Facilitation of Fas Mediated Apoptosis of Human Chondrocytes by the Proteasome Inhibitor and Actinomycin D

HYUN A. KIM, YONG H. KIM, and YEONG W. SONG

ABSTRACT. ***Objective.*** We investigated the susceptibility of chondrocytes to apoptosis induced by anti-Fas and various potentiators, and the relevant signaling pathway.

Methods. Chondrocytes were cultured from cartilages obtained at the time of joint replacement surgery for knee osteoarthritis (OA) or femur neck fracture. Fas receptor ligation was performed with agonistic anti-Fas antibody (clone CH-11) at concentrations ranging from 0.5 to 1.0 µg/ml. Mitogen activated protein kinase inhibitors SB203580 and PD98059, cycloheximide, bisindolylmaleimide, actinomycin D, or MG132 were added with anti-Fas to facilitate cell death. Chondrocyte surface expression of Fas was analyzed by FACS, and the expression of apoptosis related proteins analyzed by Western blot.

Results. Cell death increased upon coculture with 0.5 µg/ml of anti–Fas and 0.2 µg/ml of actinomycin D or 20 µM MG132. Apoptosis potentiated by actinomycin D or MG132 was effectively inhibited by caspase inhibitors, implicating the involvement of the caspase cascade in chondrocyte apoptosis. Compared with untreated cells or actinomycin D treated cells, cells treated with MG132 showed distinct shifts in the distribution of surface Fas fluorescence. Although concentrations of Bcl-2, Bax, FLICE inhibitory protein (FLIP), and Fas ligand were unaffected by MG132 or actinomycin D, both treatments led to a significant increase of p53. The expression of the p53 response proteins, MDM2 and p21, was elevated in MG132 treated chondrocytes.

Conclusion. Our results suggest that chondrocytes can be rendered sensitive to anti-Fas mediated apoptosis by the proteasome inhibitor MG132 and the transcription inhibitor actinomycin D. MG132 and actinomycin D show different characteristics in terms of apoptosis signaling. (J Rheumatol 2003;30:550–8)

Key Indexing Terms:
FAS APOPTOSIS CHONDROCYTE

Chondrocyte apoptosis has been the focus of interest recently as one of the pathologic factors leading to joint cartilage degradation in various arthritides. Increase in apoptotic chondrocytes was reported in rheumatoid arthritis (RA) and osteoarthritis (OA) cartilages *in situ*[1,2]. In addition, various stimuli relevant in the pathophysiology of arthritis, including nitric oxide (NO), Fas receptor ligation, and ceramide, have been reported to induce chondrocyte apoptosis *in vitro*[3-5]. Fas (Apo-1/CD95) is a member of the tumor necrosis factor (TNF) receptor family, which participates in the main signaling system of apoptosis induction. Fas receptor is activated by its natural ligand or by an agonistic antibody, and initiates a signaling cascade that triggers the activation of caspase-8 through the adapter molecule, Fas associated death domain (FADD)/Mort1, and subsequently the activation of caspase-3, the proteolytic engine of cell death. Fas receptor is reported to be expressed in the RA synovium, and recent investigations have revealed that exogenous treatment with agents that stimulate the Fas mediated cell death cascade induce synovial apoptosis, leading to regression of abnormal synovial proliferation[6,7]. Despite the hopes raised by the feasibility of apoptosis induction with Fas signaling molecules in the treatment of arthritis, there has been concern about the effects of this type of treatment on adjacent joint tissue. Since chondrocytes were reported to be susceptible to Fas mediated apoptosis[4], its influence upon the articular cartilage is of the utmost concern. Although the expression of Fas or Fas ligand can regulate apoptosis, Fas positive cells have variable susceptibilities to Fas mediated apoptosis, implying that the intra-

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cellular signaling process associated with apoptosis may be essential in determining whether cells undergo apoptosis after ligation of the Fas receptor. For example, activated T cells are more susceptible to Fas mediated apoptosis than are naive T cells, despite comparable levels of Fas surface expression[8]. The protein kinase C inhibitor, bisindolylmaleimide VIII, renders the Fas positive, anti-Fas resistant astrocytoma cell line susceptible to apoptosis, implying the role of an undefined factor regulating the Fas mediated apoptosis signaling pathway[9].

Our previous observations indicated the upregulation of Fas receptor in OA cartilage[10], and induction of Fas ligand upon chondrocyte anoikis caused by enzymatic degradation of cartilage matrix[11]. Here we investigated the influence of the potentiator substances on susceptibility to Fas mediated chondrocyte apoptosis and related mechanisms. The signaling pathway for Fas mediated chondrocyte death was also explored.

MATERIALS AND METHODS

Patients and chondrocyte culture. Cartilage samples were obtained from the tibial plateau of patients with knee OA and from the femoral head of patients with femur neck fracture at the time of joint replacement surgery. Pieces of articular cartilage were cut just above the calcified cartilage using a scalpel, and washed thoroughly with phosphate buffered saline (PBS). Cartilage pieces were minced and incubated with 0.25% trypsin/0.2% collagenase (Sigma, St. Louis, MO, USA) in Dulbecco's modified Eagle's medium (DMEM) until the fragments were digested. Released cells were spun, washed, and seeded at 2×10^6/plate in 10 cm culture plates in DMEM supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, 1% Fungizone (Gibco, Grand Island, NY, USA), and penicillin/streptomycin (150 U/ml and 50 mg/ml each). After about 7–10 days, confluent chondrocytes were split once and seeded at 5×10^5/ml, and these first passage chondrocytes were used in the subsequent experiments.

