Microscopy Research, 2014, 2, 67-80
Published Online October 2014 in SciRes. http://www.scirp.org/journal/mr
http://dx.doi.org/10.4236/mr.2014.24009

Repair Mechanisms in Articular Cartilage—A Porcine *in Vitro* Study

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Received 17 August 2014; revised 13 September 2014; accepted 12 October 2014

Abstract

Explants are excellent systems for studying homeostasis in cartilage. The systems are very useful in pharmacological studies involving OA-treatment and in studies of repair mechanisms during injury to hyaline cartilage. The purpose of this study was to evaluate the reparative processes occurring in a young age porcine cartilage explant model examining tissue by Light (LM) and Transmission Electron Microscopy (TEM). Explants of articular cartilage were dissected from the femoral condyles of immature one-year-old pigs and cultured in DMEM/F12 medium with FCS (stimulated explant) or in medium without FCS (control explant) for up to 4 weeks. After 1 - 4 weeks of culture with FCS, LM showed migration and proliferation of chondrocytes in cartilage close to the injured surface differentiating two areas: proliferative zone and necrotic zone. The chondrocytes present in the necrotic zone showed a polarization towards the injured surface. After budding through the injured surface, the chondrocytes formed repair tissue in an interface repair zone and in outer repair tissue. TEM showed chondrocytes in expanded lacunae involving the proliferative zone. The pericellular matrix of the expanded lacunae was partly dissolved, indicating release of matrix-degrading enzymes during proliferation and remodeling. Migratory chondrocytes were identified in oval lacunae close to the injured surface. The pericellular matrix of these oval lacunae was significantly dissolved and immunohistochemistry demonstrated strong staining with a polyclonale collagenase antibody around these units, suggesting release of matrix-degrading collagenase contributing to chondrocyte mobility. We describe an explant model comprising two different repair systems in immature articular cartilage. This model provides us with new reference points that are important in understanding the repair mechanisms.

Keywords

Cartilage Repair, EM, Explant, Migration, Proliferation

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How to cite this paper: Skagen, P.S., Kruse, H.A. and Horn, T. (2014) Repair Mechanisms in Articular Cartilage—A Porcine *in Vitro* Study. *Microscopy Research*, **2**, 67-80. http://dx.doi.org/10.4236/mr.2014.24009

1. Introduction

In vivo studies have shown that cartilage from young and adolescent animals can regenerate partial-thickness defects to produce almost normal hyaline cartilage [1] [2], whereas self-repair is not seen in similarly created defects in articular cartilage of adult animals [3]-[7] or in clinical observations of defects in human articular cartilage [8]-[11].

In vitro, articular cartilage repair mechanisms have been studied in various explant systems [12]-[18]. These *in vitro* systems are comparable to *in vivo* conditions [19] [20] and have allowed researchers to study repair mechanisms after introduction of mechanical defects imitating traumatic joint injury [21] [22]. These studies show that chondrocytes in the injured area have low survival rates thus making it difficult to establish repair mechanisms.

One of the explanations for the failure to repair, including lack of binding of tissue at the defect surface, is that chondrocytes in an approximately 150 µm wide zone close to an injured surface are damaged by mechanical injury [23]-[25]. This inhibits the repair process. The zone has been named "zone of cartilage necrosis" since chondrocytes in this area appear both necrotic [26] and apoptotic [21].

The aim of the present study was to examine the characteristics and behavior of chondrocytes by Light (LM) and Transmission Electron Microscopy (TEM) in order to understand the mechanisms underlying spontaneous repair of immature articular cartilage at the injured surface. For this purpose we used explants stimulated with growth medium for periods up to 4 weeks. In this report we describe the migration and properties of chondrocytes in the hyaline articular cartilage matrix following mechanical injury and stimulation in a growth medium. Two repair systems were seen. One system leads to proliferation of chondrocytes close to the injured cartilage. The other system involves chemotactical migration of chondrocytes towards the injured surface, followed by binding to repair tissue. Finally, we discuss the clinical relevance of this model as well as possible mechanisms in these two repair systems in the recovery of matrix components close to the injured surface.

2. Material and Methods

2.1. Explant Cultures

The left hind limbs from 4 healthy one-year-old pigs were obtained from a local slaughter-house (Steff Houlberg/Danisco, Ringsted, Denmark). These pigs are considered immature because their growth plate is clearly visible and the bones elongate up to an age of two years.

The femoral condyles were used in the experiments 3 hours after the pigs were slaughtered.

Full-depth hyaline articular cartilage specimens (1 cm^2) were dissected with a scalpel under sterile conditions from the 4 medial femoral-condyles. Each specimen was divided further into 4 pieces by cutting perpendicularly to the articular surface. Forty-eight of these explants, measuring 5 × 5 × 1, 5 - 2 mm, were used in the following experiments.

Twenty four serum stimulated explants (6 explants derived from each pig) were cultured in 75 cm^2 cell culture flasks (InVitrogen) (6 explants in each flask). Dulbecco's Modified Eagle's Medium DMEM/NUT.MIX F-12 medium with Glutamax-1 (InVitrogene) and 15% Fetal Calf Serum (FCS) (Life Technologies). Gentamycin (49 ug/ml), fungizone (1, 2 ug/ml), and ascorbic acid 2-phosphate (87 ug/ml), were added (30 ml pr culture flask). The reagents were from Sigma Aldrich.

Twenty four control-explants (6 explants derived from each pig) were cultured in a similar medium (6 explants in each flask) but without the addition of FCS.

Fresh medium was added every third day to all explants.

In order to follow the repair processes over time for a period up to 4 weeks, serum- and control-explants were cultured for either "0", 1, or 4 weeks, respectively, followed by immediate fixation (see below).

