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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[2], and G.J.V.M. van Osch[3]

20.1. ABSTRACT

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.

[1] Department of Orthopaedic Surgery, Erasmus MC, University Medical Center Rotterdam, the Netherlands
[2] Department of Orthopaedic Surgery, Erasmus MC, University Medical Center Rotterdam, the Netherlands
[3] Departments of Orthopaedic Surgery and Otorhinolaryngology, Erasmus MC, University Medical Center Rotterdam, the Netherlands

20.2. 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[1]. 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[3,4].

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[5]. Failure of repair caused by an impaired integration has been documented by several authors who studied the natural cartilage repair process [6-8], repair following transplantation of periosteal and perichondrial grafts[9,10], osteochondral grafts[11], natural[12] and tissue engineered grafts[13].

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[15].

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[16-18]. 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.

20.3. MATERIALS AND METHODS

20.3.1. 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 [19].) 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.

20.3.2. 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[20]. 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.

20.3.3. Histology

For histological evaluation sections were stained with Heamatoxylin & 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[21,22]. 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.

20.3.4. 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.

20.3.5. 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[23]. 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[24]. 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.

20.3.6. 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 (*).

20.4. RESULTS

20.4.1. 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 (Figure 20.1.). 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 (Figure 20.2). 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 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 (Figure 20.3).

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. No age-related or defect-related difference was observed in this early wound healing study (Figure 20.4.).

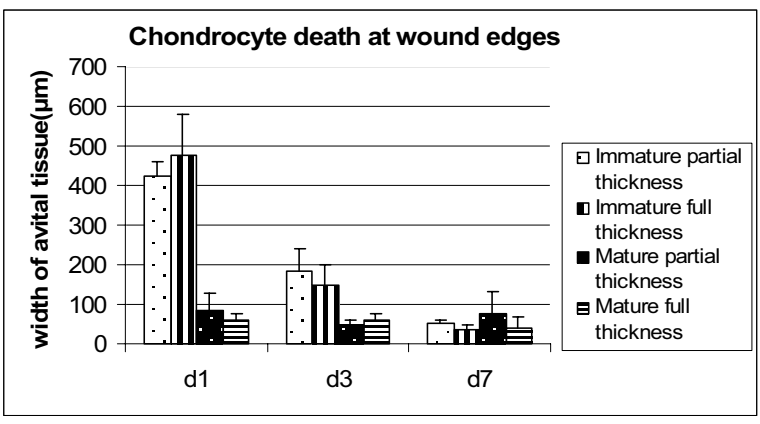

Figure 20.1. Graph representing the observed chondrocyte death in articular cartilage following experimental injury. Mean and SD of the distance between lesion edges and vital chondrocytes is shown.

