Myofibroblast Accumulation Correlates with the Formation of Fibrotic Tissue in a Rat Air Pouch Model

ANETTE BJÄRDAHLEN, PER-OLA ÖNNERVIK, GUNILLA WESTERGREN-THORSSON, ANDERS MALMSTRÖM, and BENGT SÄRNSTRAND

ABSTRACT. ***Objective.*** The pathogenesis of arthritic joints involves cartilage degradation and pannus formation. It is well known that pannus influences the cartilage; however, the mechanism behind how the degrading cartilage interacts with pannus is not well known. To investigate this interplay, the expression of extracellular matrix (ECM) components in pannus and the degrading cartilage was analyzed.
Methods. Studies were performed using a rat air pouch model where cotton with viable or killed cartilage was implanted into 7-day-old pouches for 1–28 days. The remodeling of cartilage and the formation of tissue in the cotton was characterized histologically by quantitation of infiltrated cells. The amounts of collagen, hyaluronan, and proteoglycan were estimated.
Results. Implantation of homologous femoral head cartilage in cotton resulted in extensive remodeling of cartilage and formation of ECM in the cotton. In cotton without cartilage, fibroblasts and myofibroblasts were the predominant cells in the early stage of analyses. The ECM formed in cotton was of a fibrotic type, with mainly collagen and smaller amounts of proteoglycans correlating to the presence of myofibroblasts. In the cotton with cartilage, however, inflammatory cells such as neutrophils, macrophages, and lymphocytes dominated. Delayed accumulation of collagen and increased synthesis of proteoglycans occurred early in cotton with viable as well as non-viable cartilage. In later stages, the cell pattern changed and the myofibroblasts emerged together with an increasing collagen formation.
Conclusion. The interaction between cartilage and the newly formed granulation tissue results in a faster degradation of cartilage molecules, which in turn leak into the surrounding ECM and affect the recruitment of myofibroblasts. This indicates the importance of the micromatrix. (J Rheumatol 2002;29:1698–707)

Key Indexing Terms:
AIR POUCH | ARTHRITIS | CARTILAGE
MYOFIBROBLAST | PANNUS | PROTEOGLYCAN

In vivo models are crucial to improving our understanding of the process of cartilage remodeling and granuloma/pannus formation. To study these processes, several models have been used, such as cartilage implanted in an air pouch[1], antigen induced arthritis (AIA)[2,3], and the SCID mouse model[4], where human cartilage is implanted with synovial tissue from patients with rheumatoid arthritis (RA). In these models it has been observed that extracellular matrix continuously changes during the inflammatory process. The SCID mouse model showed increased cartilage erosion when implanted with viable cartilage. The AIA model also showed a changing extracellular matrix composition in both cartilage and inflamed synovia[5]. In most of these models, with the exception of the air pouch, existing tissue has to be degraded before new tissue can be introduced. The air pouch model, using cartilage surrounded by cotton[6], offers advantages with respect to insertion of various cartilages, generation of newly formed inflammatory tissue, and possibilities for manipulating the system.

Incubation of heterologous cartilage in the pouch leads to a decrease of collagen and proteoglycan concentration in the cartilage. Inclusion of cotton around the cartilage increased both proteoglycan and collagen degradation, which indicates that the surrounding inflammatory extracellular matrix (ECM) enhances cartilage remodeling[6]. It is not known if all types of proteoglycan are affected in the inserted cartilage and how the formation of inflammatory connective tissue occurs in the surrounding cotton. The only determination until now is the measurement of the dry weight of the granulation tissue formed around the cartilage, which was shown to increase substantially. The effects of several drugs, i.e., glucocorticoids[7], nonsteroidal antiinflammatory drugs

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(NSAID)[8], and immunosuppressants[9], have been noted to inhibit this increase. Cells migrating into the cotton and the production of cytokines are central to the remodeling of the inserted cartilage, as well as the matrix formed in the cotton. The cytokines of importance, found in the lining of the air pouch wall, are initially the proinflammatory cytokines interleukin 1 (IL-1) and tumor necrosis factor-α (TNF-α). These are followed sequentially by basic fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), and epidermal growth factor (EGF). Finally, levels of transforming growth factor-ß (TGF-ß) are increased to high levels[10]. All these cytokines, together with fibroblasts, are of importance for remodeling of the cartilage and for construction of the inflammatory and finally fibrous connective tissue in the cotton.

It has been shown that platelets, polymorphonuclear cells, and lymphocytes initially occupy the pouch lining and are subsequently joined by macrophages and finally fibroblasts[10]. During inflammatory conditions, the presence of activated fibroblasts (myofibroblasts) expressing α-smooth muscle actin (α-SMA) has been described. Several myofibroblast phenotypes have been described based on the expression of cytoplasmic actin, vimentin, and desmin[11]. Myofibroblasts that express α-SMA and vimentin (VA phenotype) are considered metabolically active and produce collagen, while cells that contain α-SMA, vimentin, and desmin (VAD phenotype) are relatively inactive in producing collagen[11]. The origin of the myofibroblast remains a subject of much speculation, but it is known to be involved in wound healing, contraction, and scarring. Myofibroblasts appear during development of granulation tissue and disappear through apoptosis with resolution of healing[12,13].

