ABSTRACT. Objective. One of the most prominent alterations that characterizes osteoarthritic cartilage damage is a reduction of proteoglycan content, reflecting an imbalance between synthesis and release of proteoglycans. Both synthesis and release depend on the activity of cartilage cells. Chondrocytes in the upper layer of moderately osteoarthritic human knee cartilage appear to be phenotypically altered, including diminished proteoglycan synthesis. Transforming growth factor-β (TGF-β) as a multifunctional growth factor has differential effects believed to depend on the differentiation stage of the target cells. We tested the effect of TGF-β on phenotypically altered chondrocytes in osteoarthritic cartilage.

Methods. Human articular cartilage was cultured 4 days with or without TGF-β. Proteoglycan synthesis was determined by 35S sulfate incorporation (biochemically and by autoradiography) for the upper and deep layer separately.

Results. Osteoarthritic cartilage proved more sensitive to TGF-β than normal cartilage. Proteoglycan synthesis of osteoarthritic cartilage was stimulated significantly more by 5 ng/ml TGF-β than normal cartilage. For normal cartilage this increase was equally divided among the upper and deeper layer of the explants. For osteoarthritic cartilage the increase in proteoglycan synthesis could largely be attributed to the upper layer. Autoradiography revealed that the relative 35S sulfate incorporation in the cell clusters, present in the upper layer of osteoarthritic cartilage, was significantly increased upon the addition of TGF-β.

Conclusion. Osteoarthritic cartilage is more sensitive to TGF-β than normal cartilage because phenotypically changed chondrocytes in the damaged upper layer of osteoarthritic cartilage are more sensitive to TGF-β than chondrocytes in the more intact deep layer and are more sensitive than the chondrocytes of normal cartilage. TGF-β appears to redifferentiate the phenotypically altered chondrocytes in osteoarthritic cartilage. (J Rheumatol 1997;24:536–42)

Key Indexing Terms: OSTEOPATHY HUMAN TRANSFORMING GROWTH FACTOR-β CARTILAGE PROTEOGLYCAN CHONDROCYTE

Osteoarthritis (OA) is primarily a degenerative joint disease in which secondary inflammation may play a role. Disturbances in genetic material encoding for cartilage matrix molecules and elements that regulate turnover of matrix molecules, but also factors like instability, persistent overload of joints, and trauma, may contribute to its pathogenesis. Clinical studies on OA are obstructed, especially in the early stages of disease, by a limited correlation between the early biochemical changes in OA, such as decreased proteoglycan content at the articular surface and altered proteoglycan synthesis of the articular cartilage, and the clinical symptoms, e.g., joint pain and radiographic joint space narrowing.

The most frequently observed alteration in cartilage that characterizes early cartilage destruction in human OA is diminished proteoglycan content at the articular surface, indicating an imbalance between synthesis and release of proteoglycans. In vitro studies on human cartilage have been done mostly with cartilage obtained after total joint replacement, which reflects the end stages of the disease, including degenerative and reparative activities that have occurred over many years, and where inflammation has often been contributing to the chondropathy. This may explain the discrepancies reported with respect to proteoglycan synthesis of OA cartilage: proteoglycan synthetic activity of OA cartilage has been reported to range from enhanced, as a possible repair mechanism to compensate for increased loss of
proteoglycans, to decreased\textsuperscript{1} as a cause for the observed proteoglycan loss.

Recently, we reported that mildly to moderately osteoarthritic human knee cartilage in vitro displays both decreased and enhanced proteoglycan synthesis\textsuperscript{2}. The upper layer showed histological characteristics of OA cartilage such as decreased proteoglycan content (by safranin-O staining), chondrocyte clustering, and fibrillation extending to more severe deterioration of the matrix. It is this layer that showed strongly decreased proteoglycan synthesis. The deep layer, on the other hand, could not be distinguished histologically from normal cartilage and contained chondrocytes with enhanced proteoglycan synthesis. The chondrocytes in the upper, damaged layer were defined to be phenotypically altered ("de-differentiated"), while the chondrocytes in the deep, more intact layer of OA cartilage were considered normal\textsuperscript{2}.

