Apoptosis of Chondrocytes and Its Regulation in Pathogenesis of Osteoarthritis

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— Abstract —

골관절염 연골세포의 세포사멸과 그 조절기전

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목적 : 골관절염(osteoarthritis)은 관절연골의 점차적인 소실과 이에 따른 활액막과 연골하골의 이차적인 변화를 특징으로 하는 질환으로서 특히 노년층에서 높은 유병율을 보이는데 원인 및 병인에 대해서는 아직 뚜렷하게 규명된 바가 없다. 한편, 세포사멸(apoptosis)은 세포괴사(cell necrosis)와는 상대되는 개념의 세포 사망으로서 염증반응이 없이 진행되는 것이 특징이고 자가면역질환, 종양, 퇴행성 뇌질환 등의 기전연구에서 그 역할이 규명되고 있다. 최근 몇몇 연구에 의하면, 골관절염의 연골세포가 정상관절의 연골세포에 비해 높은 비율의 세포사멸을 보이며, 또한 Fas에 대한 항체를 처치한 경우에도 부분적으로 세포사멸을 일으킨다고 하였다. 본 연구는 골관절염의 연골세포에서 일어나는 세포사멸의 양상을 정상관절의 연골세포와 비교하고, 세포사멸과 관련한 수용체/리간드의 발현을 조사하여 골관절염의 병인에 있어서 세포사멸의 조절기전을 알아보았다.

방법 : 골관절염으로 인공슬관절 치환술을 시행받은 환자의 관절연골과 외상에 의해 슬상절단술을 시행받은 환자의 절단지에서 채취한 관절연골을 재료로, TUNEL assay를 통해 세포사멸의 정도와 위치를 비교하였고 관절연골 조직에서 RNA를 분리하여 역전사중합효소연쇄반응을통해 세포사멸의 조절에 참여

＜접수일 : 2001년 3월 24일, 심사통과일 : 2001년 6월 30일＞

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하는 Fas 관련분자 및 *bcl-2* family, TRAIL 및 그 수용체와 인터루킨-18 (IL-18)의 mRNA 발현정도를 조사하였으며 면역조직화학염색을 통하여 FasL 단백의 발현을 알아보았다.

결과 : 형광 dUTP를 이용한 TUNEL assay에서 골관절염이 없는 정상 대조군 관절연골 4례 모두에서는 세포사멸을 보이는 세포를 찾아볼 수 없었으나 골관절염의 관절연골 7례의 표본 중 6례에서 전형적인 세포사멸 세포들을 관찰할 수 있었다. 역전사중합효소연쇄반응에서는 caspase-3, caspase-8, Fas 및 FasL의 mRNA 발현정도가 골관절염의 연골에서 정상 대조군 연골에 비해 증가하였으며, 특히 FasL는 일부 골관절염에서 현저히 증가된 양상을 보였다. FasL의 유도체로 알려진 IL-18은 FasL가 증가된 환자에서 함께 강한 발현을 보였다. 면역조직화학염색에서는 정상 대조군 연골에서보다 골관절염의 연골에서 FasL 단백이 현저히 강하게 발현됨을 관찰하였다.

결론 : 본 연구는 골관절염 환자의 관절연골에서 의미있게 증가한 세포사멸과 FasL의 발현을 관찰하였으며, 이는 Fas/FasL 경로를 통한 세포사멸이 골관절염에서 일어나는 관절연골의 퇴화에 기여할 것을 시사한다. 또한 FasL의 유도체 중 하나인 IL-18의 발현도 관찰되었다. 이상의 결과는 차후 골관절염 치료에 있어서 새로운 접근을 제시하는 데 일조할 것으로 기대된다.

색인단어 : 연골세포, 세포사멸, 골관절염, Fas 리간드, 인터루킨-18

INTRODUCTION

Osteoarthritis (OA) is one of the commonest causes of disability in old age and has a huge impact on hospital costs[1]. Pathologically OA is characterized by degeneration of articular cartilage, subchondral bone thickening, marginal osteochondral outgrowth (osteophyte), and eventual joint deformity. The "degeneration" 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 the other inflammatory arthropathies in which cartilage degradation occurs as a secondary response after synovial inflammation[2].