In the air pouch model inflammatory tissue in the cotton accelerates the degradation of cartilage. How the cartilage influences the formation of inflammatory tissue and the mechanism thereof is unknown. The matrix macromolecules are of interest, especially the proteoglycans, since they have important effects on storage and activation of cytokines and proteases[14] and on cell proliferation[15], thereby enabling these molecules to influence the remodeling process of both cartilage and matrix formed in the cotton. Further, addition of glycosaminoglycans (GAG) has indeed been shown to modify the remodeling process[16]. It is therefore of interest to characterize the system with respect to effect of pannus on both viable and killed cartilage remodeling. It is also of interest to look at the effect of these cartilages on the remodeling of the surrounding inflammatory ECM and to explore the presence of potential cytoactive matrix macromolecules.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats with a rough body weight of 150–200 g were used (Möllegaard Breeding Center Ltd., Köge, Denmark). The rats had free access to food and tap water, and were kept in cages with sawdust bedding. The temperature was thermostatically maintained at 22°C, the relative humidity was 50% ± 10%, and the light was on for 12 h periods from 6:00 AM to 6:00 PM. The experiments were approved by the Animal Ethics Committee of Lund.

Preparation and implantation of cartilage and cotton. According to the method described by Bottomley, *et al*[6] a dorsal, subcutaneous air pouch was formed. Intact femoral head cartilage was removed from donor rats. Cotton squares were cut out from dental rolls. Viable cartilage with and without cotton was implanted into the air pouch. A number of cartilage samples were killed by freezing and thawing 3 times before being wrapped in cotton and implanted. Cotton without cartilage was also implanted. Intact cartilage was removed from the hip joint in a parallel experiment at comparable time points. The animals were sacrificed 1, 3, 7, 14, and 28 days after implantation. Cartilage was also incubated in a 6 well plate at 37°C in Ham's F12 medium supplemented with 10% fetal calf serum for corresponding periods.

Isolation of extracellular matrix components. The separated cartilage and cotton implants were diced and extracted at 4°C for 2 × 24 h in 500 µl extraction buffer containing 4 M guanidinium hydrochloride [technical grade, stock solution (8 M) purified with activated charcoal] 0.05 M acetate, pH 5.8 (supplemented with 1% Triton-x100, 5 mM NEM, and 5 mM EDTA) (BDH Chemicals Ltd., Poole, England).

DNA determination. An aliquot of 50 µl from the guanidinium hydrochloride buffer was precipitated with 3 volumes of 95% ethanol with 0.4% NaAc. The precipitate was centrifuged for 15 min at 10,000 g, washed with absolute alcohol, centrifuged, and dried in a Speed Vac (Savant DNA 110) for 10 min. The pellet was then dissolved in 200 µl of 0.05% trypsin (Sigma-Aldrich, Stockholm, Sweden) in phosphate buffered saline (PBS; 137 mM NaCl, 2.68 mM KCl, 8.07 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). The determination of DNA was performed by the method of Labarca and Paigen[17]. The trypsin/PBS solution was diluted in DNA buffer (0.05 M Na_2HPO_4, 2 M NaCl, 2 mM EDTA, pH 7.4). Sample solution (100 µl) was transferred to a 96 well fluorotec plate (Dynatech plates, In vitro Sweden AB, Stockholm, Sweden) and diluted with an additional 50 µl DNA buffer. Then 100 µl reagent (2.5 µg/ml DNA buffer) was added to each well and incubated in the dark for 5 min at room temperature. Plates were read with a Perkin-Elmer LS-4B luminescence spectrometer (Gammadata Burklint AB, Uppsala, Sweden) at an excitation wavelength of 356 nm and an emission wavelength at 458 nm. DNA concentration was calculated using linear regression.

Collagen determination. Implant hydroxyproline concentration was measured using the method of Stegemann and Stadler[18]. Aliquots of the guanidinium extract and the remaining pellets were incubated in 6 M HCl for 17 h at 100°C. The solution was freeze-dried and dissolved in 400 µl acetate-citrate-hydroxide buffer and 200 µl oxidant solution (300 mM in chloramine T in buffer supplemented with isopropanol). Samples were allowed to stand for 20 min at room temperature, then 200 µl aldehyde/perchloric acid reagent was added. Following incubation for 15 min at 65°C, samples were analyzed spectrophotometrically for optical density at 550 nm.

Hyaluronan determination. An aliquot of 1/10 of the guanidinium extract was diluted 20 times in buffered urea [6 M urea (Schwarz/Mann, Cambridge, England), 0.05 M HAc, pH 5.8, supplemented with 10 mM EDTA, 5 mM NEM, and 5 µg/ml OA] and loaded onto columns (0.5 × 0.7 cm) of DEAE-52 (Whatman International Ltd., Fairfield, VA, USA). The hyaluronan was eluted in 2 × 0.5 ml 6 M urea, 0.5 M HAc, pH 5.8, supplemented with 10 mM EDTA, 5 mM NEM, and 5 µg/ml OA. The elute was analyzed using a hyaluronan kit from Pharmacia AB, Uppsala, Sweden, as described by Brandt, *et al*[19].

Proteoglycan determination. Total proteoglycan content was determined by estimating the absorbance of alcian blue [8GS (IA 288) Wieslab, Lund, Sweden] at 600 nm as described by Björnsson[20]. The proteoglycans were further separated by electrophoresis.

Electrophoretic separation. Proteoglycan separation was performed using

electrophoretic techniques on 2% agarose (Biorad Laboratories, Sacramento, CA, USA)[21]. The gels were loaded with an equal extract volume. The different types of sulfated proteoglycans were quantitatively determined by scanning using a Gel-Pro Analyser densitometry system. The results were expressed as micrograms of proteoglycan per cotton or cartilage, if not otherwise stated, using small proteoglycans extracted from sclera as a standard[22].