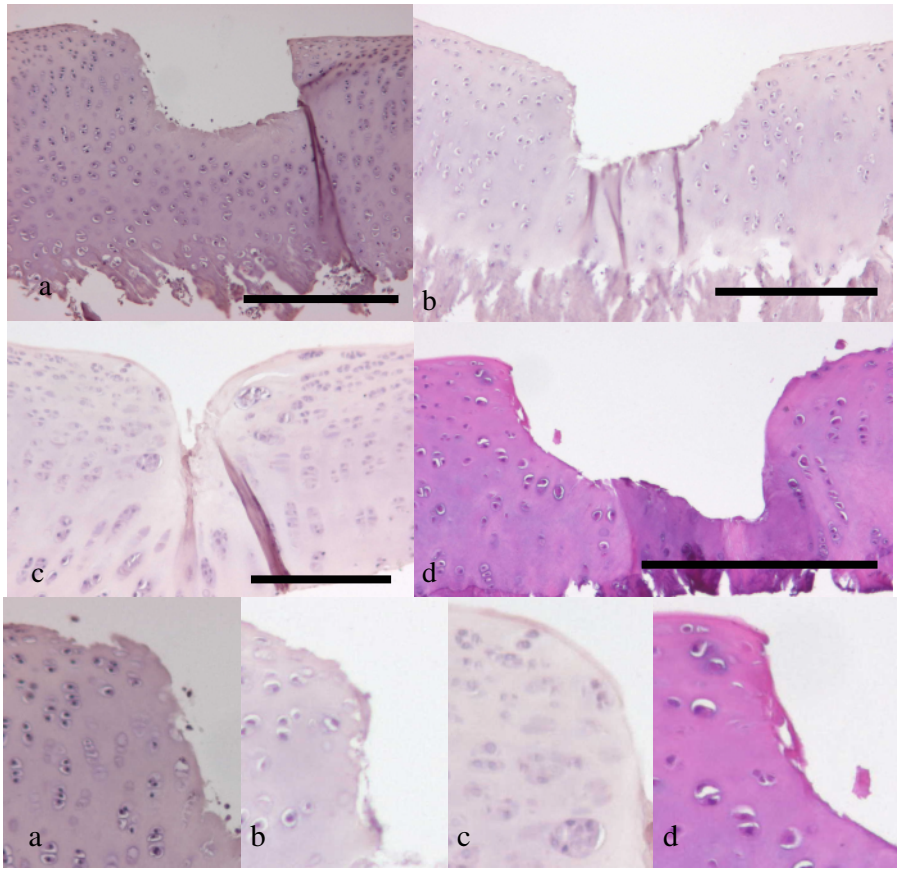

Figure 20.2. H&E stained sections from partial thickness defects are shown. Immature defects (2a) harvested 1 day after surgery show a large area of death chondrocytes. near wound edges, whereas mature defects show almost no signs of avital chondrocytes (2b) One week after surgery immature defects showed cluster formation and reduction in defect size (2c), mature defect size and shape however, was grossly unchanged (2d). Bar=500μm.

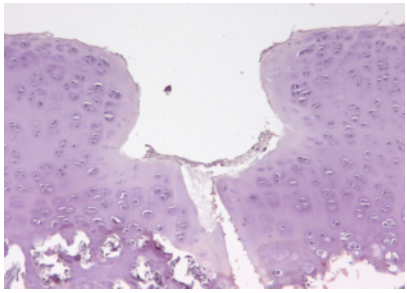

Figure 20.3. H&E stained section of an immature cartilage full-thickness defect harvested 3 days after surgery, showing a small amount of fibrous tissue at the base of the defect.

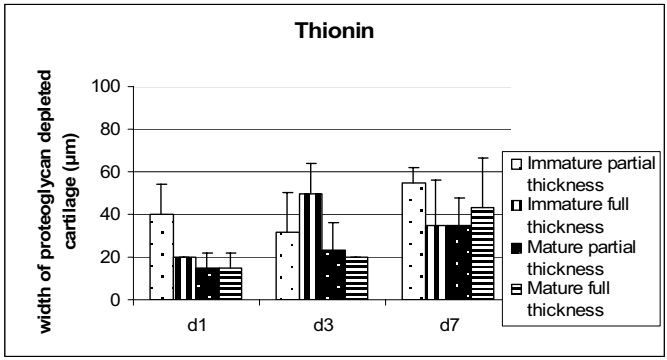

Figure 20.4. Graph representing the area of proteoglycan depletion at articular cartilage wound edges following experimental injury. Mean and SD of the distance from wound edges to normal thionin staining is shown.

