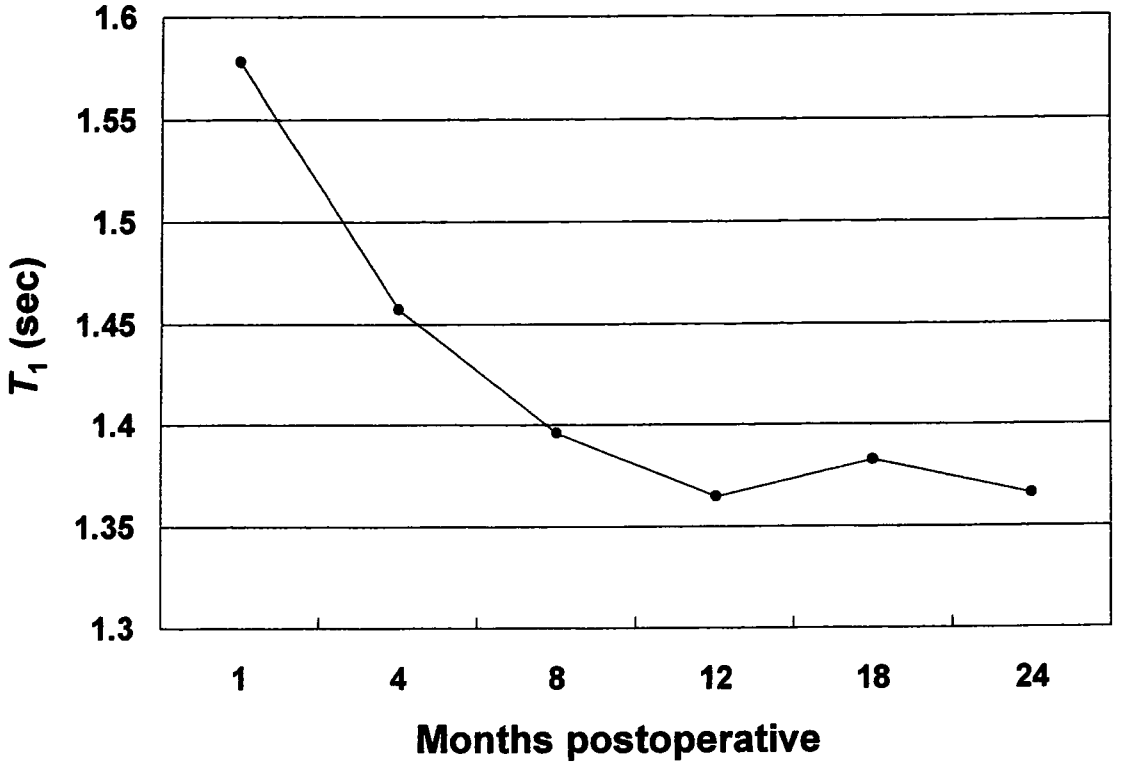

Figure 3. The T_1 in a patient after autologus chondrocytes implantation. The T_1 was at its highest level immediately after ACI, decreased with time to 9 months, and settled to almost level thereafter, which was compatible with the change of SI index.

reported to be generally superior to that after the conventional cartilage repair methods such as subchondral drilling (7), abrasion chondroplasty (8), and microfracture (18). However, histology of reparative cartilage after ACI has been reported to be inferior to normal hyaline cartilage, and has been considered to be mixed cartilage composed of hyaline-like cartilage and fibrocartilage (2,12,16). Our MR imaging findings and histological and biochemical examinations showed reparative cartilage that is definitely inferior to normal hyaline cartilage. This finding was consistent with previously reported findings. This study had a relatively small number of patients and thus statistical analysis concerning the relationship between the histology of biopsy samples and the SI indexes has not been determined. Further studies are required.

ACI is often performed in relatively young patients. Therefore, to avoid long-term degenerative changes, regeneration using more durable hyaline cartilage is desired. To improve ACI to obtain stable clinical results and good long-term outcomes, various studies targeting the acquisition of good repair cartilage tissues close to healthy hyaline cartilage are being performed (5). For the clinical application of these new ACI techniques in the future, our evaluation method using MR imaging may be useful for comparing the maturation process and long-term durability of reparative cartilage between the present and new ACI techniques. Our MR imaging method may be also useful for follow-up on each patient after ACI, but studies on additional cases are necessary.

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J Orthop Sci (2005) 10:595–607
DOI 10.1007/s00776-005-0947-z

Original article

Journal of
Orthopaedic Science

Type II collagen synthesis in the articular cartilage of a rabbit model of osteoarthritis: expression of type II collagen C-propeptide and mRNA especially during early-stage osteoarthritis

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Abstract

Background. The aim of this study was to observe time course changes in type II collagen synthesis in various regions of articular cartilage affected with osteoarthritis (OA) by examining the expression of type II collagen C-propeptide (pCOL II-C) and mRNA in a rabbit OA model.

Methods. Osteoarthritis was experimentally induced by partial lateral meniscectomy in the knees of Japanese white rabbits. The cartilage of the animals was then examined histologically over time. The degenerative area of articular cartilage was divided into three areas, according to the degree of degeneration. The ability to synthesize type II collagen was estimated by the immunohistological staining of pCOL II-C and the in situ hybridization of mRNA in type II collagen.

Results. The positive rate of pCOL II-C immunostaining in chondrocytes was highest in the central-degenerative region 1 week after surgery, and the highest rate in the para-degenerative region was observed 2 and 4 weeks after surgery. The percentage of pCOL II-C positive cells increased as the histological degeneration score increased to moderate degeneration and then decreased with further progression of the severity of cartilage degeneration. Examination by in situ hybridization revealed that the regions marked by strong pCOL II-C mRNA expression were similar to those indicated by the immunohistology results.

Conclusions. These results suggest that the type II collagen-synthesizing potential of chondrocytes is highest in moderately degenerated areas of OA articular cartilage. Cartilage repair continues to be seen even as OA advances, although the reaction varies depending on the stage of OA.

Introduction

Osteoarthritis (OA) is a degenerative disease that is initially characterized by cartilage destruction. In cases of OA, cartilage destruction is followed by cartilage repair and secondary synovitis. Chondrocytes proliferate, as seen in cluster formations, and actively synthesize and secrete extracellular matrix components, such as type II collagen and aggrecan, into the surrounding tissue. Therefore, the potential of chondrocytes to synthesize these extracellular matrix components may serve as an indicator of the potential for repair in patients with OA.

During the process of type II collagen synthesis in chondrocytes, type II procollagen synthesized in the cytoplasm is first released from the cell. The N- and C-terminals of this procollagen are then cleaved by a specific peptidase present in the extracellular matrix, leading to the formation of collagen fibrils. Considering this pathway, the cleaved C-terminal (type II collagen C-propeptide, or pCOL II-C) is likely to be at a 1:1 ratio with type II collagen, and the amount of pCOL II-C probably reflects the level of type II collagen synthesis. Clinically, the concentration of pCOL II-C in the synovial fluid has been reported to serve as a reliable marker of type II collagen synthesis in the articular cartilage.[1–5] To date, however, few articles have discussed how this molecule is expressed in the articular cartilage during the process of OA-mediated degeneration. Information about the expression of pCOL II-C in OA-affected cartilage is essential to examine whether this molecule is useful as a marker of cartilage repair in patients with OA. In the present study, changes in the distribution of pCOL II-C in experimentally induced OA-affected articular cartilage were followed throughout OA progression, from an early stage onward.

The reason an experimentally induced OA model is used is that specimens from humans with OA are primarily collected during replacement surgery in patients with terminal-stage OA; it is extremely difficult to obtain human samples during early-stage OA. Thus, the metabolic changes that occur in OA-affected cartilage

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Received: December 24, 2004 / Accepted: July 28, 2005

during the various stages of OA cannot be monitored in such human specimens. We therefore used an animal model of OA in which the cartilaginous changes are thought to resemble those seen in humans with OA. Chronological changes in the distribution of pCOL II-C in this model were examined immunohistochemically, and the expression of type II collagen mRNA was examined using in situ hybridization to determine the relation between the expression of pCOL II-C and type II collagen mRNA.

Methods

Rabbit OA model

Osteoarthritis was induced in 24 female Japanese white rabbits (Tokyo Laboratory Animals Science, Tokyo, Japan), weighing about 2.0kg each, by performing a partial lateral meniscectomy, according to the method proposed by Colombo et al.[6] First, under anesthesia with pentobarbital (0.6–0.8ml/kg body weight), an approximately 1-cm longitudinal incision was made in the lateral aspect of the left knee, followed by dissection of the lateral collateral ligament and the sesamoid ligament to expose the anterolateral aspect of the lateral meniscus. The lateral meniscus was then cut over an adequate range (up to a width of about 3mm) leading to the inner margin, and the wound was closed. After surgery, the animals were caged and raised without imposing any movement. In the control group ($n = 3$), animals that had not undergone an operation were sacrificed under deep anesthesia with pentobarbital. In the OA-induced group, the animals were sacrificed under deep pentobarbital anesthesia 1, 2, or 4 weeks after surgery ($n = 7$ for each time point). The left knee joint was removed from each sacrificed animal for subsequent histological examination. The surgery, upkeep, and disposal of individual animals were performed in accordance with National Institutes of Health (NIH) guide lines, and the research planning was recognized by the intramural research committee.

Preparation of histological specimens

The left knee joints were removed from 2 control animals and 18 OA-induced animals (6 animals from each group) and immediately immersed in 10% neutral buffered formalin for about 7 days to allow tissue fixation. This procedure was followed by decalcification in 50% EDTA solution for 6–8 weeks. The tissue specimens were then embedded in paraffin and cut into 4μm thick sections. Serial coronal sections were prepared from the femoral side of the knee, advancing backward from the sesamoid ligament attachment point. Serial coronal sections were also prepared from the tibial side, advancing backward from the anterior crucial ligament attachment point. These sections were then subjected to toluidine blue staining, immunostaining, and in situ hybridization.