Morphological characterization. Cotton-cartilage implants were fixed in 4% phosphate buffered formaldehyde, dehydrated with alcohol, embedded in paraffin, and cut into 3–4 µm sections. To study the inflammation in terms of cell type and granulation tissue development, sections were stained with Ehrlich hematoxylin and eosin. The cells were counted in morphological sections at high power magnification. Staining was also performed using specific antibodies directed towards α-SMA. Briefly, sections were incubated with swine serum (1:10) for 20 min and then incubated with primary antibody α-SMA 1:50 (M0851, Dako, Dakopatts AB, Älvsjö, Sweden). The antibody was diluted in Tris-HCl, pH 7.6, with 2% bovine serum albumin and the sections were incubated for 30 min. Then the secondary antibody, swine-anti-rabbit, was added for 30 min. The bound antibodies were then detected with avidin-biotin vectorstatin AK-500 (vector SK 5100). The background was stained with Mayers hematoxylin for 2 min and sections were finally mounted with fluorotec.

Statistical analysis. Values are expressed as the average amount per tissue if not otherwise stated. Results are shown as the standard error of the mean (SEM) 3–13 animals/point. Differences from control groups were tested for significance using Student's 2 tailed t test for unpaired data with unequal variance; $p < 0.05$ was considered significant.

RESULTS

Inflammatory response. Sections stained with hematoxylin/eosin showed clear differences in the organization and amount of ECM formed in the cotton implanted with cartilage versus without cartilage. Cotton implanted without cartilage resulted in early (day 3–7) ECM formation with collagen fibrils (Figure 1). Cotton implanted with cartilage, on the other hand, displayed a more inflammatory picture with a delayed formation of ECM (Figure 1).

Influx of cells. To evaluate the total amount of cells in the cotton, DNA analyses were performed. The granuloma formed in the cotton was heterogeneous with respect to both matrix formation and cells obtained. This precluded an exact histological evaluation of the cell numbers. Therefore a general score of the whole tissue in relation to total DNA content was performed. At day 1 a considerable amount of cells could be detected in the cotton of the 3 systems (Figure 2). At this point the 3 systems resembled one another, and the dominating cells were mainly neutrophils and to a smaller extent macrophages and lymphocytes (Figure 3A).

In cotton without cartilage the proportion of fibroblasts increased to be the dominating cell type at day 3–7. Most of these fibroblasts stained for α-SMA, which indicated the presence of active myofibroblasts. The cell number in the cotton alone remained at the same level until day 7; thereafter a decrease was noted in both total cell number and the amount of myofibroblasts. Although the myofibroblasts decreased, the predominant cell type was fibroblasts without α-SMA. From day 14 onwards the staining for α-SMA only occurred in smooth muscle cells lining the vessels (Figure 1).

In the cotton implanted around viable cartilage a somewhat larger cell number was noted, but here the cells were mainly granulocytes. As soon as day 1 the first sign of cartilage erosion was observed to occur through attacks of neutrophils (Figure 3B). Fibroblasts and myofibroblasts emerged considerably later in this system compared to plain cotton (Figures 1 and 3A). At day 14 they were found only close to cartilage. At day 28, however, they were almost uniformly distributed in the cotton compartment (Figure 3A).

The number of cells in cotton implanted with freeze-thawed (killed) cartilage increased significantly compared to the other 2 systems. As early as day 3 differences in the cell pattern were observed compared to cotton with viable cartilage. The predominant cell type was granulocytes, but considerable higher numbers of macrophages and lymphocytes were observed. From day 3 the fibroblast number increased linearly to be the predominant cell type. These fibroblasts showed no α-SMA in this early stage. However, from day 14 an 8-fold increase in the number of cells was recorded compared to the other 2 systems ($p < 0.03$) and at this stage myofibroblasts began to appear (Figure 1). At day 28 the 3 systems resembled one another regarding composition of cell types found in the surrounding cotton, with the exception of the myofibroblasts.

Remodeling of collagen in cartilage. The implanted cartilage pieces all contained about 350 µg hydroxyproline/femoral head cartilage corresponding to 2–3 mg of collagen. No significant changes in the amount of non-extractable or of total collagen were noted in any of the experiments. This indicates that only a small turnover of cartilage collagen occurred.

Formation of collagen in granulation tissue. The collagen in tissue that developed in cotton without cartilage increased from 0 to 200 µg hydroxyproline in 14 days, after which it reached a steady-state level (Figure 4A). The large increase between days 3 and 14 correlated well with the appearance of myofibroblasts. The rate of collagen accumulation in the cotton surrounding viable cartilage was slower and only equalled that of cotton without cartilage after 28 days (Figure 4A). The largest increase occurred after 14 days and here the accumulation also correlated with the appearance of the myofibroblasts. An even greater delay of collagen accumulation was recorded in cotton surrounding killed cartilage. At day 14 the amount of collagen was 4.4 times higher in cotton without cartilage than in the tissue developed in cotton with killed cartilage ($p = 0.01$; Figure 4A).