Transforming growth factor-beta-1 (TGF-\textbeta) is a multifunctional growth factor, known for its regulatory function in cell growth and matrix formation of many tissues. The role of TGF-\textbeta in inflammation mediated cartilage damage is ambiguous\textsuperscript{3}. Direct effects of TGF-\textbeta on cartilage or effects of TGF-\textbeta on interleukin 1 (IL-1) induced cartilage damage, as may play a role in OA, are less ambiguous. Almost all studies report TGF-\textbeta to restore IL-1 suppressed matrix growth\textsuperscript{4-7}. In normal cartilage, intact tissue explants, as well as isolated chondrocytes, TGF-\textbeta increases matrix formation. Increased proteoglycan and collagen synthesis, as well as decreased proteoglycan release and decreased catabolic enzyme activity, have been reported\textsuperscript{5,8-13}. Nevertheless, absence of effects\textsuperscript{14,15} and catabolic effects\textsuperscript{16,17} of TGF-\textbeta on cartilage have also been observed.

With respect to OA cartilage, fewer data are available. Livne\textsuperscript{8} reported that TGF-\textbeta stimulated synthesis of proteoglycans and collagen by chondrocytes from osteoarthritic and senescent mice. Benya\textsuperscript{13} reported that exposure to TGF-\textbeta of rabbit articular chondrocytes with a de-differentiated phenotype led to re-expression of the differentiated phenotype, indicated by a recovery of type II collagen expression and proteoglycan synthesis. Malemud\textsuperscript{18} concluded, on the basis of stimulation of proteoglycan core protein synthesis in rabbit articular chondrocytes upon addition of TGF-\textbeta, that TGF-\textbeta could be an important growth factor in the restoration of cartilage extracellular matrix after injury. We reported that TGF-\textbeta increases proteoglycan synthesis in human articular cartilage, OA cartilage being much more sensitive to TGF-\textbeta than normal cartilage\textsuperscript{19}.

In some studies it was pointed out that effects of TGF-\textbeta may depend on the differentiation state of the chondrocytes. TGF-\textbeta prevented rat epiphyseal chondrocytes in cell pellet culture from de-differentiating to hypertrophic cells: if these cells were cultured in subconfluent monolayer they de-differentiated in response to TGF-\textbeta\textsuperscript{20}. This observation was substantiated by findings for bovine chondrocytes in longterm compared to short term culture\textsuperscript{21}, suggesting that the differential effect of TGF-\textbeta may depend on variation in the TGF-\textbeta receptor between chondrocytes in distinct stages of differentiation\textsuperscript{22}. The Pujol group also reported the effects of TGF-\textbeta depended on differentiation of the chondrocytes: they studied cell proliferation and attributed the differential effects of TGF-\textbeta to differences in TGF-\textbeta receptor expression\textsuperscript{23-25}. In addition, they reported that collagen type II synthesis was stimulated by TGF-\textbeta in more de-differentiated chondrocytes, but not in differentiated cells (subsequent passages compared to the primary cultures, respectively)\textsuperscript{26}.

These studies prompted us to investigate the effects of TGF-\textbeta on mildly to moderately osteoarthritic human cartilage, with a focus on possible differential effects of TGF-\textbeta on the phenotypically altered, that is, de-differentiated cells in the upper damaged layer and the "normal" chondrocytes in the more intact deep layer of this cartilage.

\textbf{MATERIALS AND METHODS}

\textit{Cartilage.} Microscopically degenerated and normal cartilage was obtained post mortem from human knees, within 24 h after death. According to patients' charts, donors had no predisposing conditions for joint disorders. Slices of cartilage were cut aseptically, as thick as possible, but excluding the underlying bone, from the central parts of the femoral condyles (from anterior to distal side excluding the extreme ends), as described\textsuperscript{22}. Donors (7 men and 8 women, n = 15) ranged in age from 66 to 82 years with a mean age of 73 ± 2 (SEM) years. Within 1 h after dissection, the slices (kept in phosphate buffered saline) were cut into square pieces and weighed aseptically (5–15 mg). These explants were kept in Dulbecco's modified Eagle's medium (Gibco 031600-083: 0.81 mM SO\textsubscript{4}\textsuperscript{2-}, 24 mM NaHCO\textsubscript{3} supplemented with ascorbic acid (0.85 mM), glutamine (2 mM), penicillin (100 IU/ml), streptomycin sulfate (100 \mu g/ml).

\textit{Light microscopy.} To assess the histological grade of the OA, 3 degenerated and 3 normal representative specimens from each donor were fixed for light microscopy. Sections were stained with safranin-O fast green-iron hematoxylin. The sections were analyzed and graded according to slightly modified criteria\textsuperscript{2} described by Mankin, et al\textsuperscript{24}.