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[3]. 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[4]. Thus, OA is not caused simply by aging and mechanical wear from joint use[5].

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 on radicals which are entwined in the process of matrix degradation[6-12]. Articular "chondrocytes", the only cell type in articular cartilage, is the subject to control turnover 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 proteoglycans by chondrocytes. As the disease progress to the chronic phase, the cartilage reveals a decrease in thickness and cellularity[5]. Based on this finding of "hypocellularity" during the OA process, sev-

eral studies have reported that apoptosis of articular chondrocytes occurs and increases in OA[2,13]. However, the underlying mechanism or the molecules responsible for triggering the apoptosis in OA chondrocytes is still under investigation.

There are a variety of signals to initiate apoptosis: elimination of growth factors, ultraviolet or γirradiation, exposure to noxious chemicals or chemotherapeutic drugs, and activation of death receptors[14-16]. Several pathways and related molecules leading to apoptosis have been well characterized. Death receptors and ligands play a central role in instructive apoptosis[17] : Fas (also called CD95 or Apo-1) and Fas ligand (FasL, also called CD95L or APO-1L)[18] ; DR4 (also called TRAIL-R1)/DR5 (also called TRAL-R2) and TRAIL (TNF related apoptosis-inducing ligand, also called APO-2L)[19,20]. TRAIL has decoy receptors (DcRs), which interfere death signal, DcR1 (also called TRAIL-R3) and DcR2 (also called TRAIL-R4)[21-23]. Downstream signal activates adaptor molecules, for example FADD, and caspase-8 (FLICE), initiate the caspase cascade, and then evetually activate caspase-3, which degrades death substrates and dossemble cells. Cytochrome c in the mitochondria also play an important role in apoptosis in association with Apaf-1 and caspase-9 (Apaf-3). 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 promote apoptosis[24,25].

The present study aimed to investigate the apoptosis of articular chondrocytes in OA cartilage, and which apoptotic pathway and molecular mechanisms are involved in the apoptosis of articular chondrocytes in OA. Apoptotic chondrocytes in articular cartilage were determined by TUNEL (TdT-mediated dUTP-biotin nick end labeling) 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. Immunohistochemical staining was included to examine the expression of FasL in OA cartilage at the protein level, in correlation with the expression level of FasL transcripts.

MATERIALS AND METHODS

1) Sources of articular cartilage: Overall 10 specimens of OA cartilage obtained from total knee replacement surgery (ages 52-78) were included in this study. Control non-OA cartilages were obtained from 5 donors without known history of joint disease at the time of above-knee amputation surgery (ages 44-70). Cartilages were immediately washed in ice-cold phosphate-buffered saline, and cut into small pieces from the femoral condyles and tibial plateaus. One piece of cartilage was preserved in 4% paraformaldehyde for TUNEL and immunohistochemical staining; 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 increase in OA cartilage, TUNEL staining was performed in 11 cartilage, 7 OA cartilages and 4 non-OA control cartilages, using the in situ cell death detection kit (MBL, Nagoya, Japan). This kit is an apoptosis detection kit based on the TUNEL (TdT-mediated dUTP-biotin nick end labeling) method and uses flu-

orescein-dUTP to label DNA strand breaks. In cells in which apoptosis occurs, the chromatin DNA is cut by endonuclease at linker DNA site between nucleosomes. Then, DNA fragments, which are a number of multimers of nucleosomal units, exist in nuclei. In TUNEL method, 3'-OH DNA ends generated by DNA fragmentation is nick end labeled with fluorescein-dUTP, mediated by terminal deoxynucleotidyl transferase (TdT). Cartilages were immediately fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm sections. The sections were treated with a mixture of TdT and FITC-dUTP according to the manufacturer's instructions. Counterstaining was done with Propidium Iodide. After mounting, the slides were examined under a fluorescent microscopy.

