4. Coventry MB. Upper tibial osteotomy for gonarthrosis. The evolution of the operation in the last 18 years and long-term results. Orthop Clin North Am 1979;10:191–210.
5. Rudan JF, Simurda MA. High tibial osteotomy. A prospective clinical and roentgenographic review. Clin Orthop 1990;255: 251–6.
6. Insall JN, Joseph DM, Msika C. High tibial osteotomy for varus gonarthrosis. A long-term follow-up study. J Bone Joint Surg Am 1984;66:1040–8.
7. Berman AT, Bosacco SJ, Kirshner S, Avolio A. Factors influencing long-term results in high tibial osteotomy. Clin Orthop 1991;272: 192–8.
8. Yasuda K, Majima T, Tsuchida T, Kaneda K. A ten-to 15-year follow-up observation of high tibial osteotomy in medial compartment osteoarthrosis. Clin Orthop 1992;282:186–95.
9. Coventry MB, Ilstrup DM, Wallrichs SL. Proximal tibial osteotomy. J Bone Joint Surg Am 1993;75:196–201.
10. Rinonapoli E, Mancini GB, Corvaglia A, Musiello S. Tibial osteotomy for varus gonarthrosis. Clin Orthop 1998;353:185–93.
11. Naudie D, Bourne RB, Rorabeck CH, Bourne TJ. Survivorship of the high tibial valgus osteotomy. Clin Orthop 1999;367:18–27.
12. Aglietti P, Buzzi R, Vena LM, Baldini A, Mondaini A. High tibial valgus osteotomy for medial gonarthrosis. J Knee Surg 2003;16: 21–6.
13. Sprenger TR, Doerzbacher JF. Tibial osteotomy for the treatment of varus gonarthrosis. J Bone Joint Surg Am 2003;85:469–74.
14. Koshino T, Yoshida T, Ara Y, Saito I, Saito T. Fifteen to twenty-eight years' follow-up results of high tibial valgus osteotomy for osteoarthritic knee. Knee 2004;11:439–44.
15. Koshino T, Morii T, Wada J, Saito H, Ozawa N, Noyori K. High tibial osteotomy with fixation by a blade plate for medial compartment osteoarthritis of the knee. Orthop Clin North Am 1989; 20:227–43.
16. Hofmann AA, Wyatt RW, Beck SW. High tibial osteotomy. Use of an osteotomy jig, rigid fixation, and early motion versus conventional surgical technique and cast immobilization. Clin Orthop 1991;271:212–7.
17. Weber BG, Wörsdörfer O. Zuggurtungsosteosynthese bei tibiakopfosteotomie. Z Orthop 1980;118:637–43.
18. Flamme CH, Ruhmann O, Schmolke S, Wichmann R. Long-term outcome following high tibial osteotomy with tension bend principle. Arch Orthop Trauma Surg 2003;123:12–6.
19. Koshino T, Tsuchiya K. The effect of high tibial osteotomy on osteoarthritis of the knee. Clinical and histological observations. Int Orthop 1979;3:37–45.
20. Schatzker J, Burgess RC, Glynn MK. The management of nonunions following high tibial osteotomies. Clin Orthop 1985;193: 230–3.
21. Lawrence JS, Bremner JM, Bier F. Osteo-arthrosis. Prevalence in the population and relationship between symptoms and X-ray changes. Ann Rheum Dis 1966;25:1–24.
22. Ogata K. Interlocking wedge osteotomy of the proximal tibia for gonarthrosis. Clin Orthop 1984;186:129–34.
23. Ogata K, Yoshii I, Kawamura H, Miura H, Arizono T, Sugioka Y. Standing radiographs cannot determine the correction in high tibial osteotomy. J Bone Joint Surg Br 1991;73:927–31.
24. Kanamiya T, Naito M, Hara M, Yoshimura I. The influences of biomechanical factors on cartilage regeneration after high tibial osteotomy for knees with medial compartment osteoarthritis: clinical and arthroscopic observations. Arthroscopy 2002;18: 725–9.
25. Billings A, Scott DF, Camargo MP, Hofmann AA. High tibial osteotomy with a calibrated osteotomy guide, rigid internal fixation, and early motion. J Bone Joint Surg Am 2000;82:70–9.
26. Zhang Y, Shall LM, Kiritsis PG, Wolfinbarger L, Fairclots JR. Varus tension testing of fixation devices used in proximal tibial osteotomy. Contemp Orthop 1995;30:471–6.
27. Flamme CH, Kohn D, Kirsch L, Hurschler C. Primary stability of different implants used in conjunction with high tibial osteotomy. Arch Orthop Trauma Surg 1999;119:450–5.
28. Gautier E, Thomann BW, Brantschen R, Jakob RP. Fixation of high tibial osteotomy with AO cannulated knee plate. Acta Orthop Scand 1999;70:397–9.
29. Yasumoto H, Omori G, Tanabe Y, Koga Y. Mechanical stability of different fixation procedures after high tibial osteotomy (in Japanese). J Jpn Soc Clin Biomech 2001;22:319–23.
30. Holden DL, James SL, Larson RL, Slocum DB. Proximal tibial osteotomy in patients who are fifty years old or less. J Bone Joint Surg Am 1988;70:977–82.
31. Sasazaki Y, Fujikawa K, Kobayashi T, Miwa M. Clinical results of the overcorrected high tibial osteotomy for advanced osteoarthritis of the knee (in Japanese). Bessatsu Seikeigeka 2002;42: 153–58.
32. Choi HR, Hasegawa Y, Kondo S, Shimizu T, Ida K, Iwata H. High tibial osteotomy for varus gonarthrosis: a 10- to 24-year follow-up study. J Orthop Sci 2001;6:493–7.
33. Takemae T, Omori G, Nishino K, Terajima K, Koga Y, Endo N. Three-dimensional knee motion before and after high tibial osteotomy for medial knee osteoarthritis. J Orthop Sci 2006;11: 601–6.

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Regional Differences in Chondrocyte Metabolism in Osteoarthritis

A Detailed Analysis by Laser Capture Microdissection

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Objective. **To determine the change in metabolic activity of chondrocytes in osteoarthritic (OA) cartilage, considering regional difference and degree of cartilage degeneration.**

Methods. **OA cartilage was obtained from knee joints with end-stage OA, at both macroscopically intact areas and areas with various degrees of cartilage degeneration. Control cartilage was obtained from age-matched donors. Using laser capture microdissection, cartilage samples were separated into superficial, middle, and deep zones, and gene expression was compared quantitatively in the respective zones between OA and control cartilage.**

Results. **In OA cartilage, gene expression changed markedly with the site. The expression of cartilage matrix genes was highly enhanced in macroscopically intact areas, but the enhancement was less obvious in the degenerated areas, especially in the upper regions. In contrast, in those regions, the expression of type III collagen and fibronectin was most enhanced, suggesting that chondrocytes underwent a phenotypic change there. Within OA cartilage, the expression of cartilage matrix genes was significantly correlated with *SOX9* expression, but not with *SOX5* or *SOX6* expression. In OA cartilage, the strongest correlation was observed between the expression of type III collagen and fibronectin, suggesting the presence of a certain link(s) between their expression.**

Conclusion. **The results of this study revealed a comprehensive view of the metabolic change of the chondrocytes in OA cartilage. The change of gene expression profile was most obvious in the upper region of the degenerated cartilage. The altered gene expression at that region may be responsible for the loss of cartilage matrix associated with OA.**

Osteoarthritis (OA) is a disease characterized by a progressive loss of cartilage matrix that often extends over a decade. During the long course of the disease, chondrocytes undergo obvious metabolic changes. A variety of changes are known to occur that have 2 distinctive aspects. First, the anabolic activity of chondrocytes is strongly enhanced in OA. Following the initial reports more than 4 decades ago (1), an increasing number of studies have shown that the expression of virtually all cartilage components is up-regulated in OA cartilage (2–13). The increased anabolism may be a repair response of the chondrocytes that counteracts the loss of cartilage matrix (2–4). Second, in OA, chondrocytes undergo phenotypic changes. Because of this,

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chondrocytes in OA cartilage express matrix genes that are not expressed in normal cartilage, such as type I and type III collagens (5–10). Since the induction of these genes also occurs during the dedifferentiation of chondrocytes in vitro, the phenotypic changes in OA have an aspect resembling that of the dedifferentiation process (9). The phenotypic changes also show a characteristic of developmental reversal, since the expression of type IIA procollagen, a prechondrogenic splicing variant of the type II collagen gene, is observed in OA (11,12). In contrast, the presence of type X collagen in OA cartilage has persuaded investigators that chondrocytes are undergoing hypertrophic changes there (13,14).

Because of the diversity in gene expression, it is currently difficult to obtain a comprehensive idea of the metabolic changes in OA. This diversity may stem from a topographic variation of the pathology. Since cartilage pathology differs obviously from site to site within OA cartilage, it is likely that the metabolic changes in the chondrocytes also differ by areas related to that pathology (4,9,10,15). The regional differences of chondrocyte metabolism may be important to our understanding of the mechanism of disease progression. For example, a focal decline of the matrix synthesis in OA cartilage may play a critical role in the loss of cartilage matrix (3,4,9).

Conventionally, the regional differences of cellular metabolism in OA have been evaluated primarily by histologic methods, so the comparison among the areas has not been quantitative. Laser capture microdissection (LCM) is an innovative technology that enables the isolation of a specific area of tissue by its histologic features (16). Coupled with real-time polymerase chain reaction (PCR), the use of LCM allowed us to perform a quantitative evaluation of the multiple genes expressed in specific regions of OA cartilage. Thus, this study has revealed, for the first time, a comprehensive view of the changes in metabolic activity of chondrocytes in OA cartilage.

MATERIALS AND METHODS

Tissue procurement. This study was performed with the approval of the Human Ethics Review Committees of the participating institutions. For material collection, informed consent was obtained in writing from each subject or family of the donor. OA cartilage samples were obtained from 32 end-stage OA knee joints of 30 patients (mean age 70.3 years [range 56–88 years]) within 4 hours after surgery. The diagnosis of OA was based on the criteria for knee OA of the American College of Rheumatology (17). Control cartilage samples were obtained from 18 nonarthritic knee joints from 16 donors (mean age 82.3 years [range 67–89 years]) within 24 hours after death. The donors had no known history of joint disease or serious trauma, and the normality of the joint was confirmed macroscopically at the time samples were obtained. Knee cartilage in aged donors usually undergoes some degeneration, even though the donors did not have any problems with the joints. Therefore, we obtained control cartilage samples from the knees even when the cartilage showed some signs of degeneration, as long as the degeneration was superficial and limited to small areas (<20% of total cartilage area). Control ligaments, bone tissues, and menisci were also harvested from these joints.

Laser capture microdissection. In each OA joint, cartilage tissues were harvested from 2–5 sites in femoral condyles showing various degrees of cartilage degeneration. In each control joint, cartilage samples were harvested from 2–4 sites in the weight-bearing areas of the femoral condyles. The cartilage samples were cut above the calcified zone, which was confirmed under a microscope at the time of laser microdissection. Immediately after harvest, the cartilage samples were embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan), snap-frozen in liquid nitrogen, and then stored at −80°C until used.

In preparation for LCM, 20–40-μm–thick frozen sections were cut from the cartilage tissues along a plane vertical to the joint surface. The sections were first treated with 0.5*M* EDTA (pH 8.0) for 3 minutes, dehydrated with graded concentrations of ethanol, and clarified with xylene. All reagents were prepared RNase-free, and the entire process was completed within 30 minutes to minimize RNA degradation.

