Apoptosis of Chondrocytes
and Its Regulation
in the Pathogenesis of Osteoarthritis

Thesis by
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APOPTOSIS OF CHONDROCYTES
AND ITS REGULATION
IN THE PATHOGENESIS OF OSTEOARTHRITIS

Directed by Professor Jun-Seop Jahng

A dissertation submitted to the Faculty of
the Graduate School of the Yonsei University

December 31, 1999

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December 31, 1999
Acknowledgments

There are three classes of human beings: men, women, and women surgeons.
Some women are born to be surgeon.

I am very pleased to acknowledge my indebtedness to friends, colleagues, and professors who have been so good as to discuss with me many of the views expressed in this work for my PhD degree. Remarkably I should like to avail myself of this opportunity of expressing special thanks to Professor Jun-Seop Jahng, MD, PhD; Professor Eung-Shick Kang, MD, PhD; Professor Sung-Jae Kim, MD, PhD; Associate Professor Jeon Han Park, MD, PhD; and Associate Professor Byung-Seok Lee, MD, PhD.

I also would like to acknowledge the influence of the following people for their unique and individual contribution to my research and especially for their friendship. Chan Hee Lee, MD, PhD; Hyun Young Park, MD; Chang Hee Suh, MD, PhD; Jin Sung Lee, MD, PhD; Kyung Sup Kim, MD, PhD; Ha-Il Kim, MD; Suk Hyun Kim, MD; Martin Lotz, MD, PhD and Sanshiro Hashimoto, MD, PhD in the Scripps Institute; Daeho Kwon, MSc; Young Mee Kim, MSc; Yujo Jeong Choi; Jae Myun Lee, MD, PhD; Chul Hoon Kim, MD; Hoguen Kim, MD, PhD; Lucia Kim, MD; Jae Ho Han, MD; Jun Shik Kim in MBL Co.; Young Min Song in the Department of Pathology; Dong Eun Shin, MD in Pundang Cha Hospital; Kyung Jin Han, MD in Ajou University; Professor Nam Jin Yoo, MD, PhD in Catholic University; In Hong Choi, MD, PhD; and Se Jong Kim, MD, PhD. They provided me with kind advice, technical assistance, cheerful motivation, and valuable criticism. Particularly I would like to express great appreciation to Eui-Cheol Shin, MD for his extraordinary contribution and faithful friendship to my work from its beginning to completion.

Most importantly I would like to acknowledge the unconditioned love and support of my parents, Su Kyung Rho and Kuk Kyung Kim, and my sister, Jiyeon. Without them, this work would not have been possible. It is to my family whom I dedicate this work.

Kim HJ
CONTENTS

Abstract in English .................................................................................................................. 1
I. Introduction .......................................................................................................................... 3
II. Materials and Methods ...................................................................................................... 7
   1. Sources of articular cartilage ......................................................................................... 7
   2. TUNEL staining .............................................................................................................. 7
   3. RNA isolation .................................................................................................................. 8
   4. Synthesis of cDNA .......................................................................................................... 8
   5. Multiplex RT-PCR for apoptosis-related genes .......................................................... 8
   6. Semiquantitative RT-PCR for FasL, IL-18, and TRAIL-receptors .......................... 9
   7. Immunohistochemical staining for FasL ...................................................................... 10
III. Results ............................................................................................................................... 12
    1. TUNEL staining articular cartilage ............................................................................. 12
    2. mRNA expression of various apoptosis-related genes ............................................. 12
    3. mRNA expression of FasL and IL-18 ....................................................................... 15
    4. mRNA expression of TRAIL-receptors .................................................................... 15
    5. Expression of FasL in immunohistochemical staining ............................................. 16
IV. Discussion ......................................................................................................................... 19
V. Conclusion .......................................................................................................................... 23
References ............................................................................................................................... 24
Abstract in Korean .................................................................................................................. 26
LIST OF TABLES

Table 1. Apoptotic index in articular cartilage with TUNEL staining .................................. 12
Table 2. mRNA expression of apoptosis-related genes in articular cartilage ......................... 14
Table 3. FasL immunoreactivity in articular cartilage ......................................................... 16
Table 4. Correlation between mRNA and protein expression of FasL ............................... 17
LIST OF FIGURES

Figure 1. TUNEL staining in articular cartilage ........................................... 18
Figure 2. Multiplex RT-PCR for bcl-2 family genes ...................................... 13
Figure 3. Multiplex RT-PCR for Fas-related genes ........................................ 13
Figure 4. Semiquantitative RT-PCR for FasL and IL-18 ................................. 13
Figure 5. Semiquantitative RT-PCR for TRAIL-receptors .............................. 13
Figure 6. Immunohistochemical staining for FasL in articular cartilage .......... 18
ABSTRACT

Apoptosis of Chondrocytes and Its Regulation in the Pathogenesis of Osteoarthritis

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(Directed by Professor Jun-Seop Jahng)

Osteoarthritis (OA) is a disease marked by the gradual destruction of articular cartilage, accompanied by secondary change of synovium and subchondral bone. However the etiology and pathogenesis of the disease is not well understood. Recently it has been determined that apoptosis—which is a type of cell death characterized by distinct morphologic features—is involved in the development and progression of numerous degenerative diseases. It has also been reported that apoptosis is associated with the pathogenesis of OA.