3) RNA isolation from articular cartilage: Cartilage was quickly minced into thin slices with a scalpel, transferred into a TRIzol reagent with 1ml per 100mg cartilage. Approximately 1500 to 2000mg of cartilage chips in TRIzol at one time was pulverized in the Polytron (OmniMacro Homogenizer, Waterbury, CT, USA) for 15 to 30 seconds at the rate of medium impact frequency. Then, the pulverized cartilage powder in TRIzol was incubated for 30 min on a rocking plate at room temperature. For protein and tissue debris extraction, chloroform (400 μl/ml of TRIzol) was added to the mixture and centrifuged. RNA in the aqueous phase was further purified through a silica-gel-based membrane spin column (RNeasy kit, Qiagen Inc., Santa Clara, CA) according to the manufacturer's protocol. About 1-3μg total RNA could be obtained from 100mg cartilage.

4) Multiplex RT-PCR for apoptosis-related genes: Total RNA 5 μg was used to carry out a reverse transcription with the random hexamer primer in a total 20μl. The resulting reverse transcriptase product was expanded using the CytoXpress Multiple cDNA amplification kits for apoptosis panels (Biosource, Camarillo, CA) and specific primers for the sequences of interest. For the multiple PCR (MPCR) of Caspase-3 (LICE), *bcl-2, bax, bcl-xS, and bcl-xL*, the quantitative PCR detection kit (Biosource catalog #QHM0021); for the MPCR of Fas, FasL, TRAIL, and Caspase-8 (FLICE), the quantitative PCR detection kit (Biosource catalog #QHM0031) was used according to the manufacturer's instructions. To the 5 μl of total RNA, the 5μl 10x hAPO2MPCR primers, the 4μl 3.12mM dNTP, the 5μl 10× hAPO2GMPCR buffer, and the 0.5μl (5 units/μl) AmpliTaq DNA polymerase (Perkin-Elmer Cetus Corp.) were added. After an initial denaturing step at 96℃ for 1 min, the mixture was run the following temperature profile for 2 cycles: denaturing step at 96℃ for 1 min, annealing step at 57℃ for 4 min, and extension step at 72℃ for 1 min. Then it was continued the cycle program as follows for 33 cycles: denaturing step at 94℃ for 1 min, annealing step at 56℃ for 2.5 min, and extension step at 72℃ for 1 min. The PCR products were analyzed by electrophoresis in 2% agarose that contained ethidium bromide and photographed with UV light excitation.

5) 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, βactin 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'[26];
IL-18 5'-GCT TGA ATC TAA ATT ATC AGT C-3' and
5'-GAA GAT TCA AAT TGC ATC TTA T-3'[27];
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';
βactin 5'-CGT GGG CCG CCC TAG GCA CCA-3' and
5'-TTG GCC TTA GGG TTC AGG GGG G-3'.

To the 3㎕ of total RNA, the 1㎕ each primers, the 4㎕ 1.25 mM dNTP, the 2.5㎕ 10×buffer, and the 0.25㎕ (5units/㎕) AmpliTaq polymerase (Perkin-Elmer Cetus Corp.) were added. The mixture was run the following temperature profile for 24 to 35 cycles: denaturing step at 94℃ for 30 sec, annealing step at 55℃ to 56℃ for 30 sec, and extension step at 72℃ for 1 min. The PCR products were analyzed by electrophoresis in 1.5% agarose that contained ethidium bromide and photographed with UV light excitation.

6) Immunohistochemical staining for FasL: To confirm the expression of FasL at the protein level, immunohistochemical staining for FasL was performed with goat polyclonal antibody 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[28]. 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 boiling the buffer for 30 minutes in a pressure cooker (Nordic Ware, Minneapolis, MN, USA) inside a microwave oven at 700 W. For the latter, the Renaissance TSA indirect kit (NEN Life Science, Boston, MA, USA), which included streptavidin-peroxidase and biotynylated tyramide, was used. 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)[29]. Negative control slides were treated by peptide neutralization and replacement of primary antibody with non-immune goat serum.