Cartilage specimens, measuring $1mm^3$, from one control rabbit and three OA-induced rabbits (one animal from each group) were collected from a coronal section of the condyle containing the sesamoid ligament attachment point (on the femoral side) and a coronal section of the condyle containing the anterior crucial ligament attachment point (on the tibial side). These cartilage pieces were processed for immunoelectron microscopy.

Antibody

An anti-human pCOL II-C antibody (provided by Teijin Iwakuni Research Center, Iwakuni, Japan), was used for the immunostaining. This antibody was prepared by administering four subcutaneous doses of peptide (composed of 30 amino acid residues from the N-terminal of human pCOL II-C) at intervals of 2 weeks. Eight weeks after the initial administration, the antiserum from these rabbits was applied to a protein A affinity column to extract the immunoglobulin G (IgG) fractions.[5] The specificity of this antibody to rabbit pCOL II-C was confirmed by Western blotting using rabbit pCOL II-C obtained by the method reported by Choi et al.[7] (Fig. 1).

Immunostaining

Tissue sections were deparaffinized and then treated with chondroitinase ABC (0.25 units/ml) (Seikagaku, Tokyo, Japan) for 90min at 37°C. Drops of methanol solution containing 0.3% H_2O_2 were applied to the sections, which were then incubated at room temperature for 30min to eliminate endogenous peroxidase activity. The sections were then exposed to 50% diluted normal serum containing 2% bovine serum albumin (BSA) (Irvine Scientific, Santa Ana, CA, USA) for 60min at room temperature to eliminate nonspecific protein absorption. This was followed by overnight exposure to the primary antibody (i.e., anti-human pCOL II-C antibody, 33μg/ml, diluted with phosphate-buffered saline containing 0.1% BSA) (Fuji Chemical Industries, Takaoka, Japan) at 4°C. The specimens were washed with phosphate-buffered saline (PBS) and then exposed to the secondary antibody (goat anti-rabbit biotinated IgG) (Vector Laboratories, Burlingame, CA, USA) for 60min at room temperature and to avidin-biotinylated horseradish peroxidase (Vector Laboratories) for 45 minutes at room temperature. Finally, color was developed with 0.3% diaminobenzidine tetrahydrochloride

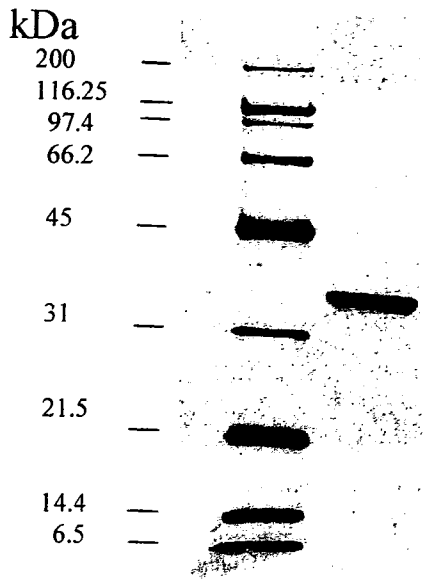

Fig. 1. Western blot of rabbit type II collagen C-propeptide (pCOL II-C) extracted from articular cartilage tissue using the method reported by Choi et al.[7] Lanes: *A*, pCOL II-C (MW 35 kDa); *B*, marker protein (MWs 6.5, 14.4, 21.5, 31, 45, 66.2, 97.4, 116.25, and 200 kDa)

dissolved in PBS containing 0.006% H_2O_2, followed by counterstaining with Meyer's hematoxylin.

Histological evaluation

The degree of articular cartilage degeneration was evaluated according to the partially modified grading criteria reported by Colombo et al.,[6] as shown in Table 1. The present criteria included loss of the superficial layer, erosion of cartilage, fibrillation or fissures (or both), loss of stainable proteoglycan, disorganization of chondrocytes, loss of chondrocytes, exposure of subchondral bone, and cluster formation. These eight categories were rated using a four-point scale (+1 to +4). Thus, the possible total scores ranged from 8 (no degeneration) to 32 (severe degeneration). The intensity of immunostaining for pCOL II-C was numerically expressed as the average percentage of positive cells among the chondrocyte population in six randomly sampled ×400-magnified visual fields, according to the method reported by Okada et al.[8]

Immunoelectron microscopy

The specimens were fixed in 4% paraformaldehyde containing 0.5% glutaraldehyde for 20 h at 4°C and then washed with 0.1 M phosphate buffer containing 8% sucrose and 0.05 M ammonium chloride. The specimens were further washed with 8% sucrose in a 0.1 M phosphate buffer and dehydrated in an ascending series of ethanol/water baths for 15 min at 4°C. The specimens were then embedded in Lowicryl K4M resin. After the embedded specimens had hardened, they were cut into

Table 1. Histological criteria for evaluating the degree of articular cartilage degeneration in an OA model

Items	Criteria for scores of 1–4			
	+1	+2	+3	+4
Loss of superficial layer	<Slight	Moderate	Focally severe	Extensively severe
Erosion of cartilage	<Detectable	Moderate	Focally severe	Extensively severe
Fibrillation and/or fissures	<1 Very small	1 Small	2 Small or 1 medium	3 Small, 2 medium, or 1 large
Loss of proteoglycan	Paler stain than control	Moderate loss of TB staining	Marked loss of TB staining	Total loss of TB staining
Disorganization of chondrocytes	Noticeable	Moderate, with some loss of column	Marked loss of columes	No recognizable organization
Loss of chondrocytes	<Noticeable decrease in cells	Moderate decrease in cells	Marked decrease in cells	Very extensive decrease in cells
Exposure of subchondral bone	<Focal exposure of bone	Moderate exposure of bone	Fairly extensive exposure of bone	Very extensive exposure of bone
Cluster formation	<3–4 Small or 1–2 medium	5–6 Small, 3–4 medium, or 1–2 large	≥7 Small, 5–6 medium, 3–4 large	≥7 Medium or 5–6 large

Eight criteria were defined by partially modifying the grading criteria reported by Colombo et al.[6] The possible total score ranged from 8 (no degeneration) to 32 (severe degeneration)
TB, toluidine blue; OA, osteoarthritis; <, including less reaction than described

ultrathin sections using an ultramicrotome (Ultracut N, Reichert-Nissei). The ultrathin sections were placed on a 300-mesh nickel grid to allow the following immune reactions to occur.

The sections were treated with PBS containing 0.1% BSA for 10min at room temperature and exposed overnight at 4°C to the primary antibody (anti-human pCOL II-C antibody, identical to the one used for immunostaining) diluted 1:100 in PBS containing 0.5% BSA with 15mM NH_4Cl. After being washed with PBS, the sections were exposed to the secondary antibody (anti-rabbit IgG gold antibody, 15nm in diameter) diluted 1:10 with PBS containing 0.5% BSA + 15mM NH_4Cl, for 120min at room temperature. After being washed again with PBS and distilled water, the sections were subjected to 2% uranyl acetate electrostaining for 3min and then washed with distilled water and exposed to Reyndol lead for 1min at room temperature. The sections were then washed again with distilled water and vapor-deposited with carbon. The sections were observed under a transmission electron microscope (JEM-120 EX II; JEOL, Tokyo, Japan) at an acceleration voltage of 80kV.

In situ hybridization

According to the procedure proposed by Takaishi et al.,[9,10] the cloning vector encoding the rabbit pCOL II-C region was digested with a limiting enzyme (BamHI or XhoI) to yield a direct chain; and T7 or SP6 RNA polymerase (Boehringer Mannheim, Mannheim, Germany) was used to prepare digoxigenin (DIG)-labeled antisense and sense cRNA probes via in vitro transcription. The labeled RNA transcripts were recovered after phenol-chloroform extraction and precipitation with ethanol and were then stored at –80°C.

Nonstained thin sections, prepared as described above, were then exposed to proteinase K 10μg/ml (TaKaRa Bio, Ohtsu, Japan) for 20min at 37°C. After the probe was added to the hybridization buffer (50% formamide, 10% dextran sulfate, 10mM dithiothreitol, tRNA 200μg/ml, 1× Denhardt's solution), the sections were further incubated for 16h at 45°C. The sections were then washed with 2 × SSC and 0.2 × SSC (each for 20min at 50°C). This procedure was followed by color development with nitroblue tetrazolium/5-bromo-4-chloro-3-idolyl-phosphate (NBT/BCIP) (Boehringer Mannheim) after exposure to an alkaline phosphatase-labeled anti-DIG antibody (Boehringer Mannheim) diluted 1:2000.

Statistical analysis

Comparison of pCOL II-C positivity in spatial discrimination over time was performed using the analysis of variance (ANOVA) followed by Scheffe's test. Differences were considered significant when $P < 0.05$.

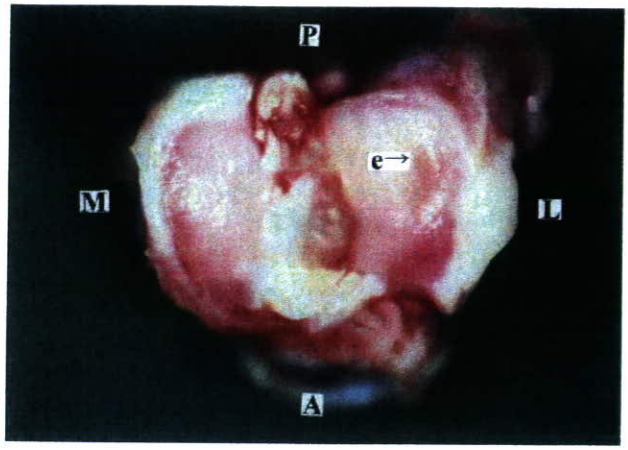

Fig. 2. Articular surface of the tibia in a rabbit osteoarthritis (OA) model 4 weeks after surgery. On the surface of the lateral tibial plateau, an area of erosion is visible in the area where the lateral meniscus was partially resected. *A*, anterior; *P*, posterior; *M*, medial; *L*, lateral; *e*, erosion

Results

Macroscopic findings

When observed macroscopically, both the lateral femoral condyle and the lateral tibial plateau showed mild erosion, along with a decrease in the superficial gloss of the articular cartilage, 1 week after surgery. Two weeks after surgery, the erosion was associated with marked depression of the cartilage surface. Four weeks after surgery, the area of erosion had grown in both size and depth. Figure 2 shows the lateral tibial plateau 4 weeks after surgery. In this OA model, the interindividual variations in the extent of erosion were less extensive on the tibial side. Therefore, the tibial side was used for subsequent histological examinations.