The present study aimed to demonstrate whether or not apoptosis occurred in chondrocytes of OA cartilage, and to determine which molecular mechanism and apoptotic pathway was involved in the apoptosis of chondrocytes in OA cartilage. For this study, OA cartilages were obtained from total knee arthroplasty and non-OA control cartilages from traumatic above-knee amputation. In TUNEL staining using fluorescein-dUTP to confirm whether or not chondrocytes of OA cartilage undergo an apoptotic process, 6 of 7 OA cartilages showed fluorescein-dUTP-tagged chondrocytes (apoptotic index: 5~30%). In contrast, all 4 non-OA control cartilages showed no dUTP tagged chondrocytes. To determine which regulatory molecules of apoptosis might work in OA cartilage, the transcript of bcl-2 family genes, caspase members, Fas-related genes, TRAIL and its receptors, and interleukin-18 (IL-18) were evaluated by RT-PCR. Furthermore to determine the expression of FasL in OA cartilage at the protein level, immunohistochemical staining for FasL was performed. Among bcl-2 family genes and caspase members, OA cartilage
demonstrated slightly higher levels of caspase-3 and caspase-8 mRNA expression than in non-OA control cartilage, while the bcl-2 family genes—bcl-2, bcl-xL, bax, and bcl-xS—were expressed with low levels in overall OA cartilage and non-OA control cartilage without significant differences. Meanwhile, similarly strong expression of Fas transcripts was shown in overall OA and non-OA cartilage, with a slight increase in OA cartilage. No or weak expression of FasL transcripts was shown in non-OA control cartilage, whereas highly increased expression of FasL transcripts was shown in OA cartilage. Immunohistochemical study also confirmed the high expression of FasL in OA cartilage at the protein level, in correlation with the expression level of FasL transcript. IL-18, known as a strong inducer of FasL and also known to be expressed in articular cartilage, was investigated to determine the correlation between the expression of FasL and IL-18. Although the expression of IL-18 transcripts did not show a clear correlation with the expression of FasL, the cartilage expressing the higher level of FasL also expressed the higher level of IL-18. Another death-inducing ligand, TRAIL, showed generally moderate expression in overall OA cartilage and non-OA control cartilage without definite differences. Among TRAIL-receptors, DR-4 was weakly expressed in overall OA and non-OA control cartilage, and DR-5 was expressed with moderate levels in some OA cartilages and with overall weak levels in other OA and non-OA cartilages. TRAIL-receptors which decoy the death signal, DcR1 and DcR2, were highly expressed in overall OA and non-OA. Although the role of the TRAIL/TRAIL-receptor pathway and bcl-2 families could not be completely ruled out, these results suggested that the Fas/FasL pathway plays a significant role in chondrocyte apoptosis of OA cartilage, and that FasL expression was probably induced by IL-18 activation.

This study documents for the first time that FasL could be expressed in chondrocytes of articular cartilage, with a particularly high level in OA cartilage. Understanding of the role of Fas/FasL in regulating chondrocyte apoptosis may contribute in developing a more rational therapeutic strategy of OA in clinical application to stop the initiation or progression of the disease condition.

Key Words: chondrocyte, apoptosis, osteoarthritis, Fas ligand, interleukin-18
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I. INTRODUCTION

Osteoarthritis (OA), also referred to as degenerative arthritis or degenerative joint disease, is one of the commonest causes of disability in old age and has a huge impact on hospital costs (Messner and Guillquist, 1996). Orthopaedic surgeons are faced with increasing numbers of OA patients as a result of overall population aging and increasing physical activity at all ages (Jackson, 1996). Clinically, OA shows recurring episodes of pain, synovitis with effusion, occasional stiffness, and progressive deformity; while roentgenographically it shows narrowing of the joint interval, increased density and thickening of the subchondral bone, subchondral cyst, and marginal bony excrescence (Turek, 1984). Pathologically it is characterized by degeneration of articular cartilage, subchondral bone thickening (sclerosis), marginal osteochondral outgrowth (osteophyte), and joint deformity. Basically, the “degeneration”—or the progressive loss of normal structure and function—of articular cartilage is the primary and main event in OA, and consequently leads to the second change of synovium and subchondral bone. This distinguishes OA from inflammatory arthropathies in which cartilage degradation occurs as a secondary response after synovial inflammation (Hashimoto et al, 1998a).
Regardless of the high prevalence, the etiology and pathogenesis of OA are not well understood. Some epidemiologic studies have reported a strong association between aging and OA (Praemer, 1992). However, the changes observed in articular cartilage from older individuals differ from those observed in articular cartilage from OA patients, and moreover, normal life-long joint use has not been shown to cause degeneration (Martin et al, 1997). Thus, OA is not caused simply by aging and mechanical wear from joint use (Buckwalter and Mankin, 1997).

To investigate the pathogenesis of OA, many studies have focused on the enzymatic degradation of “matrix” during cartilage destruction in OA, as well as in numerous cytokines and free radicals which are entwined in the process of matrix degradation (Amin et al, 1995; Hashimoto et al, 1997; Hashimoto et al, 1998b; Leistad wt al, 1998; Moos et al, 1999; Studer et al, 1999; van den Berg, 1999). Articular “chondrocyte”, the only cell type in articular cartilage, is the subject to control turn-over of matrix components and has recently come to the attention of researchers for its role in OA pathogenesis. One of the characteristics of articular cartilage in the early stage of OA is the apparent attempt of tissue remodeling, such as an increase in synthetic activity of chondrocytes and in concentration of proteoglycans. As the disease progresses to the chronic phase, the reparative attempt ultimately fails as cartilage becomes degraded. Eventually, the cartilage reveals a decrease in thickness and cellularity (Buckwalter and Mankin, 1997). Based on this finding of “hypocellularity” during the OA process, several studies have reported that apoptosis of articular chondrocytes occurs and increases in OA cartilage, and that it may play a significant role in the destruction of cartilage seen in OA. (Blanco et al, 1998; Hashimoto et al, 1998a). However, the underlying mechanism or the molecules responsible for triggering the apoptosis in OA chondrocytes is still under investigation.

Apoptosis is a type of cell death that shows distinct morphological characteristics: chromatin condensation and fragmentation as the cell shrinks, membrane bleb, and ‘apoptototic bodies’ (Kerr et al, 1994; Hale et al, 1996). Apoptosis physiologically plays a classic role in eliminating unwanted cells such as aged cells, damaged cells, and overgrown cells in tissues and organs during development and immune responses. Conversely, dysregulation of apoptosis may play a part in the etiology of cancer, autoimmune diseases,
and degenerative CNS disorders. There are a variety of signals which initiate apoptosis: elimination of growth factors, ultraviolet or γ-irradiation, exposure to noxious chemicals or chemotherapeutic drugs, and activation of death receptors (Carson and Ribeiro, 1993).

