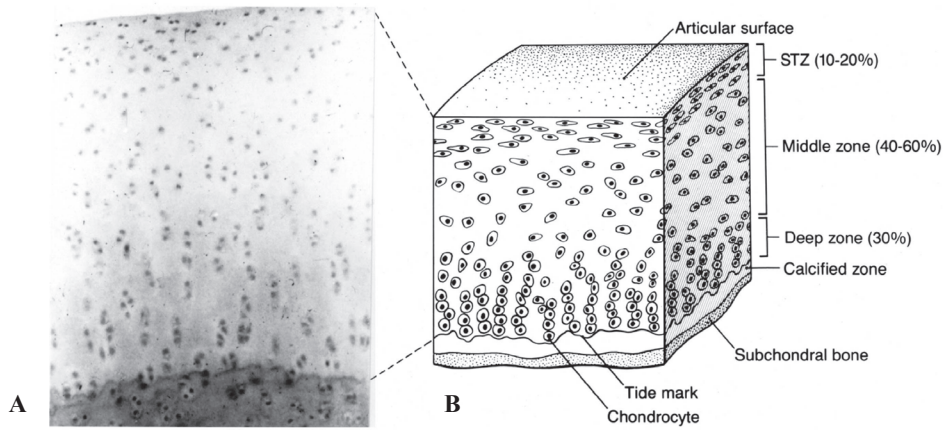


Figure 1.3 Histologic section **(A)** and schematic representation **(B)** of chondrocyte organization throughout the depth of articular cartilage. (reprinted with permission from Mow VC and Hung CT: *Biomechanics of Articular Cartilage*, in Nordin M and Frankel VD (Eds): *Basic Biomechanics of the Musculoskeletal System*, 3rd edition, 2001. Philadelphia, PA, Lippington Williams & Wilkins).

Alterations in this zone may contribute to the development of osteoarthritis by altering the mechanical behaviour of the tissue[27].

The transitional zone, or intermediate/middle zone (40-60% of total thickness[63]), is composed of spherical chondrocytes, proteoglycans, and obliquely oriented collagen fibres that primarily resist compressive forces but also serve as a transition between the shearing forces on the surface and the compressive forces placed on the deeper layers.

The deep zone, or radial zone (30% of total thickkness[63]), contains spheroidal shaped chondrocytes, aligned in columns perpendicular to the joint surface. Collagen fibres are oriented perpendicular to the surface. This zone contains the largest diameter collagen fibrils, highest concentration proteoglycans and lowest concentration of water. The collagen fibres of this zone pass into the calcified cartilage.

The calcified cartilage zone consists of a thin zone of calcified cartilage, which separates the uncalcified deep zone cartilage from the subchondral bone. (figs 1.3 and 1.4)

Chondrocytes, distributed over different cartilage zones, differ in size, shape, metabolic activity and proliferation activity[88]. A spheroidal shape, collagen type II and large aggregating proteoglycans production distinguish mature chondrocytes from other cells. Collectively, these highly specialized layers produce the superior loading and minimal friction characteristics of hyaline cartilage that make it particularly difficult to restore or duplicate once it is damaged or lost.

Injury to any part of this complex system can disrupt the biological and biomechanical properties of articular cartilage and may lead to further joint degeneration.

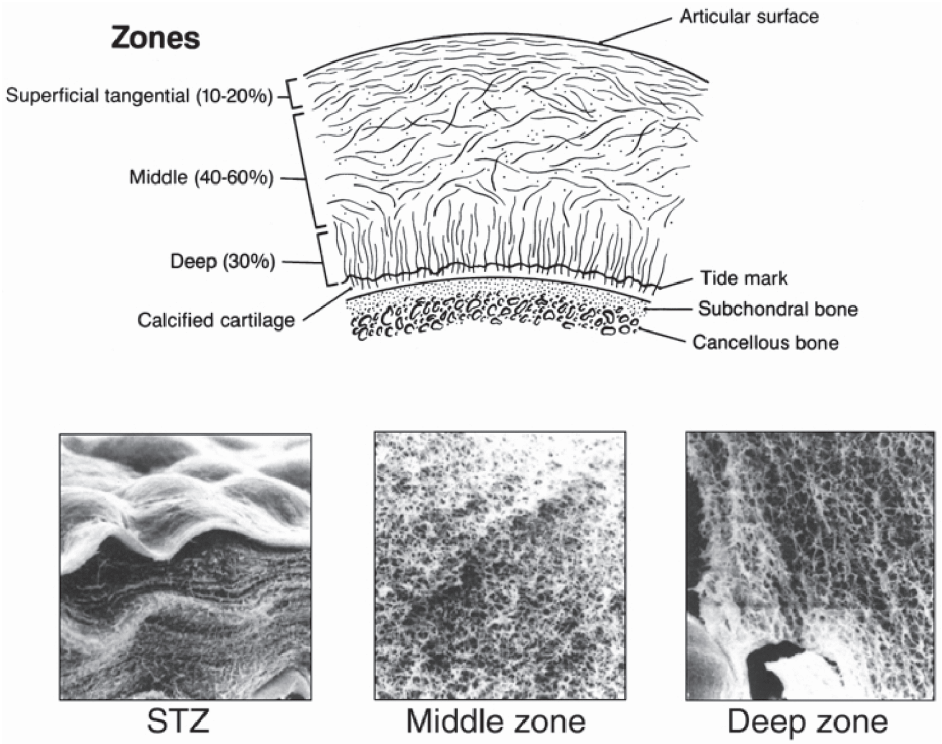


Figure 1.4 (A) Schematic representation of zonal collagen network orientation. **(B)** Scanning electron micrographs (x3000) that show actual arrangements of collagen in the 3 zones. (reprinted with permission from Mow VC and Hung CT: *Biomechanics of Articular Cartilage*, in Nordin M and Frankel VD (Eds): *Basic Biomechanics of the Musculoskeletal System*, 3rd edition, 2001. Philadelphia, PA, Lippington Williams & Wilkins).

1.2. INCIDENCE AND NATURAL OUTCOME OF CHONDRAL DEFECTS

The exact incidence of symptomatic chondral lesions (fig.1.5) and the natural outcome of joints with (osteo)chondral lesions is poorly defined.

A prospective study of 1000 consecutive knee arthroscopies revealed ICRS grade III and IV[37] chondral lesions, with an area of at least 1cm^2 in patients younger than 40, 45 and 50 years of 5.3%, 6.1% and 7.1% respectively[32]. The mean chondral or osteochondral defect area was 2.1cm^2. Another prospective study of 993 knee artroscopies[5] in patients with a median age of 35, shows 11% full-thickness articular cartilage defects (ICRS grade III and IV), 6% had a size of more than 2cm^2. Prospective arthroscopic evaluation of traumatic knee haemarthrosis patients showed 8-20% (osteo)chondral lesions[12,17], frequently associated with injury to the anterior cruciate ligament[31,50,68]. Curl et al. retrospectively reviewed 31,516 knee arthroscopies of patients in all age groups and reported chondral lesions in 19,827 (63%) of patients, with a mean of 2.7 lesions per knee. The incidence of grade III lesions was 41% and grade IV lesions

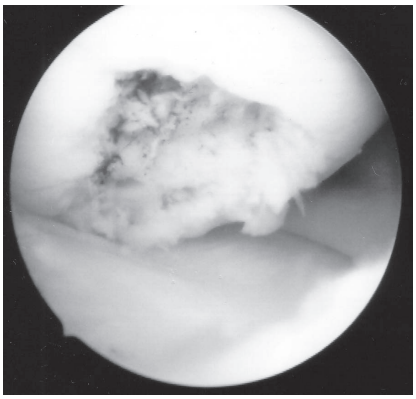


Figure 1.5 Traumatic articular cartilage defect in the medial femoral condyle of a 29 year old soccer player. The defect is debrided and multiple drill holes are made through the subchondral plate.

19%. In the younger population (age < 40 years) however, the incidence of unipolar grade IV lesions of the femoral condyle was only 5%[21].

Many of the lesions are clinically silent at the time of detection and the incidence of these asymptomatic lesions in the general population is unknown[3]. Chondral lesions have been shown to further degenerate within the knee[48,57,92], however the likelihood of articular cartilage lesions becoming symptomatic with time is unknown.

Shelbourne et al.[82] reported a series of 125 Outerbridge grade III and IV[72] cartilage defects (mean size 1.7cm^2, 60 medial, 65 lateral compartment, intact menisci), discovered during 2770 anterior cruciate ligament (ACL) reconstructive procedures. These authors showed, at a mean follow up of 8.7 years, very little difference in clinical outcome following ACL repair between patients with a chondral defect (modified Noyes score 93.4) and those without a defect (modified Noyes 95.6, $p<0.05$). Although the defect group had a significant lower subjective score than the control group, both groups had a mean score of more than 92 points, which is similar to scores found in athletes who had had an injury to the knee. There was no difference between groups with regard to radiological degenerative changes.

However, it may take up to more than 20 years before clinical and radiological degenerative changes come forward. Linden showed in a retrospective radiological study on osteochondritis dissecans of the knee in adult patients, with a 32.5 +/-7.5 year follow up, mild radiological deterioration in 14/44 joints and severe changes in 29/44 joints[48].

Animal model studies have shown that small osteochondral defects (<3mm) heal completely, while larger defects show a decreased healing potential[19,40]. However, a more recent animal study showed more initial metachromatic staining in small osteochondral defect repair (1mm), but lack of subchondral support in the smaller defects lead to an earlier breakdown of repair tissue[47].

In an extensive animal study, Shapiro et al. have described the natural repair response of 364 osteochondral defects (3mm diameter) in 122 rabbits with a follow up of 48 weeks[81]. A

fibrin blood clot fills defects within the first few days. Mesenchymal stem cells are able to grow in, extracellular matrix production is first observed after 10 days and within 3 weeks all defects are filled with scar-like cartilage tissue. The bone part of the defect ossifies, the upper part of the repair tissue does not ossify but transforms into fibrocartilage. This repair tissue does not show the typical structural organisation of hyaline articular cartilage and showed progressive prevalence and intensity of degenerative signs during follow up. In many instances, a clear gap was seen between repair and residual cartilage, demonstrating failure of integrative cartilage repair. This may be explained by the observation that spontaneously formed blood clots generally adhere well to bone surface, but only rarely to articular cartilage surface and to the defect floor and walls[35].

In conclusion, articular cartilage defect treatment is challenging for both clinicians and basic scientists, both for symptomatic relief *and* in order to prevent further joint degeneration and early osteoarthritis development. Successful repair techniques for isolated cartilage defects may be used in the future for repair of more extensive joint degeneration, such as osteoarthritis.

1.3. METHODS OF REPAIR (SHORT OVERVIEW)

As said before the primary goal in articular cartilage repair procedures should be defect filling and restoration of the articular surface with the best possible repair tissue. Long lasting biomechanical properties resembling that of hyaline cartilage and a full integration with the surrounding articular cartilage should result in pain free movement and prevent early joint degeneration. Several arthroscopic and open, experimental and clinical techniques are being developed and used in clinic.

1.3.1. Arthroscopic lavage and debridement

The initial arthroscopic treatment of isolated chondral injuries was to debride the cartilage in order to reduce mechanical symptoms and prevent further damage to the articular surfaces. However, all reports on this technique are based on subjective, retrospective studies[35]. There is no scientific evidence of any biological or repair activity resulting from arthroscopic lavage. Moreover, from a randomized, placebo-controlled study, evaluating the efficacy of arthroscopy for osteoarthritis of the knee it was shown that the outcomes after arthroscopic lavage or arthroscopic debridement were no better than those after a placebo procedure[62].

1.3.2. Subchondral bone penetration

Penetration of the subchondral bone either by Pridie drilling[38,64] or the microfracture technique[87] opens the vascular and bone marrow system. Cytokines, fibrin clot formation, growth factors and vascular invasion as well as mesenchymal stem cells are recruited to induce chon-

drogenesis. Because of its technical simplicity and low morbidity it is often used as a first-line treatment option for articular cartilage defect repair. Moreover, the microfracture technique, popularised by Steadman[85,86], has become the control treatment in several prospective studies evaluating other, more extensive surgical interventions such as autologous chondrocyte implantation (ACI)[42]. A recent prospective cohort study with a follow up of 41+/-7 months reported a good or excellent result in 67% of patients[60]. The best short-term results were observed when there was a good MRI evaluated defect filling, low body mass index and short duration of preoperative symptoms.

1.3.3. Osteochondral transplantation

In this open or arthroscopic technique osteochondral plugs are transplanted from relatively non-weigthbearing regions of the joint to debrided defect areas. Predictable bone healing guaranties solid fixation of the osteochondral plugs into defects. Osteochondral transplantation or mosaicplasty, popularised by Hangody[29] and Bobic[7], is recommended for defects limited to between 1 and 4 cm^2 [30]. The risk of donor site morbidity has gained attention and limits the size of defects that can be repaired with this technique. In a recent study using sheep, mosaicplasty was found to be associated with rapid degeneration of both transplanted and surrounding native cartilage. Finite element model analysis showed lack of mechanical support of transplanted cartilage, leading to biomechanical failure and tissue degeneration at interface regions, thereby impairing integrative cartilage repair[1].

1.3.4. Perichondrial/periosteal transplantation

With this technique chondral lesions are treated with debridement and transplantation of costal perichondrium or periosteal grafts. Progenitor cells from the cambial layer are, in a synovial joint environment, responsible for defect filling with cells and extracellular matrix. Promising short term clinical results are reported with perichondrial[34] and periosteal grafts[33,49].

However, surgical technique and tissue handling during the procedure is essential[70] and induced repair tissue not always results in complete restoration of hyaline articular cartilage and long term tissue repair[11].

1.3.5. Autologous chondrocyte transplantation (ACI)

This technique of autologous chondrocyte implantation was first described in 1984 in an animal study[76] and later in 1994 in a clinical study[12]. Articular cartilage is harvested, chondrocytes are enzymatically isolated, culture-expanded and transplanted underneath a patch of periosteum. Good to excellent initial clinical results are reported[12], durable results up to 11 years were presented by the same authors recently[75]. Histological biopsy studies[78] have shown that ACI repair can result in repair tissue of varying morphology, ranging from predominantly hyaline (22%) through mixed (48%) to predominantly fibrocartilage (30%).

Use of artificial flaps and Matrix-induced ACI (autologous chondrocytes seeded in an artificial matrix) are variations to this technique.

Knutsen et al.[42] compared ACI with microfracture in the knee in a randomised clinical trial. At 2 year follow up no significant difference in macroscopic or histological results between the two treatment groups was reported. Histological biopsy evaluation did not demonstrate relation between histology and clinical outcome. Both treatment groups had good clinical outcome, no serious complications were reported after 2 years. At 5 year follow up[43] each group had 22% (9/40 in each group) failures. At 2 and 5 years both treatment groups showed significant clinical improvement but no significant difference in clinical outcome.

1.3.6. Tissue engineering

Tissue engineering aims at reproduction of tissue with similar structural and biological properties for functional replacement of tissue. Cells, biological or artificial matrices and stimulating factors are used to form hyaline cartilage for articular cartilage repair. ACI is an example of a partially *in vitro* and partially in situ repair process. Many techniques are being developed and tested *in vitro*, in vivo and clinically. (Different cell sources are used, for example autologous chondrocytes[12,54], mesenchymal stem cells[18,97], nasal[95] or auricular[94] chondrocytes.)

1.4. WOUND HEALING / INTEGRATIVE CARTILAGE REPAIR

During the transplantation procedure of freshly harvested, *in vitro* or in situ tissue engineered cartilage the graft and host tissue need to be prepared in order to achieve a suitable fit and fixation into the defect. Thereby fresh cartilage wounds are created, therefore the integrative cartilage repair process, necessary for complete restoration of cartilage, can be regarded as a wound healing process.

1.4.1. Classic wound healing

Classic wound healing in vascularised mammalian tissue consists of a necrotic phase, inflammatory phase and remodelling phase[51,67]. *The initial phase of necrosis* is characterised by tissue damage and cell death, the amount depending on the amount of applied force to and devascularisation of the tissue. Subsequent haematoma and fibrin clot formation with platelets and blood cells is followed by the release of various cytokines, growth factors and proteolytic enzymes. These factors help to remove necrotic material, stimulate proliferation and differentiation of local cells, stimulate migration of progenitor cells into the wound area and stimulate neovascularisation. *The inflammatory phase* depends on vasodilatation and increased blood vessel permeability, transudation and exudation of fluid and proteins. This leads to a dense fibrinous network formation, containing inflammatory cells and progenitor cells. Proliferation and differentiation of these cells supplies the cells needed for tissue

replacement. *The remodelling phase* is characterised by vascular bud invasion, fibrous repair tissue formation (i.e. skin, liver, kidney, lung, brain) and in several tissues metaplasia of cells and tissue replication (i.e. bone, tendon, synovial membrane)[67].

1.4.2. Articular cartilage wound healing

Articular cartilage wound healing is different since cartilage is avascular, aneural and alymphatic[51]. The repair process of vascularized tissue is a cellular one: fibroblast or specific cells must synthesize the repair tissue (ECM). Local cell proliferation or migration of cells from the wound margin or from blood vessels entering the tissue, are responsible for local ECM production and repair tissue synthesis[51]. The absence of blood vessels and the very tight ECM, prohibiting chondrocyte migration from adjacent healthy cartilage towards the wound area, prohibit a cell-based repair in articular cartilage wounds not extending through the subchondral plate.

Factors, now identified to influence cartilage wound healing, include chondrocyte density, ECM production and collagen cross-linking, growth factors, local enzyme activity/activation and developmental stage.

Because of the highly variable and complex environment in synovial joints, it is difficult to study basic regulating mechanisms of articular cartilage repair *in vivo*. Experimental and theoretical studies of intact cartilage and (osteo)chondral defects, including studies of interface tissue between repair and normal tissue, have shown changes in tensile and shear stresses near articular cartilage wounds, influencing integrative cartilage repair processes (for review see[2]). *In vitro* explant studies and cell studies provide more controlled model systems to study basic mechanisms of cartilage wound healing and can be used to evaluate methods for improvement of integrative cartilage repair.

1.4.3. Chondrocytes

Histological studies of the cartilage-cartilage interface region, including a study presented in this thesis, have shown the importance of cell phenotype, cell density and local ECM production in the wound area[8,69,74,83,84]. Surgical preparation or the cutting of articular cartilage leads to chondrocyte loss from cartilage lesion edges[9,36,51,81,90]. Integrative cartilage repair is probably hindered by the lack of matrix-producing cells in the cartilage-cartilage interface area[2,69,77].

1.4.4. Collagen

In vitro studies, using alive and dead articular cartilage explants suggest that collagen deposition by cells in the vicinity of the cartilage-cartilage interface increases adhesive strength and therefore is involved in integrative repair[23,24].

1.4.5. Developmental stage

Differences between immature and mature cartilage influence integration between cartilage surfaces. Active proliferation and high production of ECM during development may explain the observed repair response in immature subjects[25]. Ageing has been shown to change proliferation capacity, extracellular matrix production and responsiveness to growth factor stimulation in *in vivo* studies, explant studies and in isolated chondrocyte culture studies[6,26,79]. *In vivo*, laceration of fetal lamb articular cartilage recovered completely, judged by histological disappearance of the laceration [65]. *In vitro* experiments using fetal, newborn and mature bovine articular cartilage explants, cultured following apposition for two weeks in medium, showed a 3-4 times higher biomechanical bonding strength for calf cartilage. This development associated difference did not simply depend on ECM synthesis, but appeared to depend on collagen cross-link formation[24]. However, others have shown no distinct differences in the healing response to experimental wounding *in vitro*, using embryonic sternal cartilage and immature and mature bovine articular cartilage[89].

1.4.6. Enzymes

Several investigators have used biological glues (tissue transglutaminase, fibrin) to improve the adhesive strength at the cartilage-cartilage interface, with different success[13,41,66,73]. Others have shown that enzymatic removal of proteoglycans from the surface of lesion edges can be used in cartilage repair, either as single treatment [61] or to improve the initial adhesion of transplanted cells or cartilage to cartilage wound edges[45,46,69,91]. Such treatment is most likely to have no effect on cell density itself. Synovial components, for example lubricin known to lubricate articular surfaces, has been shown to reduce adhesive strength. Addition of lubricin to an *in vitro* explant culture system was shown to reduce adhesive strength[80]. Changing local tissue composition with enzymes (articular cartilage) or environmental conditions (i.e. synovial fluid) may alter adhesive properties and subsequent integrative cartilage repair.

1.4.7. Growth factors

Growth factors appear to play an important role in wound healing and regeneration in a number of tissues, e.g. skin, skeletal muscle, gastrointestinal tract, liver and bone. Despite an increasing number of publications on the effects of various growth factors on chondrocyte proliferation, differentiation and matrix production mainly *in vitro* (for review see[20,35,44,93]) little is known about growth factors regulating cartilage wound healing. Only a few studies report an increase of growth factor expression and decrease of growth factor receptor expression in chronic hyaline cartilage damage, i.e. osteoarthritis[10,58,59] whereas no studies have documented the spatial and temporal expression of growth factors in acute cartilage wound healing.

1.5. AIMS OF THIS THESIS

The avascular status of articular cartilage and the low cell density combined with a tight ECM form important limitations for its repair capacity as compared to other tissues. The first aim of this thesis was to study the basic cellular and molecular reactions of cartilage tissue to experimental wounding. Wound healing and integrative cartilage repair was studied in various *in vivo* and *in vitro* model systems, using immature and mature animals/humans and articular and auricular cartilage.

The second aim of this thesis was to use the knowledge from these basic wound healing studies to improve articular cartilage integration. Enzyme treatment protocols were used *in vitro* to influence wound edges and chondrocyte density, growth factor addition was used to increase ECM production. The effect of treatment on cartilage-cartilage integration was evaluated histologically and biomechanically.

1.6. OUTLINE OF THIS THESIS

In the first study presented in this thesis, we have used an animal model to study the primary wound healing characteristics of cartilage tissue. Proteoglycan depletion and replenishment, chondrocyte survival and temporal and spatial growth factor expression in auricular cartilage wounds were studied in a time study in the New Zealand White rabbit *(Chapter 2)*.

In the following chapter, an articular cartilage wound healing study was presented, comparing wound healing reactions of partial-thickness and full-thickness defects in immature and mature New Zealand White rabbits. Defect morphology, cell survival and proliferation, growth factor expression and proteoglycan content were studied *in vivo* during a short term follow up study. Isolated hip and shoulder chondrocytes were cultured in an alginate culture system to determine age related differences in proliferation and glycosaminoglycan production. Furthermore, the effects of addition of the potent mitogen TGFβ1 were studied *(Chapter 3)*.

In a series of *in vitro* explant experiments using healthy bovine and human articular cartilage the effects of enzyme treatment on articular cartilage wound edges were studied. The aim of these experiments was to investigate whether specific enzymatic digestion of the extracellular matrix components in articular cartilage lesion edges could induce an integrative cartilage repair response. Wound edges of cultured explants were studied. Chondrocyte death, proliferation and tissue viability were studied in wound edges of cultured articular cartilage explants following treatment with hyaluronidase or highly purified collagenase. In order to determine the implications our findings for integrative cartilage repair, the integration of

disc-ring composites of collagenase treated bovine articular cartilage explants was studied histologically *(Chapter 4)*.

In a second series of experiments, again we have used enzymatic treatment with hyaluronidase and highly purified collagenase. We tested how this affected integrative cartilage repair of bovine articular cartilage disc-ring composites. Cartilage integration was studied histologically. The biomechanical bonding strength was tested using a disc-ring push-out testing apparatus *(Chapter 5)*.

We have observed that during long-term explant cultures articular cartilage explants are capable to regenerate hyaline-like cartilage at lesion edges. Because recent studies suggest the presence of progenitor cells in articular cartilage, it is of interest to find out which cells are responsible for chondrocyte outgrowth and neocartilage formation at wound edges. The outgrowth of cells and new tissue formation from immature and mature articular cartilage explants *in vitro* was investigated. Thionin staining and immunostaining for type II collagen were performed to characterize the tissue. The origin of chondrocytes responsible for outgrowth was investigated by determinating locations of proliferation with Ki67 immunostaining and by studying outgrowth from separately cultured superficial and deep cartilage zone explants *(Chapter6)*.

In *Chapter 7* a general discussion with remarks concerning future expectations is given.

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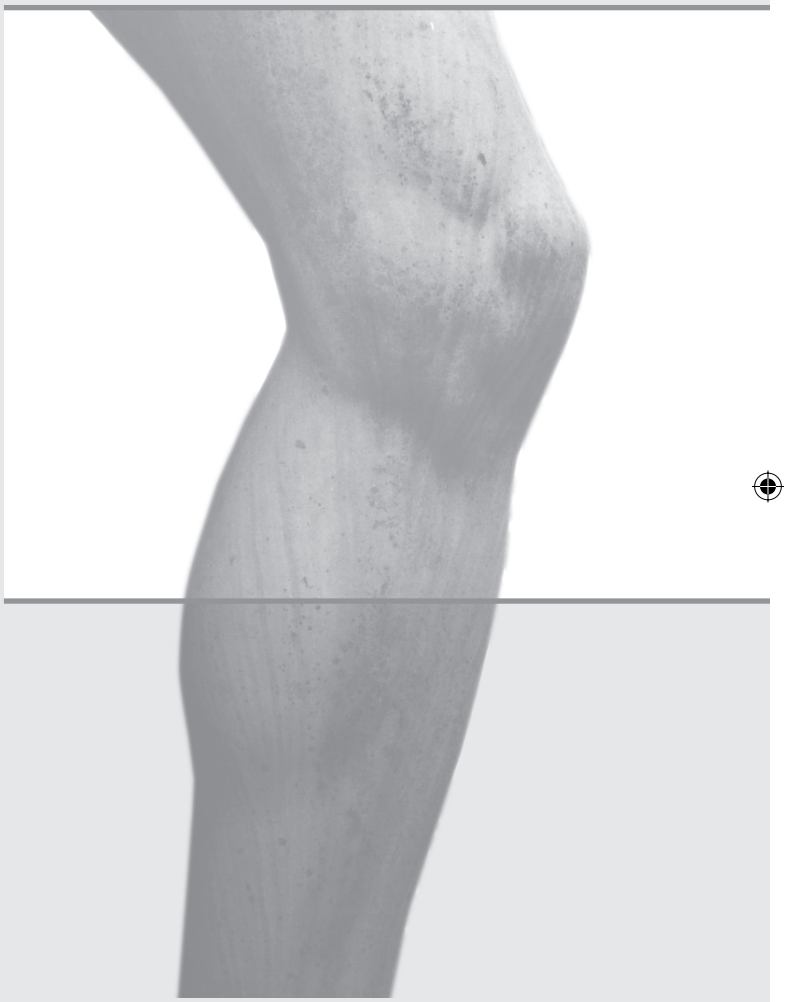
Chapter 2

Growth factor expression in cartilage wound healing: temporal and spatial immunolocalization in a rabbit auricular cartilage wound model



P.K. Bos[1], G.J.V.M. van Osch[1,2], D.A. Frenz[3], J.A.N. Verhaar[1] and H.L. Verwoerd-Verhoef[2]

Departments of Orthopaedics[1] and Otorhinolaryngology[2], Erasmus MC Rotterdam, The Netherlands.

Department of Otolaryngology and Anatomy & Structural Biology[3], Albert Einstein College of Medicine, Bronx, New York, U.S.A.

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2.1. SUMMARY

Objective: The ability of cartilage to regenerate following injury is limited, potentially leading to osteoarthritis. Integrative cartilage repair, necessary for durable restoration of cartilage lesions, can be regarded as a wound healing process. Little is known about the effects of growth factors regulating acute cartilage wound healing *in vivo*. In this study the temporal expression patterns of growth factors and proteoglycan content in cartilage wound edges *in vivo* were studied.

Design: Cartilage wounds were created in rabbit ear cartilage using a 6 mm biopsy punch. Specimens were subsequently harvested 1, 3, 7, 14 and 28 days after surgery. Paraffin sections were thionin stained to visualize proteoglycan loss and replacement. Immunohistochemical staining of TGFβ1, TGFβ3, IGF-1, IGF-II and FGF-2 was used to define growth factor expression at the cartilage wound sites.

Results: Almost no effect of cartilage wounding was observed one day after surgery. A decrease of proteoglycan content, with a maximal loss at day 7, and a subsequent restoration was observed at the wound edges. Growth factor expression increased simultaneously. Maximal immunostaining for IGF1, IGFII, FGF-2 and TGFβ3 was observed at day 7, followed by a gradual decrease. Increased expression of TGFβ1 lasted from day 3 until day 14.

Conclusion: We have demonstrated the ability of chondrocytes to increase growth factor expression and to restore the rapid decrease in proteoglycan content in the initial phase following acute wounding. A temporal increase in intracellular growth factor expression suggests an autocrine and/or paracrine metabolic stimulation, which can be regarded a sign of chondrocytes repair capacity.

2.2. INTRODUCTION

Once damaged, hyaline cartilage and elastic cartilage display a poor intrinsic repair capacity, potentially leading to osteoarthritis (OA), airway obstruction or deformation of ears and nose[1,11,21,22,26,29,30,33,35]. Merely small defects or defects in fetal or very young cartilage show healing[11,36]. Namba *et al*. demonstrated a spontaneous repair *in utero* of partial-thickness defects in articular cartilage in a fetal lamb model[26]. This repair process appears to be absent in matured animals. An important condition for durable repair of cartilage lesions is the integration of wound edges or the integration of repair tissue with the surrounding host cartilage[1]. Failure of cartilage repair caused by an impaired integration has been documented by several authors who studied the natural cartilage repair process[21,29,33]. This failure of repair was also found after transplantation of periosteal and perichondrial grafts[2,27], osteochondral grafts[32], natural[13] or bioengineered grafts[34]. During the transplantation procedure of freshly harvested or bioengineered cartilage the graft and host tissue need to be reshaped in order to achieve a suitable fit into the defect. Thereby fresh wounds are created and the integrative cartilage repair process, necessary for a long-lasting restoration of cartilage, can be regarded as a wound healing process.

Growth factors appear to play an important role in wound healing and regeneration in a number of tissues, e.g. skin[3,37], skeletal muscle[17], gastrointestinal tract[14,18], liver[4] and bone[7,19]. Transforming growth factor β (TGFβ), fibroblast growth factor 2 (FGF-2) and insulin like growth factor (IGF) play a role in various aspects of musculoskeletal tissue regeneration[17,19,20] and remodeling[10].

Despite an increasing number of publications on the effects of growth factors on chondrocyte proliferation and matrix production *in vitro* (for review see[12]) little is known about growth factors regulating cartilage wound healing. Only a few studies report an increase of growth factor expression and decrease of growth factor receptor expression in chronic hyaline cartilage damage, i.e. osteoarthritis[6,23,24] whereas no studies have documented the spatial and temporal expression of growth factors in acute cartilage wound healing.

Both hyaline cartilage and elastic cartilage are composed of chondrocytes surrounded by an extracellular matrix mainly consisting of collagen type II and aggrecan. The chondrocytes of elastic cartilage additionally produce elastin.

In the present study we report temporal and spatial growth factor expression patterns during cartilage wound healing in an auricular cartilage wound-healing model in the rabbit[31]. The immunohistochemical staining of growth factors was demonstrated using antibodies against transforming growth factor β1 and β3, insulin-like growth factors I and II and fibroblast growth factor 2. Expression of growth factors was related to post-injury changes in proteoglycan content in wound areas, as determined by thionin staining[9].

2.3. MATERIALS AND METHODS

2.3.1 Animal surgery

Nine female New Zealand White rabbits, aged 12–14 weeks (2–3 kg), were used in this experiment. Animal experiments were approved by the Erasmus University Ethics Committee. The animals were housed at the Erasmus Center for Animal Research. The surgical procedures were carried out under semi-sterile conditions.

The animals were anesthetized by an intramuscular injection of 10% ketamine-hydrochloride (Ketalin, Apharma, Arnhem, The Netherlands) 0.5 ml/kg body weight and 2% xylazine-hydrochloride (Rompun, Bayer, Leverkusen, Germany) 0.5 ml/kg body weight. After shaving and disinfecting the skin with 70% ethanol a 2×3 cm angled skin incision was made at the concave (inside) of the ear. The skin was carefully dissected from the perichondrium. An oblique incision, approximately 1cm, was made through perichondrium cartilage and opposite perichondrium and the contralateral skin was bluntly tunnelled. Through this cartilage incision a plastic device was introduced between perichondrium and contralateral skin to protect the skin and a 6 mm diameter punch hole was made through cartilage and perichondrium using a biopsy punch (Stiefel, Imported by Bipharma, The Netherlands). The cartilage/perichondrium disks were rotated 180° and reimplanted using three Vicryl 6.0 stitches through the perichondrium (Fig. 1). The skin was closed with Vicryl 6.0 and wounds were dressed with gausses (Bethadine, imported by Asta Medica, The Netherlands) which were removed one day after operation. In three rabbits wounds were created in one ear, the unwounded contralateral ear was used to harvest a control specimen. In the remaining six animals, *two* wounds were created, one wound in each ear.

The animals were killed with an intravenous injection of 1 ml Pentobarbital Natrium 200 mg/ml (Euthesate, Apharmo, Arnhem, the Netherlands). Three cartilage wound samples were harvested immediately after death at days 1, 3, 7, 14 and 28. The circular wounds, including overlying skin, were harvested by dissecting 1×1 cm specimens. Samples were fixed in Carnoy's fixative (ethanol 60%, chloroform 30%, acetic acid 10%) for 20–24 h, processed and

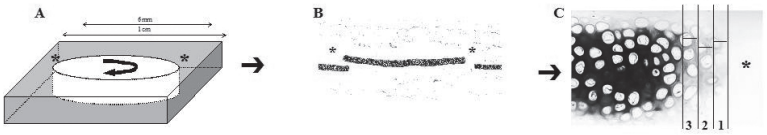

Figure 2.1 (A) Schematic drawing of experimetal wounding. A 6 mm diameter disc was punched out of the cartilage, rotated and reimplanted. **(B)** Histologic cross-section of the wounded cartilage. **(C)** Detail of cartilage lesion edge. The number of cell-layers, parallel to the wound edge, with thionin depletion or growth factor expression was scored. In this particular section thionin staining was decreased in three cell-layers.
Position of the wound in A, B and C is indicated with asterisks (*).

embedded in paraffin. Five micrometer sections were cut using a Leica (RM 2135) microtome and placed on poly L-lysine coated slides.