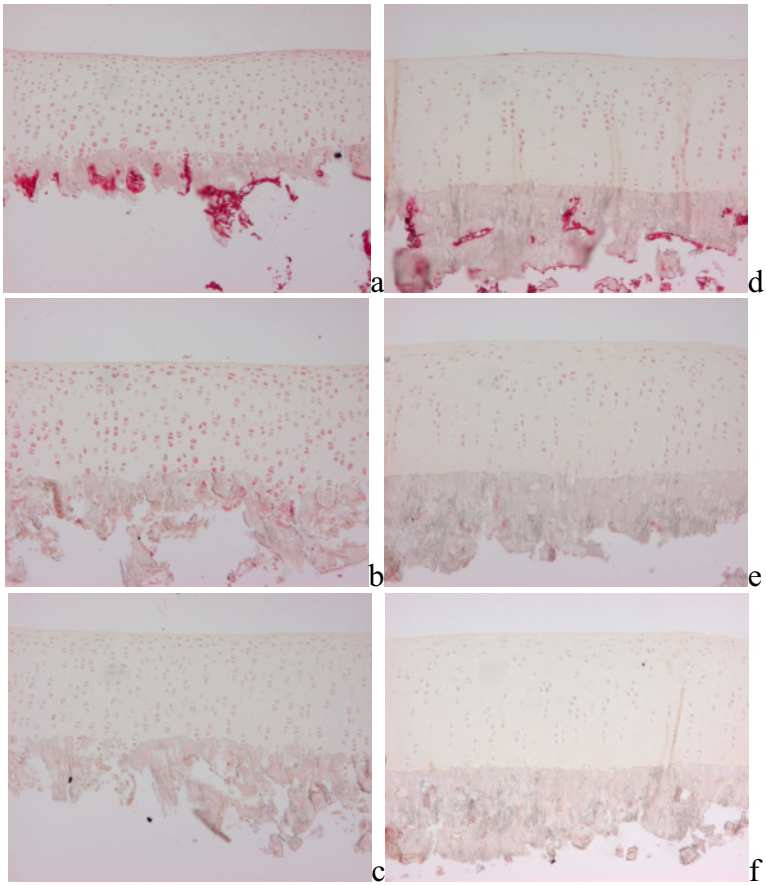

Figure 20.5. unwounded immature (a,b,c) and mature (d,e,f) hip cartilage immunostained for TGFβ1 (a and d), FGF-2 (b and e) and IGF-1 (c and f).

20.4.2. 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 (Figure 20.5).

Following experimental injury, an intense positive immunoreactivity was observed for TGFβ1 and FGF2 behind an area immunonegative cells near lesion edges. Also throughout 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 (Figure 20.6.a-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 (Figure 20.6.e). No difference in growth factor expression was observed between sections from partial-thickness or full-thickness defects.

20.4.3. 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 (Figure 20.7.). 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. 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 (Figure 20.7).

20.5. 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[11,22,25]. 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, 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[26,27] and human[16,17] 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[6]. 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.