Death receptors—cell surface receptors that transmit apoptotic signals—play a central role in instructive apoptosis (Ashkenazi and Dixit, 1998). They belong to the tumor necrosis factor (TNF) receptor gene superfamily and contain a homologous cytoplasmic sequence termed the “death domain”. The best characterized death receptor is Fas (also called CD95 or APO-1) and it binds to Fas ligand (FasL, also called CD95L or APO-1L). Fas and FasL play an important role mainly in peripheral deletion of activated mature T cells at the end of an immune response and in killing of targets such as virus-infected cells or cancer cells by cytotoxic T cells or natural killer cells (Nagata, 1997). FasL binding leads to Fas homotrimerization, with consequent clustering of death domains. Since death domains have a propensity to associate with one another, Fas ligation leads to clustering of the receptors’ death domain. Another death receptors, DR-4 (also called TRAIL-R1) and DR-5 (also called TRAIL-R2), trigger apoptosis as well by engaging with another apoptosis-inducing ligand, TRAIL (TNF-related apoptosis-inducing ligand, also called APO-2L) (Wiley et al, 1995; Pan et al, 1997b). Interestingly, TRAIL has another receptors, which interfering death signal, and they are called decoy receptors (DcRs): DcR1 (also called TRAIL-R3) lacks a cytoplasmic tail and DcR2 (also called TRAIL-R4) has a substantially truncated cytoplasmic death domain (Pan et al, 1997a; Sheridan et al, 1997; Degli-Esposti et al, 1997). Eventually, if these death receptors are trimerized by ligand binding, the death domain recruits adaptor molecules such as FADD, and caspase-8 (FLICE) are activated. Then the “caspase cascade”, consequent and parallel activation of caspases, is initiated and leads to executional caspase activation, such as caspase-3, which degrades death substrates and eventually disassembles cells.

The mitochondria, another important player in the apoptotic pathway, contributes to caspase activation by releasing cytochrome c into cytoplasm. When cytochrome c is released into cytoplasm, it is associated with Apaf-1 and caspase-9 (Apaf-3). Caspase-9 is then activated, and consequently, the caspase cascade is initiated. This mitochondrial activity can be regulated by members of the Bcl-2 family: anti-apoptotic proteins such as Bcl-
2 and Bcl-xL promote cell survival, while pro-apoptotic proteins such as Bax, Bad, and Bcl-xS induce apoptosis (Reed 1994; Korsmeyer 1995).

The present study aimed to elucidate whether or not the apoptosis of articular chondrocytes undergo and increase in OA cartilage, and if so, which molecular mechanism and apoptotic pathway are involved in the apoptosis of articular chondrocytes in OA. Apoptotic chondrocytes in articular cartilage were determined by TUNEL staining with fluorescein-dUTP. Evaluation of the various apoptosis-related genes expression—bcl-2 family genes, caspase members, Fas-related genes, TRAIL and its receptors, and IL-18—was done by RT-PCR. Similarly strong expression of Fas transcripts was shown in overall OA and non-OA cartilage, with a slight increase in OA cartilage. No or weak expression of FasL transcripts was shown in non-OA control cartilage, whereas highly increased expression of FasL transcripts was shown in OA cartilage. Immunohistochemical study also confirmed the high expression of FasL in OA cartilage at the protein level, in correlation with the expression level of FasL transcripts. These results suggest that the Fas/FasL pathway might play a significant role in inducing apoptosis of chondrocytes of OA cartilage.
II. MATERIALS AND METHODS

1. Sources of articular cartilage

Overall 10 specimens of OA cartilage obtained from total knee replacement surgery patients (ages 52-78) were included in this study. Non-OA control cartilages were obtained from 5 donors without known history of joint diseases at the time of traumatic above-knee amputation surgery (ages 44-70). Specimens were immediately washed in ice-cold phosphate-buffered saline (PBS) after removing the synovial tissues, and then cut into small pieces from the femoral condyles and tibial plateaus. One piece of cartilage was immediately preserved in 4% paraformaldehyde for TUNEL and immunohistochemical staining, and the other pieces were quickly minced into thin slices and transferred into a TRIzol reagent (Gibco BRL, Grand Island, NY, USA) for RNA isolation.

2. TUNEL staining

To determine whether apoptosis of chondrocytes occurs and increases in OA cartilage, TUNEL staining was performed in 11 cartilages, 7 OA cartilages and 4 non-OA control cartilages, using the in situ cell death detection kit (MBL, Nagoya, Japan) according to the manufacturer's instructions. Briefly, the cartilage tissue was immediately fixed in 4% paraformaldehyde. After embedding in paraffin, cartilage tissue was cut into 5 μm sections. The tissue sections were deparaffinized in a coupling jar containing xylene 3 times each for 3 min, then soaked in 100% ethanol, 90% ethanol, and 80% ethanol for 3 min each in that order. After washing with distilled water and pretreatment with PBS, the sections were treated with proteinase K solution supplied by the manufacture for 30 min at 37 °C. After washing again with distilled water and pretreatment of TdT (terminal deoxynucleotidyl transferase) buffer II, the sections were treated with a mixture of 45 μl TdT buffer II, 25 μl FITC-dUTP, and 2.5 μl TdT and incubated for 60 min at 37 °C. After removing TdT solution by tapping off, the slides were immersed in buffer solution supplied by manufacture for 15 min at room temperature. Counterstaining was done with 0.5 μg/ml propidium iodide for 15 min at 4 °C. After mounting, the slides were observed with fluorescent microscopy.
3. RNA isolation

Cartilage was quickly minced into thin slices with a scalpel, transferred into a TRIzol reagent with 1ml per 100 mg of cartilage. Approximately 1500 to 2000 mg cartilage chips in a TRIzol at one time were pulverized in the Polytron (OmiMacro Homogenizer, Waterbury, CT, USA) for 15 to 30 sec at the rate of medium impact frequency. Then the pulverized cartilage in TRIzol was incubated for 30 min on a rocking plate at RT. For protein and tissue debris extraction, chloroform (400 µl/ml of TRIzol) was added to the mixture and then centrifuged at 12,000 g, 4 °C, for 25 min (Adams et al, 1992). RNA in the aqueous phase was further purified through a silica-gel-based membrane spin column (RNeasy kit, Qiagen, Santa Clara, CA, USA) according to the manufacturer’s protocol. About 1-3 µg total RNA could be obtained from 100 mg of cartilage.