Under an LCM device (PixCell IIe; Arcturus, Mountain View, CA), each frozen section was divided into cartilage zones based on its histologic features (18,19). Cartilage samples from preserved areas contained 3 zones (superficial, middle, and deep) and were separated into these respective zones. For the cartilage from degenerated areas, the number of zones in the section differed from 3 to 1, depending on the severity of the cartilage pathology. A section containing all 3 zones was separated into the 3 respective zones. When a superficial zone was lost to the disease, the section was divided into 2 zones, the middle and deep zones (Figure 1). If a section contained only a deep zone, it was used directly for RNA extraction without microdissection. At each tissue procurement, the appropriateness of zone isolation was confirmed under a microscope.

Analysis of gene expression. Immediately after LCM, RNA was extracted from the tissues using an RNeasy Micro kit (Qiagen, Hilden, Germany) with routine use of DNase I (Qiagen). Complementary DNA (cDNA) was synthesized using Sensiscript reverse transcriptase (Qiagen). Gene expression was evaluated quantitatively by real-time PCR on a LightCycler (Roche Diagnostics, Basel, Switzerland). Gene-specific primers and probes were prepared (a list of primer and probe sequences is available at http://www.hosp.go.jp/~sagami/rinken/crc/index.html), and the process of PCR was monitored by either SYBR Green or hybridization probes. LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics) or LightCycler FastStart DNA Master Hybridization Probe (Roche Diagnostics) was used for PCR. The PCR protocol was as follows: 95°C for 10 minutes to activate *Taq* polymerase, then 40 cycles of 95°C for 10 seconds, melting temperature for the individual gene for 15 seconds (a list of melting temperatures for the individual genes is available at

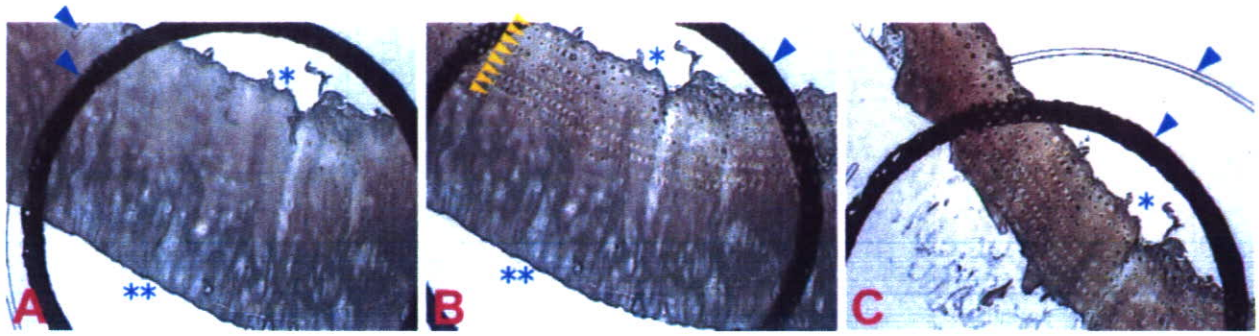

Figure 1. Separation and acquisition of cartilage zone by laser capture microdissection (LCM). **A,** A tissue section was set on an LCM device, and a transparent plastic film was placed on the section. Cartilage zones were identified through the film. **B,** The zone of interest was fixed to the film by shooting with a laser. The area was shot multiple times until the entire zone was anchored to the film. Arrays of spots indicated by **yellow arrowheads** are the laser shot marks. **C,** After laser shooting, any unnecessary area of the section was removed, and only the zone of interest that had adhered to the film was obtained. Acquisition of a middle cartilage zone from a section containing middle and deep zones is shown. The superficial zone of this section was already lost to disease. **Single** and **double asterisks** indicate the top and bottom of the section, respectively. Transparent and bold black arcs indicated by **blue arrowheads** are the marks on the plastic film. (Original magnification × 2.)

http://www.hosp.go.jp/~sagami/rinken/crc/index.html), and 72°C for 6 seconds.

The amount of specific cDNA was quantified with a standard curve based on the known amounts of PCR product. When SYBR Green I was used for monitoring, melting curves were routinely recorded to verify singularity of the product. A previous study showed that *GAPDH* is expressed at similar levels in chondrocytes in normal and OA cartilage (8). Consistently, the result of our preliminary experiment indicated that the expression of *GAPDH* and *ACTB* (a gene coding β-actin) was highly correlated in cartilage samples from OA and control knees. Thus, in this study, *GAPDH* was used as the internal standard for gene expression, and cDNA levels were expressed as the ratio of gene expression:*GAPDH* expression.

Statistical analysis. Pearson's correlation and paired *t*-tests were calculated with the SAS software package (SAS Institute, Cary, NC). For some data, statistical differences were determined by an analysis of variance followed by a Scheffe's post hoc test. *P* values less than 0.05 were considered significant.

RESULTS

Up-regulated expression of cartilage matrix molecules at different regional intensities in OA cartilage. In each OA joint, cartilage was harvested from femoral condyles, both from macroscopically intact areas and

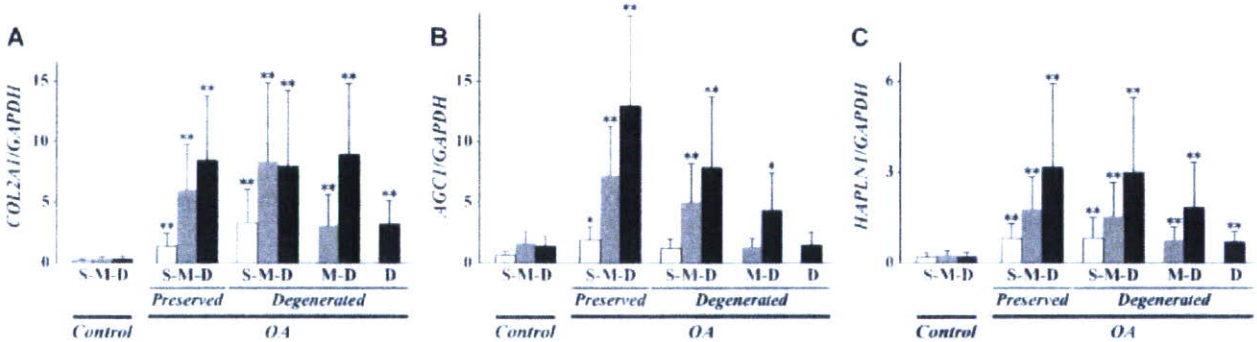

Figure 2. Expression of cartilage matrix genes in osteoarthritic (OA) and nonarthritic (control) cartilage. Cartilage samples obtained from nonarthritic knee joints and knee joints with end-stage OA were divided into superficial (S), middle (M), and deep (D) zones by laser capture microdissection, and expression of cartilage matrix genes was evaluated in the respective zones. In OA joints, cartilage samples were harvested from macroscopically intact areas (preserved) and areas with various degrees of cartilage degeneration (degenerated). The latter samples were divided into 3 groups (S-M-D, M-D, and D) according to the zones retained at the site. Expression of the genes coding type II collagen (*COL2A1*) (**A**), aggrecan (*AGC1*) (**B**), and link protein (*HAPLN1*) (**C**) is shown as ratios of the expression of *GAPDH*. Each bar represents the results from at least 16 samples. Values are the mean and SD. * = $P < 0.05$; ** = $P < 0.01$, versus the corresponding zone in control cartilage.

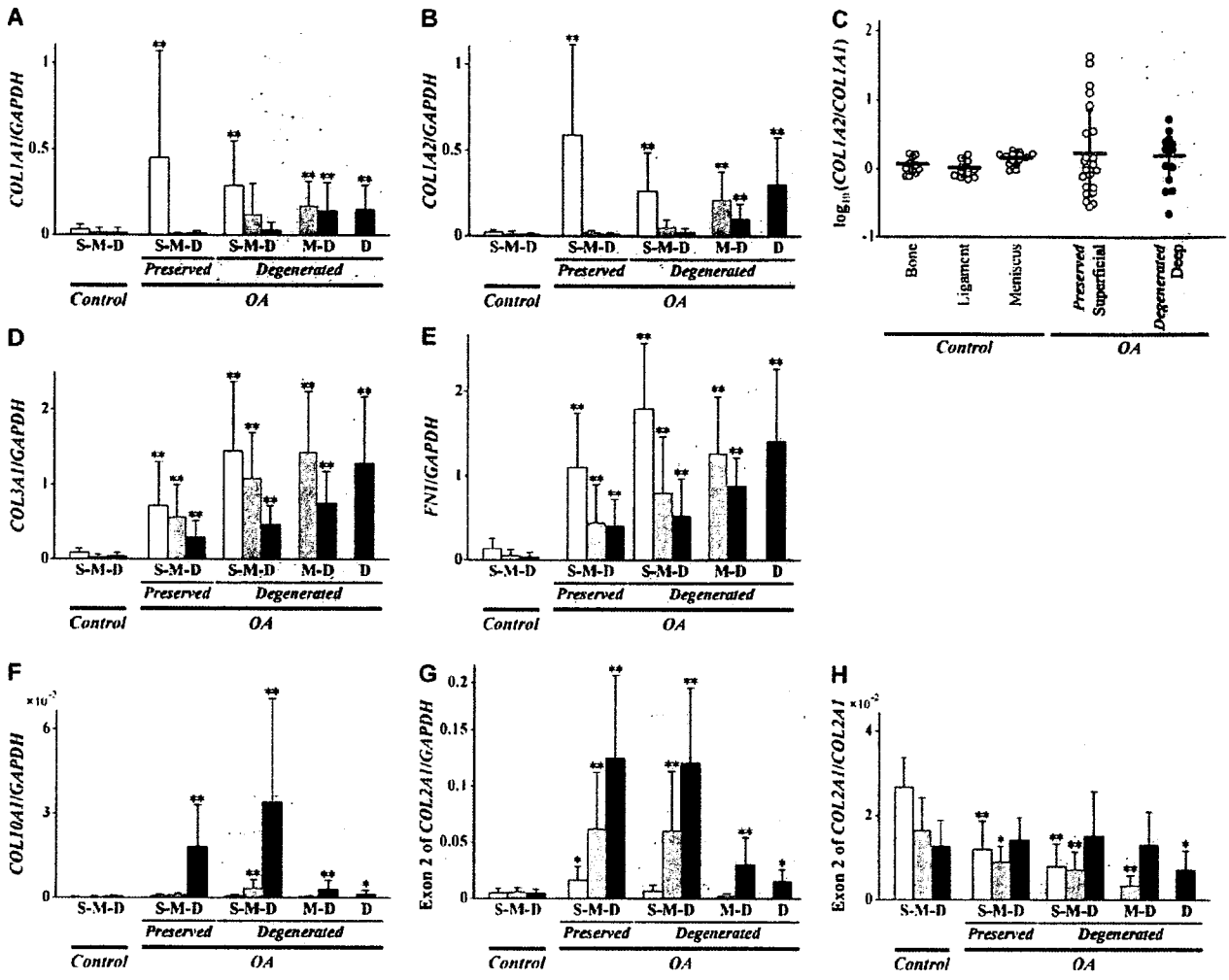

Figure 3. Expression of minor cartilaginous genes in OA and control cartilage. A and B, Expression of *COL1A1* (A) and *COL1A2* (B) in control and OA cartilage is shown as ratios of the expression of *GAPDH*, as described in Figure 2. C, The expression ratios of *COL1A2* to *COL1A1* were obtained in the superficial zone in preserved areas and in the deep zone in degenerated areas where the zone was directly exposed to the joint cavity, and were compared with those obtained in bone, ligaments, and menisci harvested from control joints. Ratios are shown in logarithmic values. D–F, Expression of genes coding type III collagen (*COL3A1*) (D), fibronectin (*FN1*) (E), and type X collagen (*COL10A1*) (F) is shown as ratios of the expression of *GAPDH*. G and H, Expression of exon 2 of *COL2A1* gene is shown as ratios of the expression of *GAPDH* (G) and by ratio to the total expression of *COL2A1* (H). S-M-D, M-D, and D under the respective groups of bars indicate the zone(s) retained in the samples. Each bar represents the results from at least 11 samples. Values are the mean ± SD. * = $P < 0.05$; ** = $P < 0.01$, versus the corresponding zone in control cartilage. See Figure 2 for definitions.

from areas showing macroscopic signs of degeneration. In this study, such areas were designated "preserved" and "degenerated" areas, respectively. OA and control cartilage samples were separated into 3 cartilage zones by LCM, and gene expression was evaluated in the respective cartilage zones by real-time PCR, considering the zonal difference and the severity of cartilage degeneration.

