

# Apoptosis and p53 Expression in Chondrocytes Relate to Degeneration in Articular Cartilage of Immobilized Knee Joints

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**ABSTRACT. Objective.** We have reported that articular cartilage showed early stage degeneration at 7 and 14 days after immobilization, moderate degeneration at 28 days, and severe degeneration at 42 days in rabbits. To test whether apoptosis occurs in association with p53 expression in chondrocytes during the process of articular cartilage degeneration, we investigated the degree of cartilage degeneration, the frequency of apoptotic cells, and the levels of p53 mRNA in rabbits and mice after knee immobilization.

**Methods.** Right knees of male Japanese white rabbits were immobilized in full extension with fiberglass casts for up to 42 days. Similarly, right knees of male p53 wild-type [p53 (+/+)] and p53 null [p53 (-/-)] mice were immobilized in full extension with bandage tape for up to 84 days. Apoptotic cells were confirmed by TUNEL staining on the sections of knee joints. Total RNA of articular chondrocytes obtained from Day 0 or immobilized knees was analyzed semiquantitatively by RT-PCR using specific primers for p53.

**Results.** Articular cartilage degenerated after immobilization of p53 (+/+) mouse knees, but not after immobilization of p53 (-/-) knees. Apoptotic cells were observed in articular cartilage in the femur and tibia of rabbits and p53 (+/+) mice after immobilization. However, only a few apoptotic cells were observed at the same sites in p53 (-/-) mice. In RT-PCR analysis, the levels of p53 mRNA obtained from immobilized groups were significantly higher than those of Day 0 groups in rabbit and p53 (+/+) mouse knees.

**Conclusion.** Apoptosis and p53 expression in chondrocytes relate to degeneration in articular cartilage of immobilized knee joints. (J Rheumatol 2003;30:559-66)

*Key Indexing Terms:*

ARTICULAR CARTILAGE  
APOPTOSIS

IMMOBILIZATION

DEGENERATION  
p53

The development and maintenance of the articular cartilage structure requires an applied load of optimal magnitude and type<sup>1</sup>. It is well known that excessive loading or unloading induces articular cartilage degeneration<sup>1,2</sup>. Articular carti-

lage of the knee degenerates after immobilization in dogs<sup>2</sup> or rats<sup>3</sup>. We have reported that articular cartilage degeneration occurs after immobilization in rabbit knee joints<sup>4</sup>. The mechanism of articular cartilage degeneration is, however, still unknown.

Apoptosis is characterized by cellular shrinkage and DNA fragmentation<sup>5</sup>. Recently, it has been reported that apoptotic chondrocytes were observed in normal articular cartilage<sup>6</sup>, and that apoptosis was a critical factor contributing to the degeneration in human osteoarthritic cartilage<sup>6-8</sup>. Although immobilization of animal knee joints can induce articular cartilage degeneration<sup>4,9</sup>, it remains unknown whether chondrocytes die by apoptosis after immobilization.

p53 is widely known as a "guardian" of the cell cycle and apoptosis<sup>10,11</sup>. p53 signaling is implicated in cellular responses to a variety of insults, such as growth factor<sup>12</sup>, heat shock<sup>13</sup>, hypoxia<sup>14</sup>, and  $\gamma$ -irradiation<sup>15</sup>. p53 expression was observed in cartilage lesions of chondrosarcoma patients<sup>16</sup>, and in articular cartilage of patients with rheumatoid arthritis (RA) or osteoarthritis (OA)<sup>17</sup>.

We hypothesized that apoptosis occurs in association

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with p53 expression in chondrocytes during the process of articular cartilage degeneration. In this study, we compared the change of cartilage degeneration and apoptosis in p53 wild-type [p53 (+/+)] and p53 null [p53 (-/-)] mice after knee immobilization. Further, we investigated the apoptosis in cartilage after immobilization of rabbit and mouse knees by terminal dUTP nick-end label (TUNEL) staining. Further, we used semiquantitative reverse transcribed-polymerase chain reaction (RT-PCR) to identify the levels of p53 mRNA in articular cartilage obtained from immobilized rabbit and mouse knees.

## MATERIALS AND METHODS

**Experiment protocol.** All experimental protocols were approved by the Ethics Committee of Animal Care and Experimentation of the University of Occupational and Environmental Health.

Forty-three male Japanese white rabbits aged 6 months and weighing roughly 3 kg were used. Immobilization of rabbit knees was performed as described<sup>4,18</sup>. Briefly, the right lower extremity from the proximal thigh to the distal end of the limb was tied by fiberglass casting tape to a straight wooden splint. The right knee joint of the rabbits was securely immobilized in full extension.

Mice carrying a disrupted, nonfunctional p53 gene, p53 (-/-), were derived by homologous recombination in an embryonic stem cell line from 129/SvJ mice, as described<sup>19-21</sup>. p53 (+/+) mice were born from the parental p53 wild-type inbred strain. Fifty-three p53 (+/+) mice and forty-seven p53 (-/-) mice were used. The right lower extremity from the proximal thigh to the distal end of the limb was tied with bandage tape.