4. Synthesis of cDNA

For the synthesis of cDNA in the 30 µl reaction volume, 5 µg total RNA, 2 µg random hexamer (Pharmacia, Uppsala, Sweden), 2 µl 10 mM dNTP (Boehringer Mannheim, Mannheim, Germany), and 200 U MMLV reverse transcriptase (Gibo BRL) were mixed and incubated at 42 °C for 1 hr. Then the mixture was heated at 95 °C for 5 min to terminate reverse transcription.

5. Multiplex RT-PCR for apoptosis-related genes

The cDNA was PCR-amplified using the CytoXpress Multiple cDNA amplification kits (Biosource, Camarillo, CA, USA) according to the manufacturer’s instructions. For the quantitative detection of bcl-2, bcl-xL, bax, bcl-xS, and caspase-3 (LICE), apoptosis set 2 (Biosource catalog #QHM0021) was used. For the detection of Fas, FasL, TRAIL, and caspase-8 (FLICE), apoptosis set 3 (Biosource catalog #QHM0031) was used. To 5 µl of cDNA, 5 µl each primer, 4 µl 3.12 mM dNTP, 5 µl 10 x buffer, and 2.5 U AmpliTaq DNA polymerase (Perkin-Elmer, Branchburg, NJ, USA) were added and PCR was done. After an initial denaturing step at 96°C for 1 min, the mixture was run through the following temperature profile for 2 cycles: denaturing step at 96°C for 1 min, annealing step at 57°C for 4 min, and extension step at 72°C for 1 min. Then it was run through the following cycle
program for 33 cycles: denaturing step at 94°C for 1 min, annealing step at 56°C for 2.5 min, and extension step at 72°C for 1 min. The PCR products were analyzed by electrophoresis in 2% agarose containing 0.5 μg/ml ethidium bromide and photographed with UV light illumination.

6. Semiquantitative RT-PCR for FasL, IL-18, and TRAIL-receptors

For the quantitation of FasL, IL-18, and TRAIL-receptors transcripts, semiquantitative RT-PCR was performed with the following primers. For the internal control, β-actin primers were used.

FasL  5'-ATG TTT CAG CTC TTC CAC CTA CAG AAG GA-3' and
     5'-CAG AGA GAG CTC AGA TAC GTT GAC-3'
     (Shin et al, 1999);

IL-18  5'-GCT TGA ATC TAA ATT ATC AGT C-3' and
     5'-GAA GAT TCA AAT TGC ATC TTA T- 3' (Olee et al, 1999);

DR4   5'-TTA CAC CAA TGC TTC CAA CAA T-3' and
     5'-AGG AGT CAA AGG GCA CGA TGT T-3';

DR5   5'-ATT GTG GCT GTG TTT GTT TGC-3' and
     5'-TGT TGG CTC TGC TGG CTC CTG-3';

DcR1  5'-GAT CCC CAA GAC CCT AAA GTT-3' and
     5'-GGT TTC CAC AGT GGC ATT GGC-3';

DcR2  5'-AGG GAT GGT CAA GGT CAG TAA T-3' and
     5'-GAT GTC AGC GGA GTC AGC GTC A-3';

β-actin  5'-CGT GGG CCG CCC TAG GCA CCA-3' and
       5'-TTG GCC TTA GGG TTC AGG GGG G-3'.

To 3 μl of cDNA, 2 μl of each pair of primers, 4 μl 1.25 mM dNTP, 2.5 μl 10 x buffer, and 1.25 U AmpliTaq polymerase (Perkin-Elmer) were added. The mixture was run through the following temperature profile for 24 to 35 cycles: denaturing step at 94°C for 30 sec, annealing step at 55°C to 56°C for 30 sec, and extension step at 72°C for 1 min. As a positive control for FasL, activated Jurkat cells—T cell leukemia cell line—with anti-CD3 antibody was used. The PCR products were analyzed by electrophoresis in 1.5% agarose containing 0.5μl/ml ethidium bromide and photographed with UV light illumination.
7. Immunohistochemical staining for FasL

To confirm the expression FasL at the protein level, immunohistochemical staining for FasL was performed, with goat polyclonal antibodies for human FasL (N-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 11 cartilages. To amplify the signal on immunohistochemistry, two strategies were used, i.e. antigen retrieval in citrate buffer, and signal amplification with biotinylated tyramide (Lee et al, 1999). For the former, heat-induced epitope retrieval was conducted by immersing the slides in Coplin jars filled with 10 mM/L citrate buffer (pH 6.0) and by boiling the buffer for 30 min in a pressure cooker (Nordic Ware, Minneapolis, MN, USA) inside a microwave oven at 700 W; the jars were then cooled for 20 min. For the latter, the Renaissance TSA indirect kit (NEN Life Science, Boston, MA, USA), which included streptavidin-peroxidase and biotinylated tyramide, was used. After rinsing with PBS, the tissue sections were treated with 1% H₂O₂ in PBS for 15 min at RT to abolish endogenous peroxidase activity. After washing with TNT buffer (0.1 M/L Tris-HCl, pH 7.4, 0.15 M/L NaCl and 0.05% Tween 20) for 20 min, the tissue sections were treated with TNB buffer (0.1 M/L Tris-HCl, pH 7.4, 0.15 M/L NaCl and 0.5% blocking reagent). Sections were incubated for 1 h at 37°C with 2 µg/ml goat polyclonal antibody for human FasL. They were then incubated with biotinylated secondary antibodies (Santa Cruz Biotechnology) for 50 min at 37°C; streptavidin-peroxidase was then applied for 30 min at RT, followed by three washes for 5 min in TNT buffer. The reaction products were developed with diaminobezidine (Sigma, St. Louis, MO, USA) and counterstained with hematoxylin. The results were reviewed independently by two pathologists. Cartilages were interpreted as positive for FasL by immunohistochemistry when at least weak to moderate cytoplasmic staining was seen in greater than 30% of the chondrocytes. The immunoreactivity for FasL was graded as 0 (0-10% of the cells with a very weak signal), 1+ (11-30% of the cells with a weak to moderate signal), 2+ (31-60% of the cells with a moderate to strong signal), or 3+ (>60% of the cells with a strong signal) (Lee et al, 1998). The immunostaining with FasL antibody was judged to be antibody-specific by several criteria, including: (a) use of normal goat serum at the same dilution produced no consistent immunostaining of any cells; (b) intensity of signal diminished as the dilution of antibody was increased; and (c) preincubating FasL antibody with blocking peptide abrogated the
positive immunostaining (Lee et al 1999). As negative controls, three slides were treated by peptide neutralization and replacement of primary antibody with non-immune goat serum. In the neutralization assay, goat polyclonal antibody for FasL was incubated with a 10-fold (by weight) excess of the peptide antigen (Santa Cruz Biotechnology), which had been used to raise each antibody, in PBS overnight at 4°C. After incubation, the neutralized antibody was used instead of primary antibody.
III. RESULTS