2.3.2. Thionin staining

The sections were deparaffinized in xylene, rehydrated through graded ethanol and stained in 0.04% thionin in 0.01 M aqueous sodium acetate, pH 4.5 for 5 min[9].

2.3.3. Immunohistochemistry

Antibodies directed against growth factors that play a role in musculoskeletal tissue regeneration and remodelling were selected. Not all the members from the TGFβ superfamily were tested; we selected TGFβ1 and TGFβ3 because an opposite effect has been suggested[3]. All steps were performed at room temperature. The sections were deparaffinized in xylene and rehydrated through graded ethanol. After preincubation for 30 min with phosphatebuffered saline (PBS) containing 1% bovine serum albumin (BSA) and 10% normal goat serum to block non-specific antigens, sections were incubated for 2 h at room temperature with antibodies against TGFβ1 (Antihuman TGFβ1, 5 µg/ml, monoclonal mouse IgG, Serotec Ltd, Oxford, U.K.) and TGFβ3 (Antihuman TGFβ3, monoclonal mouse IgG, 5 µg/ml, R&D Systems, Minneapolis, MN, U.S.A.), FGF2 (Antibovine FGF-2, monoclonal mouse IgG, 5 µg/ml, Upstate Biotechnology, Campro Scientific, Veenendaal, The Netherlands), IGF-I (Antihuman IGF-I, mouse monoclonal IgG, 5 µg/ml, Upstate Biotechnology) and IGF-II (Antirat IGF-II, mouse monoclonal IgG, 5 µg/ml, Upstate Biotechnology) diluted in PBS containing 1% BSA and 2% normal goat serum. The antibodies were linked with a secondary antibody, biotinylated rabbit antimouse immunoglobulins (Super Sensitive Concentrated Detection Kit, Bio Genex, San Ramon, U.S.A.). The secondary antibody was labeled using streptavidine-alkalin phosphatase (Super Sensitive Concentrated Detection Kit, Bio Genex, San Ramon, U.S.A.). Alkaline phosphatase activity was demonstrated by incubation with a New Fuchsine substrate (Chroma, Kongen, Germany), resulting in a red colored signal. In control sections the primary antibody was omitted. For isotype control a mouse monoclonal negative control antibody (mouse IgG1 negative control, Dako A/S) was used. The slides were not counterstained to enable semiquantitative analysis of growth factor expression.

2.3.4. Histological analysis

The sections were separately scored by two independent observers at a magnification of 200×. In rabbit auricular cartilage chondrocytes are organized in layers perpendicular to the horizontal surface and parallel to the cutting surface [Fig. 1(c)]. Changes in proteoglycan content or growth factor expression can therefore be quantified by counting the number of cell layers. The number of cell layers with decreased or lost thionin staining starting from the wound surface was scored.

In serial sections the cartilage area containing immunopositive chondrocytes was determined by counting (number of positive cell layers) or measuring (distance in µm) starting from the wound edge.

Observed differences in growth factor expression and proteoglycan content were evaluated using Mann-Whitney U-test. Spearman's correlation was calculated for growth factor expression and proteoglycan depletion. The interobserver variability was tested using Pearson's correlation coefficient.

2.4. RESULTS

All animals recovered well from the operation. All wounds healed well, no signs of infection or perforation of the skin covering the punch wounds were observed. The interobserver reproducibility of scoring cell layers with thionin depletion or growth factor expression was good: the calculated correlation coefficient was 0.885 (two-tailed *P*-value <0.01). The average width of one cell layer calculated for all nine animals was 37.7 (±4.6) µm.

2.4.1. Thionin staining

The extracellular matrix in normal, unwounded auricular cartilage showed a homogeneous thionin staining of the extracellular matrix.

One day after surgery almost no effect of cartilage wounding was observed, i.e. there was minimal reduction in thionin staining. After 3 days, a decrease in thionin staining was observed and after 7 days a maximal thionin depletion with an average of 7.6 cell layers was observed, indicating a considerable loss of proteoglycans in the cartilage wound edges. However, at day 14 there was a recurrence in thionin staining, starting in pericellular areas. After 28 days, an almost complete proteoglycan replenishment was observed (Figs 2 and 3).

2.4.2. Immunohistochemical growth factor expression

Growth factor expression in the cartilage was primarily observed intracellularly whereas almost no staining was observed in the extracellular matrix. Omission of the primary antibodies and isotype control, using a mouse IgG1 control antibody confirmed specificity of the immunohistochemical procedure.

Chondrocytes in unwounded, freshly harvested cartilage showed no expression of TGFβ, IGF or FGF-2 [Fig. 4(a)]. One day after surgery minimal growth factor expression could be noticed at the cartilage wound site. After 3 days positive staining for growth factors was found in the chondrocytes of the cartilage wound edges [Figs 4(b) and 5]. The number of cartilage cell layers expressing immunostaining for TGFβ1 and TGFβ3 increased between day 1 and 3, TGFβ1 maintained around that level until day 14. An increase of IGF-I, IGF-II and FGF-2 was observed between days 3 and 7. Staining for IGF-I, IGF-II, FGF-2 and TGFβ3 reached a peak

level at day 7 [Figs 4(c) and 5], and showed a gradual decrease afterwards. At day 28 growth factor expression returned to basal levels [Fig. 4(d) and 5].

A small zone of chondrocytes (one to two cell layers) directly bordering the cartilage cutting line did not demonstrate antibody reactivity. From day 7 clear empty lacunae were observed in this zone.

2.4.3. Correlation between thionin depletion and growth factor expression

A simultaneous pattern of growth factor expression and loss of thionin-staining was observed in the cartilage wound area. The area containing positive cell-layers for TGFβ1, IGF-I, IGF-II

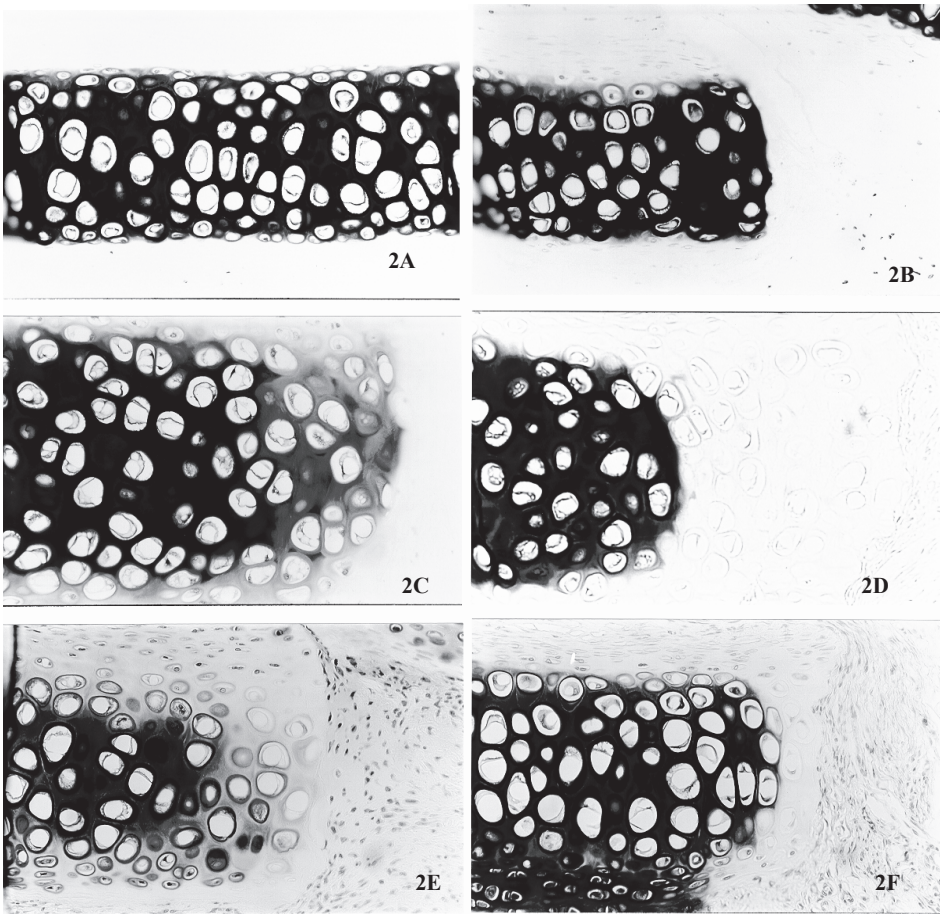

Figure 2.2 Thionin staining of paraffin embedded rabbit auricular cartilage (original magn.200X). **(A)** Unwounded cartilage shows an evenly distributed purple staining. **(B)** No matrix changes were noticed one day after surgery. **(C)** At day 3 a decrease in staining was observed at the wound edges, indicating a proteoglycan loss. **(D)** After 7 days the loss of thionin staining reached its maximum in this sample 7 cell layers. **(E)** Note the pericellular restorage of staining at day 14. **(F)** An almost complete restorage of matrix staining was observed at day 28.

Table I Spearman's correlation coefficient for the area of growth factor expression and the area of thionin depletion

TGFβ1	TGFβ3	IGF-I	IGF-II	FGF2
0.73**	0.44	0.60*	0.69**	0.71**

***P*<0.01 **P*<0.005.

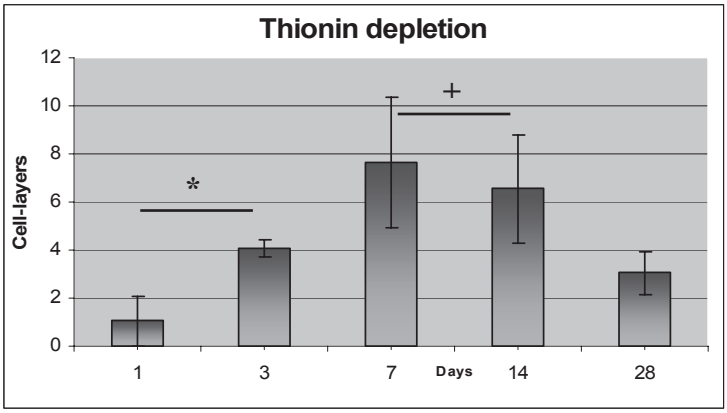

Figure 2.3 Number of cell layers partially or completely depleted of thionin staining (mean +/- SD of three rabbits). * P<0.05, + P=0.1

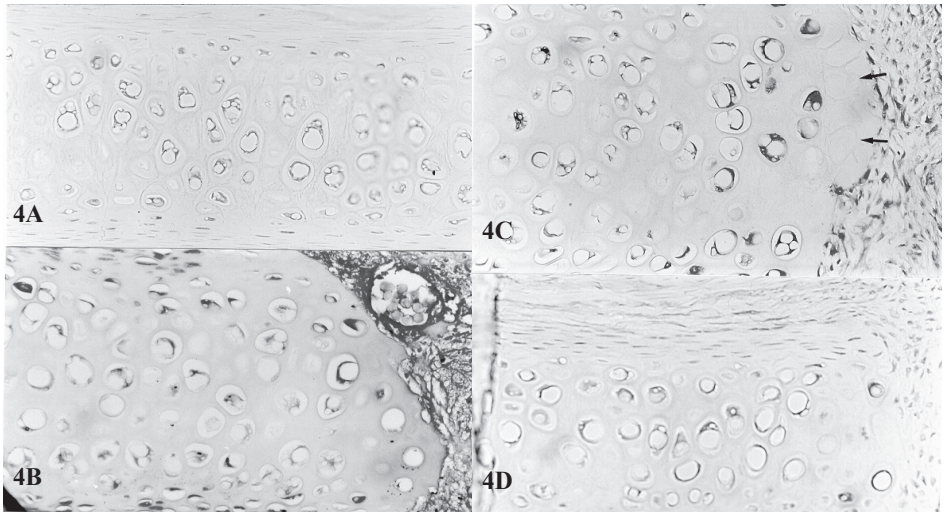

Figure 2.4 Immunohistochemical staining for FGF2 on paraffin embedded rabbit auricular cartilage (original magn.200X). Note the intracytoplasmatic localization of growth factor signal. **(A)** In unwounded cartilage no growth factor expression can be observed in chondrocytes. Note that a part of the perichondrium cells show expression of FGF2. **(B)** At day 3 chondrocytes at the cartilage wound edges start to express FGF2. **(C)** Maximal expression was noticed 7 days after surgery. Chondrocytes directly bordering the cartilage cutting line do not show expression of FGF2 (arrows). **(D)** After 28 days the number of cell layers expressing FGF2 was reduced. (See also: Colour figures section)

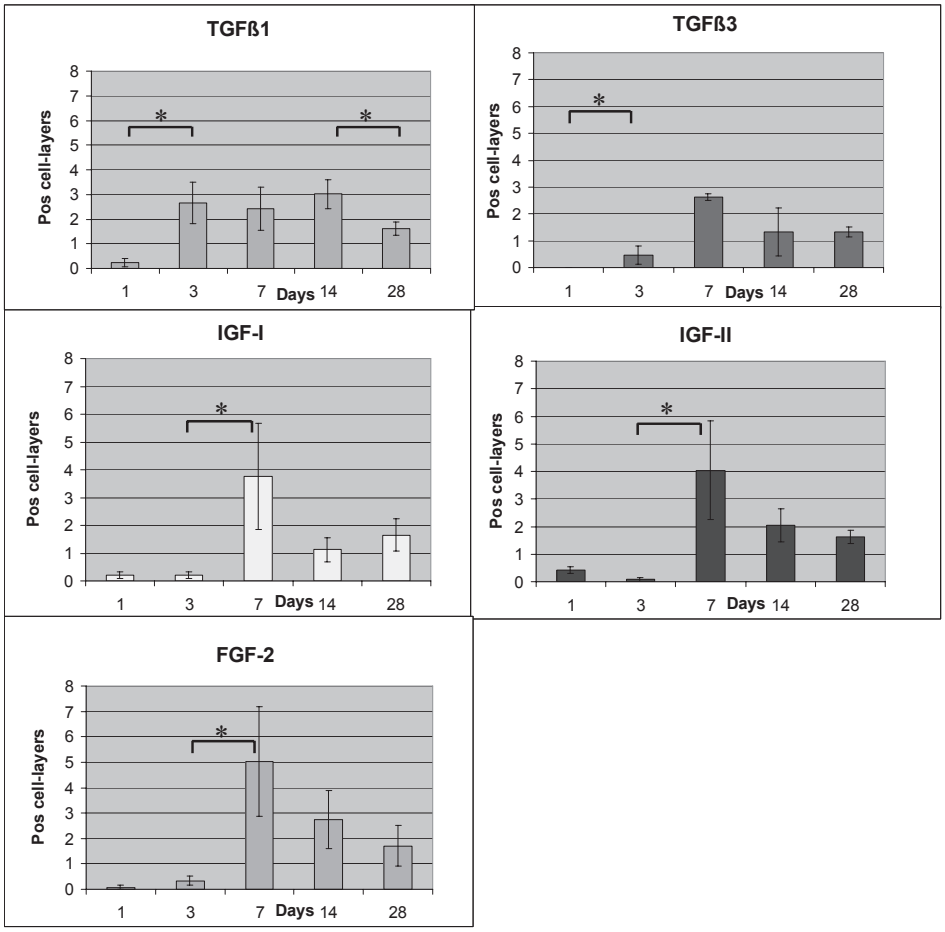

Figure 2.5 The number of cell layers positively stained for growth factors (mean ± SEM of 3 rabbits) is represented. A relatively fast increase in expression of TGFβ1 and TGFβ3 was observed between day 1 and 3. Expressions of IGF-I, IGF-II and FGF2 increased between day 3 and 7. Note the clear peak level expression of IGF-I, IGF-II, FGF2 and TGFβ3 at day 7. Peak level expression of TGFβ1 lasted from day 3 to 14. *: $p<0.05$

and FGF-2, as measured from the cartilage wound edges, correlated significantly with the number of cell layers depleted from proteoglycans (Table I).

2.5. DISCUSSION

The absence of sufficient natural repair following cartilage injury prohibits durable tissue restoration. In the present study we have shown signs of repair in cartilage exposed to acute wounding. A gradual decrease of proteoglycans at the wound site was followed by a repair

reaction demonstrated by a renewed pericellular proteoglycan production. Simultaneously, a temporal increase in growth factor expression was shown in these cells depleted from their normal surrounding matrix. In the final phase, when virtually all lost proteoglycans were restored, growth factor expression was reduced to basal levels. Knowledge on *in vitro* effects of growth factors on matrix production and cell proliferation of chondrocytes and cartilage explants[12] suggest that the temporal expression of endogenous growth factors indicates an autocrine and/or paracrine stimulation of chondrocyte metabolism following acute cartilage injury.

In articular cartilage, the relation between effects of wounding on proteoglycans and chondrocyte metabolism is difficult to study due to the continuous presence of mechanical loading, which influences cell metabolism[28]. Exclusion of mechanical loading by joint immobilization however, will lead to a reduction of proteoglycan content[16]. In this study, we have used ear cartilage to investigate post-injury changes in proteoglycan content and chondrocyte metabolic response. Ear cartilage is not subjected to the levels of mechanical loading occurring in joints and does not have the limitations of *in vitro* experiments. Additional advantages of our model are the ability to study more than one condition per animal, which requires less extensive surgical procedures and causes less discomfort to the animals.

This is the first report in which the temporal and spatial expression of growth factors, known to be important in musculoskeletal tissue regeneration[10,17,19], were studied in an acute cartilage injury model. A relatively fast increase in TGFβ1 and TGFβ3 expression was observed 3 days after injury. IGF-I, IGF-II and FGF-2 increased to a peak expression at day 7. Previous studies have also demonstrated elevated intracellular expression of growth factors and cytokines in chondrocytes, however these studies were carried out in chronic injured, i.e. osteoarthritic cartilage from patients or animal models[6,23-25]. The experimental setup we have used models the situation encountered following cartilage transplantation.

Currently, much effort is expanded in developing tissueengineered cartilage transplants to fill up cartilage defects[8]. Successfully developed cartilage transplants, containing the proper amounts and types of collagens and proteoglycans with the appropriate biomechanical properties, still need to integrate into the host cartilage in order to achieve a permanent and durable restoration of the cartilage defect. Integrative cartilage repair is required to enable cartilage transplants to stay in position in the initial (healing) phase, allowing both cartilage wound sites to integrate, and to withstand biomechanical loading patterns in the final phase. For this integration, a wounded edge of the cartilage defect has to form a solid connection with the edges of the transplant.

In spite of the signs of repair we demonstrated after injury, we did not observe integrative repair. Because of the chondrocytes inability to migrate through the matrix, the cells closest to the wound edges are responsible for the integration of both wound sides. However, a part of these cells appeared to die, i.e. first no increased growth factor expression was seen and later empty lacunae were observed. In agreement with our findings other investigators

have described cell death following cartilage injury[25]. It is suggested that, like in skin wound healing[15], cells in the wound area of experimentally injured articular cartilage explants die by apoptosis[32]. Recently it has been shown that at least a part of the chondrocytes in osteoarthritic cartilage die by apoptosis[5]. A positive effect in the healing process can be expected from interventions aiming at protecting the viability of chondrocytes aligning the wound site.

In conclusion, we have shown the effects of injury on extracellular matrix and chondrocytes. The suggested relation between growth factor expression and proteoglycan depletion during wound healing and the subsequent replenishment of proteoglycans indicate that cartilage possesses some intrinsic repair capacity. The temporal expression pattern of the growth factors reported here provides the opportunity to study whether adding extra or neutralizing endogenous growth factors can stimulate extracellular matrix production needed for integrative cartilage repair.

2.6. ACKNOWLEDGMENTS

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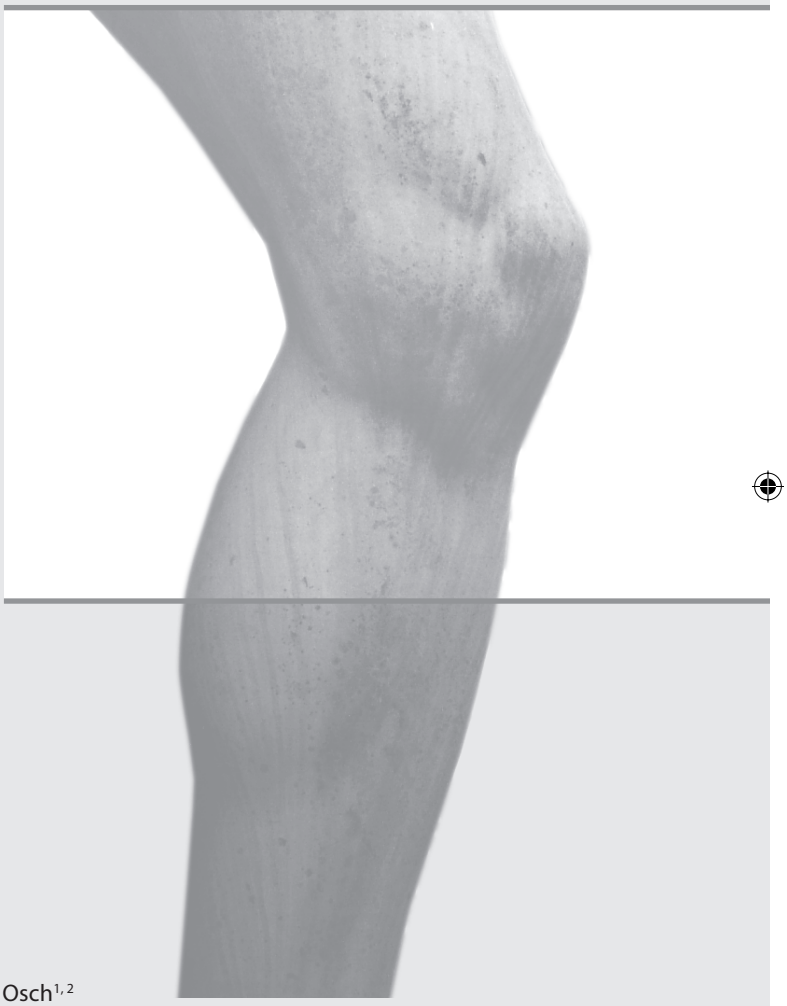
Chapter 3

Age related differences in articular cartilage wound healing. A potential role for transforming growth factor β1 in adult cartilage repair.



P.K. Bos[1], J.A.N. Verhaar[1], G.J.V.M. van Osch[1, 2]

1 Department of Orthopaedic surgery, Erasmus MC, University Medical Center Rotterdam, the Netherlands

2 Department of Otorhinolaryngology, Erasmus MC, University Medical Center Rotterdam, the Netherlands

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3.1. SUMMARY

Objective of this study was to investigate the early wound healing reactions of immature and mature articular cartilage on experimental wound healing in the New Zealand White rabbit. The proliferation potential and glycosaminoglycan production of isolated chondrocytes of these animals was studied in an alginate culture system. A band of tissue with death chondrocytes was observed at wound edges of immature articular cartilage, whereas mature cartilage showed a significant smaller amount of dead chondrocytes. A general increase in TGFβ1, FGF2 and IGF1 was observed throughout cartilage tissue with the exception of lesion edges. The observed immunonegative area appeared to correlate with the observed cell death in lesion edges. Repair in immature cartilage was indicated by chondrocyte proliferation in clusters and a decrease in defect size. No repair response was observed in mature articular cartilage defects. The alginate culture experiment demonstrated a higher proliferation rate of immature chondrocytes. Addition of recombinant TGFβ1 increased proliferation rate and GAG production of mature chondrocytes. We were not able to further stimulate immature chondrocytes. These results indicate that TGFβ1 addition may contribute to induce cartilage repair responses in mature cartilage as observed in immature, developing cartilage.

3.2. INTRODUCTION

Articular cartilage displays a limited repair response following injury. Partial-thickness articular cartilage defects, limited to the cartilage itself, are not repaired and full thickness defects are repaired with fibrocartilage. Complete repair of partial-thickness cartilage injury has only been reported in one study on fetal lamb[24]. This repair process appears to be absent in matured animals. Current clinical and experimental treatment methods do not result in durable and predictable restoration of the articular surface in damaged joints[7,8].

An important prerequisite for durable repair of cartilage lesions is the integration of wound edges or the integration of repair tissue with the surrounding host cartilage[2]. Failure of repair caused by an impaired integration has been documented by several authors who studied the natural cartilage repair process [22,27,32], repair following transplantation of periosteal and perichondrial grafts[3,25], osteochondral grafts[28], natural[10] and tissue engineered grafts[33].

Knowledge of the mechanisms by which immature, developing cartilage is able to repair defects may help in developing repair strategies for mature cartilage.

Sufficient amounts of chondrocytes and extracellular matrix (ECM) production are important for new hyaline cartilage tissue formation, defect filling and possibly for integrative repair. Active proliferation and high production of ECM during development may explain the observed repair response in immature subjects[14]. Age-related differences have been shown for concentrations of proteoglycans and TGFβ1 in synovial fluid of knee joints of immature and mature New Zealand White rabbits. An increased concentration of TGFβ1 in immature joint fluid was suggested to be a reason for the observed better healing capacity[34].

Ageing has been shown to change proliferation capacity, extracellular matrix production and responsiveness to growth factor stimulation in *in vivo* studies, explant studies and in isolated chondrocyte culture studies[4,15,26]. However, varying results were reported in literature.

Aim of this study was to investigate the early wound healing reactions (chondrocyte survival, histological changes, and immunohistochemical expression of growth factors) of immature and mature articular cartilage on experimental cartilage injury *in vivo* and to study the potential of isolated chondrocytes from these animals to proliferate and produce ECM *in vitro*. The possibility of stimulating these chondrocytes with a potent growth factor, TGFβ1, was studied in a three dimensional alginate culture system.

3.3. MATERIALS AND METHODS

3.3.1. Surgery and tissue sampling

All animal experimental procedures in this study were approved by the Animal Ethics Committee (protocol no. 1169803) and carried out in accordance with the guidelines of the Erasmus University Rotterdam (The Netherlands).

Nine immature (age 6 weeks) and 9 adult (age 48-50 weeks) New Zealand White rabbits were used for these experiments (Physial growth plate closure in New Zealand White rabbits: distal femur 19-24 weeks, proximal tibia 25-32 weeks [18].) The animals were anesthetized by an intramuscular injection of 10% ketamine-hydrochloride (Ketalin, Apharma, Arnhem, the Netherlands) 0.5 ml/kg body weight and 2% xylazine-hydrochloride (Rompun, Bayer, Leverkusen, Germany) 0.5 ml/kg body weight. Both knees were shaved and disinfected with 70% alcohol prior to surgery. A midline incision was used to approach the knee, a medial arthrotomy was performed, the patella was lateralized and the knee flexed. In the weigthbearing area of the medial femoral condyle of both limbs an anterior-posterior partial-thickness cartilage defect was created using a specially designed 0.5mm wide gouge, deepness 0.3mm. Length of the defect was controlled manually and approximately 5mm. Care was taken not to penetrate the subchondral plate, no bleeding was observed in the defects. In knee cartilage of one side the subchondral bone was cut using a scalpel, thereby opening the subchondral bone. Knees were rinsed thoroughly with saline, wounds were closed in layers. Animals were allowed to move freely in their boxes. The animals were killed at 1, 3 and 7 days following surgery. Areas of the defects in articular cartilage including a small part of the calcified cartilage were harvested using a scalpel, fixed in phosphate buffered formalin 4% for 24 hours, processed and embedded in paraffin. Three to four 5µm thick sections were cut and mounted on poly-L lysine coated slides. Articular cartilage from non-operated hips served as control tissue (n=3 per age group).

From the same animals, 3 mature and 3 immature, hip and shoulder cartilage was harvested aseptically and used for culture experiments.

3.3.2. Cell cultures

Chondrocytes were isolated with pronase E (2 mg/ml saline; Sigma, St Louis, MO) followed by overnight incubation at 37 °C with collagenase B (1,5 mg/ml medium with 10%FCS; Roche diagnostics Mannheim, Germany) Cells were suspended in 1.2% low-viscosity alginate (Keltone LV, Kelco) at a density of 4 x 10^6 cells / ml and alginate beads were prepared as described before[31]. Beads were cultured for 14 days in 24-well plates with or without 10 ng/ml TGFβ1 (recombinant human TGFβ1, R&D systems, Oxon, UK) in DMEM/Ham's F12 medium (Life Technologies, Breda, the Netherlands) with 10% FCS, 50 µg/ml gentamicin, 1,5 µg/ml fungizone and 25 µg/ml L-ascorbic acid freshly added (n=3 animals per condition, 3 beads per animal). Medium was changed three times a week.

Alginate beads were harvested directly and after 7 and 14 days of culture. Beads were snap-frozen in liquid nitrogen and stored in -80^0 until processing.

3.3.3. Histology

For histological evaluation sections were stained with Haematoxylin & Eosin (H&E). For evaluation of proteoglycan content sections were stained in 0.04% thionin in 0.01 M aqueous sodium acetate, pH 4.5 for 5 minutes.

Proteoglycan depletion at the wound edges was measured at a 400x magnification using a grid containing 50x50µm boxes. Defect size was evaluated by measuring the maximal defect diameter at day 7 in serial sections. Chondrocyte death at wound edges was determined by measuring the distance from the wound edge until vital chondrocytes. Nuclear and cytoplasmatic changes were analyzed to judge cell viability/death: chondrocyte death was defined as a cell with a condensed, pyknotic nucleus and either shrunken or deeply eosinophylic cytoplasm or fragmentation of the nucleus/cytoplasm[5,19]. Cluster formation was semiquantified by counting the amount of clusters in wound edge areas at day 7. A cell-cluster was defined as 5 or more chondrocytes grouped together. The wound edge was defined as a band of 200µm of tissue bordering the lesion, both vertical and horizontal. One representative section was used to count the clusters.

3.3.4. Immunohistochemical staining for growth factors

All steps were performed at room temperature. The sections were deparaffinized, preincubated for 30 minutes with normal goat serum to block non-specific antigens and incubated with antibodies against TGFβ1 **(**Anti Human TGFβ1 / 5µg/ml / monoclonal mouse IgG / Serotec Ltd, Oxford OX5 1JE, UK), FGF2 (Anti bovine FGF-2 / monoclonal mouse IgG / 5µg/ml / Upstate biotechnology, Campro Scientific, Veenendaal, the Netherlands) and IGF-I (Anti human IGF-I / mouse monoclonal IgG / 5µg/ml / Upstate biotechnology). The antibodies were linked with biotinylated Rabbit anti Mouse immunoglobulins and streptavidine-alkaline phosphatase (Super Sensitive Concentrated Detection Kit, Bio Genex, San Ramon 94583, USA). Alkaline phosphatase activity was demonstrated by incubation with a New Fuchsine substrate (Chroma, Kongen, Germany), resulting in a red colored signal. The slides were not counterstained with Haematoxylin and Eosin.

In control sections the primary antibody was omitted. For isotype control a mouse monoclonal negative control antibody (mouse IgG1 negative control / Dako A/S) was used.

3.3.5. Assessment of proliferation and GAG production

Beads were dissolved in sodium citrate and digested with papain (Sigma, St Louis, MO). The amount of DNA in the beads was measured using Hoechst 33258 dye[20]. Calf thymus DNA (Sigma, St Louis, MO) was used as a standard. Extinction (365 nm) and emission (440 nm) were measured with a spectrofluorometer (Perkin-Elmer LS-2B).

The amount of GAG was quantified using a modified Farndale assay in microtiter plates[13]. In short, the metachromatic reaction of GAG with dimethylmethylene blue is monitored using a

spectrophotometer. The ratio A_{540}/A_{595} is used to calculate the amount of GAG in the samples. Chondroitinsulfate C (Shark; Sigma, St Louis, MO) is used as a standard.

3.3.6. Statistical analysis

Results are expressed as mean ± SD. Differences between groups were calculated using Mann-Whitney U test, $p \leq 0.05$ was considered statistically significant (*).