A considerable amount of the accumulated collagen in cotton was extractable by guanidinium hydrochloride, indicating that the collagen had a low degree of cross-linking (Figure 4B), especially during the initial stage of implantation. At day 14 the synthesis of new collagen was increased 3-fold in the plain cotton compared to cotton with cartilage. The proportion of newly synthesized collagen constituted

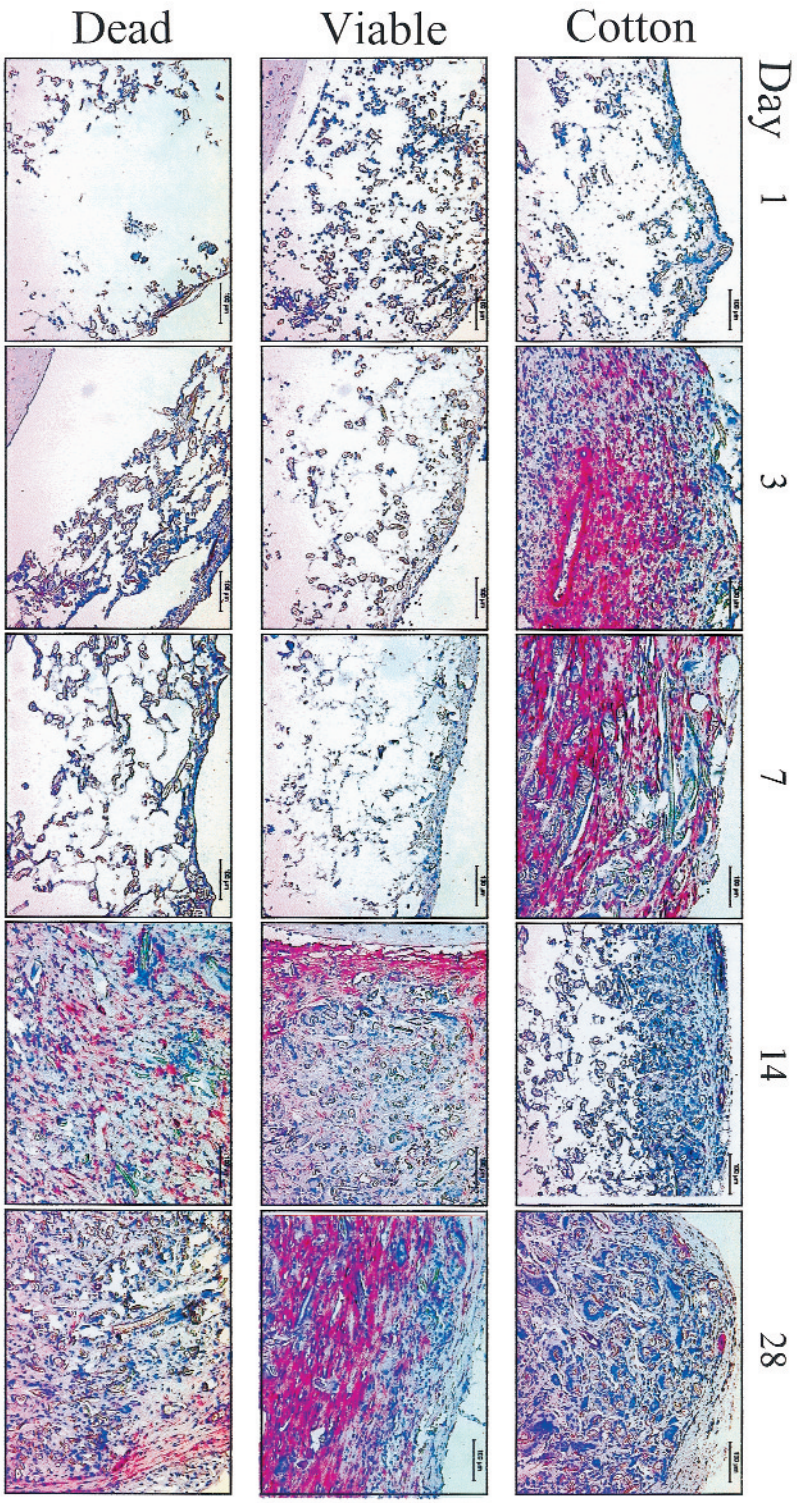

Figure 1. Myofibroblast staining of cartilage/cotton implants. Intact femoral head cartilage from donor rats was implanted into a 7-day-old air pouch for 1–28 days. Three different implant variants were made: cotton with viable cartilage, cotton with killed cartilage, and cotton without cartilage. According to predetermined time points the implants were removed and fixed in formaline, dehydrated with alcohol, and embedded in paraffin. The paraffin embedded sections of 3–4 µm were stained with α-smooth muscle actin, and the bound antibodies were detected with avidin-biotin vectorstatin and subsequently vector red. The background was stained with Mayers' hematoxylin (original magnification ×20).

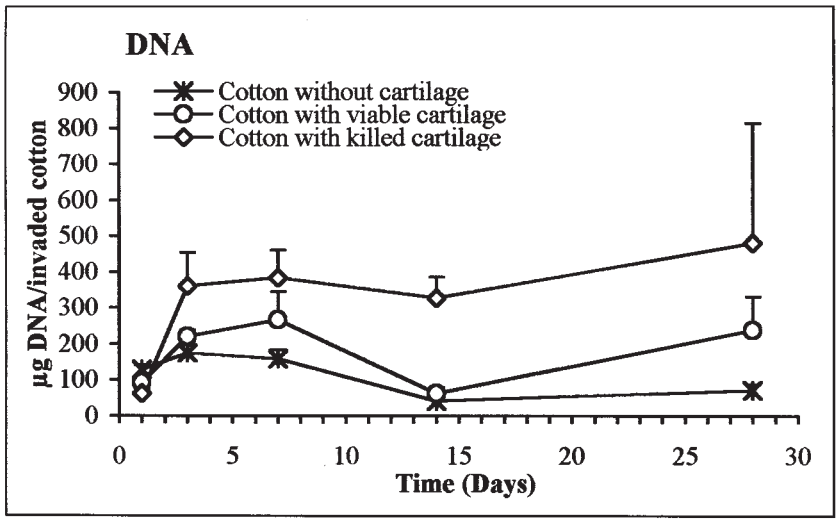

Figure 2. DNA analysis of invaded cells in cotton/cartilage implants in a rat air pouch. At different time points the rats were sacrificed and the implants were removed and diced separately. The cotton was extracted with guanidinium hydrochloride buffer and an aliquot was removed and analyzed as described in Materials and Methods. The values are shown as mean ± SEM, where n = 8 for cotton alone and n = 5 for cotton with viable and killed cartilage.

almost one-third of the total amount of collagen observed, which indicates a high synthesis in all of the cotton compartments.