Induction and inhibition of apoptosis. Fas receptor ligation was performed with agonistic anti-Fas antibody (clone CH-11; Medical & Biological Laboratory Co., Nagoya, Japan) at a concentration range from 0.5 to 1.0 µg/ml. This antibody was very effective in inducing cell death in a susceptible cell line, yielding more than 70% death in Jurkat cells after overnight incubation. The control culture was incubated with equivalent concentration of control mouse monoclonal IgM. The potentiation of apoptosis was attempted with the following compounds — SB203580, PD98059, cycloheximide, actinomycin D (all from Calbiochem, San Diego, CA, USA), bisindolylmaleimide VIII (Alexis, San Diego, CA, USA), and MG132 (Biomol, Plymouth Meeting, PA, USA). For caspase inhibition experiments, cells were preincubated for 60 min, before addition of anti-Fas, with 100 µM of pan-caspase inhibitor (z-VAD-FMK; Biomol), caspase-3-specific inhibitor (z-DEVD-FMK), caspase-8-specific inhibitor (z-IETD-FMK), or caspase-9-specific inhibitor (z-LEHD-FMK; all from R&D Systems, Minneapolis, MN, USA).

Quantification of cell death and apoptosis. Cell death was quantitated by the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay, as described[12]. Briefly, chondrocytes were seeded at 5×10^4/100 µl/well in 96 well microtiter plates and incubated with anti-Fas and various potentiators. After incubation, MTT was added to each well to a final concentration of 0.125 mg/ml, and the plate was incubated at 37°C for 3 h. The formazan product thus obtained was solubilized with 100 µl of dimethylsulfoxide (DMSO) and the optical density was read using a test wavelength of 595 nm.

Percentage cell survival was calculated by dividing the optical density of cells under a particular condition by the optical density of untreated control cells, and then multiplying by 100. Cell death was also determined by the trypan blue exclusion assay, in which the medium containing the floating cells was collected and combined with adherent cells that were collected after trypsinization and centrifugation at 1500 rpm for 5 min. After resuspending the pellet in DMEM containing 1% FBS and staining with trypan blue, viable cells in 5 random fields of view were counted. The percentage of treated viable cells was determined as a percentage of control viability.

To analyze apoptosis, flow cytometry using annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) labeling and a DNA fragmentation ELISA were used. For flow cytometry, floating and adherent cells were collected, washed, and resuspended in cold binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at the final concentration of 1×10^6/ml. Aliquots of 1×10^5 cells were incubated with 5 µl annexin-V-FITC and 5 µg/ml PI (both from Pharmingen, San Diego, CA, USA) for 15 min at room temperature, and 400 µl of binding buffer was added before flow cytometric analysis; 10^4 cells were analyzed on a FACS II flow cytometer (Becton Dickinson, Mountain View, CA, USA), and FITC and PI fluorescences were measured through 520 and 630 nm bandpass filters, respectively. DNA fragmentation ELISA was performed as recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany). Briefly, after incubation, floating and adherent cells were collected and the pellets, containing about 5×10^4 cells, were lysed in 0.2 ml of lysis buffer; 20 µl of lysates were used in the cell death ELISA.

Flow cytometry analysis of Fas receptor expression. Cells were seeded at 4×10^5/well in 12 well tissue culture plates and incubated with MG132 and actinomycin D for 14 h. After incubation, the cells were briefly trypsinized, washed twice with PBS, and resuspended in FACS buffer (1% bovine serum albumin, 0.5% sodium azide in PBS); then 2.5×10^5 cell aliquots were incubated with FITC conjugated anti-Fas antibody (Coulter Immunotech, Marseille, France) for 1 h, washed twice with PBS, and analyzed on a FACS II flow cytometer. FITC labeled mouse control IgG stained cells were used as the control.

Western blot. Cellular protein was extracted in lysis buffer containing 50 mM sodium acetate, pH 5.8, 10% v/v sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 µg/ml of aprotinin at 4°C. Protein concentrations were determined using the BCA protein assay kit (Pierce Chemical, Rockford, IL, USA) and bovine serum albumin was used as the standard; 20–40 µg samples were combined with gel loading buffer (50 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue), boiled for 5 min, electrophoresed on a 12% SDS-PAGE gel, and then electrophoretically transferred. Ponceau S staining of polyvinylidene fluoride membrane was routinely performed to roughly confirm equal loading of protein before every Western blot procedure. The blot was blocked with Tris buffered saline containing 5% nonfat milk at room temperature for 1 h, and incubated with anti-Bcl-2 (Transduction Laboratory, Lexington, KY, USA), anti-Bax, anti-Fas ligand, anti-FLIP, anti-caspase-8, anti-caspase-3 (all from Pharmingen), anti-p53, anti-p21, and anti-MDM2 (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blot was then rinsed and incubated with 1:5000 peroxidase conjugated goat anti-mouse or anti-rabbit IgG (Biorad, Hercules, CA, USA) for 1 h. Bound immunoglobulin was detected using an enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK).

Data analysis. Data are expressed as means ± SD. Statistical analysis was carried out using the paired t test to compare each treatment condition versus the control. Statistical significance was established at the 95% confidence level ($p < 0.05$).

RESULTS

Induction of chondrocyte apoptosis with anti-Fas treatment. In contrast to a previous observation[4], the treatment of chondrocytes with anti-Fas antibody failed to reveal any signifi-

cant degree of cell death by the MTT assay or the trypan blue exclusion assay. Extending the incubation period to 96 h, increasing the anti-Fas concentration up to 2 µg/ml, decreasing the cell density to 50% and 25%, and culturing the chondrocytes in pellet culture system did not cause any significant difference (data not shown). In selected samples, cell death was analyzed using MTT, trypan blue, annexin V-PI FACS, and DNA fragmentation ELISA simultaneously, and the results were compared (Table 1). Annexin V-PI FACS revealed a slight increase in early apoptosis (annexin V positive/PI negative) in anti-Fas treated chondrocytes in a limited number of samples (2 of 15 tested), but statistical significance was not reached. On the other hand, the DNA fragmentation ELISA detected a small but consistent increase in DNA fragments in anti-Fas treated chondrocytes.

