Fibroblast-like synoviocyte-chondrocyte interaction in cartilage degradation

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Abstract

Objective

In vitro models for joint diseases often focus on a single cell type, such as chondrocytes in osteoarthritis (OA) or fibroblast-like synoviocytes (synoviocytes) in rheumatoid arthritis (RA). However, these joint diseases affect the whole joint and interaction between chondrocytes and synoviocytes may play an important role in disease pathology. The current study was designed to study the use of the alginate recovered chondrocyte method as a model for cartilage degradation and to study interaction between chondrocytes and synoviocytes.

Methods

Bovine chondrocytes were cultured in alginate beads for 1 week, subsequently chondrons were retrieved and seeded into transwells. Every two days cartilage-slices were analysed for proteoglycan content (colorimetric, Blyscan GAG kit), collagen content (HPLC) and collagen HP and LP crosslinking (HPLC). For degradation experiments, monocultures of cartilage-slices labelled with 35S and cocultures with synoviocytes were stimulated with IL-1β or TNF-α. After 7 days, 35S release was measured taken as a measure of cartilage degradation.

Results

After biochemical analysis, three week old cartilage-like slices were chosen to perform cartilage-degradation experiments. Synoviocytes were able to induce cartilage degradation only in the presence of living chondrocytes. In addition, the cytokines interleukin 1 (IL-1β) and tumor necrosis factor (TNF-α) were only able to induce cartilage degradation by chondrocytes, not by synoviocytes.

Conclusion

These data indicate that the alginate recovered chondrocyte method provides a novel model for cartilage degradation in which the interaction between synoviocytes and chondrocytes can be studied.

Key words

Alginate recovered chondrocytes, cartilage, cartilage degradation, rheumatoid arthritis, fibroblast-like synoviocytes, in vitro model.
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Introduction
Rheumatoid arthritis (RA) is characterised by hyperplasia and inflammation of the synovial tissue, which forms the so-called pannus tissue and degrades the articular cartilage at the cartilage-pannus junction, leading to loss of joint function and disability (1, 2). Fibroblast-like synoviocytes (synoviocytes) of the synovial lining layer are thought to play a key role in the hyperplasia of the synovial tissue and the degradation of the cartilage (3, 4). During inflammation, immune cells diffusing into the synovial fluid (SF) and synovial tissue produce inflammatory mediators that are able to stimulate cartilage degradation by synoviocytes and chondrocytes and that cause the hyperplasia of the synovial tissue. For example, macrophages produce matrix metalloproteinases themselves, but are also an important source of interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α), cytokines that stimulate matrix metalloproteinase production by chondrocytes and synoviocytes (5, 6). Although the contribution of the different cell types in joint homeostasis and pathology is generally recognized, not many studies address the interaction between the cell types involved. To study RA pathology, both in vivo and in vitro models are applied to elucidate pathways and study treatment efficacy. Of the in vivo models, the most common are collagen-induced arthritis (CIA) in rats and mice, adjuvant-induced arthritis (AIA) in rats, and the transplantation of human tissues in severe combined immune deficient (SCID) mice (7-9).

In vitro models are potentially more suitable and convenient to study cell-cell interactions. Most of the in vitro disease models that are used to study RA disease mechanisms focus on synoviocytes or the complete synovial tissue. To study synoviocyte invasive-ness, Transwell invasion systems with Matrigel™ (collagen type IV) or Invitrogel (collagen type I) are used. One of the downsides of these models is that the Matrigel and Invitrogel are both composed of different components than cartilage, which primarily contains collagen type II and proteoglycans along with minor quantities of other proteins. It is desirable to use a matrix that resembles real cartilage because different mechanisms are involved in the degradation of different proteins and tissues. Cartilage type II collagen degradation, for example, is mediated by a different panel of proteases than type I collagen in bone or skin. Therefore, to understand the mechanism involved in cartilage degradation, a matrix consisting of the same components as articular cartilage should be used. Another important aspect of cartilage degradation is the presence or absence of chondrocytes. The interaction between chondrocytes and synoviocytes may play an important role in disease pathology, since chondrocytes are able to degrade their surrounding cartilage. In addition, both chondrocytes and synoviocytes, and also infiltrating macrophages, are able to produce inflammatory mediators (e.g. IL-1β and/or TNF-α) and can be activated by these cytokines (5, 6, 10, 11).

