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OSTEOARTHRITIS and CARTILAGE

Characterization of a model of osteoarthritis in the rabbit knee

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Summary

A new computerized method of histomorphometry was used to assess the development of osteoarthritis (OA) in a rabbit model. Three groups of 10 New Zealand White rabbits with closed epiphyses underwent unilateral anterior cruciate ligament transection (ACLT) and contralateral arthrotomy (sham). Groups were killed at 4, 8 and 12 weeks. At the time of death the femoral condyles were assessed grossly following the application of India ink using the following grading scale. Grade 1: intact surface; grade 2: minimal fibrillation; grade 3: overt fibrillation; grade 4: erosion. All histological sections were assessed using a color image analysis system. The mean thickness and area were measured for a defined cartilage region. The root mean square surface roughness (based on deviations from an idealized smooth surface) was calculated to assess the surface profile of the articular cartilage. The results were as follows. After ACLT, no full-thickness ulceration was noted at 4 weeks. Four of the medial femoral condyles at 8 weeks and six at 12 weeks showed full-thickness ulceration of the articular cartilage. The per cent cartilage area and cartilage thickness (ACLT divided by sham) in almost all regions showed decreases with time, indicating progressive erosion. The surface of the ACLT knees was much rougher than that of sham of the knees. These results demonstrate the usefulness of a quantitative methodology using a computerized video analysis system to assess the articular cartilage following ACLT in a rabbit model for the development of OA.

Key words: Osteoarthritis, Articular cartilage, Image analysis, Histomorphometry.

Introduction

SINCE osteoarthritis (OA) in humans is most commonly a slowly progressive disease and its onset is not definable ordinarily, the early stage of the disease is difficult to study. Thus, an appropriate animal model is essential for studying the pathogenesis and treatment of OA. Such an animal model must satisfy the requirements of reproducibility of the disease, ease of animal handling, amount of tissue available for study, and cost [1].

Experimentally-induced OA in animals has been employed for many years using a variety of techniques including immobilization of a joint [2, 3], a surgical alteration and destabilization of the joint structure [4–10], intra-articular injection of an agent [11] and limb denervation [12]. Immobilization of a joint has been shown to produce articular cartilage degeneration histologically. Evans *et al.* [2] reported changes such as thinning of cartilage, matrix fibrillation, cleft and ulceration as a result of immobilization of the rat knee, and this was felt to resemble the osteoarthritic process of human OA. However, no attempt of cartilage repair as characterized by the proliferation of chondrocytes in clones and an increase in proteoglycan synthesis, which are ordinarily seen in human OA, was noted in this model. The induced lesions with immobilization may be different from spontaneous OA in human. This model has been studied on the basis of the clinical observation that cartilage degeneration occurs after meniscectomy. Histologic and biochemical changes in the partial medial meniscectomy model presented by Moskowitz *et al.* [7] are similar to human OA. This model might be useful, but the progression of the osteoarthritic lesion seems to be very slow. Shapiro *et al.* [13] reported that lesions rarely progressed to the stage of severe destructive OA. The lesions also tended to be focal, and were limited to the medial compartment. It was noted that regeneration of the meniscus could interfere with the progression of osteoarthritic change.

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Anterior cruciate ligament transection (ACLT) in the dog is a recognized model for OA and has been studied by many investigators [6, 8–10, 14, 15]. There are, however, conflicting studies regarding the progression of the disease and the extent of the degenerative changes. MacDevitt *et al.* [8] found consistent fibrillation of the medial tibial plateau by 8 weeks, whereas Johnson [14] reported less severe change even after 27 weeks. Studies by Brandt *et al.* [15] showing a full-thickness loss of articular cartilage 54 months after ACLT, which included a process of hypertrophic repair as described by Vignon *et al.* [9], is persuasively similar to human OA. However, weight, age and breed of their animals as well as surgical technique, cage activity and exercise in these studies are considerably different. In addition, utilizing the dog limits extensive study because of the high cost of purchase and daily board [1, 15]. Small animals such as rats do not provide enough cartilage tissue for multidisciplinary study, and the rats have open epiphyses. While the rabbit has been used frequently in orthopedic research [16], little has been described about the effect of ACLT in this animal [17].

This manuscript describes the use of a computerized image analysis system to characterize the cartilage histomorphometry in a rabbit model of OA following ACLT during the different stages of the progression.

Materials and methods

Thirty New Zealand White (NZW) female rabbits (14 ± 2 months old) weighing 4.4 ± 0.5 kg and with closed epiphyses were used in this study. Closed epiphyses were confirmed by X-ray. The rabbits were divided into three groups of 10 each after surgical ACLT and simple arthrotomy of their contralateral knees.

SURGICAL PROCEDURE

All rabbits were anesthetized by an intramuscular injection of ketamine (100 mg/kg) and xylazine (8 mg/kg). Both knees were shaved and disinfected with Betadine solution. A medial parapatellar incision was made through the skin and an arthrotomy performed. The patella was dislocated laterally and the knee placed in full flexion. The ACL was visualized and transected with a no. 15 blade. The joint was then irrigated with sterile saline and closed. The capsule and the synovium were closed together with a running structure of 4-0 prolene. The skin was closed with a running 3-0 nylon mattress suture supplemented with interrupted sutures. The contralateral knee was opened, the patella dislocated, the knee irrigated and closed, but the ACL was not cut.

Post-operatively, the animals were permitted cage activity (60 cm × 60 cm × 40 cm) without immobilization. The animals were closely monitored for infections and other complications. The average weight of the rabbits at surgery was 4.42 ± 0.53 kg, and at death 4.45 ± 0.28 kg. A group of 10 rabbits each was killed at 4, 8 and 12 weeks by intracardiac injection of T-61 euthanasia solution.