\textit{Localization of proteoglycan synthesis.} Cartilage explants, taken at random and handled individually, were cultured (37°C, moisturized, 5% CO\textsubscript{2} in air) 4 days in 96 well microtiter plates in 200 \mu l culture medium (as above) containing 10% human serum (pooled from male AB+ donors, decomplemented for 2 h at 56°C). After culturing, part of the degenerated and normal tissue explants were sliced into 2 halves. The upper layer included the articular half with the articular surface, and in the case of degenerated cartilage the fibrillated segment of the specimen. The deep layer near the bone was the macroscopically intact half, for normal as well as degenerated cartilage. From these specimens proteoglycan synthesis rate and DNA content were determined.

\textit{Effects of TGF-\textbeta.} In a 2nd set of experiments, normal and degenerated cartilage tissue explants were cultured 4 days in culture medium without addition of serum. Human TGF-\textbeta (5 ng/ml; British Biotechnology Limited, Oxford, UK) was added during the last 24 h of the 4 day culture period or during the entire 4 day culture period. Because of the TGF-\textbeta neutralizing activity of serum proteins, no serum was added to these cultures. Tissue explants cultured 4 days without addition of TGF-\textbeta were used as controls.

Proteoglycan synthesis rate was determined.

\textit{Localization of the TGF-\textbeta effect.} In a 3rd set of experiments, normal and degenerated cartilage was cultured with TGF-\textbeta as described above, whereupon the explants were sliced into 2 halves as described in the first set of experiments. Proteoglycan synthesis rate and DNA content were deter-
Autoradiographic localization of incorporated label. For autoradiographic examinations, cartilage tissue explants after the 4 h labelling period (see above) were rinsed 3 times (45 min) in 1.5 ml culture medium and washed at 0.5 ml ice cold PBS. Subsequently, they were fixed in phosphate buffered 4% formalin (pH 7.0) containing 2% sucrose. Standard processing of the tissue in an automatic tissue processing apparatus was followed by embedding the specimens in paraffin wax. Histological sections were prepared and stained as described above. Dry deparaffined sections were then covered with a photographic emulsion (K5, Ilford); after exposure for 7 days the autoradiographs were developed. Autoradiography was evaluated independently by 2 persons.

Calculations and statistical analysis. Because of focal differences in composition and bioactivity of the cartilage on the femoral condyles, the results of 10 cartilage specimens of one donor, taken at random and handled individually, were averaged and taken as a representative value for the cartilage of that donor. Reproducibility of these representative values was within a 5% range. Means of average values obtained from several donors (n ± SEM are given. Statistical evaluation of differences was performed by the Mann-Whitney U test. Significance was accepted for p < 0.05.

RESULTS

Histological grading. For the degenerated cartilage the mean grade was 5.8 ± 0.2 (SEM; n = 15, varying from 3 to 8). Normal cartilage had a mean grade of 0.5 ± 0.1 (SEM; n = 15, varying from 0 to 2). Figure 1 shows a representative light micrograph of normal and degenerated cartilage. The degenerated cartilage in this micrograph was scored grade 7; the normal cartilage was scored 0. The thickness of the normal and degenerated cartilage from the articular surface to the deep zone on average was comparable. The damaged articular surface of degenerated cartilage was evident. In specimens of degenerated cartilage, cell clusters were present in the upper layer at the articular surface and close to the tears perpendicular to the surface. These clusters were not observed in normal cartilage. Furthermore, a strong reduction of safranin-O staining, although not distinctly visible in the black and white micrographs, was observed at the articular surface of degenerated specimens. The deep layer of degenerated cartilage tissue explants was histologically intact and comparable to normal cartilage.

Localization of proteoglycan synthesis. As can be inferred from Table 1, after 4 days of culture sulfate incorporation...
Table 1. Sulfate incorporation rate as a measure for proteoglycan synthetic activity of OA and normal cartilage after 4 days of culture under 10% serum conditions, expressed as nmol of sulfate incorporated/mg of cellular DNA. Mean values ± SEM are given (n = 4). Total: incorporation of an intact specimen. Sum: calculated incorporation rate of the upper layer and the deep layer. * Statistically significant differences between normal and OA cartilage. The average DNA contents of specimens were 5.1, 5.3, 5.9, and 5.0 μg for deep and upper layer for normal and OA cartilage, respectively.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Sum</th>
<th>Upper Layer</th>
<th>Deep Layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>21.41 ± 5.67</td>
<td>18.09 ± 5.12</td>
<td>22.91 ± 6.87</td>
<td>10.95 ± 1.09</td>
</tr>
</tbody>
</table>

Rate was lower for degenerated cartilage than for normal cartilage; 14.4 and 21.4 nmol/h/mg, respectively. This difference was due to a lower sulfate incorporation rate at the upper layer of the degenerated cartilage. The calculated sum of the sulfate incorporation rate of the upper layer and the deep layer, normalized for cellular DNA, did not differ from that of the undivided tissue explant. The lower sulfate incorporation rate in the upper layer of degenerated cartilage did not originate from a higher cellular DNA content. No significant differences in cellular DNA content were observed between the upper layer and the deep layer, neither for degenerated cartilage nor for normal cartilage (5.1 and 5.3, 5.9 and 5.0 μg for deep and upper layers for normal and degenerated cartilage, respectively).