In the present study, we investigate the use of cartilage-like slices obtained by the alginate recovered chondrocyte (ARC) method in an in vitro model for cartilage degradation. To validate the model, we investigated the interplay between chondrocytes and synoviocytes. In addition, the effect of IL-1β and TNF-α, the most important cytokines produced by macrophages, on the degradation of the cartilage-like slices by chondrocytes and synoviocytes was studied.

Material and methods
Chondrocyte isolation and culture
Chondrocytes were isolated from the metacarpophalangeal joints of 6 month old calves. Cartilage was harvested and digested with 0.2% pronase for one hour, followed by overnight digestion with 0.025% collagenase. Cells were separated from the undigested tissue using a 200 μm filter (Braun Medical, Oss, The Netherlands) and cultured in a 1:1 mixture of DMEM (Gibco-BRL, Paisley, UK) and HAM’s F12 (BioWhittaker, Versiers, Belgium), supplemented with 0.28 mM ascorbic acid (Sigma-Aldrich, Steinheim, Ger-
many), 100 IU/ml penicillin (Bionwit-thaker, Versiers, Belgium), 100 μg/ml streptomycin (BioWiththaker) and 20% foetal calf serum (FCS, GibcoBRL). After one week, beads were dissolved in sodium citrate (55 mM in 0.9% NaCl) for 20 minutes. Chondrocytes with their cell associated-matrix were centrifuged for 10 minutes at 110 g and resuspend-ed at a concentration of 300 beads per 2.5 ml of medium. 0.5 ml of cell sus-pension was transferred to the inner compartment of a 12 mm Transwell. 1.5 ml of culture medium was added in the outer compartment. This procedure results in the formation of cartilage-like slices during three weeks of culture, and two times per week slices (n = 3 per time point) were frozen at -20°C for biochemical analysis. Medium was re-freshed three times per week.

**Proteoglycan measurement**

Cartilage-like slices were digested for 24 hr at 56°C in papain buffer con-taining 3% v/v papain, 5 mM cysteine HCl, 50 mM EDTA and 0.1 M sodium acetate, the buffer was adjusted to pH 5.5. The amount of sulphated gly-cosaminoglycans (GAGs) was deter-mined in an aliquot (1 μl) of the papain digest by dimethylene blue staining (Biocolor Ltd, Belfast, N. Ireland), using chondroitin 4-sulfate as a standard.

**Collagen content and crosslinking**

A second aliquot (500 μl) of the papain digests was hydrolyzed by the addition of 500μl of 12 M HCl at 110°C for 20-24 h for cross-link and amino acid analysis. Hydrolyzates of the papain digests were dissolved in water containing the internal standards pyridoxine (10 μM; Sigma) and homoarginine (2.4 mM; Sigma). For collagen cross-link analy-sis [hydroxylysyl pyridinoline (HP) and lysylpyridinoline (LP)], samples were diluted 100-fold with 50% acetic acid and analyzed by HPLC (12). For amino acid analysis, an aliquot of the cross-link samples was diluted 25-fold with 0.1 M borate buffer (pH 11.4), derivatized with 9-fluorenylmethyl chloroformate (Fluka) and analyzed by HPLC (13). HP, LP, and hydroxylysine (Hyl) are expressed as mol per mol col-lagen, assuming 300 hydroxyproline (Hyp) residues per triple-helical colla-gen molecule (12).