RESULTS

1) TUNEL staining in articular cartilage: TUNEL-positive cells were seen in 6 among 7 cases of OA examined by TUNEL staining (apoptotic index: 5~30%, mean: 11%) (Table 1, Fig. 1). In contrast, no TUNEL-positive cells were seen in all 4 cases of control non-OA.

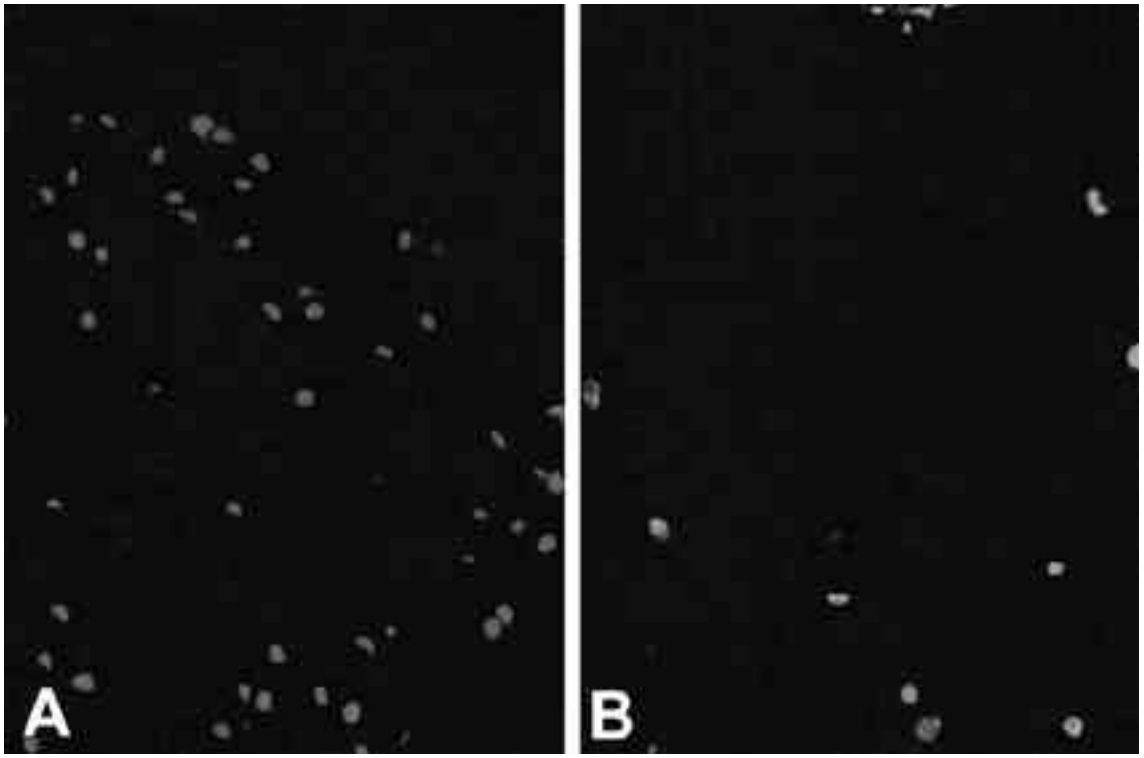

Fig. 1. TUNEL staining in articular cartilage (×200). Negative red cells are seen in control non-OA cartilage (A), while positive green cells in OA cartilage. Note relatively low number of cells in OA (B).

Table 1. Apoptotic index in articular cartilage through TUNEL staining

	Control Non-OA Cartilage (N=4)	OA Cartilage (N=7)
Apoptotic index[a]	0%	11±8.8%[b] (range, 5-30%)

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

2) mRNA expression of various apoptosis-related genes in OA cartilage: Expression of *bcl-2, bcl-xL, bax, bcl-xS*, caspase-3, caspase-8, Fas, FasL, and TRAIL was detected by using commercially available multiplex RT-PCR kits. Caspase-3 and caspase-8 were constitutively expressed in overall cartilages, whereas their expression was slightly increased in OA cartilages compared with control non-OA cartilages (Fig. 2, Table 2). The expression of bcl-2 family genes was weak in overall cartilages and no significant difference between OA and control non-OA. Meanwhile, Fas expression was overall strong in both OA and control non-OA carti-

lages, with a slight increase in OA cartilage. FasL expression was generally weak but showed a tendency to increase in some OA cartilages (Fig. 3). Another death-inducing ligand, TRAIL, showed generally moderate expression and no definite difference between OA and control cartilage (Fig. 3).