Compared with that in the control cartilage, the expression of type II collagen was strongly up-regulated in all areas in OA cartilage (Figure 2A). The up-regulation was most apparent in the deep zone, where the expression was ~20-fold that in the corresponding zone of the control cartilage. In contrast, the level of up-regulation was considerably reduced in the upper part of the degenerated cartilage. Where the zones were

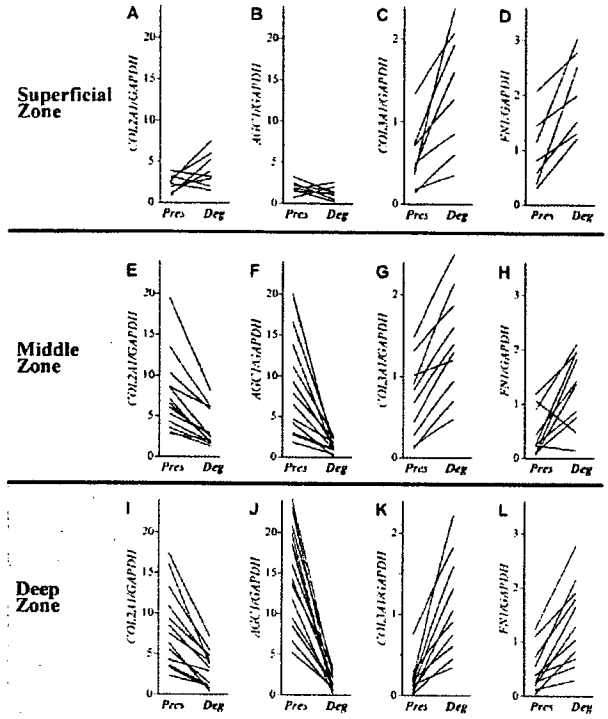

Figure 4. Comparison of gene expression between preserved (Pres) and degenerated (Deg) areas. In each osteoarthritic joint, the expression of 4 genes was compared in the respective cartilage zones between the preserved and degenerated areas. For the middle and deep zones, expression in the degenerated area was determined where the zones were directly exposed to the joint cavity due to the loss of the upper zone(s) to the disease. Expression of *COL2A1* (A, E, and I), *AGC1* (B, F, and J), *COL3A1* (C, G, and K), and *FN1* (D, H, and L) in the superficial, middle, and deep zones is shown. In these graphs, each line represents the expression in a single joint. Results from 7–13 joints are shown as the ratio of gene expression to *GAPDH* expression.

directly exposed to the joint cavity due to the loss of the upper zone(s) to the disease, the expression levels in the middle and deep zones were almost half of those in the preserved areas.

The expression of aggrecan was also enhanced in OA cartilage (Figure 2B). Similar to type II collagen, the increase was most obvious in the deep zone of the preserved area but was less intense in the degenerated area. In this gene, the regional change of expression was more obvious than that in type II collagen. Thus, in the middle and deep zones exposed to the joint cavity in degenerated areas, the expression was virtually unenhanced, and the expression levels were similar to those in the control cartilage. The expression of link protein presented a regional change similar to that of aggrecan, although the decline in the degenerated area was less apparent (Figure 2C).

Spatially distinctive patterns in OA cartilage shown by expression of minor cartilaginous genes induced by OA. In OA, there is enhanced expression of several genes that are not expressed at substantial levels in normal cartilage. Types I, III, and X collagen and fibronectin are among those genes (5,9,13,14,20–22), which are termed minor cartilaginous genes in this report. A change in alternative splicing also occurs in OA, and there is induced expression of exon 2 of type II collagen gene, which is not expressed in healthy adult cartilage (11,12). Therefore, we evaluated the expression of these genes and the exon in OA and control cartilage, paying special attention to regional differences.

In accordance with previous reports (6–8,23), the expression of type I collagen genes, *COL1A1* and *COL1A2*, was induced in OA cartilage (Figures 3A and

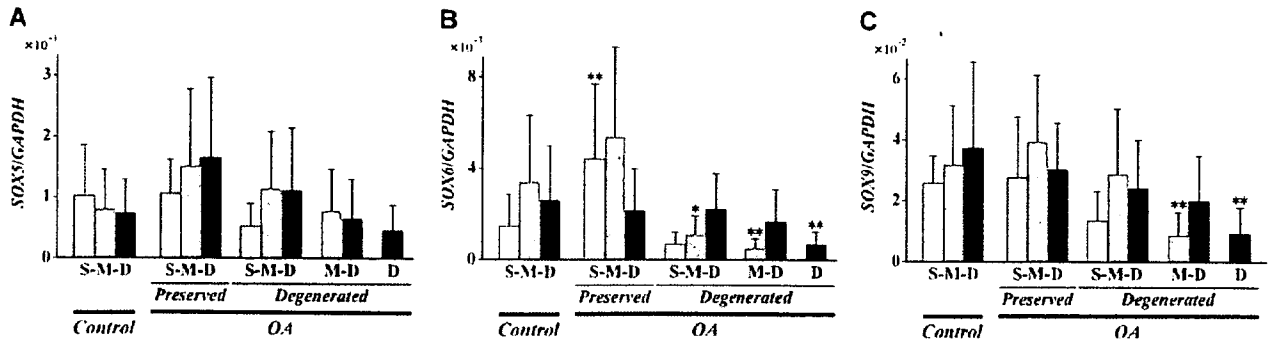

Figure 5. Expression of *SOX* genes in OA and control cartilage. Expression of *SOX5* (A), *SOX6* (B), and *SOX9* (C) in control and OA cartilage is shown as ratios of the expression of *GAPDH*, as described in Figure 2. S-M-D, M-D, and D under the respective groups of bars indicate the zone(s) retained in the samples. Each bar represents the results from at least 13 samples. Values are the mean and SD. * = $P < 0.05$; ** = $P < 0.01$, versus the corresponding zone in control cartilage. See Figure 2 for definitions.

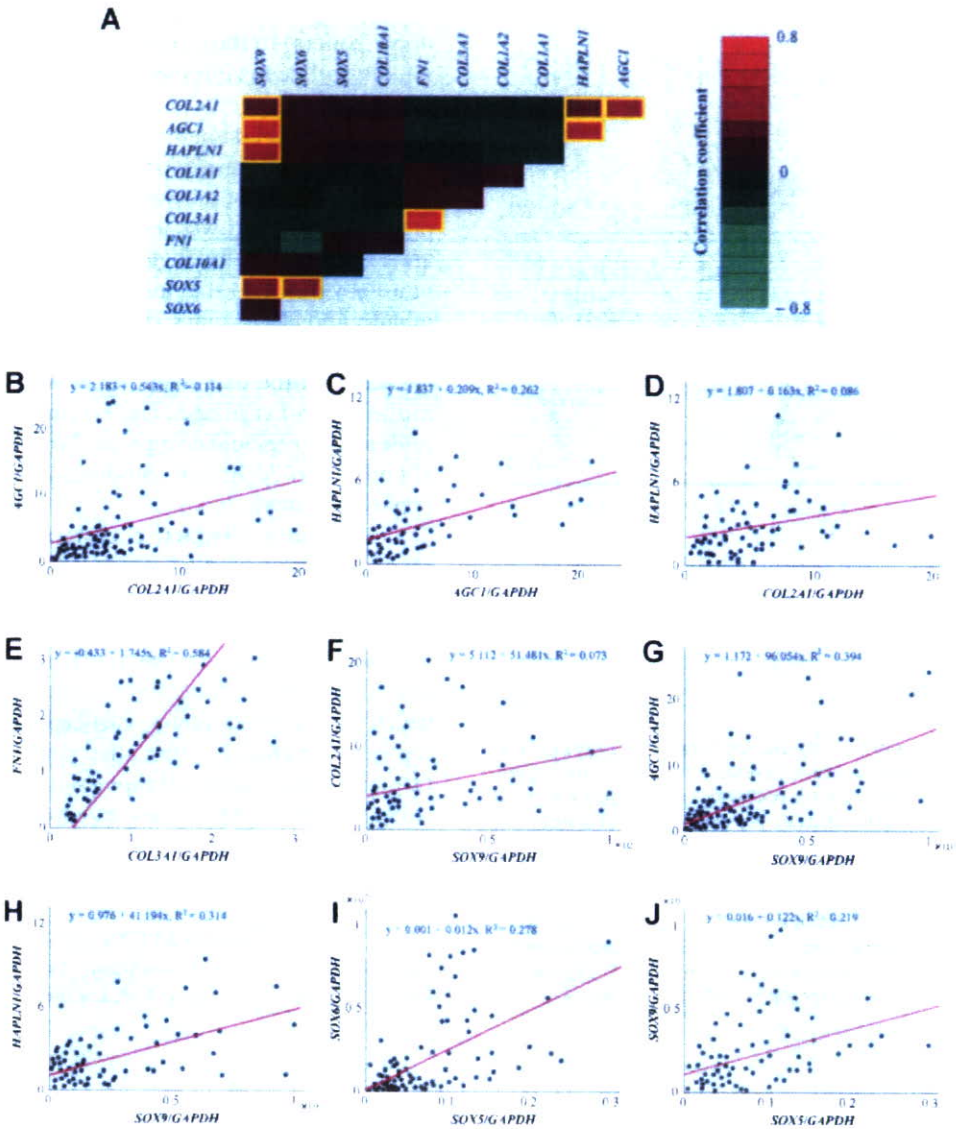

Figure 6. Correlation of gene expression in osteoarthritic (OA) cartilage. Expression of cartilage matrix genes, minor cartilaginous genes induced by the disease, and 3 cartilage-related *SOX* genes was determined at various sites of OA cartilage, and a correlation of expression was investigated among the genes. **A,** Correlation coefficients among the genes are shown by a heat map. Red and green colors indicate positive and negative correlations, respectively. Yellow square frames indicate significant correlations of expression. **B–J,** Correlation of gene expression is shown by scattergrams. Significant correlations were found between *COL2A1* and *AGC1* (**B**), *AGC1* and *HAPLN1* (**C**), *COL2A1* and *HAPLN1* (**D**), *COL3A1* and *FN1* (**E**), *SOX9* and *COL2A1* (**F**), *SOX9* and *AGC1* (**G**), *SOX9* and *HAPLN1* (**H**), *SOX5* and *SOX6* (**I**), and *SOX5* and *SOX9* (**J**), with the strongest correlation between *COL3A1* and *FN1*.

B). However, their induction levels varied markedly among samples, and practically no induction was observed in approximately half of the samples. Within the samples with detectable expression, these genes were expressed in the superficial zones in less degenerated areas and in the middle and deep zones in severely degenerated areas. Interestingly, although these genes showed similar patterns of expression within OA cartilage, their expression levels often differed considerably. The loss of coordinated expression was apparent when

the expression ratio of *COL1A2* to *COL1A1* was compared between OA cartilage and other normal tissues containing type I collagen as a major component (Figure 3C). While the expression ratio of *COL1A2* to *COL1A1* was between 0.7 and 1.7 in the bone, ligament, or meniscus tissues obtained from nonarthritic joints, the ratio in OA cartilage ranged widely from 0.2 to 44. The poor coordination in expression suggests that the expression of type I collagen genes could be induced by an aberrant mechanism(s) in OA cartilage.