**Isolation and propagation of rheuma-toid fibroblast-like synoviocytes**

Synoviocytes were obtained at joint replacement surgery or synovectomy from RA patients after obtaining their informed consent. Tissue was collect-ed in sterile phosphate buffered saline (PBS). Fat and connective tissue were removed and tissue was digested with collagenase (CLS2, Worthington Biochemical Corporation) for 2-3 hours at 37°C. Cells were then separated from the undigested tissue using a 200 μm filter (B-Braun Medical, Oss, The Neth-erlands) and cultured in 162 cm² culture flasks (Costar, Cambridge, NY, USA) with Iscove’s Modified Dulbecco’s me-dium (IMDM; Biowiththaker, Versiers, Belgium) supplemented with glutamax (Biowhittaker), 100 U/ml penicillin and streptomycin (Biowiththaker) and 10% foetal calf serum (FCS, GibcoBRL) in a humid atmosphere of 5% CO₂ in air at 37°C. Upon reaching confluence, cells were detached with 0.25% trypsin and split in a 1:3 ratio. For all experiments 3rd-5th passage v were used. Using light microscopy > 95% of cells were judged to be synoviocytes.

**Radioactive labelling with sulphate**

To establish a sensitive method for the detection of cartilage degradation of the cartilage-like slices, the matrix was labelled with ³⁵S sulphate (Amersham, Braunschweig, Germany). 0.25 μCi of ³⁵S in sulphate solution was added per ml medium during the first week of cul-ture in Transwells. During the follow-ing two weeks culture medium without the radioactive tracer was used.

**Co-culture of rheumatoid fibroblast-like synoviocytes with cartilage-like slices**

Isolated RA synoviocytes were added on top of the cartilage-like slices in the inner compartment of the Transwells in a volume of 0.5 ml of serum-free DMEM:HAM F12 medium. The outer compartment of the Transwells was filled with 1.5 ml DMEM:HAM F12 medium containing 10% FCS and 10% normal human serum (NHS) as well as penicillin/streptomycin. Radioactivity in pooled culture medium (inner + outer compartment) and cartilage-like slices was determined after 7 days of co-culture (without intermediate re-freshment of the culture medium). Car-tilage-like slices were digested in 0.5 ml Soluene (Amershamb) for 3 hours in a 50°C shaking incubator. This digest was added to 9.5 ml of UltimaGold scintillation fluid (Amershamb) and radioactivity was measured by a liquid scintillation analyzer (Tri-Carb 1900 CA, Packard). The same was done for 0.5 ml of medium. The percentage of matrix degradation was calculated as: \[ \frac{\text{dpm}_{\text{medium}}}{\text{dpm}_{\text{medium}} + \text{dpm}_{\text{matrix}}} \times 100\% \]

**Statistical analysis**

For statistical analysis, means and standard deviations were calculated. Differences between conditions were tested for statistical significance using the Kruskall-Wallis test with a post hoc Mann-Whitney U-test. Differences were considered statistically significant at \( p < 0.05 \). All statistical analyses were performed using SPSS 11.5 Software (SPSS, Chicago, IL).

**Results**

**Biochemical analysis of cartilage-like slices**

Cartilage-like slices were analysed biochemically to study their resemblance to native cartilage. Some known differ-ences with native cartilage were ob-served, probably due to the relatively high cell numbers and relatively short culture period (Table 1). As expected, the cellularity of the matrix was high-er in cultured slices compared to the amount in native cartilage (2.74 ± 0.24 μg DNA/mg tissue after 3 weeks of culture and 0.24 ± 0.05, respectively) (Fig. 1A). To determine the amount of extracellular matrix that was formed, the levels of proteoglycans (assessed as GAG) and collagens (assessed as hydroxyproline) were measured. The amount of GAG/dry weight increased from 26.6 ± 3.6 μg/mg immediately after transfer to Transwells, to around 90-100 μg/mg after 9 days of culture (Fig. 1B). After these 9 days in culture, the amount of GAGs was relatively
stable, but still lower than in normal articular cartilage (145.4 ± 96.4). In addition, normal bovine articular cartilage contained more collagen per dry weight (550 ± 163 μg/mg) than the slices in culture (112 μg/mg) (Fig. 1C). The amount of collagen per total dry weight increased in the first week after the transfer to Transwells and remained relatively stable from thereon (Fig. 1D).