Toluidine blue staining

Semimacroscopically, the region of cartilage erosion showed gradual expansion after surgery. In this study, the articular cartilage of the OA model was divided into three regions on the basis of the regional relation with the site of maximum erosion. The region distant from the erosive site that did not show evidence of degeneration when stained with toluidine blue was defined as "the intact region." The central part of the erosive site, showing the most advanced degeneration, was defined as "the central-degenerative region". The region adjacent to the erosive site was defined as "the para-degenerative region."

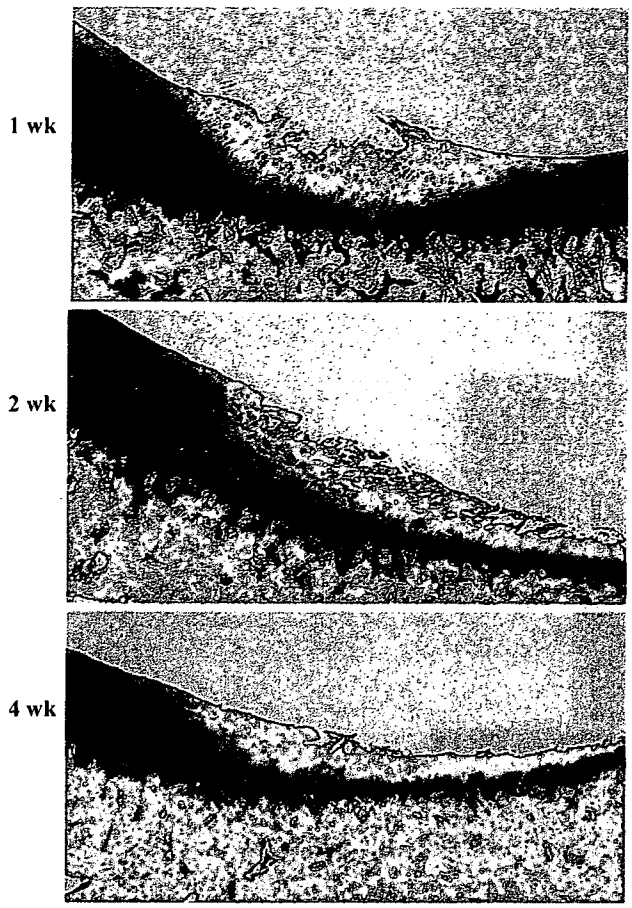

Fig. 3. Toluidine blue staining of the articular cartilage in a rabbit OA model 1, 2, and 4 weeks after surgery. Fibrillation, reduced staining, and cluster formation are visible in the areas of erosion 1 week after surgery. These changes had advanced even further at 2 and 4 weeks after surgery

When the lateral tibial plateau of the rabbit OA model was examined 1 week after surgery, the central-degenerative region exhibited fibrillation of the cartilage surface, reduced chromatic response to toluidine blue (TB), and formation of chondrocyte clusters. Two weeks after surgery, the area of cartilaginous erosion had advanced and the articular cartilage had become thinner, concomitant with an increase in the depth of the superficial fibrillation fissures in the central-degenerative region and a decrease in the chromatic response to TB in the same region. Four weeks after surgery, these changes had advanced further, and the number of chondrocytes in the central-degenerative region had decreased markedly (Fig. 3).

One week after surgery, the mean histological degeneration score was 8 at all points in the intact region, 10.0 ± 1.6 in the para-degenerative region, and 17.0 ± 7.8 in the central-degenerative region. Two weeks after surgery, the mean score was still 8 at all points in the intact region but had increased to 11.7 ± 2.1 in the para-degenerative region and to 23.3 ± 2.3 in the central-degenerative region. Four weeks after surgery, the score remained at 8 at all points in the intact region but had increased to 12.0 ± 1.1 in the para-degenerative region and to 25.8 ± 1.7 in the central-degenerative region.

Immunohistological findings

The localization of pCOL II-C in the cartilage from the lateral tibial plateau of the OA model was analyzed immunohistologically. One week after surgery, intense staining was seen in the chondrocytes of the central-degenerative region, whereas almost no staining was seen in the chondrocytes of the intact region (Fig. 4). Positive staining was present in the para-degenerative region but was weaker than that in the central-degenerative region. The extracellular matrix of the area from the central-degenerative region to the para-degenerative region showed staining at sites close to the degenerated superficial layers. Two weeks after surgery, pCOL II-C staining was more intense in the chondrocytes of the para-degenerative region than in those of the central-degenerative region (Fig. 5). At this time point, almost no staining was seen in the intact region. The extracellular matrix of the area from the central-degenerative region to the para-degenerative region showed staining at sites near the superficial layers 2 weeks after surgery. The sites that showed immunostaining 4 weeks after surgery were similar to those seen 2 weeks after surgery. In other words, immunostaining for pCOL II-C was negative in the intact region and was stronger in the para-degenerative region than in the central-degenerative region (Fig. 6). Also at 4 weeks after surgery, positive staining of the extracellular matrix was seen at sites close to the superficial layers, although staining of the cells themselves and of the extracellular matrix in the deeper layers was weaker at 4 weeks than at 2 weeks after surgery.

The intensity of anti-pCOL II-C immunostaining in the chondrocytes was evaluated using the method described by Okada et al.[8] One week after surgery, the percentage of pCOL II-C-positive cells averaged 2.5% ± 1.8% in the intact region, 18.4% ± 14.4% in the para-degenerative region, and 32.9% ± 19.7% in the central-degenerative region. Two weeks after surgery, the corresponding percentages were 1.9% ± 2.2%, 47.0% ± 11.6%, and 23.9% ± 19.0% in the intact, para-degenerative, and central-degenerative regions, respectively. Four weeks after surgery, the values were 9.8% ± 5.9%, 52.0% ± 12.8%, and 15.3% ± 14.0% in the intact, para-degenerative, and central-degenerative regions, respectively (Fig. 7). We then analyzed the relation between the histological degeneration score and the per-

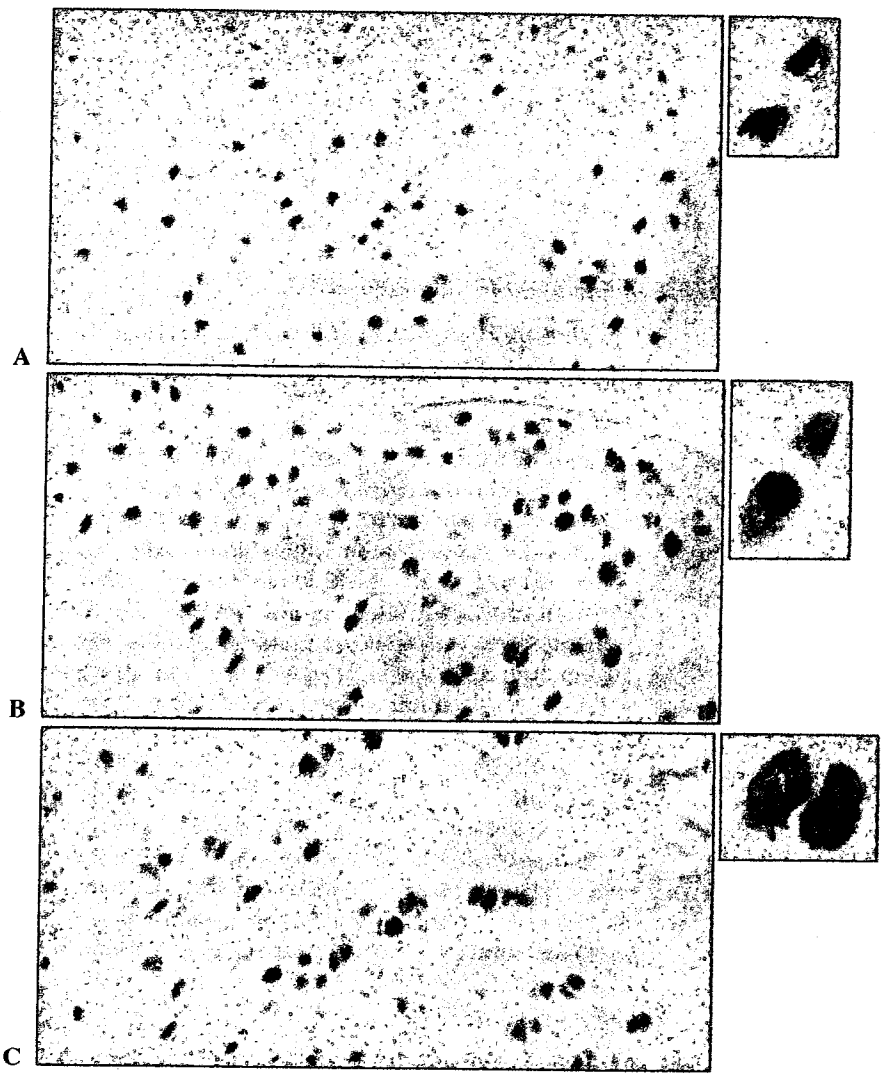

Fig. 4. pCOL II-C immunostaining in the chondrocytes of the articular cartilage in a rabbit OA model 1 week after surgery. Intense immunostaining is more visible in the central-degenerative region of the cartilage specimen than in the other regions. **A** Intact region. **B** Para-degenerative region. **C** Central-degenerative region. **A–C** ×100. **Insets** ×400

centage of pCOL II-C-positive cells at 1, 2, and 4 weeks after surgery (Fig. 8). Between the scores of 8 and 16, an increase in the score was associated with an increase in the percentage of pCOL II-C-positive cells. At scores >16, however, an increase in the score was associated with a reduction in the percentage of pCOL II-C-positive cells.