Effect of TGF-β on cartilage tissue. Upon addition of TGF-β, significant stimulation of sulfate incorporation rate as a measure of proteoglycan synthesis was observed for degenerated and normal cartilage (Table 2). When TGF-β was added for 4 days instead of only the last 24 h of the culture period, the effect was statistically significantly stronger. During both exposure times, the effect on degenerated cartilage was statistically significantly stronger than for normal cartilage. Note that the differences in values between Table 2 and Table 1 originate from the normalization of sulfate incorporation by cartilage tissue wet weight in combination with 10% serum culture conditions, in contrast to normalization by cellular DNA and culture conditions in the absence of serum, respectively.

Biochemical localization of proteoglycan synthesis upon TGF-β addition. Figure 2 depicts the localized effects of

Table 2. Effect of TGF-β on sulfate incorporation rate as a measure for proteoglycan synthesis rate of OA and normal cartilage after 4 days of culture in the absence of serum, expressed per mg DNA. Mean values ± SEM are given (n = 4). TGF-β was added (5 ng/ml) during the last 24 h of culture (1 day) or during the entire 4 day culture period (4 days). * Statistically significant differences between normal and OA cartilage. ** Statistically significant differences between 1 and 4 day cultures.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>TGF-β</th>
<th>%</th>
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<tbody>
<tr>
<td>1 Day</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>TGF-β</td>
<td>OA</td>
<td>3.62 ± 1.67</td>
<td>5.52 ± 1.51*</td>
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<tr>
<td></td>
<td>Normal</td>
<td>3.47 ± 1.02</td>
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<tr>
<td>4 Day</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TGF-β</td>
<td>OA</td>
<td>3.54 ± 1.74</td>
<td>7.89 ± 3.29*</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>3.43 ± 0.46</td>
<td>5.91 ± 0.9</td>
</tr>
</tbody>
</table>

Figure 2. Effect of TGF-β on normal (N) and osteoarthritic cartilage (OA) sulfate incorporation rate as a measure for proteoglycan synthetic activity after 4 days of culture in the absence of serum, corrected for cellular DNA content, and expressed as percentage of control sulfate incorporation in the absence of TGF-β (23.9 ± 2.3 and 16.4 ± 0.7 nmol/h/mg cellular DNA for normal and OA cartilage, respectively). Mean values ± SEM (n = 7) are given for intact tissue explants (T), the upper layer (U), and the deep layer (D). Asterisks indicate statistically significant difference between the increase in sulfate incorporation under the influence of TGF-β between the articular and bone side.
DISCUSSION

The classification of early OA was found. No specific localization of these few "active" cell clusters is evident in our patients. However, some clusters, remarkably, do not show pronounced surface deformation. The majority of cell clusters did not show pronounced surface deformation in patients with higher OA. In patients with higher OA, the presence of cell clusters did not show pronounced surface deformation. We have observed similar to those described for cartilage damage during joint overload. Even in the absence of TG-1-6, the ability to produce collagen synthesis was retained in the cartilage. The cartilage in the joint showed no signs of degeneration. The surface of TG-1-6, even in the absence of TG-1-6, showed no signs of degeneration.

Layer of degenerated cartilage.