3.5. RESULTS

3.5.1. Wound healing

One day after surgery, a band of tissue with avital chondrocytes was observed at wound edges of immature articular cartilage. Mature cartilage also showed chondrocyte death in lesion edges, however this band of avital tissue was significantly smaller (fig.1). In immature tissue defect size rapidly decreased during the 7 days of this study. Chondrocyte clusters were observed at lesion edges, clusters seem to grow towards the defect. Also defect walls appear to be pushed to the centre of the defect, thereby decreasing the volume of the defects. Mature defects remained significantly larger during the 7 days of this experiment as compared to immature defects (fig.2). Maximum defect diameter after 7 days in mature animals was 424 +/-42µm as compared to 163 +/-83µm in immature animals (p=0.03). Very few chondrocyte clusters were observed in mature cartilage wound edges. Wound edges of immature animals

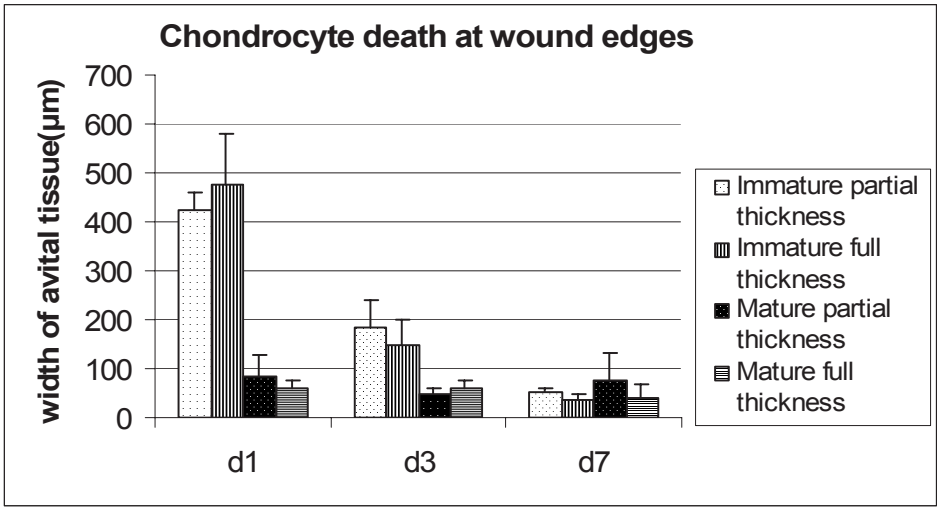

Figure 3.1 Graph representing the observed chondrocyte death in articular cartilage following experimental injury. Mean and SD of the distance between lesion edges and vital chondrocytes is shown

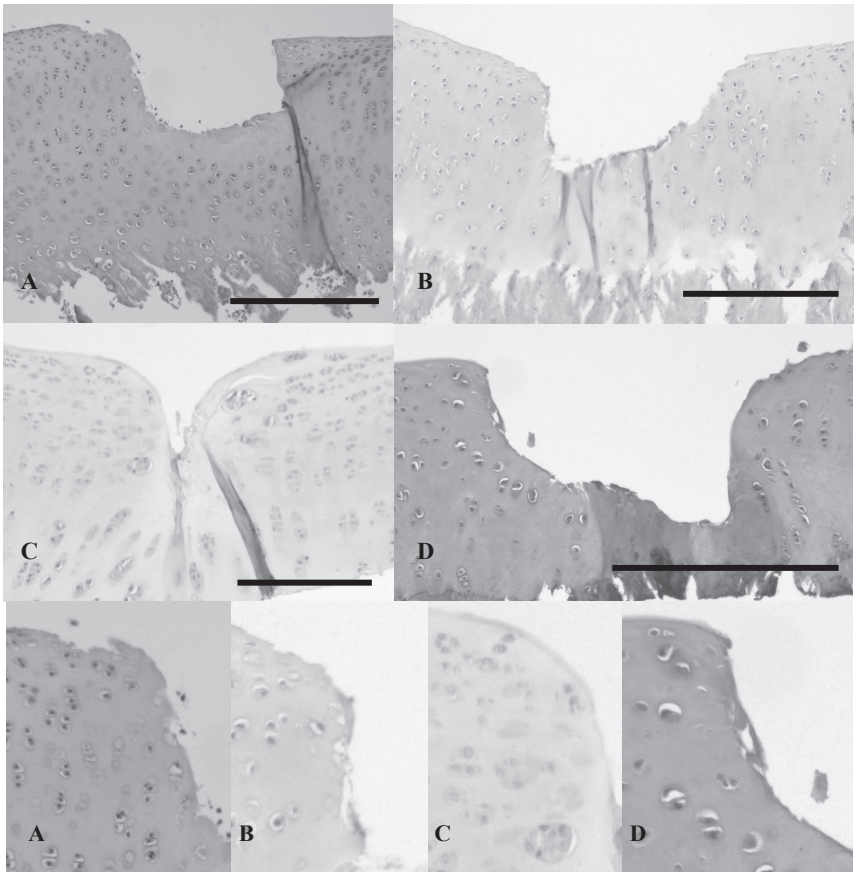

Figure 3.2 H&E stained sections from partial thickness defects are shown. Immature defects **(2a)** harvested 1 day after surgery show a large area of death chondrocytes. near wound edges, whereas mature defects show almost no signs of avital chondrocytes **(2b)** One week after surgery immature defects showed cluster formation and reduction in defect size **(2c)**, mature defect size and shape however, was grossly unchanged **(2d)**. Bar=500µm

showed significant more chondrocyte clusters as compared to mature animal wound edges (6.8 +/-0.4 versus 0.3 +/-0.5; p=0.03).

Hardly any filling with new tissue was observed in partial and full-thickness defects. Small amounts of fibrous tissue were observed at the base of defects in immature tissue in 1 of 3 defects after 3 days (partial and full-thickness) and 1 of 3 defects after 7 days (full-thickness only). In mature animals in which the subchondral bone was opened, a small amount of fibrous tissue was observed in 1 of 3 defects after 7 days. In the rest of the mature defects no filling was observed at all (fig.3).

A small band of tissue directly bordering the cartilage defects showed decreased proteoglycan staining in all animals. This band of proteoglycan depleted wound edges was relatively small, as compared to the zone of chondrocyte death, and remained stable during the study.

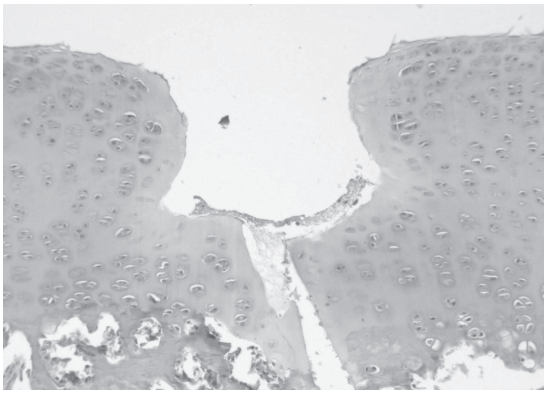

Figure 3.3 H&E stained section of an immature cartilage full-thickness defect harvested 3 days after surgery, showing a small amount of fibrous tissue at the base of the defect.

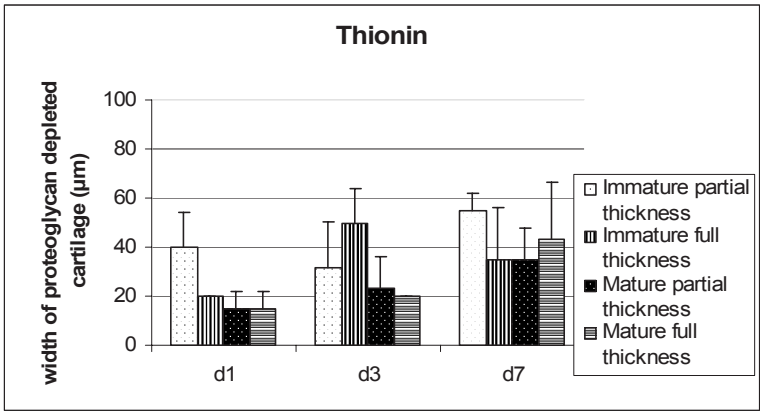

Figure 3.4 Graph representing the area of proteoglycan depletion at articular cartilage wound edges following experimental injury. Mean and SD of the distance from wound edges to normal thionin staining is shown.

No age-related or defect-related difference was observed in this early wound healing study (fig.4).

3.5.2. Immunohistochemistry

In unwounded articular cartilage from femoral heads immunohistochemical staining of immature cartilage showed an equally distributed weak positive signal for transforming growth factor β1 (TGFβ1) and fibroblast growth factor-2 (FGF2) and a very weak signal for insulin like growth factor-1 (IGF1). Mature tissue demonstrated merely a weak signal for TGFβ1 and FGF2 in one animal, whereas no immunoreactivity for these growth factors could be detected in the remaining 2 animals (fig 5).

Following experimental injury, an intense positive immunoreactivity was observed for TGFβ1 and FGF2 behind an area immunonegative cells near lesion edges. Also throughout

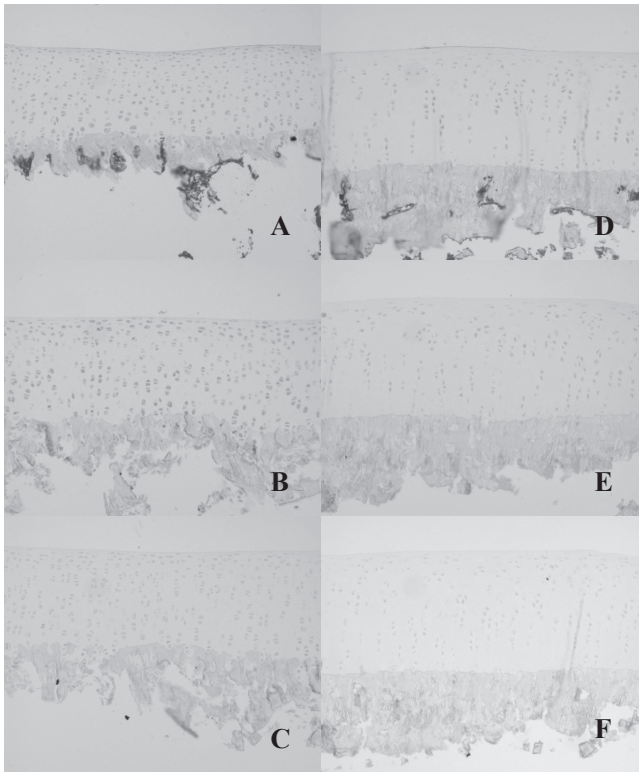

Figure 3.5 Unwounded immature **(a,b,c)** and mature **(d,e,f)** hip cartilage immunostained for TGFβ1 **(a and d)**, FGF-2 **(b and e)** and IGF-1 **(c and f)**. (See also: Colour figures section)

the cartilage tissue of the entire sections, away from the wound area, an intense positive signal for TGFβ1 and FGF2 was observed. The intensity for IGF1 staining was less, however, also stronger than in unwounded cartilage (fig.6a-f). The absence of immunoreactivity for the growth factors in lesion edges appears to correlate with the occurrence of chondrocyte death in articular cartilage wound edges. The observed cell clusters in lesion edges were positive for the growth factors tested (fig.6e). No difference in growth factor expression was observed between sections from partial-thickness or full-thickness defects.

3.5.3. Cell proliferation and GAG production

Chondrocyte proliferation was observed in alginate beads containing immature cells, cultured for two weeks in medium with 10% FCS. However, no increase in DNA content was observed in alginate beads with mature chondrocytes (fig.7). GAG content gradually increased in beads with mature and immature chondrocytes, although the increase was larger with immature chondrocytes.

Addition of TGFβ1 to cultures of alginate beads containing immature chondrocytes did not result in an increase in the observed proliferation and GAG production.

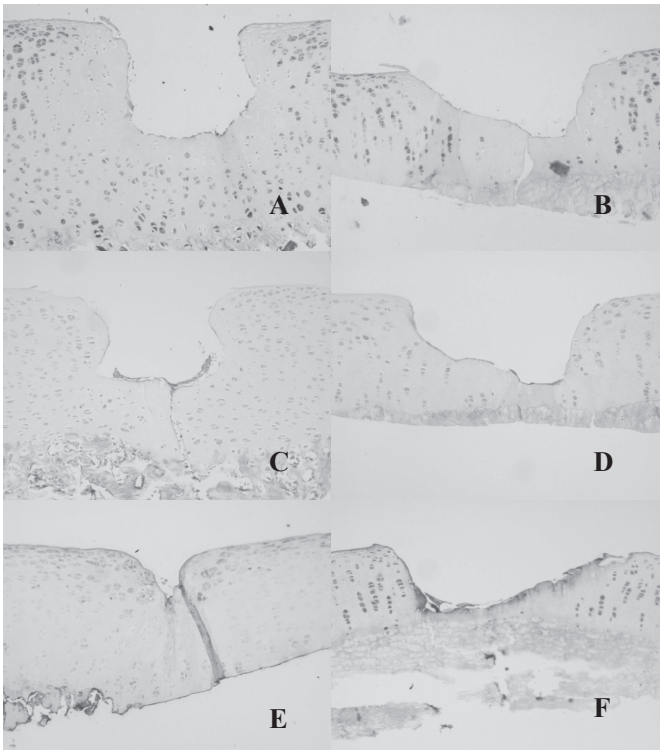

Figure 3.6 Figure 6: Immunohistochemical staining for TGFβ1 and FGF2 and IGF1 was studied in wounded articular cartilage. **a**=FGF-2, immature cartilage, day1; **b**=FGF-2, mature cartilage, day1; **c**=IGF-1, immature cartilage, day3; **d**=IGF-1, mature cartilage, day3; **e**= TGFβ1, immature cartilage, day7; **f**=TGFβ1, mature cartilage, day7. The area of tissue with immunonegative chondrocytes in wound edges correlated with the absence of immunopositive cells in wound edges. (Compare figures 6a,b to 2a,b). (See also: Colour figures section)

However, addition of TGFβ1 to mature chondrocyte cultures resulted in a significant increase in DNA content in alginate beads as compared to control cultures. At the same time a significant increase in total GAG production was found (fig 7).

3.6. DISCUSSION

In the present study, we have shown an early repair response in immature rabbit articular cartilage defect repair *in vivo*, and almost no repair response in mature articular cartilage defects. Immediately after wounding, chondrocyte death was induced in wound edges of both immature and mature cartilage, as is described previously in other studies[5,17,28]. Subsequent repair was initiated in immature cartilage, indicated by chondrocyte proliferation in clusters, a decrease in defect size and in some defects fibrous tissue formation. In addition to this,

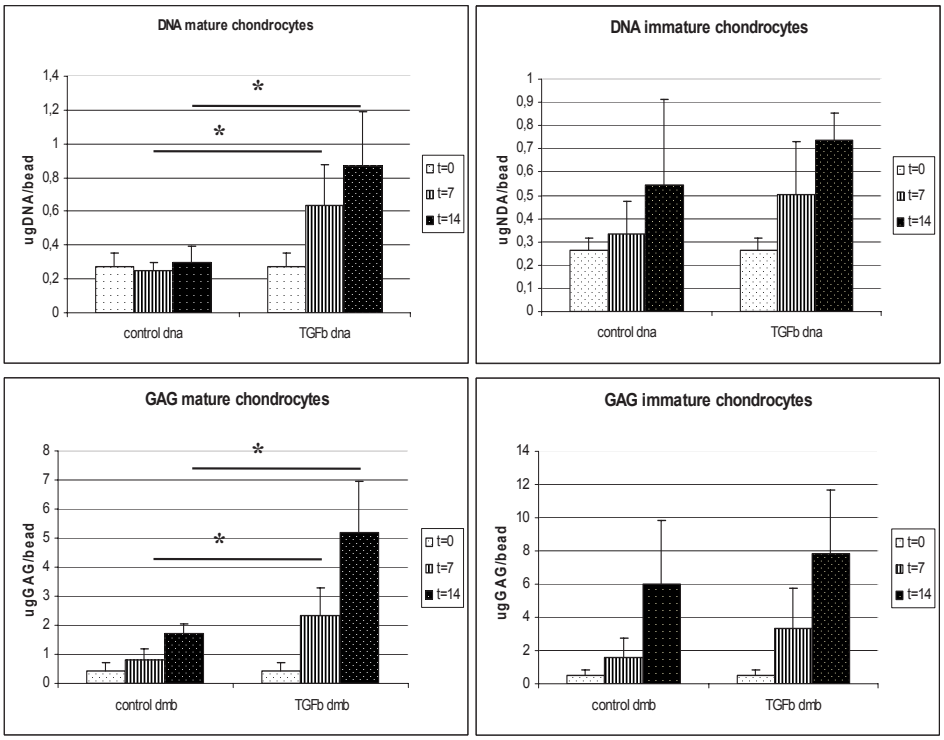

Figure 3.7 Glycosaminoglycan production and DNA content of alginate beads with immature and mature chondrocytes cultured for 14 days in medium with and without addition of 10ng/ml TGFβ1. TGFβ1 induced an increase in proliferation rate of mature chondrocytes in culture, whereas the observed proliferation rate of immature chondrocytes was not further stimulated.

we observed that immature defects rapidly decreased in size during this early wound healing study, whereas mature defects remained stable in size. In a subsequent alginate culture experiment we have confirmed that immature chondrocytes possess better proliferation capacity. This is in accordance with the results of animal[1,12] and human[4,15] articular chondrocyte culture studies. The early wound reactions observed in deep wounds are in accordance with the described early observations by Shapiro et al.(1993) in their extensive study on the repair of full-thickness defects of articular cartilage in New Zealand White rabbits[27]. The observed absence of defect filling with fibrous tissue in most of our full-thickness defects may be explained by the short follow-up period of 1 week. Another explanation might be that opening the subchondral bone using a scalpel is not sufficient for ingrowth of mesenchymal stem cells.

Immunohistochemical staining of wounded articular cartilage showed an increased expression of TGFβ1, FGF2 and IGF1 throughout the entire cartilage sections of immature and mature cartilage, with the exception of the lesion edges. The immunonegative cartilage area appeared to correlate with chondrocyte death in wound edges. The observed increase

in immunohistochemical reaction for growth factors in chondrocytes throughout cartilage tissue following experimental injury as compared to unwounded control cartilage may be explained by the occurrence of haemarthrosis induced by the arthrotomy. This general increase in immunoreactivity may hide a spatial or temporal difference in growth factor expression near wound edges as observed in auricular cartilage[6], osteoarthritic cartilage[23] or wound healing in other tissues[9,21].

Wei and Messner (1998) studied maturation-dependent changes of TGFβ1 and proteoglycans concentrations in rabbit synovial fluid in unwounded knees and during osteochondral defect repair. A decreased TGFβ1 concentration was demonstrated in unwounded joint synovial fluid from adult rabbits as compared to young and adolescent rabbits. Following injury, TGFβ1 levels were unchanged in young animals, whereas a minor increase was observed in adolescent and adult animals. As in our study, a better filling of defects was also observed in young and adolescent animals compared to adult animals. It was suggested that an increase in TGFβ1 levels in synovial fluid in young animals leads to a higher healing capacity. However, more cartilage degenerative signs and osteophyte formation was observed in this age group.

Barbero et al.(2004) reported that addition of a growth factor combination of TGFβ1, FGF2 and PDGF-BB to chondrocytes cultures, increased the proliferation rates of chondrocytes of all mature ages (20-91 years) and only a slight decrease with age was reported[4]. Guerne et al. showed a better response of young donor cells (ages 10-20) to recombinant PDGF-AA than to recombinant TGFβ, while the inverse pattern was observed in cells from adult donors. In this study TGFβ was the most potent stimulus in all cell preparations (compared to PDGF and FGF2), and in a large number of older donors, it was the only factor which significantly stimulated chondrocyte proliferation[15].

Fibroblast growth factor-2 was used in a partial-thickness articular cartilage defect repair study using immature and mature rabbits[35]. Defects in immature cartilage were almost completely repaired with hyaline-like cartilage following repeated intra-articular injections with FGF-2. However, no effect was observed in mature rabbits.

After having read the studies mentioned above, we have chosen to use recombinant human TGFβ1 in an attempt to stimulate proliferation and GAG production in the alginate culture system of immature and mature chondrocytes. We showed that addition of recombinant TGFβ1 to culture medium increased proliferation rate and GAG production of chondrocytes derived from mature articular cartilage. We were not able to further stimulate immature chondrocytes. These results indicate that TGFβ1 addition may contribute to induce cartilage repair responses in mature cartilage as observed in immature, developing cartilage.

In a similar experimental setup to the present study, Hunziker et al.(2001) studied the potential of various growth factors to induce chondrogenesis in a partial thickness articular cartilage defect model, using mature minipigs (age 2 to 4 years) [16]. Evaluation of defects after 6 weeks showed that TGFβ1 was able to induce chondrogenesis, although this is described to

be due to synovial cells that are attracted to the defect. Possible stimulation of chondrocytes at the wound edges is not mentioned. Concentrations above 1000ng/ml however, induced side effects such as synovitis, pannus formation, cartilage erosion and joint effusion. No osteophyte formation was observed. Similar chondrogenesis, with higher cellularity without undesired side effects was observed with the use of bone morphogenetic protein 2 (BMP-2) and BMP-13.

Care must be taken to use TGFβ-superfamily proteins in high concentrations in joints. The adverse osteoarthritis-like effects of administering TGFβ-superfamily proteins into joints has been shown by many researchers (inflammatory synovitis, pannus formation, cartilage erosion and osteophyte formation)[11,29,30].

In summary, differences in early wound healing response of immature and mature articular cartilage are described in this study. Immature cartilage has a higher intrinsic proliferative potential and is therefore able to decrease the size of a defect. We further demonstrated that addition of TGFβ1 can restore proliferative response of mature chondrocytes in culture. Further studies need to elaborate the possibility to stimulate mature chondrocytes in explant studies and *in vivo* to proliferate, thereby increasing their ability to produce ECM needed for cartilage repair.

3.7. ACKNOWLEDGEMENTS

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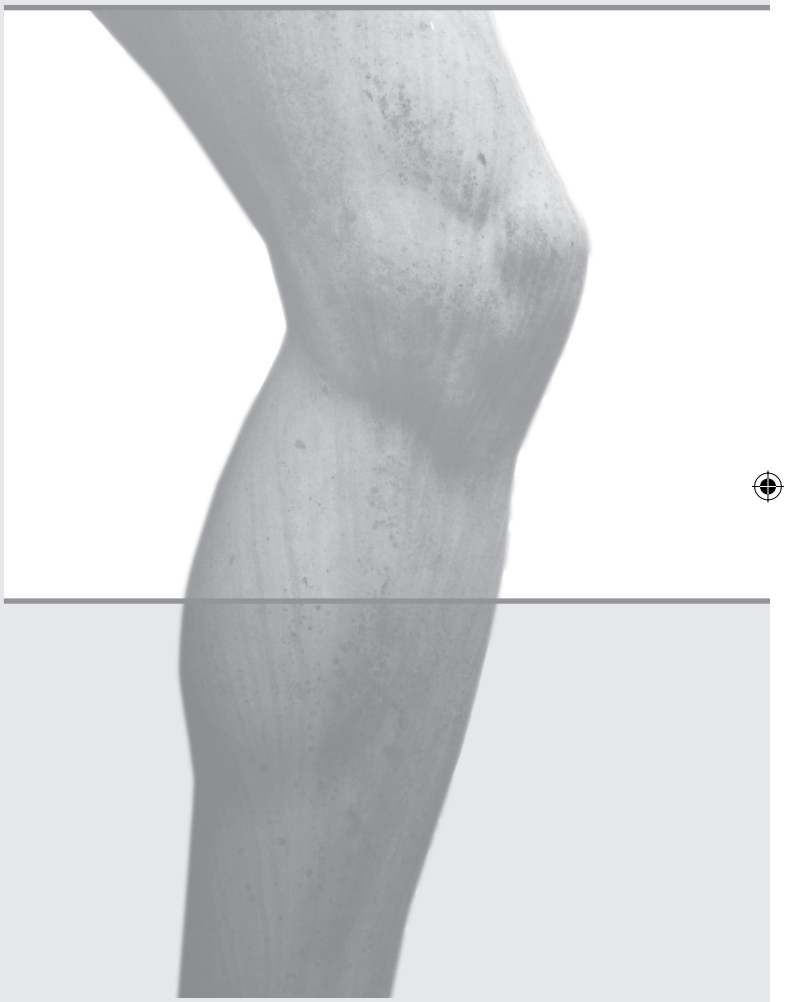
Chapter 4

Specific enzymatic treatment of bovine and human articular cartilage. Implications for integrative cartilage repair



PK Bos[1], J DeGroot[2], M Budde[2], JAN Verhaar[1], GJVM van Osch[1,3]

Departments of Orthopaedics[1] and Otorhinolaryngology[3] , Erasmus MC Rotterdam

TNO Prevention and Health[2], Leiden

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4.1. SUMMARY

Objective. Chondrocyte death in articular cartilage wound edges and the subsequent lack of matrixproducing cells in the interface area are considered to be a major cause of impaired cartilage wound healing and poor integrative cartilage repair. This study was undertaken to investigate whether enzymatic matrix digestion can be used to stimulate integrative cartilage repair via a mechanism of local increase in the amount of vital chondrocytes in cartilage wound edges.

Methods. Full-thickness bovine articular cartilage samples were cultured *in vitro* for 14 days in standard medium. Samples were either left untreated or treated for 48 hours with 0.3% hyaluronidase or 30 units/ml highly purified collagenase VII. Nuclear and cytoplasmic changes were analyzed to determine cell viability, and the number of vital chondrocytes in wound edges was determined. Subsequently, we investigated whether increased chondrocyte density in the lesion edges resulted in better wound healing. Finally, full-thickness human tibial plateau cartilage explants were tested with similar enzyme treatment protocols to determine the clinical value of our results.

Results. In bovine explants a rapid onset of chondrocyte death was observed in wound edges in all treatment groups. This led to low chondrocyte density in a band of 0–150 μm from the lesion edges in untreated and hyaluronidase-treated explants. Treatment with 30 units/ml collagenase resulted in a significant increase in chondrocyte density in this area. The integration experiments demonstrated improved integration of the lesion edges after treatment with collagenase. In human articular cartilage an increase in chondrocyte density at the lesion edges could also be achieved, but only when proteoglycans were depleted from the wound edges prior to collagenase treatment.

Conclusion. Treatment with highly purified collagenase improves integrative cartilage repair, possibly by increasing the cell density at cartilage wound edges.

4.2. INTRODUCTION

Articular cartilage is characterized by limited intrinsic repair capacity following injury. Partial- and full-thickness wounds or defects are not repaired with hyaline cartilage, and this may lead to the development of osteoarthritis. Current clinical and experimental treatment methods do not result in durable and predictable restoration of the articular surface in damaged joints[6,7]. In general, two strategies can be used for articular surface restoration: (osteo)chondral transplantation or in situ induction of new cartilage. However, every surgical intervention in cartilage involves chondrocyte death in wound edges. This results in the formation of an acellular wound edge, next to a zone of chondrocyte proliferation[5,8,13,30]. Recent *in vitro* experiments have shown a rapid onset of cell death in experimentally wounded fetal, young, and mature hyaline cartilage[31,36].

An important prerequisite for long-term repair or regeneration of articular cartilage is the integration of transplanted cartilage or locally induced repair tissue with the native cartilage at the recipient site[1,14,29]. Integrative cartilage repair is probably hindered by the lack of matrix-producing cells in the cartilage–cartilage interface area[1,25,27]. The acellularity is due to a combination of chondrocyte loss from lesion edges, avascularity, the absence of multipotent progenitor cells, and the inability of chondrocytes to migrate through the tight extracellular matrix.

Classic wound healing in vascularized mammalian tissue consists of an inflammatory phase, a proliferation phase, and a regeneration phase. During the inflammatory phase, proteolytic enzymes are released and activated. Damaged extracellular matrix components are selectively removed so that newly formed proteoglycans and collagen can fill in the discontinuity in the tissue. Integrative cartilage repair is considered to be greatly impaired by chondrocyte death in lesion edges and the absence of removal of necrotic tissue. In cartilage wound edges, spontaneous proteolytic enzyme activity is not sufficient for necrotic tissue removal, which is necessary to provide wound edges with integration capacity.

The aim of the present study was to investigate whether specific enzymatic digestion of the extracellular matrix in articular cartilage lesion edges can induce integrative cartilage repair via a mechanism of local increase in the number of vital chondrocytes in cartilage wound edges. In a bovine articular cartilage explant culture model, chondrocyte death and tissue viability were studied in full-thickness explant edges of acutely wounded cartilage, after treatment with hyaluronidase or highly purified collagenase. In order to determine the implications of our results for integrative cartilage repair, we studied the integration of bovine articular cartilage explants after collagenase treatment. Finally, similar (enzymatically induced) processes were investigated in human articular cartilage explants from 3 healthy donors.

4.3. MATERIALS AND METHODS

4.3.1. Tissue cultures

Full-thickness articular cartilage samples were harvested from the metacarpophalangeal joints of five 6-month-old calves, within 6 hours after slaughter (T. Boerand Son, Nieuwerkerk a/d IJssel, The Netherlands), under sterile conditions using 4-mm dermal biopsy punches (Stiefel; imported by Bipharma, Veendam, The Netherlands). First the cartilage was cut from the articular surface by gently pushing and rotating a new and sterile biopsy punch for every 6–10 explants. Then a sharp scalpel was used to cut the cartilage loose from the subchondral bone, resulting in full-thickness discs. Explants were collected in medium, and wet weight was measured. When indicated, 6-mm full-thickness discs of tibial plateau cartilage from healthy human donors (3 donors ages 25, 37, and 39 years, with no previous joint disease), obtained at autopsy within 12 hours after death, were used.

The discs were randomly divided into treatment groups. Prior to culture, cartilage was treated (a) without enzyme (control), (b) with 0.3% hyaluronidase (Sigma, St. Louis, MO), or (c) with 30 units/ml highly purified collagenase VII (Sigma)[17,21,33] in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium with 2% fetal calf serum (FCS) for 48 hours. Alternatively, cartilage was treated with 0.1% hyaluronidase followed by 10 units/ml or 30 units/ml collagenase (24 hours each). Explants were subsequently washed thoroughly and cultured for 14–28 days in separate wells in DMEM/Ham's F-12 medium (Gibco, Grand Island, NY) supplemented with 10% FCS and 25 µg/ml ascorbic acid. Medium was changed 3 times per week in all experiments. Samples were harvested before treatment (time <1 hour), after enzymatic treatments (time 48 hours), and after 7, 14, or 28 days of culture.

4.3.2. Experimental design

Experiment I. In experiment 1, bovine explants were treated for 48 hours (control or with hyaluronidase or collagenase) and cultured for 14 days. Each group consisted of 10 discs: 4 for histologic evaluation, 3 for Farndale assay, and 3 for determination of collagen damage.

Experiment 2. Experiment 2 was conducted to investigate the implications of treatment with 30 units/ml collagenase VII for integrative cartilage repair. Two-millimeter center cores were punched out of 8-mm full-thickness bovine cartilage samples. Cores were reimplanted immediately (group A; n = 5), after 48 hours (group B; n = 8), or after the core and outer ring were treated for 48 hours with collagenase (group C; n = 8) and cultured for 28 days.

Experiment 3. In experiment 3, human explants (2 donors) were treated for 48 hours (control or with collagenase) and cultured for 14 days. Each study condition included 3 discs for histologic analysis.

Experiment 4a. The purpose of experiment 4a was to test whether proteoglycan depletion prior to collagenase treatment can enhance the effects of collagenase (as observed in experiment 1). Bovine explants were treated for 48 hours (control, with 0.1% hyaluronidase for 24

hours followed by 10 units/ml collagenase for 24 hours, or with 0.1% hyaluronidase for 24 hours followed by 30 units/ml collagenase for 24 hours) and cultured for 14 days. Each study condition included 3 discs for histologic analysis.

Experiment 4b. In experiment 4b, bovine explants were treated for 48 hours (control, with 0.1% hyaluronidase for 24 hours followed by 10 units/ml collagenase for 24 hours, or with 0.1% hyaluronidase for 24 hours followed by 30 units/ml collagenase for 24 hours) and cultured for 7 days. Each study condition included 4 discs: 1 for confirmation of histologic findings (experiments 1 and 4a) and 3 for determination of collagen damage.

Experiment 5. Human explants (1 donor) were treated for 48 hours (control or with 0.1% hyaluronidase for 24 hours followed by 10 units/ml collagenase for 24 hours) and cultured for 14 days. Each study condition included 3 discs for histologic analysis.

4.3.3. Biochemical analysis of proteoglycan depletion

Discs were frozen in liquid nitrogen and stored at -80°C prior to use. To evaluate the effect of hyaluronidase and highly purified collagenase on proteoglycan content, discs were digested overnight in papain (Sigma), samples were diluted 5-fold, and the amount of glycosaminoglycan (GAG) was quantified using the Farndale assay (in 3 explants per condition)[11]. Briefly, in this assay the metachromatic reaction of GAG with dimethylmethylene blue is monitored using a spectrophotometer. Absorbance at 540 nm and 595 nm was determined with microtiter plates. The ratio A540:A595 was used to calculate the amount of GAG per 0.5-ml extract. Chondroitin sulfate C (from shark; Sigma) was used as a standard. The amount of GAG per disc is presented as µg GAG/mg wet weight.

4.3.4. Evaluation of collagen damage

Damage to the collagen network was assessed by selective proteolysis (by α-chymotrypsin) of denatured collagen as described previously[3]. Cartilage samples (3 cryopreserved samples per condition) were extracted twice with 4*M* guanidinium chloride to remove proteoglycans. After washing, denatured collagen was digested overnight at 37°C with α-chymotrypsin (Sigma). The supernatant was separated from the insoluble collagen, and hydroxyproline levels in both fractions were determined colorimetrically after acid hydrolysis[9]. Collagen damage was calculated as the percentage of hydroxyproline released into the supernatant. Total collagen content was calculated from the hydroxyproline content in tissue and supernatant (assuming 300 hydroxyproline residues per triple helix and a molecular weight of 300,000 g/mole), and normalized to the wet weight of the cartilage sample.

4.3.5. Histologic analysis

Discs from each study condition were fixed in 4% phosphate buffered formalin, processed, and embedded in paraffin. Four 5-µm sections (100-µm intervals) were cut and mounted on poly-L-lysine–coated slides. Sections were stained in 0.04% thionin in 0.01*M* aqueous sodium

acetate for evaluation of proteoglycan depletion[32]. Spontaneous and enzymatically induced proteoglycan depletion at lesion edges was measured at a magnification of 200x, using a grid with 50 x 50–µm boxes. Serial sections were stained with hematoxylin and eosin (H&E) for evaluation of chondrocyte viability and quantification of cell density.