For both safranin O staining and TUNEL staining, 3 to 5 mice in each group at 0 (Day 0, baseline), 14, 28, 56, and 84 days were sacrificed. For both safranin O staining and TUNEL staining, 3 to 6 rabbits in each group at 0, 7, 14, 21, 28, and 35 days after immobilization were sacrificed. For determination of the levels of p53 mRNA, 3 to 6 rabbits in each group were used at 0, 7, 14, 21, 24, and 28 days after immobilization, and 3 to 10 mice were used at 0, 14, 28, 56, and 84 days after immobilization.

**Histological analysis.** Frontal blocks of the knee joints were excised from the weight-bearing region of the femur and the tibia. Specimens for histology were fixed with 10% formalin for one week for rabbits and for 2 days for mice and decalcified with Morse solution (20% citric acid, 45% formic acid, 1:1, v/v) at 4°C for one week for rabbits and for 24 h for mice. Specimens were embedded in paraffin.

**Safranin O staining.** Sections (4 µm thickness) were stained with safranin O. Briefly, after deparaffinization, tissues were immersed for a few seconds in Weigert's hematoxylin, which is 1% hematoxylin (Merck, Darmstadt, Germany) in 95% ethyl alcohol, and 2% iron (III) chloride (Nacalai Tesque, Kyoto, Japan) with 1 ml hydrochloric acid (Nacalai Tesque) (1:1, v/v). After washing, tissues were immersed in 0.02% fast green FCF (Wako Pure Chemical Industries Ltd., Osaka, Japan) for 5 min, 1% acetic acid for a few seconds, and 0.1% safranin O (Chroma-Gesellschaft Schmid GmbH, Münster, Germany) for 20 min. Sections were graded according to Mankin's scoring system<sup>22</sup>.

**TUNEL staining.** Apoptotic cells were confirmed by TUNEL staining. Sections of 4 µm thickness were stained with the TUNEL method using the TACS in situ apoptosis detection kit (Travigen Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. Briefly, after deparaffinization and rehydration, 20 mg/ml proteinase K solution was placed on tissues at room temperature for 15 min, optimal time for the digest. Specimens were immersed in 2% H<sub>2</sub>O<sub>2</sub> solution at room temperature for 5 min. Terminal deoxynucleotidyl transferase (TdT) labeling reaction mix was added inside a humidity chamber at 37°C for 90 min. Subsequently, tissues were exposed to stop solution, phosphate buffered saline, streptavidin-horseradish peroxidase, and diaminobenzidine working solution.

Finally, we proceeded with hematoxylin-eosin counterstaining. We included a positive control obtained by DNase treatment and sections from murine spleen after radiation with a high rate of apoptosis as a second independent control.

**Semiquantitative RT-PCR.** Semiquantitative RT-PCR was performed as described<sup>23</sup>. The articular cartilage sample was obtained from the lateral and medial condyle of the femur and tibia and was cut into small pieces with a scalpel. The pieces of cartilage tissues were analyzed separately per each rabbit and mixed together from 3 to 10 mice in each group. Total RNA was extracted by Sepasol-RNA I super (Nacalai Tesque) according to the manufacturer's instructions. Briefly, cartilage was lysed in Sepasol-RNA I super and chloroform was added. After centrifugation, isopropanol was added into the aqueous phase. After centrifugation, the precipitate was suspended in 70% ethanol, centrifuged, and dried. To remove any genomic DNA, the total RNA solution was treated with RNase-free DNase (TaKaRa, Shiga, Japan) in the presence of 20 units RNasin (Promega, Madison, WI, USA). First-strand cDNA from 0.5 mg total RNA was synthesized using the Superscript preamplification system (Gibco BRL) according to the manufacturer's instructions.

The p53 sequences of rabbit<sup>24</sup> and mouse<sup>25</sup> were identified, and the following PCR primer sets specific to a selected coding region with Genetyx-Mac 7.0.3 (Software Development, Tokyo, Japan) were used. PCR primer sets of rabbit p53 were sense 5'-AAG AAG TCA CAG CAC ATG AC-3' (nucleotides 481-500), antisense 5'-AGG TAG CTG GAG TGA GCC CT-3' (nucleotides 1081-1100). The sequence for glyceraldehyde-6-phosphate dehydrogenase (GAPDH) of rabbit was cloned<sup>26</sup>, and the PCR primer sets specific to a selected coding region with Genetyx-Mac 7.0.3 were as follows: GAPDH sense 5'-GAG ACT TTA TTG ATG GTT TC-3' (nucleotides 456-476), antisense 5'-ATG TTT GTG ATG GGC GTG AA-3' (nucleotides 1253-1273). Specific PCR primers of mouse were designed with Genetyx-Mac 7.0.3: p53 sense 5'-TAC TCT CCT CCC CTC AAT AA-3' (nucleotides 367-386), antisense: 5'-CTT GTA GTG GAT GGT GGT AT-3' (nucleotides 677-696). Specific PCR primers of mouse were designed from published sequences: β-actin<sup>27</sup> sense: 5'-CCT AAG GCC AAC CGT GAA AAG-3' (nucleotides 256-276), antisense: 5'-TCT TCA TGG TGC TAG GAG CCA-3' (nucleotides 881-901).