GROSS MORPHOLOGY

After death, both knees including the synovium (except for the synovium at the incision site) were harvested. Gross morphological changes of the femoral condyles were assessed according to the following criteria after the application of India ink [8, 18]. Grade 1 (intact surface): surface appears normal and does not retain any ink; grade 2 (minimal fibrillation): site appears normal before staining, but retains the India ink as elongated specks or light gray patches; grade 3 (overt fibrillation): the cartilage is velvety in appearance and retains ink as intense black patches; grade 4 (erosion): loss of cartilage exposing the underlying bone.

HISTOLOGICAL PREPARATION

The condylar tissues were fixed in 10% neutral buffered formalin with 1% cetylpyridinium chloride (CPC) for 72 h and then decalcified with 14% EDTA. CPC was used to prevent loss of glycosaminoglycan (GAG) from the tissues during processing [19]. Completed decalcification was confirmed by X-ray. After decalcification, the femoral condyles were cut into four pieces (regions A, B, C and D from lateral condyle to medial condyle, Fig. 1) along the sagittal plane, and all pieces were embedded in paraffin. Six micron sections were cut and stained with hematoxylin and eosin (H&E), and safranin O/fast green.

Synovium from the infrapatellar fat pad of all 60 rabbit knees was also embedded in paraffin and sectioned. They were stained with H&E and Lillie's DNA stain [20] for assessment by image analysis.

HISTOMORPHOLOGY

The histological sections were assessed using a color image analysis system which allowed detection of the shape or the number of objects by means

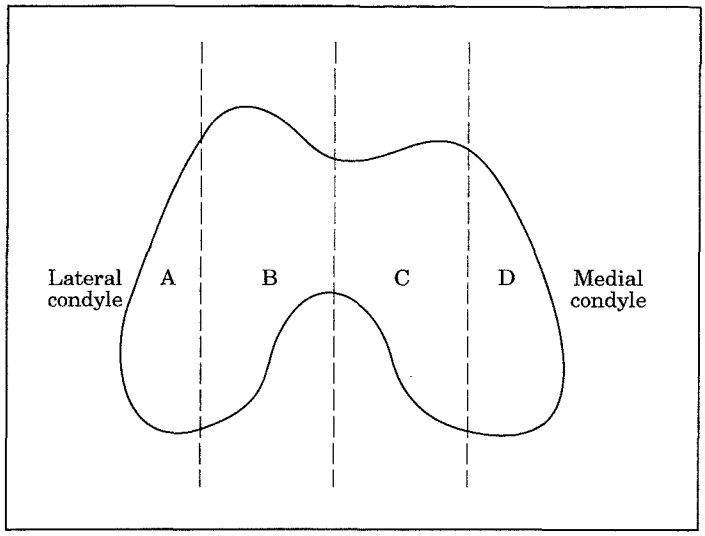

FIG. 1. Schematic representation of the femoral condyle indicating the divided regions used for histology.

of color thresholding (Oncor instrument systems, U.S.A.). Histologic sections were visualized with a Nikon Microphot microscope and an attached high resolution Hitachi HV-C10 color video camera. Video images were captured by high definition video processing boards within the computer of the image analysis system. Processed images were viewed on a Sony high resolution color monitor. Customized analysis software was developed to measure the following parameters: (1) cartilage thickness; (2) cartilage area; (3) surface roughness; (4) thickness of synovial lining cell layer; and (5) cell density of synovium.

GEOMETRIC PARAMETERS

The geometric parameters of the cartilage specimens were measured on a 5 mm long weight-bearing area of the inferior surface (femorotibial joint surface of the femoral condyle) of condyles at 32× magnification (Fig. 2). The thickness of the cartilage from the surface to the tidemark was calculated from the mean of five measurements made perpendicular to the surface of each section at five equally spaced points along the 5 mm width. The area of the cartilage present between the monitor screen edges (5 mm) was likewise calculated.

Synovial tissues were assessed by measuring the thickness of the synovial lining cell layer. This thickness was determined by averaging the measured thickness at 100 equally spaced locations along the length of the histological specimen.

SURFACE ROUGHNESS

The surface profile of each specimen was assessed by determining the surface roughness. Cartilage roughness is expressed by calculation of the root mean square (RMS) surface roughness. The RMS is

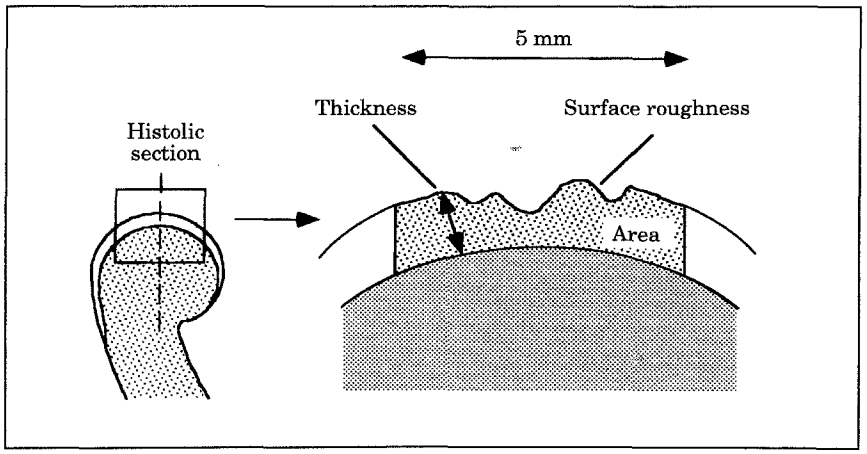

FIG. 2. Schematic representation of typical histology specimen displayed on the image analysis monitor. The geometric parameters of the cartilage specimens were measured on condylar weight-bearing areas of 5 mm in width.