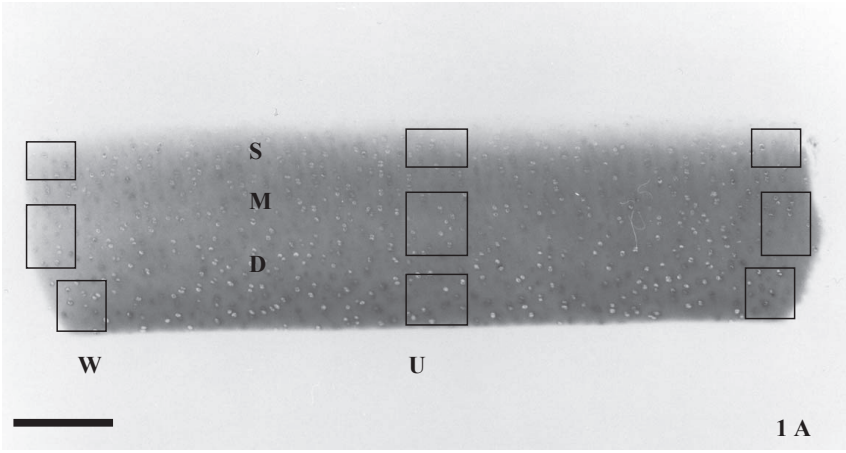
4.3.6. Type II collagen immunostaining

Sections were deparaffinized in xylene and rehydrated through graded ethanol. They were then incubated for 30 minutes with 0.2% pronase (Sigma) for antigen retrieval and for 30 minutes with 1% hyaluronidase (Sigma) for better antibody penetration, with subsequent incubation for 2 hours at room temperature with specific anti–type II collagen monoclonal antibody IIII6B3 (1:100; Developmental Studies Hybridoma Bank, Johns Hopkins University School of Medicine, Baltimore, MD). Alkaline phosphatase–labeled secondary antibodies were used. Alkaline phosphatase activity was demonstrated by incubation with a New Fuchsin substrate (Chroma, Kongen, Germany), resulting in a red-colored signal. In control sections, the primary antibody was omitted. The slides were counterstained with hematoxylin and mounted in gelatin glycerin.

4.3.7. Evaluation of chondrocyte viability

H&E-stained sections were used for determination of cell death and viability. Nuclear and cytoplasmic changes, as described by Kim and Song[16], were analyzed to assess cell viability/death: chondrocyte death was defined as a cell with a condensed, pyknotic nucleus and either a shrunken or deeply eosinophilic cytoplasm or fragmentation of the nucleus/cytoplasm. Only cells with visible nuclei were evaluated and scored. The number of vital cells and the percentage of cells with nuclear and cytoplasmic changes were determined with visualization at 400x magnification, using a grid containing 50 x 50–µm boxes. Cell counts are presented per mm^2.

In bovine explants, a band of 0–150 µm along the lesion edges, showing signs of cell death, was defined as wounded cartilage. The middle parts of the explants (~2 mm from the lesion edge) were defined as the unwounded cartilage and served as controls. The cartilage (average total thickness 600 µm) was divided into a superficial zone (100 µm from the surface down), a deep zone (150 µm from the base up), and a middle zone (between superficial and deep zones) (Figure 1A), based on morphologic characteristics[24]. To evaluate the effect of proteoglycan removal prior to collagenase treatment, an area was selected in which there was no loss of thionin staining observed in the control samples, and complete thionin destaining in the 0.1% hyaluronidase–treated samples (Figure 1B). Vital chondrocyte density was scored in a 250 x 250–µm section of this area, overlapping the superficial and middle zones. In human explants, the band 0–200 µm from the lesion edges was defined as wounded cartilage[31]. The human cartilage (average total thickness 1.3 mm) was divided into a superficial zone (200 µm from the surface down), a deep zone (250 µm from the base up), and a middle zone (between superficial and deep zones), based on morphologic characteristics[24].



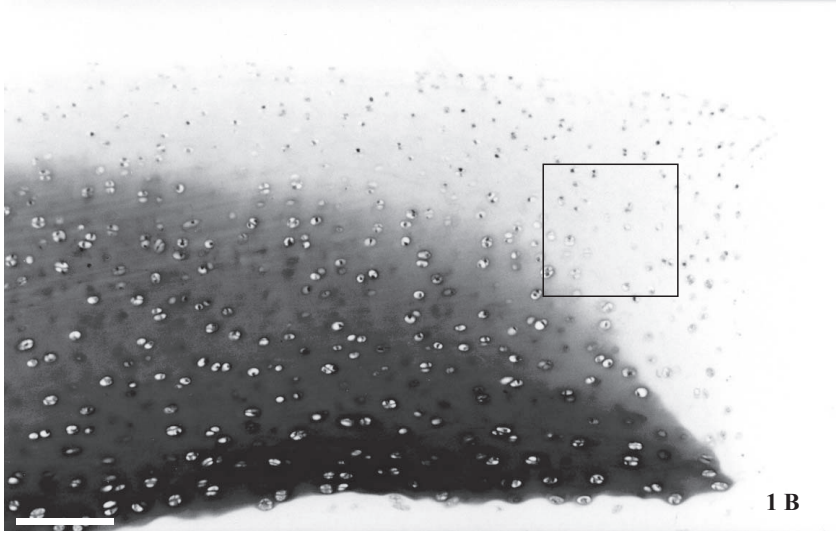


Figure 4.1 (A) Full-thickness bovine articular cartilage explant fixed within one hour after explantation. Vital chondrocytes were counted in the superficial (s), middle (m) and deep (d) zones of wounded (w) and unwounded (u) cartilage. Thionin staining, bar ≈ 300µm
(B) Thionin staining of a cartilage wound edge after 24 hours of hyaluronidase treatment and 24 hours of collagenase treatment, showing proteoglycan depletion at the wound edge and superficial zone. The box in this figure indicates the area in which vital chondrocytes were analysed. Thionin staining, bar ≈ 200µm

4.3.8. Statistical analysis

Results are expressed as the mean ± SD. Vital cell densities per mm^2 were calculated from cell counts in the superficial, middle, and deep zones of wounded and unwounded cartilage for all conditions and time points. Vital cell counts were grouped together: enzyme treated versus untreated cartilage, successive time points after wounding, wounded versus unwounded cartilage, and proteoglycan-depleted versus nondepleted. The significance of mean vital cell

counts, GAG content, collagen release, and percentage of damaged collagen was assessed by one-way analysis of variance. Differences between groups were calculated using the Mann-Whitney U test. *P* values less than 0.05 were considered significant.

4.4. RESULTS

In unwounded areas of the cartilage explants, no cell death was observed throughout the study. The average number of vital cells in unwounded bovine cartilage remained constant (superficial zone 886 ± 219/mm2, middle zone 760 ± 120/mm2, deep zone 400 ± 175/mm2 [mean ± SD]). At the wound edges, an immediate decrease in the amount of vital chondrocytes was observed compared with the unwounded (central) part of the explants (Figure 2). Early nuclear and cytoplasmic changes in the superficial and middle zones of the explants were already visible at time 0 (i.e. explants fixed within 1 hour after harvesting) (Figure 3A). The reduced amount of vital cells in the wound edges of control explants remained constant during the entire tissue culture period and led to the formation of a low chondrocyte density band of ~150 µm after 7 and 14 days (Figure 3B). Hyaluronidase treatment before culture had no effect on the number of vital cells.

Strikingly, after 48 hours of collagenase VII treatment, the number of vital chondrocytes in the lesion edges was significantly higher than in control explants (Figure 2). Vital cell counts in collagenase-treated wound edges further increased during culture, leading to chondrocyte densities comparable with those in unwounded cartilage (Figures 2 and 4). In the collagenase treatment group, no difference between wounded and unwounded cartilage could be demonstrated in the deep zone after 7 days ($P = 0.77$) or 14 days of culture ($P = 0.25$) or in the superficial zone after 14 days of culture ($P = 0.38$).

A gradual depletion of thionin staining at lesion edges in control, hyaluronidase-treated, and collagenase VII–treated bovine explants was observed starting immediately after explantation. Proteoglycan depletion in the control group was comparable with that in the collagenase group, with a maximum depletion depth of 75 µm. An almost complete loss of thionin staining was observed after 48 hours of hyaluronidase treatment, followed by a gradual replenishment starting in the pericellular regions. Biochemical analysis in control experiments demonstrated a 5.8% decrease in GAG per milligram wet weight and 0.33 ± 0.12% (mean ± SD) collagen release into the medium after 48 hours without treatment. Collagenase treatment did not affect the decrease in GAG content, whereas 48 hours of hyaluronidase treatment resulted in a significant decrease in GAG content (83.0% decrease; $P = 0.025$). Hyaluronidase treatment did not affect collagen release into the medium, whereas 48 hours of collagenase treatment resulted in a significant increase in collagen release (14.4% increase; $P = 0.05$).

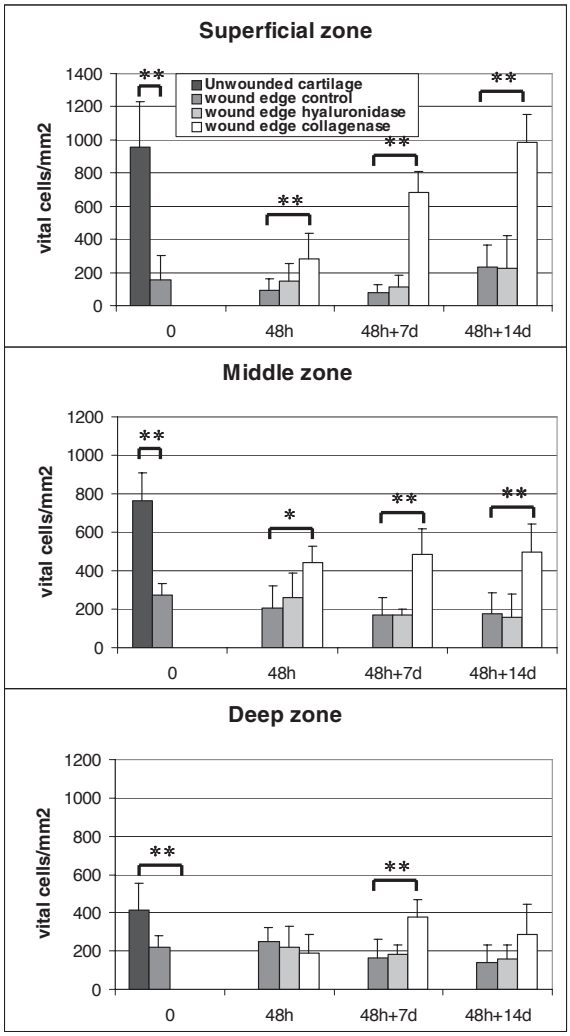


Figure 4.2 Number of vital chondrocytes per mm^2 in wound edges (0 to 150µm from lesion edges) of full-thickness bovine articular cartilage explants. Vital cell counts are presented from unwounded (central) parts of control explants and wound edges of control explants, hyaluronidase treated explants and collagenase treated explants. Vital chondrocytes were counted before treatment (t=0), directly after treatment (t=48 h) and after 7 and 14 days of culture. Mean ± SD of 4 explants is presented. * : p≤0.05 ** : p≤0.01

Subsequently, the effects of collagenase treatment on integrative cartilage repair were tested. Core punches (2 mm) were obtained from large (8-mm) bovine cartilage explants and reimplanted immediately or after 48 hours of treatment with or without collagenase. One explant (from group B) was damaged during reimplantation of the central disc and expelled before culture, and 3 discs appeared to be reimplanted upsidedown after 48 hours of treat-

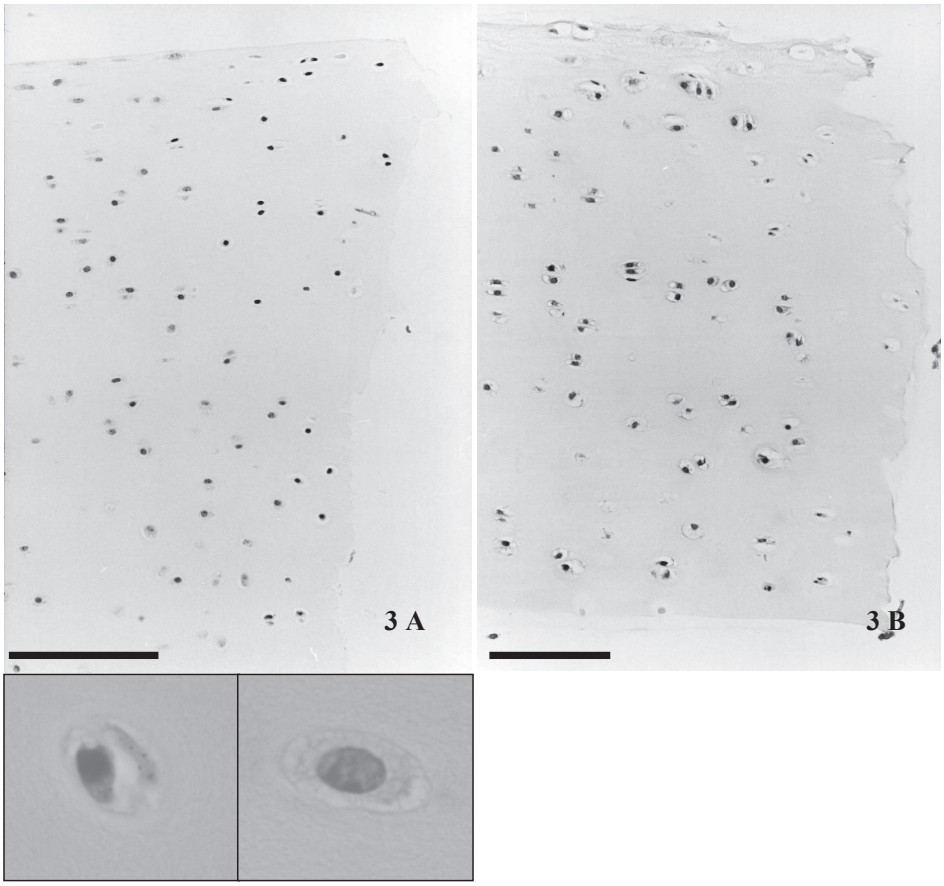


Figure 4.3 (A) Bovine articular cartilage wound edge of an explant fixed in formalin within one hour after explantation. Chondrocytes with pyknotic nuclei and shrunken cytoplasm are visible, mainly in the upper part of the cartilage. Further removed from the wound edge vital chondrocytes can be seen with normal haematoxylin staining of the nucleus and no shrinkage or fragmentation of the cytoplasm. H&E staining, bar ≈ 200 µm. Inserts: chondrocyte with condensed, pyknotic nucleus and shrunken fragmented cytoplasm. Normal, vital chondrocyte.
(B) Untreated bovine cartilage explant after 14 days of culture showing an acellular band at the cartilage wound edge. H&E staining, bar ≈ 100µm (See also: Colour figures section)

ment (2 from group B, 1 from group C); all composite explants remained intact during the subsequent 28 days of culture.

No signs of integration between central discs and accessory explants after 28 days of culture could be observed in either control group (immediate reimplantation or reimplantation after 48 hours without collagenase treatment). In these samples, either a gap between the wound edges was observed or the surgical cutting line remained visible (Figure 5A). Small pieces of tissue, with poor cellularity, were formed in or close to the interface region in 6

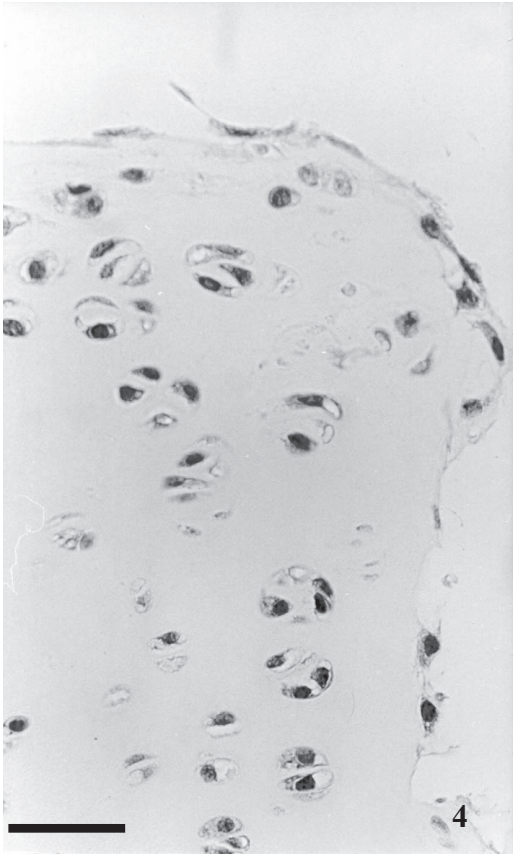


Figure 4.4 Bovine explant treated with 30U/ml highly purified collagenase VII for 48 hrs and subsequently cultured for 14 days. The figure shows an increased chondrocyte density in the superficial and upper part of the middle zone in the cartilage wound edge. Note cluster formation in the wound edge. H&E staining, bar ≈ 40µm (See also: Colour figures section)

composite explants (1 of 5 from group A, 5 of 8 from group B). This tissue exhibited positive staining for type II collagen (Figure 5B).

In contrast, in the collagenase-treated group, integration between superficial zones or superficial zones and a part of the middle zone was observed in 4 of 7 composite explants after 28 days of culture (Figures 5C and D). In this group, outgrowth of tissue in or close to the interface region was observed in 1 of the integrated and 3 of the non-integrated composite explants. The extracellular matrix of this tissue was positive for type II collagen, but, in contrast to the new tissue formation in the controls, this tissue demonstrated normal chondrocyte density. The explants with upside-down reimplanted cores provided evidence that the outgrowth originates from the upper part of the cartilage.

Since young bovine cartilage probably has a larger regenerative capacity than adult human cartilage, the effect of enzymatic treatment was tested on human cartilage from healthy

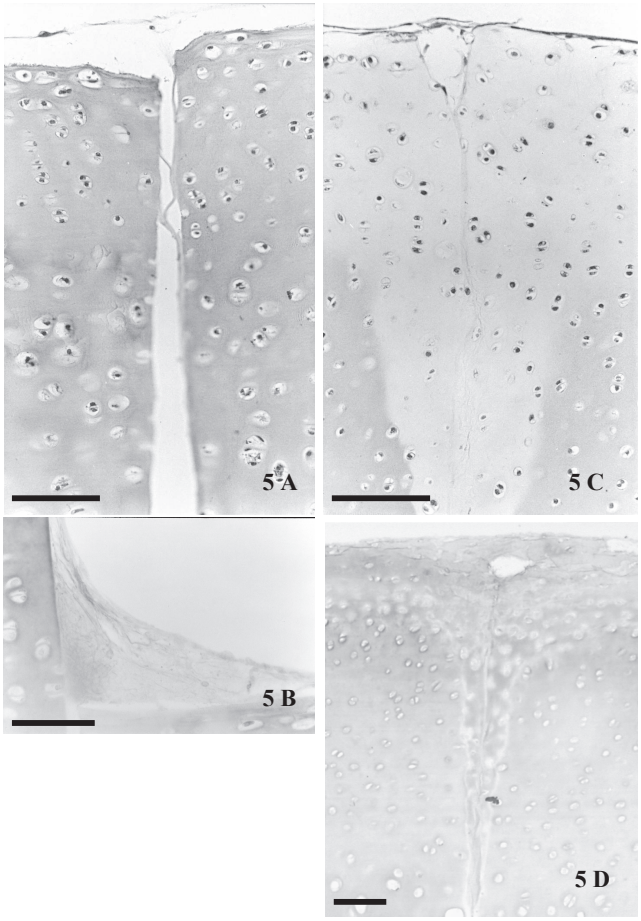


Figure 4.5 Effect of collagenase treatment on integrative cartilage repair. Central discs (2 mm) were punched out of large (8-mm) full-thickness bovine explants, reimplanted after 48 hours of collagenase treatment or 48 hours without treatment (controls), and cultured for 28 days. (**A)**, Central disc that was reimplanted without treatment. No integration is observed (hematoxylin and eosin [H&E] stained). (**B)**, Immunostaining for type II collagen, showing that newly formed tissue in or close to the interface region is positive for type II collagen. (**C)**, Explant that was reimplanted after 48 hours of collagenase treatment, demonstrating integration (H&E stained). (**D)**, Immunostaining for type II collagen in integration area, showing that integrated cartilage is positive for type II collagen. (Bars ~100 µm.) (See also: Colour figures section)

donors (ages 25 and 37 years) (experiment 3). In explants from both donors, no degenerative changes were observed; only very few chondrocytes, primarily located in the direct vicinity of the cutting line, exhibited nuclear and cytoplasmic changes. No significant decrease in the number of vital chondrocytes in the superficial, middle, or deep zone could be demonstrated after 14 days of explant culture. Moreover, only a small band (<25 µm) at the cutting line of

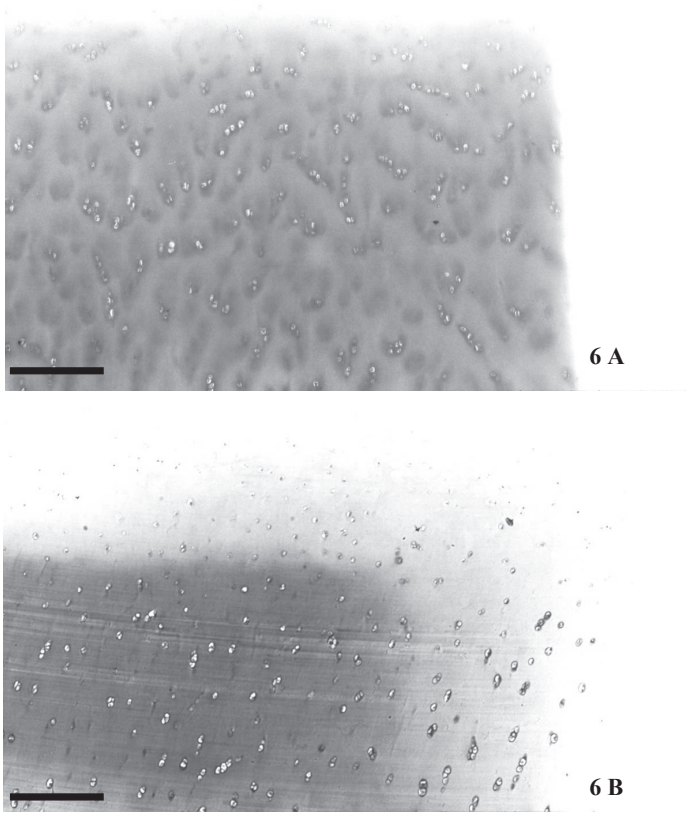


Figure 4.6 Wound edges of human articular cartilage explants without enzymatic treatment (**A**) and after sequential treatment with 0.1% hyaluronidase followed by 10 units/ml collagenase (**B**). Explants were treated for 48 hours, cultured for 14 days, and than fixed for histologic analysis. (Thionin stained; bar ~300 µm.)

the explants showed depletion of thionin staining (Figure 6A). This area was observed both in control and in collagenase-treated explants and did not change during 14 days of culture.

Differences between the results of experiments with bovine samples and those with human samples may be explained by a difference in spontaneous proteoglycan depletion in cartilage wound edges following explantation and culture. Proteoglycan depletion might be a prerequisite for highly purified collagenase to be able to exert its effect on cartilage. To investigate this, we studied the effect of collagenase treatment on cell numbers in native and proteoglycan-depleted bovine explants. Treatment for 24 hours with 0.1% hyaluronidase resulted in an increased area with loss of thionin staining. Subsequent treatment for 24 hours with 10 units/ml collagenase or 30 units/ml collagenase resulted in an increase in the number of vital cells in this area (33.3% increase and 48.4% increase, respectively, after 14 days of

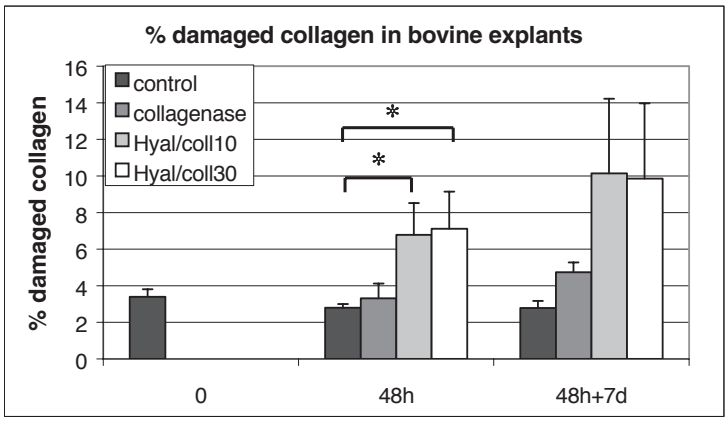


Figure 4.7 Percentage of damaged collagen in bovine explants determined by selective proteolysis of denatured collagen in explants directly after explantation, after enzymatic treatment with collagenase or 0.1% hyaluronidase followed by 10U/ml collagenase (coll10) or 30U/ml collagenase (coll30) and after 7 days of culture.

culture). Treatment with 30 units/ml collagenase for 48 hours (experiment 1) without extra proteoglycan depletion resulted in a 24.7% increase in vital chondrocytes.

To evaluate whether an increase in cell density correlates with increased collagenase activity, we determined the percentage of collagen that was damaged (experiment 4). A significant increase in damaged collagen was observed in bovine explants that were treated with collagenase after hyaluronidase treatment (Figure 7), but not in explants treated with collagenase alone. This indicates that there is an increased effect of collagenase on collagen after proteoglycan depletion. There was no increase in the percentage of collagen damage caused by treatment with high-dose collagenase (30 units/ml) compared with low-dose collagenase (10 units/ml).

In addition to induction of collagen damage in the tissue, treatment with collagenase may result in release of collagen from the tissue. Therefore, release of collagen into the medium was monitored. Hyaluronidase treatment did not increase the release of collagen into the medium as compared with that seen in untreated controls. As noted above, treatment with 30 units/ml collagenase alone resulted in increased collagen release into the medium (14.4%; $P = 0.05$). Hyaluronidase treatment prior to treatment with 10 units/ml or 30 units/ml collagenase resulted in a further increase in collagen release (38.1%; $P = 0.05$ and 46.3%; $P = 0.05$, respectively), indicating that proteoglycan density is an important factor in collagen susceptibility to proteolytic degradation. Removal of enzymes stopped the release of collagen.

To further substantiate that the effects observed in young bovine tissue also occur in adult human articular cartilage, the effect of enzymatic treatment on cell density was tested in human tibial plateau cartilage. In human cartilage explants, sequential treatment with hyaluronidase and collagenase (experiment 5) had effects similar to those found with bovine

samples. Wound edges demonstrated loss of thionin staining in a band of 0–200 μm after treatment with 0.1% hyaluronidase followed by 10 units/ml collagenase (Figure 6). Collagenase treatment resulted in an increased number of vital cells in the superficial zone after 14 days of culture in proteoglycan-depleted wound edges compared with nondepleted wound edges (mean ± SD 396 ± 104 cells/mm2 versus 183 ± 56 cells/mm2; $P = 0.05$).

4.5. DISCUSSION

Restoration of the damaged articular joint surface presents a challenge for both basic scientists and clinicians. Complete and durable repair of an articular cartilage defect requires integration of cartilage wound edges or integration between transplanted cartilage or locally induced repair tissue and the surrounding cartilage at the recipient site. Long-term success of articular cartilage repair techniques relies on integrative cartilage repair. Failure of repair caused by impaired integration has been documented by several authors who studied the natural cartilage repair process[22,28,34], or repair following transplantation of periosteal and perichondral grafts[2,26], osteochondral grafts[31], natural grafts[10], or bioengineered grafts[35]. Integration is probably hindered by a lack of matrix-producing cells in the interface region, caused by chondrocyte death and inability of chondrocytes to migrate through the extracellular matrix toward lesion edges.

Recently, Silverman et al studied the adhesion between native cartilage discs, using fibrin glue polymer alone or mixed with fresh articular chondrocytes. Their results demonstrated that adhesion of cartilage to cartilage can be improved by an increased amount of chondrocytes in the interface region[29].

Obradovic et al.[25] have demonstrated that integration between native cartilage and immature "tissue-engineered" cartilage constructs (5 days) was better than integration between native cartilage and more mature "tissue-engineered" cartilage constructs (5 weeks). They observed increased cellularity in the interface region in experiments with immature constructs compared with mature constructs, and histologic and biomechanical analysis showed improved bonding and a stronger adhesion between construct and native cartilage with immature constructs. In the present study, we have demonstrated that treatment of articular cartilage explants with highly purified collagenase can result in a significant increase in chondrocyte density at the wound edges where initial cell death occurs. Furthermore, in an integration experiment, we have shown that this "vitalization" of wound edges, providing vital, matrixproducing cells at the cartilage–cartilage interface, benefits integrative cartilage repair. The increase in cell density in wound edges is one possible explanation for the observed improved integrative repair.

In our experiments, human articular cartilage explants from healthy donors showed considerably less chondrocyte death in lesion edges than was observed in bovine explants.

Therefore, in human articular cartilage, the reduced amount of cell death in reaction to wounding may require less adjustment for integrative repair. However, since chondrocyte density in adult human cartilage is low compared with the cell density in most animal models, increased chondrocyte density may improve integration in human cartilage as well. In bovine experiments, collagenase treatment resulted in increased cell density. In our initial experiments with human cartilage, however, no effects of collagenase on cell density were found. In human cartilage, proteoglycan depletion occurred only in a small band at the wound edges. No further depletion or replenishment was observed during culture. Since spontaneous proteoglycan depletion was almost absent in human cartilage wound edges, proteoglycans had to be removed enzymatically from the wound edges to induce an effect of collagenase on chondrocyte density. Sequential treatment with hyaluronidase and collagenase resulted in the desired increase in cell density at the wound edges. This suggests that for human articular cartilage, enzymatic treatment may also be beneficial for the integration of wound edges.

Several studies have shown that enzymatic removal of proteoglycans from the surface of lesion edges can be used in cartilage repair, either as single treatment[23] or to improve the initial adhesion of transplanted cells or cartilage to cartilage wound edges[15,19,20,25]. Such treatment probably has no effect on cell density itself; this requires action of collagenase. Lee et al have investigated the effects of surrounding matrix, growth factors, and cell shape on control of chondrocyte division in articular cartilage. With the use of ^{3}H-thymidine labeling, they showed that disruption of the collagen architecture induces DNA synthesis and cell proliferation[18]. These findings are consistent with our observations that collagenase treatment can induce an increase in chondrocyte density in cartilage wound edges. Moreover, we have shown that hyaluronidase treatment prior to collagenase treatment can enhance the effects of collagenase on bovine cartilage and is necessary in order to increase chondrocyte density in human articular cartilage wound edges.

After removal of the enzymes and further culture, we did not see an increase in the percentage of damaged collagen and or in the amount of collagen secreted into the medium. This indicates that collagenase might be used safely, since there was no prolonged enzyme activity. The observed increase in chondrocyte density at the lesion edges is at least partly a result of repopulation, rather than a gradual breakdown of nonvital tissue by extended enzymatic activity. Repopulation can occur due to cell migration, local proliferation in lesion edges, or a combination of the two. Our results did not allow us to draw final conclusions on this; however, the absence of a decrease in cell density in the areas behind the wound edges, the observed chondrocyte clusters in wound edges, and evidence from the literature showing a correlation between collagen destruction and chondrocyte proliferation[18] suggest that proliferation contributes more to the revitalization of the lesion edges than does migration.

Further research is needed to establish whether integration can also be induced *in vivo* and whether the integration between explants observed histologically can withstand bio-

mechanically relevant forces. Obviously, before application *in vivo*, enzyme treatment has to be shortened[15,23] and integrative repair has to be optimized. We did not demonstrate integration between deep cartilage zones. Deep-zone cartilage is probably less susceptible to proteoglycan depletion. Therefore, collagenase has less effect and cell density is not increased. Furthermore, chondrocyte proliferation as well as extracellular matrix production at the interface can probably be enhanced with the application of appropriate growth factors.

Recent investigations have focused on the mechanisms of chondrocyte cell death in cartilage injury and disease. Apart from necrosis, several studies have shown that chondrocytes in human osteoarthritis[4], experimentally induced osteoarthritis[12], rheumatoid arthritis[16], and experimental wound healing[31] can die by apoptosis. Further study to elucidate the regulatory mechanisms of chondrocyte apoptosis and the use of apoptosis inhibitors may help to improve cartilage wound healing. The exact mechanism of chondrocyte death remains of great interest from a scientific point of view; however, the rapid onset of changes apparently leaves little time to intervene in this process. Local induction of chondrocyte proliferation, as shown in our experiments, may bypass this phenomenon and provide vital cartilage wound edges with sufficient integrative repair capacity.

In summary, the present results show that treatment of articular cartilage with highly purified collagenase alone or in combination with hyaluronidase has beneficial effects on cell number and integration of cartilaginous wound edges. These findings suggest that surgery aimed at the facilitation of repair of articular cartilage defects may benefit from pretreatment with these enzymes and thereby result in better healing.