Amplification was performed on a PC-800 temperature control system (Astec, Fukuoka, Japan) using Ready-To-Go PCR beads (Amersham Pharmacia Biotech, Uppsala, Sweden). The cycling sequence for rabbit p53 was 95°C for 20 s, 61°C for 20 s, 72°C for 40 s for 27 cycles with an additional step for 3 min at 72°C. The cycling sequence for rabbit GAPDH was 95°C for 20 s, 50°C for 40 s, 72°C for 40 s for 35 cycles with an additional step for 3 min at 72°C. The thermal cycling sequence for mouse p53 amplification was 95°C for 20 s, 56°C for 20 s, 72°C for 40 s for 37 cycles with an additional step for 3 min at 72°C. The thermal cycling sequence for mouse β-actin was 32 cycles of the same with an additional step for 3 min at 72°C.

To verify that amplifications were in the mid-linear range of each PCR analysis, we performed PCR amplification over a range of 20-40 cycles with each amplifier set, using samples prepared from Day 0 or immobilized rabbits and mice. Bands were scanned with a Sharp JX-350 (Sharp, Osaka, Japan), quantified with NIH Image v.1.62 software (National Institutes of Health, Bethesda, MD, USA) on a Macintosh G3 computer, and normalized with GAPDH expression or β-actin, respectively.

**Statistical analysis.** Data are expressed as the mean ± SD and were evaluated by analysis of variance. If significant F values were found, Fisher's test for multiple comparisons was performed. A p < 0.05 was considered significant.

## RESULTS

**Safranin O staining.** In p53 (+/+) mice, the specimens in the Day 0 group showed a normal view of the articular cartilage, with a smooth joint surface, regular cartilage cell columns, and good staining of the matrix metachromasia (Figure 1A). Loss of metachromasia was found in the specimens 14 days

after immobilization (Figure 1B). In specimens taken 28 days after immobilization, the loss of metachromasia, irregularity of cell columns, cluster formation, and fissures in the cartilage were evident (Figure 1C). Mankin grade increased markedly from 2 points at 14 days to 9 points at 28 days after immobilization (Table 1). Focal complete loss of cells and cloning of others without loss of the cartilage layer appeared, and cluster formation of cartilage cells progressed 56 and 84 days after immobilization (Figure 1D). Nuclear pyknosis, a morphologic sign of cell death and therefore also of apoptosis, was found, along with a decrease in cartilage cell number (Figure 1D). These findings were observed in both tibia and femur.

In p53 (-/-) mice, the articular cartilage specimens had a normal appearance from 0 to 84 days after immobilization, similar to the specimens from p53 (+/+) mice at Day 0. Articular cartilage did not appear to degenerate the entire 84 day period of immobilization (Figures 1E, 1F).

**TUNEL assay.** In rabbit femur and tibia, TUNEL positive cells were observed in articular cartilage 21 days after immobilization. The number of TUNEL positive cells in the superficial layer of articular cartilage was increased compared to the middle to deep layer (Figures 2A, 2B). TUNEL positive cells increased from 21 days after immobilization (Table 1). There were no significant differences in the number of TUNEL positive cells between the immobilized groups at 7 ( $5.29 \pm 1.77\%$ ) or 14 days ( $4.50 \pm 1.34\%$ ) after immobilization and the Day 0 group ( $1.22 \pm 0.22\%$ ). At 21, 28, and 35 days after immobilization, TUNEL stained cells were significantly ( $p < 0.05$ ) increased compared to the Day 0 group. The values in the immobilization group were  $13.5 \pm 2.5\%$ ,  $9.7 \pm 2.5\%$ , and  $10.2 \pm 3.5\%$ , respectively; these percentages were 11.1, 8.0, and 8.4 times greater than the Day 0 group, respectively. There were no significant

differences among the values 21, 28, and 35 days after immobilization (Figure 3A).

The finding of TUNEL positive cells did not differ between p53 (+/+) and p53 (-/-) mice at Day 0, indicating the equal status of both p53 (+/+) and p53 (-/-) mice. In p53 (+/+) mice, TUNEL positive cells were observed in articular cartilage (of both femur and tibia) from 14 days after immobilization (Figure 2C, Table 1). The image shows the loaded region, where the TUNEL positive cells were concentrated. We counted cells above and below the tidemark, and from central to marginal region within the articular cartilage. However, in p53 (-/-) mice, only a few cells were stained by the TUNEL method in articular cartilage after immobilization (Figure 2D). Thus, after immobilization, the number of TUNEL positive cells increased markedly in p53 (+/+) mice, but not significantly in p53 (-/-) mice (Figure 3B). The percentage of TUNEL positive cells ( $\pm$  SD) in p53 (+/+) mice increased from  $3.1 \pm 0.7\%$  at 0 days to  $5.4 \pm 1.4\%$  at 14 days,  $14.6 \pm 3.6\%$  at 28 days, and  $16.1 \pm 2.9\%$  at 56 days, and decreased thereafter to  $12.2 \pm 0.6\%$  at 84 days. For p53 (-/-) mice, the percentage of apoptotic cells ( $\pm$  SD) for the same immobilization periods was  $1.2 \pm 0.6\%$ ,  $1.5 \pm 0.7\%$ ,  $2.1 \pm 0.4\%$ ,  $3.0 \pm 0.9\%$ , and  $4.4 \pm 1.4\%$ , respectively (Figure 3B).