In situ hybridization

The expression of type II procollagen mRNA in the articular cartilage of the lateral tibial plateau of the OA models was examined using in situ hybridization. One week after surgery, mRNA expression was seen in the chondrocytes of all three regions (intact, para-degenerative, and central-degenerative) but was most marked in the central-degenerative region, as shown in Fig. 9. Two weeks after surgery, mRNA expression was stronger in the chondrocytes of the para-degenerative region than in those of the intact or central-degenerative regions (Fig. 10). Four weeks after surgery, the expression of mRNA followed a tendency similar to that seen at 2 weeks after surgery (Fig. 11). Table 2 summarizes the pCOL II-C immunostaining results and the expression of type II collagen mRNA in relation to the time after surgery (in weeks) and the specimen regions.

Immunoelectron microscopy

The distribution of pCOL II-C molecules in the cells was examined using immunoelectron microscopy. When chondrocytes from the control and the OA-induced rabbits were examined, gold particles were mainly observed along the rough endoplasmic reticulum in the cytoplasm and nucleus. The pCOL II-C distribution pattern in the cells was the same for cartilage obtained 1, 2, and 4 weeks after surgery. The

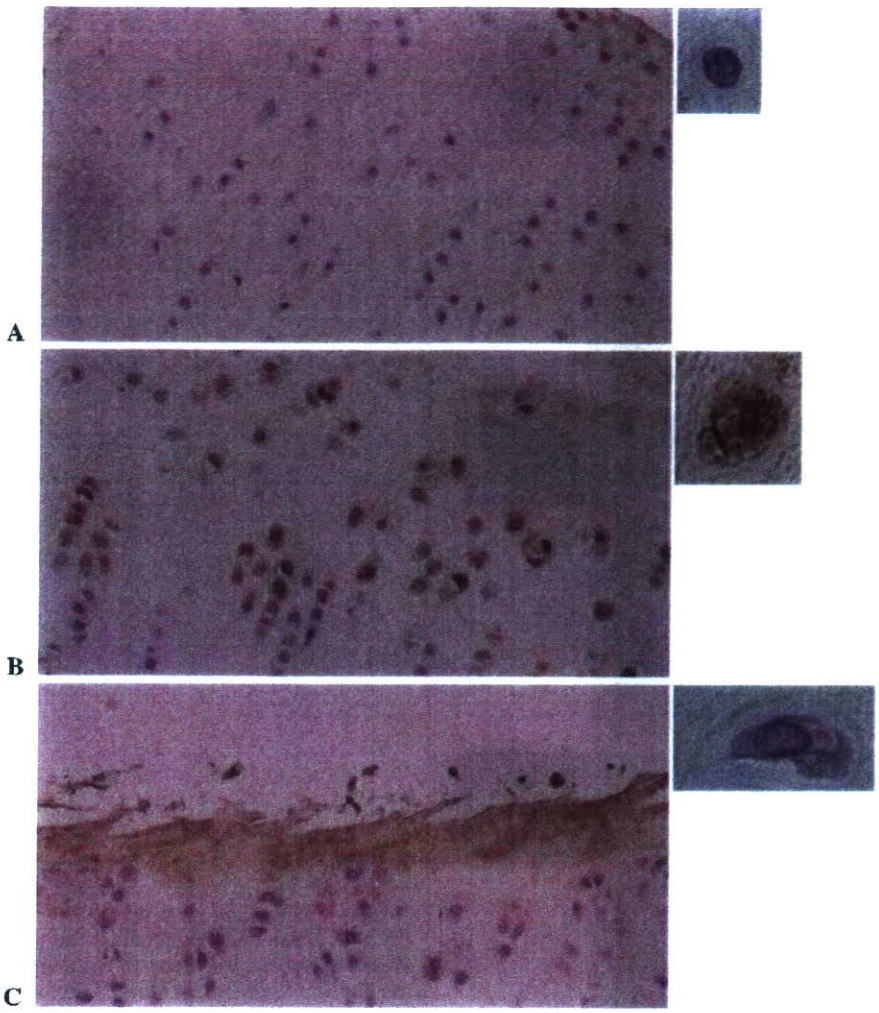

Fig. 5. pCOL II-C immunostaining in the chondrocytes of the articular cartilage in a rabbit OA model 2 weeks after surgery. The staining is more intense in the chondrocytes of the para-degenerative region than in the central-degenerative region. **A** Intact region. **B** Para-degenerative region. **C** Central-degenerative region. **A–C** ×100. **Insets** ×400

Table 2. Intensity of pCOL II-C staining and type II collagen mRNA expression in cartilage

	OA model, 1 week			OA model, 2 weeks			OA model, 4 weeks		
Procedure	A	B	C	A	B	C	A	B	C
pCOL II-C immunostaining	–	+	++	–	+++	++	–	++	+
Type II collagen mRNA (in situ hybridization)	+	++	++	+	+++	++	+	++	+

A, intact region; B, para-degenerative region; C, central-degenerative region
–, none; +, positive, but weak; ++, moderate; +++, strong

immunoelectron microscopy findings for a sample obtained 2 weeks after surgery are shown in Fig. 12.

Discussion

The present study using a rabbit OA model revealed that the chromatic responses of chondrocytes to pCOL II-C staining changes as the degeneration of articular cartilage advances. The distribution of pCOL II-C in the chondrocytes, as observed by immunohistochemistry, can be interpreted as reflecting the presence of the C-terminal type of II procollagen synthesized in the cells.

One of the reasons we used the animal OA model was that we wanted to know the changes in the chondrocytes of articular cartilage at an early stage of OA. Therefore, we investigated these changes from 1 week after surgery on the animal model. For degenera-

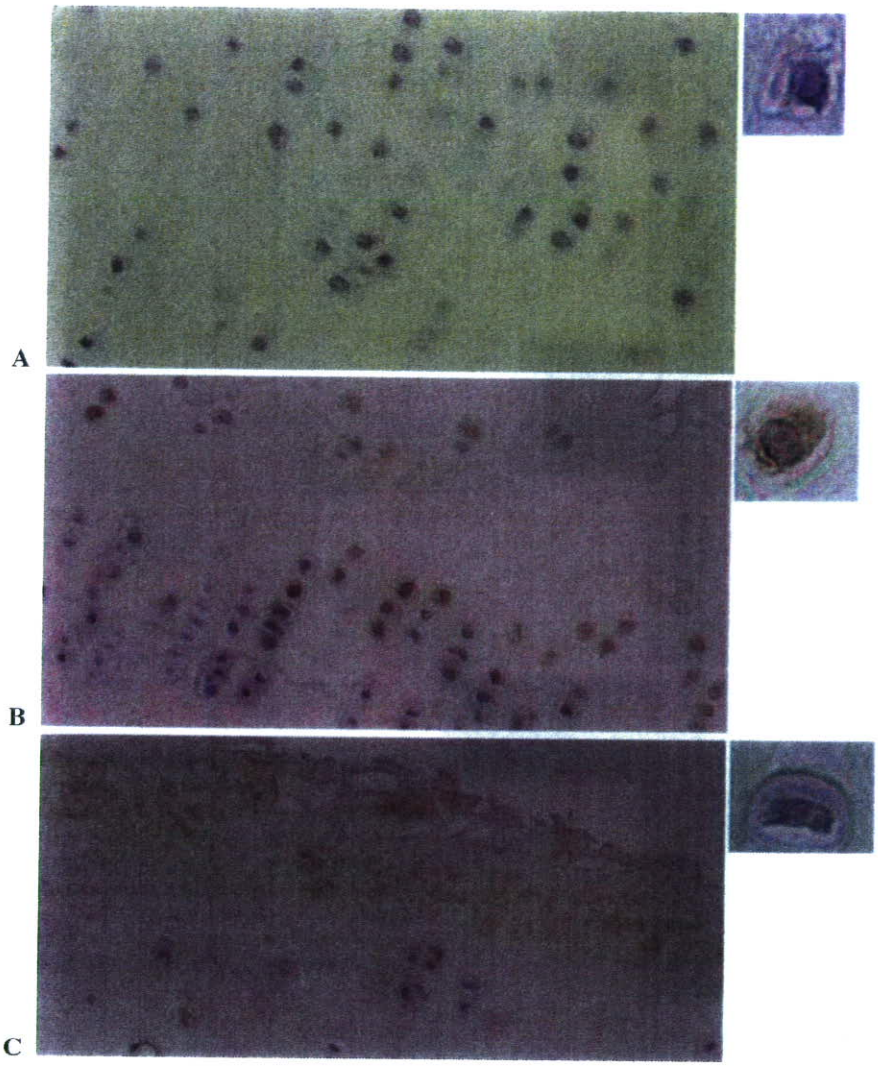

Fig. 6. pCOL II-C immunostaining in the chondrocytes of the articular cartilage in a rabbit OA model 4 weeks after surgery. The staining is markedly more intense in the chondrocytes of the para-degenerative region than in the central-degenerative region. **A** Intact region. **B** Para-degenerative region. **C** Central-degenerative region. **A–C** ×100. **Insets** ×400