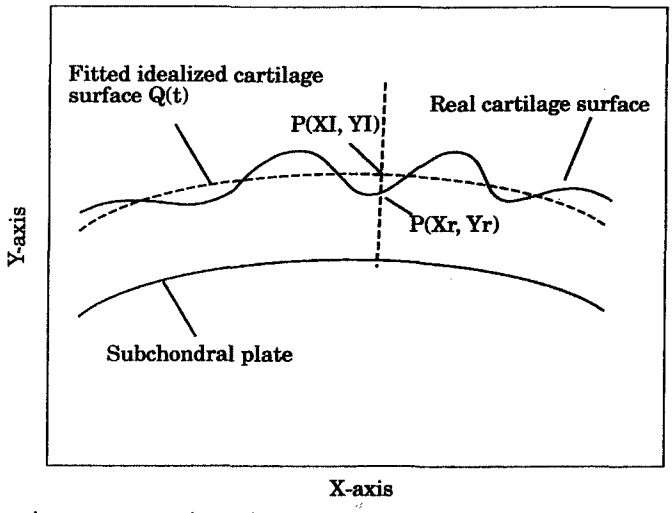

FIG. 3. Schematic representation of root mean square surface roughness calculation.

based on the deviations of the eroded cartilage surface from an idealized smooth surface, and is determined by the following equation [21, 22]:

$$\left(\frac{1}{N}\sum_{i=1}^{N}(Y\,idealized_i - Y\,real_i)^2\right)^{1/2}$$

where

N = the number of digitized points;
$Y\,idealized_i$ = the theoretical coordinate of the ideal smooth surface of articular cartilage, determined from the coordinates of the surrounding normal cartilage surface;
$Y\,real_i$ = the actual coordinate of the articular cartilage surface.

Calculation of surface roughness

The surface roughness measurement was performed utilizing a calibrated digital image processing system with custom software. Each femoral condyle section was digitally captured and a 5 mm portion displayed. The two-dimensional coordinates of the real cartilage surface (Xr, Yr) at each pixel of the digitized image were determined by the computer after the operator set threshold levels so the computer could distinguish between the background and the cartilage surface. A third order polynomial [Q(t)] was fitted to the real cartilage surface (Xr, Yr) using a least squares method for nonlinear curves. The surface roughness was calculated as the difference between the idealized surface Q(Xr) and the real surface (Xr, Yr) (Fig. 3). The surface roughness could then be calculated using the above equation.

CELL DENSITY

Synovial cell density was measured at high magnification (256×) using the sections stained with Lillie's nuclear stain. A color threshold for the darkly stained nuclei was established by identifying the color of the nuclei and allowing the computer system to filter out all images outside this threshold. After filtering the image only cell nuclei were visible on the display. These nuclei, treated as objects by the system, were then counted. The area of tissue visible on the screen was measured, and the cell density was calculated. The cell density from five different views was determined, and the results averaged to obtain a measurement for each specimen.

STATISTICAL ANALYSIS

All data from both knees were subjected to statistical analysis using two-way ANOVA with the level of significance at $a = 0.05$ and Bonferroni/Dunn post hoc comparisons.

Results

The assessment of the changes observed were compared to the following definition of OA: a disease characterized by deterioration and localized erosion of articular cartilage, accompanied by bone remodeling and changes of the periarticular tissues, and characterized by a variable rate of progression.

GROSS MORPHOLOGY

All specimens from the ACLT group exhibited complete transection of the ACL at death. All ACLT knees demonstrated osteoarthritic changes. The severity depended upon the time from ACLT to

death. At various time points, all the gross characteristics of OA were seen, including fibrillation, erosion, subchondral bone eburnation, and osteophyte formation. Gross thickening of the capsules was also seen.

No full-thickness ucleration was noted in the 4-week group. Eight of 10 specimens at 8 weeks, and nine of 10 specimens at 12 weeks showed moderate to severe OA. This was characterized by deterioration and localized erosion of articular cartilage, accompanied by bone remodeling and changes of periarticular soft tissue. Four of the 8-week specimens and six of the 12-week specimens showed full-thickness ulceration of the articular cartilage [Figs 4 and 5(a)–(c)]. Full-thickness ulceration was seen typically on the medial condyles rather than on the lateral condyles [Fig. 5(c)]. Osteophytes were seen along the ridge of the patellofemoral joint which sometimes extended to the femoral condyle.

The synovium around the supra- and infrapatellar fat pads and posterior capsules was brown colored with diffuse hypertrophy.

Posterior medial meniscal tears were noted in some rabbits, accompanied by severe osteoarthritic changes, with exposure of eburnated subchondral bone. The lateral meniscus in some rabbits was also torn.

HISTOLOGY: ACLT EFFECTS OVER TIME

Qualitative observations

Femoral condyle. Varying degrees of osteoarthritic change were present in all ACLT knees. Degenerative change such as cartilage fibrillation was seen typically on the medial condyle rather than the lateral condyle. No full-thickness ulceration was seen in the 4-week group. Vertical clefts into the transitional zone were found in some specimens [Fig. 6(a)]. The fibrillated condylar surface of the 4-week group was, however, less rough than that of the 8-week group. Two of the 4-week specimens showed increased cartilage thickness when compared to contralateral sham operated knees, possibly as a result of cartilage swelling. The tidemark of all the 4-week specimens of the ACLT knees was preserved with normal appearance. A slight decrease in safranin-O staining was seen in the fibrillated areas.

Four of the 10 specimens at 8 weeks showed full-thickness ulceration, however the region (length) of ulceration at 8 weeks was smaller than that at 12 weeks. Specimen surfaces at 8 weeks were rougher than that of 12 weeks. Saw-tooth-like fissures were seen on the periphery of the full thickness ulceration, and hypocellularity with cloning of cartilage cells was observed at the margin of the ulceration [Fig. 6(b)]. Extensive loss of safranin O stain was seen in the fibrillated area.

Six of the 12-week ACLT group exhibited full-thickness ulceration of the cartilage, exposing the smooth surface of the subchondral bone as viewed in Fig. 6(c).