As a measure of the quality of cartilage, often the ratio of GAGs per collagen is determined. The level of GAG/collagen in normal bovine articular cartilage was 0.3 (± 0.08) μg/μg. After an initial drop in GAG/collagen content, when chondrocytes were transferred from alginate to the Transwells system, an increase in GAG/collagen was observed (Fig. 1E). After 7-10 days in Transwell culture, the level of GAG/collagen remained relatively stable at approximately 0.9 μg/μg (three times higher than in native cartilage). As another measure of the amount of collagen per total protein, the amount of hydroxyproline (a collagen-specific amino acid) was compared to the amount of proline (which is present in all proteins). The ratio of hydroxyproline over proline is 0.72 (± 0.16) pmol/pmol in native articular cartilage, while in our in vitro engineered cartilage-like slices this ratio was 0.6 times lower. After 2 days of culture in Transwells, the ratio was 0.43 (± 0.01) and remained stable thereafter (Fig. 1F).

Two measures of the proper post-translational modification of the newly formed collagen are the level of hydroxylation (hydroxylysine/collagen) and the ratio of HP over LP crosslinking. The amount of collagen modification is an indication of the quality of the collagen that is formed. In our cartilage-like slices we found a lower ratio of HP/LP crosslinking than that found in normal bovine articular cartilage (around 17-18 and 59 ± 5.4, respectively) (Fig. 1H). The ratio of crosslinking in the cartilage-like slices was still increasing, however. The number of hydroxylysine residues per collagen was 63 (± 6.2) pmol/pmol collagen in normal articular cartilage, while in our in vitro engineered cartilage-like slices it was around 77 pmol/pmol collagen (Fig. 1G). This indicates that the lysyl hydroxylation of the collagen type II resembles that of normal bovine articular cartilage.

### Table I. Biochemical analysis of native bovine cartilage compared to in vitro engineered cartilage-like slices after 3 weeks of culture in Transwells, the time at which they are used for co-culture and stimulation experiments.

<table>
<thead>
<tr>
<th></th>
<th>Native bovine cartilage</th>
<th>Cartilage-like slices:</th>
<th>Statistics (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA/dry weight (μg/mg)</td>
<td>0.24 ± 0.05</td>
<td>2.74 ± 0.24</td>
<td>0.036</td>
</tr>
<tr>
<td>GAG/dry weight (μg/mg)</td>
<td>145.4 ± 24.9</td>
<td>96.4 ± 8.3</td>
<td>0.036</td>
</tr>
<tr>
<td>Collagen/dry weight (μg/mg)</td>
<td>549.1 ± 162.7</td>
<td>112.5 ± 12.8</td>
<td>0.036</td>
</tr>
<tr>
<td>GAG/collagen (μg/μg)</td>
<td>0.28 ± 0.08</td>
<td>0.87 ± 0.15</td>
<td>0.036</td>
</tr>
<tr>
<td>Hydroxyproline/proline (pmol/pmol)</td>
<td>0.72 ± 0.16</td>
<td>0.44 ± 0.02</td>
<td>0.036</td>
</tr>
<tr>
<td>Hydroxylysine/collagen (pmol/pmol)</td>
<td>62.9 ± 6.2</td>
<td>77.0 ± 1.2</td>
<td>0.036</td>
</tr>
<tr>
<td>Ratio HP/LP</td>
<td>59.1 ± 5.4</td>
<td>16.7 ± 1.0</td>
<td>0.036</td>
</tr>
</tbody>
</table>

**Co-culture of cartilage-like slices with RA synoviocytes**

To study the interaction between chondrocytes and synoviocytes, RA synoviocytes were cultured on top of
of the cartilage-like slices for 7 days. After 7 days of co-culture without medium refreshment, increased \( ^{35} \text{S} \) release was observed when the RA synoviocytes were co-cultured with living chondrocytes (123 ± 27\%, n = 7, p = 0.035), indicating an increased cartilage matrix degradation by chondrocytes, synoviocytes, or both (Fig. 2). To investigate whether the increased cartilage release was caused directly by active synoviocytes or resulted from the interaction between synoviocytes and chondrocytes, synoviocytes were seeded on cartilage in which chondrocytes were freeze-killed. After the culturing of synoviocytes on cartilage with killed chondrocytes, no increase in \( ^{35} \text{S} \) release was seen (97 ± 18\%, n = 7, p = not significant), indicating that an interaction between synoviocytes and living chondrocytes is essential for the synoviocyte-induced cartilage degradation of live cartilage.