1. TUNEL staining in articular cartilage

In TUNEL staining study to examine whether chondrocytes of OA cartilage undergo apoptotic process, almost all OA cartilage showed definite apoptotic feature. Among 7 cases of OA examined by TUNEL staining, 6 cases showed fluorescein-dUTP tagged chondrocytes (apoptotic index: 5–30 %, mean: 11 %) (Table 1 and Fig. 1). In contrast, all 4 non-OA control cartilages showed no tagged chondrocytes. With independent examination by two pathology specialists, the results were interpreted in a blind fashion.

<table>
<thead>
<tr>
<th>Table 1. Apoptotic index in articular cartilage with TUNEL staining</th>
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<tbody>
<tr>
<td>Non-OA Control Cartilage (N=4)</td>
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<tr>
<td>Apoptotic index $^a$</td>
</tr>
</tbody>
</table>

a. The apoptotic index was calculated as the percentage of morphologically identified apoptotic cells and apoptotic bodies in the total number of cells shown on a section (Heatley, 1995).

b. Mean ± SD of 6 cases of OA cartilage showed apoptotic chondrocytes.

2. mRNA expression of various apoptosis-related genes in OA cartilage

For the detection of bcl-2 family genes, caspase members, and Fas-related genes, multiplex RT-PCR was performed using commercially available kits. By using these kits expression of bcl-2, bcl-xL, bax, bcl-xS, caspase-3, caspase-8, Fas, FasL, and TRAIL could be detected. Caspase-3 and caspase-8 were constitutively expressed in overall cartilage, whereas their expression was slightly increased in OA cartilage compared with non-OA control cartilage (Fig. 2 and Table 2). The expression of bcl-2 family genes was weak in overall cartilage as well as showing no definite differences between OA and non-OA control. Meanwhile, Fas expression was strong in overall OA and non-OA control cartilage, with a slight increase in OA cartilage (Fig. 3). The FasL expression was generally weak but showed a tendency to increase in some OA cartilage (Fig. 3 lane 5-7). Another death-inducing ligand, TRAIL, showed generally moderate expression and no definite differences between OA and control cartilage (Fig. 3).
Figure 2. Multiplex RT-PCR for bcl-2 family and caspase-3. Samples from 1 to 3 were control cartilage and from 4 to 10 were OA cartilage.

Figure 3. Multiplex RT-PCR for Fas, FasL, TRAIL, and caspase-8 genes. Sample from 1 to 3 were control cartilage and from 4 to 10 were OA cartilage.

Figure 4. Semiquantitative RT-PCR for FasL and IL-18. Samples from 1 to 3 were control cartilage and from 4 to 10 were OA cartilage. In sample 4, part of the cartilage grossly normal-looking (4N) and the typically degenerated cartilage (4O) were separately harvested. RNA was extracted from each cartilage and RT-PCR was done.

Figure 5. Semiquantitative RT-PCR for TRAIL-receptors. Samples from 1 to 3 were control cartilage and from 4 to 10 were OA cartilage.
Table 2. mRNA expression of apoptosis-related genes in articular cartilage

<table>
<thead>
<tr>
<th></th>
<th>Non-OA Control Cartilage (N=3)</th>
<th>OA Cartilage (N=7)</th>
</tr>
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<tbody>
<tr>
<td><strong>bcl-2 family genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bcl-2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>bcl-xl</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>bax</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>bcl-xS</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td><strong>Caspase members</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caspase-3*</td>
<td>+++</td>
<td>++++</td>
</tr>
<tr>
<td>Caspase-8*</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td><strong>Fas-related genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fas*</td>
<td>+++</td>
<td>+++/++++</td>
</tr>
<tr>
<td>FasL*</td>
<td>+</td>
<td>+ ~ ++++</td>
</tr>
<tr>
<td>IL-18</td>
<td>+ ~ ++++</td>
<td>+ ~ ++++</td>
</tr>
<tr>
<td><strong>TRAIL-related genes</strong></td>
<td></td>
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<tr>
<td>TRAIL</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>DR-4</td>
<td>±</td>
<td>±</td>
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<tr>
<td>DR-5</td>
<td>+</td>
<td>+/+</td>
</tr>
<tr>
<td>DcR1</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>DcR2</td>
<td>+++</td>
<td>+++</td>
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</table>

The grading of mRNA expression of these genes was based on the values measured by densitometer and converted by the relative % ratio to the expression level of house-keeping genes (GAPDH or β-actin) as 100%: ±, < 30% intensity of with very weak levels; 1+, 30-60% of intensity with weak levels; 2+, 60-90% of intensity with moderate levels; 3+, 90-120% of intensity with moderate to strong levels; 4+, 120-150% of intensity with strong levels; and 5+, > 150% intensity with very strong levels.