The injured surface involving the upper, middle and lower cartilage zones as well as articular surface and chondrocytes located centrally were extensively studied with LM. TEM was used to study the injured surfaces and repair tissue involving the middle zone as well as chondrocytes located centrally in the explant (middle zone) after "0" and 4 weeks of culturing.

2.2. Light Microscopy (LM)

Twenty one explants in medium containing FCS, and 21 control-explants, were fixated in 10% neutral buffered

formaldehyde in saline for 48 hours and processed conventionally for LM (three explants from both stimulated and control, after "0" and 4 weeks of culturing were processed for TEM, see below). Explants were embedded in paraffin with the articular surface placed perpendicular to the surface of the paraffin block. Ten 3 μm specimens were cut from each experiment for LM. Each specimen represented the full area of the explant including the articular surface and 2 injured surfaces. The specimens were stained with Hematoxylin and Eosin (H&E).

One representative explant from each time point was used for figure legends. Representative samples were defined as free floating explants with no adherence to other explants or to the surface of the culture flask. For serum stimulated explants cultured for 4 weeks a representative sample was defined as having repair tissue on all injured surfaces.

2.3. Immunohistochemistry

Immunohistochemical studies of 3 μm paraffin specimens derived from stimulated and control-explants were carried out with use of a polyclonale sheep antibody against porcine collagenase-2 (MMP-1) (Cambio Ltd., Cambrigde, UK, Cat no: CA-1188-AS) and a mouse monoclonal antibody to rat Proliferating Cell Nuclear Antigen (PCNA) (Clone PC10, Signet Laboratories, MA, USA). The PCNA antibody recognizes PCNA from all vertebrate species.

Parafin was removed from specimens with xylen and re-hydrated with decreasing concentration of ethanol. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide. Before incubation with the primary antibodies, sheep anti porcine collagenase antibodies were absorbed with normal goat antiserum (DAKO, Code No. X0907) and specimens were immersed 3 hours in 10% normal goat serum to block nonspecific reactions. Labeling was visualized with DAKO's ChemMate Detection kit (Code No. K 5001) using streptavidin/peroxidase in accordance with the manufacturer's instructions.

Images of each stained sections were captured with a digital camera (Nicon Coolpix 955) attached to a Leitz Laborlux S microscopy.

2.4. Transmission Electron Microscopy (TEM)

Three FCS stimulated explants (4 weeks of growth) and three control explants (0, one sample and 4 weeks, two samples) of growth were processed for TEM. The specimen was cut with two razor blades into small pieces (about 1 mm) and fixated overnight in 2.5% glutaraldehyde (Merck) in Dulbecco's Phosphate Buffered Saline (PBS) (Sigma Aldrich). Post-fixation was done in 2% osmium tetraoxide for 1 hour and samples were embedded in Epon resin. Semi-thin sections were stained with toluidine blue to facilitate the orientation.

Subsequently, ultra-thin sections were mounted onto 150-mesh copper grids and stained with uranyl acetate/lead citrate before examination in a Transmission Electron Microscope (TEM) (Phillips EM208, Philips; Eindhoven, The Netherlands).

2.5. Semi-Quantitative Analysis of Proliferating Chondrocytes

After final cell culturing of explants, analysis was made on 2 × 21 explant of the numbers of proliferative chondrocytes at the injured surfaces from weeks "0", "1", and "4 weeks" (+/−FCS). Seven H&E stained sections from each explant were photographed on a Leitz Laborlux S microscopy at magnification ×100 with a Nicon Coolpix camera model 995. Each digital picture representing one injured surface involving the upper-lower cartilage zones was transfer to a photo-editor program (Corel Photo House/Print House, Corel, USA) and scaled to full size on the monitor, with the articular surface pointing upwards and the injured surface perpendicular to the ground.

From the upper-middle cartilage zones, each injured surface was divided into 7 sections measuring 200 μm (length) × 200 μm (deep) (**Figure 1A**). Seven sections correspond (7 × 200 μm = 1, 4 mm) to the distance measured from the articular surface to approximately the middle cartilage zone (see **Figure 1A**).

Total numbers of chondrocytes (with defined nucleus) and the numbers of lacunae were counted. Total number of proliferative chondrocytes per lacuna was determined: total numbers of chondrocytes pr 0.04 mm^2 (200 μm × 200 μm) recorded, divided by numbers of lacunae recorded pr. 0.04 mm^2.

The lower hyperthropic zone was not included in the analysis. This was due to technical problems in defining each lacuna after culturing.

Numbers of proliferative condrocytes were analysed according to time and FCS treatment by a factorial ANOVA design applied to mean number of condrocytes/lacuna (n = 7) within each explant. Weeks (week 0, 1 and 4) and FCS treatment (−FCS, +FCS) served as main factors. Tests employ a significance level at 5% adjusted for type I error rate by the Bonferoni method in post hoc multiple comparisons of means. In total, forty nine (49) observations were made for each data point.

2.6. Semi-Quantitative Analysis of Repair Tissue

Repair tissue at the injured surfaces was divided into 200 µm zones as shown in **Figure 1**. Chondrocytes in seven different zones were counted by using the method described above. In total, 14 observations were made for each data point. Mean value and standard derivation were calculated for each data point as shown in **Figure 6**.

3. Results

3.1. Light Microscopy

Before culture (time "0"): the degree of structural anisotropy of porcine immature cartilage is low (**Figure 1A**). Chondrocytes are organized into 3 layers: the upper zone (A: superficial zone), middle zone (B: transitional zone), and lower hyperthropic zone (C: radial zone) (**Figure 1A**). During culture new zones develop close to the surfaces injured by cutting with a scalpel. Chondrocyte polarization increases towards the injured surfaces (**Figures 1B-D**).