Formation of hyaluronan in granulation tissue. Cotton without cartilage contained 1 µg hyaluronan/tissue as soon as day 1, and only small changes occurred throughout the experiment (Figure 4C). The amount occurring in cotton surrounding viable cartilage was similar to that in cotton alone, but after 7 days a rapid rise took place, resulting in an 8-fold increase by day 28 ($p = 0.03$). The level of hyaluronan in cotton with killed cartilage was significantly higher at day 1 than in cotton with viable cartilage and cotton without cartilage ($p = 0.047$). Only small changes were noted later in the experiment (Figure 4C).

Remodeling of proteoglycans in cartilage. The amount of proteoglycans in viable cartilage without cotton was 350 µg/femoral head cartilage, decreasing by 40% over 28 days (Figure 5A). The content of proteoglycans in the viable cartilage with cotton decreased at a higher rate during the first 7 days compared to cartilage alone and killed cartilage. At this point the amount of total proteoglycans in the viable cartilage with cotton was significantly decreased compared to viable cartilage without cotton ($p = 0.001$). The rate of degradation diminished, and by day 28 cartilage with cotton had lost 70% of its proteoglycan content. To investigate if the cartilage chondrocytes themselves mediated the decrease of proteoglycans, cartilage was incubated in cell culture medium. During these conditions no decreasing of proteoglycan levels occurred (Figure 5B). Further, no age changes in cartilage occurred *in vivo* during the observation period (Figure 5B).

Remodeling of cartilage involved both aggrecan and the small leucine-rich proteoglycans decorin and biglycan. Aggrecan decreased faster than the small proteoglycans. In 3 days, 50% of the aggrecan was lost, whereas decorin/biglycan decreased by only 30% (Figures 5C and 5D). The aggrecan remaining in the cartilage was further processed in both viable and killed cartilage, presumably proteolytically, resulting in smaller fragments with lower retardation values (Figure 5D). The proportion of biglycan and decorin was 70% and 30%, respectively, in all 3 experiments as elucidated by sodium dodecyl sulfate-polymerase chain reaction (data not shown).

Formation of proteoglycans in granulation tissue. Proteoglycans accumulated almost linearly in the inserted cotton plug up to 28 days (Figure 5E). The proteoglycan content in cotton surrounding viable and killed cartilage differed substantially. Already at day 1 the amount of proteoglycans obtained was 6.4-fold higher in cotton surrounding cartilage than in cotton without cartilage ($p = 0.001$) (Figure 5E). The proteoglycan content in cotton surrounding viable cartilage increased continuously up to 21 days, showing a 2-fold elevation compared to the level in cotton alone (Figure 5E; $p = 0.036$). The proteoglycan content in cotton surrounding cartilage increased up until day 7 and then remained constant at this level.

DISCUSSION

Our main findings are that cartilage and the surrounding tissue mutually influence the remodeling of extracellular matrix, and that myofibroblasts are involved in the process. Under cell culture conditions no clear remodeling occurs