4.6. ACKNOWLEDGEMENTS

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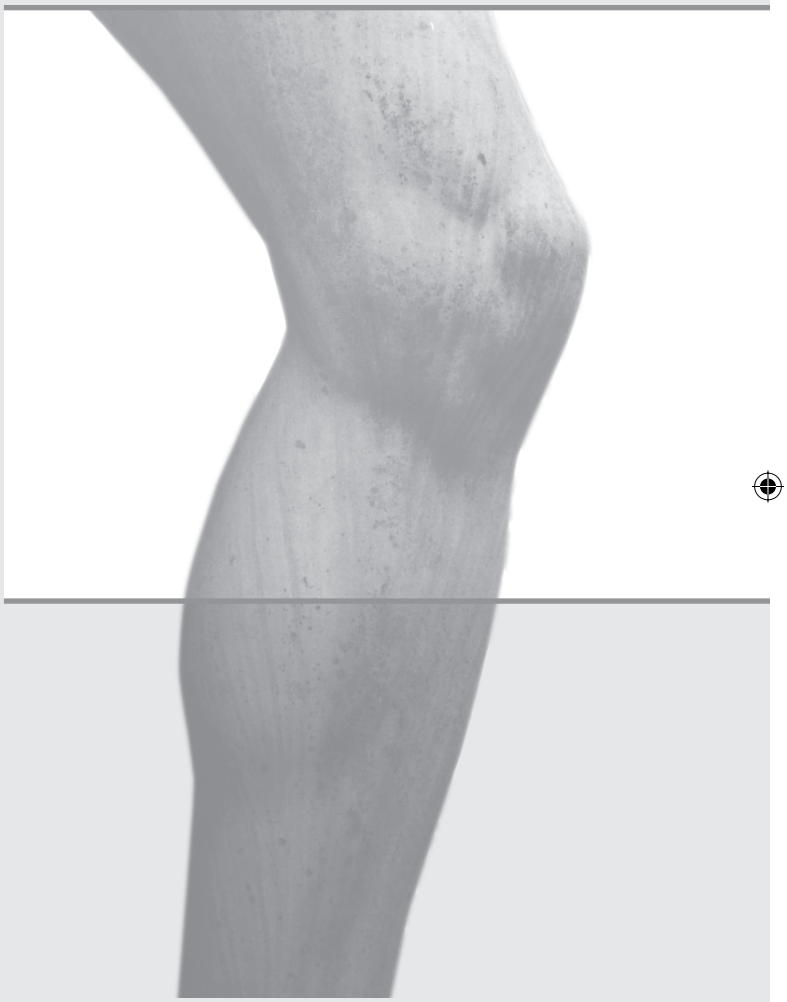
Chapter 5

Improved cartilage integration and interfacial strength after enzymatic treatment in a cartilage transplantation model



J. van de Breevaart Bravenboer[1], C.D. In der Maur[2], P.K. Bos[1], L. Feenstra[2], J.A.N. Verhaar[1], H. Weinans[1], G.J.V.M. van Osch[1,2]

Departments of Orthopaedics[1] and Otorhinolaryngology[2] , Erasmus MC Rotterdam

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5.1. SUMMARY

The objective of the present study was to investigate whether treatment of articular cartilage with hyaluronidase and collagenase enhances histological and mechanical integration of a cartilage graft into a defect. Discs of 3 mm diameter were taken from 8-mm diameter bovine cartilage explants. Both discs and annulus were either treated for 24 hours with 0.1% hyaluronidase followed by 24 hours with 10 U/ml collagenase or left untreated (controls). Discs and annulus were reassembled and implanted subcutaneously in nude mice for 5 weeks. Integration of disc with surrounding cartilage was assessed histologically and tested biomechanically by performing a pushout test. After 5 weeks a significant increase in viable cell counts was seen in wound edges of the enzyme-treated group as compared with controls. Furthermore, matrix integration (expressed as a percentage of the total interface length that was connected; mean ± standard error) was 83 ± 15% in the treated samples versus 44 ± 40% in the untreated controls. In the enzyme-treated group only, picro-Sirius Red staining revealed collagen crossing the interface perpendicular to the wound surface. Immunohistochemical analyses demonstrated that the interface tissue contained cartilage-specific collagen type II. Collagen type I was found only in a small region of fibrous tissue at the level of the superficial layer, and collagen type III was completely absent in both groups. A significant difference in interfacial strength was found using the push-out test: 1.32 ± 0.15 MPa in the enzyme-treated group versus 0.84 ± 0.14 MPa in the untreated controls. The study shows that enzyme treatment of cartilage wounds increases histological integration and improves biomechanical bonding strength. Enzymatic treatment may represent a promising addition to current techniques for articular cartilage repair.

5.2. INTRODUCTION

Localized articular cartilage defects are a major problem for orthopaedic surgeons. Articular cartilage shows a poor intrinsic repair capacity[15,16,24], chondral defects do not heal and may increase the risk for early osteoarthritis. A number of different treatment techniques, such as subchondral penetration[19,23,27], osteochondral transplantation and mosaïcplasty[10-12], perichondrium covering of the defect [3,13]and autologous chondrocyte transplantation[4,5], as well as various enzymatic treatment techniques[6,17,21,28], have been tried in either clinical or laboratory settings in an attempt to restore the articular surface. Until now none of these techniques has resulted in a durable and predictable repair of the articular cartilage. Many researchers focus on the production, or local induction, of hyaline-like cartilage; however, these techniques are generally not directly aimed at local integration with the surrounding healthy cartilage. Variable and suboptimal wound healing and integration may be a cause of potential failure of otherwise promising techniques.

Injury to cartilage results in the formation of an acellular and thus metabolically inactive zone adjacent to the wound interface[2,18,32], thereby prohibiting significant matrix deposition at the wound interface area and subsequently limiting integration. Ideally, the biochemical composition of the integrative matrix should equal that of native cartilage, with high contents of collagen type II and proteoglycans, and low amounts of collagen types I and III. Furthermore, the biomechanical properties of the interfacial tissue should be within the range of native cartilage in order to prevent excessive strain[33] and mechanical failure. We previously showed that enzymatic treatment with collagenase increased cell density at the wound edges of cartilage explants after 2 weeks of *in vitro* culture[1]. This treatment method could improve cartilage integration in chondral defects and potentially could confer benefit in clinical applications. In the present study we used enzymatic treatment with hyaluronidase and collagenase, and tested how this would affect wound healing and cartilage integration in terms of matrix composition and biomechanical properties. Specifically, we applied a combination hyaluronidase and collagenase treatment on both sides of a cartilage explant, and tested the effect of this treatment on cell viability at the wound edge, production of collagens types I, II and III, collagen fibre orientation, and biomechanical bonding strength.

5.3. METHODS

5.3.1. Tissue cultures

Articular cartilage samples were harvested from the metacarpophalangeal joints of calves aged 6–12 months. Fullthickness cartilage explants of 8 mm diameter and with a thickness of 0.9–1.2 mm were prepared using a dermal biopsy punch and scalpel. The explants were then randomly divided into two groups. From the centre of the explants, 3-mm cores were

punched out, using a custom built device to ensure punching in the exact middle of the explant. Group 1 (n = 12) specimens (both outer ring and inner core) were incubated for 24 hours in 0.1% hyaluronidase type I-S from bovine testes (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) followed by 24 hours in 10 U/ml highly purified collagenase VII (Sigma-Aldrich Chemie BV), both in Dulbecco's modified eagle's medium/ Hams' F12 with 2% foetal calf serum. Specimens from group 2 (controls; n = 12) were incubated in Dulbecco's modified eagle's medium/Ham's F12 culture medium (Gibco, Grand Island, NY, USA) supplemented with 2% foetal calf serum at 37°C for 48 hours (controls). The choices of enzymes, enzyme concentrations and treatment times were based on the findings from our previous *in vitro* study[1]. After 48 hours the samples were washed three times for 10 min in culture medium, and the 3-mm inner cores were reimplanted in their accompanying 8-mm outer rings. Constructs were then implanted in four subcutaneous pockets on the backs of six nude mice (BALB-C nu/nu; Harlan, Horst, The Netherlands), for which approval was obtained from the local animal ethical committee (DEC no.126-01-01). Each mouse carried two enzyme-treated constructs (group 1) and two control constructs (group 2). After 5 weeks the mice were killed by cervical dislocation and constructs were harvested.

5.3.2. Histology

From each mouse, one control and one enzyme-treated construct were processed for histology. Constructs were divided into two halves. One half was fixed in 4% phosphate- buffered formalin and embedded in paraffin, and the other half was frozen in liquid nitrogen and stored at -80°C for later cryosection preparation. Sections (6 µm) were cut using a standard microtome (paraffin) or cryomicrotome and mounted on Starfrost slides (Knittel, Braunschweig, Germany). Paraffin sections were haematoxylin and eosin stained as well as immunostained for collagen type II. Cryosections were used for thionin (proteoglycan) stain, picro-Sirius Red stain and immunohistochemical stains for procollagen type I, collagen type I and collagen type III.

Evaluation of chondrocyte viability

The number of vital chondrocytes was counted in surface, middle and deep zones in both wounded and unwounded areas using haematoxylin and eosin coloured slides at 400× magnification using a 50 × 50 µm boxed grid. Nuclear and cytoplasmic changes, as described by Kim and coworkers[20], were analyzed to assess cell viability/ death. Only cells with visible nuclei were evaluated. Values for wounded cartilage were scored from a 150-µm broad band on both sides of the lesion, and the values for both sides of the interface were then averaged to obtain one value per interface. Furthermore, the cartilage was divided into superficial (100 µm from the surface down), deep (150 µm from the bottom up) and middle (between superficial and deep) zones, as previously described[1]. For comparison, values from unwounded tissue were obtained from the middle part of the outer ring, which is more then 1 mm from

the wound edge. Chondrocyte densities are represented as vital cells/mm2. For each explant the amount of viable chondrocytes was calculated from the values obtained from two to four sections. Subsequently, the averages for the control and enzyme-treated groups were calculated and used for statistical evaluation.

Evaluation of integration

Cryosections were fixed in acetone and stained with 0.04% thionin in 0.01 M sodium acetate for 5 min. For each sample we assessed the percentage of total interface length that had a matrix–matrix connection using a microscope with a 50 µm square grid. A clear distinction could be made between parts with a matrix connection and parts of the cartilage touching each other but without a clearly connected matrix, which were scored as parts with a gap. Interface integration percentages were obtained from measurements of two to four different sections from each sample, resulting in one average value for each interface.

Picro-Sirius Red stain

Cryosections were fixed in acetone and stained with 0.1% Sirius Red F3BA (Direct Red 80; Fluka Chemie, Zwijndrecht, The Netherlands) in a saturated picric acid solution for 1 hour. Brief washing in 0.1% acetic acid was followed by rapid dehydration in 100% alcohol (three changes for 3 min each), after which a xylene bath (two changes for 5 min each) was used to prepare the slides for mounting with Entellan (Merck, Darmstadt, Germany). Slides were analyzed using a polarized light microscope (Dialux 20; Leitz, Wetzlar, Germany) to evaluate fibre orientation in the interface area. The relative sign of birefringence was determined using the analyzer filter. For semiquantitative analyses, samples from both groups were classified as follows: 0 = no fibres crossing; 1 = occasional fibre crossing; and 2 = many fibres crossing.

Collagen immunostaining

Cryosections were fixed in acetone for procollagen type I, and collagen types I and III staining. For collagen type II staining paraffin sections were deparaffinized using xylene and rehydrated through a graded series of ethanol, after which they were incubated with 0.2% pronase for 30 min to retain antigenicity. Treatment with 1% hyaluronidase (Sigma-Aldrich Chemie BV) was used to unmask the epitopes. Nonspecific binding was blocked using 10% normal goat serum (CLB, Amsterdam, The Netherlands) followed by incubation with the respective antibody for 2 hours. Antibodies used were M38 and II-6B3 (both 1:100; Developmental Studies Hybridoma Bank) for procollagen type I and collagen type II, respectively; ab6308 (mouse monoclonal IgG antibody, 1:500; Abcam Ltd, Cambridge, UK) for collagen type I; and ab6310 (mouse monoclonal IgG antibody, 1:500, Abcam Ltd) for collagen type III. All primary antibodies were previously complexed with goat Fab fragment against mouse conjugated with alkaline phosphatase (GAMAP, 1:400; Immunotech, Marseilles, France) at 4°C overnight. After coupling, 0.1% normal mouse serum was used for 2 hours before usage

to capture the unbound GAMAP, after which the antibody solution was used on the slides. Sections were subsequently incubated for 30 min with alkaline phosphatase anti-alkaline phosphatase (APAAP, 1:100 for procollagen I and collagen II, 1:75 for collagen types I and III; Dakopatts, Copenhagen, Denmark). New Fuchsine substrate (Chroma, Kongen, Germany) was used for colour development and haematoxylin for counterstaining, after which slides were mounted using Vectamount (Vecto Laboratories Inc., Burlingame, CA, USA). Negative controls were subjected to the same protocol with omission of the primary antibody.

5.3.3. Mechanical testing

After harvesting of the constructs, the surrounding fibrotic tissue was carefully removed. From each of the six mice, one control and one enzyme-treated construct were frozen using liquid nitrogen and stored in airtight tubes at -80°C for later mechanical testing. Immediately before testing constructs were slowly thawed in airtight tubes. Thickness of the sample was measured to an accuracy of 50 µm using calipers. Constructs were then mounted in a specially designed push-out setup (Fig. 1) on a materials testing machine (LRX; Lloyd Instruments, Fareham, UK) equipped with a 500 N load cell. Push-out tests were performed by leading the push-out rod on top of the 3 mm inner core through the specimen at 10 µm/s. During the test constructs were kept moist by adding a few drops of phosphate-buffered saline on top before starting the test, which on average took 4–5 min. During the test both displacement and load were monitored at a sample frequency of 18 Hz and the output of these values was read out and stored on a desktop computer. For each specimen the peak loadto-failure (maximum observed load) was used to calculate the interface stress-to-failure (maximum load normalized to interface area) as a representative marker of interfacial strength. Furthermore, we also performed push-through tests of intact cartilage for comparison ($n = 4$) and pushout tests of constructs immediately after reassembly of core and annulus ($n = 8$) to determine the friction component of our setup; all this was done in a manner similar to that described above.

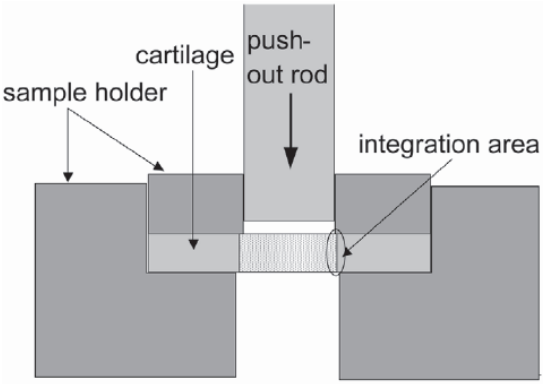

Figure 5.1 Schematic representation of the push-out setup. Displacement transducer and load cell are connected to the push-out rod.

5.3.4. Statistical analysis

Values shown are mean ± standard deviation unless otherwise specified. Statistical analyses for both viable cell count and mechanical testing were done using Student's t test for independent samples. Matrix integration scores and results from polarized light microscopy were analyzed using the Mann–Whitney U test. $P \leq 0.05$ was considered statistically significant.

5.4. RESULTS

5.4.1. Histology

Cell counts in the integration area revealed significantly more vital cells near the wound edges in the enzymetreated group than in the untreated group in all three layers (Table 1), with the largest increase in the superficial layer. Many cells were located in the interface region of the enzyme-treated group (Fig. 2a,2b), but despite the apparently normal average vital cell count in the 150-µm broad band in the untreated control samples, the tissue in the interface region was almost acellular (Fig. 2c,2d). Measurement of matrix integration on thionin stained sections (Fig. 3) revealed an average matrix–matrix connection percentage of 83 ± 15% of wound interface length in the enzymetreated constructs, as compared with 44 ± 40% in the untreated group ($P < 0.05$), with variability between sections of the same interface typically being less then 15%.

To assess the quality of the newly formed interface matrix we evaluated which types of collagen were present in this new tissue. Immunohistochemical staining revealed the presence of limited amounts of (pro-)collagen type I in the interfaces, which was limited to the area of ingrowth of fibrous tissue from the top surface (Fig. 4a; four out of 10 interfaces in the treated group and three out of 10 interfaces in the control group). Typically, this ingrowth was around 10% of the interface length, with Fig. 4a showing the worst case. Furthermore,

Table I Effect of enzymatic treatment on cell viability in cartilage wound edges 5 weeks after subcutaneous implantation in nude mice

Zone	Unwounded (n = 9)	Non-enzyme treated (n = 5)	Enzyme-treated (n = 4)
Surface	1440±175	1151±133	2316±209*
Middle	787±160	866±27	1097±59*
Deep	646±75	589±16	960±45*

Table 1: Cartilage was treated with hyaluronidase and collagenase or left untreated and implanted subcutaneously into nude mice for 5 weeks. The number of vital cells were counted in a 150-mm broad band along both sides of the wound edges, as well as in unwounded control areas in surface, middle and deep zones. Chondrocyte densities are represented as vital cells/mm^2. *: $P < 0.05$ (versus unwounded and non-enzyme-treated tissue).

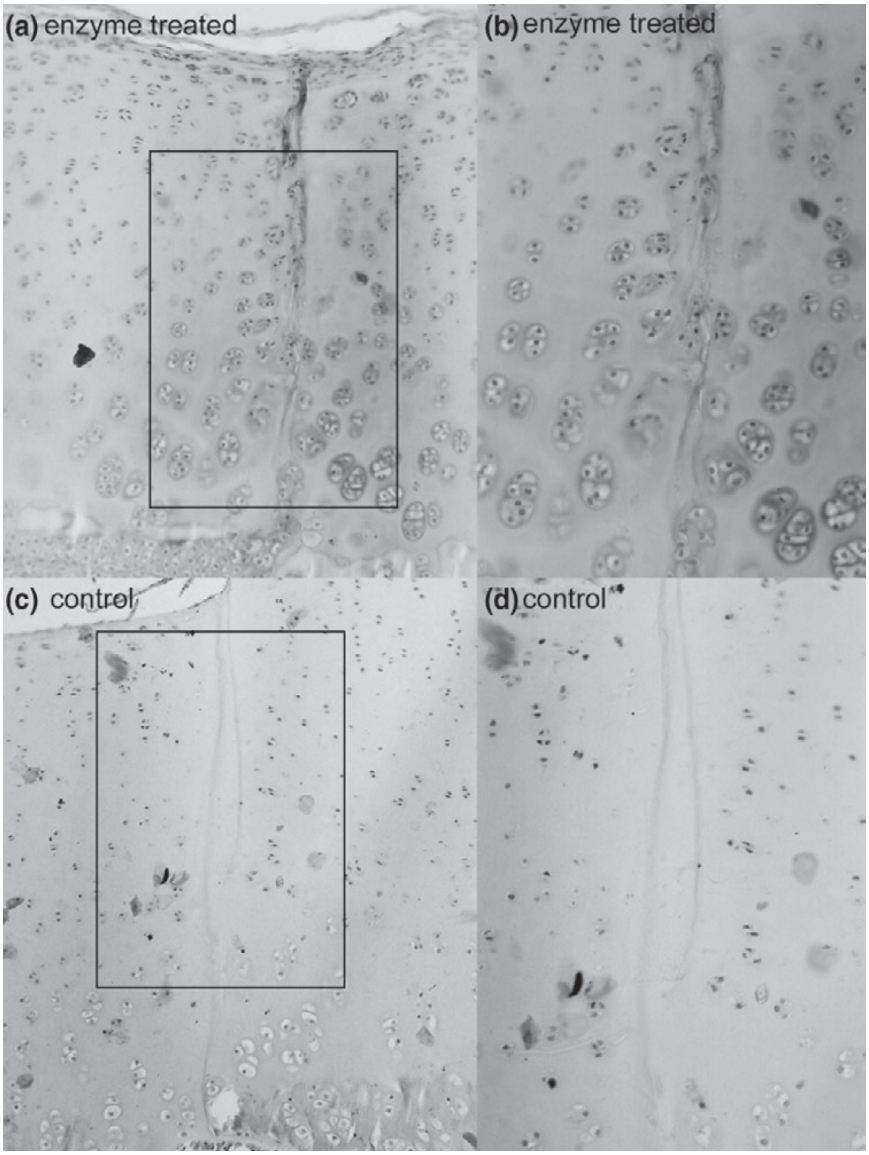

Figure 5.2 H&E - stained sections of enzyme-treated and untreated control constructs 5 weeks after implantation. **(a)** Enzyme treated construct that shows good integration, with cells located in the interfacial region [**(b)** enlargement]. **(c)** Untreated construct that shows a poor integration, with no cells present in the interfacial tissue [**(d)** enlargement]. Magnifications: panels a and c 100×; panels b and d 200×.

an abundance of cartilagespecific collagen type II was found in all interfacial matrices (Fig. 4b), whereas no collagen type III was found in any of the interface areas (Fig. 4c). No clear differences in immunohistochemical staining were observed between the two groups.

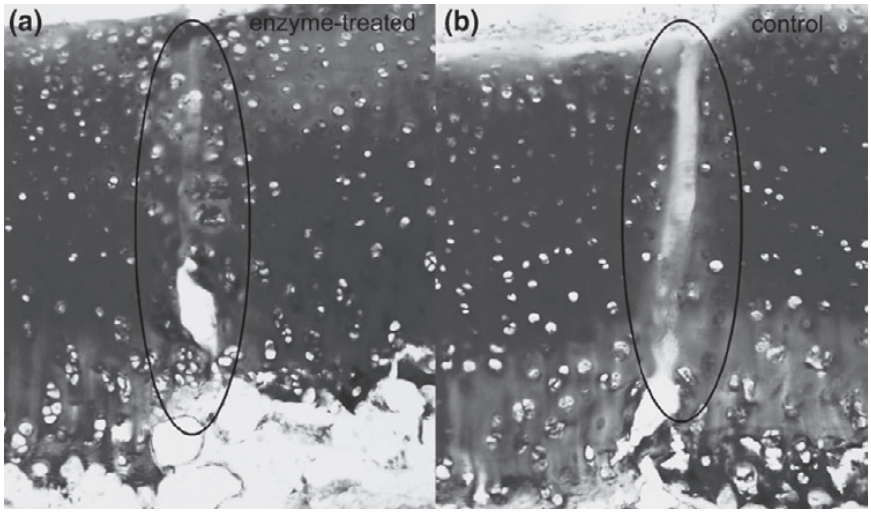

Figure 5.3 Thionin stained sections 5 weeks after implantation in nude mice. **(a)** Enzyme treated and **(b)** untreated control section. Note the clear difference in thionin staining of the interfacial tissue between the enzyme treated and control section. Interfaces are encircled.

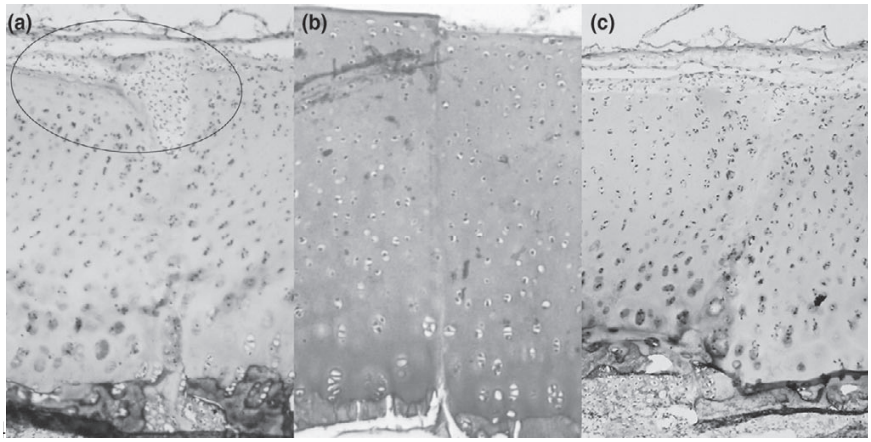

Figure 5.4 Immunohistochemical stainings for collagens present in the interfacial area of enzyme-treated constructs. **(a)** Collagen type I, with light staining (in red) in the area of fibrous ingrowth (circled). **(b)** Collagen type II, showing medium intensity staining (in red) in the entire matrix of the interfacial area. **(c)** Collagen type III; staining (in red) only present in the surrounding capsule. (See also: Colour figures section)

Polarized light microscopy of picro-Sirius Red stained sections indicated that collagen fibres in the wound interface were mainly directed perpendicular to the interface. Many fibres were seen crossing the interface in three out of five treated samples and in none of the control samples. Occasional fibre crossing was observed in two out of five treated samples and in three out of five control samples; in two out of five control samples no fibre crossing was observed. Most of the perpendicularly running fibres in the untreated control group

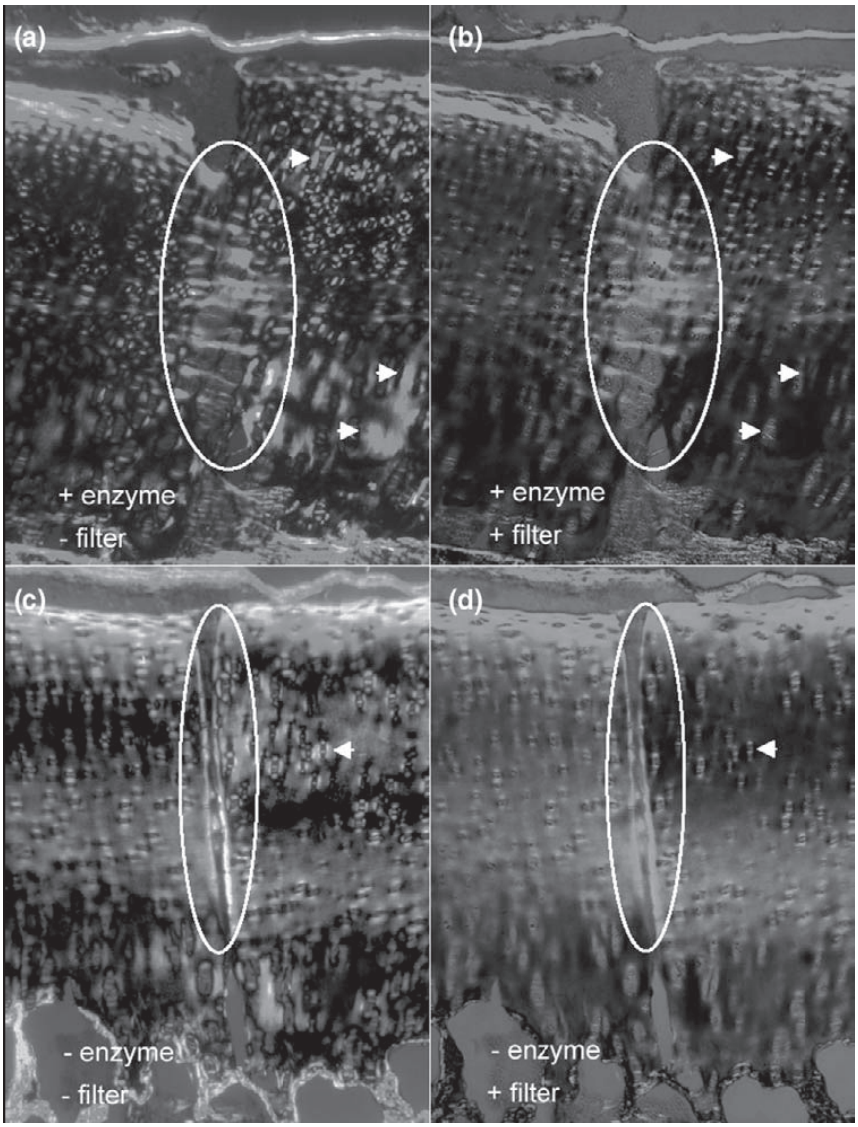

Figure 5.5 Picro-Sirius Red stained sections of the interface regions (circled). **(a, b)** Enzyme treated group, well integrated. **(c, d)** Untreated control group, not integrated. In panels a and c show crossed polarizing filters without analyzer filter; fibres run in parallel and perpendicular directions relative to the interface. Note the squares around individual chondrocytes, signifying pericellular collagen shell (arrowheads). In panels b and d the same field of view is shown as in panels a and c, but this time with the analyzer filter in place, revealing only those fibres that run in a perpendicular direction relative to the interface (circled), pointed out by the fact that the pericellular fibres that run in the parallel direction have disappeared (arrowheads), as well as the lightening up of the superficial cartilage layer. Clearly visible are the fibres crossing through the interface area, thus connecting both pieces of cartilage (panel b) and fibres along the wound edge projecting into the interface area (panel d). Original magnification: 25×.

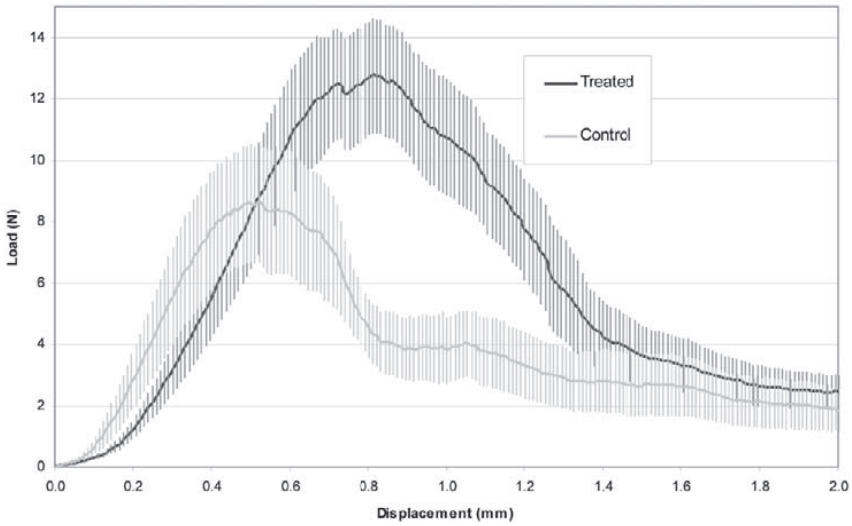

Figure 5.6 Average force-displacement curves of push out tests with standard error bars for untreated ($n = 5$) and enzyme-treated ($n = 6$) curves, respectively. Failure strength in the enzyme-treated group was significantly higher (+58%). The failure of the curve to return to zero can be explained by friction between pushed-out core and sample holder.

protruded only into the interface (Fig. 5). Statistical analyses showed a significant difference between groups ($P < 0.05$).

5.4.2. Mechanical testing

Mechanical assessment of the cartilage interface between inner core and outer ring by push-out test revealed that the interface connection was stronger in the treated group; the enzyme-treated group exhibited a 58% increase in stressto- failure over the untreated controls (1.32 ± 0.15 MPa versus 0.84 ± 0.14 MPa). Average force–displacement curves, including standard errors, are shown in Fig. 6.

Furthermore, the push-through strength of intact articular cartilage was 8.8 ± 0.52 MPa, with failure occurring in an annular manner, as with the integrated constructs. Pushout tests performed immediately after reinsertion of the core into the annulus revealed a maximum friction stress of 22.2 ± 9.4 kPa, which is only 1.7–2.6% of the stress measured in the integrated constructs.

5.5. DISCUSSION

In the present study we found an improvement in histological and biomechanical integration of articular cartilage after treatment with a combination of hyaluronidase and collagenase, a protocol that was previously shown to increase chondrocyte densities in wound edges *in*

vitro[1]. Our setup of a 3-mm disc placed in an annulus provides a reasonable representation of the *in vivo* situation, in which cartilage is transplanted into a defect with wound edges perpendicular to the surface. Because an *in vitro* culture system might not provide the optimal environment for tissue growth and repair[25], we decided to perform our experiments in the well established nude mouse model[26,31], creating an environment in which there is an ample supply of nutrients.

In this setup, cellularity in nontreated wound edges reached the levels of unwounded cartilage, which is in contrast to results from our previous *in vitro* study [1]. We believe that this is due to the nutrient-rich *in vivo* environment. However, in this model we confirmed[1] that the enzymatic treatment protocol enhanced the number of cells near the wound edges as compared with nontreatment, and resulted in better histological integration, as assessed by the percentage of matrix connection in the interfacial area. Furthermore, the repair tissue exhibited collagen fibres crossing the wound edges, and the matrix in both experimental groups exhibited cartilage specific collagen type II, limited (pro-)collagen type I and no collagen type III. This improved integration following enzymatic treatment was further supported by pushout tests, which are similar to tests described by others[14,25].

Although enzymatic treatment significantly increased mechanical strength to 1.32 MPa, the interfacial strength was still almost sevenfold less than the 8.8 MPa intrinsic failure strength values observed for intact cartilage. It should be appreciated that the fairly simple normalization to interface area is a rather crude method because the interface stress is not uniformly distributed. Therefore, tests using different sizes or shapes of specimens cannot readily be compared. Because the average thickness of our samples was 1.14 ± 0.28 mm for the treated group and 1.14 ± 0.21 mm for controls, and no correlation could be found between sample thickness and failure strength, we may compare strength values within the present study.

Our findings indicate a relation between interfacial strength and cellular activity at the interface. This confirms the results reported by DiMicco and coworkers[9], who used fetal, calf and adult bovine cartilage; after 14 days of culture those investigators found the highest failure stress in calf cartilage at 77 kPa in a single lap shear test. However, Reindel and coworkers[29] found an interface strength of 34 kPa after 3 weeks of culture, and showed that integrative strength was highly dependent on the use of fetal bovine serum in culture, which can influence cellular activity. Dependence of integration on active cell processes is also demonstrated by lack of adhesive strength when combining two lyophilized explant blocks[8]. In an 8-week bioreactor culture of tissue engineered cartilage core constructs with surrounding native cartilage, Obradovic and coworkers[25] found better mechanical integration of very young (5 days) constructs (254 kPa) as compared with more mature constructs (5 weeks; approximating 150 kPa). Peretti and coworkers[26] also used lyophilized explants, which were seeded with chondrocytes and then held together using fibrin glue and placed subcutaneously in nude mice for up to 6 weeks. Tensile testing showed a clear increase of failure strength to 77 kPa, which is 10 times higher than unseeded control explants, with failure always occurring at

the interface between new tissue and devitalized matrix. Because cellular activity is clearly an important factor in integration, we should appreciate that in most studies young bovine cartilage is used, which is more cellular than human cartilage. In a previous study, however, we did see similar effects of enzymatic treatment on cell density in human adult articular cartilage[1]. It can be anticipated that, because of the lower cell numbers, the overall repair process might be slower than in the present study but can still be stimulated using enzyme treatment.

Our findings suggest that enzymatic treatment may be a promising technique with which to improve cartilage integration, in addition to currently developing clinical and experimental articular cartilage repair techniques. The cell counts along the wound edge in the control group were comparable to those of native tissue. However, a close look at the histological pictures (Fig. 2c,2d) shows that a thin acellular band is still visible. In the enzyme-treated group cell counts were even higher than those in native cartilage, and histology did not reveal a large acellular band, as seen in the controls (Fig. 2a,2b), thus fulfilling one of the prerequisites for integration, namely the presence of active chondrocytes close to the lesion site. The high cellularity at the wound edge observed in the present study probably resulted in the increased collagen fibre deposition across the wound gap of adjacent cartilage surfaces, as shown in the picro-Sirius Red slides (Fig. 5). Normally, cross-gap deposition of collagen between native and repair tissue is insufficient in the reparative process that occurs after fullthickness defects[22,24,30]. The observed cross-gap deposition of collagen in the present study coincides with increased interfacial strength, as shown previously in integration experiments using fetal, calf and adult bovine cartilage explants[9]. Those studies showed a correlation between increased adhesive strength and an increased hydroxyproline incorporation in the interface area. Furthermore, inhibition of collagen cross-link formation by β-aminopropionitrile resulted in almost complete loss of integrative repair.