Next, we investigated the role of various potentiators in terms of facilitation of Fas mediated cell death. The potentiators we used were as follows: the p38 mitogen activated protein kinase (MAPK) inhibitor, SB203580, the MAPK of the extracellular signal-regulated kinase (MEK) inhibitor, PD98059, protein kinase C inhibitor bisindolylmaleimide VIII, protein synthesis inhibitor, cycloheximide, proteasome inhibitor, MG132, and transcription inhibitor, actinomycin D. The addition of SB203580, PD98059, or bisindolylmaleimide VIII to anti-Fas did not result in any significant increase in chondrocyte death (Figure 1). However, coincubation with 20 µM cycloheximide or MG132 or with 0.2 µg/ml actinomycin D led to significant cell death. The degree of cell death induced by cycloheximide/anti-Fas was less than that induced by either MG132/anti-Fas or actinomycin D/anti-Fas (mean cell death after 24 h was 25.6% in cycloheximide/anti-Fas vs 54.8% and 44.0% in MG132/anti-Fas, actinomycin D/anti-Fas, respectively).

To assess the extent of cell death over time, cell viability was determined by MTT assay after 12, 24, 36, and 48 h of incubation (Figure 2). Cotreatment with MG132 or actinomycin D with anti-Fas significantly affected cell viability in a time dependent manner, and cell death was significantly increased 24 h after these cotreatments ($p < 0.0001$). MG132 or actinomycin D alone significantly decreased viability starting at 36 h of incubation, but their effects were less compared to those observed when cells were cotreated with anti-Fas (Figure 2).

To verify that MG132 or actinomycin D potentiates anti-Fas mediated chondrocyte apoptosis, and not necrosis, we analyzed cell death by dual annexin V-PI staining. In both MG 132/anti-Fas and actinomycin D/anti-Fas treated samples, increased early apoptosis was evident after 24 h of culture, as it was by MTT assay (Figure 3, Table 1).

Inhibition of anti-Fas mediated apoptosis by caspase inhibitors. To verify the involvement of the caspase cascade in MG132 or actinomycin D potentiated anti-Fas mediated chondrocyte apoptosis, we performed an inhibition assay using various caspase inhibitors. As illustrated in Figure 4, the pan-caspase inhibitor z-VAD-FMK effectively inhibited both MG132 and actinomycin D potentiated cell death. Although Z-IETD-FMK (caspase-8 inhibitor), Z-LEHD-FMK (caspase-9 inhibitor), and Z-DEVD-FMK (caspase-3 inhibitor) all led to significant inhibition of cell death induced by MG132/anti-Fas and actinomycin D/anti-Fas ($p < 0.001$ by paired t test), the degrees of this inhibition were generally lower for actinomycin D potentiated cell death than for MG132 potentiated cell death, implying the involvement of a cell death pathway independent of the classic caspase cascade under these conditions. Caspase-3 processing was observed in immunoblot as early as 6 h after MG132/anti-Fas and actinomycin D/anti-Fas treatment, which preceded apoptotic cell death (Figure 5). However, we were unable to detect either baseline expression or processing of caspase-8 by immunoblot in the control or any of the treated chondrocytes (data not shown).

Modification of cell surface Fas by proteasome inhibitor. The influence of MG132 and actinomycin D on chondrocyte

Table 1. Proportions of dead cells assayed by the trypan blue exclusion assay, MTT assay, annexin V-PI flow cytometry, and DNA fragmentation ELISA after 24 h of incubation (mean ± SD). Chondrocytes from 6 different donors were treated and assayed at the same time. Anti-Fas (CH11) was administered at a concentration of 0.5 µg/ml, actinomycin D at 0.2 µg/ml, and MG132 at 20 µM. The percentage death of control chondrocytes was set at 0% for trypan blue and MTT assay. Negative values denote cell death lower than control. Annexin V positive, PI negative, early apoptotic cells are counted by annexin V-PI flow cytometry. For DNA fragmentation assay, OD at 405 nm indicative of fragmented DNA in the cell lysate is shown.

	Trypan Blue, %	MTT, %	Annexin V-PI Flow Cytometry, %	DNA Fragmentation ELISA (OD 405 nm)
Control	0	0	5.7 ± 3.2	0.004 ± 0.01
Anti-Fas	2.0 ± 5.6	–0.3 ± 5.8	6.9 ± 3.1	0.021 ± 0.03
MG132	14.6 ± 8.2	–3.0 ± 9.5	8.4 ± 3.1	0.016 ± 0.02
MG132/anti-Fas	37.2 ± 14.7	23.1 ± 6.8	22.7 ± 15	0.122 ± 0.07
Actinomycin D	16.1 ± 11.1	10.3 ± 11.0	14.0 ± 5.9	0.183 ± 0.07
Actinomycin D/ anti-Fas	36.7 ± 11.5	26.2 ± 13.2	22.8 ± 12.7	0.390 ± 0.11

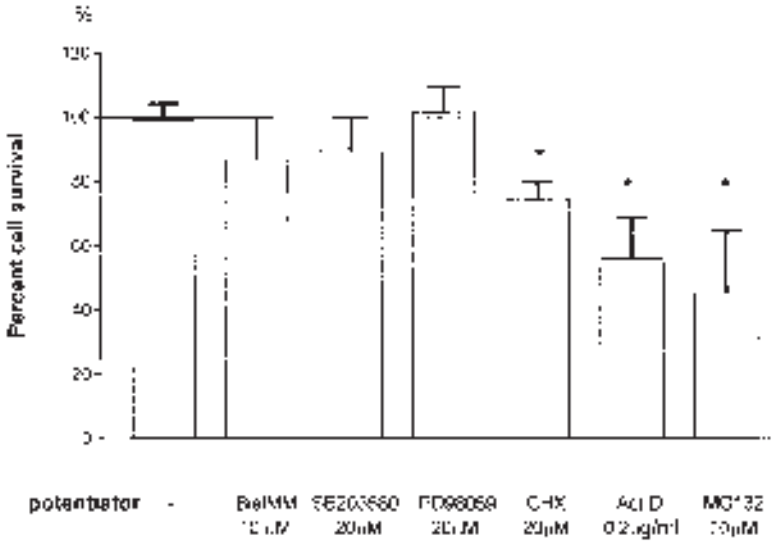
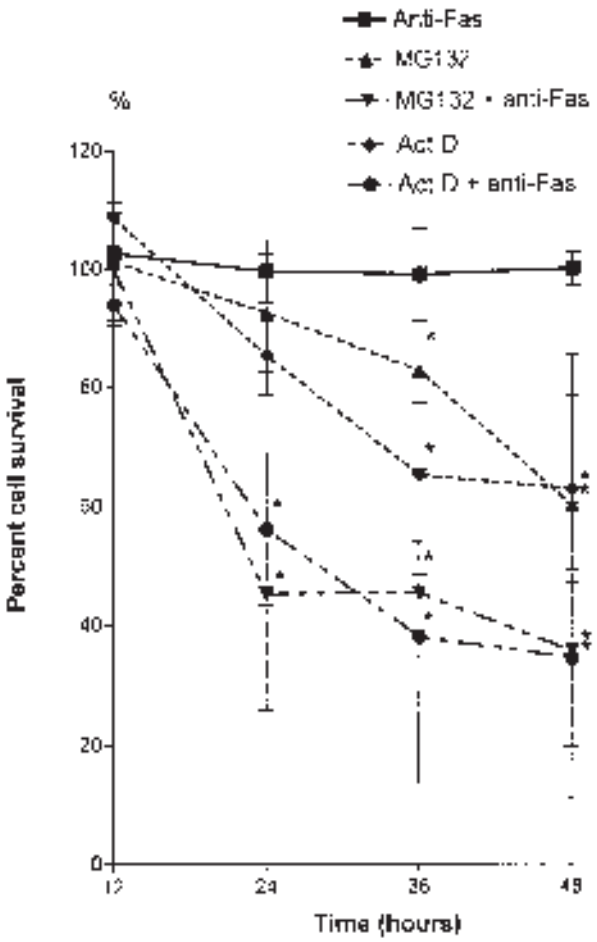