In addition, the effect of IL-1\( \beta \) and TNF-\( \alpha \), both macrophage-derived cytokines, was studied (Fig. 3). Stimulation of living cartilage-like slices with IL-1\( \beta \) and TNF-\( \alpha \) resulted in an upregulation of \( ^{35} \text{S} \) release of 250 ± 110\% (n = 7, p = 0.004) and 190 ± 64\% (n = 7, p = 0.004) respectively. No additional effect of synoviocytes was seen (238\% ± 99 and 200\% ± 75 respectively) (Fig. 4). This absence of induction by synoviocytes could be caused by two factors: (i) there are 40-50 times more chondrocytes than synoviocytes in each culture, so the induction of degradation by synoviocytes could be lost in the response by the chondrocytes, or (ii) the amount of IL-1\( \alpha \) and TNF-\( \alpha \) added to the culture has reached a threshold level so the cytokines produced by the synoviocytes do not have additional effect. When synoviocytes were seeded on cartilage with freeze-killed chondrocytes, again no increase of \( ^{35} \text{S} \) release was seen. Addition of IL-1\( \beta \) and TNF-\( \alpha \) to the culture of synoviocytes on dead cartilage also had no effect on \( ^{35} \text{S} \) release.

Together these results show an interplay between synoviocytes and chondrocytes in the breakdown of cartilage, as well as a strong induction of chondrocyte-mediated cartilage-breakdown by the proinflammatory cytokines IL-1\( \beta \) and TNF-\( \alpha \).

**Discussion**

In the present study we describe the use of a novel, medium-throughput version of the alginate recovered chondrocyte method employing human synoviocytes and bovine chondrocytes. In view of its relevance for human pathology the use of human cells is preferred over animal cells, but adult human articular cartilage is difficult to come by. Bovine articular chondrocytes, on the other hand, are available in virtually unlimited quantities and are widely accepted and used as a substitute for human articular chondrocytes in in vitro studies (14-16). Therefore, in this model we combined human fibroblasts with bovine articular chondrocytes, making it possible to test many different conditions of cartilage destruction. Our in vitro engineered cartilage resembled native bovine cartilage quite well, although in our system as well the actual amount of collagen was relatively low, a known problem in cartilage tissue engineering. The ratio of HP/LP crosslinking was lower in our cultured cartilage, resembling the level of crosslinking ratio in adult human cartilage rather than young bovine cartilage (unpublished observation).

The major benefits of our in vitro engineered cartilage over existing in vitro models are the composition of the matrix, which resembles real cartilage with its most abundant components being collagen type II and proteoglycans, and the presence of chondrocytes instead of fibroblasts.
of a collagen type I or type IV matrix without cells. One of the reasons collagen type II is preferred is because while several MMPs are able to degrade the collagen type IV present in Matrigel, the most important MMPs in the degradation of cartilage are the collagenases MMP-1 and MMP-13. MMP-1 and -13 are the only MMPs that are able to initiate the degradation of collagen type II (17). MMP-1 can be produced by both synoviocytes and chondrocytes, while MMP-13 is characteristically produced by activated chondrocytes (17-20). In addition, the breakdown of proteoglycans may be a crucial step in cartilage degradation; *in vivo* data showed that active ADAMTS-5, an enzyme involved in proteoglycan degradation, was essential for cartilage degradation in an osteoarthritis mouse model (21). This again emphasizes the importance of including all components of native cartilage.

Previously, Pap et al. (11) showed that the storage of cartilage, or the inhibition of chondrocyte protein synthesis, reduced the degradation of cartilage matrix in the presence of synoviocytes. Our study confirms these data by showing that synoviocytes are unable to degrade *in vitro* engineered cartilage in the absence of live chondrocytes. Some existing explant models use cartilage explants in combination with synoviocytes or macrophages (6, 22). The drawback of using explants of cartilage slices is that the cartilage often has to be stored, which influences the reaction of chondrocytes. In addition, because the matrix synthesis rate in our engineered cartilage is greater than in cartilage explants, the amount of radioactive tracer molecules incorporated into the proteoglycans of the matrix is higher. Therefore the sensitivity in our model will be greater than in models using cartilage explants.