In contrast to type I collagen, the induction of type III collagen messenger RNA (mRNA) was consistently observed in OA samples. Within OA cartilage, the expression of type III collagen was most intense in the upper region of degenerated cartilage (Figure 3D). The expression of another gene, fibronectin, was consistently induced in OA cartilage. The regional change of fibronectin expression was very similar to that of type III collagen expression (Figure 3E).

Unlike type I or type III collagen, the induction of type X collagen was observed primarily in the deep zone (Figure 3F). The induction was weaker than that of type I or type III collagen as judged by the ratios of expression to that of *GAPDH*, and the level of induction was considerably different among OA samples; the expression was virtually absent in approximately half of the samples. Interestingly, the expression of type X collagen was more obvious in the less degenerated areas than in the more degenerated areas where the superficial zone was lost to the disease.

Consistent with previous reports, the expression of exon 2 of the *COL2A1* gene was obviously increased in OA cartilage when evaluated by the ratio of its expression to that of *GAPDH* (Figure 3G). However, the expression of exon 2 relative to total *COL2A1* expression was rather reduced in OA cartilage (Figure 3H). Thus, it was assumed that the appearance of type IIA procollagen might not be the result of a phenotypic change in the chondrocytes as previously speculated (11,12), but is more likely to be associated with the up-regulation of type II collagen expression.

Chondrocytes at the upper part of degenerated cartilage undergo a phenotypic change. Next, we compared gene expression between preserved areas and degenerated areas in the respective cartilage zones of the respective OA joints. In the superficial zone, the expression was compared in each sample between the preserved and degenerated areas (i.e., between the 2 regions in the superficial zone without and with macroscopic degeneration). In the middle and deep zones, the comparison was performed in each sample between the preserved areas and the degenerated areas where the zones were directly exposed to the joint cavity.

The result clearly indicated that a shift occurred in the pattern of gene expression at the upper region of degenerated cartilage (Figure 4). In the degenerated areas in the middle and deep zones, the expression of cartilage matrix genes (type II collagen and aggrecan) was suppressed, while the expression of minor cartilaginous genes (type III collagen and fibronectin) was enhanced. In the superficial zone, the expression of minor cartilaginous genes was induced similarly in the degenerated areas, although the suppression of cartilage matrix gene expression was not apparent. In spite of considerable differences in expression levels among the samples, the shift of gene expression was consistently observed in almost all OA samples. Thus, the chondrocytes are considered to undergo a phenotypic change at the upper region of degenerated cartilage, no matter in which cartilage zone the cells reside.

Expression of *SOX* genes in OA and control cartilage. During chondrogenic differentiation, the expression of cartilage matrix genes is regulated by the transcriptional factors *SOX5*, *SOX6*, and *SOX9* (24). In order to estimate the involvement of these molecules in the change of chondrocyte metabolism in OA, their expression was investigated (Figure 5). In OA cartilage, the expression of *SOX* genes tended to be reduced in the degenerated areas, particularly in the upper region of the degenerated cartilage. The reduction was most obvious with *SOX6*, followed by *SOX9*, and was least apparent with *SOX5*. In the preserved areas, the expression of *SOX5* and *SOX6* tended to be increased above control levels, although this trend was not observed with *SOX9*. These regional changes of *SOX* expression within OA cartilage suggested that the altered *SOX* gene expression might be related to the change in matrix gene expression in OA.

Correlation of gene expression in OA cartilage. In an attempt to understand the mechanism(s) underlying the altered gene expression in OA cartilage, a possible correlation of gene expression was investigated (Figure 6A). The expression of 3 cartilage matrix genes correlated significantly. The expression of type II collagen was significantly correlated with that of aggrecan ($r = 0.110$, $P = 0.0081$) (Figure 6B), and a stronger correlation was observed between aggrecan and link protein ($r = 0.512$, $P < 0.0001$) (Figure 6C). A significant correlation was also observed between type II collagen and link protein ($r = 0.294$, $P < 0.0001$) (Figure 6D), implying that the expression of these genes might be modulated by a common factor(s) in OA cartilage.

In contrast, no significant correlation was found between the expression of cartilage matrix genes and minor cartilaginous genes induced by the disease in any combination (from $P = 0.102$ to $P = 0.991$) (Figure 6A).

Among the 5 minor cartilaginous genes evaluated, a significant correlation was observed only between type III collagen and fibronectin ($r = 0.764$, $P < 0.0001$) (Figure 6E). Therefore, the expression of minor cartilaginous genes was assumed to occur without any association in OA cartilage, except for that of type III collagen and fibronectin. Interestingly, the correlation between type III collagen and fibronectin was stronger than any other relationship observed in this study, suggesting the presence of certain link(s) in their expression. In fact, we have obtained data indicating that the expression of type III collagen in human OA cartilage could be induced, at least partly, through the activation of $\alpha 5\beta 1$ integrin by fibronectin (Fukui N: unpublished observation).

Next, a possible correlation of expression was investigated between the *SOX* genes and the 3 cartilage matrix genes. Although no significant correlation was found between *SOX5* or *SOX6* and the matrix genes (from $P = 0.072$ to $P = 0.857$) (Figure 6A), the expression of all 3 matrix genes was significantly correlated with that of *SOX9* (Figures 6F–H). The correlation was strongest with aggrecan ($r = 0.627$, $P < 0.0001$), followed by link protein ($r = 0.560$, $P < 0.0001$), and was weakest with type II collagen ($r = 0.270$, $P = 0.013$). The expression of *SOX* genes was not correlated with that of the minor cartilaginous genes in any combination (from $P = 0.436$ to $P = 0.959$) (Figure 6A). Meanwhile, the expression of *SOX* genes was mutually correlated. Significant correlations were observed between *SOX5* and *SOX6* ($r = 0.527$, $P < 0.0001$) (Figure 6I) and between *SOX5* and *SOX9* ($r = 0.468$, $P = 0.001$) (Figure 6J), although the correlation between *SOX6* and *SOX9* was not significant ($P = 0.728$).

DISCUSSION

The result of this study has provided a comprehensive view of the change in metabolic activity of the chondrocytes in OA. The profile of gene expression differed considerably with the site, depending on the cartilage zone and the extent of cartilage degeneration. In the macroscopically intact areas of OA cartilage, the expression of cartilage matrix genes was markedly enhanced, particularly in the middle and deep zones. This observation was consistent with the results of previous studies using in situ hybridization (3,4,6,9), in which the enhanced matrix synthesis was considered to be a reparative response that attempts to reconstitute the impaired cartilage matrix (2–4). Meanwhile, the up-regulation of cartilage matrix genes was less obvious in the degenerated areas, particularly in the upper regions. Instead, at those regions, the expression of type III collagen and fibronectin was most enhanced. The shift in gene expression was apparent when the profile of gene expression was compared between preserved and degenerated areas in each OA joint (Figure 4).

This shift in gene expression could be significantly involved in the progression of the disease. First, in OA, cartilage matrix is lost primarily from the surface of degenerated cartilage (25), and that loss of matrix could be accelerated by the reduced cartilage matrix synthesis in the surface region (4,9). Second, matrix loss may be facilitated by the induction of type III collagen synthesis. Although this collagen could be a minor component of normal articular cartilage (26–28), it may diminish the quality of cartilage matrix when expressed in excess through the inhibition of proper matrix organization (28,29). Third, fibronectin is known to cause an intense catabolic response in chondrocytes and synoviocytes when cleaved into fragments (30). Therefore, the induction of this protein at the site of enhanced catabolism may be even more significant in the progression of the disease. Taking these findings together, the shift in matrix gene expression at the upper region of degenerated cartilage could be a critical event in OA pathology. Since the shift of gene expression was observed in virtually all OA samples, the regulation of cellular metabolism at that site may be an effective strategy in the future to delay or inhibit disease progression.

Compared with type III collagen and fibronectin, the expression of the other minor cartilaginous genes was less pronounced in OA cartilage in terms of areas, intensities, and frequencies. The induction of type I collagen mRNA was highly variable among OA samples, and, even when expressed, *COL1A1* and *COL1A2* mRNA were often induced at different intensities. The expression of type I collagen in human OA cartilage has remained controversial in previous studies. Although our result of *COL1A1* expression was consistent with several reports (4,6,9), it was discordant with another report regarding the area of expression (31). Further, while we observed the expression of *COL1A2* in human OA cartilage, it was not detected in an earlier study (9). The revealed discrepancy between *COL1A1* and *COL1A2* expression may account for these contradictions in the literature. Likewise, there has been a controversy regarding the induction of *COL10A1* ex-

pression in human OA cartilage: some investigators observed the expression in the upper part of OA cartilage (12,32), whereas others reported it in the deep zone (13,14,33–35). Our result is consistent with the latter finding, in that we identified its expression primarily in the deep zone. However, because the expression of *COL10A1* was relatively weak and fairly inconsistent among OA cartilage samples, we assume that the expression of type X collagen in OA cartilage might be of limited significance in the pathology of OA.

Previously, the appearance of type IIA procollagen mRNA or exon 2 of *COL2A1* in OA cartilage was considered to be the result of a phenotypic reversal of chondrocytes (11,12). However, this speculation is not supported by the present result. Since a result consistent with our own was reported in another recent study (23), a phenotypic reversal of chondrocytes may not be a dominant event in OA cartilage.

In light of these findings, the metabolic change of the chondrocytes in OA may be understood as follows. In the degenerated areas, a major change in the metabolism occurs in the upper region of degenerated cartilage. Such a change resembles that of the dedifferentiation process in the decline of type II collagen and aggrecan expression and the induction of type III collagen expression (Figures 2 and 3) (an illustration of the sequential changes of gene expression in articular chondrocytes during dedifferentiation is available at http://www.hosp.go.jp/~sagami/rinken/crc/index.html). However, the change is different from that process in the expression of link protein, fibronectin, and type I collagen genes. Thus, the metabolic change in the degenerated areas of OA cartilage was considered to be unique and not closely related to the one during the dedifferentiation process. Meanwhile, in the preserved areas, the expression of cartilage matrix genes is highly up-regulated. Although the phenotypic deviation is less obvious in those areas, the expression of type I collagen and type X collagen genes may be induced there in the superficial and deep zones, respectively.

Although the mechanism(s) for these metabolic changes remains entirely unknown, the change in *SOX9* expression may be related to the altered chondrocyte metabolism in OA. As shown in the correlation study, the regional difference in matrix gene expression within OA cartilage could be ascribed, at least partly, to the change in SOX9 expression. However, the present result also indicates that the general up-regulation of matrix gene expression in OA chondrocytes was not associated with the increase in *SOX9* expression. In this study, the amounts of SOX proteins were not assessed. Furthermore, the transcriptional activity of SOX9 is known to be modulated by the level of phosphorylation (36) and by the presence of coregulators (37,38). Thus, taking these factors into account may provide a better explanation of the significance of SOX proteins in the altered chondrocyte metabolism in OA.

Although the present study has clarified the metabolic change of chondrocytes in OA cartilage, it also has several limitations. First, the metabolic change was evaluated primarily by mRNA expression, and protein synthesis was not determined. The major difference in mRNA expression levels among the samples posed another problem. A large variation among human cartilage samples has been reported repeatedly in previous studies (7,8,23). For OA samples, this might reflect the diversity of the pathology, while the variation among the controls might have stemmed from differences in joint physiology that could be related to the donor's condition before death. These points should be clarified by future studies. Despite these limitations, we believe that our study has revealed several novel aspects of OA pathology. We hope that the current results may offer another clue to eventually establishing a novel strategy to treat this tenacious disease.

AUTHOR CONTRIBUTIONS

Dr. Fukui had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Fukui.

Acquisition of data. Ikeda, Ohnuki, Tanaka, Hikita, Mitomi, Juji, Katsuragawa, Yamamoto, Sawabe, Yamane, Suzuki.