Figure 1. Cell survival (%) after 24 h incubation with various potentiators at the indicated concentrations. Anti-Fas (CH 11) was administered at a concentration of 0.5 µg/ml and the potentiators were added simultaneously. MG132 was added 2 h before anti-Fas because preincubation led to a significant increase in cell death compared to simultaneous incubation. Percentage cell survival was analyzed by MTT assay. Values are mean ± SD of at least 4 samples from different donors. BisIMM: bisindolylmaleimide, CHX: cycloheximide, Act D: actinomycin D. *Statistical significance compared to the control ($p < 0.05$).



Fas surface concentrations was investigated. Cells were incubated with MG132 or actinomycin D for 14 h and the levels of cell surface Fas were determined by flow cytometry after staining with FITC conjugated monoclonal UB2 recognizing human Fas. Compared with untreated cells or actinomycin D treated cells, cells treated with MG132 showed small, but distinct shifts in the distributions of Fas fluorescence (Figure 6).

Effects of MG132 and actinomycin D on expression of apoptosis related molecules in chondrocytes. The Fas cascade is directly inhibited by FLIP, whereas overexpression of Bcl-2 inhibits Fas mediated apoptosis in some cell types[13]. We investigated whether MG132 or actinomycin D influenced the concentration of FLIP and Bcl-2, as well as Bax, the potentiator of cell death in the Bcl-2 family, and Fas ligand, the natural ligand of Fas. Cells were incubated 6, 8, and 14 h under various treatment conditions and the levels of Bcl-2, Bax, FLIP, and Fas ligand were determined by Western blot analysis (Figure 7). Although the expression level of Bax was highest and the expressions of Bcl-2 and Fas ligand were very low in chondrocytes, treatment with MG132 or actinomycin D, alone or in combination with anti-Fas, did not appreciably change the amount of any of these proteins.

Figure 2. Time dependence of MG132/anti-Fas and actinomycin D/anti-Fas on the potentiation of cell death. Anti-Fas (CH 11) was administered at a concentration of 0.5 µg/ml, actinomycin D at 0.2 µg/ml, and MG132 at 20 µM, and percentage cell survival was analyzed by MTT assay. Values are mean ± SD of 8 samples from different donors. Act D: actinomycin D. *Statistical significance compared to the control ($p < 0.05$).

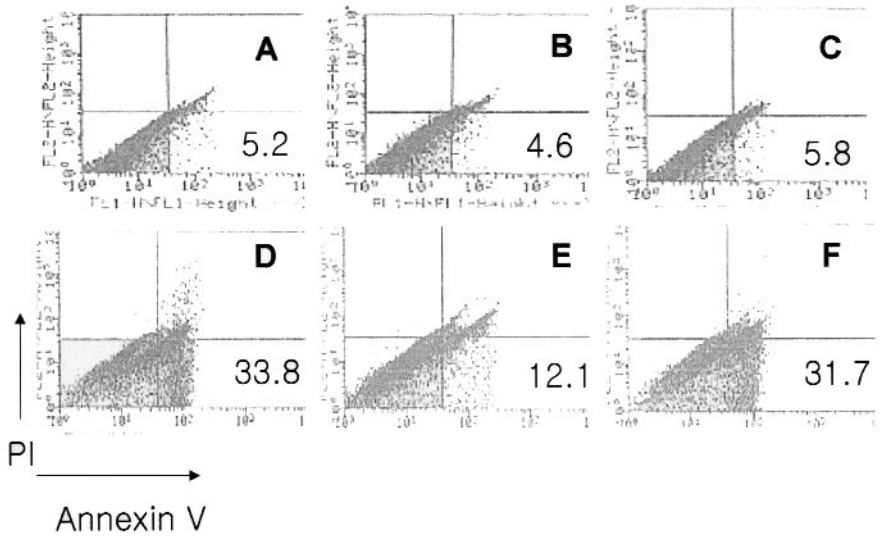
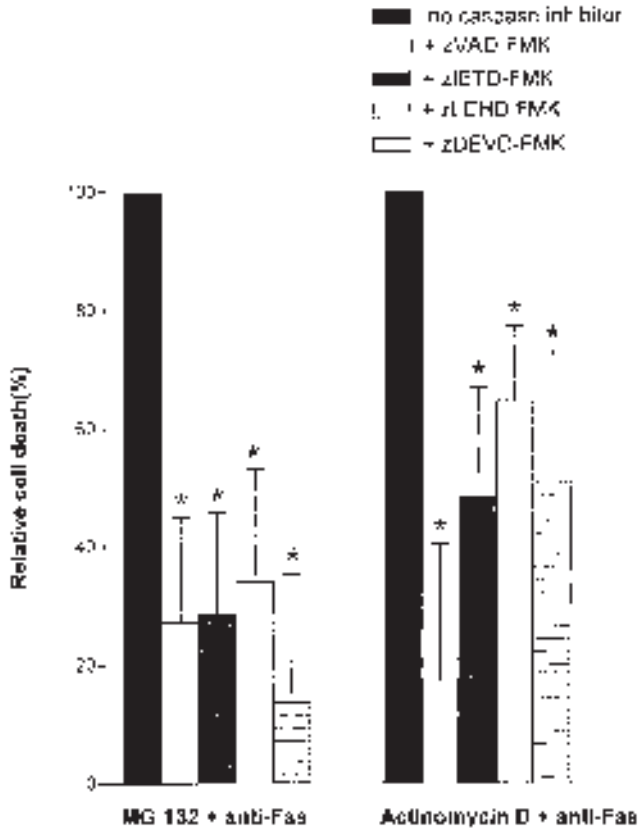