Synovium. Histologically, synovium from the infrapatellar fat pads of the ACLT knees at 4 weeks exhibited hyperplasia of the synovial lining cells with a mononuclear cell infiltration in the subsynovial tissue. The synovial lining cell layer from ACLT knees was two to three times thicker than that from contralateral sham knees. Comparison of each time period revealed that the lining cell layer of the synovium decreased in thickness over time. Concurrently, the fibrosis under the

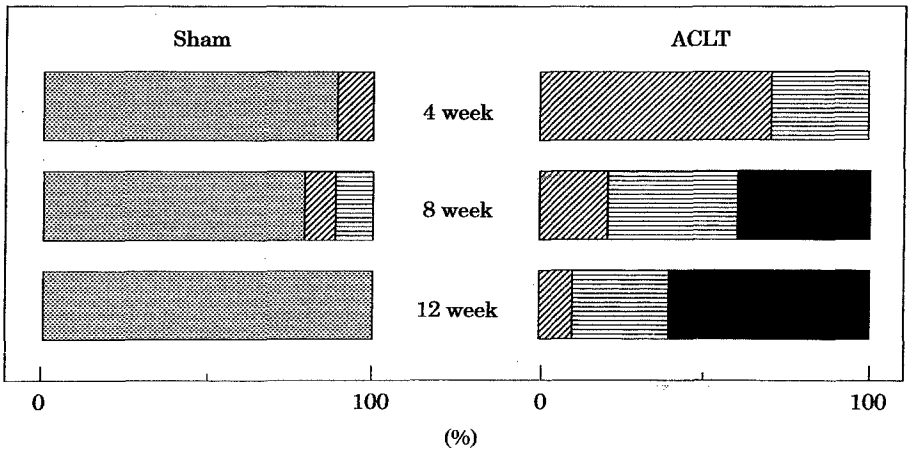

FIG. 4. Gross morphological assessment of femoral condyles of anterior cruciate ligament transection of (ACLT) and sham specimens at 4, 8 and 12 weeks ($n = 10$ each). No full thickness defects were noted in the 4-week group. Eight at 8 weeks and nine at 12 weeks showed moderate to severe cartilage degeneration. ⊡: Grade 1; ▨: grade 2; ▤: grade 3; ■: grade 4.

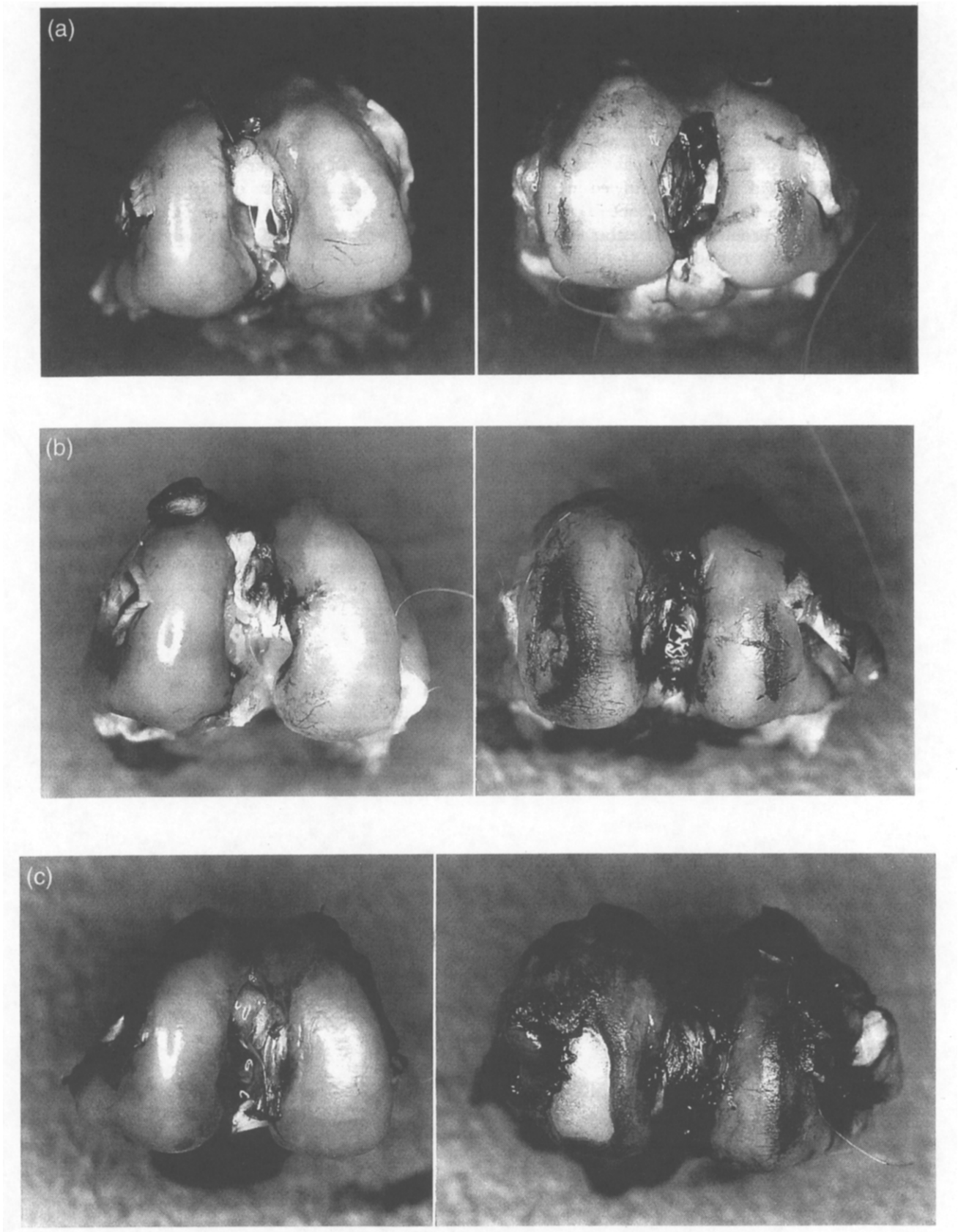

FIG. 5. (a) Gross morphological finding at 4 weeks after surgery and (b) 8 weeks after surgery. Right: experimental: left: sham operated control. Minimal fibrillation is seen in (a) both in the medial and lateral condyles. The retained intense black patches of ink in (b) indicate overt fibrillation of articular cartilage in the medial condyle. Gross morphological finding at 12 weeks after surgery. Right: experimental; left: sham operated control. Full-thickness ulceration is marked in the medial condyle.