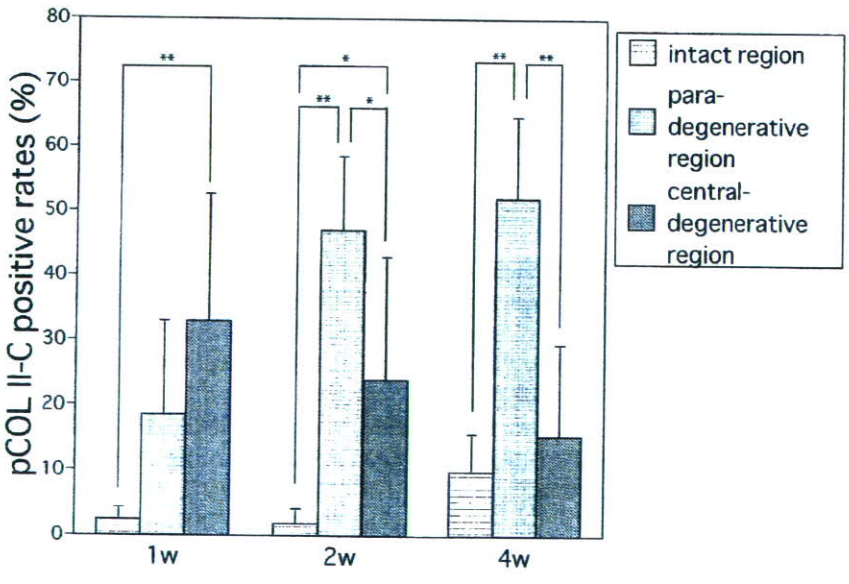

Fig. 7. Changes in the percentage of pCOL II-C-positive chondrocytes in discrimination over time. pCOL II-C positivity is highest in the central-degenerative region 1 week after surgery and highest in the para-degenerated regions at 2 and 4 weeks after surgery. $^{*}P < 0.05$. $^{**}P < 0.01$

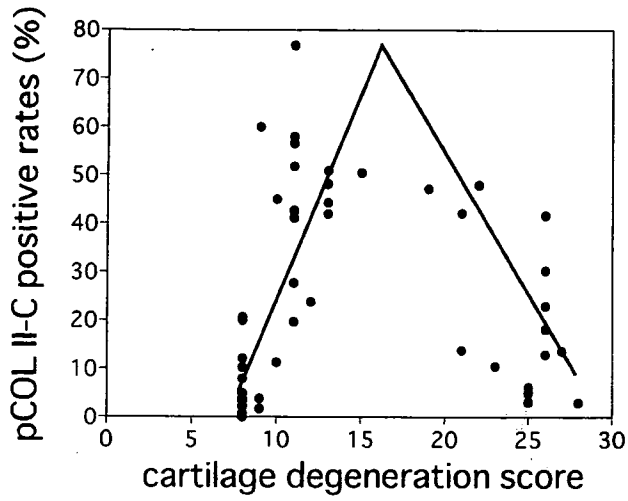

Fig. 8. Histological degeneration scores and pCOL II-C positivity. For histological degeneration scores of 8–16, pCOL II-C positivity increases with the increasing degeneration score. For degeneration scores higher than 16, however, pCOL II-C positivity decreases with increasing degeneration scores

tion scores of ≤16 points, an increase in the score was associated with an increase in the percentage of pCOL II-C-positive cells. For scores >16 points, an increase in the score was associated with a reduction in the percentage of pCOL II-C-positive cells. These results suggest that when the degeneration is moderate or mild the synthesis of type II collagen in chondrocytes is stimulated by the progression of degeneration; but when degeneration is more severe, further degeneration is associated with a reduction in the synthesis of type II collagen.

Nakajima et al. examined human cartilage collected during total knee replacement surgery and examined the pCOL II-C immunostaining pattern of OA-affected articular cartilage. Their study revealed that as OA advanced from mild to moderate severity the pCOL II-C staining intensified; but when OA became more severe, pCOL II-C staining decreased. Nakajima et al. therefore concluded that in humans with OA the synthesis of type II collagen is more active when OA is moderate than when it is mild or severe.[11] Some investigators have

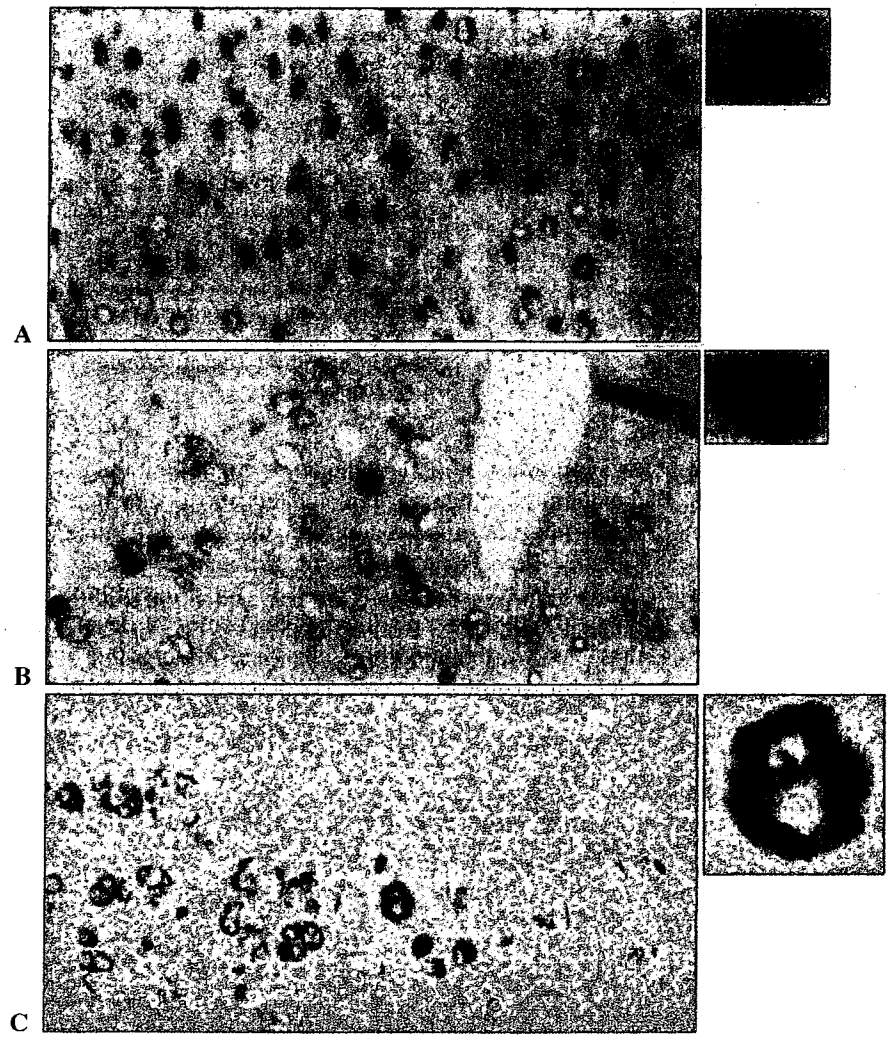

Fig. 9. In situ hybridization of type II collagen mRNA in articular cartilage 1 week after surgery. mRNA expression is most apparent in the chondrocytes of the central-degenerative region and weaker in the para-degenerated and intact regions. **A** Intact region. **B** Para-degenerative region. **C** Central-degenerative region. **A–C** ×100. **Insets** ×400

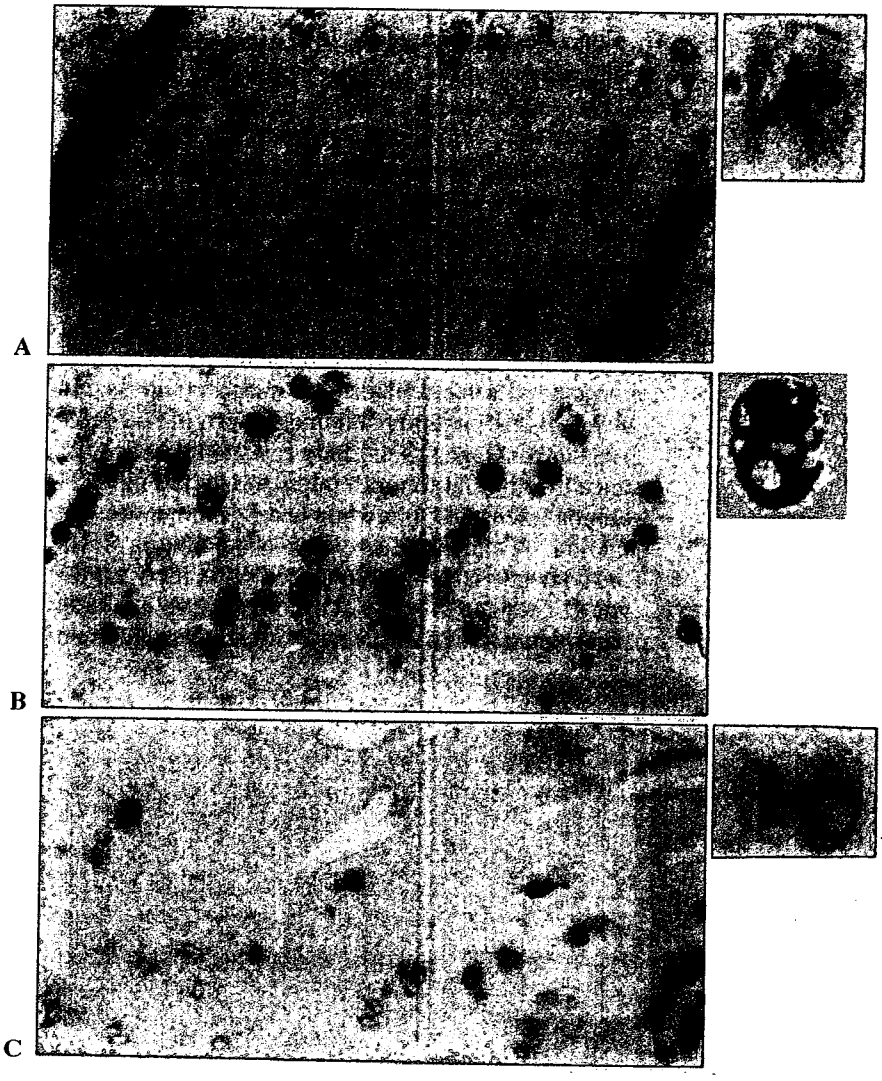

Fig. 10. In situ hybridization of type II collagen mRNA in articular cartilage 2 weeks after surgery. mRNA expression is stronger in the chondrocytes of the para-degenerative region than in those of the intact and central-degenerative regions. **A** Intact region. **B** Para-degenerative region. **C** Central-degenerative region. **A–C** ×100. **Insets** ×400

analyzed the relation between the progression of OA and the metabolism of type II collagen. When the pCOL II-C level in synovial fluid from the knee joints of humans with OA was analyzed, the progression of early OA to moderate OA was found to be associated with an elevated concentration of pCOL II-C in the synovial fluid; as the OA increased in severity, however, the pCOL II-C level decreased.[4,5,12] Related to these findings, Kobayashi et al. reported that the degree of pCOL II-C staining and the synovial fluid concentration decreased in accordance with the progression of articular cartilage regeneration observed after surgical treatment using a high tibial osteotomy for human OA.[13]

A conclusion similar to that made by Nakajima et al. can be drawn from the histopathological findings of the present study using a rabbit OA model. The present study revealed that the main site of pCOL II-C expression shifts from the center of degeneration toward the para-degenerative region, suggesting that the potential to synthesize type II collagen is higher in the para-degenerative region than in the most degenerated central-degenerative region. During the early stages of degeneration, when the extent of degeneration is mild even in the central-degenerative region, the synthesis of type II collagen is enhanced at the center of degeneration. As the degeneration increases in severity, however, the synthesis of type II collagen increases in the peripheral regions (where degeneration is still relatively mild) and decreases in the central-degenerative region (where the degeneration has already become severe).