Analysis and interpretation of data. Fukui, Mori, Sandell, Ochi.

Manuscript preparation. Fukui.

Statistical analysis. Fukui.

REFERENCES

1. Collins DH, McElligott TF. Sulphate ($^{35}SO_4$) uptake by chondrocytes in relation to histological changes in osteoarthritic human articular cartilage. Ann Rheum Dis 1960;19:318–30.
2. Sandell LJ, Heinegard D, Hering TM. Cell biology, biochemistry, and molecular biology of articular cartilage in osteoarthritis. In: Moskowitz RW, Altman RD, Hochberg MC, Buckwalter JA, Goldberg VM, editors. Osteoarthritis: diagnosis and medical/surgical management. 4th ed. Philadelphia: Lippincott Williams & Wilkins; 2007. p. 73–106.
3. Aigner T, Gluckert K, von der Mark K. Activation of fibrillar collagen synthesis and phenotypic modulation of chondrocytes in early human osteoarthritic cartilage lesions. Osteoarthritis Cartilage 1997;5:183–9.
4. Aigner T, Vornehm SI, Zeiler G, Dudhia J, von der Mark K, Bayliss MT. Suppression of cartilage matrix gene expression in upper zone chondrocytes of osteoarthritic cartilage. Arthritis Rheum 1997;40:562–9.
5. Adam M, Deyl Z. Altered expression of collagen phenotype in osteoarthrosis. Clin Chim Acta 1983;133:25–32.

6. Miosge N, Hartmann M, Maelicke C, Herken R. Expression of collagen type I and type II in consecutive stages of human osteoarthritis. Histochem Cell Biol 2004;122:229–36.
7. Aigner T, Fundel K, Saas J, Gebhard PM, Haag J, Weiss T, et al. Large-scale gene expression profiling reveals major pathogenetic pathways of cartilage degeneration in osteoarthritis. Arthritis Rheum 2006;54:3533–44.
8. Aigner T, Zien A, Gehrsitz A, Gebhard PM, McKenna L. Anabolic and catabolic gene expression pattern analysis in normal versus osteoarthritic cartilage using complementary DNA-array technology. Arthritis Rheum 2001;44:2777–89.
9. Aigner T, Bertling W, Stoss H, Weseloh G, von der Mark K. Independent expression of fibril-forming collagens I, II, and III in chondrocytes of human osteoarthritic cartilage. J Clin Invest 1993;91:829–37.
10. Nerlich AG, Wiest I, von der Mark K. Immunohistochemical analysis of interstitial collagens in cartilage of different stages of osteoarthrosis. Virchows Arch B Cell Pathol Incl Mol Pathol 1993;63:249–55.
11. Aigner T, Zhu Y, Chansky HH, Matsen FA III, Maloney WJ, Sandell LJ. Reexpression of type IIA procollagen by adult articular chondrocytes in osteoarthritic cartilage. Arthritis Rheum 1999; 42:1443–50.
12. Nah HD, Swoboda B, Birk DE, Kirsch T. Type IIA procollagen: expression in developing chicken limb cartilage and human osteoarthritic articular cartilage. Dev Dyn 2001;220:307–22.
13. Girkontaite I, Frischholz S, Lammi P, Wagner K, Swoboda B, Aigner T, et al. Immunolocalization of type X collagen in normal fetal and adult osteoarthritic cartilage with monoclonal antibodies. Matrix Biol 1996;15:231–8.
14. Von der Mark K, Kirsch T, Nerlich A, Kuss A, Weseloh G, Gluckert K, et al. Type X collagen synthesis in human osteoarthritic cartilage: indication of chondrocyte hypertrophy. Arthritis Rheum 1992;35:806–11.
15. Poole RA, Guilak F, Abramson SB. Etiopathogenesis of osteoarthritis. In: Moskowitz RW, Altman RD, Hochberg MC, Buckwalter JA, Goldberg VM, editors. Osteoarthritis: diagnosis and medical/surgical management. 4th ed. Philadelphia: Lippincott Williams & Wilkins; 2007. p. 27–49.
16. Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, et al. Laser capture microdissection. Science 1996;274:998–1001.
17. Altman R, Asch E, Bloch D, Bole G, Borenstein D, Brandt K, et al. Development of criteria for the classification and reporting of osteoarthritis: classification of osteoarthritis of the knee. Arthritis Rheum 1986;29:1039–49.
18. Buckwalter JA, Mankin HJ, Grodzinsky AJ. Articular cartilage and osteoarthritis. In: Pellegrini VD Jr, editor. AAOS instructional course lectures. Vol. 54. Rosemont (IL): American Academy of Orthopedic Surgeons; 2005. p. 465–80.
19. Poole RA. Cartilage in health and disease. In: Koopman WJ, Moreland LW, editors. Arthritis and allied conditions: a textbook of rheumatology. 15th ed. Philadelphia: Lippincott Williams & Wilkins; 2004. p. 223–69.
20. Miller DR, Mankin HJ, Shoji H, D'Ambrosia RD. Identification of fibronectin in preparations of osteoarthritic human cartilage. Connect Tissue Res 1984;12:267–75.
21. Goldwasser M, Astley T, van der Rest M, Glorieux FH. Analysis of the type of collagen present in osteoarthritic human cartilage. Clin Orthop Relat Res 1982;(167):296–302.
22. Burton-Wurster N, Horn VJ, Lust G. Immunohistochemical localization of fibronectin and chondronectin in canine articular cartilage. J Histochem Cytochem 1988;36:581–8.
23. Gebhard PM, Gehrsitz A, Bau B, Soder S, Eger W, Aigner T. Quantification of expression levels of cellular differentiation markers does not support a general shift in the cellular phenotype of osteoarthritic chondrocytes. J Orthop Res 2003;21:96–101.
24. De Crombrugghe B, Lefebvre V, Behringer RR, Bi W, Murakami S, Huang W. Transcriptional mechanisms of chondrocyte differentiation. Matrix Biol 2000;19:389–94.
25. Hollander AP, Pidoux I, Reiner A, Rorabeck C, Bourne R, Poole AR. Damage to type II collagen in aging and osteoarthritis starts at the articular surface, originates around chondrocytes, and extends into the cartilage with progressive degeneration. J Clin Invest 1995;96:2859–69.
26. Young RD, Lawrence PA, Duance VC, Aigner T, Monaghan P. Immunolocalization of type III collagen in human articular cartilage prepared by high-pressure cryofixation, freeze-substitution, and low-temperature embedding. J Histochem Cytochem 1995;43: 421–7.
27. Wotton SF, Duance VC. Type III collagen in normal human articular cartilage. Histochem J 1994;26:412–6.
28. Eyre DR, Weis MA, Wu JJ. Articular cartilage collagen: an irreplaceable framework? Eur Cell Mater 2006;12:57–63.
29. Young RD, Lawrence PA, Duance VC, Aigner T, Monaghan P. Immunolocalization of collagen types II and III in single fibrils of human articular cartilage. J Histochem Cytochem 2000;48:423–32.
30. Homandberg GA. Cartilage damage by matrix degradation products: fibronectin fragments. Clin Orthop Relat Res 2001;(391 Suppl):S100–7.
31. Wagner S, Hofstetter W, Chiquet M, Mainil-Varlet P, Stauffer E, Ganz R, et al. Early osteoarthritic changes of human femoral head cartilage subsequent to femoro-acetabular impingement. Osteoarthritis Cartilage 2003;11:508–18.
32. Kirsch T, Swoboda B, Nah H. Activation of annexin II and V expression, terminal differentiation, mineralization and apoptosis in human osteoarthritic cartilage. Osteoarthritis Cartilage 2000;8: 294–302.
33. Gibson GJ, Verner JJ, Nelson FR, Lin DL. Degradation of the cartilage collagen matrix associated with changes in chondrocytes in osteoarthrosis: assessment by loss of background fluorescence and immunodetection of matrix components. J Orthop Res 2001; 19:33–42.
34. Boos N, Nerlich AG, Wiest I, von der Mark K, Ganz R, Aebi M. Immunohistochemical analysis of type-X-collagen expression in osteoarthritis of the hip joint. J Orthop Res 1999;17:495–502.
35. Aigner T, Reichenberger E, Bertling W, Kirsch T, Stoss H, von der Mark K. Type X collagen expression in osteoarthritic and rheumatoid articular cartilage. Virchows Arch B Cell Pathol Incl Mol Pathol 1993;63:205–11.
36. Huang W, Zhou X, Lefebvre V, de Crombrugghe B. Phosphorylation of SOX9 by cyclic AMP-dependent protein kinase A enhances SOX9's ability to transactivate a Col2a1 chondrocyte-specific enhancer [published erratum appears in Mol Cell Biol 2000;20:7838]. Mol Cell Biol 2000;20:4149–58.
37. Tsuda M, Takahashi S, Takahashi Y, Asahara H. Transcriptional co-activators CREB-binding protein and p300 regulate chondrocyte-specific gene expression via association with Sox9. J Biol Chem 2003;278:27224–9.
38. Kawakami Y, Tsuda M, Takahashi S, Taniguchi N, Esteban CR, Zemmyo M, et al. Transcriptional coactivator PGC-1α regulates chondrogenesis via association with Sox9. Proc Natl Acad Sci U S A 2005;102:2414–9.

A Novel Cell Delivery System Using Magnetically Labeled Mesenchymal Stem Cells and an External Magnetic Device for Clinical Cartilage Repair

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Purpose: The purpose of this study was to investigate whether it is possible to successfully accumulate magnetically labeled mesenchymal stem cells (MSCs), under the direction of an external magnetic force, to the desired portion of osteochondral defects of the patellae after intra-articular injection of the MSCs. **Methods:** MSCs were cultured from bone marrow and were labeled magnetically. Osteochondral defects were made in the center of rabbit and swine patellae, and magnetically labeled MSCs were injected into the knee joints either under the direction of an external magnetic force or with no magnetic force applied. In the rabbit model we evaluated the patellae macroscopically and histologically, and in the swine model we observed the patellae arthroscopically. **Results:** Accumulation of magnetically labeled MSCs to the osteochondral defect was shown macroscopically and histologically in the rabbit model and was shown by arthroscopic observation to be attached to the chondral defect in the swine model. **Conclusions:** We showed the ability to deliver magnetically labeled MSCs to a desired place in the knee joint. **Clinical Relevance:** Our novel approach is applicable for human cartilage defects and may open a new era of repairing cartilage defects caused by osteoarthritis or trauma by use of a less invasive technique. **Key Words:** Cell delivery system—Mesenchymal stem cell—Magnetic force—Cartilage repair—Arthroscopic observation.

Articular cartilage has very limited healing potential. Although there are several treatment options for cartilage defects, no treatment has been established as a gold standard procedure. One recent strategy for cartilage repair is by transplantation of mesenchymal stem cells (MSCs).[1-4] MSCs are the cell population of undifferentiated cells isolated from adult tissue that have the capacity to differentiate into mesodermal lineages, such as bone, cartilage, fat, muscle, or other tissues.[5,6] The MSCs from bone marrow can be cultured and differentiated into the desired lineage in vitro with the application of specific growth factors or bioactive molecules. We previously examined the effectiveness of a cell delivery system using an internal magnet and magnetic

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liposomes, or magnetic beads, in vitro.[7-10] However, for clinical applications, it is indispensable to develop an external magnetic device, as well as to use materials that have been safely used in humans. Recently, there have been many reports of labeling MSCs with ferumoxides.[11-13] Ferumoxides are dextran-coated superparamagnetic iron oxide nanoparticles approved by the US Food and Drug Administration as a magnetic resonance contrast agent for hepatic imaging of humans. By use of this technique, it has become easy to make magnetically labeled MSCs. We originally made an external magnetic device that generated a high magnetic force of 0.6 T (Tamagawa, Miyagi, Japan) for the purpose of human tissue repair. In this study, using this device, we hypothesized that we could successfully accumulate magnetically labeled MSCs, which have been shown to have a capacity to differentiate into chondrocytes,[12] to the desired portion of the osteochondral defect of the rabbit's patella after intraarticular injection of MSCs. We also hypothesized that this novel, less invasive approach could be done under arthroscopic control in swine knees.