Figure 3. Annexin V–FITC/propidium iodide (PI) dual staining of chondrocytes analyzed by flow cytometry. Cells were incubated with A: control mouse IgM, B: 0.5 µg/ml anti-Fas, C: 20 µM MG132, D: 0.5 µg/ml anti-Fas + 20 µM MG132, E: 0.2 µg/ml actinomycin D, F: 0.5 µg/ml anti-Fas + 0.2 µg/ml actinomycin D, and after 24 h cells were stained as described in the text. Numbers in the right lower quadrant denote percentages of early apoptotic chondrocytes (annexin V positive, PI negative). Data are representative of 15 samples from different donors.



Because recent reports demonstrate p53 induction by proteasome inhibitor or RNA synthesis inhibitor in various cell lines, which in turn is associated with an extensive biological response including the induction of the apoptotic cell death, we investigated the influence of MG132 and actinomycin D on chondrocyte p53 expression[14,15]. We found that treatment with either MG132 or actinomycin D led to significant increases of p53 (Figure 8). Increase of p53 was also evident in samples cotreated with MG132/anti-Fas and actinomycin D/anti-Fas. Thus the expressions of the p53 response proteins MDM2 and p21 in response to MG132 or actinomycin D were determined. As observed with p53, treatment of chondrocytes with MG132 led to a prompt increase (within 1 h) of both MDM2 and p21 (Figure 8). However, treatment of chondrocytes with actinomycin D did not lead to any significant induction of MDM2 or p21.

DISCUSSION

Apoptosis occurs in the articular cartilages in human OA and RA as well as in aging animal cartilages[16], and is recognized as an important process not only in the remodeling of the cartilage endplate, but also in joint diseases. We focused

Figure 4. Inhibition of MG132/anti-Fas and actinomycin D/anti-Fas mediated chondrocyte apoptosis by various caspase inhibitors. Chondrocytes were incubated with 100 µM of each caspase inhibitor 1 h before anti-Fas treatment and cell death was analyzed after 24 h using the DNA fragmentation ELISA. Optical density (OD) at 405 nm indicating the amount of DNA fragment induced by MG132/anti-Fas and actinomycin D/anti-Fas was set at 100%. Values are means ± SD of 4 samples from different donors. *$p < 0.001$ compared to control by paired t test.

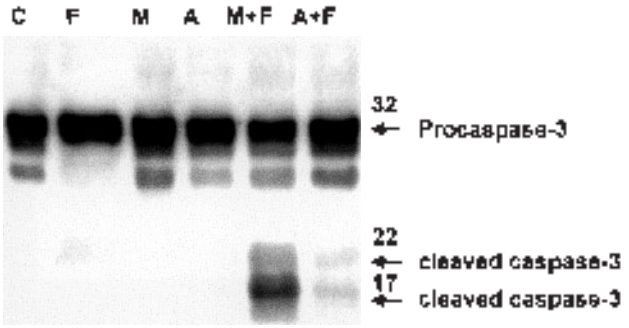


Figure 5. Processing of caspase-3 by MG132/anti-Fas and actinomycin D/anti-Fas. Anti-Fas (CH 11) was administered at a concentration of 0.5 µg/ml, actinomycin D at 0.2 µg/ml, and MG132 at 20 µM. Cells were incubated 6 h and the lysates subjected to Western blot analysis with antibody, which recognizes both the pro and active form of caspase-3. C: control IgM incubation, F: anti-Fas, M: MG132, A: actinomycin D. Molecular sizes are indicated as kDa.

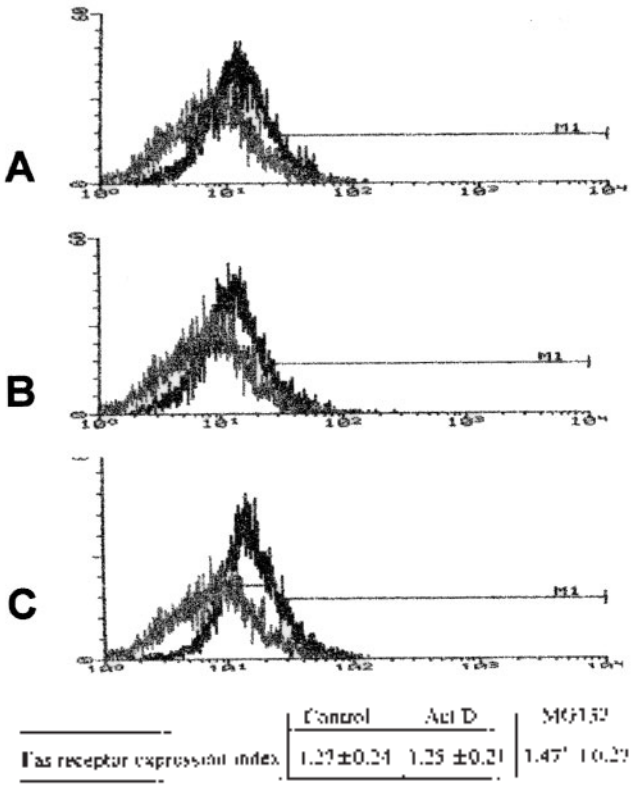


Figure 6. Effects of MG132 and actinomycin D on cell surface Fas. Chondrocytes were incubated 14 h with A: control mouse IgM, B: 0.2 µg/ml actinomycin D, and C: 20 µM MG132. Cells were detached from the culture plate and stained for Fas with FITC conjugated UB2 antibody. FITC labeled control mouse IgG stained chondrocytes were used as the unstained control (gray line trace). Fluorescence was measured by flow cytometry. Data are representative of 6 samples from different donors. The Fas receptor expression index is the ratio of mean fluorescence index from treated chondrocytes (black line trace) and the control IgG stained chondrocytes (gray trace). * p = 0.004 compared to control.