**RT-PCR analysis.** The levels of p53 mRNA in rabbit chondrocytes 7 and 14 days after immobilization were found to be similar to the level of p53 mRNA in chondrocytes from the Day 0 group. However, the levels of p53 mRNA in chondrocytes obtained from the immobilized groups at later stages of the procedure (21, 24, and 28 days) were significantly ( $p < 0.01$ ) higher than those of the Day 0 group. In particular, the levels of p53 mRNA in rabbit chondrocytes peaked at 21 days after immobilization. The levels of p53 mRNA (with significant differences of  $p < 0.001$ ) were

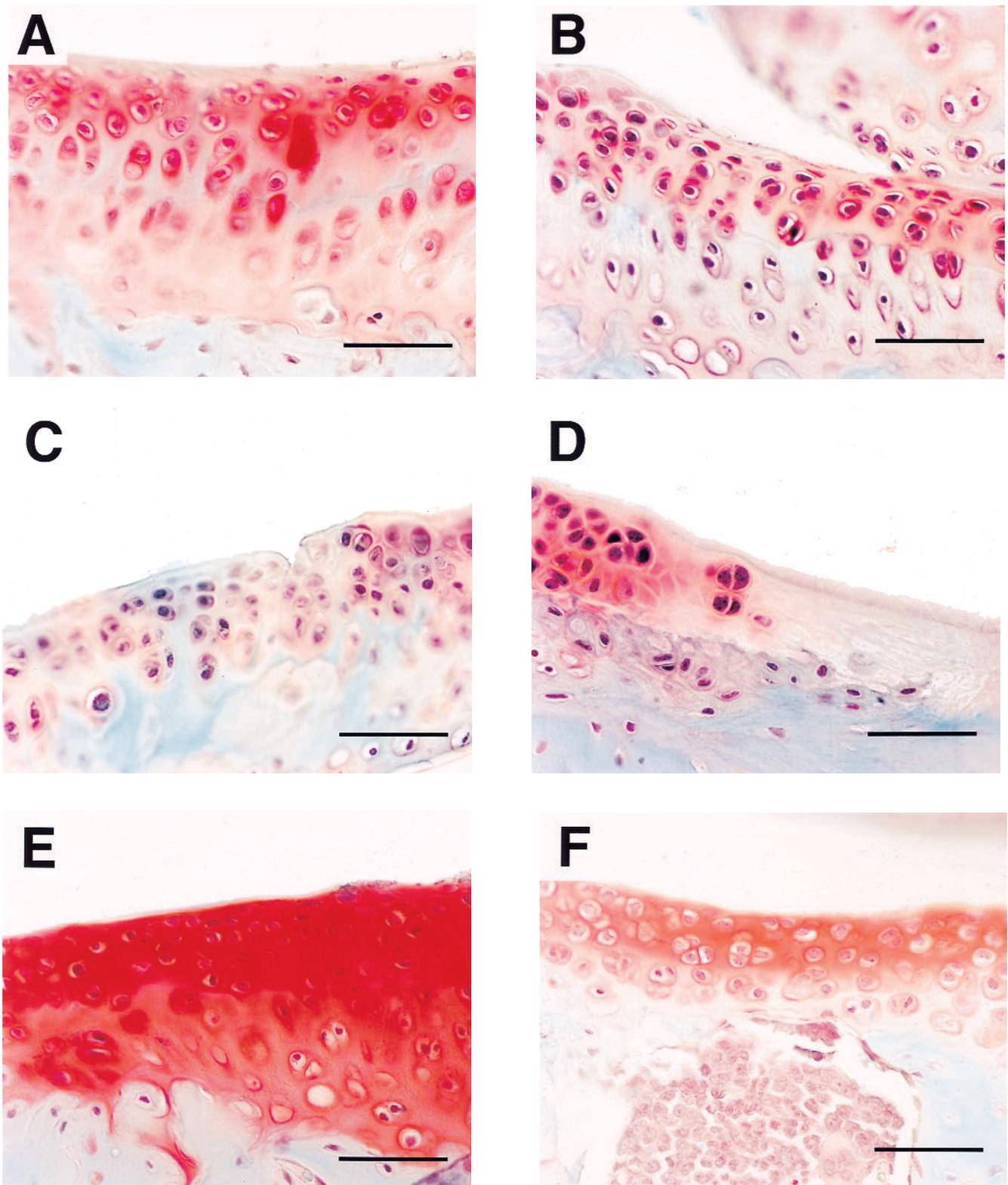
Table 1. Changes of histological findings, frequency of apoptotic cells, and p53 expression in articular cartilage after immobilization.