3) mRNA expression of FasL and IL-18: There were no previous reports describing about the expression of FasL in articular cartilage, however FasL transcript was detected in multiplex RT-PCR system (Fig. 3). For more precise quantitation of FasL

Fig. 2. Multiplex RT-PCR for bcl-2 family and caspase-3. Samples from 1 to 3 are control cartilage and from 4 to 10 were OA cartilage.

Table 2. mRNA expression of apoptosis-related genes in articular cartilage

		Control Non-OA Cartilage (N=3)	OA Cartilage (N=7)
bcl-2 family genes			
	bcl-2	+	+
	bcl-xL	++	++
	bax	+	+
	bcl-xS	±	±
Caspase members			
	Caspase-3*	+++	++++
	Caspase-8*	++	+++
Fas-related genes			
	Fas*	+++	+++ /++++
	FasL*	+	+~+++++
	IL-18	+~+++++	+~+++++
TRAIL-related genes			
	TRAIL	++	++
	DR-4	±	±
	DR-5	+	+
	DcR1	++++	++++
	DcR2	+++	+++

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 βactin) 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. *, different expression levels between OA cartilage and control non-OA cartilage.

expression, semiquantitative RT-PCR of FasL was separately performed. FasL expression was significantly different in OA cartilage compared to control non-OA cartilage (Fig. 4, Table 2). No or weak FasL expression was seen 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). Although the expression of IL-18, known as

Fig. 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.

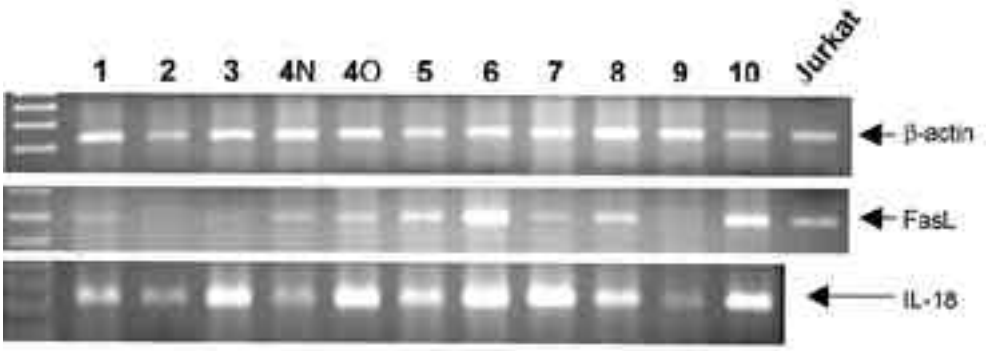

Fig. 4. Semiquantitative RT-PCR for FasL and IL-18. Sample from 1 to 3 were control cartilage and from 4 to 10 were OA cartilage. In sample 4, part to the cartilage grossly normal-looking (4N) and the typically degenerated cartilage (4O) were separately harvested. RNA was extracted from each cartilage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

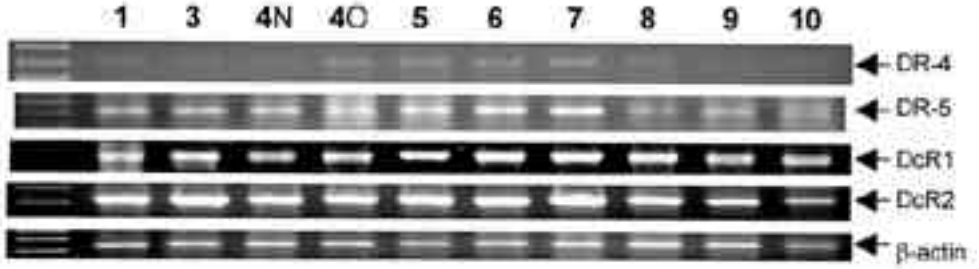

Fig. 5. Semiquantitative RT-PCR for TRAIL-receptors. Samples from 1 to 3 were control cartilage and from 4 to 10 were OA cartilage.