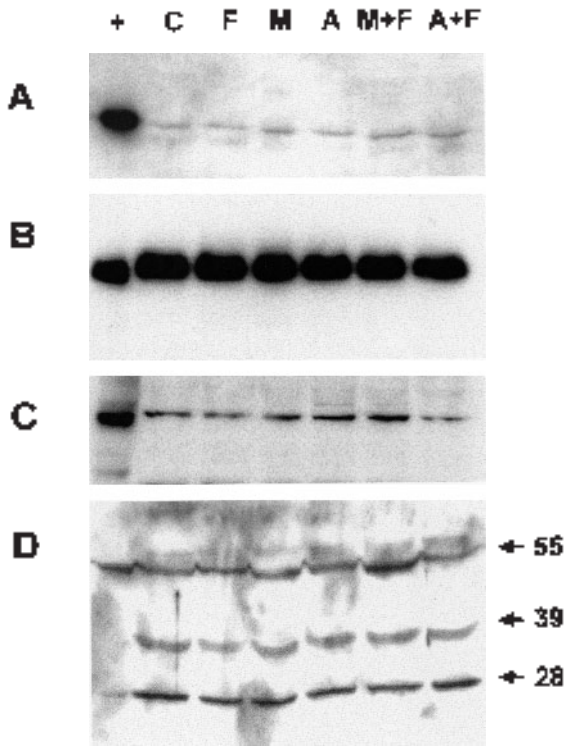


Figure 7. Western blot analysis of (A) Bcl-2, (B) Bax, (C) Fas ligand, (D) FLIP. Anti-Fas (CH 11) was administered at a concentration of 0.5 µg/ml, actinomycin D 0.2 µg/ml, and MG132 20 µM. Cells were incubated 6 h and lysed and the lysates were subjected to Western blot analysis, as described in Materials and Methods. +: positive control, C: control IgM incubation, F: anti-Fas, M: MG132, A: actinomycin D. For FLIP, molecular sizes indicated as kDa.

on cellular factors that make human articular chondrocytes susceptible to Fas mediated apoptosis. Although signal transduction of the Fas mediated cell death pathway has been extensively studied, there are few data regarding the mechanism of Fas mediated apoptosis in articular chondrocytes. In contrast to previous reports[4,17-19], we identified little chondrocyte death induced by agonistic anti-Fas antibody alone. Because the MTT colorimetric assay used might not have detected low levels of apoptosis induced by anti-Fas, we also used the trypan blue exclusion assay, annexin V-PI dual flow cytometry, and DNA fragmentation ELISA, and compared results from the same samples. Although the MTT assay and the trypan blue exclusion assays failed to show significant cell death in anti-Fas treated chondrocytes versus the control, even after prolonged incubation, flow cytometry detected minute numbers of annexin V positive-PI negative early apoptotic chondrocytes in some of the anti-Fas treated samples. In addition, DNA fragmentation ELISA revealed a small but consistent increase of DNA fragments in anti-Fas treated chondrocytes. These methodological differences for detection of apoptosis might explain the discrepancy in reports on anti-Fas mediated chondrocyte apoptosis[4,17-20]. It is notable that the majority of reports

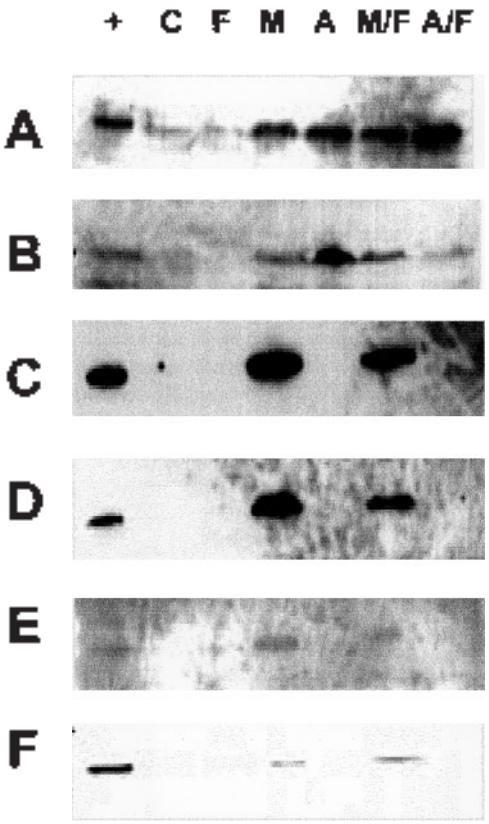


Figure 8. Western blot analysis of (A, B) p53, (C, D) p21, (E, F) MDM2. Cells were incubated with the indicated agents for 1 h (A, C, E) or 6 h (B, D, F). Anti-Fas (CH 11) was administered at a concentration of 0.5 µg/ml, actinomycin D 0.2 µg/ml, and MG132 20 µM. The cells were then lysed and the lysates were subjected to Western blot analysis, as described in Materials and Methods. +: positive control, C: control IgM incubation, F: anti-Fas, M: MG132, A: actinomycin D. Molecular sizes indicated as kDa.

showing positive results for anti-Fas mediated chondrocyte apoptosis utilized methodologies based on the detection of DNA fragmentation, such as DNA fragmentation ELISA, or TUNEL analysis[17-19]. It has been reported that the processes of DNA fragmentation and cell death may be independent in some apoptotic processes[21], and the significance of DNA fragmentation in the absence of a metabolic disturbance indicative of cell death is uncertain. We do not believe that agonistic anti-Fas alone induces a significant amount of cell death in chondrocytes *in vitro*, and this is in accord with animal studies that showed no sign of cartilage degradation in anti-Fas injected arthritic mouse joints[22]. Anti-Fas therapy for inflammatory arthritis may not have a significant influence on cartilage integrity in this regard.