Rabbit	Immobilization time (days)					
	0	7	14	21	28	35
Histological findings	Normal	Loss of metachromasia Undulated surface	Loss of metachromasia Undulated surface	Loss of metachromasia Undulated surface	Irregularity of cell columns Cell clusters Fissuring	Irregularity of cell columns Cell clusters Fissuring
Mankin grade	0	2	2	4	9	10
Apoptosis	No increase	No increase	No increase	Increase*	Increase*	Increase*
p53 expression	No increase	No increase	No increase	Increase*	Increase*	No data

p53 (+/+) mouse	Immobilization time (days)				
	0	14	28	56	84
Histological findings	Normal	Loss of metachromasia Undulated surface	Irregularity of cell columns Cell clusters Fissuring	Focal complete loss of cells Cell clusters Fissuring	Focal complete loss of cells Cell clusters Fissuring
Mankin grade	0	2	9	11	11
Apoptosis	No increase	No increase	Increase*	Increase*	Increase*
p53 expression	No increase	Increase*	Increase*	No increase	No increase

\*Compared to day 0 value.



*Figure 1.* Histological changes in articular cartilage obtained from p53 (+/+) and p53 (-/-) mouse knees after immobilization. All sections were obtained from the loaded regions of tibias. Safranin O staining. (A) Day 0 in p53 (+/+) mice. (B) 14 days after immobilization in p53 (+/+) mice. (C) 28 days after immobilization in p53 (+/+) mice. (D) 84 days after immobilization in p53 (+/+) mice. (E) Day 0 in p53 (-/-) mice. (F) 84 days after immobilization in p53 (-/-) mice. Scale bar = 50  $\mu$ m.