one of the FasL-inducing factors, did not show a clear correlation with the expression of FasL, the cartilage that showed higher expression of FasL also showed higher expression of IL-18 (Fig. 4). Interestingly, in the same patient, a part of grossly normal-looking cartilage (Fig. 4, lane 4N) showed weak expression of IL-18, while the other typically degenerated part of cartilage (Fig. 4, lane 4O) showed strong expression of IL-18.

4) mRNA expression of TRAIL-receptors: Among TRAIL-receptors, DR-4 was overall weakly expressed in both OA and control non-OA cartilages. DR-5 was expressed with moderate levels in some OA cartilage, but with overall weak levels in other OA and non-OA cartilages (Fig. 5 and Table 2). Whereas, DcR1 and DcR2 were highly expressed in overall OA and non-OA (Fig. 5, Table 2).

5) FasL immunohistochemistry: The expression of FasL was detected in all 7 samples of OA cartilage in which we performed immunohistochemistry, with grade 1+ in 2 samples and grade 3+ in 5 samples, whereas non-OA cartilage showed grade 0 immunoreactivity for FasL in 3 samples and grade 1+ in 1 sample (Fig. 6, Table 3). In positive chondrocytes, detectable cell surface staining was accompanied by strong granular cytoplasmic reactivity. There was no preference for 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 car-