The explanation for the success of the enzymatic treatment technique may be found by examining wound healing in vascularized tissues. In nonvascularized articular cartilage, proteolytic enzyme activity is either lacking or insufficient to degrade and remove the observed acellular band in the wounded areas, as occurs with debris and necrotic tissue in vascularized tissues. Application of enzymes may remove this layer, uncovering an activated area of chondrocytes that are capable of integration. Another possible underlying mechanism of this enzymatic treatment may be the partial degradation of extracellular matrix surrounding the wound edge chondrocytes, which frees chondrocytes from the tight extracellular matrix in which they were entrapped. Because chondrocytes have been shown to have the ability to migrate[7], this may enable them to move to the wound edge in need of repair. A third possible mechanism of the enzymatic degradation of wound edge extracellular matrix may be the stimulation of local chondrocyte proliferation, which can be seen by looking at the cell clusters in the histological images (e.g. Fig. 2). Although we did observe cell division, the

exact mechanism by which the enzymatic treatment exerts its effects is still unclear. A more detailed mechanistic study is needed to further elucidate this.

In the present study we demonstrated the potential of hyaluronidase and collagenase treatment in a screening '*in vivo*' environment. Animal experiments with actual articular cartilage defects are needed to determine the value of our findings. Further studies must be undertaken to optimize the enzymatic treatment protocol (e.g. shorter treatment duration) and learn more about the mechanisms involved, such as cell migration to the wound area and matrix deposition, and to improve mechanical interface strength further to the level of intact cartilage, which is still almost an order of magnitude higher. Therefore, longer term studies are required to judge the success of different integration enhancing techniques against the mechanical strength of intact cartilage, and to develop protocols that may become clinically applicable, which in our view could be a valuable addition to existing repair strategies.

5.6. CONCLUSION

The present study shows that enzymatic treatment of cartilage wounds increases histological integration and improves biomechanical bonding strength. Enzymatic treatment may represent a promising addition to current techniques for articular cartilage repair.

5.7. ACKNOWLEDGEMENTS

Monoclonal antibodies II-6B3 and M38 were obtained from the Developmental Studies Hybridoma Bank, which is maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA and the Department of Biological Sciences, University of Iowa, Iowa City, Iowa, USA under contract N01-HD-6-2915 from the National Institute of Child Health and Human Development. The authors thank Nicole Kops for immunohistochemistry, Inge van Rensen for her work on cell counting, and Corrina de Ridder for her help in the nude mice experiments. Further thanks go to the company T. Boer & Zn., Nieuwerkerk a/d IJssel, The Netherlands for their kind supply of bovine joints.

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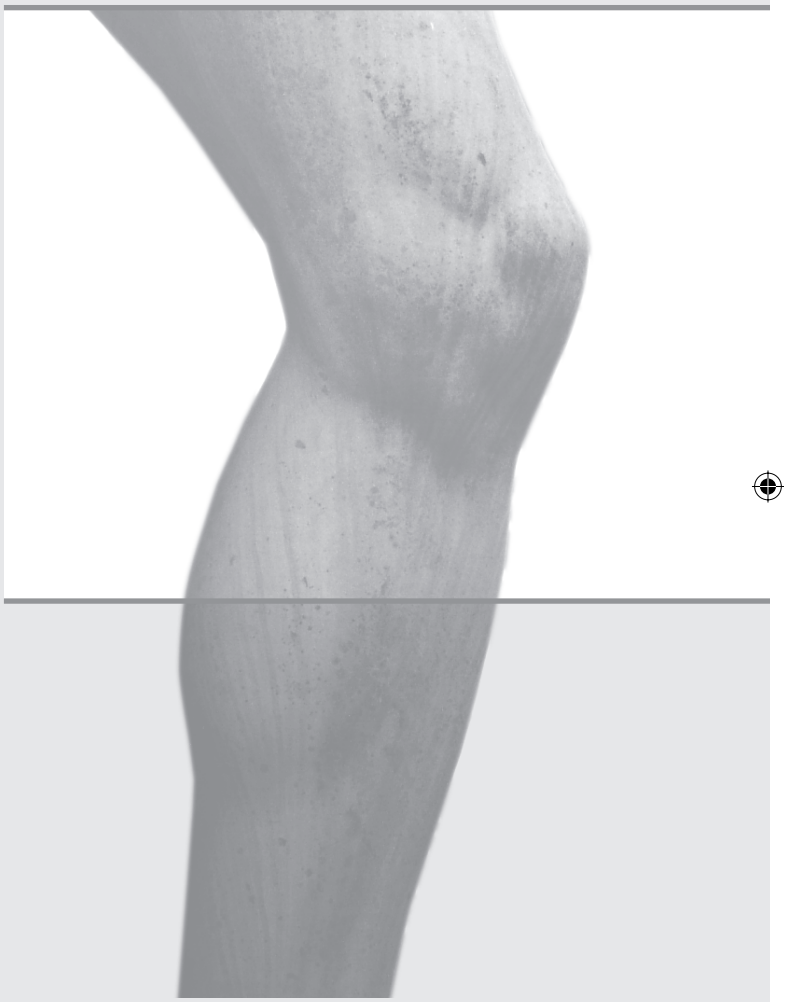
Chapter 6

Cellular origin of neocartilage formed at wound edges of articular cartilage in a tissue culture experiment



P.K. Bos[1], N. Kops[1], J.A.N. Verhaar[1], G.J.V.M. van Osch[1,2]

Department of Orthopaedics[1] and Department of Otorhinolaryngology[2], Erasmus MC, Rotterdam, the Netherlands

Submitted

6.1. SUMMARY

The regeneration capacity of cartilage in general is limited. Complete repair of partial thickness articular cartilage has only been reported in a fetal sheep model.

However, in long-term culture studies of articular cartilage explants we have observed outgrowth of chondrocytes and neocartilage formation at wound edges. This illustrates that under optimal circumstances articular cartilage is capable to regenerate hyaline cartilage. Recent studies suggest the presence of mesenchymal stem cells in articular cartilage. In the present study we investigated the origin of chondrocyte outgrowth and neocartilage formation at wound edges from immature and mature articular bovine cartilage explants *in vitro*, in order to understand which cells are responsible for repair.

Full-thickness explants from immature and mature animals were cultured for four weeks and superficial and deep zone cartilage explants of immature animals were separately cultured. Significant more outgrowth was observed from immature explants as compared to mature explants. At wound edges of immature explants, this outgrowth consisted of hyaline-like cartilage. We found proliferation activity both in the superficial zone and deep zone chondrocytes in immature explants, using the Ki67 proliferation marker. No expression was observed in the middle zone. In mature cartilage, only a few proliferating cells were seen, located throughout the explants. In the experiment culturing immature superficial and deep zone cartilage explants separately, there was abundant new tissue formation originating from deep cartilage and almost no outgrowth from the superficial cartilage. This indicates that neocartilage originates from chondrocytes in the deep zone cartilage and not from chondrocytes in the superficial zone cartilage.

Present data can help to understand wound healing in partial-thickness and full-thickness defects of immature and mature cartilage and can be of help in finding methods to stimulate the regeneration of articular cartilage.

6.2. INTRODUCTION

The regeneration capacity of cartilage in general is considered to be limited. Articular cartilage is avascular and chondrocytes are sitting isolated in a dense extracellular matrix and display a limited proliferation potential. Complete repair of partial articular cartilage injury has only been reported in one study on fetal sheep[18]. More mature cartilage does not repair in cases of partial-thickness cartilage defects.

For a long time this was considered to be due to the fact that cartilage is lacking progenitor cells and consists of one cell type (chondrocytes). It is now clear that articular chondrocytes do not form a uniform population; Differences in proliferation capacity and extracellular matrix (ECM) synthesis rates between chondrocytes from the superficial, middle and deep layer of articular cartilage have been shown. Also, distinct morphological differences can be observed between layers[4,5,15,25]. It is hypothesized that articular cartilage develops by appositional growth[13] and that the surface of (immature) articular cartilage contains progenitor cells[9]. Others have demonstrated an increased amount of mesenchymal stem cells in human OA cartilage as compared to healthy young donor cartilage, in both normal-appearing and lesional tissue (up to Mankin grade IV) [1,6,12,21].

In long-term culture experiments using young bovine articular cartilage we have observed outgrowth of cells from explants and neocartilage formation at the wound edges. It is of interest to find out which cells are responsible for the observed neocartilage formation at wound edges in this tissue culture experiment: either the suggested progenitor cell population in the superficial zone [9] or the suggested mesenchymal stem cells in healthy and OA articular cartilage [1]. Stimulation of these cells *in vivo* could supply chondrocytes to cartilage defects, needed to induce defect filling and cartilage-cartilage integration.

In the present study our research questions were: which cells are responsible for the observed outgrowth of chondrocytes and neocartilage formation in-vitro? Are these cells capable to form hyaline-like cartilage and is this capacity preserved throughout maturation? We investigated the outgrowth of neocartilage from immature and mature articular cartilage explants *in vitro*. Thionin staining and immunostaining for type II collagen were performed to characterize the newly formed tissue. The origin of chondrocytes responsible for outgrowth was investigated by determinating locations of proliferation with Ki67 immunostaining and by studying outgrowth from separately cultered superficial and deep cartilage explants.

6.3. MATERIALS & METHODS

6.3.1. Culture experiments

In the first experiment full thickness cartilage discs were harvested aseptically from the metacarpophalangeal joints of 6 months old calves (immature)(8 legs) and 30 month old

cows (mature)(8 legs), using 4mm circular biopsy punches (Stiefel, Imported by Bipharma, the Netherlands) and a sharp scalpel to cut the cartilage loose from the subchondral bone. A new biopsy punch and scalpel was used for every 4 to 6 explants for all experiments. Tissue sampling was conducted in accordance with the rules and regulations of the Erasmus MC animal ethics committee (DEC). Cartilage explants were cultured for 48 hours in separate wells in DMEM/Ham's F12 medium with 2% FCS (n=8 for immature cartilage, n=10 for mature cartilage). Explants were subsequently washed thoroughly and cultured for 4 weeks in DMEM/F12 medium with 10% FCS and 25µg/ml ascorbic acid. Medium was changed three times per week. Samples were harvested, 4% formalin fixed and embedded in paraffin for H&E, thionin and collagen II staining.

To identify the locations of chondrocyte proliferation in the explants full-thickness discs of both age groups were cultured for 3, 7 and 14 days (n=5 per condition). Discs were harvested, snap-frozen in liquid nitrogen and embedded in Tissue-Tek (Sakura Finetek Europe bv, The Netherlands) for cryosectionning and Ki67 immunostaining.

A second culture experiment was performed to investigate the origin of chondrocytes responsible for the outgrowth of chondrocytes and neocartilage formation. Cartilage explants from different depths were harvested using a technique similar to techniques described by others [3,4]. Parallel to the surface the superficial layer was cut with a sharp scalpel (depth: approximately one third of full-thickness), this was followed by a full-thickness circular cut with a 4mm dermal biopsy punch, extending through the deep zone cartilage, not into the subchondral bone. Again with a scalpel the remaining middle and deep layers were cut from the subchondral bone, parallel to the surface (approximately two thirds of full-thickness). Because the metacarpophalangeal joint cartilage of 30 month old cows is too thin to obtain reliable separation of superficial and deep cartilage, this experiment was performed on 6 month old bovine cartilage only.

Both superficial and deep cartilage explants were harvested and cultured in separate wells in DMEM/F12 medium with 10% FCS and 25µg/ml ascorbic acid. Discs were harvested after 7 days, 2 weeks and 4 weeks (n=10 per condition), fixed in 4% formalin and embedded in paraffin for H&E and thionin staining.

6.3.2. Histology

For evaluation of proteoglycan content paraffin sections were deparaffinized in xylene, rehydrated through graded ethanol and stained in 0.04% thionin in 0.01 M aqueous sodium acetate, pH 4.5 for 5 minutes. For histological evaluation serial sections were stained with Haematoxylin & Eosin (H&E). Outgrowth was measured in serial sections at a magnification of 200x, using a Sony 3CCD Color video camera, Leica light microscope and Leica Qwin imaging software. Outgrowth at wound edges was semiquantified by measuring the area of outgrowth at the explant wound edges. The area (μm^2) was devided by the height (µm) of the lesion edge. Results are expressed as outgrowth in $\mu m^2/\mu m \pm SD$.

6.3.3. Collagen type II immunostaining

The sections were deparaffinized in xylene and rehydrated through graded ethanol. Sections were incubated with 0.2% pronase (Sigma, St Louis, MO) for antigen retrieval and 1% hyaluronidase (Sigma) for better antibody penetration. The sections were incubated for 2 hours at room temperature with monoclonal II-II 6B3 antibody (1:100; Developmental Studies Hybridoma Bank, Iowa City, IA, under contract N01-HD-6-2915 from the NICHD). Control was performed by using an isotype IgG1 monoclonal antibody. Alkaline phosphatase labeled secondary antibody was used. Alkaline phosphatase activity was demonstrated by incubation with a New Fuchsine substrate (Chroma, Kongen, Germany), resulting in a red colored signal. The slides were counterstained with haematoxylin.

6.3.4. Von Willebrand Factor immunostaining

To determine whether bloodvessels are present in immature bovine explants, immunostaining for von Willebrand Factor was performed on formalin fixed and paraffin embedded tissue. The sections were deparaffinized in xylene and rehydrated through graded ethanol. Unspecific reactions were blocked with 10% normal goat serum. Sections were incubated with von Willebrand Factor monoclonal antibody in 2% serum (1:30; VW1-2, QED bioscience Inc., San Diego, USA) for one hour at room temperature. Isotype control was performed by using Mouse isotype IgG1 monoclonal antibody (1:30) (DakoCytomation, X0931). Alkaline phosphatase conjugated goat anti-mouse IgG (Immunotech, IM0818) and alkaline phosphatase (APAAP, DakoCytomation, D0651) were subsequently added for 30 minutes. Visualisation was performed with an alkaline phosphatase substrate.

6.3.5. Ki67 immunostaining

To determine locations of chondrocyte proliferation in the explants, immunohistochemical staining for expression of Ki67 was performed on frozen tissue. 6 µm cryosections were cut and mounted on slides. Sections were fixed in acetone 100%, rinsed in steps with Phosphate buffered saline (PBS) followed by 1% H_2O_2 in methanol and PBS. Preincubated with block buffer and 10% serum, followed by incubation with primary monoclonal antibody for Ki67 in 2% serum (1:25; Sigma, St Louis, MO) for two hours at room temperature. Isotype control was performed by using Mouse isotype IgG1 monoclonal antibody (1:25). The secondary antibody was labeled using streptavidin-alkaline phosphatase (Super Sensitive Concentrated Detection Kit, Bio Genex, San Ramon 94583, USA). Alkaline phosphatase activity was demonstrated by incubation with NovaRED (Vector, NovaRED substrate kit, cat no. SK-4800). Slides were not counterstained.

6.3.6. Data analysis

Differences between groups were calculated using Mann-Whitney U test, $p<0.05$ was considered statistically significant (*), $p<0.001$ highly significant (**).

6.4. RESULTS

6.4.1. Tissue cultures

In the initial experiment, using cartilage explants of 6 month old calves cultured for 4 weeks, we observed outgrowth and neocartilage formation at the wound edges of 75% (6/8) of the explants after 4 weeks of tissue culture. Neocartilage formation at wound edges of explants was 43.06 ± 34.40 $\mu m^2/\mu m$ (fig.1). The newly formed tissue showed a high cell density. The cells had a round shape and the extracellular matrix contained proteoglycans, indicated by an intense thionin positive staining (fig.2a). Immature cartilage explants showed positive staining of the newly formed tissue for collagen type II, indicating that the outgrown tissue contains hyaline cartilage properties (fig.2b).

In the same experiment, using articular cartilage explants of 30 month old cows, little outgrowth was observed after 28 days as compared to outgrowth from immature cartilage explants (fig.1). At wound edges of the explants very little outgrowth was observed (5.12± 6.29 $\mu m^2/\mu m$) after 28 days of culture. The outgrowth was significant smaller at wound edges of mature compared to immature cartilage explants ($p<0.001$). Immunohistochemical staining of tissue outgrowth from mature cartilage explants was negative for collagen type II (fig.2d).

6.4.2. Proliferating cells

Ki67 immunostaining performed on cryosections of explants cultured for 3, 7 and 14 days showed positive staining in superficial zone chondrocytes and in deep zone chondrocytes in immature explants. This was a consistent observation. In none of the sections chondrocytes in the middle zone chondrocytes showed immunoreactivity (fig.3). In mature cartilage explants

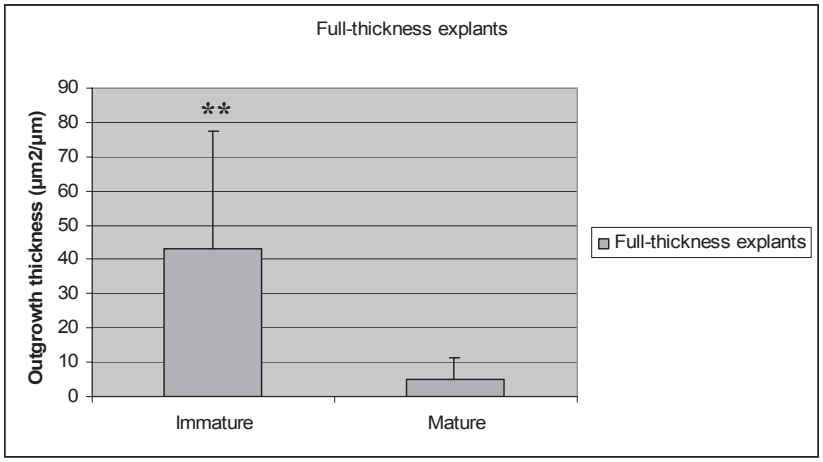


Figure 6.1 Graph representing outgrowth of tissue from immature and mature bovine full-thickness articular cartilage explants cultured for 4 weeks. Outgrowth at wound edges was semiquantified and expressed as area of outgrowth devided by height of explants at wound edges ($\mu m^2/\mu m$).

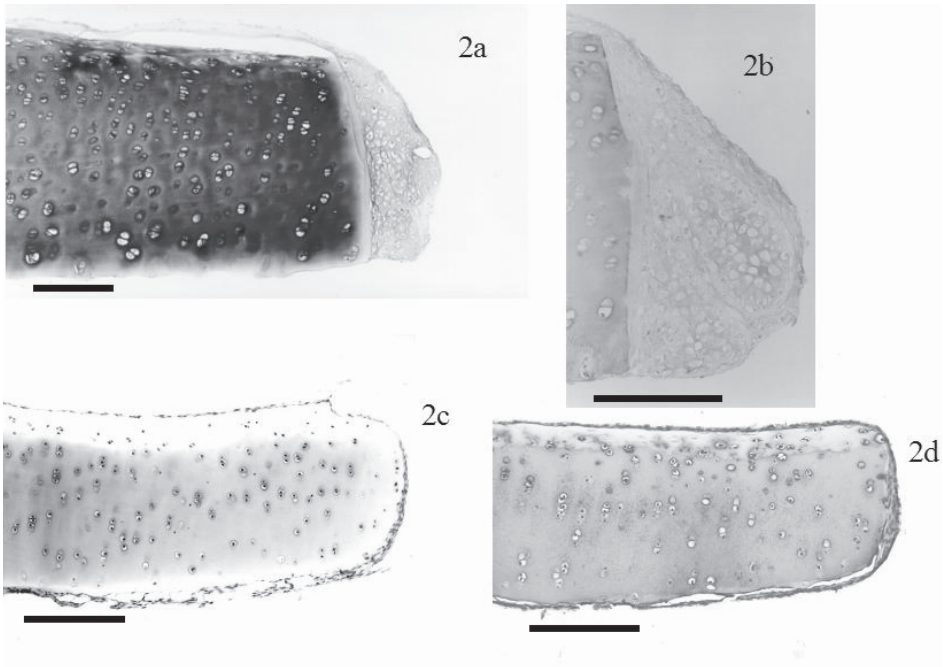


Figure 6.2 Wound edge of full-thickness explant.
Immature **(a&b)** and mature **(c&d)** bovine articular cartilage with newly formed cartilage covering the lesion edge after 4 weeks in culture. **(2a&c)** Thionin staining; **(2b&d)** Immunohistochemical staining for collagen type II; Bar =200µm. (See also: Colour figures section)

no pattern of positive immunoreaction was observed in the explants after 3, 7 and 14 days. Sporadic immunopostive cells were observed throughout the explants. A positive staining with Ki67 was observed in newly formed tissue cells in both immature and mature explants.

Haematoxylin & Eosin staining and immunostaining for von Willebrand factor did not reveal any tube-like structures throughout cartilage explants. Blood vessels were absent throughout the explants, indicating no vascular contribution to the tissue outgrowth.

6.4.3. Cellular origin

In the second culture experiment, using immature cartilage explants, we cultured the superficial and deep zone explants separately. After 7 days in culture we observed very little outgrowth of chondrocytes from the superficial explants and more outgrowth of chondrocytes from the deep cartilage explants (1.41±2.36 and 4.05±5.59, not significant). After 14 and 28 days of culture, significant more outgrowth ($p<0.001$) was observed from deep cartilage explants (Fig.4).

Neocartilage formation often completely surrounding the explants was observed in 7/10 deep cartilage explants after 14 days and in all deep cartilage explants after 28 days of culture (fig.5).

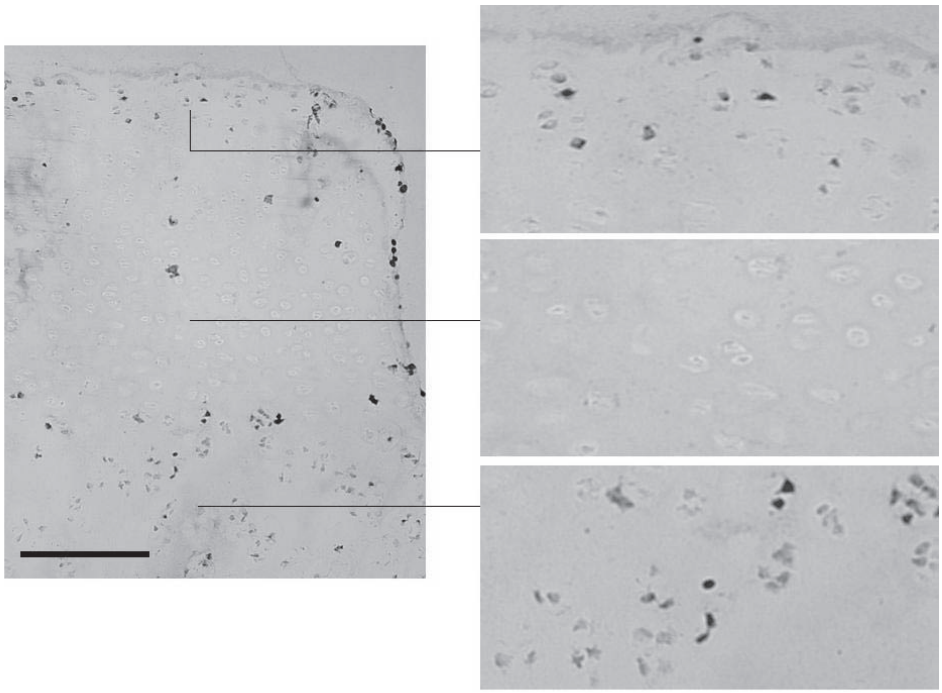


Figure 6.3 The location of cell proliferation by immunohistochemical staining for proliferation marker Ki67. In full-thickness immature explants a positive immunoreaction was observed in superficial zone and deep zone chondrocytes, as well as in cells covering the lesion edge. No proliferation activity was observed in the middle zone chondrocytes. Bar =200µm. (See also: Colour figures section)

Significant more outgrowth was observed at lesion edges of deep cartilage explants as compared to full thickness explants in immature tissue (figs.1 and 4, p=0.001), possibly explained by larger wound surfaces or more damage sustained to the tissue.

As a sign of proliferation capacity, in immature cartilage explants we observed a positive Ki67 immunoreactivity in both superficial and deep zone cartilage throughout the culture experiments (after 3, 7 and 14 days). Cluster formation, was observed at wound edges of superficial explants in 2/10 after 14 days and 7/10 after 28 days. Cluster formation was observed only once in a deep cartilage explant after 14 days. This may indicate that superficial zone chondrocytes are capable to proliferate following injury but the amount of cells that grow out and the location of cell division is not sufficient to form new cartilage tissue.

6.5. DISCUSSION

We have shown in a bovine articular cartilage culture experiment that chondrocytes are able to grow out of explants cultured for four weeks and form new cartilage with hyaline-like char-

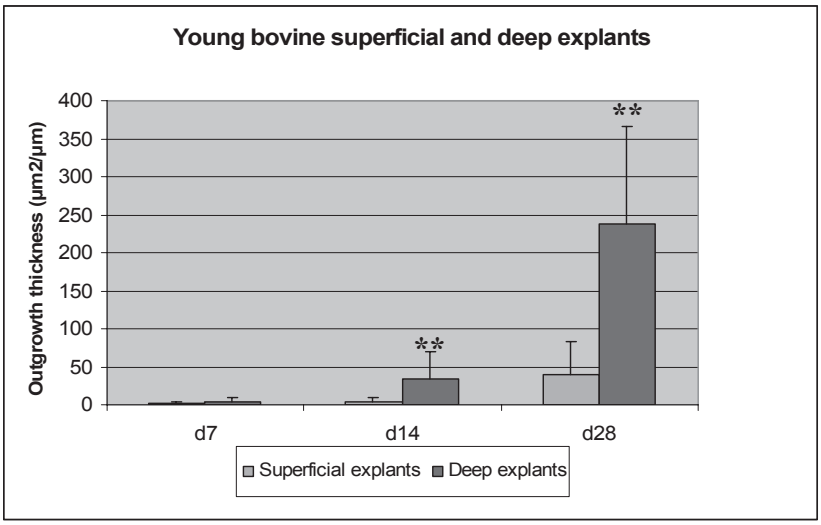


Figure 6.4 Graph representing tissue formation at wound edges and surrounding immature bovine articular cartilage explants cultured for 4 weeks.
Superficial and deep cartilage was explanted separately before culture. After 14 days very little outgrowth was observed at lesion edges of superficial cartilage, whereas at lesion edges of deep cartilage a significant larger amount of neocartilage is formed. Significant more outgrowth was observed after 28 days.

acteristics. The new tissue formation starts at the wound edges. In an attempt to identify the cell source responsible for outgrowth and neocartilage formation we have shown proliferation activity both in the superficial zone and deep zone chondrocytes in immature explants. Cartilage from the middle zone did not show proliferation activity. This is in accordance with studies using tritiated thymidine injection in neonate rabbits, that demonstrated two proliferative zones in the joints, one above the subchondral bone and one just beneath the articular surface[26,27]. A second experiment was performed and showed that the neocartilage originates from chondrocytes in the lower zones of the cartilage and not from the superficial zone cartilage. Abundant new tissue formation was observed originating from deep cartilage and almost no outgrowth occurred from the superficial cartilage. Furthermore, new formed tissue from the deeper cartilage zones shows hyaline-like properties (immunopositive for collagen type II and positive proteoglycan staining). Whereas, the few cells observed at wound edges of mature explants show fibrous tissue characteristics (immunonegative for collagen type II and less intense proteoglycan staining).

Outgrowth of chondrocytes from explant wound edges *in vitro* with formation of monolayers has been described before[16,19]. However, the origin of chondrocytes responsible for the outgrowth and the formation of neocartilage with hyaline-like cartilage properties has not been demonstrated before. Healing of cartilage lesions with fibrocartilage only appears to occur when progenitor cells from the bone marrow enter an articular cartilage lesion after

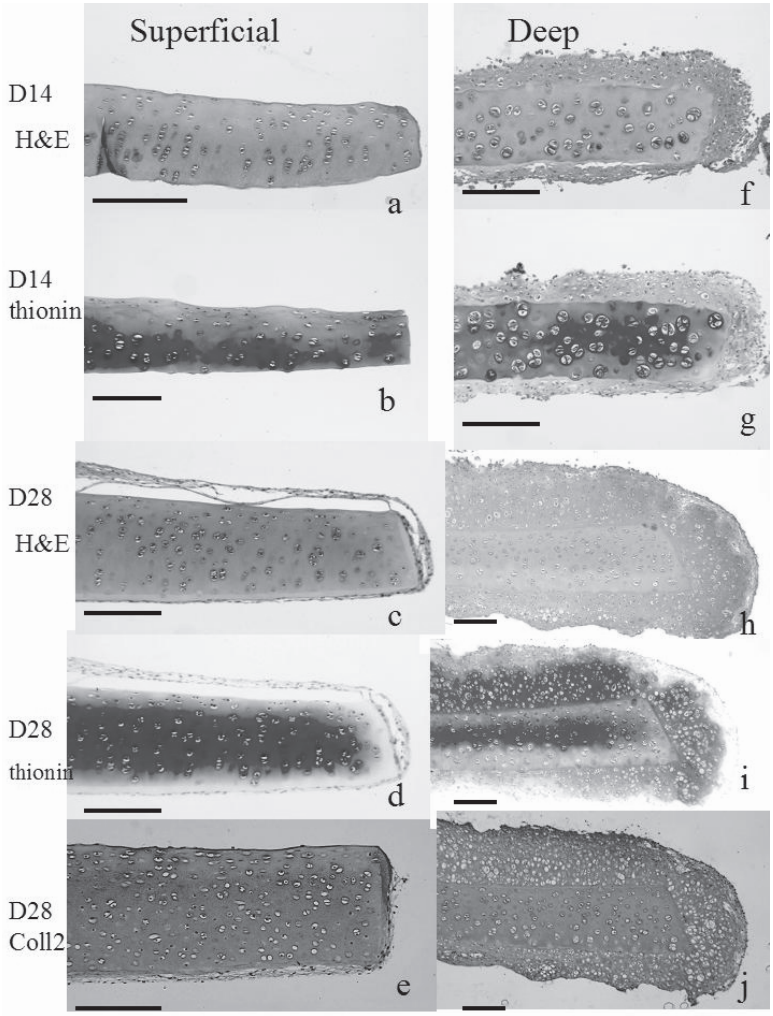


Figure 6.5 Wound edges of superficial and deep explants of immature bovine articular cartilage. **(a)** superficial cartilage, 14 days of culture, H&E; **(b)** superficial cartilage, 14 days of culture, thionin; **(c)** superficial cartilage, 28 days of culture, H&E; **(d)** superficial cartilage, 28 days of culture, thionin; **(e)** superficial cartilage, 28 days of culture, collagen typeII immunostaining; **(f)** deep cartilage, 14 days of culture, H&E; **(g)** deep cartilage, 14 days of culture, thionin; **(h)** deep cartilage, 28 days, H&E; **(i)** deep cartilage, 28 days, thionin; **(j)** deep cartilage, 28 days, collagen typeII immunostaining. Bar =200µm. (See also: Colour figures section)

penetration of the subchondral bone[20]. In our culture set-up using full-thickness articular cartilage explants from 6 month old calves or 30 month old cows progenitor cells from the bone marrow are not present. Another cell source suggested by Hunziker to be able to repair articular cartilage defects is the synovial lining[14]. This type of cells is also not present in our culture set-up. We therefore were initially surprised that the cultured cartilage explants

expressed formation of new hyaline-like cartilage tissue attached to the explant in the initial culture experiment (Fig.2). The newly formed tissue showed a high cell density. The cells had a round shape and the extracellular matrix contained proteoglycans and collagen type II.

The neocartilage formation was surprising and at first the similarity with previous culture experiment of ear cartilage with perichondrium occurred to us. Despite the absence of repair or regeneration of damaged articular cartilage by chondrocytes, cartilage structures in the head and neck region are known to show some healing capacity. The neocartilage formed here originates from proliferation of progenitor cells in the inner layer (cambium layer) of perichondrium[10,11]. Articular cartilage is not lined by perichondrium and repair of articular cartilage injury by the chondrocytes generally is considered not to happen. However, recently it has been suggested that the superficial zone cartilage plays an important role in morphogenesis of diarthrodial joints[2]. Furthermore, in enzymatically isolated chondrocytes of 7 day old calves the presence of a progenitor cell population in the superficial zone has been postulated[9]. These cells may form an unlimited cell source and may therefore be the ideal cell population for expansion in culture to obtain the high amounts of cells needed for tissue engineering graft development. However, for techniques aiming at in situ induction of repair tissue (neocartilage formation in defects or cartilage wound healing) the question remains which cells are capable to grow out of whole tissue explants (or lesion edges *in vivo*), form new cartilage tissue and retain the ability to form collagen type II and proteoglycans.

In a recent study the presence of mesenchymal progenitor cells in normal and osteoarthritic adult human articular cartilage was shown by analyzing isolated chondrocytes for the expression of cell surface markers known to define mesenchymal stem cells in bone marrow and perichondrium. Even in severe OA cartilage lesional tissue (Mankin grade IV, where most of the superficial zone is damaged or disappeared[17]) up to 15% mesenchymal progenitor cells were identified[1].

We suggest that deep zone articular cartilage in young individuals contain chondrocytes capable of proliferation while remaining their cartilage phenotype. These cells are able to form new cartilage tissue.