The time curve of cell death showed that anti-Fas mediated chondrocyte death, potentiated by MG132 or actinomycin D, is a slow process. Chondrocyte death induced by both MG132/anti-Fas and actinomycin D/anti-Fas was mainly apoptotic in character, as revealed by a preponderance of annexin V positive-PI negative cells during the early period of cell death, the processing of caspase-3, and the inhibition by caspase inhibitors. The pan-caspase inhibitor zVAD-FMK was efficient at inhibiting the chondrocyte death induced by both MG132/anti-Fas and actinomycin D/anti-Fas. The slightly lower inhibition by other caspase inhibitors compared to pan-caspase inhibitor may be due to the requirement of multiple caspases for cell death or different efficiency of entry into the cell of each tetrapeptide. Inhibition by z-IETD-FMK (caspase-8 inhibitor), z-LEHD-FMK (caspase-9 inhibitor), and z-DEVD-FMK (caspase-3 inhibitor) of actinomycin D/anti-Fas induced apoptosis tended to be less efficient than that of MG132/anti-Fas induced apoptosis. This phenomenon could be explained by: (1) necrosis partially accounting for actinomycin D/anti-Fas induced chondrocyte death; or (2) actinomycin D/anti-Fas induced apoptosis being mediated by a pathway independent of classical caspase cascade; or (3) chondrocyte apoptosis induced by actinomycin D/anti-Fas being switched to necrotic death in the presence of caspase inhibitors.

The elevation of cell surface Fas enhances death by Fas L in some cell types[23], but it is unknown whether an increase in cell surface Fas is sufficient to trigger the death of chondrocytes by anti-Fas antibody. This study shows that MG132 leads to a slight but significant increase of chondrocyte surface Fas expression after 14 h incubation. Actinomycin D did not increase surface Fas expression, implying that a surface Fas increase may not be the sole mechanism responsible for the facilitation of anti-Fas mediated apoptosis.

Masuko-Hongo, *et al* suggested that the constitutive expression of FLIP is responsible for the resistance of chondrocytes to Fas mediated apoptosis[20]. In our experiment, neither MG132 nor actinomycin D treatment was found to affect the levels of FLIP expression. The expressions of Bcl-2, Bax, and Fas ligand were also unaffected. On the other hand, the expression of p53 was markedly increased after both MG132 and actinomycin D treatment. We then studied the expression of MDM2 and p21, which are induced by the activation of p53, and found that MG132 increased the expressions of MDM2 and p21 in chondrocytes. However, actinomycin D did not affect the MDM2/p21 concentrations, and as noted above, another p53 responsive gene product, Bax, was unaffected by either MG132 or actinomycin D. The function of p53 is well delineated, and it is accepted that once activated by cellular stress, p53 mediates growth arrest and apoptosis by activating a number of cellular genes[24]. The ubiquitin-proteasome pathway may play several important roles in the p53 pathway, leading to cell cycle arrest or apoptosis. Transcriptional targets of p53 include the gene $p21^{WAF1/CIP1}$ (p21), which encodes a protein that functions as a cell cycle inhibitor, by complexing with and inactivating cyclin dependent kinase complexes[25]. The overexpression of p21 inhibits cell growth by causing cell

cycle arrest. As well, p21 has a potential role in the regulation of DNA replication and repair[26]. It is uncertain whether p21 is required in the promotion of p53 dependent apoptosis, because mice harboring mutant p21 alleles are competent in terms of this process[27]. Similarly, it is uncertain whether MDM2, which encodes an inhibitor of p53 transactivation, and thus acts to create a feedback loop that regulates p53 function[28], acts as an effector molecule of chondrocyte apoptosis. Indeed, no p53 target identified and tested to date (for example, p21, MDM2, Fas, and Bax) is *singly* responsible for p53 dependent apoptosis[26]. Because actinomycin D did not affect the expression of MDM2 or p21 despite the accumulation of p53, another divergent pathway, in addition to surface Fas expression and the caspase cascade, might act in the MG132 or actinomycin D facilitated Fas mediated signaling of chondrocyte apoptosis.

Recently, the role of nuclear factor-κB activation in anti-Fas induced chondrocyte apoptosis has been suggested by a study that employed the proteasome inhibitors MG132 and PS1[29]. Since nuclear factor-κB (NF-κB) activation is known to protect cells from the cytotoxic effects of tumor necrosis factor-α (TNF-α) in a variety of cell lines[30], and since some cells respond to agonistic CD95 antibody by the translocation of NF-κB[29] to the nucleus, the inhibition NF-κB inhibitory protein (IκB) degradation by proteasome inhibitor seems a plausible mechanism for the potentiation of anti-Fas mediated chondrocyte apoptosis. However, proteasome inhibitors including MG132 possess a wide spectrum of cellular target molecules and, as in the case of p53 and MDM2/p21 accumulation, demonstration of NF-κB inhibition *per se* is not sufficient evidence to prove the direct executionary role of proteasome inhibitors in chondrocyte apoptosis. Recently, we employed a strategy involving transfection of a chondrocyte cell line with adenovirus IκB super-repressor, which suppresses NF-κB specifically, and observed that suppression of NF-κB does not render this cell line susceptible to anti-Fas mediated death (unpublished observations).

Our results suggest that chondrocytes can be rendered sensitive to anti-Fas mediated death by MG132 and actinomycin D. MG132 and actinomycin D show different characteristics in terms of apoptosis signaling: (1) Although pan-caspase inhibitor effectively inhibits both MG132 and actinomycin D facilitated apoptosis, caspase-3, -8, -9 inhibitors more effectively inhibit the MG132 facilitated pathway. (2) MG132 moderately increases surface Fas expression, whereas actinomycin D does not. (3) Both MG132 and actinomycin D increase p53 expression, but only MG132 leads to accumulation of the p53 targets MDM2 and p21. These results suggest that proteasome inhibition and RNA synthesis inhibition sensitize chondrocytes to anti-Fas induced apoptosis through distinct cell death pathways.