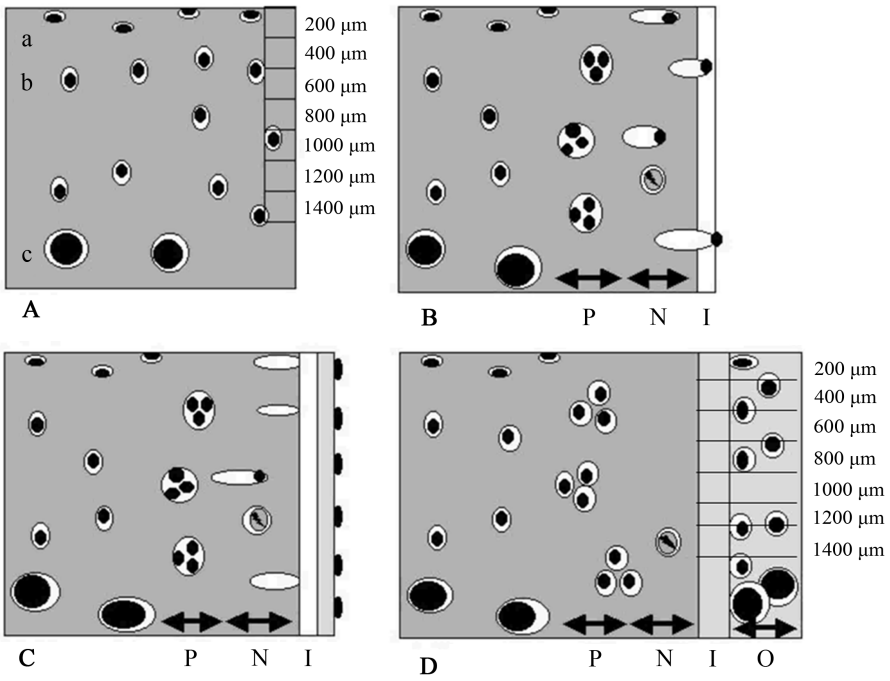


Figure 1. Model of cartilage repair mechanism in an explant system. A. Porcine cartilage explant before culturing (time "0"): a. upper cartilage zone; b. middle cartilage zone; c. lower hyperthropic cartilage zone. Squares, measuring 200 µm × 200 µm, indicates sections at the injured surface in which chondrocyte proliferation was determined; B. Stimulated explant cultured 1 week. Proliferation of chondrocytes in the proliferative zone (P) and migration of chondrocytes in the zone of necrosis (N), towards the injured surface. Matrix molecules are released from the injured surface, which cause the interface repair zone (I); C. Stimulated explant cultured 1 - 4 weeks. Chondrocytes have penetrated the interface repair zone and further repair tissue is synthesized towards this zone; D. Stimulated explant cultured 4 weeks. The outer repair tissue (O) contains hyperthropic chondrocytes in the lower zone. Chondrocytes in the proliferative zone (P) are separated from each other during matrix secretion and further remodeling. Zones (200 µm) in outer repair tissue relates to quantitative determination of chondrocytes.

3.2. The Proliferative Zone

After 1 week of culture, a zone (50 - 100 µm wide) was identified with lacunae containing two or more chondrocytes (proliferative) in all explants. This zone continued to develop during 4 weeks of culture (**Figure 2C**). Lacunae with proliferative chondrocytes appeared in larger number in stimulated explants (**Figure 6**) than in controls (**Figure 2C**). The proliferative zone was present at a distance of 40 - 80 um from the injured surface (**Figure 3A**, **Figure 2D**). This zone was prominent in the upper middle region of cartilage and was followed by a zone of lacunae containing single chondrocytes (zone of necrosis) closer to the surface (**Figure 2D**). Proliferation of chondrocytes was also evident in the lower hyperthropic zone but their number was not determined. After 4 weeks of culture, several explants showed major chondrocyte proliferation deeper into the explant (200 - 300 µm measured from the surface).

3.3. Semi-Quantitative Analysis

Number of chondrocytes per lacuna appearing over 3 time periods of FCS treatment (weeks 0, 1, 4) are shown in **Figure 6**. Results of ANOVA revealed a significant effect of time on FCS treatment ($F_{1, 79} = 17, 4$). Multiple comparisons showed different chondrocyte mean values among all explant groups ($p < 0.001$). These results show that proliferation increases at the injured surface after the first week in culture and continues by extending the time of culture with or without addition of FCS to the medium.