Young and adolescent animals with open physes have a greater spontaneous healing response and earlier specialization of repair-tissue in full-thickness defects than do mature animals[23]. It is suggested that the improved repair and durability of repair tissue in young subjects is the result of superior initial healing response[24]. Using autoradiography after labelling with 3H-thymidine and 3H-cytidine it is demonstrated that chondrocytes from cartilage bordering defects did not participate in the repopulation of full-thickness osteochondral cartilage defects *in vivo*. It is suggested that repair originates wholly by proliferation and differentiation of mesenchymal cells of the marrow[20]. Shapiro et al observed no label in chondrocytes immediately adjacent to cartilage defects at periods of one to ten days. This may be explained by cell death observed in cartilage wound edges[7,8,22]. However, in all of the other chondrocytes, normal and repair cartilage, incorporated label was demonstrated.

From the present study we suggest that *in vivo*, in the younger subjects, a part of the repair can originate from proliferation of chondrocytes in the cartilage bordering full-thickness defects. Repair of partial thickness wounds in fetal animals may also be explained by this repair process.

The fact that spontaneous repair of partial-thickness articular defects in non-fetal cartilage is never seen *in vivo* can have several causes. First, the progenitor cell properties can be lost due to further maturation or due to disappearing of the deeper zone at the moment of secondary ossification in the epiphysis. In our experiment with mature cartilage explants, the loss of proliferation capacity in mature cartilage was confirmed by immunonegative Ki 67 proliferation marker staining.

On the other hand, it may very well be that the chondrocytes lack a certain stimulus to induce proliferation and differentiation. The nutritional and biomechanical circumstances in a moving joint *in vivo* may not allow the formation of neocartilage in-vivo, whereas in-vitro the circumstances are more optimal to induce proliferation and differentiation. Pro-inflammatory factors such as IL1, IL6 and TNFa or cytokines present in synovial joints following joint trauma, synovitis and effusion, may limit proliferation and tissue formation *in vivo*.

Present data can help to understand cartilage defect filling and wound healing in partial-thickness and full-thickness defects observed in immature animals.

Although it remains questionable whether our results are of value for adult subjects with isolated articular cartilage defects, we have shown that articular cartilage is able to regenerate hyaline cartilage tissue. With proper stimulation, chondrocytes in young adults may be stimulated to express such regenerative response.

Further research has to reveal the answers to the questions mentioned above and to the question how this knowledge can be implemented in current strategies to stimulate articular cartilage repair. It also remains to be seen if in OA cartilage, where the superficial cartilage is cracked, grooved or completely disappeard, chondrocytes from remaining deeper layers can be stimulated to proliferate and form new tissue.

6.6. CONCLUSION

In the present study we demonstrate the capacity of chondrocytes in immature explants to grow out after experimental wounding and form new tissue with hyaline-like properties. Chondrocytes from deep zone cartilage in these explants are able to proliferate and form abundant amounts of new tissue. Repair of partial-thickness defects in immature animals *in vivo* may be explained by these observations. In full-thickness articular cartilage defects and in patients treated with debridement and subchondral penetration repair is considered to originate from bone marrow mesenchymal stem cells alone. The present study suggests that a part of the repair may originate from cells grown out of wound edges. Further study

on outgrowth of these cells and stimulation of this process may help to improve cartilage wound healing and integrative cartilage repair.

6.7. ACKNOWLEDGEMENTS

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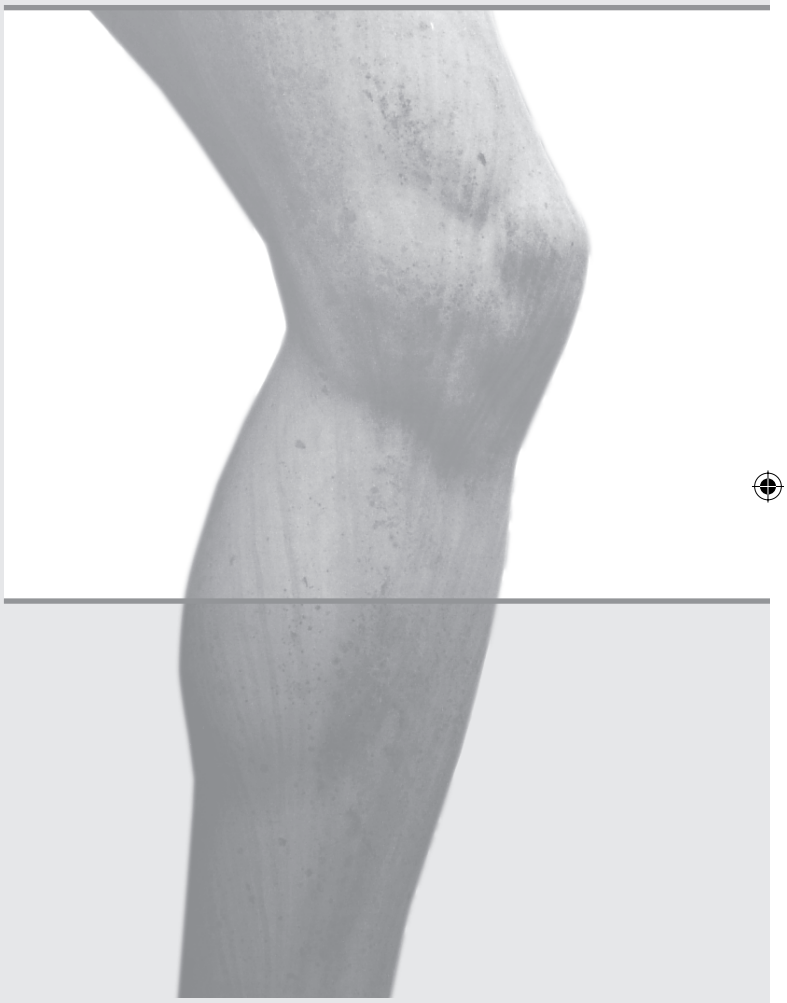
Chapter 7

General Discussion



GENERAL DISCUSSION

The general aim of this thesis was to study the cellular and molecular reactions of cartilage tissue to experimental wounding and to use this knowledge to develop methods to improve articular cartilage integration.

Articular cartilage wound healing or integrative cartilage repair is an important prerequisite for successful treatment of cartilage defects. Isolated articular cartilage lesions cause symptoms such as pain, effusion, locking phenomena and disturbed function mainly in young, healthy subjects. Articular cartilage defect repair must relieve symptoms and prevent further joint degeneration.

As described in the introduction, poor integrative repair with host cartilage has been shown by many authors. Successful cartilage repair techniques depend on an optimal integration of transplanted or in situ generated new cartilage tissue with the surrounding (healthy) cartilage and subchondral bone[1,2]. Osteochondral transplantation or other techniques that require opening of the subchondral bone and transplanting tissue into subchondral bone and cartilage result in a successful and strong bonding of bone, tissue engineered construct or repair tissue at bone level. This is comparable to bone healing in fracture repair. However, as described in the introduction chapter, the composition of articular cartilage with its avascular status, low cell density, tight ECM hampers a successful healing at the cartilage level.

Other techniques designed for repair of partial-thickness defects not extending through the subchondral bone, as published in literature and presented during live surgery and video-presentations (at the International Cartilage Repair Society meetings and recent Orthopaedic Research Society meetings) require complete debridement of the defects up to the subchondral bone before transplantation[23]. This practically always results in bleeding from the subchondral bone to a certain extent, which means that there is access to the vascular system and perhaps to the subchondral bone and mesenchymal stem cells. Furthermore, spontaneously formed blood clots generally adhere well to bone surface, but only rarely to articular cartilage surface and to the defect floor and walls[12].

Transplanted tissue or in situ formed new tissue must bond to the subchondral bone and, at the cartilage level, to the surrounding cartilage. Bonding to the subchondral bone in these repair techniques appears not to form a clinical problem. However, the integration of cartilage is less predictable. Insufficient integration may lead to biomechanical failure and tissue degeneration at interface regions.

In our first study we have used the New Zealand White Rabbit *auricular cartilage* as a model to study the natural reaction of cartilage tissue to experimental wounding in a time study **(chapter 2)**.

We have shown the effects of injury on extracellular matrix and chondrocytes. The suggested relation between growth factor expression and proteoglycan depletion during wound healing and the subsequent replenishment of proteoglycans indicate that cartilage possesses some intrinsic repair capacity. The temporal expression of endogenous growth factors in reaction to acute cartilage injury suggests an autocrine and/or paracrine stimulation of chondrocyte metabolism. Chondrocyte death was observed in the area directly bordering the cartilage cutting line. Dead cells in this area are not replaced. The acellular, inactive zone remains and hampers integrative cartilage repair.

Considering these observations we investigated the early wound healing reactions of immature and mature *knee articular cartilage* in New Zealand White rabbits *in vivo* **(Chapter 3).** Furthermore, the potential of isolated chondrocytes from these animals to proliferate and produce ECM with and without TGFβ1 stimulation was tested *in vitro*. We showed an early repair response in immature cartilage and almost no repair response in mature cartilage defects *in vivo*. Chondrocyte death was induced in wound edges of both immature and mature cartilage, as observed in the auricular wound healing study **(chapter 2).** Immature cartilage repair was indicated by chondrocyte proliferation in clusters and decrease in defect size. An increased expression of TGFβ1, FGF2 and IGF1 immunostaining was observed throughout the entire cartilage sections of immature and mature cartilage, with the exception of the lesion edges. The immunonegative cartilage area appeared to correlate with chondrocyte death in wound edges. No decrease in growth factor immunoexpression was observed during this short term wound healing study.

In a subsequent alginate culture experiment we have confirmed that immature chondrocytes possess better proliferation capacity. However, addition of recombinant TGFβ1 increased proliferation rate and GAG production of mature, but not immature chondrocytes.

In conclusion, our studies confirmed that immature cartilage has a higher intrinsic proliferative potential and is able to decrease the size of articular cartilage defects *in vivo*. Furthermore, we showed that with addition of a potent growth factor proliferation and subsequent ECM production of mature chondrocytes can be restored *in vitro*.

The observed cell death in the studies presented in chapters 2 and 3 is in accordance with results of others[14,24,28]. The loss of cells in lesion edges is now considered to be an important factor influencing cartilage wound healing. The previously termed zone of necrosis, extending approximately 150-200μm from the wound edge has been shown to consist of both necrosis at the mechanically injured wound edge and apoptosis extending into the tissue[28]. Behind this zone of dead chondrocytes and empty lacunae there is zone of cellular proliferation and altered ECM metabolism.

Chondrocyte death in wound edges can be of multifactor cause: for example direct mechanical injury to the ECM and cells, inflammatory reaction following injury, apoptosis. Use of

sharp surgical instruments[24], local or systemic inflammation inhibition, apoptosis inhibition[28], timing of defect treatment, construct development/cell-density[22], local ECM digestion[7] may be used to reduce this cell loss or increase local cell density in lesion edges.

The acellularity can be due to a combination of chondrocyte loss from lesion edges, avascularity, the absence of multipotent progenitor cells, absence of necrotic tissue removal and the inability of chondrocytes to migrate through the tight ECM.

Low chondrocyte density in adult articular cartilage itself, combined with cell death in lesion edges leads to a relative absence of matrix producing cells in the cartilage-cartilage interface where repair tissue is needed. A temporary increase of chondrocytes, with the capacity to rapidly produce ECM is probably needed to fill wounds and defects with sufficient amounts of repair tissue. Silverman et al.[26] have studied the adhesion between native cartilage discs using fibrin glue polymer alone or mixed with fresh articular chondrocytes. This study demonstrated that adherence of cartilage to cartilage can be improved by an increased amount of chondrocytes in the interface region. An *in vitro* bioreactor study by Obradovic et al.[21] demonstrated an increased integration of native cartilage with immature 'tissue engineered' cartilage constructs (5-days) compared to more matured 'tissue engineered' cartilage constructs (5-weeks). In their study, an increased cellularity was observed in the interface region using immature constructs compared to mature constructs, leading to an improved histological and biomechanical integration. In our enzyme treatment studies, we have also shown an increased histological integration with increased cellularity in the interface region of cartilage explants **(chapters 4 and 5)**.

Another method to increase local ECM production besides increasing the local cell density is by stimulation of cells to produce more collagens and proteoglycans. In **chapter 3** we have shown that adult articular chondrocytes can be stimulated to produce more ECM, using the potent growth factor TGFβ1. In this study immature chondrocytes, that already produced significant more ECM than mature chondrocytes during culture, could not be further stimulated. In this study we showed that adult chondrocytes can be activated by a potent stimulating factor.

Several investigators have studied the effects of superficial enzymatic treatment of cartilage surfaces in order to achieve a local and temporal decrease in antiadhesive properties of matrix proteoglycans[13,17-19,22]. Thereby promoting adhesion of cells and tissue and possibly facilitate the circumstances for cross-linking of ECM across the interface. In pilot articular cartilage explant culture experiments, using chondroitinase ABC, hyaluronidase and highly purified collagenase, we observed differences in ECM composition during long-term culture follow up, as expected. However, also a distinct difference in chondrocyte density was observed following treatment with collagenase as compared to proteoglycan digesting enzymes. In **Chapter 4** we demonstrated that treatment of articular cartilage explants with highly puri-

fied collagenase resulted in a significant increase in chondrocyte density at the wound edges. Furthermore, in an integration experiment, we showed that this "vitalization" of wound edges improves histological integration. We suggest that an increase in cell density in wound edges explains the observed improved cartilage integration/wound healing. An increased biomechanical bonding strength was confirmed using a push out test. We suggest that there is a relation between increased interfacial strength and cellular activity at the cartilage-cartilage interface (**chapter 5**).

Differences in proliferation capacity, ECM synthesis rates and distinct morphological differences between chondrocytes from the superficial, middle and deep layer of articular cartilage have been shown[4,5,16,29].

Articular chondrocytes do not form a uniform population and the presence of progenitor cells is suggested[3,6,9,10,27]. It was hypothesized that articular cartilage develops by appositional growth[11] and that progenitor cells are present in the surface of (immature) articular cartilage[9]. Others have demonstrated an increased amount of mesenchymal stem cells in human OA cartilage as compared to healthy young donor cartilage, not limited to the superficial zone[3,6,10,27].

In our long-term culture experiments using young bovine articular cartilage we have observed outgrowth of cells from explants and neocartilage formation with hyaline cartilage properties at the wound edges. Thus under optimal, *in vitro* circumstances articular cartilage is able to spontaneously regenerate hyaline-like cartilage. We were interested to find out which cells are responsible for this process. Stimulation of these cells *in vivo* could supply the chondrocytes needed to induce defect filling and cartilage-cartilage integration.

In **chapter 6**, we present a bovine articular cartilage culture experiment. We showed that chondrocytes are able to grow out of explants cultured for four weeks and form new cartilage with hyaline-like characteristics. Proliferation activity, demonstrated by a positive Ki67 immunoreactivity, was observed in both superficial and deep zone cartilage of immature cartilage explants. A second experiment, in which superficial and deep cartilage explants were cultured separately, showed that the neocartilage originates from chondrocytes in the lower zones of the cartilage and not from the superficial zone cartilage. In conclusion, we found that deep zone articular cartilage in young individuals contain chondrocytes capable of proliferation and new cartilage tissue formation *in vitro*. Superficial zone cartilage may contain progenitor cells suitable for tissue engineering, when the cells are released from their matrix[9]. In our experimental setup however, these cells were not able to grow out and form new cartilage tissue needed for wound healing and defect repair.

From this study we suggest that *in vivo*, in the younger subjects, a part of osteochondral defect repair can originate from proliferation of chondrocytes in the cartilage bordering these full-thickness defects. Repair of immature partial thickness defects[20] may be explained by this repair process.

Our first aim was to study the basic cellular and molecular reactions of cartilage tissue to experimental wounding. Natural wound healing reaction of auricular and articular cartilage was studied *in vivo* and *in vitro*. Rabbit, bovine and human articular cartilage was used. Age-related, location related and temporal differences were shown.

In vivo rabbit auricular cartilage wound healing showed chondrocyte death in wound edges, decrease and restoration of proteoglycan staining and temporal, local increase in growth factor expression. A comparable wound healing reaction was observed in our *in vivo* rabbit articular cartilage model, with the exception of the growth factor expression pattern. In this short term (7days) defect repair study, a general increase in growth factor expression was observed throughout the cartilage tissue. The *in vitro* bovine explant studies showed a similar amount of initial chondrocyte death in wound edges. The observed local decrease in proteoglycan staining, followed by replacement was in accordance with the *in vivo* studies. Adult human articular explants demonstrated less chondrocyte death and proteoglycan loss during culture follow up. Low cell density and tight ECM may explain this difference compared to mature and immature bovine articular cartilage explant studies.

Furthermore, in a long-term explant study we demonstrated the outgrowth of chondrocytes from immature bovine articular cartilage explants. The cells originated from the deep zone cartilage and were able to form new hyaline-like cartilage tissue. These observations suggest that a part of the repair of partial and full-thickness defects originates from cells grown out of wound edges.

We have studied articular cartilage wound healing *in vivo* in a small animal model (New Zealand white rabbit). Although this is a frequently used animal for articular cartilage repair experiments, relatively large defects are created in small joints. We do not know whether this model is the best to represent the human situation. A larger animal model, with relatively smaller defects may well form a better representation of a human articular cartilage defect.

Our second aim was to use this knowledge to develop strategies to improve cartilage wound healing. In relation to possible cartilage wound healing treatment, we have shown that collagenase can be used to stimulate proliferation and to increase chondrocyte densities in articular cartilage wound edges. The integration experiments demonstrated improved integration of the lesion edges after treatment with collagenase. In adult human articular cartilage an increase in chondrocyte density at the lesion edges could also be achieved, but only when proteoglycans were actively depleted from the wound edges prior to collagenase treatment.

Since chondrocyte density in adult human cartilage is low compared with the cell density in most animal models, increased chondrocyte density may improve integration in human cartilage as well.

The length of collagenase treatment in our experiments (48 hours) forms a limitation for its use in clinical practice. Recently, we have shown that similar results can be reached with a one hour treatment protocol using a combination of hyaluronidase and collagenase[15].

ECM production and proliferation of adult articular chondrocytes can be stimulated, up to immature chondrocyte levels, by addition of TGFβ1. Deep zone cartilage contains chondrocytes able to proliferate, grow out of wound edges and form new hyaline-like cartilage under optimal (*in vitro*) circumstances.

Regarding outgrowth of chondrocytes from cartilage wound edges, further study on the outgrowth of cells and stimulation of this process may help to improve cartilage wound healing and integrative cartilage repair.

Results from successful articular cartilage defect repair may in the future be extrapolated to osteoarthritis repair. An isolated cartilage defect in an otherwise healthy joint may be regarded as a different entity when compared to general degeneration in osteoarthritic joints. Although the extended inflammatory and general synovial joint changes result in a different biological environment[12,25], one could regard isolated cartilage defect repair as a first step (or *in vivo* human model) towards a biological approach for extended osteoarthritis repair.

FUTURE RESEARCH

Future research has to elaborate whether our *in vitro* results, that improved integrative cartilage repair, can be reached in an *in vivo* synovial joint environment. Biomechanical loading, variation in inflammatory reaction of articular cartilage and synovial tissue, local diffusion and nutrition under synovial fluid conditions are examples of differences.

Furthermore, there are limitations to the length of *in vitro* cartilage tissue experiments. Long-term effects of interventions on integrative cartilage repair have to be evaluated *in vivo*. For example, improved histological and biomechanical integration of cartilage following collagenase treatment has to proof its value for clinical outcome in long-term *in vivo* experiments.

Chondrocyte death inhibition (for example by inhibition of caspases), cell delivery to the cartilage-cartilage interface region (using tissue engineering approaches), rapid local ECM production in sufficient amounts (using growth factors) and modulation of ECM cross-linking/integration might improve articular cartilage wound healing.

Temporal inhibition of collagen cross-linking during the ECM production might improve the interdigitation of collagen fibrils before cross-linking takes place[8]. Enzymes and growth factors may be stored in a vehiculum, for example in collagen or fibrin matrix/glue, with slow release activity.

New strategies can be tested in our articular cartilage explant culture model and integration model. Histological and biomechanical analysis can be used to value possible beneficial interventions/factors.

In vivo effects of intervention and long-term effects are probably best tested in large animal models with the possibility of arthroscopic evaluation of tissue morphology and biomechanical testing of the repair tissue characteristics and integration by indentation/probing. Conventional MRI or contrast-enhanced MRI can be used for noninvasive assessment of cartilage repair in a time manner for animal model studies, human experiments and clinical treatment evaluation. Development of new techniques in MRI evaluation of cartilage, including use of contrasts, cell labelling, ECM composition evaluation is important for cartilage repair investigation.

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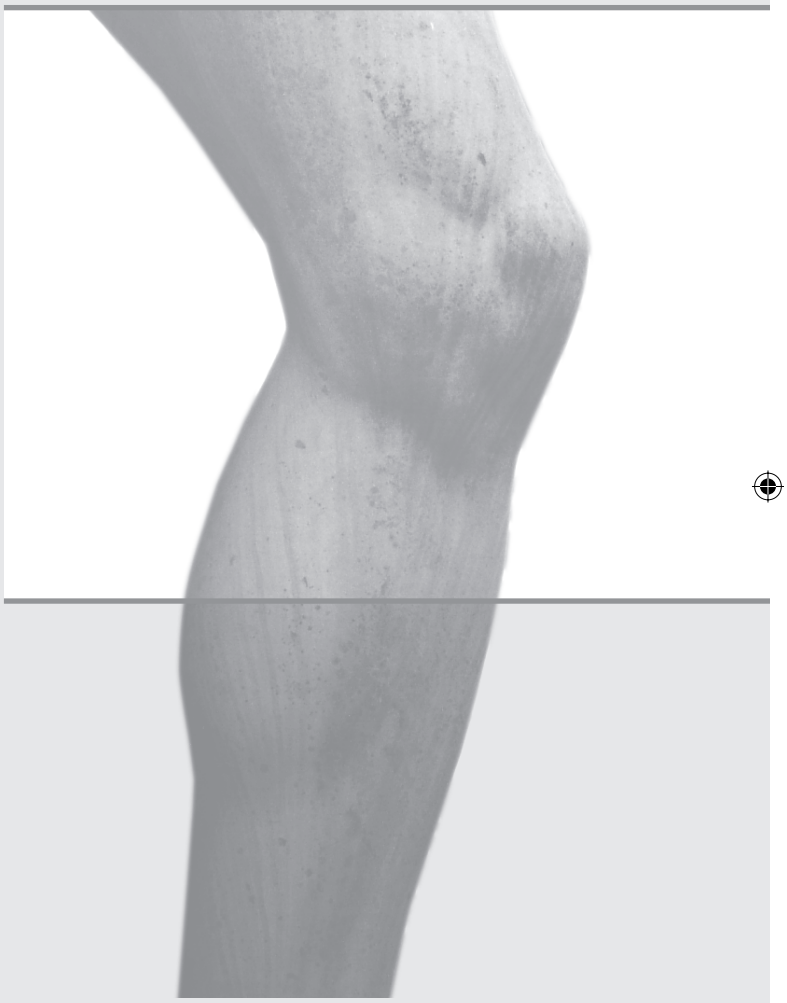
Chapter 8

Summary



The intrinsic regeneration capacity of articular cartilage following injury is limited. Partial-thickness defects are not repaired and full-thickness defects are repaired with fibrocartilage. Untreated, these defects may progress to early osteoarthritis. The goal of surgical treatment of (osteo)chondral defects is to reduce symptoms, improve joint congruence by restoring the joint surface with the best possible tissue, and to prevent joint degeneration. Current clinical and experimental treatment methods, for cartilage repair and regeneration, do not result in durable and predictable restoration of the articular surface in damaged joints. An important prerequisite for durable repair of cartilage lesions is the integration of wound edges or the integration of repair tissue with the surrounding host cartilage.

In the general introduction chapter **(chapter 1)** the morphology and molecular composition of articular cartilage is described. The incidence and natural outcome of articular cartilage defects is given as well as a short overview of current clinical and experimental surgical treatment techniques.

The composition of articular cartilage with its avascular status, low cell density and tight extracellular matrix (ECM) hampers a successful healing at the cartilage level. The general aim of this thesis was to study the cellular and molecular reactions of cartilage tissue to experimental wounding and to use this knowledge to improve articular cartilage integration.

In **chapters 2 and 3,** the primary wound healing characteristics of cartilage tissue *in vivo* was investigated. In **chapter 2,** we studied wound healing of auricular cartilage wounds in the New Zealand White rabbit. Proteoglycan depletion and replenishment, chondrocyte death and temporal and spatial growth factor expression were demonstrated following experimental wounding of the auricular cartilage.

In conclusion, we have demonstrated the ability of chondrocytes to increase growth factor expression and to restore the initial proteoglycan decrease following acute wounding. The temporal expression of endogenous growth factors in reaction to acute cartilage injury suggests an autocrine and/or paracrine stimulation of chondrocyte metabolism. This can be regarded a sign of chondrocytes repair capacity. Chondrocyte death was observed in cartilage wound edges, this acellular tissue interferes with the wound healing process and prevents proper repair.

In **chapter 3** we studied articular cartilage wound healing reactions of partial-thickness and full-thickness defects in immature and mature New Zealand White rabbits. Furthermore, the potential of isolated chondrocytes from these animals to proliferate and produce ECM with and without TGFβ1 stimulation was tested *in vitro.*

In conclusion, we demonstrated that immature articular cartilage has a higher intrinsic proliferative potential than mature cartilage. Immature cartilage is able to decrease the size of articular cartilage defects *in vivo*. We showed that with addition of a potent growth factor

proliferation and subsequent ECM production of mature chondrocytes could be restored *in vitro.*

In **chapter 4,** the use of enzymatic matrix digestion to stimulate articular cartilage repair is described. In a bovine articular cartilage explant study we demonstrated that treatment of explants with highly purified collagenase resulted in a significant increase in chondrocyte density at the wound edges during culture follow up. In an integration experiment, we showed improved histological integration after treatment with collagenase.

We concluded that treatment with highly purified collagenase could improve integrative cartilage repair, probably by increasing the cell density at cartilage wound edges.

In **chapter 5,** we studied whether treatment with enzymes (hyaluronidase and collagenase) could improve histological and biomechanical integration of cartilage. Integration of bovine articular cartilage explants following enzyme treatment and subcutaneous implantation in nude mice for 5 weeks was evaluated histologically and biomechanically.

In conclusion, we demonstrated that enzyme treatment of cartilage explants improved histological integration and biomechanical bonding strength.

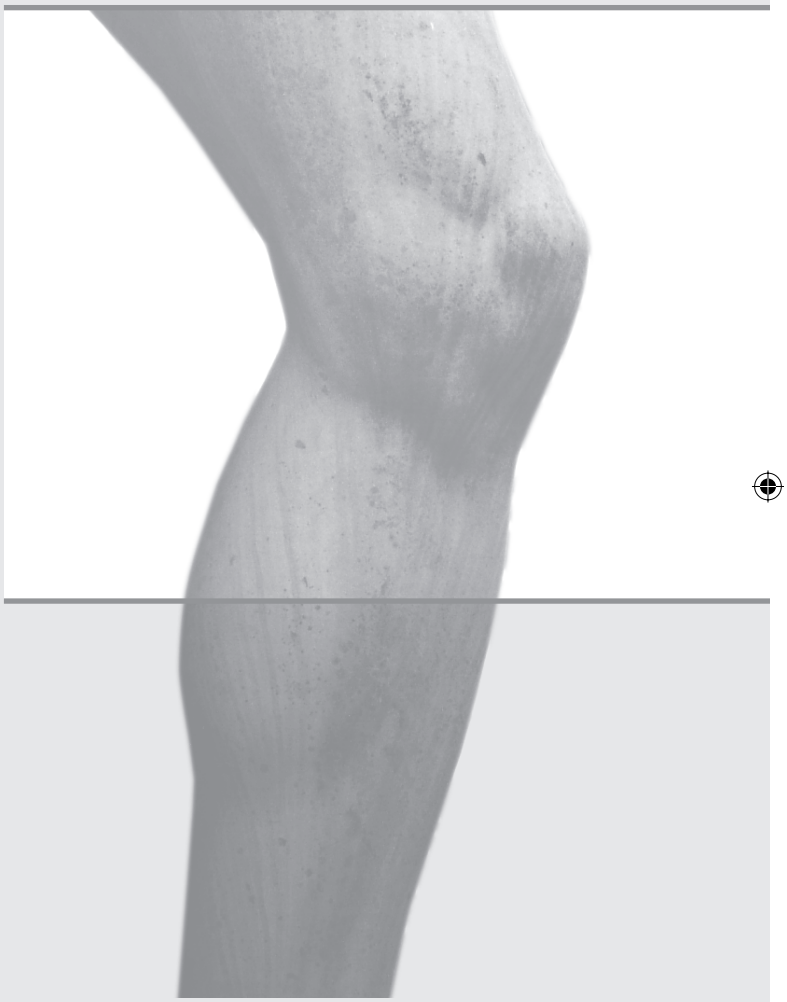
In **chapter 6,** we further studied the outgrowth of chondrocytes and neocartilage formation at wound edges of immature and mature bovine articular cartilage explants that was noticed *in vitro*. We showed that chondrocytes are able to grow out of explants cultured for four weeks and form new cartilage with hyaline-like characteristics. Significant more outgrowth was observed from immature explants. Proliferation activity, demonstrated by a positive Ki67 immunoreactivity, was observed in both superficial and deep zone cartilage of immature cartilage explants. A second experiment showed that the neocartilage originates from chondrocytes in the lower zones of the cartilage and not from the superficial zone cartilage.

In conclusion, we suggested that deep zone articular cartilage in young individuals contain chondrocytes capable of proliferation and new cartilage tissue formation *in vitro*. We suggest that *in vivo*, in the younger subjects, a part of (osteo)chondral defect repair can originate from proliferation of chondrocytes in the cartilage bordering defects.

In **chapter 7,** the basic cellular and molecular *in vivo* and *in vitro* wound healing reactions of cartilage are discussed. Wound healing reactions *in vivo* show similar characteristics to our *in vitro* articular cartilage explant culture model. The composition of cartilage with its avascular status, low cell density and tight ECM limit cartilage wound healing. Age-related and location-related chondrocyte proliferation characteristics were reported. We have shown that enzyme treatment and TGFβ1 addition can be used to increase chondrocyte densities in wound edges and stimulate ECM production needed for integrative cartilage repair.

Chapter 9

Nederlandse samenvatting



De intrinsieke herstel capaciteit van gewrichts-kraakbeen na beschadiging is beperkt. Chondrale defecten (beperkt tot het kraakbeen) herstellen niet en osteochondrale defecten (reikend tot voorbij de subchondrale plaat) herstellen met fibreus kraakbeen. Onbehandeld kunnen deze defecten de ontwikkeling van vroege artrose tot gevolg hebben. Het doel van de chirurgische behandeling van (osteo)chondraal defecten is: verminderen van symptomen, verbeteren van de gewrichts congruentie door herstel van het gewrichtsoppervlak met weefsel van een zo optimaal mogelijke kwaliteit, en voorkomen van verdere gewrichts degeneratie. De huidige klinisch en experimenteel toegepaste behandelmethoden voor kraakbeendefecten resulteren niet in een voorspelbaar en duurzaam herstel van het gewrichtsoppervlak in aangedane gewrichten. Een belangrijke voorwaarde voor duurzaam herstel van kraakbeen defecten is de integratie van kraakbeen wondranden of integratie van reparatieweefsel met het omliggende kraakbeen.

In **hoofdstuk 1** (algemene introductie) wordt de morfologie en moleculaire samenstelling van gewrichtskraakbeen beschreven. Een overzicht van de incidentie en het natuurlijk beloop van gewrichts kraakbeen defecten wordt beschreven. Een kort overzicht van de huidige klinische en experimentele chirurgische behandelmethoden wordt gegeven.

De samenstelling van gewrichtskraakbeen met zijn avasculaire status, lage cel dichtheid en dichte extracellulaire matrix structuur beperkt succesvol herstel op kraakbeen niveau. Het algemene doel van dit proefschrift is het bestuderen van cellulaire en moleculaire reacties van kraakbeen tijdens experimentele wondgenezing en het gebruiken van opgedane kennis om kraakbeen integratie te verbeteren.

In **hoofdstuk 2 en 3,** werd de primaire wondgenezing van kraakbeen weefsel na experimentele verwonding *in vivo* bestudeerd. In **hoofdstuk 2,** beschrijven wij de wondgenezing van kraakbeen wonden in oorkraakbeen van New Zealand White konijnen. Proteoglycaan depletie en herstel, chondrocyten celdood en tijd- en plaats gerelateerde groeifactor expressie werd gedemonstreerd na experimentele verwonding van oorkraakbeen.

Concluderend hebben wij laten zien dat kraakbeencellen tijdelijk meer groeifactoren tot expressie kunnen brengen in combinatie met herstel van initieel proteoglycaan verlies na acute experimentele verwonding. De tijdelijke expressie van endogene groeifactoren in reactie op acuut kraakbeenletsel suggereert een autocriene en/of paracriene stimulatie van het chondrocyten metabolisme. Dit kan beschouwd worden als een teken van kraakbeen herstel capaciteit. Chondrocyten celdood werd vastgesteld in kraakbeen wondranden, dit acelulair weefsel interfereert met het wondgenezingsproces en voorkomt succesvol herstel.

In **hoofdstuk 3,** werd de wondgenezing van chondrale en osteochondrale defecten in niet-volwassen en volwassen New Zealand White konijnen knieën bestudeerd. Tevens werd *in vitro* bestudeerd: het vermogen van geïsoleerde chondrocyten van deze dieren om te prolifereren en extracellulaire matrix te vormen met en zonder TGFβ1 stimulatie.

Concluderend hebben wij aangetoond dat niet-volwassen gewrichts kraakbeen een betere intrinsieke proliferatie capaciteit bezit dan volwassen kraakbeen. Het lichaam is in staat kraakbeen defecten in niet-volwassen dieren te verkleinen *in vivo*. Wij hebben laten zien dat met toevoeging van een potente groeifactor de proliferatie en dientengevolge extracellulaire matrix productie door volwassen chondrocyten kan worden hersteld *in vitro*.