REFERENCES

1. Kim HA, Song YW. Apoptotic chondrocyte death in rheumatoid arthritis. Arthritis Rheum 1999;42:1528-37.
2. Blanco FJ, Guitian R, Vazquez-Martul E, de Toro FJ, Galdo F. Osteoarthritis chondrocytes die by apoptosis. A possible pathway for osteoarthritis pathology. Arthritis Rheum 1998;41:284-9.
3. Blanco FJ, Ochs RL, Schwarz H, Lotz M. Chondrocyte apoptosis induced by nitric oxide. Am J Pathol 1995;146:75-85.
4. Hashimoto S, Setareh M, Ochs RL, Lotz M. Fas/Fas ligand expression and induction of apoptosis in chondrocytes. Arthritis Rheum 1997;40:1749-55.
5. Sabatini M, Rolland G, Leonce S, et al. Effects of ceramide on apoptosis, proteoglycan degradation, and matrix metalloproteinase expression in rabbit articular cartilage. Biochem Biophys Res Commun 2000;267:438-44.
6. Sakai K, Matsuno H, Morita I, et al. Potential withdrawal of rheumatoid synovium by the induction of apoptosis using a novel in vivo model of rheumatoid arthritis. Arthritis Rheum 1998; 41:1251-7.
7. Zhang H, Yang Y, Horton JL, et al. Amelioration of collagen-induced arthritis by CD95 (Apo-1/Fas)-ligand gene transfer. J Clin Invest 1997;100:1951-7.
8. Ehl S, Hoffmann-Rohrer U, Nagata S, Hengartner H, Zinkernagel R. Different susceptibility of cytotoxic T cells to CD95 (Fas/Apo-1) ligand-mediated cell death after activation in vitro versus in vivo. J Immunol 1996;156:2357-60.
9. Zhou T, Song L, Yang P, Wang Z, Lui D, Jope RS. Bisindolylmaleimide VIII facilitates Fas-mediated apoptosis and inhibits T cell-mediated autoimmune diseases. Nature Med 1999;5:42-8.
10. Kim HA, Lee YJ, Seong SC, Choe KW, Song YW. Apoptotic chondrocyte death in human osteoarthritis. J Rheumatol 2000;27:455-62.
11. Kim HA, Suh D, Song YW. Relationship between chondrocyte apoptosis and matrix depletion in human articular cartilage. J Rheumatol 2001;28:2038-45.
12. Xu Y, Bialik S, Jones BE, et al. NF-kappa B inactivation converts a hepatocyte cell line TNF-alpha response from proliferation to apoptosis. Am J Physiol 1998;275:C1058-66.
13. Roy S, Nicholson D. Cross-talk in cell death signaling. J Exp Med 2000;192:F21-5.
14. Hietanen S, Lain S, Krausz E, Blattner C, Lane DP. Activation of p53 in cervical carcinoma cells by small molecules. Proc Natl Acad Sci USA 2000;97:8501-6.
15. Ljungman M, Zhang F, Chen F, Rainbow AJ, McKay BC. Inhibition of RNA polymerase II as a trigger for the p53 response. Oncogene 1999;18:583-92.
16. Adams CS, Horton WE. Chondrocyte apoptosis increases with age in the articular cartilage of adult animals. Anat Rec 1998;250:418-25.
17. Kuhn K, Hashimoto S, Lotz M. Cell density modulates apoptosis in human articular chondrocytes. J Cell Physiol 1999;180:439-47.
18. Kuhn K, Hashimoto S, Lotz M. IL-1b protects human chondrocytes from CD95-induced apoptosis. J Immunol 2000;164:2233-9.
19. Lisignoli G, Grassi F, Zini N, et al. Anti-Fas induced apoptosis in chondrocytes reduced by hyaluronan. Arthritis Rheum 2001;44:1800-7.
20. Masuko-Hongo K, Sakata M, Yuan GH, et al. Expression of Fas-associated death domain-like interleukin 1b converting enzyme (FLICE) inhibitory protein (FLIP) in human articular chondrocytes: possible contribution to the resistance to FAS-mediated death of in vitro cultured chondrocytes. Rheumatol Int 2001;21:112-21.
21. Lai JH, Ho LJ, Lu KC, Chang DM, Shaio MF, Han SH. Western and Chinese anti-rheumatic drug-induced T cell apoptotic damage uses different caspase cascades and is independent of Fas/Fas ligand interaction. J Immunol 2001;166:6914-24.

22. Fujisawa K, Asahara H, Okamoto K, et al. Therapeutic effect of the anti-Fas antibody on arthritis in HTLV-1 tax transgenic mice. J Clin Invest 1996;98:271-8.
23. Itoh N, Yonehara S, Ishii A, et al. The polypeptide encoded by the cDNA for human surface Fas antigen can mediate apoptosis. Cell 1991;66:233-43.
24. Chang Y, Lee Y, Tejima T, et al. Mdm2 and bax, downstream mediators of the p53 response, are degraded by the ubiquitin-proteasome pathway. Cell Growth Diff 1998;9:79-84.
25. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 1993;75:805-16.
26. Reinke V, Lozano G. The p53 targets mdm2 and Fas are not required as mediators of apoptosis in vivo. Oncogene 1997; 15:1527-34.
27. Deng C, Zhang P, Harper JW, Eledge SJ, Leder P. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. Cell 1995;82:675-84.
28. Barak Y, Juven T, Haffner R, Oren M. Mdm2 expression is induced by wild type p53 activity. EMBO J 1993;12:461-8.
29. Kuhn K, Lotz M. Regulation of CD95-induced apoptosis in human chondrocytes. Arthritis Rheum 2001;44:1644-53.
30. Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM. Suppression of TNF-a-induced apoptosis by NF-kB. Science 1996;274:787-9.