METHODS

Our research methods were reviewed and approved by the Ethical Committee of Hiroshima University, Hiroshima, Japan.

Cell Culture

The method for isolation and in vitro expansion of bone marrow–derived MSCs is well known and has been previously described. A modification of the culture method of Kotobuki et al.[14] was used. In brief, 5 mL of bone marrow from the tibia of adult human donors was aspirated with 1 mL of heparin sodium when they underwent anterior cruciate ligament reconstruction, and this was centrifuged for 5 minutes at 1,500 rpm; the subsequent supernatant, including heparin sodium, was discarded. The extract was resuspended in 6 mL of culture medium, composed of Dulbecco's modified Eagle medium (Invitrogen [Gibco], Paisley, Scotland) with 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO) and 1% antibiotics (penicillin, streptomycin, and Fungizone; BioWhittaker, Walkersville, MD). We seeded 2 mL of the suspension onto 100-mm culture dishes (Falcon; BD Biosciences, Franklin Lakes, NJ), and 8 mL of culture medium was added to each dish. The dishes were incubated for 3 weeks under a humidified atmosphere and 5% carbon dioxide at 37°C. The medium was not changed for the first 7 days. When the medium was changed, the suspended cells and the supernatant were discarded, and fresh culture medium was added to the dish, where the adherent cells were left. After the first change of medium, the medium was then changed every 3 days. About 2 weeks after seeding, the cells had proliferated and reached confluence. The cells were then harvested by treatment with 0.25% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) (2.5-g/L trypsin/1-mmol/L EDTA solution; Nacalai Tesque, Kyoto, Japan). To expand the MSCs, 2 to 3 • 10^5 of the harvested cells were seeded on 100-mm culture dishes. On reaching confluence again, the cells were reseeded under the same conditions. We referred to these adherent cells as MSCs. We confirmed that these cells had the capacity to differentiate into osteocytes, adipocytes, and chondrocytes (data not shown) as shown in previous experimental studies.[8,14]

Magnetic Labeling of MSCs

MSCs were labeled overnight with 25 • g Fe/mL ferumoxides and 375 ng/mL poly-L-lysine (PLL) (poly-L-lysine hydrobromide; molecular weight, 388 kd [P-1524; Sigma-Aldrich]) as a transfection agent.[11] In brief, 2.2 • L of ferumoxide stock solution (11.2 mg Fe/mL; Tanabe Seiyaku, Osaka, Japan) was added per milliliter of culture medium, without cells, and mixed well. PLL was then added at 3.75 • L/mL from a 0.1-mg/mL stock solution. The medium was mixed and incubated for 60 minutes at room temperature with occasional gentle mixing. Labeling was initiated by removal of the medium from the adherent MSCs and then adding to the medium containing the ferumoxides-PLL mixture. After incubation overnight in the medium, the MSCs were collected after trypsinization, and some cells were stained with Prussian blue to evaluate magnetic labeling of MSCs.

External Magnetic Device

A variable DC electromagnet (model TM-SP12010SC-014; Tamagawa) was manufactured for the purpose of generating an external magnetic force (Fig 1A). The disk-shaped electromagnet consists of a solenoid iron coil and is designed to be able to generate a magnetic field efficiently. The generating magnetic field is symmetric about the arbor of the disk (Fig 1B). In brief, the magnetic field is directed to the center of the disk surface, and its magnitude decreases away from the surface (Fig 1C). The magnitude of the magnetic field

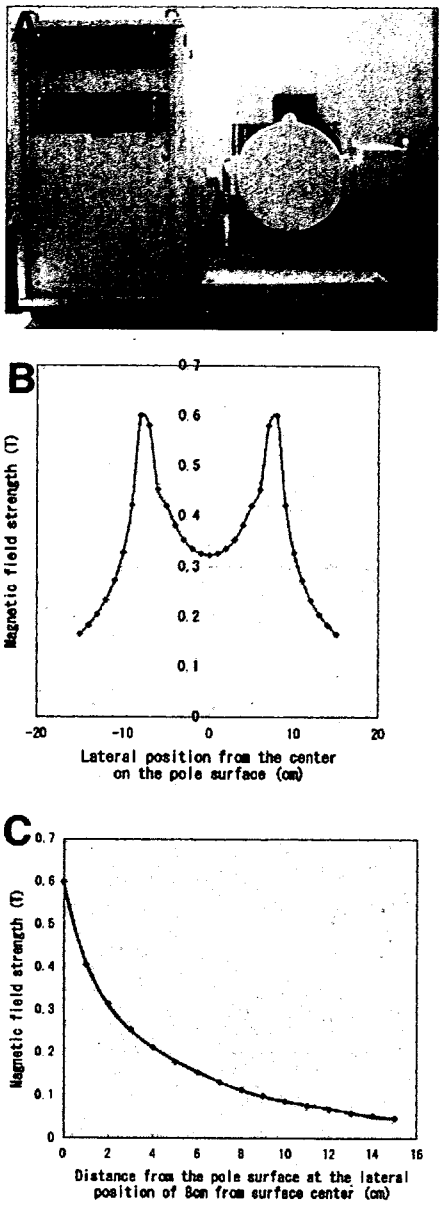

FIGURE 1. (A) An external magnetic device generated a magnetic force (maximum, 0.6 T) to the round wall (40 cm in diameter). (B) The maximum magnetic field is 0.6 T, and (C) the magnitude of the magnetic field decreases away from the surface.

is increased by intensifying the electric current through the electromagnet, and is limited by the temperature of the coil. When a sample lies 8 cm from the center of the pole, the maximum magnetic field is 0.6 T (Fig 1B), and the maximum gradient of the amplitude of the magnetic field is 25 T/m. The disk is able to change height and direction.

Accumulation Capacity of Magnetically Labeled MSCs Under Influence of Magnetic Force

Magnetically labeled MSCs (9 • 10^6/300 • L Dulbecco's phosphate-buffered saline solution [PBS]) were injected into a tissue culture flask two thirds full with PBS. In the magnetic force group, injection of magnetically labeled MSCs was performed under the influence of an external magnetic force (0.6 T). In the control group, cell injection was performed without the influence of a magnetic force.

Rabbit Model

Surgery and Treatment: Six male Japanese white rabbits weighing 2.5 to 3.0 kg were used in this study. All rabbits were killed by the intravenous administration of pentobarbital sodium, and knee joints were opened through a medial parapatellar approach. Patellae were turned over, and an osteochondral defect (3 mm in diameter and 2 mm in depth) was made in the center of the patellae with a cylindrical drill. The joint capsules were closed in a routine manner, and the knees were removed. After surgery, 100 • L of PBS was injected into the knee joint. The knees were put on the external magnetic device (8 cm from the pole center, with patellae in the direction of the generated magnetic force). In the magnetic force group, magnetically labeled MSCs (3 • 10^6 cells/50 • L PBS) were injected into the knee joints under the influence of a magnetic force (0.6 T) and were kept in position by use of the magnetic force for 4 hours. In the control group, magnetically labeled MSCs were injected into the knee joints without the influence of a magnetic force and were kept in the same position for 4 hours.

Evaluation: After treatment, the knees were fixed with 4% formalin for 2 days, and the patellae were then removed with the joint capsule. The knee joints were opened through a lateral parapatellar approach and were observed macroscopically. The patellae were removed and immersed in 4% formalin for 24 hours, decalcified with 10% EDTA for 4 weeks, and then embedded in paraffin. Histologic sections were stained with H&E, and Prussian blue staining was used for microscopic analysis.

Swine Model

In this study, 4 fresh-frozen porcine knees were used (age range, 3 to 6 months). Before testing, the knees were stored at • 20°C and thawed for 48 hours at 4°C. The knee joints were opened through the medial parapatellar approach. Patellae were turned over, and full-thickness, critical-sized chondral de-

fects (10 mm in diameter) were made in the center of the patellae. The joint capsules were closed in a routine manner, and an arthroscope (Smith & Nephew Endoscopy, Andover, MA) was inserted into the knee joints. After surgery, the knees were put on the external magnetic device (with the patellae being in the direction of the generated magnetic force). In the magnetic force group, magnetically labeled MSCs ($8 \cdot 10^5$ cells/100 • L PBS) were injected into the knee joint under the influence of a magnetic force (0.6 T) by use of a 1-mL injector (whose needle was bent to the chondral defects). When magnetically labeled MSCs were injected into the knee joints, arthroscopic findings were recorded, and then the arthroscope was removed. At 90 minutes after injection under the influence of the magnetic force, the chondral defect sites were again observed arthroscopically and recorded. During arthroscopy, the inflow of fluid was turned on intermittently. In the control group, magnetically labeled MSCs were injected into the knee joints under arthroscopic observation without the influence of a magnetic force, and the arthroscopic findings were recorded.

RESULTS

Magnetic Labeling of MSCs

We confirmed that almost 100% of MSCs were stained blue with Prussian blue stain.

Accumulation Capacity of Magnetically Labeled MSCs Under Influence of Magnetic Force

In the control group (Fig 2B and Video 1 [online only, available at www.arthroscopyjournal.org]), magnetically labeled MSCs fell down vertically with gravity. In the magnetic force group (Fig 2C and Video 2 [online only, available at www.arthroscopyjournal.org]), magnetically labeled MSCs fell down not vertically but diagonally, moved in the direction of the generated magnetic force, and accumulated to the side wall of the flask under the influence of gravity and the magnetic force.

Rabbit Model

Macroscopic Examination: In the control group, magnetically labeled MSCs, visible as brown particles, were scattered in the joints and were attached to the bottom side of the joint capsules (6/6 knees) (Fig 3C). In the magnetic force group, magnetically labeled MSCs had accumulated to the osteochondral defect of the patellae and the capsule around the patellae (6/6 knees) (Fig 3D).

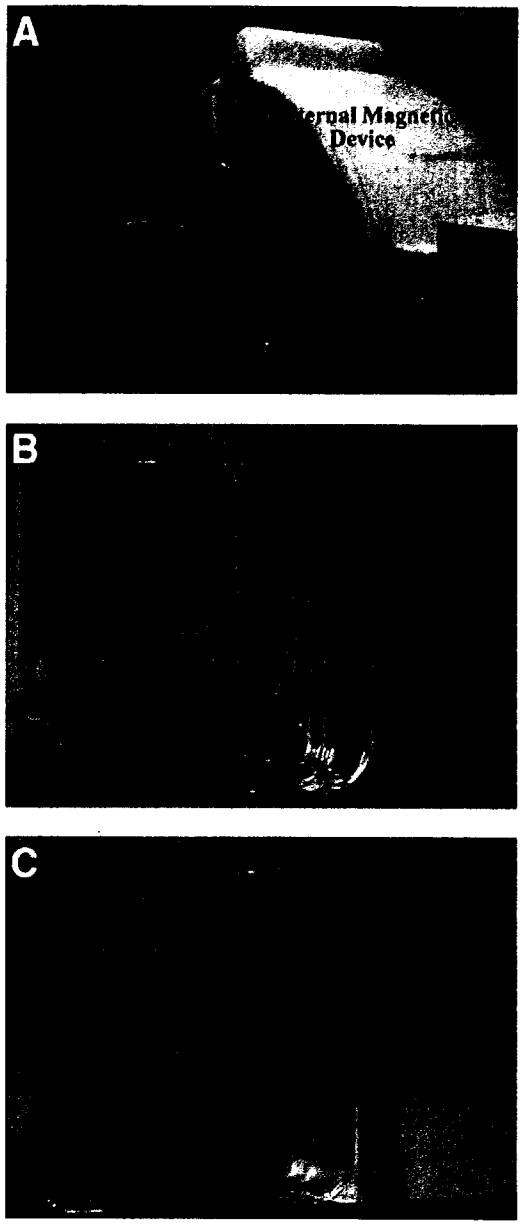

FIGURE 2. (A) External magnetic device. (B) Magnetically labeled MSCs ($9 \cdot 10^6$ cells/300 • L PBS) were injected into a flask without a magnetic force, and the MSCs fell down vertically (Video 1 [online only, available at www.arthroscopyjournal.org]). (C) Under the influence of a magnetic force (Video 2 [online only, available at www.arthroscopyjournal.org]), magnetically labeled MSCs were induced to the side wall of the flask, where the magnetic force was directed.