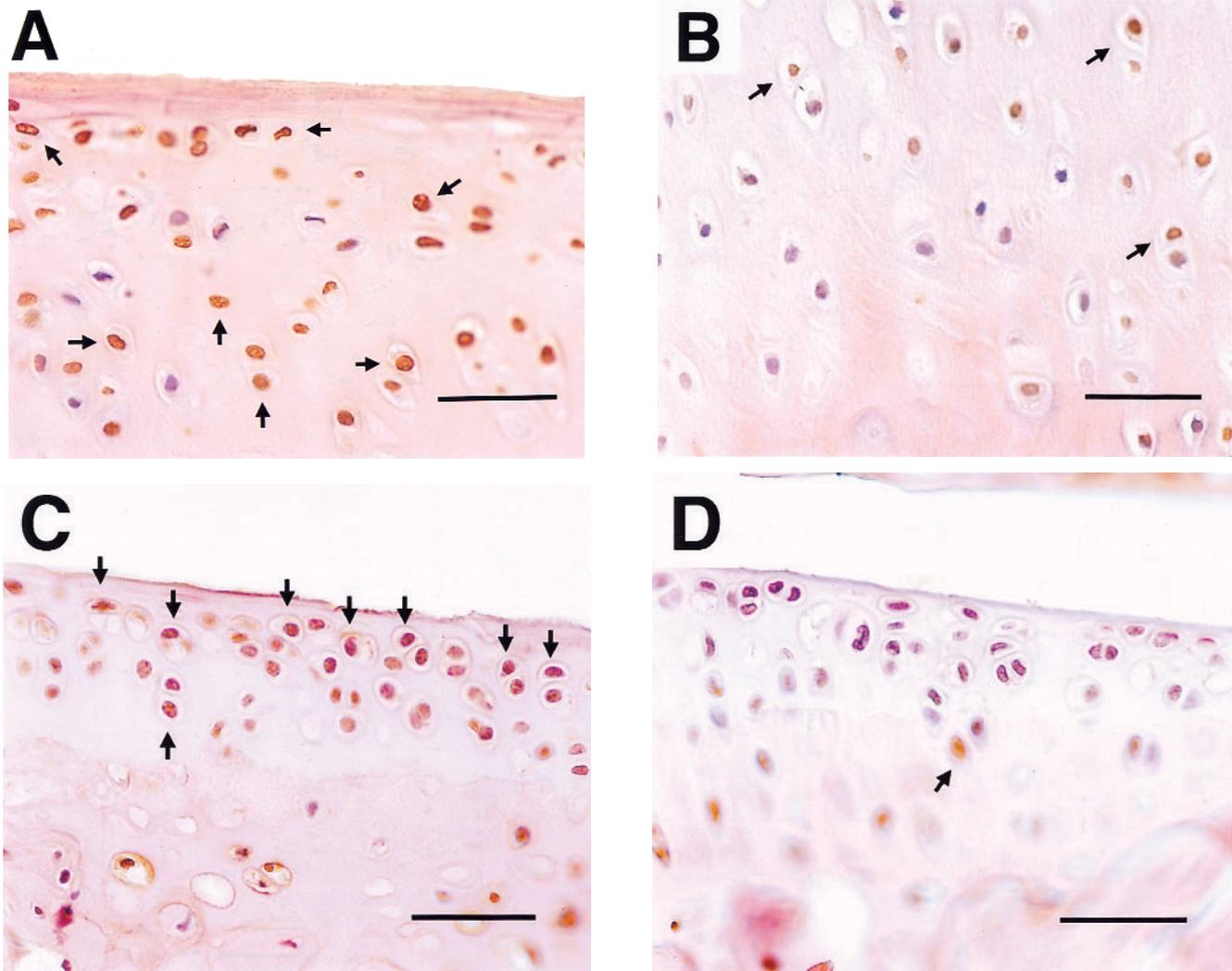


Figure 2. Apoptotic cells in articular cartilage 28 days after immobilization visualized by labeling DNA with the TUNEL method. (A) Superficial layer in rabbit. (B) Middle to deep layers in rabbit. (C) p53 (+/+) mice. (D) p53 (-/-) mice. Arrows show representative apoptotic cells. Scale bar = 50  $\mu$ m.

found to be decreased at Days 24 and 28 after immobilization compared to levels of p53 mRNA at 21 days after immobilization, by roughly 50% (Figure 4A, Table 1).

The levels of p53 mRNA obtained from mouse knee joints at 14 and 28 days after immobilization were higher than those of the Day 0 group: the number of arbitrary units were 1.89 and 2.20 times that of the Day 0 group. However, the levels of p53 mRNA were no longer increased by 56 and 84 days after immobilization (Figure 4B, Table 1), but in fact were declining.

## DISCUSSION

We have previously reported that, in rabbit knee joints, loss of metachromasia was shown in articular cartilage 7 days after immobilization, fissuring and cell clusters were revealed 28 days after immobilization, and ulceration of joint surfaces appeared and chondrocyte numbers decreased 42 days after immobilization<sup>4</sup>. In this study, we showed that

the course of cartilage degeneration in p53 (+/+) mice was similar to that in rabbits. It has been reported that in mouse knee joints immobilization for 7 days also led to increased damage to the articular cartilage<sup>28</sup>, and chondrocyte death and matrix depletion were observed from 3 to 5 days after immobilization<sup>29</sup>. Reduced joint loading after immobilization does not maintain normal articular cartilage in dogs<sup>1,2</sup>, since both hyaluronan and aggrecan in the articular cartilage decreases<sup>2</sup>. We previously observed, in synovial fluid of rabbit knee joints, that the concentration of both transforming growth factor- $\beta$  and basic fibroblast growth factor changed after immobilization<sup>18</sup>. Immobilization caused the unusual change in articular cartilage and synovial fluid. Prolonged immobilization may reduce mechanical stress on the cartilage and aggravate articular cartilage degeneration.

The degenerative matrix changes in safranin O staining were seen at Day 7 in rabbits and at Day 14 in p53 (+/+) mice, while significant differences were found in the

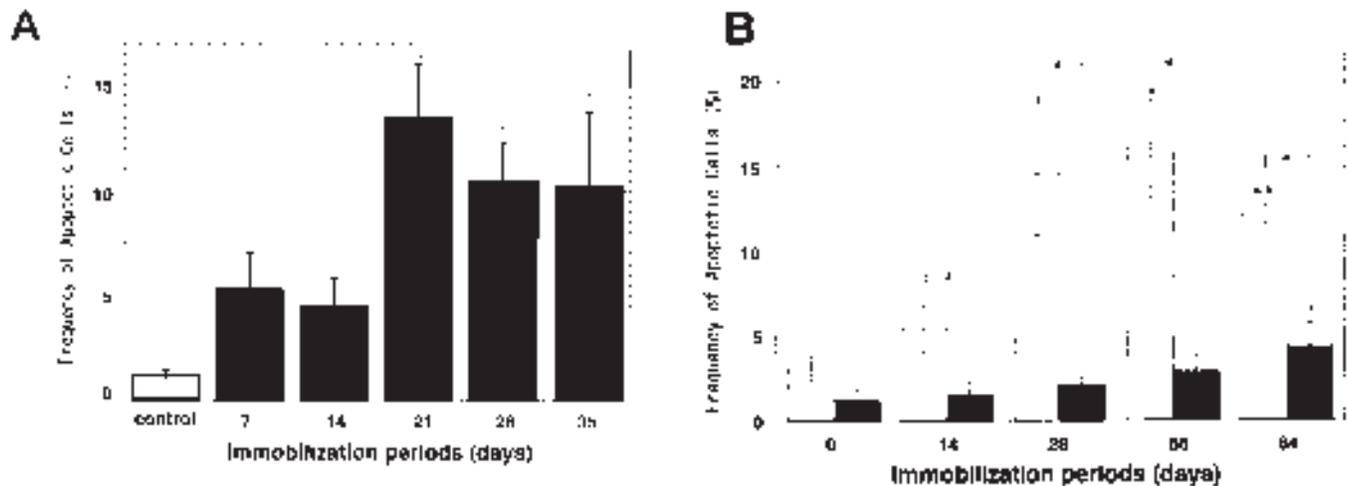


Figure 3. Frequency of apoptotic cells in articular chondrocytes after immobilization in rabbits, and in p53 (+/+) and p53 (-/-) mice. Data (in percentages) are mean  $\pm$  1 SD. (A) At least 3 rabbits in each group were sacrificed. \* $p < 0.05$ , Fisher F test. (B) At least 3 mice in each group were sacrificed. a: Value compared with the Day 0 group of p53 (+/+) mice,  $p < 0.001$ . b: Value at 56 days compared with 84 days after immobilization,  $p < 0.01$ . c: Value compared with Day 0 group of p53 (-/-) mice,  $p < 0.05$ . d: Value compared with p53 (-/-) mice,  $p < 0.05$ , at each time point. Fisher's test for multiple comparisons was performed.