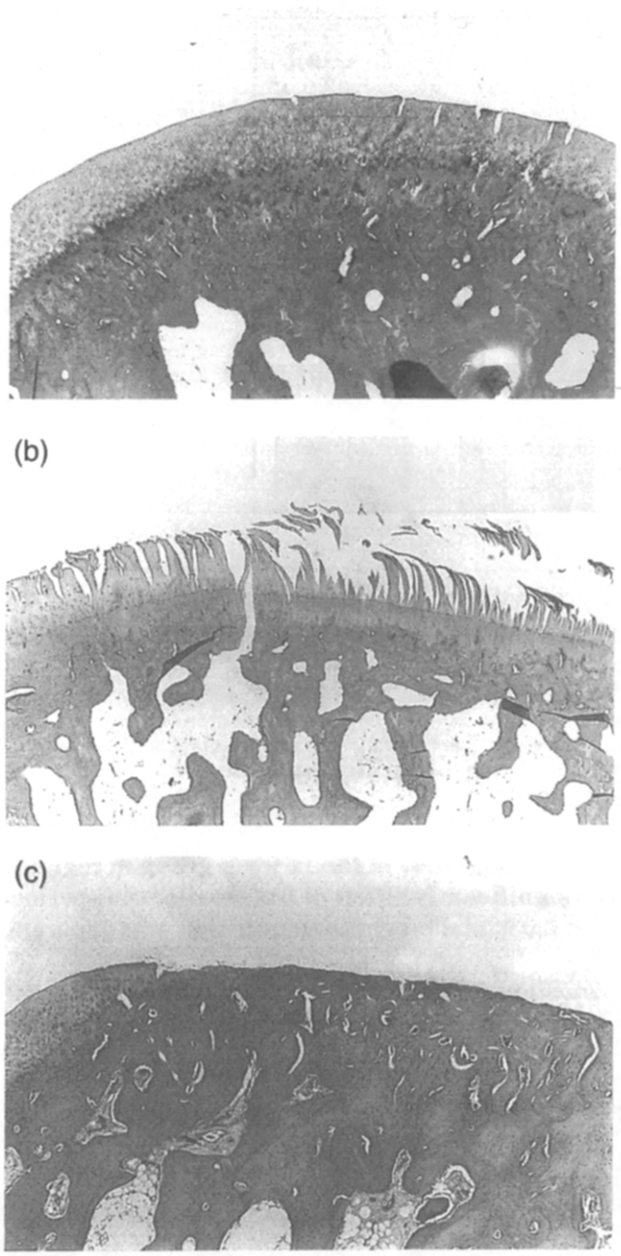

FIG. 6. Histological finding of femoral condyle from an animal killed (a) at 4 weeks after surgery; (b) at 8 weeks after surgery; and (c) at 12 weeks after surgery (hemotoxylin and eosin magnification 48×). In (a) vertical clefts are observed in the transitional zone of the articular cartilage. Saw-tooth-like fissures reaching the tidemark of articular cartilage are seen in (b). Ulceration of the cartilage exposing the smooth surface of the subchondral bone is seen in (c).

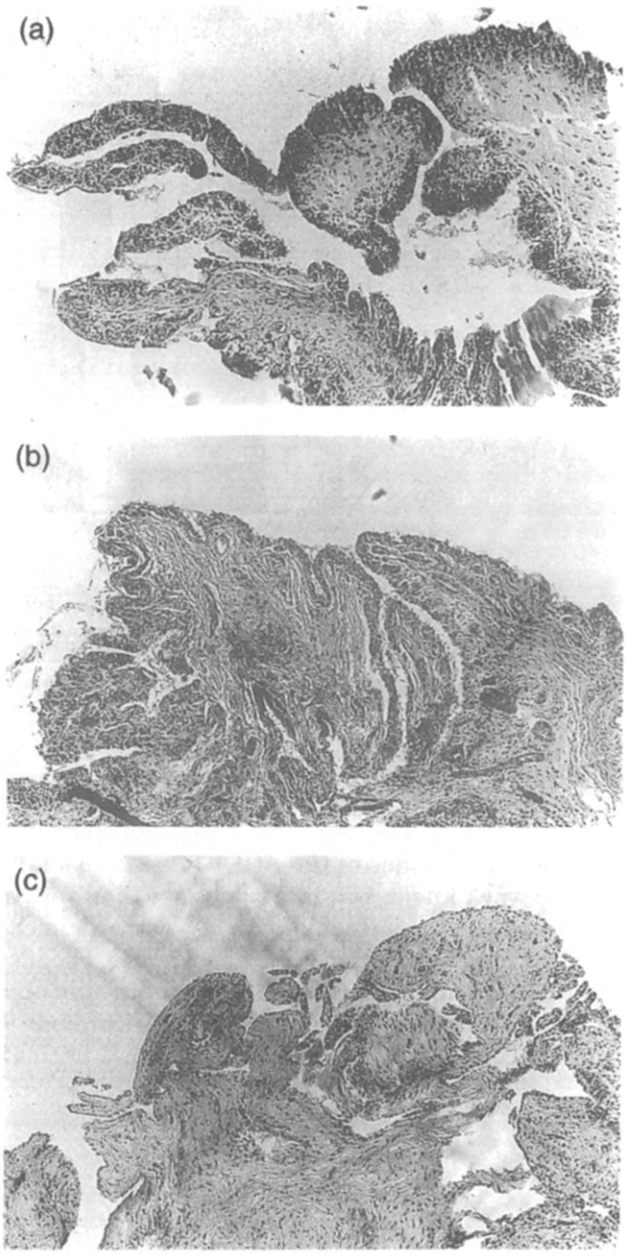

FIG. 7. (a) Histological finding of synovium from an animal killed (a) at 4 weeks after surgery; (b) at 8 weeks after surgery; and (c) at 12 weeks after surgery (hemotoxylin and eosin magnification ×19). Hyperplasia of synovial lining cells is marked in (a). The lining cell layer of the synovium decreased in thickness (b) when compared to the 4-week specimen. Concurrently, the fibrosis under the synovial lining cells increased. Gross fibrotic proliferation of subsynovial tissue is seen in (c).

synovial cell layer increased over time after ACLT [Fig. 7(a)–(c)].