Paper and other experimental materials in the upper layers of TG-1-6 were dependent on the concentration in the upper layers of TG-1-6. However, there was no significant difference in the concentration of TG-1-6 in the upper layers of TG-1-6. A similar increase in TG-1-6 was seen in TG-1-6. The increase in TG-1-6 was seen in TG-1-6. The increase in TG-1-6 was seen in TG-1-6.
The preclinical mildly to moderately osteoarthritic human knee cartilage can be divided histologically and biochemically. The upper layer close to the articular space is damaged, has a reduced proteoglycan content, determined both histologically and biochemically, and chondrocytes are joined in cell clusters. By contrast, the deep layer close to the bone is histologically indistinguishable from healthy cartilage. Corroborating our previous findings, we show that after culturing, proteoglycan synthesis is lower in osteoarthritic cartilage than normal cartilage. This difference has previously been attributed to decreased vitality of the chondrocytes in osteoarthritic cartilage. Chondrocytes in the osteoarthritic cartilage were less capable of responding to the serum growth factors. This is also the reason for equal proteoglycan synthesis of normal and osteoarthritic cartilage in the absence of serum. The difference in anabolic activity of normal and osteoarthritic cartilage during culture under serum conditions may explain the discrepancies in the literature, where decreased as well as increased proteoglycan synthesis of osteoarthritic compared to normal cartilage has been reported. Our study localizes the inadequacy in the upper layer of the osteoarthritic cartilage. Proteoglycan synthesis in the deep layer of cartilage is similar for osteoarthritic and normal cartilage. This phenomenon cannot be explained by hypoxia or hypercellularity of the deep or upper layer of the cartilage, respectively, but is based upon a difference in chondrocyte proteoglycan synthetic activity. From the reduced proteoglycan synthesis, in combination with increased proliferation and aberrant collagen synthesis, we concluded that the chondrocytes in the upper layer of osteoarthritic cartilage were phenotypically changed or de-differentiated.

TGF-β is able to enhance proteoglycan synthesis in articular cartilage, which corroborates findings of many others. Our study shows, however, that preclinical osteoarthritic knee cartilage is more sensitive to TGF-β than normal cartilage, an effect evident after a 24 h exposure to TGF-β, but more clearly after 4 day exposure. We previously showed that this effect is not caused by endogenous production of TGF-β, which could have been higher for osteoarthritic than for normal cartilage. Anabolic activity of TGF-β on osteoarthritic cartilage corroborates studies using cartilage of osteoarthritic and senescent animals. This beneficial effect makes TGF-β a theoretical candidate for intraarticular treatment of OA, although less favorable effects, like osteophyte formation in the joint, have been reported. Moreover, osteoarthritic joint fluid contains high levels of TGF-β. Active TGF-β is present in concentrations equal to the concentrations used in the present study, but it is possible that inhibitors of TGF-β are also present. Further research is indicated in this respect, in addition to investigation of whether local activation of the high concentrations of latent TGF-β produced in the osteoarthritic joint is a possibility.

Of interest, we found TGF-β predominantly stimulates the de-differentiated chondrocytes joined in cell clusters, which without TGF-β did not significantly contribute to proteoglycan synthesis, an effect that was obvious after 24 h stimulation with TGF-β and even more striking after 4 day exposure. The biochemical data discriminating between an upper and a deep layer is substantiated by autoradiographic observations. In particular, cell clusters that without TGF-β did not contribute significantly to proteoglycan synthesis were sensitive for TGF-β. This corroborates results of investigators who reported that effects of TGF-β depended upon the differentiation state of the chondrocytes. It should be noted that a few cell clusters did contribute to proteoglycan synthesis in the absence of TGF-β and that after TGF-β treatment there were some clusters, in small numbers, that did not contribute significantly to proteoglycan synthesis. An explanation for this phenomenon can only be speculative; it could originate from variations in the differentiation state of the chondrocyte clusters. It is tempting to consider that a difference in the TGF-β II receptor isoform could be involved in the differential effect of TGF-β on proteoglycan synthesis of normal and de-differentiated chondrocytes of osteoarthritic cartilage. It has been reported that freshly isolated (differentiated) and prolonged culture (de-differentiated) bovine articular chondrocytes expressed different forms of the type II receptor. However, using mRNA polymerase chain reaction techniques, this could not be confirmed for intact human normal and osteoarthritic articular cartilage.

Although TGF-β predominantly stimulates proteoglycan synthesis of phenotypically changed chondrocytes of osteoarthritic cartilage, this does not prove that TGF-β actually re-differentiates the de-differentiated chondrocytes. Notwithstanding the reported suppressive effects of TGF-β on proteoglycan release and catabolic activity in intact specimens, it remains unknown if these effects are directed at the de-differentiated chondrocytes in the upper layer of osteoarthritic cartilage. Moreover, the quality and retention characteristics of the newly formed proteoglycans under the control of TGF-β remain to be investigated.

We conclude that with respect to proteoglycan synthesis osteoarthritic cartilage is more sensitive to TGF-β than normal cartilage because the phenotypically changed chondrocytes in the damaged upper layer of osteoarthritic cartilage are more sensitive to TGF-β than the chondrocytes in the more intact deep layer.

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