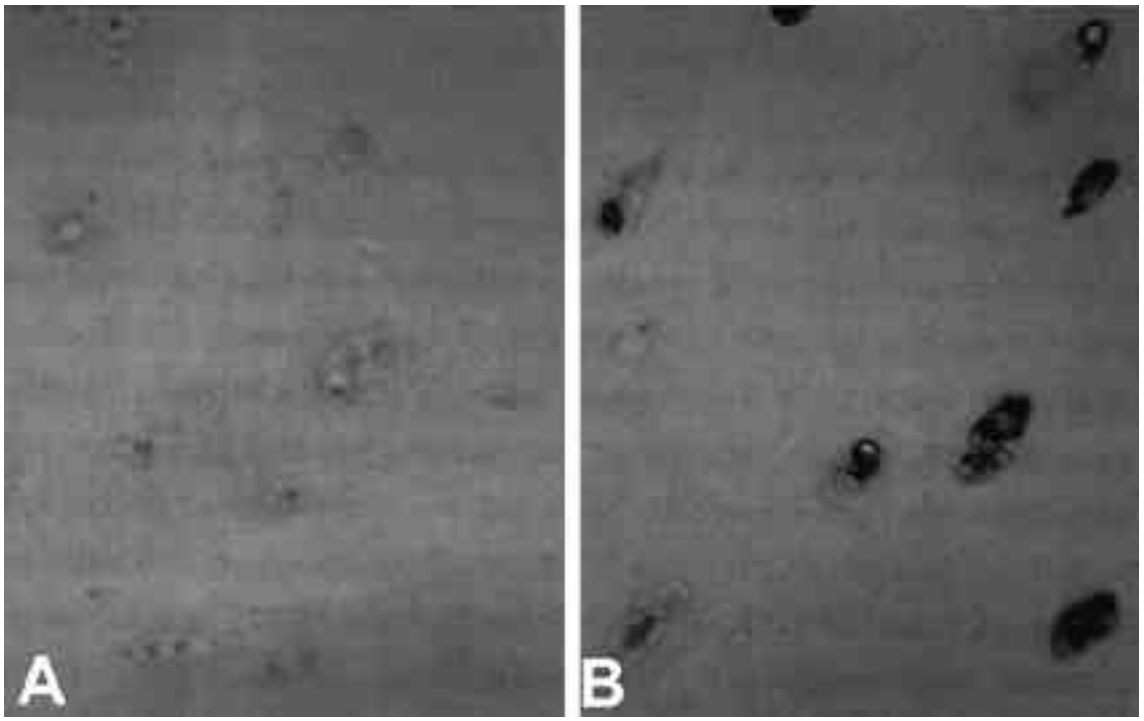

Fig. 6. Immunohistochemical staining for FasL in articular cartilage (×400). Negative cells with bluish pale cytoplasm are seen in control non-OA cartilage (A), while positive cells with brown granular cytoplasmic reactivity are seen in OA cartilage (B).

Table 3. FasL Immunoreactivity in articular cartilage

	Control Non-OA Cartilage (N=4)	OA Cartilage (N=7)
Positive cartilages[a]		
n/N (%)	0%	86%
Immunoreactivity		
0	3 cartilages	-
1+	1 cartilage	2 cartilages
2+	-	-
3+	-	5 cartilages

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.

Table 4. Correlation between mRNA and protein expression of FasL

	Case No.	FasL		IL-18
		Transcript level[a]	Protein level[b]	Transcript level[a]
Control Non-OA cartilage (N=5)	1	+	NI	++
	2	±	0	+
	3	±	+	+++++
	14	NI	0	NI
	15	NI	0	NI
OA cartilage (N=10)	4N	++	NI	+
	4O	++	+++	+++++
	5	++++	+++	++++
	6	+++++	+++	+++++
	7	++	+	+++++
	8	+++	NI	++++
	9	±	NI	+
	10	+++++	NI	+++++
	11	NI	+++	NI
	12	NI	+	NI
	13	NI	+++	NI

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.

tilage was well correlated with the level of expression of FasL transcript (Table 4).

DISCUSSION

Recent OA research has mainly followed two lines: one line concentrates on repair of localized cartilage injuries and 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[1]. Current treatment modality of OA varies from conservative measures to surgical interventions, how-

ever these are not relied on a certain understanding of the etiology or pathogenesis of OA. Based on this idea, the present study has been designed to investigate the apoptosis of articular chondrocytes in OA cartilage, especially which apoptotic molecules are involved in the apoptosis of articular chondrocytes in OA to propose the underlying mechanism regulating apoptosis of chondrocytes in OA pathogenesis.

The primary and central event of OA is progressive degradation of articular cartilage. The etiology and pathogenesis of this commonest disease is not yet well understood. Recent reports have showed evidences that the apoptosis of articular chondrocytes occurred and increased in OA[8,13] and apoptosis of articular chondrocytes might be related to decreased collagen type II in the matrix[30]. These studies have suggested that apoptosis plays an important role in the OA pathogenesis and is linked with cartilage destruction seen in OA. The present study also documented compatible results with the previous reports, that 6 of 7 cases of OA cartilage revealed fluorescein-dUTP tagged positive chondrocytes through TUNEL staining (apoptotic index: 5~30%) but all 4 cases of control non-OA cartilage showed no positive chondrocytes. The apoptotic index was lower in this study through TUNEL staining than in previous report through flowcytometry because the enzymatic digestion process might accelerate apoptosis in chondrocytes[13]. New technique measuring active form caspase-3 by immunohistochemical method can be tried to detect apoptosis of chondrocytes with more specificity.

Meanwhile, investigations about the molecular mechanisms and underlying pathway, regulating apoptosis in OA chondrocyte, are quite limited in quantity. A study have 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 inhibitory role of *bcl-2* in chondrocytes apoptosis[31]. In the present study, among *bcl-2* family genes, both anti-apoptotic and pro-apoptotic members showed overall similar level of expression, without significant difference between OA and control non-OA cartilage. It suggests that they do not play a crucial role in OA pathogenesis.