Quantitative histomorphometry

Femoral condyles. Per cent cartilage area and cartilage thickness (the values of ACLT divided by sham) in almost all regions showed decreases with time, as shown in Figs 8 and 9. Decreases in cartilage area and cartilage thickness at 12 weeks in region C ($P < 0.02$) and region D ($P < 0.02$) were statistically significant when compared with the contralateral sham operated knee. In region B, the cartilage area of ACLT knees of 8-week specimens is larger than that of contralateral sham operated knees.

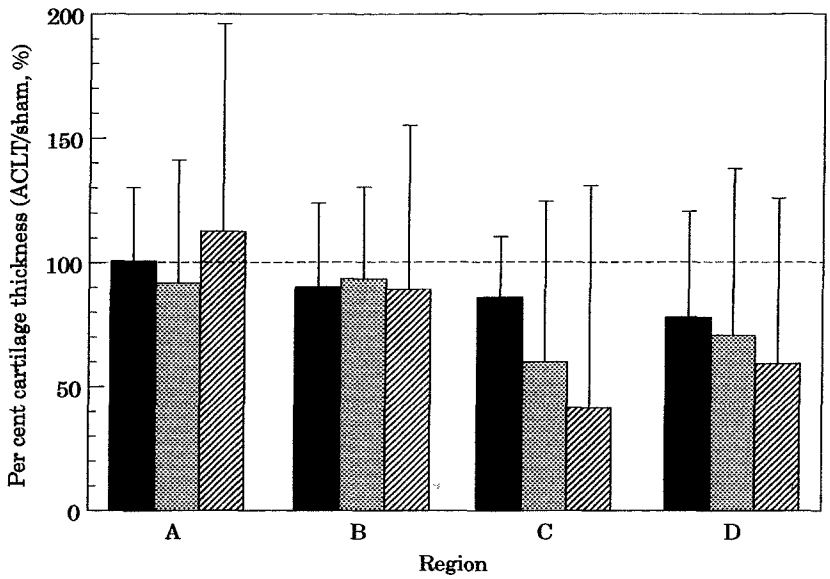

FIG. 8. Per cent cartilage thickness [anterior cruciate ligament transection (ACLT)/sham] of the femoral condyle for all regions at each time period (mean ± S.D., $n = 8$). Per cent cartilage thickness in almost all regions showed decreases with time (significant effect of time in region B and C; $P < 0.02$). Decreases in cartilage thickness at 12 weeks in region C ($P < 0.02$) and region D ($P < 0.02$) were statistically significant when compared to the contralateral sham operated knee. ■: 4 weeks; ⊡: 8 weeks; ▨: 12 weeks.

Cartilage roughness of ACLT knees in each time period was compared with that of the contralateral sham knees (Fig. 10). In regions C and D (medial condyle), the surface of the ACLT knees, especially of the 8-week knees, was much rougher than that of sham knees. The surface of the 12-week specimens was less rough than the 8-week specimens, because cartilage erosion had exposed the smooth subchondral bone on the 12-week condyles. To provide a better measurement of this observation, a normalized measure of surface roughness was established by dividing the surface roughness by the cartilage thickness, as shown in Fig. 11. The normalized surface roughness of the 12-week group in region C was significantly different from earlier time periods ($P < 0.05$), and progression over time was apparent.

Synovium. The thickness of the synovial lining cell layer and the cell density of synovium from ACLT knee were two to three times higher than those

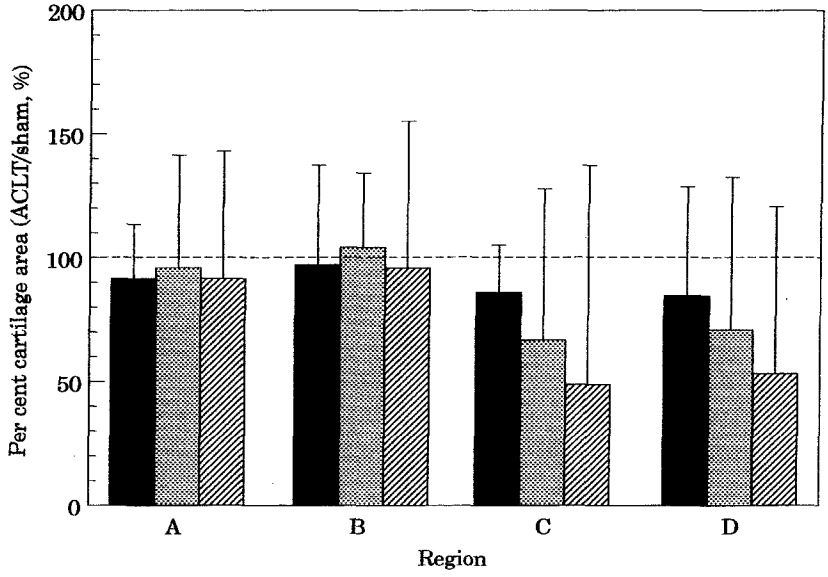

FIG. 9. Per cent cartilage area (anterior cruciate ligament transection (ACLT)/sham] of the femoral condyle for all sections at each time period (mean ± S.D., $n = 8$). Statistically significant differences of effect of time in region B and C ($P < 0.01$) were noted. Decreases in cartilage area at 12 weeks in region C ($P < 0.02$) and region D ($P < 0.02$) were statistically significant when compared to the contralateral sham operated knee. ■: 4 weeks; ⊡: 8 weeks; ▨: 12 weeks.