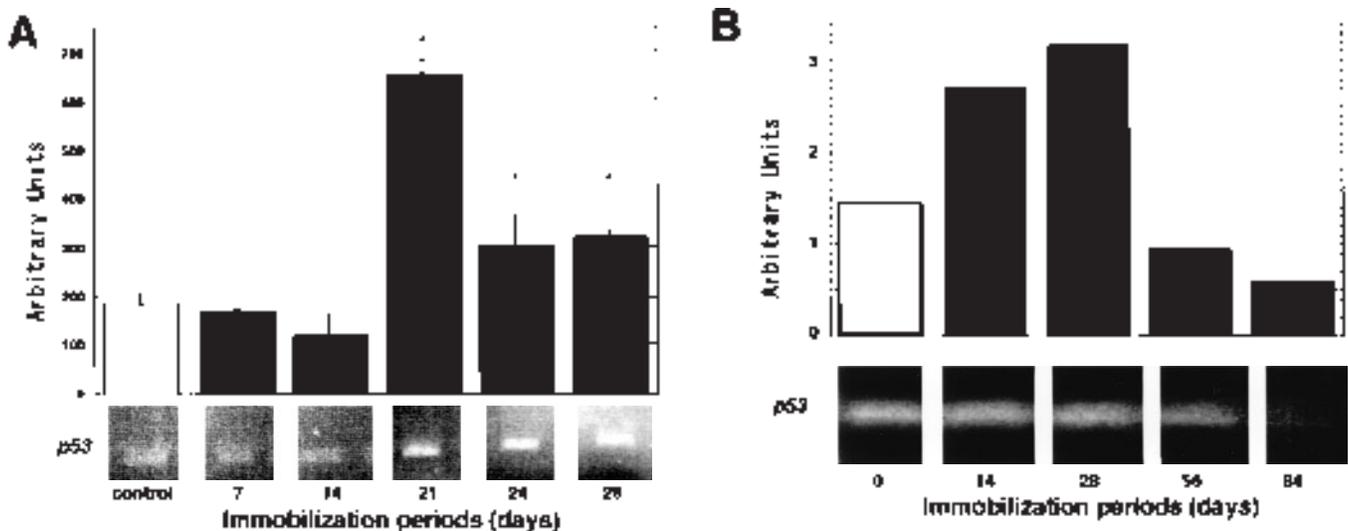


Figure 4. Levels of mRNA for p53 from the articular cartilage of rabbit and mouse knees after immobilization for the indicated periods. (A) RT-PCR analysis of p53 expression in rabbit chondrocytes obtained from 0 to 28 days after immobilization. Densitometric scanning of signals of p53 expression was normalized to GAPDH. At least 3 rabbits in each group were used. Data show mean  $\pm$  1 SD. \* $p < 0.01$ , Fisher F test. Similar results were obtained in at least 3 independent experiments, and representative bands for p53 are shown. (B) RT-PCR analysis of p53 expression in p53 (+/+) mouse chondrocytes obtained from 0 to 84 days after immobilization. Densitometric scanning of signals representing p53 expression was normalized to  $\beta$ -actin. At least 3 mice in each group were used. All pieces of cartilage tissue from each group were pooled. Similar results were obtained in at least 3 independent experiments; a representative band for p53 is shown.

number of apoptotic chondrocytes in rabbits at 21 days and in p53 (+/+) mice 28 days after immobilization, and found in the amount of detectable p53 expression in rabbits at 21 days and in p53 (+/+) mice 14 days after immobilization. Matrix degeneration preceded the cellular abnormalities. On the other hand, the frequency of apoptotic cells and p53 expression significantly increased during the development

of cartilage degeneration. In p53 (-/-) mice, the frequency of apoptotic cells was lower compared with that in p53 (+/+) mice and matrix degeneration was not experienced. From these observations, we consider that apoptosis in chondrocytes cannot be a substantial initiator of matrix degeneration, but apoptosis follows the initial reaction to injury (fissure formation, matrix proteoglycan depletion) just as

cell clusters at 28 days (an indicator of chondrocyte proliferation) follow chronologically the peak of apoptosis. Thus, we consider that cartilage degeneration is associated with apoptosis and p53 overexpression.