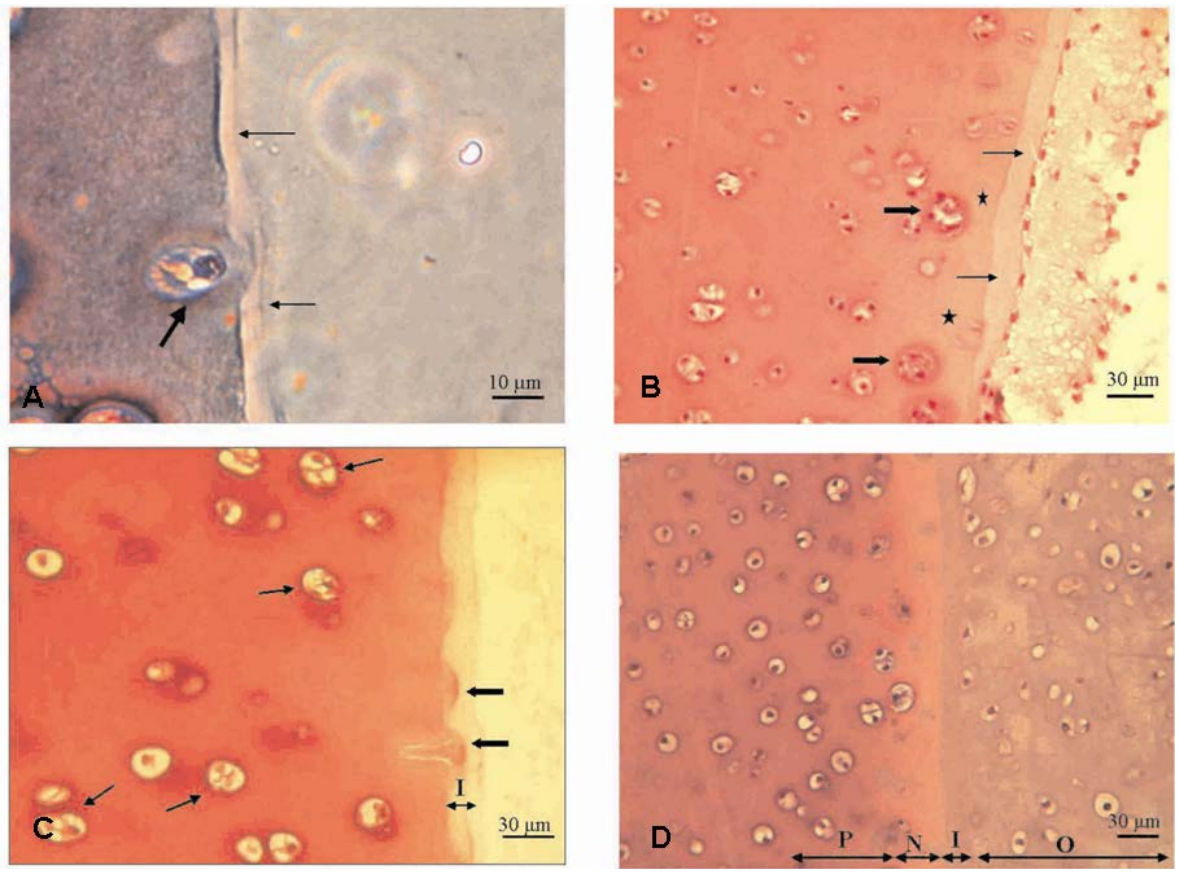


Figure 2. A. Stimulated explant cultured for 1 week. A migratory chondrocyte is penetrating the interface repair zone. A corresponds to **Figure 1B** in the cartilage repair model; B. Stimulated explant cultured for 4 weeks. Proliferative chondrocytes (bold arrow), the interface repair zone (thin arrow) and empty lacunae (asteric) close to the injured surface are visible. The outer repair tissue is reduced in this explant sample. B corresponds to **Figure 1C**; C. Control explant cultured for 4 weeks. Chondrocytes show budding at the injured surface (bold arrow) into a faintly stained interface repair zone (I). Chondrocytes also show proliferation (thin arrow) in the explants; D. Stimulated explant cultured for 4 weeks. This explant sample shows a repair tissue resembling hyaline cartilage. The four different zones (P, N, I and O) are highly visible. D corresponds to **Figure 1D**.

3.4. Zone of Necrosis

The zone of necrosis was 40 - 80 µm broad and stretched from the upper to the lower parts of the explant after 1 - 4 weeks of culture. This zone, present in both stimulated and control explants, contained numerous oval shaped lacunae orientated towards the injured surface (**Figure 3A**). However, stimulated explants contained a much larger number of such oval shaped lacunae. With time the number of oval shaped lacunae containing chondrocytes increased in all explants. Stimulated explants were covered by a zone of repair tissue (**Figure 2B** and **Figure 3A**) whereas in control explants chondrocytes were only budding through the injured surface after 1 - 4 weeks (**Figure 2C**).

Pycnotic nuclei or empty lacunae (indicative of dead chondrocytes) were seen occasionally in both stimulated and control explants (**Figure 3A**).

3.5. Repair Zones

In stimulated explants an interface repair zone appeared that was invaded by chondrocytes after 1 - 4 weeks (**Figure 2A**) and increased in thickness with the appearance of a layer of cells involving all zones of the injured surface (**Figure 2B**). After 4 weeks of stimulation, further repair tissue (outer repair tissue) developed towards the interface repair zone.

In control explants this interface repair zone appeared as a thin (about 10 - 20 µm) slightly eosinophilic and amorphous matrix substance.