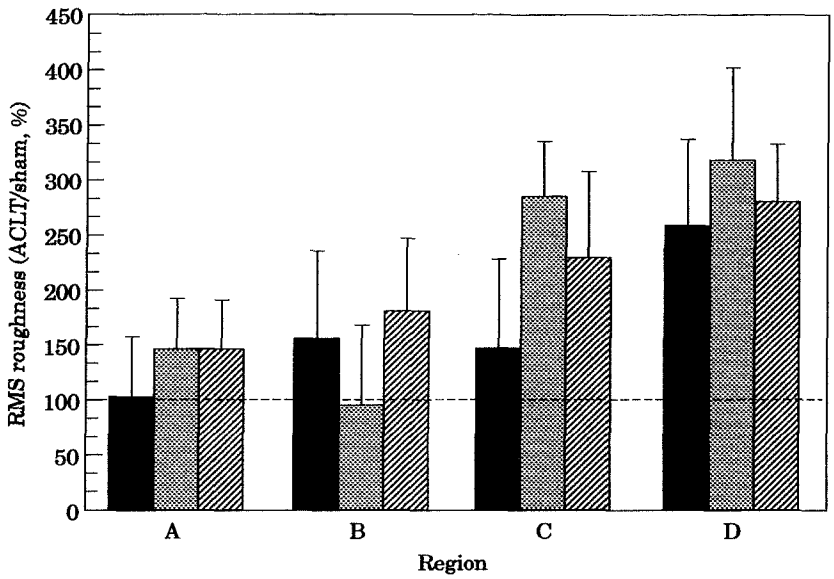

FIG. 10. Per cent root mean square (RMS) roughness [anterior cruciate ligament transection (ACLT)/sham] of the femoral condyle for all sections at each time period (mean ± S.D., $n = 8$). In regions C and D (medial condyle) the surface of ACLT knees was much rougher than that of sham knees ($P < 0.01$). Statistically significant differences of effect of time between 8 and 12 weeks in region B ($P < 0.05$) and 4 and 12 weeks in region C ($P < 0.05$) were noted. ■: 4 weeks; ⊡: 8 weeks; ▨: 12 weeks.

from the contralateral sham knees. This difference was statistically significant ($P < 0.02$, Figs 12 and 13). The thickness of the synovial lining cell layer showed a decrease with time ($P < 0.02$).

Discussion

Cartilage and synovium from ACL transected knees in the rabbit were studied morphologically, histologically and histomorphometrically. The results showed that degenerative changes of the articular cartilage occurred and were progressive with time.

By gross morphology the degenerative changes such as cartilage fibrillation and erosion of the articular cartilage in this model were easily definable and progressive; eight of 10 rabbits of the 8-week group and nine of 10 rabbits of the 12-week group developed advanced osteoarthritic changes. Degenerative changes were detected in the 4-week group, but they were less marked. No full-thickness ulceration was seen in the 4-week rabbits. Although

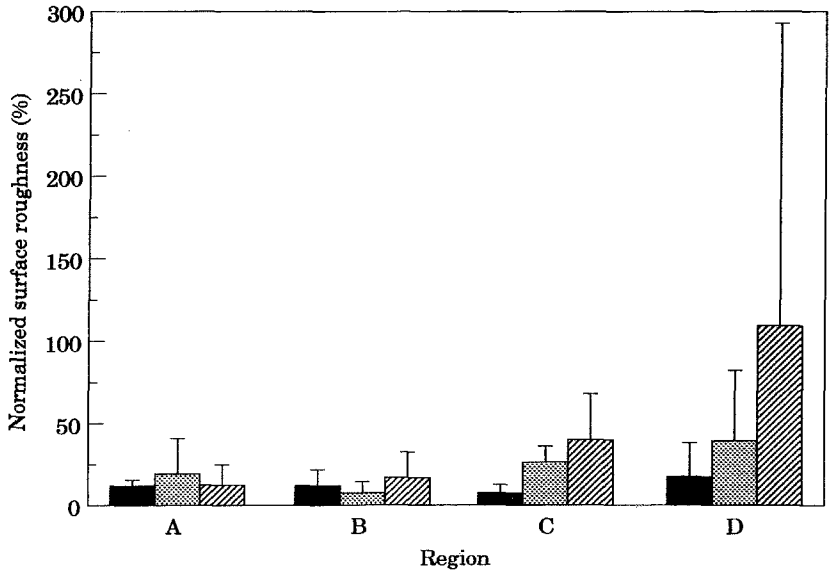

FIG. 11. Normalized cartilage roughness (anterior cruciate ligament transection) of the femoral condyle for all sections at each time period (mean ± S.D., $n = 8$). The normalized surface roughness of the 12-week group in region C was significantly different from earlier time periods ($P < 0.01$). ■: 4 weeks; ⊡: 8 weeks; ▨: 12 weeks.

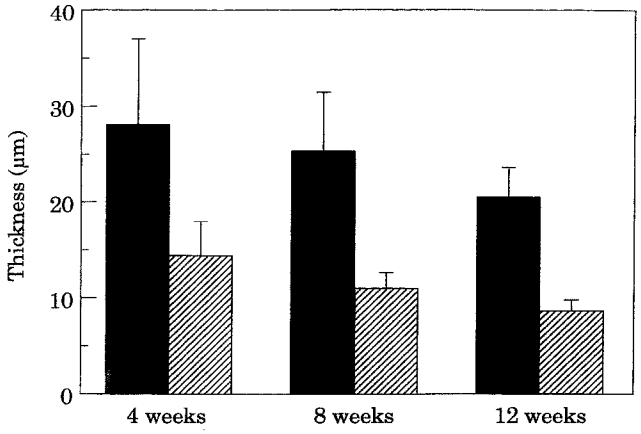

FIG. 12. Thickness of synovial lining cell layer at each time period (mean ± S.D., $n = 8$). Significant effect of surgery ($P < 0.02$) and time from 4–12 weeks ($P < 0.02$) and 8–12 weeks ($P < 0.001$) were noted. ■: Anterior cruciate ligament transection; ▨: sham.

there was some degree of biologic variability regarding the morphological assessment, the 4-week time period was considered to be an early stage in the development of OA.