We demonstrated by safranin O staining that articular cartilage degeneration after immobilization is different in p53 (+/+) than in p53 (-/-) mice; cartilage degenerated in p53 (+/+) mice, but not in p53 (-/-) mice. To our knowledge, this is the first description of cartilage degeneration after immobilization of p53 (+/+) and p53 (-/-) mouse knees. In this study, chondrocytes in p53 (-/-) mice resisted degeneration caused by immobilization, and retained their phenotypes. An interpretation of the protection against apoptosis of p53 (-/-) mice might be that chondrocytes have a defective intracellular signal that inhibits cells from entering cell cycle arrest or apoptotic pathways. p53 may play a critical role in setting or determining the finite lifespan of chondrocytes<sup>30</sup>. Since apoptosis follows loss of metachromasia in cartilage of p53 (+/+) mice, apoptosis could not entirely account for no matrix degeneration after immobilization in p53 (-/-) mice. There is one possibility — that cartilage matrix of p53 (-/-) mice resists initial matrix degeneration caused by immobilization.

In this study, we showed that apoptotic rabbit chondrocytes were significantly increased 21 days after immobilization at the joint surface regions. We previously reported that bone cells decreased with immobilization after sciatic neurectomy in mice<sup>31,32</sup> and that skeletal unloading significantly increased the percentage of hypoploid bone marrow cells, which are considered to reflect predominantly apoptotic cells, relative to normally loaded mice<sup>21</sup>. The basic mechanism underlying the promotion of apoptosis remains to be identified. Within the context of the “use it or lose it” concept of skeletal homeostasis, the development of apoptosis may be a natural and appropriate response in the immobilized joint. Apoptosis is a physiological phenomenon that occurs during embryonic development, cell maturation, and endocrine induced atrophy. In the neural tubes of embryos, few apoptotic cells were observed in p53 (-/-) mice after X-irradiation; on the other hand, apoptotic cells were increased in p53 (+/+) mice<sup>20</sup>. Apoptosis can be initiated by various events and is thought to be related to p53 expression. After immobilization, chondrocytes may have an efficient system for detecting DNA damage induced by lack of physiological mechanical stress and for transducing the mechanical signal into promotion of apoptosis.

From these results, we considered how apoptosis fits into the overall scheme of cartilage homeostasis. Limb immobilization stems synovial fluid flow in the knee joint and causes a malnutritional microenvironment for articular cartilage. Cartilage matrices, including type II collagen, proteoglycan, and hyaluronan, are not well maintained and initiate degeneration. Apoptosis of chondrocytes follows the initial reaction to matrix degeneration just as cell clusters follow

the peak of apoptosis. Focal complete loss of chondrocytes due to apoptosis accelerates matrix degeneration. The frequency of apoptotic cells and p53 expression significantly increase during the development of cartilage degeneration. Our results are consistent with the report that apoptosis of articular chondrocytes in RA and OA correlates with degree of cartilage destruction and expression of apoptosis related proteins of p53 and c-myc<sup>17</sup>. There are few chondrocytes detected by the *in situ* nick-end labeling (ISNEL) method in normal articular cartilage, but many chondrocytes detected by ISNEL exist in the degenerated cartilage of the superficial layer with highly expressed proteins of p53 and c-myc. Takahashi, *et al*<sup>33</sup> reported that hyaluronan protects against chondrocyte apoptosis during the development of experimental OA, and these inhibitory effects of hyaluronan on chondrocyte apoptosis may play a role in its mechanism of action in chondroprotection. We can consider that protection against chondrocyte apoptosis contributes to preservation of matrix.

We have shown that p53 mRNA in articular chondrocytes was increased following immobilization of the knee joint in both rabbits and p53 (+/+) mice. In rabbits, the levels of p53 peaked at 21 days, and then declined from 28 days after immobilization. The percentages of apoptotic cells were significantly higher at 21 days compared to levels at up to 14 days after immobilization. In p53 (+/+) mice, the levels of p53 peaked at 28 days, and then declined from 28 days after immobilization. The percentages of apoptotic cells were significantly higher 28 days after immobilization compared to the Day 0 group. Lu, *et al*<sup>34</sup> have reported that, in epidermal cells of hairless SHK-1 mice, the number of p53 positive cells after ultraviolet B (UVB) light exposure gradually increased, reached peak levels, and then decreased markedly. Although after UVB exposure the increase in p53 positive cell numbers preceded that of apoptotic cells, the time course of both increases was almost parallel. Similar changes in p53 protein expression have been reported after a variety of cellular insults, such as cerebral ischemia in rats<sup>35</sup> and  $\gamma$ -irradiation of a human lymphoblastoid cell line<sup>36</sup>. We previously reported that the levels of p53 mRNA of bone marrow cells is enhanced 7 days after skeletal unloading<sup>21</sup>. We consider that limited motion with splint immobilization facilitates signaling of p53 in articular chondrocytes, and that the subsequent increase of apoptotic chondrocytes may depend on p53. However, this study has limited information about p53 protein in terms of function and about potential colocalization of p53 signals and TUNEL positive cells.

We conclude that apoptosis and p53 expression in chondrocytes relate to degeneration in articular cartilage of immobilized knee joints. These results are not immediately applicable to all other forms and models of cartilage degeneration, but regulation of the p53 signal might permit prevention of cartilage degeneration.

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