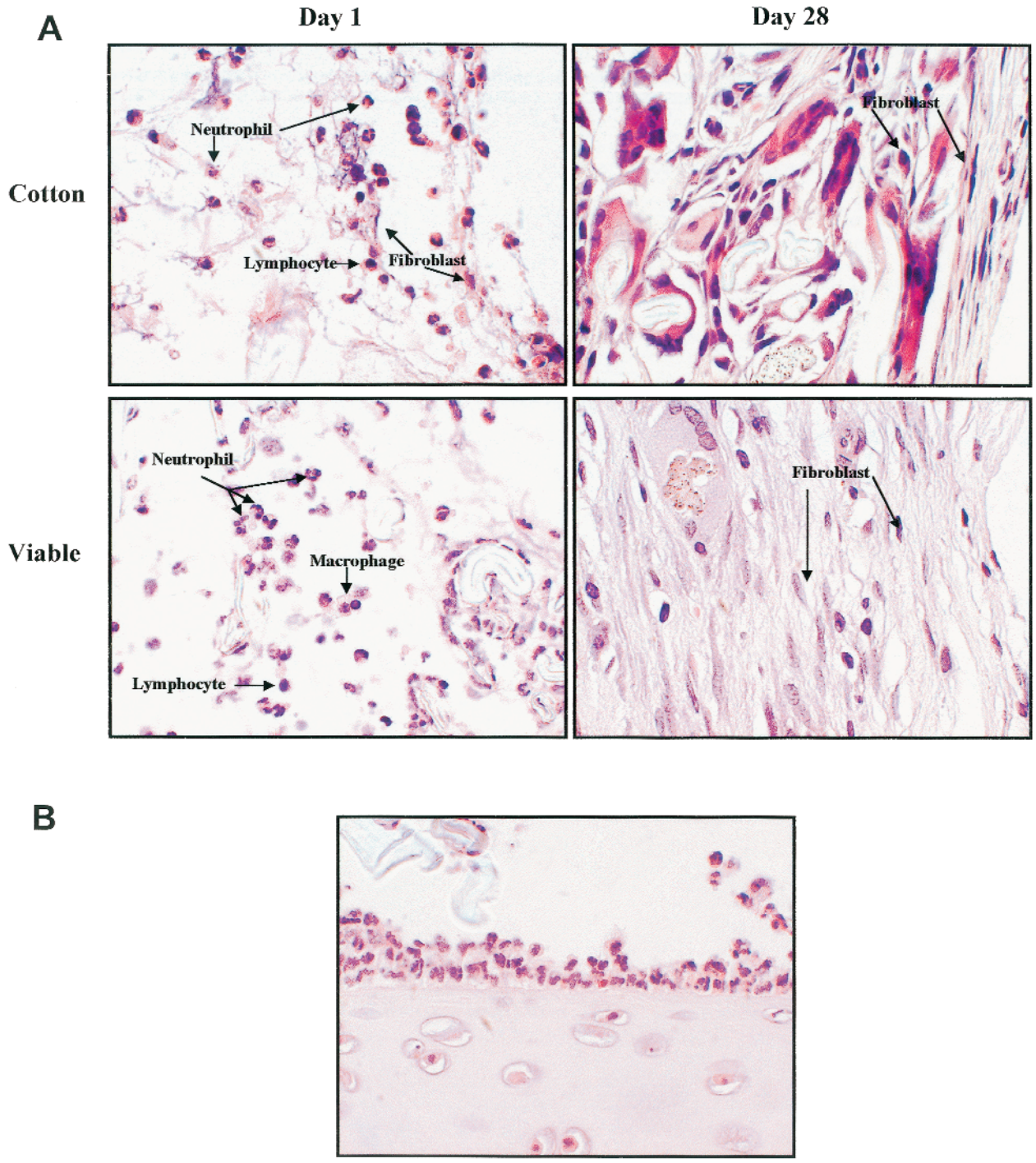

Figure 3. Infiltration of cell types in cotton/cartilage implants in rats. Cotton with or without cartilage was implanted into a 7-day-old air pouch for 1–28 days. At different time points the implants were removed and fixed in formaline, dehydrated with alcohol, and embedded in paraffin. The paraffin embedded sections of 3–4 µm were stained with Mayers' hematoxylin/eosin, and the different cell types were studied at a magnification ×60. A. Different cell types occurring with (lower panel) and without (upper panel) cartilage during the acute inflammatory phase (day 1) and the fibrous phase (day 28). The different cell types are indicated by arrows. B. The first degradation attack on the cartilage by neutrophils at day 1.

compared to cartilage in the air pouch, where an extensive remodeling occurs. A developing inflammatory matrix around the cartilage in cotton enhances the process, which agrees with the findings of Bottomley, *et al*[6]. Chondrocyte activity is, however, also of importance for the reduction in proteoglycans, as they decrease faster in living than in killed cartilage (Figure 5A).

The mechanism for the observed remodeling is most

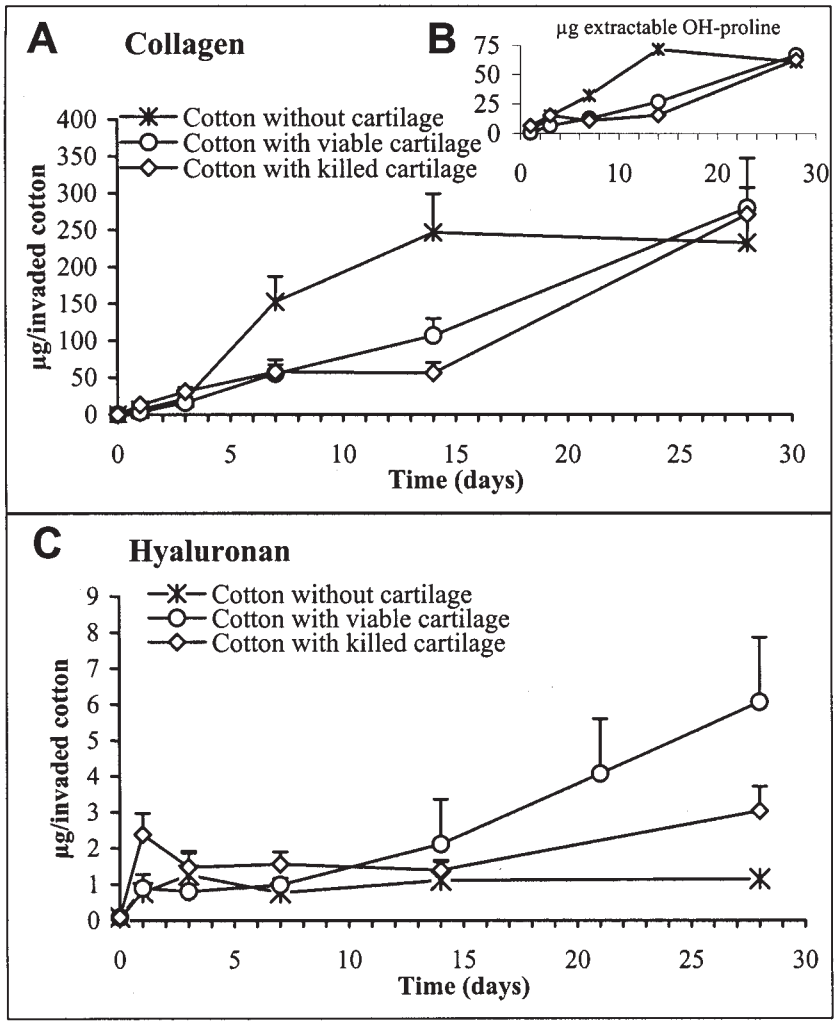

Figure 4. Extracellular matrix formation in invaded cotton surrounding intact femoral head cartilage in a rat air pouch model. Cotton with and without cartilage or plain cotton was implanted into a 7-day-old air pouch. The different implants were removed from the pouches at different time points and extracted separately. Aliquots of the extract were analyzed for their hydroxyproline and hyaluronan levels. The remaining pellets were also analyzed for their hydroxyproline content. A. Non-extractable hydroxyproline in the 3 sets of implants at different time points. B. Total hydroxyproline content of extractable collagen deposited in the cotton (insert). Mean values ± SEM are shown and the number of animals analyzed for both extractable and non-extractable collagen were the following: only cotton n = 12, with viable cartilage n = 10, and with killed cartilage n = 8. C. The amounts of hyaluronan detected in the tissue; number of animals: n = 3 with cotton only, n = 5 with viable cartilage, and n = 7 with killed cartilage.