Caspase-8 is a kind of initiator caspase associated with apoptosis involving death receptor and caspase-3 is an effecter caspase to degrade death substrates and eventually disassemble cells. There was a report that caspase-3 expression was detected in cartilage chondrocytes through immunostaining[32]. Consistently, the present study has also demonstrated caspase-3 and caspase-8 highly expressed in articular cartilage, and with slight increase in OA cartilage than in control non-OA cartilage. Caspase regulation is currently targeted as potential treatment strategy in neurodegenerative diseases, ischemia-reperfusion injury, graft-versus-host disease, and autoimmune disorders by manipulation of apoptosis with caspase inhibitor of small-peptide[33]. OA also can be a candidate disease for application of this type of therapy.

TRAIL, one of death receptor ligand mediating apoptosis, is recently documented[19,34]. TRAIL expression in articular cartilage has not yet shown in literature. In the present study, TRAIL transcript demonstrated similar level of expression in overall OA and control non-OA cartilage. Its death-mediating receptors, DR-4 and DR-5, were expressed with weak levels on overall OA and control

non-OA cartilage, whereas its death-inhibiting decoy receptors, DcR1 and DcR-2, were expressed with high levels on overall OA and control non-OA cartilage. This result suggests that the TRAIL/TRAIL-receptor pathway does not play a significant role in apoptosis of chondrocytes in OA cartilage.

As for Fas/FasL expression in articular chondrocytes, there are 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[7]. However, recently the expression of FasL transcripts has been reported in articular chondrocytes from a cell culture system[35]. In the present study, Fas transcript expressed overall at high levels in cartilages of OA and non-OA, and showed slight increased expression in OA cartilage than in control non-OA cartilage. The expression of FasL transcripts and protein was both documented in articular cartilages, at strongly high level on OA cartilage. Unlike expression of Fas, which is constitutive in many tissues throughout human body[19,34], the expression of FasL is restricted mainly to in activated T-cells and natural killer (NK) cells, and to immune-privilege sites such as testis, eye chamber, and parts of the nervous system[18]. The documentation of FasL expression in articular cartilage is for the first time, in our knowledge. The verification of FasL from cartilage tissue reflects the in vivo status more closely than from culture system. For the primary cell culture, chondrocytes are mandatory to pass through enzymatic digestion process, a kind of selection step for survival. Besides, the enzymatic digestion process itself can induce apoptosis.

There are several molecules known as inducer of FasL, including oxidative stresses, parts of viral proteins, and IL-18[36]. IL-18, originally identified as interferon-?-inducing-factor, is a new member of the IL-1 family of cytokines[37] and is also known as one of inducer of FasL in T-cells and NK cells[38,39]. 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[27]. In the present study, although the expression of IL-18 transcript did not show the clear correlation with the expression of FasL, the cartilage expressing the higher level of FasL also expressed the higher level of IL-18. It suggests IL-18 may induce FasL in chondrocytes of OA cartilage.

On the basis of these results, normal chondrocytes do not undergo apoptosis because they express quite amount of Fas but 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, leads to "kiss of death", and result in apoptotic cell death. FasL expression may be a critical limiting factor for the acceleration of chondrocytes death during the course of OA pathogenesis, because Fas is constitutively expressed in normal chondrocytes. To tell this story, many further studies are necessary. One of them is to determine whether IL-18 treatment induces the expression of FasL and cell death in chondrocytes culture system. While, the bcl-2 family and TRAL/TRAIL-receptor pathway seem not to play a significant role in apoptosis seen in OA cartilage.

In summary, this study implies Fas/FasL pathway may play an important role in apoptosis of chondrocytes during OA pathogenesis, so that OA can be targeted by therapeutic prevention of apoptosis to stop the

initiation or progression or to reverse the condition.

CONCLUSIONS

The present study has elucidated that apoptosis occurred and was increased in OA cartilage and documented that FasL was expressed in articular cartilage, at high levels in OA cartilage, and was probably triggered by IL-18. In conclusion, the Fas/FasL pathway may play an important role in the apoptosis of chondrocytes in OA pathogenesis. Therefore, the Fas/FasL mediated apoptotic pathway in OA can be targeted by therapeutic prevention of apoptosis to modify the disease process.

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