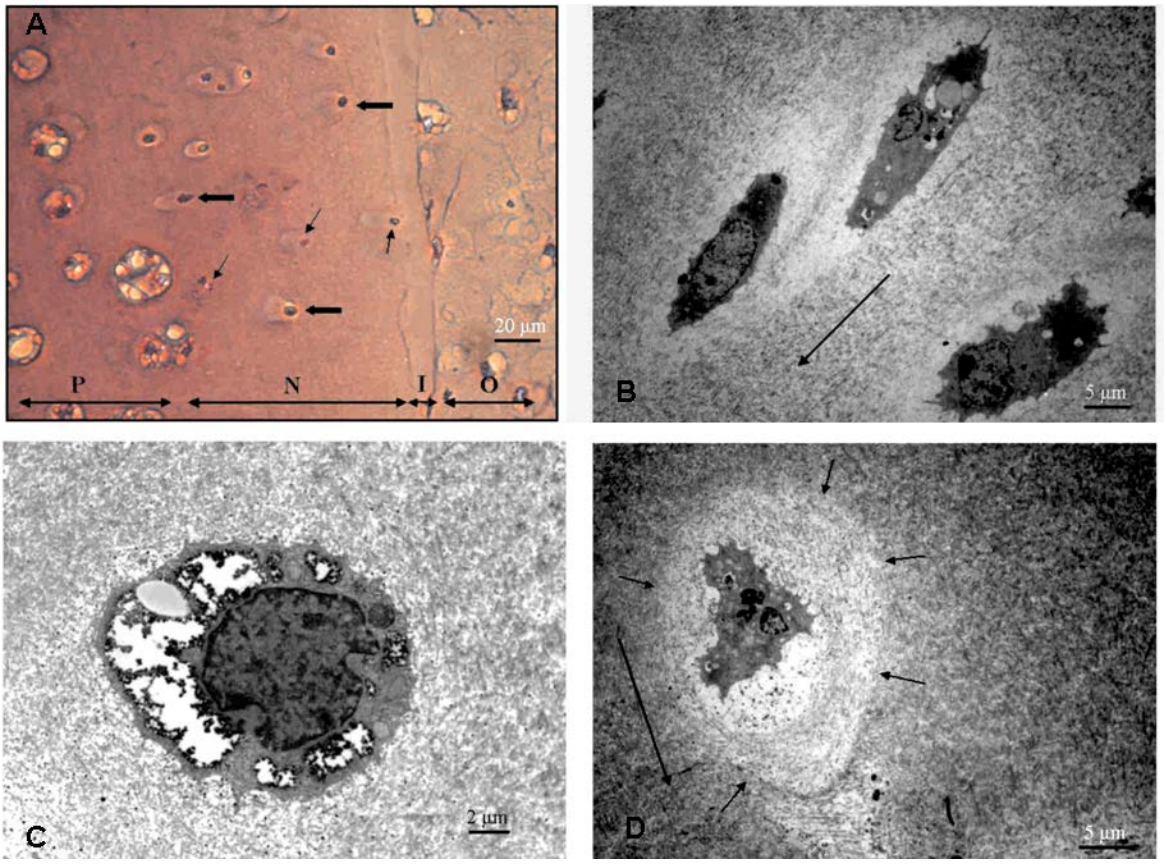


Figure 3. A. Stimulated explant cultured for 4 weeks showing; the proliferative zone (P), the zone of necrosis (N), the interface repair zone and the outer repair tissue (O). This explant contains many migratory chondrocytes at the injured surface. A corresponds to **Figure 1D**; B. (TEM) Stimulated explant; showing migratory chondrocytes in the zone of necrosis. The arrow indicates direction of migration. B corresponds to **Figure 1B** and **Figure 1C**; C. Chondrocyte located centrally in stimulated explants; D. Stimulated explant; a migratory chondrocyte in the zone of necrosis is moving towards the injured surface (long arrow). Matrix outside the lacuna (short arrows) is significantly dissolved. D corresponds to **Figure 1B** and **Figure 1C**.

All stimulated explants cultured for 4 weeks developed repair tissue at the injured surfaces emphasizing that these cartilage pieces gives fairly reproducible results in terms of studying repair tissue. However, a few explants (3) from this group did not develop repair tissues at the upper zones.

Semi-quantitative analysis of repair tissue revealed an increase from 8 cells (mean) in the upper zone to about 43 cells (mean) in the lower zone indicating that the middle-lower zones has a higher capacity for development of repair tissue (**Figure 7**).

3.6. The Articular Surface of Explant

Proliferation of chondrocytes below the articular surface was limited in both stimulated and control explants. Neither repair tissue nor chondrocytes were present on the articular surface.

3.7. Immunohistochemistry

A strong signal for collagenase was seen in all explants stimulated with FCS. The migratory zone but also the proliferative zone and areas deeper into the cartilage revealed some staining signals. Areas stained for collagenase showed an oval staining pattern with diameters of up to 50 - 100 µm (**Figure 4A**).

A weak staining for collagenase was also observed in the prolifeative and migratory zones of control-explants, cultured 1 - 4 weeks (data not shown). Control explants (t = 0) were without staining for collagenase.

Intense nuclear staining for PCNA was observed in the proliferative zone of stimulated explants. Staining was observed within expanded lacuna containing chondrocytes. Numerous chondrocytes were also stained in the outer repair zone while a few chondrocytes were stained deeper into the cartilage (**Figure 4C**). A low number of lacunas in the proliferative zone of control explants (cultured 1 and 4 weeks) showed also staining for PCNA (data not shown).