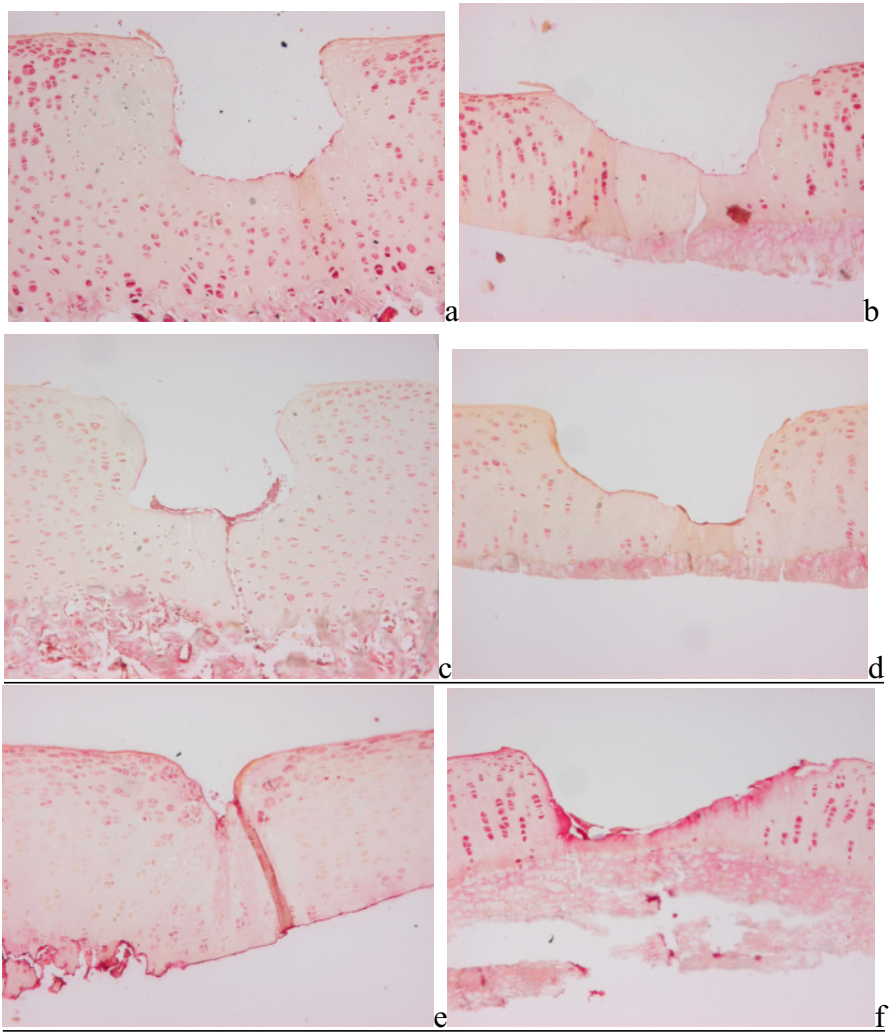

Figure 20.6. Immunohistochemical staining for TGFβ1 and FGF2 and IGF1 was studied in wounded articular cartilage. a=FGF-2, immature cartilage, day 1; b=FGF-2, mature cartilage, day 1; c=IGF-1, immature cartilage, day 3; d=IGF-1, mature cartilage, day 3; e= TGFβ1, immature cartilage, day 7; f=TGFβ1, mature cartilage, day 7. The area of tissue with immunonegative chondrocytes in wound edges correlated with the absence of immunopositive cells in wound edges. (Compare Figure 20.6.a, b. to 20.6.2a, b).

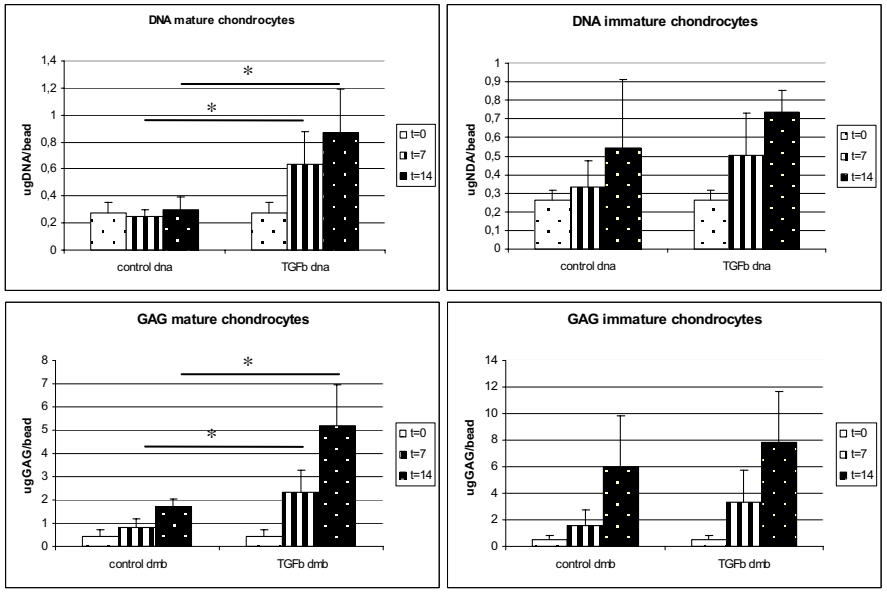

Figure 20.7. Glycosaminoglycan production and DNA content of alginate beads with immature and mature chondrocytes cultured for 14 days in medium with and without addition of 10ng/ml TGFβ1. TGFβ1 induced an increase in proliferation rate of mature chondrocytes in culture, whereas the observed proliferation rate of immature chondrocytes was not further stimulated.

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 heamarthros 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[28], osteoarthritic cartilage[29] or wound healing in other tissues[30,31].

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[17]. 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[16].

Fibroblast growth factor-2 was used in a partial-thickness articular cartilage defect repair study using immature and mature rabbits[32]. 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) [33]. 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)[34-36].

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.

20.6. ACKNOWLEDGMENTS

The authors would like to thank the Animal Experimental Center of the Erasmus MC, for housing and taking care of the rabbits. Simone van der Veen is acknowledged for her help with DNA and DMB-assays, Esther de Groot for her assistance with immunohistochemical work. This project has been supported by the Foundations "De Drie Lichten" and "Vereniging Trustfonds Erasmus Universiteit Rotterdam" in The Netherlands.

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