None of the previously reported studies simultaneously analyzed the responses to immunostaining for pCOL II-C and the expression of type II collagen mRNA in the articular cartilage of either OA-affected humans or OA animal models. Of the studies reported to date, reports on the distribution of type II collagen synthesis in the articular cartilage of humans with OA

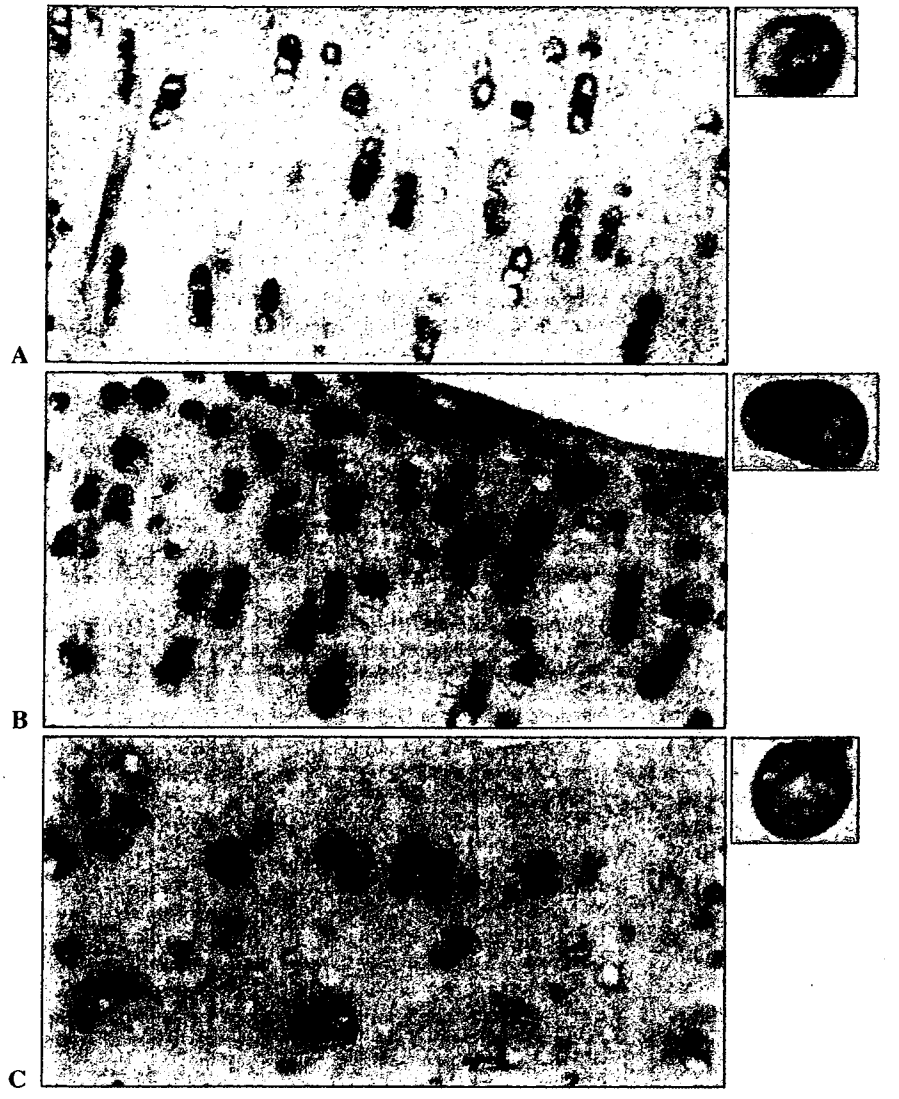

Fig. 11. In situ hybridization of type II collagen mRNA in articular cartilage 4 weeks after surgery. mRNA expression is stronger in the para-degenerative region than in the intact and central-degenerative regions. **A** Intact region. **B** Para-degenerative region. **C** Central-degenerative region. **A–C** ×100. **Insets** ×400

have revealed that in patients with moderate degeneration the expression of type II collagen mRNA in the chondrocytes increased from the intermediate to the deeper layers of articular cartilage[14] and that the area showing enhanced mRNA expression was approximately identical to that in the extracellular matrix region, which exhibited increased immunostaining for type II collagen.[15,16] Furthermore, an increase in type II collagen mRNA expression has been reported in the osteophytes of articular cartilage in humans with OA, and this increase in mRNA expression was related to an increased response of the extracellular matrix in the same region to immunostaining for type II collagen.[17,18] In OA models, the increase of immunostaining intensity of degenerated type II collagen has been also reported in the degenerated area of articular cartilage.[19–22] In the present study, the regions showing increased rates of pCOL II-C immunostaining in the chondrocytes were almost identical to the regions showing enhanced expression of type II procollagen mRNA. This finding provides additional support to the view that the increase in pCOL II-C staining in the chondrocytes reflects an increase in the synthesis of type II procollagen.

When examined using immunoelectron microscopy, gold particles, which represent the anti-pCOL II-C antibody, were detected on the rough endoplasmic reticulum (RER) of the chondrocytes. This finding suggests that this antibody is bound to the C-peptide of type II procollagen being synthesized on the RER. Gold particles were also observed in the nuclei, although the significance of this finding remains obscure. If this finding is interpreted as being suggestive of the active uptake of pCOL II-C by the nuclei, pCOL II-C may have some regulatory function, such as acting as a transcription factor. Nakata et al. reported that one of the DNA-binding proteins (binding to the type II collagen

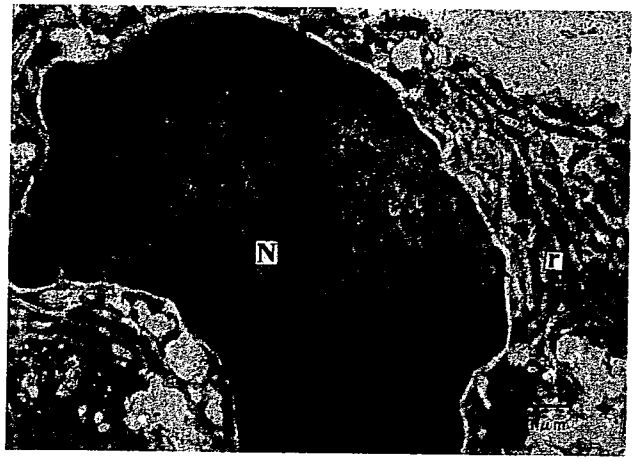

Fig 12. Immunoelectron microscopy image of the ultrastructural distribution of pCOL II-C in the chondrocytes of the articular cartilage in an OA-induced rabbit model 2 weeks after surgery. Gold particles are visible in the cytoplasm and nuclei along the rough endoplasmic reticulum. *N*, nucleus; *r*, rough endoplasmic reticulum. *Bar* 1 μm

enhancer) was identified as pCOL II-C and that pCOL II-C suppresses transcription of the type II collagen gene.[23]

Osteoarthritis may be understood as a condition where the balance between the destruction and repair of articular cartilage is biased toward destruction. The results of the present study using a rabbit model of OA also suggest that the repair potential of chondrocytes continues to exist even as cartilage destruction advances, and that this potential varies according to the location of the cartilage relative to the point of maximum destruction.

Acknowledgments. The authors are indebted to Prof. Emeritus Yutaka Yabe (Keio University, Department of Orthopedics) and the late Prof. Masayuki Shinmei (National Defense Medical College, Department of Orthopedic Surgery) for the opportunity to conduct this research. The authors also express their sincere gratitude to Mr. Tadaaki Yoshida (formerly of Kureha Chemical Industry) for his assistance with immunoelectron microscopy and all the staff members of the Orthopedic Surgery Department of the National Defense Medical College, who provided support and advice during this study.

The authors did not receive and will not receive any benefits and funding from any commercial party related directly or indirectly to the subject of this article.