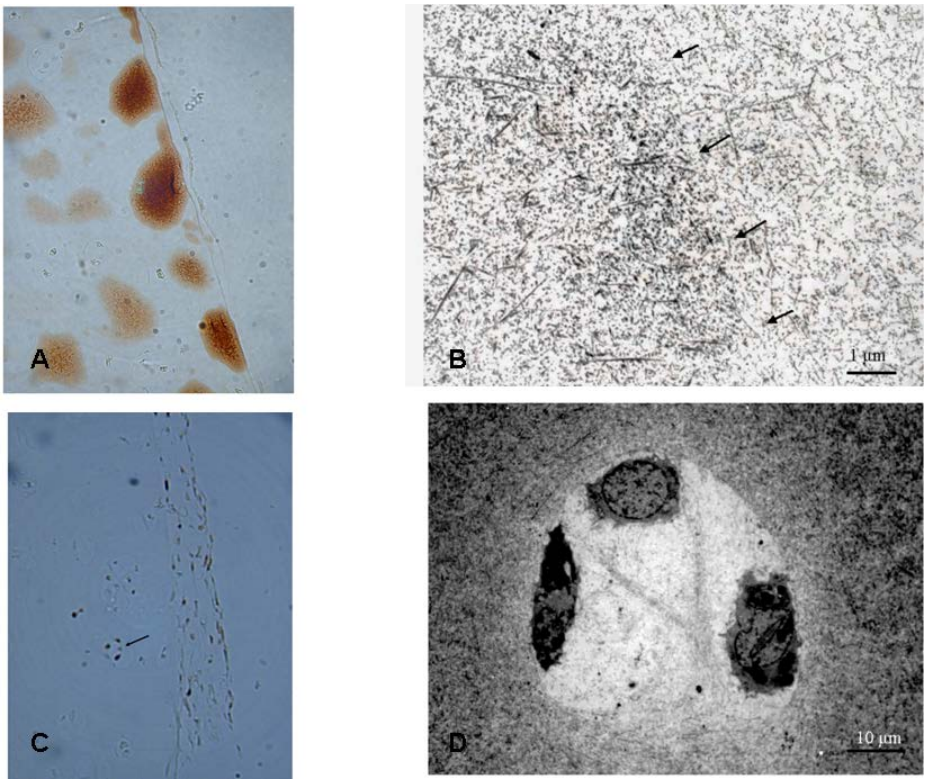


Figure 4. A. Immunostaining of collagenase-2 in cartilage; B. Stimulated explant; the interface repair zone shows merging of new and existing (arrows) tissue at the injured surface. B corresponds to **Figure 1D**; C. Immunostaining of PCNA in chondrocytes; D. Stimulated explant; showing proliferative chondrocytes (the proliferative zone) in an expanded lacuna. D corresponds to **Figure 1C** and **Figure 1D**.

3.8. Transmission Electron Microscopy

Toluidine blue stained sections were selected to study the different zones described above.

3.9. Before Culture (Time "0")

A typical feature of porcine immature cartilage was an almost random distribution of chondrocytes in a homogeneous matrix substance that was not structured in compartments or regions. Collagen type II heterofibrils were randomly oriented in the cartilage (diameters of about 80 - 120 nm, 60 nm D-banding pattern, fibril length up to 8 μm). Chondrocytes appeared predominantly ellipsoidal in shape and contained filopodia that protruded into the pericellular matix of the cartilage. Chondrocytes contained large nuclei and appeared normal (data not shown).

3.10. The Proliferative Zone (after 4 Weeks)

The proliferative zone showed from 2 - 6 proliferative chondrocytes per lacuna in treated explants (**Figure 4D**) whereas in control explants, only 2 - 3 chondrocytes per lacuna were found. Individual chondrocytes contained numerous lysosomes, glycogen granules, fat droplets, and rough endoplasmic reticulum (**Figure 4D**).

In this zone the lacunae in all explants were highly expanded. For example, lacunae containing 3 chondrocytes were about 20 - 25 μm in diameter while those with a single chondrocyte located centrally were approximately 10 um in diameter (**Figure 3C**). The pericellular matrix of expanded lacunae was partly dissolved (**Figure 4D**). Within the dissolved zone, although no type II collagen heterofibrils were seen, filaments and larger electron dense components were present.

3.11. The Zone of Necrosis (after 4 Weeks)

The pericellular matrix of oval shaped lacunae was dissolved in a zone of about 5 - 10 μm from the chondrocyte surface. Within this zone only thin filaments were seen (**Figure 3B**, **Figure 3C**). Chondrocytes present in oval shaped lacunae in both stimulated and control explants contained numerous lysosomes and rough endoplasmic reticulum (**Figure 3B**, **Figure 3D**).

3.12. Repair Zones

In control explants the interface repair zone was composed of type II collagen hetero-fibrils and aggregates of matrix components. Fibrils had diameters of about 20 - 40 nm and lengths up to 3 μm and contained a regular D cross-banding pattern (periodicity of approximately 60 nm). In stimulated explants a similar cell-free interface repair zone was seen towards the injured surface (**Figure 4B**). Thin type II collagen hetero-fibrils and aggregates of matrix components were identified.

In all explants the interface repair zone showed merging with the matrix at the injured surface (**Figure 4B**). The continuous layer of cells towards the interface repair zone contained chondrocytes with Golgi complex, lysosomes, secretory vacuoles and vesicles, and a prominently developed rough endoplasmic reticulum (**Figure 5B**).

Highly contrasted globular aggregates of matrix components were seen at the protrusions of the plasma membrane. These aggregates were associated with collagen hetero-fibrils (**Figure 5B**).

The outer repair tissue consisted of single cells in lacunae surrounded by a matrix similar to the interface repair zone indicating that these cells are still chondrocytes after 4 weeks of culture.

4. Discussion

In this paper we report that chondrocytes migrate in intact cartilage and that repair tissue binds to injured cartilage in porcine articular cartilage explants. These observations have not been reported before. Previous reports indicated that proliferation of chondrocytes was limited to the proliferation zone during *in vivo* repair of immature, mature, or osteoarthritic articular cartilage [26]-[32].

Proliferation of chondrocytes in explants was primary estimated by semi-quantitative method using histology but also immunohistochemistry with a monoclonal antibody towards PCNA demonstrated proliferation, within expanded lacuna in the proliferative zone and chondrocytes in the outer repair zone (**Figure 4C**).