* Genes showing different expression levels between OA cartilage and non-OA control cartilage.
3. mRNA expression of FasL and IL-18

There were no previous reports describing about the expression of FasL in articular cartilage, however mRNA of FasL was detected in multiplex RT-PCR using articular cartilage in this study (Fig. 3). For more precise investigation of the expression and quantitation of FasL, semiquantitative RT-PCR of FasL was performed. FasL expression was significantly different in OA cartilage compared to non-OA control cartilage (Fig. 4 and Table 2). No or weak Fas expression was shown in non-OA cartilage, while showing highly increased expression in OA cartilage, even stronger than in the positive control, activated Jurkat cell. Particularly strong expression was shown in several OA cartilages (Fig. 4, lanes 4-6, 8, 10). Since IL-18, known as one of the FasL-inducing factors, can be expressed in articular cartilage, the correlation of the expression of FasL and IL-18 was investigated in this study. Although the expression of IL-18 did not show a clear correlation with the expression of FasL, the cartilage showing the higher expression of FasL also showed a higher expression of IL-18 (Fig. 4, lanes 4-6, 8, 10). Interestingly, in the same patient, the part of grossly normal-looking cartilage (Fig. 4, lane 4N) showed weak expression of IL-18, while the typically degenerated part of cartilage (Fig. 4 lane 4O) showed strong expression of IL-18. These results suggest that the Fas/FasL pathway plays a significant role in apoptosis of chondrocytes in OA, possibly through the induction of FasL by IL-18.

4. mRNA expression of TRAIL-receptors

Since the multiplex RT-PCR showed that TRAIL, another apoptosis-inducing ligand, was expressed in overall cartilage (Fig. 3), the mRNA expression level of TRAIL-receptors was investigated with semiquantitative RT-PCR to determine the role of the TRAIL pathway in the apoptosis of chondrocytes. Among TRAIL-receptors, DR-4 was weakly expressed in overall OA and non-OA control cartilage, and DR-5 was expressed with moderate levels in some OA cartilages (Fig. 5 lane 4-7) with overall weak levels in other OA and non-OA cartilages (Fig. 5 and Table 2). TRAIL-receptors which decoy the death signal, DcR1 and DcR2, were highly expressed in overall OA and non-OA (Fig. 5 and Table 2). Since DR-5 was moderately expressed in some OA cartilage, a possible role of the TRAIL/DR-5 apoptotic pathway could not be completely excluded. But the strong expression of DcRs suggested that the TRAIL/DR-5 pathway could not transmit the death signal, or if so, it played a minor role in apoptosis of chondrocytes of OA cartilage.
5. Expression of FasL in immunohistochemical staining

To confirm the expression of FasL at the protein level, immunohistochemical staining for FasL was performed in 11 cartilages. The expression of FasL was detected in all 7 samples of OA cartilage with grade 1+ in 2 samples and grade 3+ in 5 samples (Figure 6 and Table 3), whereas non-OA control cartilage showed grade 0 immunoreactivity for FasL in 3 samples and grade 1+ in 1 sample. In positive chondrocytes, detectable cell surface staining was accompanied by strong granular cytoplasmic reactivity. There was no preference for the distribution of FasL-positive chondrocytes from the superficial layer to deep layer. Negative controls, using competing epitope peptide, for FasL showed no signal. The grading of immunoreactivity for FasL in cartilage was well correlated with the level of expression of FasL transcript (Table 4).

<table>
<thead>
<tr>
<th>Table 3. FasL immunoreactivity in articular cartilage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Non-OA Control Cartilage</td>
</tr>
<tr>
<td>(N=4)</td>
</tr>
<tr>
<td>OA Cartilage</td>
</tr>
<tr>
<td>(N=7)</td>
</tr>
<tr>
<td>Immunoreactivity grade*</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>1+</td>
</tr>
<tr>
<td>2+</td>
</tr>
<tr>
<td>3+</td>
</tr>
</tbody>
</table>

a. Quantitation of FasL positivity: 0, 0-10% of the cells with a very weak signal; 1+, 11-30% of the cells with a weak to a moderate signal; 2+, 31-60% of the cells with a moderate to strong signal; 3+, >60% of the cells with a strong signal.

b. Number of cartilage samples showing each grade of FasL immunoreactivity.
<table>
<thead>
<tr>
<th>Case No.</th>
<th>FasL</th>
<th>IL-18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Transcript level</td>
<td>Protein level</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>NI</td>
</tr>
<tr>
<td>2</td>
<td>±</td>
<td>0</td>
</tr>
<tr>
<td>Non-OA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>control cartilage</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>(N=5)</td>
<td>NI</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>NI</td>
<td>0</td>
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<td>15</td>
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<td>0</td>
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</tr>
<tr>
<td>4O</td>
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<td>+++</td>
</tr>
<tr>
<td>5</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>6</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>OA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cartilage</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>(N=10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>+++</td>
<td>NI</td>
</tr>
<tr>
<td>9</td>
<td>±</td>
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<tr>
<td>10</td>
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<td>NI</td>
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<tr>
<td>11</td>
<td>NI</td>
<td>+++</td>
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<tr>
<td>12</td>
<td>NI</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>NI</td>
<td>+++</td>
</tr>
</tbody>
</table>

NI: Not informed.

a. The criteria of grading is same as the one in Table 2.
b. The criteria of grading is same as the one in Table 3.
Figure 1. TUNEL staining in cartilage (x 200). Control cartilage showed only negatively stained cells with red colors (left) and OA cartilage showed positively stained cells with green fluorescent (right).

Figure 6. Immunohistochemical staining for FasL in articular cartilage. Control cartilage showed negatively stained cells with bluish or pale cytoplasm (left) and OA cartilage showed positively stained cells with brown granular cytoplasmic reactivity (right).
IV. DISCUSSION

Recent research on OA has mainly followed two lines (Messner and Gillquist, 1996). One line concentrates on repair of localized cartilage injuries, while the other line examines possible causes of the disease with biologic or molecular methods in order to find ways to stop its initiation or progression or to reverse the condition. Current treatment modalities of OA include conservative measures such as non-steroid anti-inflammatory drugs (NSAIDs) and physiotherapy; arthroscopic lavage or debridement; occasional abrasion arthroplasty, multiple drilling, or microfracture to induce metaplasia of fibrocartilage; and realignment osteotomy procedures to delay the progress of disease. Eventually, the only solution for overt OA is total joint replacement with artificial prosthesis. Although these procedures have been revolutionized by many orthopaedic surgeons’ pioneer work and have improved the quality of life for many long-suffering patients, they are not based on a clear understanding of the etiology or pathogenesis of OA. Thus, new research on articular cartilage should ultimately be aimed at developing new biologically-based therapeutic strategies as alternatives to current procedures. Based on this idea, the present study was designed to elucidate whether the apoptosis of articular chondrocytes occurs and increases in OA cartilage, and if so, which apoptotic pathways are involved. In doing so, underlying pathways and the molecular mechanisms regulating apoptosis of chondrocytes in OA pathogenesis is proposed.