The first detailed study of ACLT in the rabbit knee was performed by Vignon *et al.* in 1987 [17]. They reported histological changes including cartilage hypertrophy, reduced cell density, and matrix alterations preceding cartilage fibrillation 12 weeks after surgery. We also found increases in cartilage thickness in some specimens in 4- and 8-week groups, but it was not significant, whereas significant decreases were found in cartilage thickness at 12 weeks. The same breed of rabbits (NZW) was used in both studies, however the ranges of the weight of their rabbits and ours were different and were, respectively, 1.8–2.5 and 3.8–4.9 kg (14 months old). Their rabbits were probably immature and had open epiphyses according to a weight vs maturity study that was published by Woo *et al.* [23].

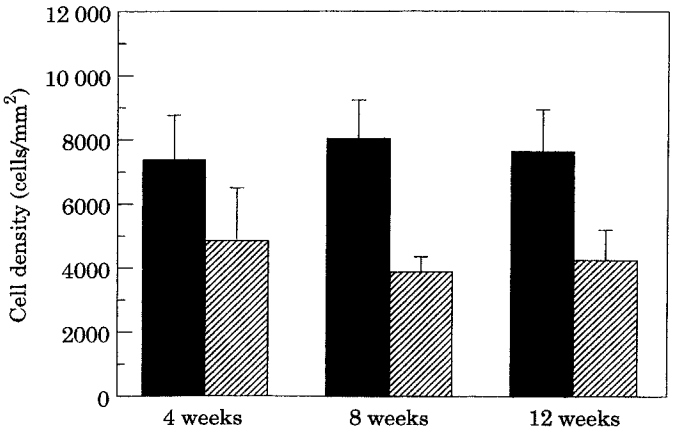

FIG. 13. Cell density of synovium at each time period (mean ± S.D., $n = 8$). Significant effect of surgery ($P < 0.02$) was noted. ■: Anterior cruciate ligament transection; ▨: sham.

The use of mature animals is considered to be important for the study of experimental OA, since immature animals with their higher reparative abilities might produce results different from the adult with a diminished reparative capacity.

When compared to the meniscectomy model in the rabbit studied by Moskowitz *et al.* [7], the ACLT model seems to lead to relatively more rapid progression of osteoarthritic changes. However, there was no description of full-thickness ulceration exposing eburnated subchondral bone in their model. There is some question as to whether the meniscectomy model leads to the terminal stage of osteoarthritic lesions.

Hulth *et al.* [5] studied a rabbit model of OA in which there was severance of the medial cellateral ligament (MCL) and both cruciate ligaments, and excision of the medial meniscus. Despite a surgical intervention which seems to be drastic, some parts of both femoral and tibial cartilages maintained a normal appearance with no osteophyte formation, even 12 weeks after surgery. Other investigators have found the osteophyte formation precedes cartilage change [7, 10, 17]. Simple comparison of models is difficult to perform because of a lack of detail related to the pathology of the OA condition; i.e. animal model, species and time of OA. Excessive surgical intervention like the Hulth model may cause extensive inflammation in the joint, which in turn may lead to peri-articular contracture and result in self-limitation of progression of degenerative changes in the joint. In contrast, ACLT is advantageous because it is a simple procedure that enables control of intra-articular bleeding and minimizes post-operative synovitis [24]. Furthermore, and most importantly, is that ACLT in the rabbit knee leads to progressive changes of cartilage degeneration.

Histomorphometric analysis provided an assessment and quantification of the histological findings. Geometric measurement revealed more cartilage breakdown on the medial femoral condyles (regions C and D) than on the lateral condyles (regions A and B). Normalized cartilage roughness parameters quantified a dramatic profile of degenerating cartilage. Because complete erosion on a condyle produced a smooth surface when measured by RMS roughness, it was elected to calculate a quantity that combined the thickness and roughness measurements, which hopefully would reflect the relationship of these two parameters. This 'normalized roughness' proved to describe the findings quite accurately.

The grading system by Mankin [25] is widely used for evaluating histologic findings of osteoarthritic specimens, and its usefulness is not debatable. It has,

however, a limitation for representing the cartilage profile (the width and extent of the cartilage lesion) as well as some subjective error of observers, because it consists of a ranking scale (for example, full thickness lesions of the cartilage are ranked in the same score) rather than a quantitation evaluation. On the other hand, histomorphometric parameters are interval scaled, and enable demonstration of the cartilage changes in detail.

Although the causes of OA are multiple and complicated, it is known that synovitis as well as instability will lead to its development. This ACL transection model showed intensive synovitis. While gross fibrotic proliferation of subsynovial tissue was seen at 12 weeks histologically, analysis of the synovium revealed a diminution in the thickness of the synovial lining cell layer which correlated with the advancing term of the osteoarthritic change. This synovial change may reflect a transition from an active phase of synovitis, which may be in response to the progressive loss of articular cartilage (presumably through release of chemical mediators, such as interleukin-1 [26]), to a chronic phase.

Conclusions

In summary, it was found that simple ACL transection leads to a progressive degenerative arthritis in the rabbit knee. This progressive OA has been characterized by gross appearance, and histological parameters as measured with a new computerized analysis system. It was also noticed that the measured changes on the femoral condyles (specifically the medial femoral condyle) were good indicators of cartilage degeneration. The early changes of degeneration were detectable by 4 weeks post-ACL transection, and became progressively worse over time.

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