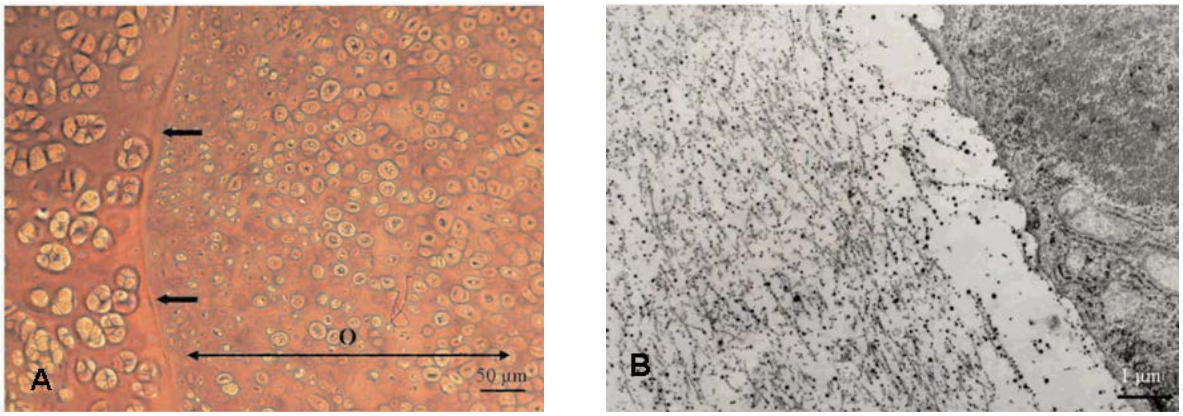


Figure 5. A. Stimulated explant cultured for 4 weeks. Hyperthropic chondrocytes are present in the outer repair tissue (O). The interface repair zone (bold arrow) is hardly visible. A corresponds to the lower hyperthropic cartilage zone in **Figure 1D**; B. Stimulated explant; extensive chondrocyte secretion of matrix proteins at the transition of the interface repair zone and the outer repair tissue. B corresponds to **Figure 1D**.

Chondrocytes proliferated into the proliferative zone in both stimulated and control explants but the number of chondrocytes per lacuna in stimulated explants were significantly higher after both 1 and 4 weeks of culture. This proliferation of chondrocytes in control explants must be induced either by metabolites in the medium or by endogenous mitogenic factors released during mechanical injury [33]. The higher number of chondrocytes per lacuna in stimulated explants suggests that FCS functions as an additional stimulant.

With regard to cell proliferation, which was essentially determined by the number of cells/lacunae, one might assume that this counting under-represent the numbers of proliferating cells as cells that are in initial stages of cell cycle would not be included.

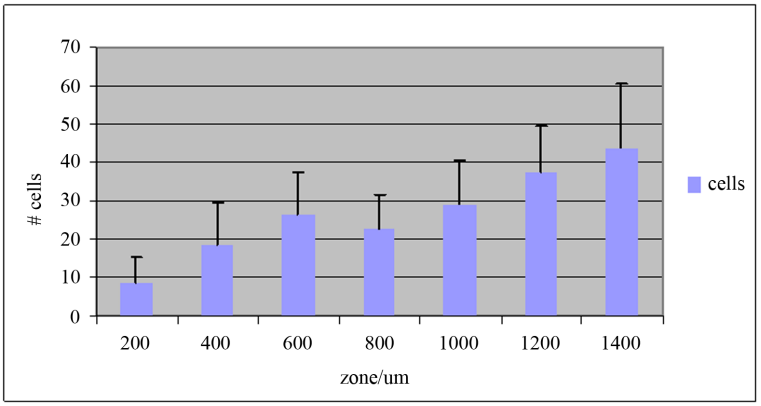
Previous failures to observe chondrocyte migration and binding of repair tissue to the defect surface have been suggested to be due to chondrocyte death in the zone of necrosis [34]. Alternatively the absence of repair tissue has been attributed to the composition of the growth medium even when FCS is added. Chondrocyte death in the region of the injured surface results from increased pressure that induces apoptosis and necrosis. When cartilage is removed with a trephine, significant cell death occurs at the injured surface [21] [35]. This can be avoided by using a sharp scalpel thereby favoring the migratory zone over the necrotic zone at this surface. The clinical relevance of this observation suggests that attention to this detail should be paid when removing wounded cartilage during transplantation procedures.

With regard to binding repair tissue at the defect surfaces we observed that some stimulated explants were not covered with repair tissue at the upper zones, after 4 weeks of culturing. As we did not perform quantitative analysis of cell behavior such as cell death and cell migration, we can only speculate that missing repair tissue could relate to cell damage when cutting through the articular surface with a scalpel.

Quantitative analysis indicated that there were a higher number of cells in the repair relating to the middle-lower zone (**Figure 7**). This result most likely reflected the fact that the corresponding zones in the explants contain a higher number of chondrocytes compared to the upper zone.

Chondrocyte migration to the injured surface is the most critical aspect of the repair process. Since the migratory pattern was polarized in this direction in both stimulated and control explants (**Figure 3A**, **Figure 2C**) it appears that chondrocytes are sensitive to chemotactic factors entering the matrix from the culture medium or released from the injured surface [34] (Vincent *et al*., 2002). Several mitogens and matrix molecules have been reported to act as chemoattractants for chondrocytes. These include IGF [36], BMP [37], HGF/SF [38], fibronectin, type I and type II collagens [39] and bFGF [40]. The growth factors including fibronectin are potentially present in medium containing FCS. It was therefore surprising to find that control explants cultured without FCS also showed chondrocytes migrating close to the injured surface. This result might indicate that these chondrocytes sense metabolites (glucose, amino acids, etc.) and vitamins as chemoattractants. Alternatively the attraction may be the result of substances released from the injured surface such as bFGF [33] and type II collagens [39].

Stimulated explants show a higher migratory response in the number of oval lacunae as well as a higher number of migratory chondrocytes on the injured surface after 1 - 4 weeks of culture, an effect attributable to



o: +FCS , •: −FCS.

Figure 6. Mean of mean numbers of proliferating chondrocytes/lacuna within explants at the injured surface according to week and −/+FCS. Sticks indicate 95% Cl of the means.