The primary and central event of OA is progressive degradation of articular cartilage. The etiology and pathogenesis of this common disease is not yet well understood. Recent reports have shown evidence that the apoptosis of articular chondrocytes occurred and increased in OA (Blanco et al, 1998; Hashimoto et al, 1998a) and that apoptosis of articular chondrocytes may be related to decreased collagen type II in the matrix (Yang et al, 1997). These studies suggested that apoptosis plays an important role in OA pathogenesis and is linked with the cartilage destruction seen in OA. The present study also documented results compatible with previous reports, that 6 of 7 cases of OA cartilage revealed apoptotic chondrocytes with TUNEL staining (apoptotic index: 5–30%), but that all 4 cases of non-OA control cartilage showed no apoptotic chondrocytes. The apoptotic index was lower in this
study through TUNEL staining than in previous studies through flowcytometry and this might be due to the enzymatic digestion process that could accelerate apoptosis in chondrocytes during the cell isolation procedure (Blanco et al, 1998).

Meanwhile, investigations concerning the molecular mechanisms and underlying pathways regulating apoptosis in OA chondrocytes and cartilage have been few. One study reported that Bcl-2 in articular chondrocytes was down-regulated by serum withdrawal or retinoic acid treatment, while the level of Bax expression remained unchanged compared with control cells, suggesting a role of Bcl-2 in chondrocyte apoptosis (Feng et al, 1998). In the present study, bcl-2 family genes, both anti-apoptotic and pro-apoptotic, showed similarly low levels on overall cartilage, without any significant difference between OA and non-OA control cartilage, which suggested they do not play a crucial role in OA pathogenesis.

Caspase-8 is a kind of initiator caspase associated with apoptosis involving a death receptor. Caspase-3 is an effector caspase to degrade death substrates and eventually disassembles cells. There has been a report that caspase-3 expression was shown in cartilage chondrocytes with immunostaining (Krajewska et al, 1997). Consistently, the present study also demonstrated that caspase-3 and caspase-8 were highly expressed in articular cartilages, with a slight increase in OA than in non-OA control. These results support that the articular cartilage undergoes apoptosis, in which the death receptor-mediated pathway may be associated. Caspase regulation is currently targeted as a potential treatment strategy in neurodegenerative diseases, ischemia-reperfusion injury, graft-versus-host disease, and autoimmune disorders by regulating apoptosis with caspase inhibitor of a small-peptide permeable plasma membrane (Thornberry and Lazebnik, 1998). OA can also be a candidate disease for application of this type of therapy.

TRAIL, one of the death receptor ligands mediating apoptosis, has recently been documented (Wiley et al, 1995; Pitti et al, 1996). TRAIL expression in articular cartilage has not been shown in the literature yet. In this study, TRAIL transcript was demonstrated with similar levels in overall OA and non-OA control cartilages. However, as for its death-mediating receptors, DR-4 was expressed at similarly weak levels on overall OA and non-OA control cartilage, while DR-5 was expressed with moderate levels in some OA cartilages with overall weak levels in other OA and non-OA cartilages. Meanwhile, its death-inhibiting
decoy receptors, DcR1 and DcR-2, were expressed with similarly high levels in overall OA and non-OA control cartilage. Since DR-5 was moderately expressed in some OA cartilage, a possible role of the TRAIL/DR-5 apoptotic pathway could not be completely excluded. But the strong expression of DcRs suggested that the TRAIL/DR-5 pathway could not transmit the death signal, or if so, it played a minor role in apoptosis of chondrocytes of OA cartilage.

As for Fas/FasL expression in articular chondrocytes, there has been a report that a subpopulation of chondrocytes expressed Fas and was susceptible to Fas-induced apoptosis, but FasL transcript was not detectable in resting or activated, normal or OA, chondrocytes (Hashimoto et al, 1997). Very recently, the expression of FasL transcripts has been reported in articular chondrocytes grown in a cell culture system (Kuhn et al, 1999). However, a report documenting FasL in articular cartilage has not been reported in the literature yet. In the present study, Fas transcript was expressed with high levels in cartilages of OA and non-OA, and showed slightly increased expression in OA cartilage than in non-OA control cartilage. Both FasL transcripts and protein were documented with no or weak expression in non-OA control cartilages, with a highly increased level in OA cartilage. This contrast between OA cartilage and non-OA control cartilage was more apparent at the protein level. Unlike the expression of Fas, which is constitutively expressed in many tissues throughout the human body (Wiley et al, 1995; Pitti et al, 1996), the expression of FasL is restricted mainly to activated T cells and natural killer (NK) cells, and to immune-privilege sites such as testis, eye chamber, and parts of the nervous system (Nagata, 1997). This study is the first documentation of FasL expression in articular cartilage, to my knowledge. The verification of FasL expression from cartilage tissue reflects the in vivo status more closely than that of a cell culture system. For the primary cell culture, it is mandatory that chondrocytes pass through an enzymatic digestion process, a kind of selection step for survival. Besides, the enzymatic digestion process itself can be harmful to chondrocytes.

There are several molecules or pathways known as inducers of FasL, including oxidative stresses, parts of viral proteins, and IL-18 (Walker et al, 1998). IL-18, originally identified as the interferon-γ-inducing-factor, is a new member of the IL-1 family of cytokines (Okamura, 1995) and is also known as an inducer of FasL in T cells and NK cells.
Articular chondrocytes produce the IL-18 precursor and, in response to IL-1 stimulation, secrete the mature form of IL-18, as well as contribute to cartilage degradation (Olee et al, 1999). In the present study, although the expression of IL-18 transcripts did not show a clear correlation with the expression of FasL, the cartilage expressing the higher level of FasL also expressed the higher level of IL-18, suggesting that IL-18 might induce FasL in chondrocytes of OA cartilage.