In **hoofdstuk 4,** wordt het gebruik van enzym behandeling ter stimulatie van kraakbeen herstel beschreven. In een runder-gewrichts kraakbeen explant studie hebben wij aangetoond dat behandeling met sterk gezuiverd collagenase resulteert in een significante toename van chondrocyten dichtheid in kraakbeen wondranden tijdens weefsel kweken. In een integratie experiment, werd een verbeterde histologische integratie aangetoond na behandeling met collagenase.

Geconcludeerd werd dat behandeling van kraakbeen met sterk gezuiverd collagenase leidt tot verbetering van kraakbeen integratie, waarschijnlijk door toename van chondrocyten dichtheid in kraakbeen wondranden.

In **hoofdstuk 5,** werd bestudeerd of met gebruik van enzymbehandeling (hyaluronidase en collagenase) de histologische en biomechanische integratie van kraakbeen kon worden verbeterd. Integratie van runder-gewrichts kraakbeen explants, na enzym behandeling en subcutane implantatie in een naakte muizen model gedurende 5 weken, werd histologisch en biomechanisch geëvalueerd.

Geconcludeerd werd dat enzym behandeling van kraakbeen explants leidt tot een verbeterde histologische en biomechanische kraakbeen integratie.

In **hoofdstuk 6,** wordt een studie beschreven waarin de uitgroei van chondrocyten en nieuw kraakbeen vorming bij kraakbeen wondranden van niet-volwassen en volwassen runder explants *in vitro* werd bestudeerd. Aangetoond werd dat chondrocyten in staat zijn te groeien uit explants die gedurende vier weken worden gekweekt. Deze cellen zijn in staat nieuw kraakbeen te vormen met hyalien kraakbeen kenmerken. Significant meer uitgroei werd aangetoond bij niet-volwassen kraakbeen explants. Proliferatie activiteit, aangetoond door positieve immunokleuring voor de proliferatiemarker Ki 67, werd gedetecteerd in zowel de superficiële als de diepe laag van niet-volwassen kraakbeen explants. Een tweede experiment toonde aan dat het nieuw gevormde kraakbeen ontstaat uit chondrocyten afkomstig uit de diepe laag en niet uit de superficiële kraakbeen laag.

Concluderend suggereren wij dat de diepe kraakbeen laag van jonge patiënten chondrocyten bevat die in staat zijn te prolifereren en nieuw kraakbeen te vormen *in vitro*. Wij suggereren dat *in vivo,* in de jonge patiënt, een deel van (osteo)chondraal defect herstel kan ontstaan door proliferatie van chondrocyten in het kraakbeen grenzend aan het defect.

In **hoofdstuk 7,** worden de basale celreactie en moleculaire reactie tijdens kraakbeen wondgenezing *in vivo* en *in vitro* bediscussieerd. Kraakbeen wondgenezing *in vivo* vertoont vergelijkbare kenmerken met ons *in vitro* gewrichts kraakbeen explant model. De samenstelling van kraakbeen (afwezigheid van bloedvaten, lage cel dichtheid, dichte extracellulaire matrix) beperkt de wondgenzings capaciteit. Leeftijds-afhankelijke en locatie-afhankelijke verschillen in proliferatie capaciteit werden gerapporteerd. Wij hebben aangetoond dat behandeling met enzymen en toevoeging van TGFβ1 gebruikt kan worden om chondrocyten dichtheid in het wondgebied te verhogen en extracellulaire matrix productie te verhogen, benodigd voor kraakbeen integratie.

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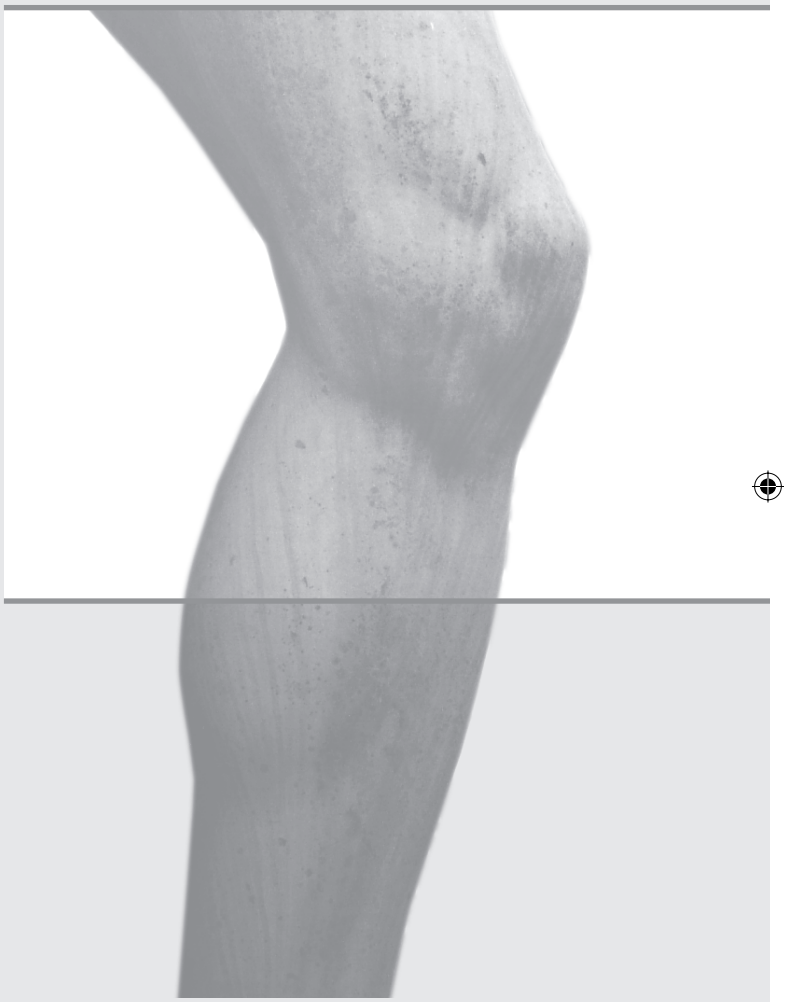
Lieve Ellen, onze beste beslissingen namen wij in de Caribbean! Voorlopig zit ik niet meer achter het muurtje. Kus, dank voor je liefde en steun.

Curriculum Vitae

Koen Bos werd geboren op 30 oktober 1971 te Leiden. Op 8 jarige leeftijd verhuisde hij met ouders, broer en zusje van Leiderdorp naar Munstergeleen. In 1990 behaalde hij zijn VWO-B diploma aan de Serviam Scholengemeenschap te Sittard. In hetzelfde jaar startte hij de studie geneeskunde aan de Erasmus Universiteit te Rotterdam. In 1996 werd de doctoraalfase afgerond. In 1998 verbleef hij voor een keuze-coschap orthopaedie en traumatologie in het Royal Orthopaedic Hospital / University Hospital in Birmingham, Groot Brittanië (o.l.v. Mr.JL Plewes) In 1998 werd het artsexamen succesvol afgelegd. In september 1998 begon de auteur zijn promotieonderzoek en werkte als onderzoeksassistent in het kraakbeen laboratorium (o.l.v. dr. GJVM van Osch en prof.dr. JAN Verhaar). In 2001 startte hij met de chirurgische vooropleiding in het Albert Schweitzer Ziekenhuis te Dordrecht (opleider: dr. KG Tan). De orthopaedie opleiding werd voortgezet in het Erasmus MC (opleider: prof.dr. JAN Verhaar). Voor zijn perifere opleidingsstage werkte hij in 2005 op de afdeling orthopaedie en traumatologie van de Reinier de Graaf Groep te Delft (opleider: dr.RM Bloem). Per januari 2007 zal hij werkzaam zijn als orthopaedisch chirurg in het Erasmus MC, te Rotterdam.

De auteur woont samen met Ellen van den Berg, samen hebben zij een dochter Maud en een zoon Jilles.

Colour figures



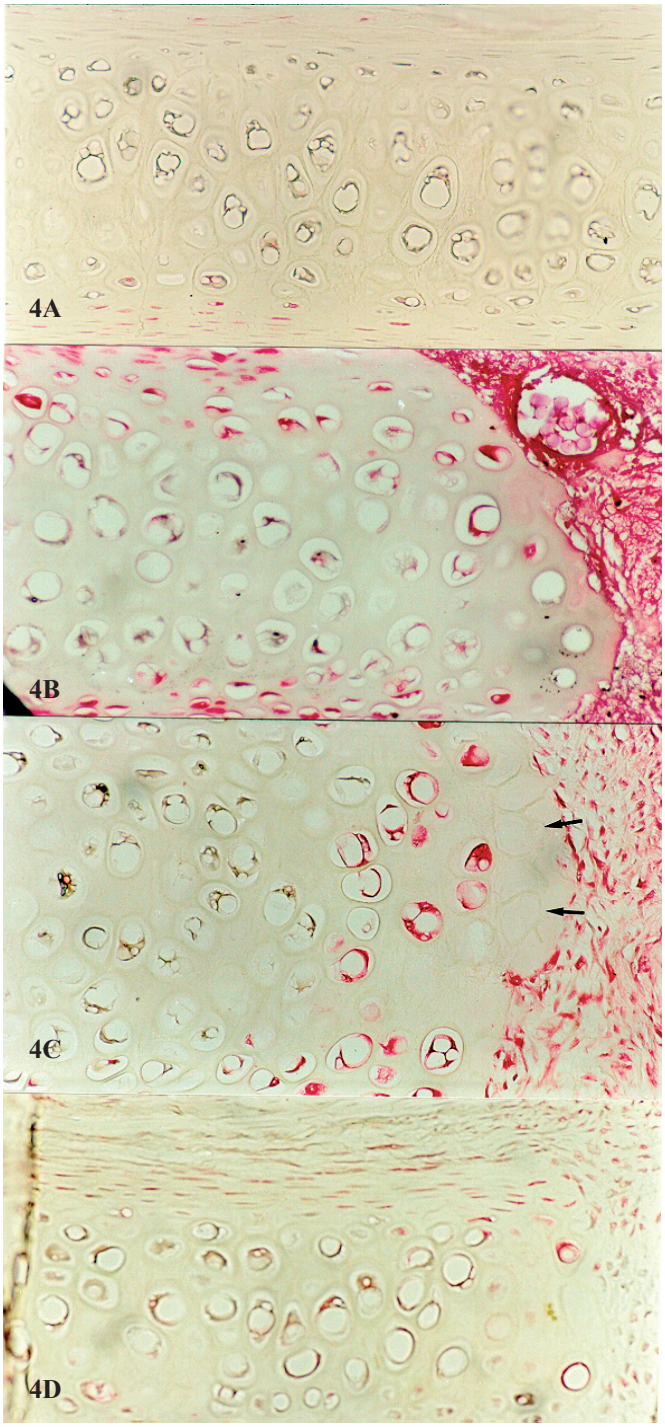


Figure 2.4, page 36

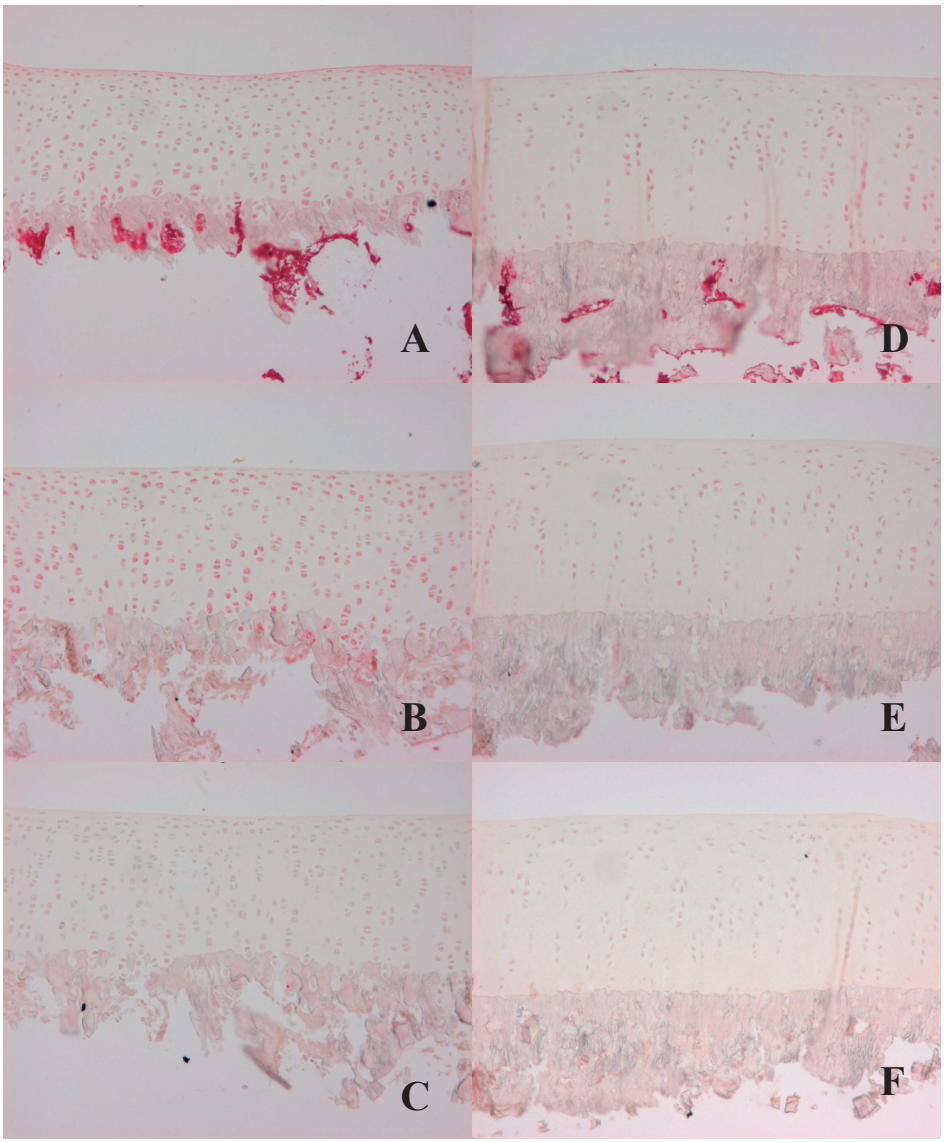


Figure 3.5, page 51

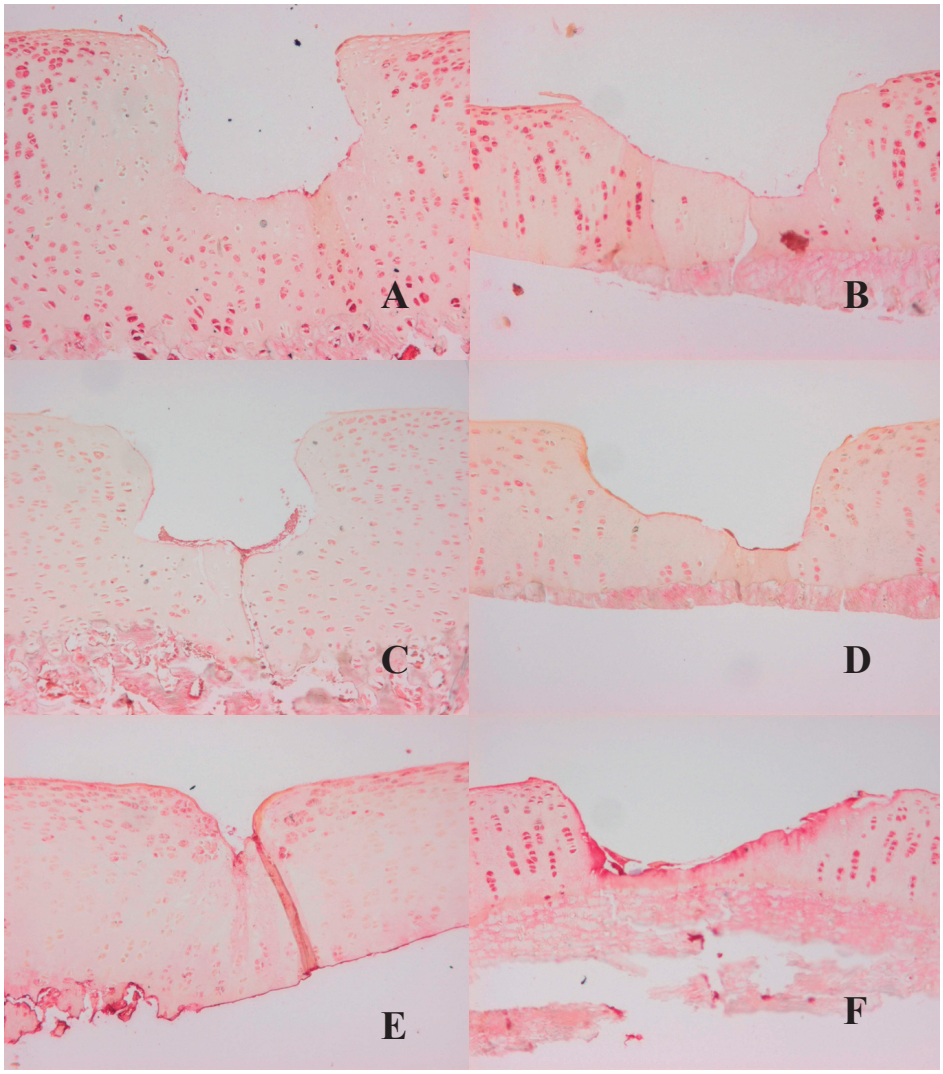


Figure 3.6, page 52

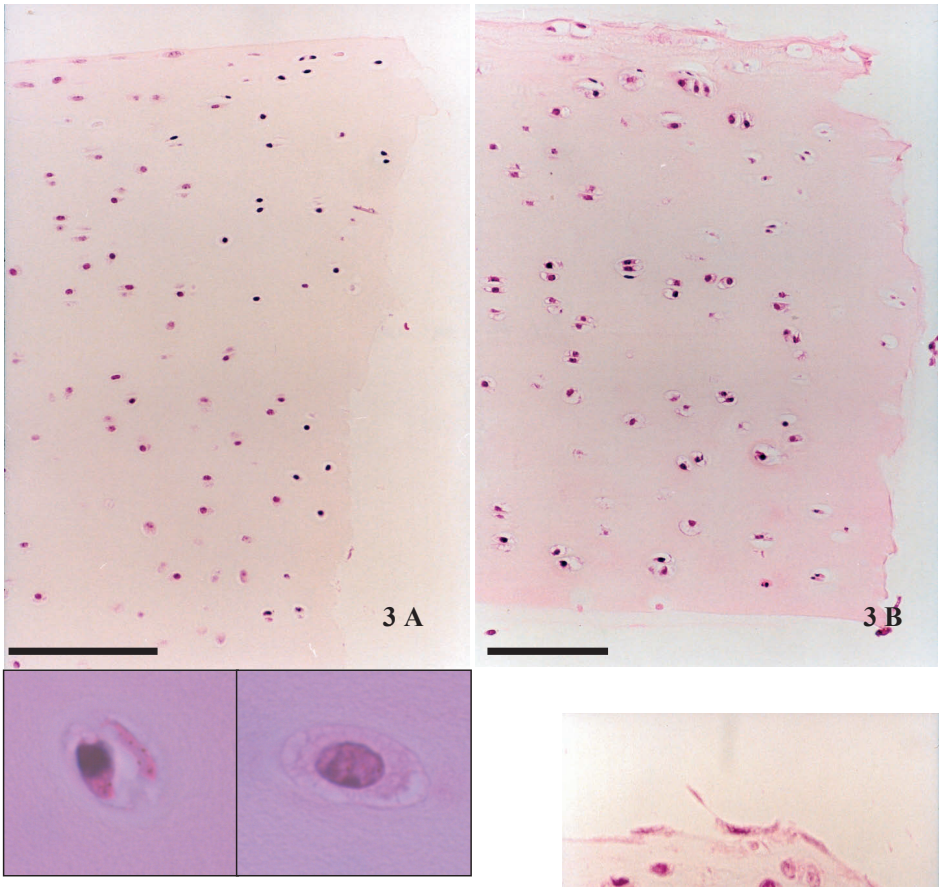


Figure 4.3, page 68

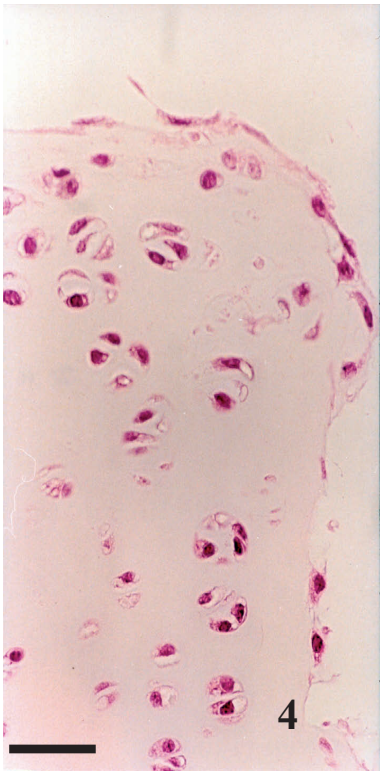
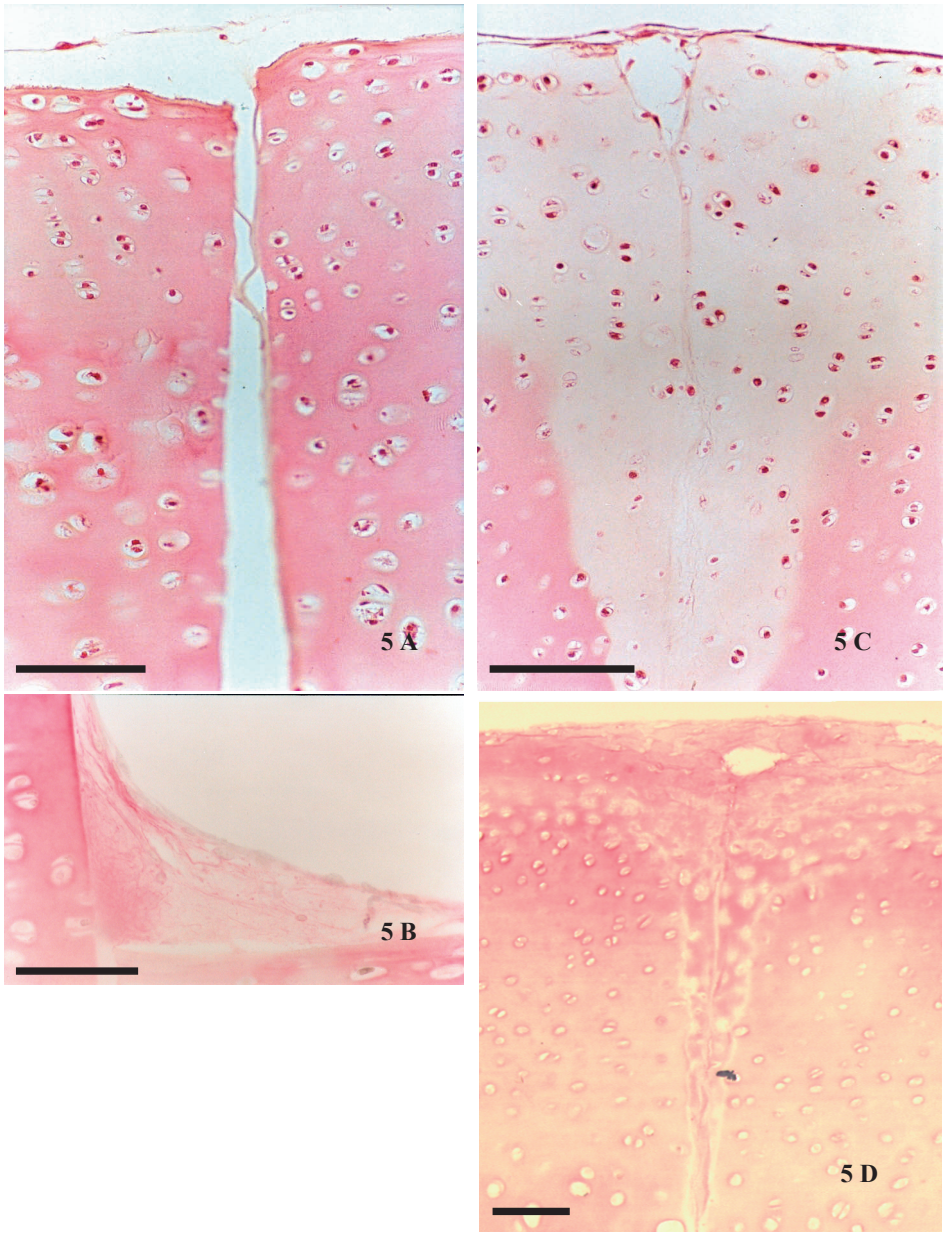


Figure 4.4, page 69



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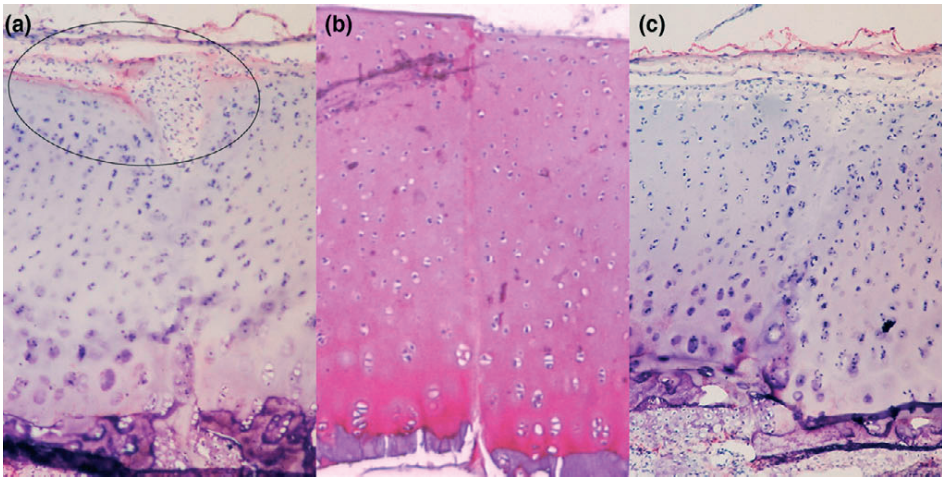

Figure 5.4, page 87

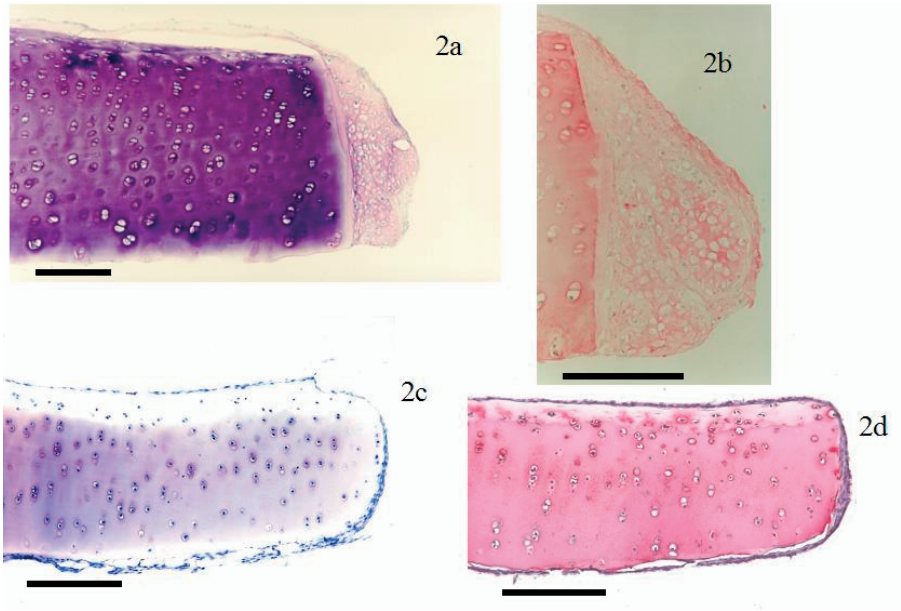

Figure 6.2, page 101

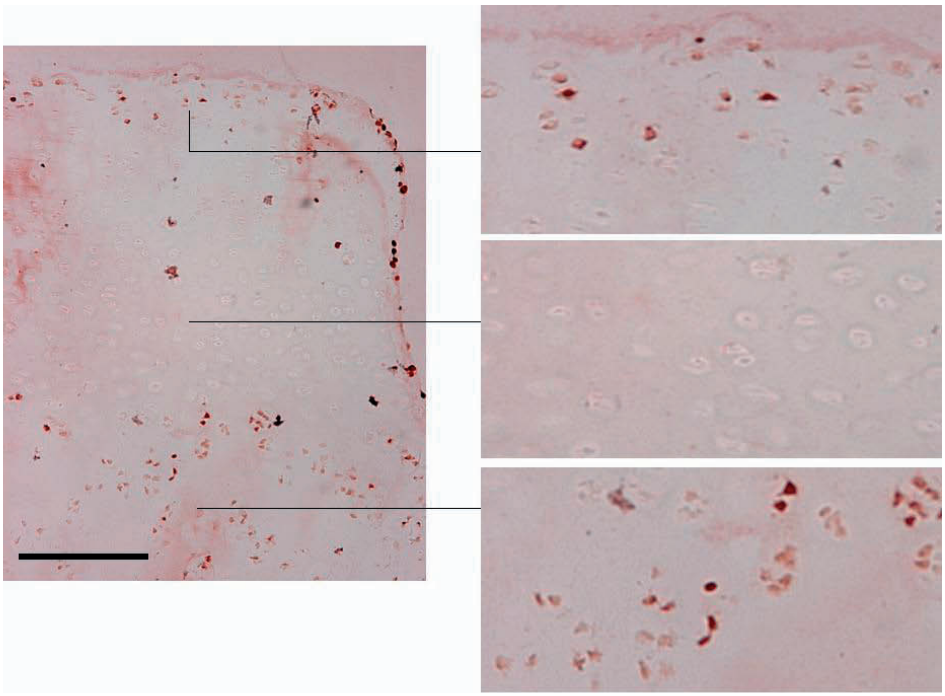

Figure 6.3, page 102

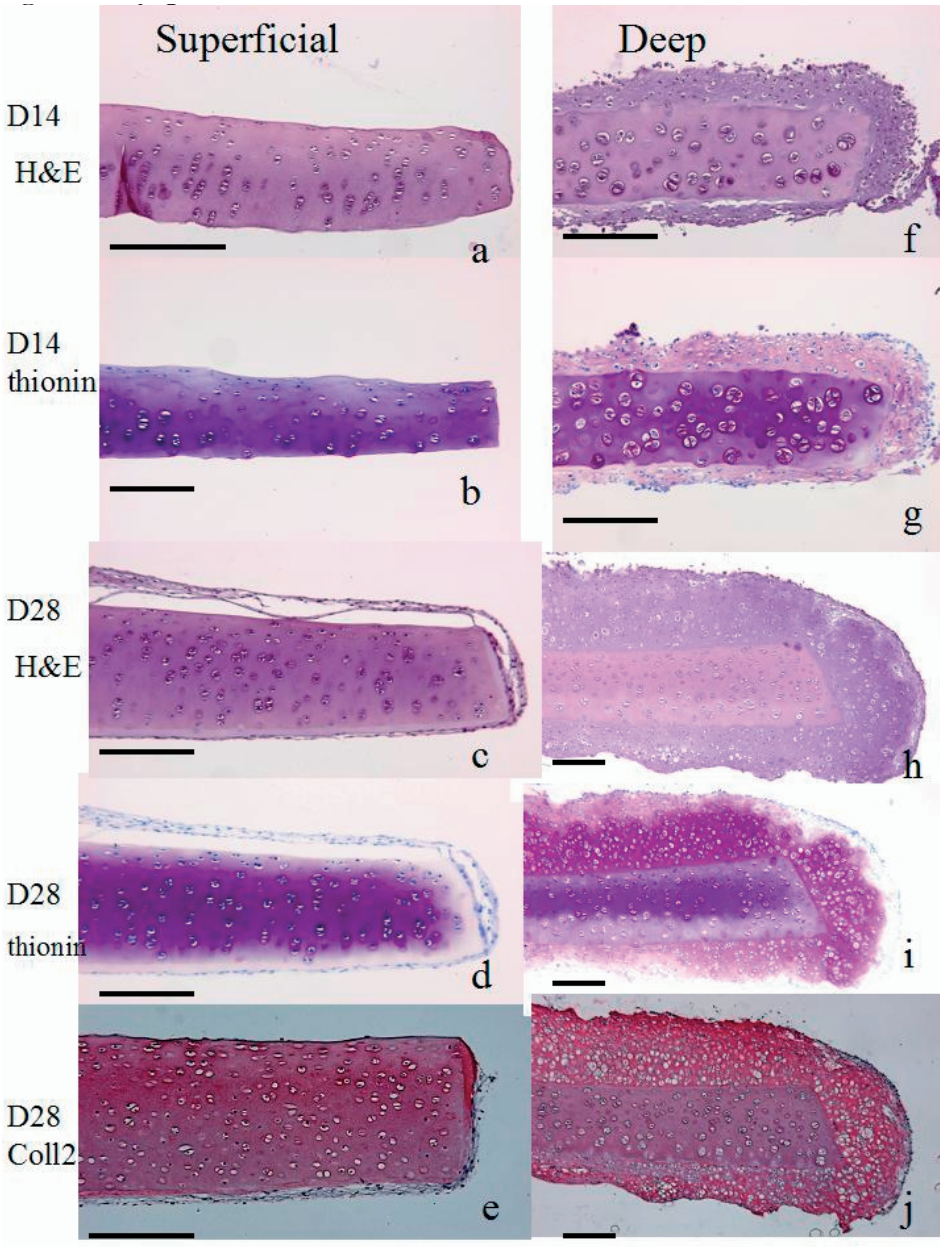

Figure 6.5, page 104