Histologic Findings: In the control group, no cells, excluding red blood cells, were shown to be on the osteochondral defect of the patellae as de-

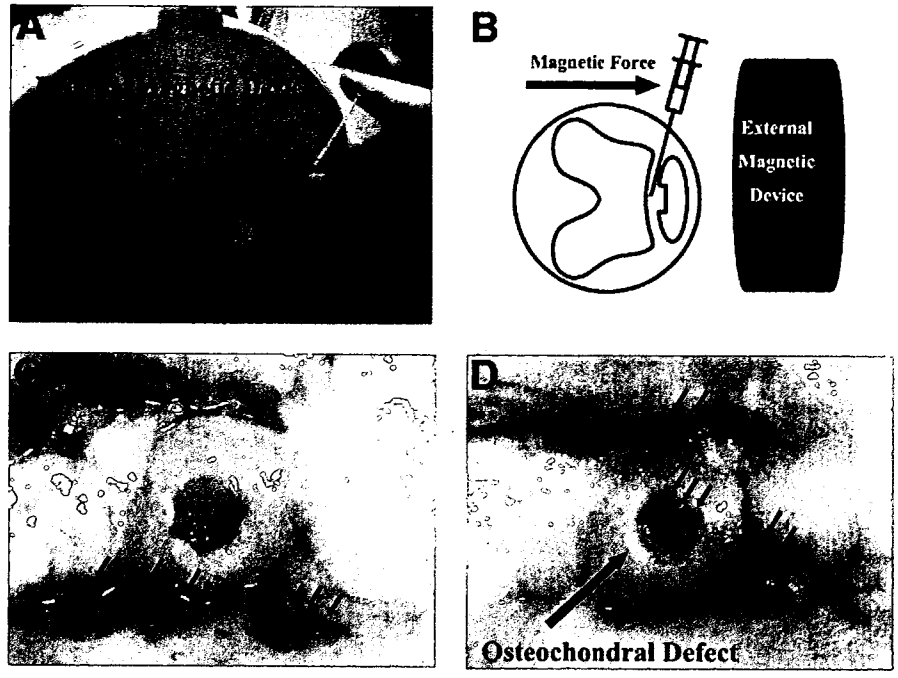

FIGURE 3. Rabbit model. (A, photograph of the experimental procedure; B, schema of the procedure.) Osteochondral defects were made in the center of the patellae, and magnetically labeled MSCs ($3 \cdot 10^6$ cells/50 • L PBS) were injected into the knee joint with or without the influence of a magnetic force. Macroscopic findings are shown 4 hours after injection, and magnetically labeled MSCs are visible as brown particles (arrows). In the control group, the MSCs did not accumulate to the defect and were attached to the bottom side of the capsules as a result of gravitation (C). In the magnet force group, magnetically labeled MSCs accumulated to the osteochondral defect of the patellae (D).

termined by H&E staining (Fig 4A and 4B) and Prussian blue staining (Fig 4C and 4D). In the magnetic force group, many cells were visible on the osteochondral defect of the patellae with H&E staining (Fig 5A and 5B) and were stained blue with Prussian blue, indicating that the cells contained iron and were magnetically labeled MSCs (Fig 5C and 5D).

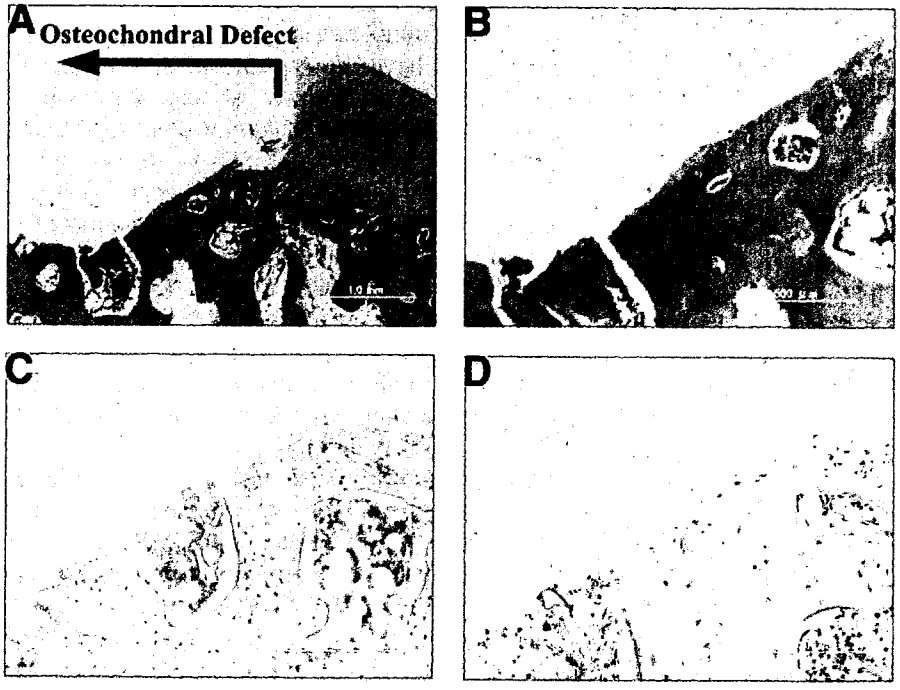

FIGURE 4. Microscopic findings of control group (rabbit model). There were no cells, excluding red blood cells, on the osteochondral defect of the patellae stained with (A, original magnification • 40; B, original magnification • 100) H&E and (C, original magnification • 100; D, original magnification • 200) Prussian blue stain.

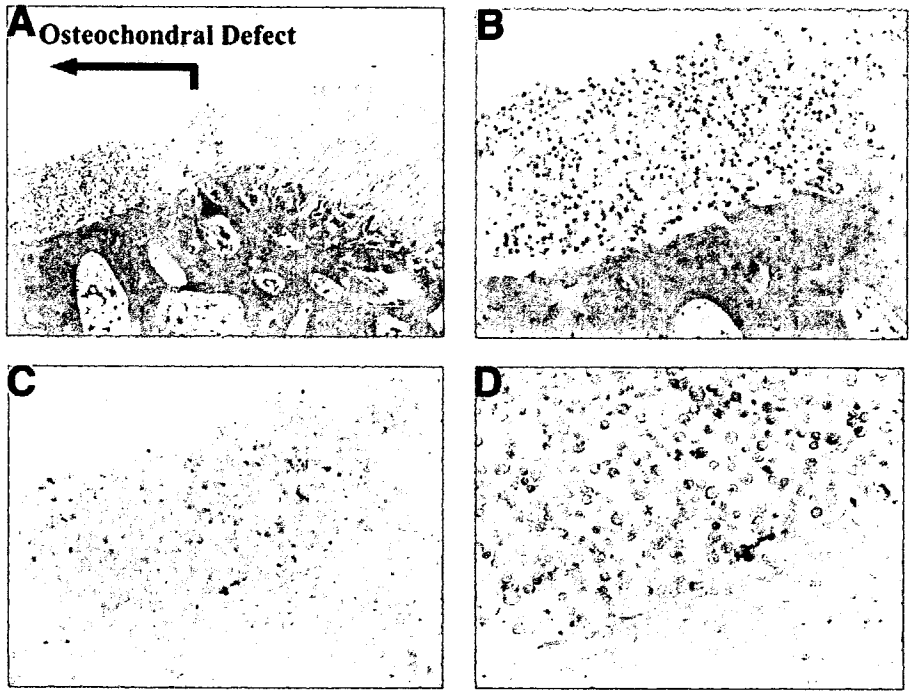

FIGURE 5. Microscopic findings of magnetic force group (rabbit model). Many cells were seen on the osteochondral defect with H&E stain (A, original magnification • 40; B, original magnification • 100), and the cells were stained blue with the Prussian blue stain (C, original magnification • 100; D, original magnification • 200), indicating that the cells contained iron and magnetically labeled MSCs.

Swine Model

In the control group, MSCs were immediately scattered in the joints after injection (Fig 6B and Video 3 [online only, available at www.arthroscopyjournal.org]). In the magnetic force group, arthroscopic observation showed that magnetically labeled MSCs had accumulated to the chondral defect of the patellae (Fig 6C and Video 4 [online only, available at www.arthroscopyjournal.org]). Under the influence of the magnetic force, metallic equipment (arthroscopic system and injector's needle) was not affected by the magnetic force, because we used nonmagnetic metals as arthroscopic tools. Only the video camera for recording arthroscopic observation was affected, if the camera was within 30 cm of the external magnetic device. At 90 minutes after MSC injection, MSCs were attached to the chondral defect site against gravity and inflow of the fluid (Fig 6D and Video 5 [online only, available at www.arthroscopyjournal.org]).

DISCUSSION

This study clearly showed that our system using arthroscopic control and an external magnetic device could deliver magnetically labeled MSCs to an osteochondral defect of the patellae. This is the first report to visualize targeting of magnetically labeled MSCs under the influence of a magnetic force to accumulate to the desired place in the knee joint.

Recently, many researchers have reported methods of transplanting MSCs to repair cartilage defects.[1-4] MSCs can be easily gathered from the marrow of an ilium or tibia, and large amounts of cells can be obtained by culture outside the human body. Furthermore, MSCs have the capacity to differentiate into mesodermal lineages, such as bone, cartilage, fat, muscle, or other tissue.[5,6] Murphy et al.[1] reported that intra-articular injection of MSCs to an osteoarthritic knee stimulated regeneration of articular cartilage in the goat model, and Tatebe et al.[4] reported that MSCs were able to survive on the osteochondral defects and differentiated to cartilage. Nishimori et al.[2] reported that intra-articular injection of MSCs along with a bone marrow stimulation procedure repaired chronic osteochondral defects in the rat model. In a clinical trial, Wakitani et al.[3] reported that the implantation of MSCs together with high tibial osteotomy for the human osteoarthritic knee was better than only high tibial osteotomy on the arthroscopic and histologic grading scale. On the other hand, Agung et al.[15] reported that injured cartilage was repaired after intra-articular injection of MSCs in the rat model but that the injection of too many MSCs generated free bodies of scar tissue in the joint. We therefore conceived a