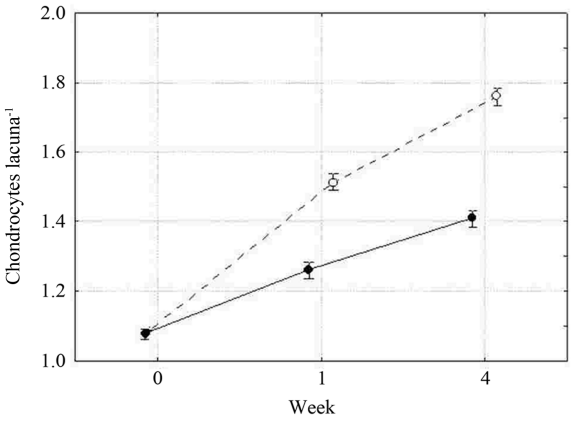


Figure 7. Numbers of chondrocytes in different zones of the repair tissue.

chemoattractants only present in FCS.

In our comparison of stimulated versus control explants one might assume that chondrocytes in control samples has a relatively lower cell viability because the medium does not contain essential metabolites and growth factors important for normal chondrocyte metabolism. Surprisingly, we did not observe differences in cell behavior and cell viability for control explants. Additionally, when a few control explants, which had been cultured for 4 weeks, were transferred to medium containing FCS numerous chondrocytes were budding from the injured surfaces after 1 week of culturing (data not shown). This emphasizes that chondrocytes remain viable at the injured surfaces in the absence of serum and that the chondrocytes are protected by the extracellular matrix.

Digestion of the cartilage matrix that is rich in type II collagen molecules is necessary but does not appear to be sufficient for chondrocytes to migrate in the matrix and bud through the injured surface. Budding chondrocytes (**Figure 2A**, **Figure 2C**) are expanding the lacunae as if trying to push into the medium. Others [41] have shown that migratory chondrocytes express a contractile actin isoform during outgrowth of enzymatic pretreated cartilage explants. It would therefore be of interest to determine if such an actin isoform is expressed by chondrocytes during their migration and the expansion of lacunae.

The EM figures involving lacunae expansion during proliferation (**Figure 4D**) and migration (**Figure 3B**, **Figure 3D**) demonstrate that chondrocytes expand the lacunae and migrate after digesting the dense matrix. As demonstrated by immunohistochemistry with a polyclonal collagenase antibody it suggests that chondrocytes produce and secrete enzymes such as collagenase-2 (MMP1) during migration and cell proliferation (**Figure 4A**).

The most important enzymes in type II collagen degradation in cartilage are collagenase-1 (MMP-1) [42] [43],

collagenase-2 (MMP-8) [43] [44], and especially collagenase-3 (MMP-13) [45]-[49]. Chondrocyte expression of matrix MMP-13 has also been reported by others [28] to participate in pericellular proteolysis during lacunae expansion. As our purchased antibody is raised against the native form of porcine collagenase-2 it is likely that this polyclonal antibody also recognize other forms of collagenase expressed in porcine cartilage.

Since both proliferation and migration take place in almost the same area, why do some chondrocytes proliferate while others show chemotactic behavior? The answer lies in matrix-chondrocyte interaction. Stimulation is controlled by the matrix [50] [51]. Disturbance of tissue-homeostasis induces local chondrocytes to respond to endogenous and/or exogenous stimuli related to repair. Chondrocytes in the zone of necrosis lose numerous matrix components (into the growth medium) and tissue-homeostasis changed dramatically. These chondrocytes are more prone to chemotactic response. In contrast, chondrocytes in the proliferative zone undergo only limited loss of matrix proteins that serve as mitotic stimuli.

The interface repair zone originates from matrix components released from the injured surface. These components may come from viable chondrocytes in the zone of necrosis. Matrix components secreted into the injured area are important for proper binding to the repair tissue [52].

Chondrocyte migration from the zone of necrosis through the interface repair zone is followed by extensive matrix synthesis towards this zone (**Figure 2A**, **Figure 2B**). Loss of matrix components at the injured surface favors chondrocyte migration, and this may improve cartilage repair.

TEM of the interface repair zone and outer repair zone showed that both were primarily composed of thin type II collagen hetero-fibrils forming a network with small and large aggregates of PGs [53] (**Figure 5B**). The 20 - 40 nm diameter of these fibrils is similar to the fibril diameter (25 - 50 nm) found in embryonic cartilage [54].

Development of large hyperthropic chondrocytes in the outer repair tissue involving the deep cartilage zone (**Figure 5A**) indicates their commitment as pre-hyperthropic chondrocytes before stimulation and migration. This development emphasizes that differentiation in repair tissue of the upper-lower zones is related to the positions of migratory chondrocytes.

Mature articular chondrocytes are resting cells with low mitotic activity. However, when injured explants are immediately transferred to cell culture, chondrocytes are spontaneously reactived for repair.

5. Conclusions

In summary, we present evidence derived from an articular cartilage explant model of two repair systems that operate independently. The first repair system is induced by the mechanical injury that stimulates chondrocytes to proliferate and lacunae to expand. The proliferation may depend on the local release of matrix bound mitogenic factors and MMPs [33] such as collagenase. The second repair system is dependent on chemotactic factors in the growth medium or released from the injured surface. The chemotactic response induces chondrocytes from upper-lower zones to migrate towards the injured surface. This is followed by cell proliferation and binding to repair tissue to the injured surface.

The clinical relevance of these repair mechanisms further depends on such factors as mechanical forces, components of synovial fluid, and the presence of subchondral bone. *In vivo* studies, rather than explants, will be required to evaluate the contributions of these additional factors.

Acknowledgements

The authors thank; Steff Houlberg, Danisco (Ringsted/Denmark) for providing articular cartilage from slaughted pigs, and Mogens Bildsøe (PhD) at ZooNox for statistical analysis.

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