likely due to the secretion of cytokines, IL-1, TNF-α, and interferon-γ from the surrounding inflammatory cells and fibroblasts from the surrounding tissue[10]. The findings of Pap, *et al*[2] show that IL-1ß drives the fibroblast-like synoviocytes to erode articular cartilage much faster than cells without any stimulation. As both viable and killed cartilage contained, with time, aggrecan of considerably smaller size (Figure 5D), it is conceivable that the induction of matrix metalloproteinases such as MMP3[23] and MMP9[24] is a key factor for the degradation of proteoglycans. The source of these enzymes should be inflammatory cells[25] and fibroblasts/myofibroblasts[26] in the surrounding cotton. The faster degradation of aggrecan in viable cartilage, as shown in this paper, indicates that chondrocytes are also active and of importance in the remodeling process. This is strengthened by the fact that this occurs although cotton surrounding killed cartilage contains considerably more inflammatory cells. When aggrecan degrades, the free GAG chains may attract granulocytes to migrate into the cotton compartment. The binding of GAG chains to neutrophils is mediated by the binding to L-selectin[27]. This phenomenon was mainly observed in the system with killed cartilage. The killed carti-

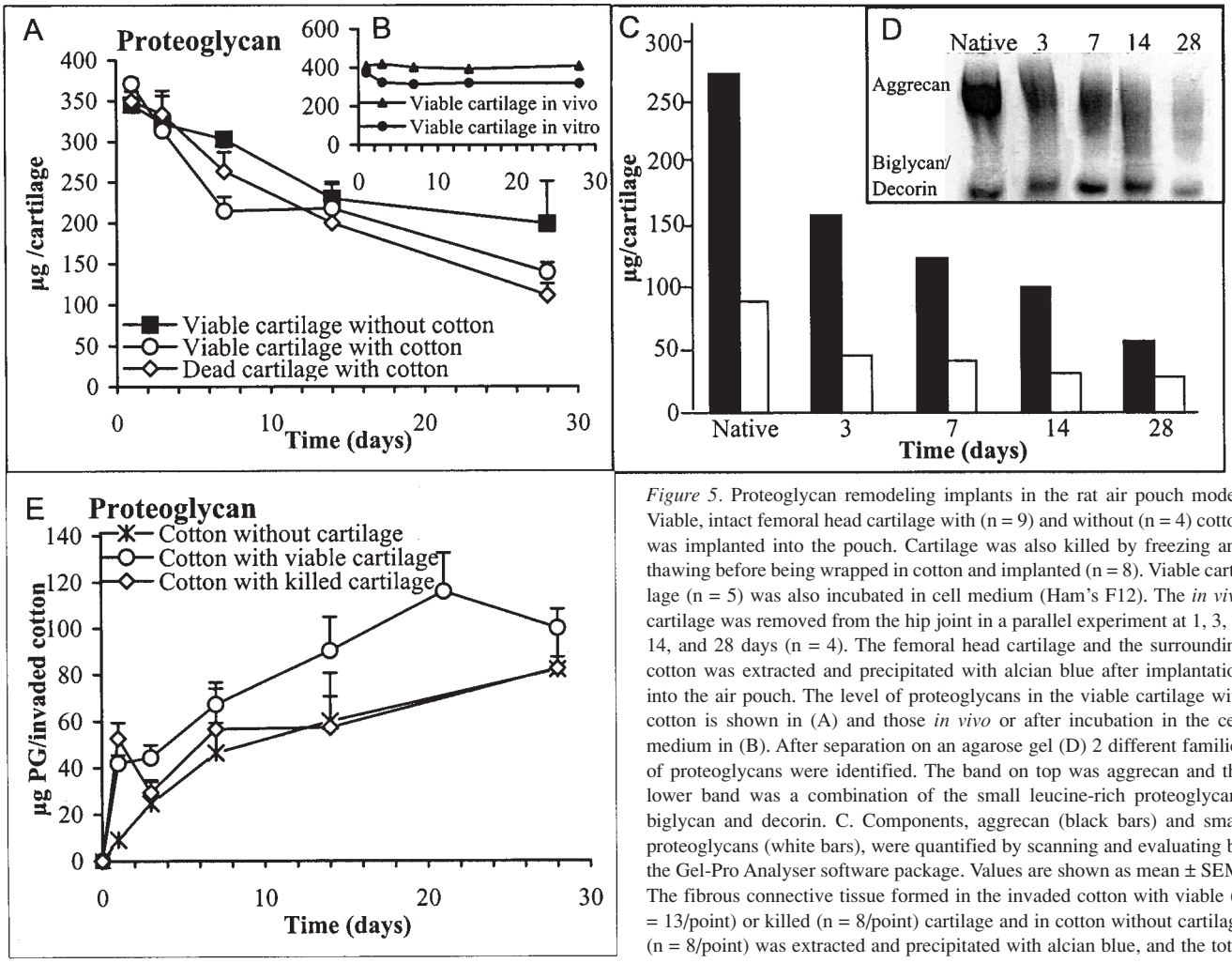

Figure 5. Proteoglycan remodeling implants in the rat air pouch model. Viable, intact femoral head cartilage with (n = 9) and without (n = 4) cotton was implanted into the pouch. Cartilage was also killed by freezing and thawing before being wrapped in cotton and implanted (n = 8). Viable cartilage (n = 5) was also incubated in cell medium (Ham's F12). The *in vivo* cartilage was removed from the hip joint in a parallel experiment at 1, 3, 7, 14, and 28 days (n = 4). The femoral head cartilage and the surrounding cotton was extracted and precipitated with alcian blue after implantation into the air pouch. The level of proteoglycans in the viable cartilage with cotton is shown in (A) and those *in vivo* or after incubation in the cell medium in (B). After separation on an agarose gel (D) 2 different families of proteoglycans were identified. The band on top was aggrecan and the lower band was a combination of the small leucine-rich proteoglycans biglycan and decorin. C. Components, aggrecan (black bars) and small proteoglycans (white bars), were quantified by scanning and evaluating by the Gel-Pro Analyser software package. Values are shown as mean ± SEM. The fibrous connective tissue formed in the invaded cotton with viable (n = 13/point) or killed (n = 8/point) cartilage and in cotton without cartilage (n = 8/point) was extracted and precipitated with alcian blue, and the total amount of proteoglycan was quantitated after estimating the absorbance at 600 nm (E).

lage released the largest amount of chondroitin sulfate-bearing molecules already by day 1 (Figure 5E), which leads to a 2-fold increase in inflammatory cells.

The importance of fibroblasts for immune response and the inflammation process is gradually gaining acceptance, especially with respect to the switch from acute to chronic inflammation[28]. In this study this concept is of great interest, since the formation of inflammatory extracellular matrix is dependent upon the presence of cartilage with or without viable chondrocytes. In the absence of cartilage, a connective tissue dominated by fibroblasts was formed. A steady-state level was obtained after 14 days with respect to both collagen and proteoglycan concentration. In cotton surrounding killed cartilage a different picture emerged, where at day 1 an 8-fold higher concentration of proteoglycans occurred. These most likely originate from the cartilage. In this environment the fibroblasts, which are present in smaller quantities than in cotton alone, display a much higher production of proteoglycans and lower synthesis of collagen than in cotton alone. The resulting ECM is thus very different, with a considerable 45-fold difference in the collagen/proteoglycan ratio and an increased ratio of hyaluronan/collagen.