On the basis of these results, normal chondrocytes do not undergo apoptosis because they express a significant amount of Fas, but a negligible amount of FasL. However, increased expression of FasL, which may be induced by IL-18 activation in OA cartilage, can lead to a simultaneous expression of Fas and FasL, leading to the "kiss of death" and resulting in apoptotic cell death. FasL expression induced by IL-18 may be a critical limiting factor for the acceleration of chondrocyte death during the course of OA pathogenesis because Fas is constitutively expressed in normal chondrocytes. Further study is needed to determine whether IL-18 treatment induces the expression of FasL and cell death in a chondrocytes culture system.

The present study was designed with the intention of throwing a fishing net into the puddle of apoptosis-regulating genes, because few investigations have thoroughly dealt with this subject in OA cartilage. As a result, a notable fish—FasL—was caught; cartilage can be listed as one of the tissues expressing FasL. As a basic question, if apoptosis does occur in OA cartilage and if it is a main mechanism in OA pathogenesis, then when does apoptosis play a role during the disease process of OA, at the initiation of OA or during cartilage destruction? There is no definite answer and no direct evidence yet, however it seems to involve the cartilage destruction process. In summary, the results of this study imply that apoptosis of chondrocytes does occur and increases in OA cartilage, and that the Fas/FasL pathway may play an important role in the apoptosis of chondrocytes during OA pathogenesis, probably inducing expression of FasL by IL-18. Therefore, an understanding of the role of Fas/FasL in regulating chondrocyte apoptosis may provide a more rational treatment strategy for OA for clinical application by therapeutic prevention of apoptosis to stop the initiation or progression of, or to reverse, the disease condition.
V. CONCLUSION

The present study has elucidated that apoptosis occurred and was increased in OA cartilage and documented that FasL was expressed in articular cartilage, at very high levels in OA cartilage, and was probably triggered by IL-18. In conclusion, the Fas/FasL pathway may play a significant role in the apoptosis of chondrocytes during OA pathogenesis. Therefore, the Fas/FasL mediated apoptotic pathway in OA can be targeted by therapeutic prevention of apoptosis to stop the initiation or progression of, or to reverse, the disease condition.
REFERENCES


국문요약

골관절염 병인에 있어서 연골세포의 세포사멸과 그 조절기전

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연세대학교 대학원 의학과

김 현 정

골관절염(osteoarthritis)은 관절연골의 점차적인 소실과 이에 대한 이차적인 변화인 활액막과 연골하골의 변화를 특징으로 하는 질환으로서, 특히 노년층에서 높은 유병률을 보이는 질환이다. 골관절염은 정형외과 영역에서 진료하는 임상 의에게는 가장 흔히 접하게 되는 질병이나, 원인 및 병인에 대해서는 아직 뚜렷하게 규명된 바가 없다. 한편, 세포사멸(apoptosis)은 programmed cell death라고도 하며 세포피사(cell necrosis)와는 상대적인 개념의 세포 사망으로서 염증반응이 없이 진행되는 것이 특징이며 자가면역질환, 중앙, 퇴행성 뇌질환 등의 기전연구에서 그 역할이 규명되고 있다. 최근 발표된 연구에 의하면, 골관절염의 연골세포가 정상관절의 연골세포에 비해 높은 비율의 세포사멸을 보이며, 또한 Fas에 대한 항체를 처치한 경우에도 부분적으로 세포사멸을 일으킨다고 최근 보고된 바 있다. 이에 본 연구는 골관절염의 연골세포에서 일어나는 세포사멸의 양상을 정상관절의 연골세포와 비교하고, 촉음 수용체/리간드의 발현을 조사하여, 골관절염의 병인에 있어서 세포사멸의 역할을 규명하고 그 조절기전을 밝히는 데에 목적이 있다.

본 연구에서는 골관절염으로 인골슬관절 치환술을 시행받은 환자의 관절연골과 의상에 의해 슬상절단술을 시행받은 환자의 절단지에서 채취한 관절연골을 재료로 in situ TUNEL assay를 이용한 형태학적 연구를 통해 세포사멸의 정도와 위치를 비교하고, 관절연골 조직에서 RNA를 분리하여 역전사증합효소 연쇄반응을 통해 apoptosis 조절에 관여하는 Fas 관련분자 및 bcl-2 family, TRAIL 및 그 수용
체와 IL-18 (인터루킨-18)의 RNA 발현정도를 알아보았다. 그 결과 형광 dUTP를 이용한 in situ TUNEL assay에서 골관절염이 없는 정상 대조군 관절연골 4례 모두에서에서는 세포사멸을 보이는 세포를 찾아볼 수 없었으나 골관절염의 관절연골 7례의 표본 중 6례에서 전형적인 세포사멸 세포들을 관찰할 수 있었다 (평균 11%). 발현량을 조사한 유전자들 중에서는 caspase-3, caspase-8, Fas 및 FasL의 RNA 발현정도가 골관절염의 연골에서 정상 대조군 연골에 비해 증가하였으며, 특히 FasL는 일부 골관절염에서 현저히 증가된 양상을 보였다. 또한 FasL의 유도체로 알려진 IL-18은 FasL가 증가된 환자에서 역시 강한 발현을 보였다. 한편, 면역조직화학법으로 FasL 단백의 발현을 조사하여 보았을 때, 역시 정상 대조군 연골에서보다 골관절염의 연골에서 현저히 강하게 발현됨을 관찰할 수 있었다.

결론적으로, 본 연구는 관절연골에서 FasL의 발현을 처음으로 보고하는 것이며, 이상의 결과는 Fas/FasL 경로가 골관절염에서 일어나는 관절연골의 퇴화에 관련될 것을 시사한다. 더욱이 IL-18이 FasL의 유도체로 작용하여 그 전단계에서 발현하는 것으로 사료된다. 이는 차후 골관절염 치료에 있어서 새로운 접근을 제시하는 데 일조할 것으로 기대된다.

핵심되는 말: 연골세포, 세포사멸, 골관절염, Fas 리간드, 인터루킨-18