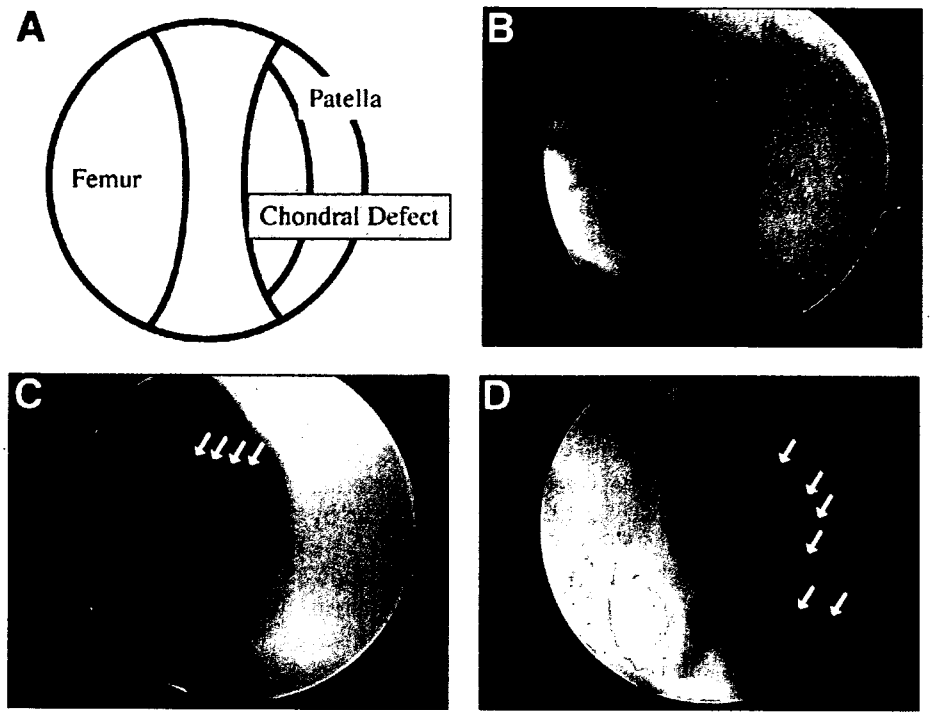

FIGURE 6. Arthroscopic findings of swine model. Magnetically labeled MSCs are visible as brown particles (arrows). (A) Chondral defects of the patellae are shown on the right side of the photographs and videos. (B) In the control group, magnetically labeled MSCs were scattered in the joint (Video 3, online only, available at www.arthroscopyjournal.org). (C) In the magnetic force group, magnetically labeled MSCs accumulated in the chondral defect of the patellae (Video 4, online only, available at www.arthroscopyjournal.org). (D) At 90 minutes after the injection of MSCs, MSCs were attached biologically to the chondral defect site (Video 5 [online only, available at www.arthroscopyjournal.org]).

new method of accumulating a relatively small number of MSCs to a desired area using a magnetic force with the aim of avoiding side effects such as the production of free bodies. We have previously reported a drug delivery system and cell delivery system using a magnetic force.[7-10,16] Tanaka et al.[16] reported that a magnetic liposomal delivery system using transforming growth factor • 1 is useful for cartilage repair of the rabbit knee, and Yanada et al.[8] concluded that MSC-RGDS (arginine [R]–glycine [G]–asparatic acid [D]–serine [S]) peptide–bead complexes are useful for cartilage repair in vitro. These studies used a permanent neodymium magnet. For our study, we developed a new external magnetic device for the treatment of humans.

There are some limitations to our study, however. First, we used ferumoxides to make magnetically labeled MSCs. Ferumoxides are approved by the US Food and Drug Administration as a magnetic resonance contrast agent, but it is not confirmed whether MSCs labeled with ferumoxides have the same chondrogenic capacity as nonlabeled MSCs. Arbab et al.[12] reported that labeling with ferumoxides does not inhibit the chondrogenic differentiation capacity of MSCs, but Kostura et al.[13] reported contradictory results. We confirmed that magnetically labeled MSCs have the capacity to differentiate into chondrocytes in the chondrogenic differentiation medium that contained transforming growth factor • 1 and bone morphogenetic protein 2 (data not shown). Second, this study was conducted over a short time. We have only shown that magnetically labeled MSCs accumulated in the osteochondral defect of the patella and did not confirm whether articular cartilage was formed in the osteochondral defect. A further long-term study is needed. Third, we used human MSCs, because we aimed at clinical applications in humans. We investigated whether magnetically labeled human MSCs accumulated in the desired place. For another long-term study in animals, it would be necessary to use immunodeficient animals or the host's MSCs. Fourth, we used a magnetic field of 0.6 T, but we do not know the most effective strength of the magnetic field. However, we confirmed that our external magnetic device was able to control the direction of magnetically labeled MSCs. If we use a greater magnetic force, there may be effects on operation tools, such as probes, arthroscopic equipment, and television monitors. In this study, however, the operation tools were not affected by the magnetic field. (It should be noted that we used nonmagnetic tools for arthroscopy and an arthroscopic system that did not use a magnet in the arthroscope.) We definitely need to perform another study to investigate the optimal strength of the mag-

netic field for clinical applications. The last aspect is the efficiency of magnetically labeling in magnetic resonance imaging (MRI). Because ferumoxides act as a magnetic resonance contrast agent for hepatic imaging in humans, there may be some artifacts on MRI. Given that this labeling procedure was originally developed for monitoring MSCs,[17] magnetic labeling may have the merit of monitoring implanted MSCs on MRI.

Another important factor is the location of the osteochondral defect. Although it is relatively easy to apply this new method to lesions in the patella or patella groove, it is not easy to apply it to femoral condyle lesions or tibial plateau lesions. However, we think that this new method may be applicable for these lesions by managing the direction of the external magnetic force and by effectively using gravity by changing the knee position of the patients.

Although there were some limitations to our study, this system has the potential to become a novel stem cell delivery method in humans. Our novel, less invasive approach is applicable to human cartilage defects and may open a new era of repairing cartilage defects caused by osteoarthritis or trauma. Furthermore, we think that this new system may be applicable not only for the treatment of osteochondral defects but also for the treatment of brain or spinal cord injuries, fractures, and myocardial infarctions.

CONCLUSIONS

We have shown the ability to deliver magnetically labeled MSCs to a desired place in the knee joint. This supports the hypothesis of our study.

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REFERENCES

1. Murphy JM, Fink DJ, Hunziker EB, Barry FP. Stem cell therapy in a caprine model of osteoarthritis. Arthritis Rheum 2003;48:3464-3474.
2. Nishimori M, Deie M, Kanaya A, Exham H, Adachi N, Ochi M. Repair of chronic osteochondral defects in the rat. A bone marrow-stimulating procedure enhanced by cultured allogenic bone marrow mesenchymal stromal cells. J Bone Joint Surg Br 2006;88:1236-1244.
3. Wakitani S, Imoto K, Yamamoto T, Saito M, Murata N, Yoneda M. Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees. Osteoarthritis Cartilage 2002;10:199-206.
4. Tatebe M, Nakamura R, Kagami H, Okada K, Ueda M. Differentiation of transplanted mesenchymal stem cells in a large osteochondral defect in rabbit. Cytotherapy 2005;7:520-530.
5. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143-147.
6. Jiang Y, Jahagirdar BN, Reinhardt RL, et al. Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002;418:41-49.
7. Ochi M, Adachi N, Nobuto H, Yanada S, Ito Y, Agung M. Articular cartilage repair using tissue engineering technique—Novel approach with minimally invasive procedure. Artif Organs 2004;28:28-32.
8. Yanada S, Ochi M, Adachi N, Nobuto H, Agung M, Kawamata S. Effects of CD44 antibody- or RGDS peptide-immobilized magnetic beads on cell proliferation and chondrogenesis of mesenchymal stem cells. J Biomed Mater Res A 2006;77:773-784.
9. Nakashima Y, Deie M, Yanada S, Sharman P, Ochi M. Magnetically labeled human natural killer cells, accumulated in vitro by an external magnetic force, are effective against HOS osteosarcoma cells. Int J Oncol 2005;27:965-971.
10. Hamasaki T, Tanaka N, Ishida O, et al. Characterization of labeled neural progenitor cells for magnetic targeting. Neuroreport 2005;16:1641-1645.
11. Bulte JW, Arbab AS, Douglas T, Frank JA. Preparation of magnetically labeled cells for cell tracking by magnetic resonance imaging. Methods Enzymol 2004;386:275-299.
12. Arbab AS, Yocum GT, Rad AM, et al. Labeling of cells with ferumoxides-protamine sulfate complexes does not inhibit function or differentiation capacity of hematopoietic or mesenchymal stem cells. NMR Biomed 2005;18:553-559.
13. Kostura L, Kraitchman DL, Mackay AM, Pittenger MF, Bulte JW. Feridex labeling of mesenchymal stem cells inhibits chondrogenesis but not adipogenesis or osteogenesis. NMR Biomed 2004;17:513-517.
14. Kotobuki N, Hirose M, Takakura Y, Ohgushi H. Cultured autologous human cells for hard tissue regeneration: Preparation and characterization of mesenchymal stem cells from bone marrow. Artif Organs 2004;28:33-39.
15. Agung M, Ochi M, Yanada S, et al. Mobilization of bone marrow-derived mesenchymal stem cells into the injured tissues after intraarticular injection and their contribution to tissue regeneration. Knee Surg Sports Traumatol Arthrosc 2006;14:1307-1314.
16. Tanaka H, Sugita T, Yasunaga Y, et al. Efficiency of magnetic liposomal transforming growth factor-beta 1 in the repair of articular cartilage defects in a rabbit model. J Biomed Mater Res A 2005;73:255-263.
17. Daldrup-Link HE, Rudelius M, Piontek G, et al. Migration of iron oxide-labeled human hematopoietic progenitor cells in a mouse model: In vivo monitoring with 1.5-T MR imaging equipment. Radiology 2005;234:197-205.

卒後
研修講座

変形性膝関節症のリスクファクター*

大森　豪**

[整形外科 59巻3号：297～304，2008]

はじめに

変形性膝関節症（膝OA）は年齢に伴う膝関節の退行性疾患であり，日常診療の場において変形性関節症の中では腰椎に次いで頻度が高い．本症の病態は，関節軟骨の変性と摩耗を主体として骨や軟骨下骨，滑膜，半月板，靱帯といった関節構成体に炎症反応や増殖性変化，変形性変化が生じ，結果的に関節破壊の進行にいたる一連の過程として理解される．膝OAは“common disease”であり，その発症と進行には多数の因子が関与している．これらの因子は，膝関節に限局する局所因子と全身性因子あるいは遺伝要因と環境要因などに区分され，疫学や生体力学，生化学，画像解析などさまざまなアプローチで研究が行われている．

膝OAの治療や予防を考えるうえでリスクファクターを理解することはきわめて重要であり，本稿では膝OAの発症・進行因子について，これまでに明らかになっているものおよび残された課題を含めて概説する．なお，ここで述べる膝OAとはX線像にて診断されたradiographic OAであり，膝痛などの症状を有するsymptomatic OAではない．

1 年齢と性別

男女とも40歳代以降年齢とともに膝OAの頻度は増加し，70歳代では男性で30～40%，女性で50～60%に達する[1]（図1）．40歳以降の各年代では女性が1.5～2.5倍発症率が高くなっており，これらの点から加齢および女性は膝OAの危険因子といえる．興味深いのは40歳以下の年代では，Lawrenceら[2]は男性で5.5%，女性で3.9%，NHANES-I[3]でも35～44歳の群で男性1.75%，女性1.44%と逆に男性の発症率がわずかながら高く，比較的若年者の膝OA発症に靱帯，半月，軟骨損傷といった膝外傷が潜在的に影響している可能性を示唆する所見と考えられる．

2 人　種

これまでの報告では，欧米の白人，日本人，中国人における年代別の膝OA発生率はいずれも男女とも加齢とともに増加する（図1）．また，NHANES-I[3]では米国内の黒人は白人に比べて男性で1.4倍，女性で2.8倍膝OAに対する危険度が大きいことも示されている．近年，異なる二つの人種を同一の解析手法で比較した研究が行われ，Zhangら[4]はFramingham studyのプロトコルを用いて調査を行い，中国人女性が白人女性に比べて有意に膝OAが多いことを示した．また，Yoshidaら[5]も同様の手法で日本人女性は白人女性に比べて1.9倍膝OAの危険度が高いと述べており，今後，同様の研究がすすむに従い人種間の相違や特徴が明らかになることが期待される．

Key words：medial knee osteoarthritis, risk factor, review

* Risk factor of knee osteoarthritis

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