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Effect of GDF-5 on Ligament Healing

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Received 20 August 2004; accepted 17 June 2005

Published online 7 October 2005 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jor.20002

ABSTRACT: The effects of growth and differentiation factor-5 (GDF-5) on ligament healing were studied using a gap injury model of the medial collateral ligament in rat knee joints. The administration of GDF-5 once at the time of surgery significantly improved the mechanical properties of the femur–ligament–tibia complex. At 3 weeks after surgery, 30 μg of GDF-5 improved the ultimate tensile strength of the complex by 41%, and the stiffness by 60%, compared with the vehicle control ($p < 0.05$ for both; Fisher's PLSD test). The observation with a transmission electron microscopy revealed that GDF-5 increased the diameter of collagen fibrils in the repair tissue, which was considered to be a possible mechanism for the positive result in the biomechanical testing. Quantitative PCR and in situ hybridization revealed enhanced type I procollagen expression by GDF-5, and the PCR analysis also revealed that the GDF-5 treatment reduced the expression of type III procollagen relative to type I procollagen. The PCR analysis further showed that the expression of decorin and fibromodulin was relatively reduced against type I procollagen by the growth factor, which was considered to be responsible for the increase of collagen fibril diameter in the repair tissue. No adverse effects were observed, and the use of GDF-5 was considered a promising approach to facilitate ligament healing. © 2005 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 24:71–79, 2006

Keywords: ligament healing; growth and differentiation factor-5; biomechanical evaluation; transmission electron microscopy; mRNA expression

INTRODUCTION

Various attempts have been made to facilitate ligament healing to date. Since the discovery of growth factors, their use has become a focus of research because they are known to play pivotal roles in tissue healing. Several growth factors have been used for ligament healing, namely, platelet-derived growth factor-BB (PDGF-BB),[1,2] insulin-like growth factor-1 (IGF-1),[2] fibroblast growth factor-2 (FGF-2),[2,3] and transforming growth factor-beta1 and beta2 (TGF-β1 and β2),[1,4] alone or in combination. Promising results have been reported with some of them, but the best protocol to improve ligament healing has not yet been established.

Growth and differentiation factor-5 (GDF-5) in mice, or cartilage-derived morphogenetic protein-1 (CDMP-1) in humans, is a growth factor that belongs to the TGF-β superfamily.[5,6] During embryonal development, GDF-5 is expressed in cartilaginous tissue and plays important roles in the formation of joints and long bones.[5,6] Recent studies on GDF-5–deficient mice revealed that the factor is also involved in the development of tendons.[7,8] An earlier study showed that GDF-5 induces a tendon- or ligament-like tissue when ectopically administered in rat muscles,[6] suggesting that the factor may reintroduce the process of tendon development in mature individuals. Considering that the healing process is closely related to the developmental steps, it seems plausible that GDF-5 facilitates tendon or ligament healing. In

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fact, several investigators have applied GDF-5 on injured Achilles tendons and reported favorable results.[9–11] Based on these facts, we hypothesized that the addition of exogenous GDF-5 would facilitate the healing of an injured ligament, and examined the hypothesis using a gap injury model in a medial collateral ligament (MCL) in rat knee joints.

MATERIALS AND METHODS

Animal Model and Application of GDF-5

A total of 97 male Sprague–Dawley rats, 6 weeks old and weighing 215 ± 18 g (mean ± SD), were used for the study. All experiments were performed under the approval of the institutional review boards. Following anesthesia, a 1.5-cm skin incision was made in the medial side of the right knee, and an MCL was exposed. A 2-mm segment of the ligament centered on the joint line was sharply removed with a scalpel to create a full-width ligament gap, based on a reported model for delayed ligament healing.[12] This model had an advantage that the volume of the repair tissue was larger than that in a simple laceration model, which made the analysis easier. The rats were assigned to one of four groups. In Group I, the gap in the ligament was left untreated and served as a nontreatment control ($n = 36$). In Group II, the gap was filled with 10 μL of fibrin sealant (Beriplast® P, Aventis Pharma, Tokyo, Japan) mixed with 10 μL of 1 mM HCl solution to evaluate the effect of the carrier and vehicle on ligament healing ($n = 10$). In Groups III and IV, 10 μL of fibrin sealant was mixed with 10 μL of HCl solution containing 3 or 30 μg of recombinant mouse GDF-5 (R&D systems, Minneapolis, MN), respectively, and applied to the ligament gap ($n = 15$ and 36, respectively). Following the treatment, the fascia and skin were closed with 5-0 sutures. After surgery, the animal was housed in a cage with the operated limb free until the time of evaluation.

Histological Analysis

Eighteen rats in Groups I and IV were used for histological analysis. At 1, 3, and 6 weeks after surgery, three animals in each group were sacrificed, and medial collateral ligaments were obtained from the right knee joints. Coronal sections (4 μm thick) were made and stained with hematoxylin and eosin, and then the cellularity, cell morphology, and orientation of the tissue matrix were evaluated under a light microscope.

Biomechanical Testing

Biomechanical testing was performed using 10 animals in each of Groups I through IV. The animals were sacrificed at 3 weeks after surgery, and the right hind limbs were obtained by disarticulation at the hip and ankle joints. The limbs were wrapped in a plastic film and stored at −80°C until use. Before measurement, the specimen was thawed overnight at 4°C, and all soft tissues were carefully dissected free, sparing the cruciate ligaments, lateral collateral ligament, menisci, and MCL. The specimen was mounted in custom-built clamps with acrylic resin and attached to a uniaxial tensile testing machine (Tensilon® UTM 2.5T, Toyo Baldwin, Tokyo, Japan). Prior to the measurement, the positions of the clamps were adjusted so that the knee was flexed at 60 degrees, and the MCL was aligned in parallel with the load axis of the actuator. Then all knee ligaments except the MCL and the menisci were carefully removed. The femur–MCL–tibia complex was loaded at a rate of 20 mm/min until failure, and the load–deformation curve was recorded by an X-Y plotter (SS 207-EP, Toyo Baldwin). The site of failure was recorded for each specimen. The stiffness of the femur–MCL–tibia complex was obtained based on the linear slope of the load–deformation curve between 1 and 3 mm of elongation.

Transmission Electron Microscopy Analysis

Ultrastructural analysis of the healing tissue was performed on three animals in each of Groups I and IV at 6 weeks after surgery. For the evaluation, the animals were sacrificed and MCLs were obtained. The samples were then prepared according to a previously reported method[13] with some modifications. In brief, immediately after sacrifice, the operated MCL was drip-fixed by modified Karnovsky's fixative while the knee joint was held at 30 degrees flexion. An entire MCL was detached from bones and processed by 2% osmium tetroxide in the cacodylate buffer for 2 h at 4°C. After dehydration with a series of graded ethanol, the central part of the repair tissue was cut out in the form of a 2-mm cube. The tissue was then embedded in Epon 812 with careful attention to the orientation. Sections were cut at 100-nm thickness and stained with aqueous uranyl acetate and lead citrate. The examination was performed under a Hitachi H-7000 electron microscope. From each ligament, five photomicrographs of fibril cross sections were taken at the magnification of ×50,000. Locations of all photomicrographs were randomly selected, but those with artifacts and cellular components were carefully excluded. These images were scanned into a computer, and quantitative assessment was performed using an image analysis software (NIH Image version 1.61; National Institutes of Health, Bethesda, MD). To evaluate fibril diameters, the minimum fibril diameter was always measured to prevent errors due to obliquity in the sectioning. Fibrillar structures with a diameter less than 20 nm were excluded to avoid measurement of microfibrils or of noncollagen fibrillar components. Next, for each specimen, the total area occupied by the collagen fibrils and the total number of fibrils were counted on all five

photomicrographs, and the averaged percentage occupied by the fibrils and the averaged number of fibrils per 1 μm^2 were calculated.

In Situ Hybridization

Eighteen animals in Groups I and IV were used for in situ hybridization to evaluate the expression of pro α1(I) collagen mRNA. At 1, 3, and 6 weeks after surgery, three animals in each group were sacrificed and the ligaments were obtained. The method of in situ hybridization was described previously.[3] Briefly, after harvest, the ligaments were immediately fixed by immersion in 4% paraformaldehyde solution at 4°C overnight. The tissues were then embedded in OCT compound (Miles Laboratories, Elkhart, IN) and coronal sections of 10-μm thickness were prepared and mounted on glass slides coated with gelatin and poly-L-lysin. The sections were treated with proteinase K, then acetylated and prehybridized at 50°C for 3 h in the hybridization buffer. The hybridization was then allowed to proceed at 50°C for 24 h in the hybridization buffer containing the digoxigenin-labeled RNA probe. The probe was synthesized by T7 RNA polymerase using a pBluescript vector containing partial human pro α1(I) collagen cDNA as a template. In the transcription process, the probe was labeled with digoxigenin using DIG RNA labeling kit (SP6/T7) (Roche Diagnostics, Basel, Switzerland).

After hybridization, the sections were washed under an appropriate condition, and the hybridized probe was visualized by the color reaction for digoxigenin. The nuclei were stained with methyl green, and the sections were observed under a light microscope. The intensity of gene expression was assessed in a semiquantitative manner by a blinded observer (H.H.).

Quantitative Reverse Transcription-Polymerase Chain Reaction Analysis

Five animals in each of Groups I, III, and IV were sacrificed and served for quantitative PCR analysis at 1 week after surgery. After sacrifice, the ligament repair tissue was harvested, carefully avoiding contamination of the surrounding tissue. The tissue was then minced with a scalpel, and RNA was extracted using a commercially available kit (RNeasy® Micro; Qiagen, Valencia, CA). The synthesis of cDNA was performed with Sensiscript® reverse transcriptase (Qiagen). The cDNA was then used for real-time quantitative PCR on a LightCycler® (Roche Diagnostics) using gene specific primers and probes (Table 1). The expression of pro α2(I) collagen, pro α1(III) collagen, decorin, fibromodulin, and lumican was evaluated together with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard. For some genes, the amplification was monitored by the fluorescence of SYBRE® Green I dye that bound to the double-strand DNA. For the others, the probes carrying fluorescent dyes were used for monitoring. The initial amount of cDNA for the gene was determined with a standard curve based on the known amounts of the PCR product.