IL-1β is an important mediator of RA and a known stimulator of both chondrocytes and synoviocytes (10, 23-26). In chondrocytes it upregulates the production of MMP-1 and MMP-13 (26) and in synoviocytes IL-1β induces the production of inflammatory cytokines and pro-MMP-1 and also stimulates proliferation (27). IL-1β is mainly produced by monocytes/macrophages, but it has also been suggested that IL-1β may play a role in the interaction between synoviocytes and chondrocytes (11). Neidhart and colleagues found that the inhibition of IL-1β in a co-culture of chondrocytes with synoviocytes inhibited the degradation induced by IL-1β, also indicating a role for IL-1β in the interaction between chondrocytes and synoviocytes (10). Although we found an effect of IL-1β on chondrocytes, we did not find an additional effect on synoviocytes. This could be the result of the difference in cell numbers (around 6x10⁶ chondrocytes versus 1x10⁵ synoviocytes in our model) or because of the relatively high concentration of IL-1β added to the culture. Possibly, chondrocytes are already maximally activated by IL-1β, so the addition of synoviocytes cannot induce any increase in cartilage degradation.

Similar to IL-1β, TNF-α plays an important role in RA pathology. While IL-1β is thought to play a role in the later stages of disease, TNF-α is predominantly detected in the early stages (2). TNF-α plays an important role in the invasive behaviour, proliferation and cytokine production of synoviocytes. In addition, it downregulates matrix production and upregulates cytokine and MMP production in chondrocytes (28, 28-31). In our model, TNF-α is able to induce cartilage degradation comparable to IL-1β. Again no additional stimulation of cartilage degradation was observed in the co-culture setup, suggesting that TNF-α might play a role in our co-culture system similar to that of IL-1β and similar to its role in RA pathology. This indicates that TNF-α could be one of the cytokines through which synoviocytes and chondrocytes communicate with each other and with macrophages. Since macrophages are the main producers of both IL-1β and TNF-α, the role of macrophages in cartilage degradation should not be neglected. Therefore it seems that in models such as the one described in this paper, the inclusion of macrophages or the addition of cytokines produced by activated macrophages could lead to a further optimization of the model.

Although the model described here was designed to study the direct interaction of synoviocytes and chondrocytes in RA, it can easily be adjusted to study cartilage degradation in osteoarthritis. In the current view, in RA the contact of the pannus tissue with the articular cartilage is responsible for the invasion and degradation of the articular cartilage (18). In contrast, it is thought that in osteoarthritis, chondrocytes are primarily responsible for cartilage degradation (32). A hyperplastic synovium however is also found in patients with osteoarthritis; therefore the interaction between chondrocytes and synoviocytes might play a role in osteoarthritis, but perhaps through soluble factors rather than cell-cell contact (33). In concordance with such a view, synoviocytes could be separated from the chondrocytes in our model by seeding them in the inner compartment of the transwell. Using this procedure, interaction via soluble factors remains possible while cell-cell contact is absent.

In conclusion, the use of the alginate-recovered chondrocyte method provides a novel model for cartilage degradation. Comparable to the findings in other *in vivo* models and *in vitro* models with real cartilage, in this model the role for synoviocytes in cartilage degradation in RA seems to be dependent on the presence of living chondrocytes. Moreover, the addition of proinflammatory cytokines stimulated cartilage breakdown only when living chondrocytes were present, but not in the presence of living synoviocytes. Therefore, the effect of cytokines on this interaction seems to be mostly directed to chondrocytes. This indicates that in choosing models to study cartilage degradation, it is crucial to utilise a matrix resembling real cartilage with living chondrocytes and to include more than one cell type, especially since our data suggest that synoviocytes might not be able to degrade cartilage themselves.

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