The mechanism behind these differences is unclear. The increased hyaluronan synthesis may be essential for the recruitment of cells and in particular fibroblasts[29,30]. Both viable and killed cartilage secreted matrix components and/or cytokines, which influence the recruitment of granulocytes, lymphocytes, and myofibroblasts. In addition, the metabolic activity of fibroblasts in the cotton compartment is influenced to produce a larger amount of proteoglycans. Substances diffusing from the cartilage are mainly aggrecan and degradation products thereof. Zhang, *et al* showed that the G1 domain of aggrecan can induce arthritis[31], resulting in pannus formation and cartilage and bone degradation.

Another common component of cartilage that is diffusing

out is cartilage oligomeric matrix protein (COMP), which is mainly found in cartilage[32]. Under inflammatory conditions an efflux to synovial fluid[33] and to the bloodstream is notable, and COMP can therefore be used as an inflammatory marker. In addition, it has also been shown that it is possible to induce arthritis by immunization of COMP into the tail roots of rats[34]. Other possible candidates for affecting matrix formation in cotton are osteopontin[35] and cytokines. As high amounts of aggrecan diffuse into the surrounding tissue it is most likely the candidate for influencing remodeling of the cotton compartment[36]. The increased amount of proteoglycans may change fibroblast activities such as cell proliferation and migration[37], matrix production, and cytokine binding, e.g., TGF-ß, bFGF, PDGF, IL-4, and IL-8[38]. Increased amounts of chondroitin sulfate chains, generated from aggrecan both in cotton surrounding viable cartilage and in particular killed cartilage, have a direct effect on the recruitment and survival of neutrophils[27]. This is reflected in an 8-fold increase of cells in cotton surrounding killed cartilage. The effect of matrix is further underlined by the fact that exogenously added highly sulfated GAG influence the remodeling process in the cartilage in the air pouch. Indeed, addition of heparan sulfate inhibits the formation of the fine fibrotic tissue (A. Bjärdahlen, unpublished observation).

Of specific interest is the cell pattern that showed a delay of nearly 14 days in the presence of myofibroblasts in the cotton surrounding cartilage. The origin of these cells is unclear, which makes it difficult to find a mechanism for this appearance or the delay of these cells. They may be recruited from primitive mesenchymal cells[39] or from fibroblasts[12]. Their relation to fibroblasts is also unclear, but it is possible that activation by cytokines, especially TGF-ß, may, as part of an activation process, induce increased expression of actin, thus generating a myofibroblast phenotype[40]. In the systems with cartilage, the appearance of myofibroblasts together with collagen formation was delayed. It is therefore possible that the increased number of inflammatory cells secrete more cytokines, such as interferon-γ, that abrogate TGF-ß activity, thereby delaying myofibroblast appearance and matrix production[41]. Further studies need to be performed to delineate the new findings that components from the extracellular matrix of cartilage influence remodeling of the surrounding tissue. Emphasis should be put on the recruitment of inflammatory cells and recruitment/induction of myofibroblasts by factors released from cartilage. Of specific interest here are also the degradation products of the proteoglycan protein core as well as those of the polysaccharides.

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