Table 1. Primer and Probe Sequences Used for Quantitative PCR

Gene	GenBank Accession Number	Primer/Probe Sequence	Amplicon Size (bp)
Col1A2[a]	AF121217	Forward: 5′- TACAACGCAGAAGGGGTGTC -3′	193
		Reverse: 5′- CCTCAGCAACAAGTTCGACG -3′	
		Probe Flu: 5′- GCTTCTCAGAACATCACCTACCACTGCA -3′	
		LC: 5′- GAACAGCATTGCGTACCTGGACGA -3′	
Col3A1	X70369	Forward: 5′- ATGGTGGCTTTCAGTTCAGC -3′	284
		Reverse: 5′- TGTCTTGCTCCATTCACCAG -3′	
Decorin[a]	Z12298	Forward: 5′- TGGACTGAACCGTATGATTG -3′	208
		Reverse: 5′- GCTGGCTGCATCAACTTT -3′	
		Probe Flu: 5′- GCAGGGAATGAAGGGTCTCGGATACA -3′	
		LC: 5′- CCGCATCTCAGACACCAACATAACTGC -3′	
Fibromodulin	NM080698	Forward: 5′- TGTCCGGCTGTCTCACAACA -3′	198
		Reverse: 5′- TCCACCACCGTGCAGAAACT -3′	
Lumican	BC061878	Forward: 5′- GGCGTGCCTGGAAACTCATT -3′	198
		Reverse: 5′- GCGCAAATGCTTGATCTTGG -3′	
GAPDH[a]	AF106860	Forward: 5′- TGAACGGGAAGCTCACTGG -3′	307
		Reverse: 5′- TCCACCACCCTGTTGCTGTA-3′	
		Probe Flu: 5′- CTGAGGACCAGGTTGTCTCCTGTGA -3′	
		LC: 5′- TTCAACTGCAACTCCCATTCTTCCACC -3′	

[a]Gene-specific probes were used for quantitative PCR. Flu: probe conjugated with Fluorescein; LC: probe conjugated with LC red 640.

Statistics

For the results of biomechanical measurement and quantitative PCR, the data were first analyzed by one-way factorial analysis of variance (one-way factorial ANOVA), and when a significant variance was observed, Fischer's PLSD test was performed as a post hoc test. For the result of ultrastructural analysis, unpaired t-test was performed to compare the data between the two experimental groups. A p-value of <0.05 was used for statistical significance.

RESULTS

All rats survived the surgery well and resumed normal cage activity within 1 or 2 days after surgery. At sacrifice, it was confirmed that the skin wound had healed normally and the operated knee had resumed a normal range of motion equal to the unoperated side. The average weight of the animals did not show a significant difference among the groups at any evaluation period.

Gross Appearance and Histological Evaluation

At 1 week after surgery, a larger volume of the repair tissue was formed at the ligament gap in Groups III and IV, compared with the control groups. The tissue in these GDF-5–treated groups tended to have a firmer consistency, although there was no obvious difference in their appearance. At 3 weeks, the difference in the volume became less obvious, but the repair tissue was still thicker in Group IV at 6 weeks.

On hematoxylin and eosin-stained sections, the effect of GDF-5 was most obvious at 1 week (Fig. 1A and B). In the animals treated with 30 μg of GDF-5, the repair tissue showed higher cellularity, and

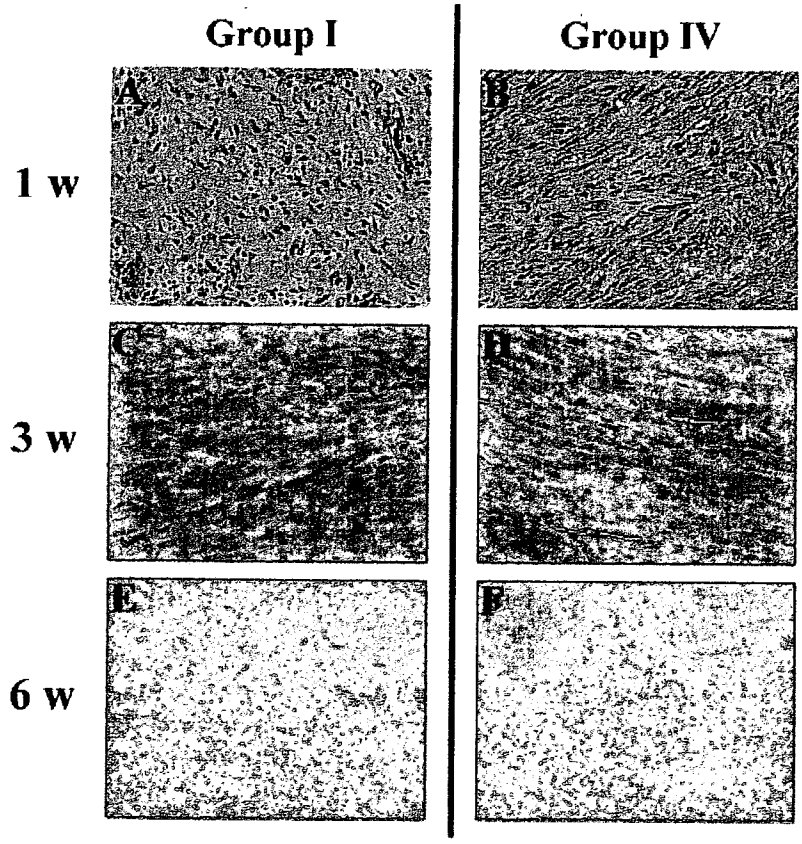

Figure 1. Histology of the repair tissue formed at the ligament gap 1, 3, and 6 weeks after surgery. The left and right columns show the results of the control animals (Group I; A, C, and E) and those treated with 30 μg of GDF-5 (Group IV; B, D, and F), respectively. Three animals in each group were evaluated at each time point, and representative photomicrographs are shown. Hematoxylin and eosin, original magnification ×20. [Color scheme can be viewed in the online issue, which is available at http://www.interscience.wiley.com]

the cells presented elongated forms while those in the control group were rounder and more spherical. These trends were observed at 3 weeks to a lesser extent, and the matrix in Group IV had better alignment and clearer orientation compared with the control animals (Fig. 1C and D). At 6 weeks after surgery, the difference between the treated and control groups was less obvious, and the repair tissues in both groups presented a similar histology (Fig. 1E and F). Unlike previous studies on tendon healing,[9–11] we did not find cartilaginous tissue formation by the GDF-5 treatment.

Biomechanical Testing

In all specimens, failure occurred at the ligament midsubstance. The ultimate tensile strength of the femur–MCL–tibia complex was 7.6 ± 3.3, 9.1 ± 3.0, 9.5 ± 2.9, and 12.9 ± 3.0 N for Groups I, II, III, and IV, respectively. The variation among the 4 groups was significant ($p < 0.01$; one-way factorial ANOVA), and the strength of Group IV was significantly greater than that of Group I or II ($p < 0.05$ for either group; Fischer's PLSD test) (Fig. 2A). The stiffness of Group I, II, III, and IV was 3.6 ± 1.8, 3.8 ± 1.7, 5.4 ± 1.7, and 6.2 ± 1.9 N/mm, respectively, and their variation was statistically significant ($p < 0.05$; one-way factorial ANOVA). Again, the stiffness of Group IV was significantly higher than that of Group I or II ($p < 0.05$ for either group; Fischer's PLSD test) (Fig. 2B).

Transmission Electron Microscopic Study

In Group I, the repair tissue contained a rather uniform population of collagen fibrils with small diameters (Fig. 3A and B), while the tissue in Group IV contained some thicker fibrils (Fig. 3C and D). The mean fibril diameters for Groups I and IV were 36.5 ± 8.0 and 45.9 ± 15.7, respectively, and the difference between the two groups was statistically significant ($p < 0.01$; unpaired t-test) (Fig. 3E). The numbers of fibrils per 1 μm^2 were 171 ± 21 and 224 ± 43 for Groups I and IV, respectively, and the repair tissue in Group IV contained significantly higher number of fibrils than Group I ($p < 0.01$; unpaired t-test) (Fig. 3F). Consequently, the percentages of the area occupied by collagen fibrils in Groups I and IV were 26.3 ± 4.9 and 49.1 ± 4.0, respectively, and the value in Group IV was significantly higher than that in Group I ($p < 0.01$; unpaired t-test) (Fig. 3G).

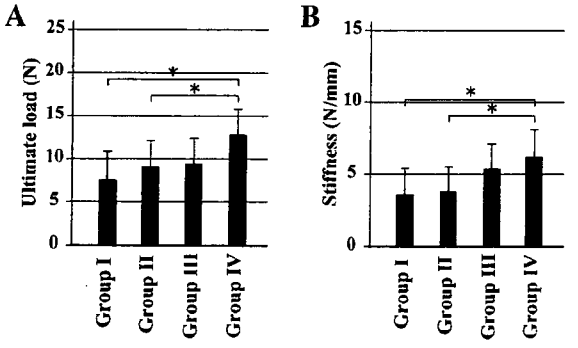

Figure 2. Ultimate tensile strength (A) and stiffness (B) of the femur–MCL–tibia complex 3 weeks after surgery. Each bar represents the average of 10 specimens. Values are expressed by means + SD. *$p < 0.05$.

In Situ Hybridization Study

In an attempt to elucidate the mechanisms for the advantageous effect of GDF-5, the expression of pro α1(I) procollagen mRNA was evaluated by in situ hybridization at 1, 3, and 6 weeks after surgery. At 1 week, abundant gene expression was observed in the repair tissue in both groups, but the expression in Group IV was more enhanced than in Group I (Fig. 4). The gene expression in the remnants of original MCL was barely detectable in both groups. At 3 weeks after surgery, the mRNA expression in the repair tissue was reduced in both groups, but the expression in Groups IV was still stronger than that in Group I. The expression in the repair tissue was hardly detectable at 6 weeks for both groups.

Quantitative PCR Study

To clarify the difference in the repair process, the levels of mRNA expression were studied on two dominant procollagens together with three small leucine-rich proteoglycans (SLRPs) that are known to affect collagen fibrillogenesis. Compared with Group I, the expression of pro α2(I) collagen was significantly higher in Groups III and IV ($p < 0.01$ for both groups; Fischer's PLSD test) (Fig. 5A), while the expression of pro α1(III) collagen was similar for all three groups (Fig. 5B). Thus, the expression of type III collagen relative to type I was significantly reduced in both GDF-5 treated groups ($p < 0.01$ for both groups; Fischer's PLSD test) (Fig. 5C). Among the three SLRPs studied, the expression of decorin was