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Fas-Mediated Stimulation Induces IL-8 Secretion by Rheumatoid Arthritis Synoviocytes Independently of CPP32-Mediated Apoptosis

Chiyoko Sekine,* Hideo Yagita,* Tetsuji Kobata,* Tomoko Hasunuma,†
Kusuki Nishioka,† and Ko Okumura*

**Department of Immunology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113, Japan; and †St. Marianna University, Kawasaki, Japan*

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In this study, we investigated the IL-1β converting enzyme (ICE) family cysteine proteases responsible for the Fas-mediated apoptosis of rheumatoid arthritis (RA) synoviocytes and their involvement in proinflammatory cytokine production. CPP32 inhibitor, but not ICE inhibitor, was capable of inhibiting the Fas-mediated apoptosis of RA synovial cells. CPP32, but not ICE, was activated in response to anti-Fas stimulation. IL-8, but not IL-1β, was secreted from the anti-Fas-stimulated RA synoviocytes even in the presence of CPP32 inhibitor. These results demonstrated that CPP32, but not ICE, is the predominant cysteine protease that mediates the Fas-mediated apoptosis of RA synovial cells. We also demonstrated that anti-Fas stimulation of RA synoviocytes leads to IL-8 secretion independently of the CPP32-mediated apoptosis, which would accelerate inflammation. © 1996 Academic Press, Inc.

Rheumatoid arthritis (RA) is considered to be a proliferative disorder of synovial tissue, which is accompanied by inflammatory cell infiltration and bone erosion. RA is also characterized by regression of the hyperplastic synovial cells with subsequent replacement by fibrotic tissue that leads to the joint dysfunction, but the mechanisms of regression and replacement remain obscure. We previously demonstrated that typical apoptotic cell death was evident in synovial tissues from RA patients and that RA synoviocytes underwent apoptosis upon ligation of the Fas (APO-1/CD95) antigen *in vitro* (1). This suggests that Fas-mediated apoptosis may be involved in the regression of synovial cells in RA.

Recently, several members of a new family of cysteine proteases have been implicated in various modes of apoptotic cell death, which include ICE (2), CPP32 (apopain) (3), and the *Caenohabditis elegans Ced-3* cell death gene product (4). Based on structural homology, these enzymes have a similar and unique structure that is unrelated to classical cysteine proteases (5, 6). These enzymes are synthesized as inactive proenzymes and activated by proteolytic cleavage to generate two polypeptide subunits. ICE was first identified as a cysteine protease that cleaves pro-IL-1β to generate mature, biologically active IL-1β (7, 8). Emerging evidences indicated that these ICE family proteases play an important role in controlling apoptosis, including that mediated by Fas. It has been reported that Fas-mediated apoptosis of some cell lines was inhibited by ICE-specific inhibitors(9, 10). Moreover, thymocytes from ICE-deficient mice were resistant to the Fas-mediated apoptosis(11).

If ICE is generally involved in the Fas-mediated apoptosis, we speculated that it may lead to IL-1β release from apoptotic RA synoviocytes by promoting the processing of pro-IL-1β, which would accelerate the inflammation. In this context, we examined the ICE family proteases involved in the Fas-mediated apoptosis of RA synoviocytes in association with proinflammatory cytokine production. Although ICE was not critical for the Fas-mediated apoptosis of RA

All correspondence should be addressed to Dr. Ko Okumura. Fax: 81-3-3813-0421.

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synoviocytes and IL-1β was not released, we found that RA synoviocytes secret IL-8 upon anti-Fas stimulation independently of the CPP32-mediated apoptosis.

MATERIALS AND METHODS

Cells. Cultured synovial cells were prepared from several RA patients as described previously (1) and maintained in Dulbecco's modified Eagle's medium (DMEM ; Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS ; JRH Biosciences, Lenaxa, KS), 2 mM L-glutamine (WAKO, Osaka, Japan), 100 units/ml penicillin, and 100 μg/ml streptomycin.

Cytotoxicity assay. RA synovial cells (10^4 cells/well) were allowed to attach overnight in 96-well flat-bottom microtiter plates. On the following day, anti-Fas monoclonal antibody (mAb) (CH-11 ; MBL, Nagoya, Japan) or control mouse IgM (PharMingen, San Diego, CA) was added at a concentration of 1 μg/ml. To some wells, an ICE-specific inhibitor, Ac-Tyr-Val-Ala-Asp-aldehyde (YVAD-CHO ; Peptide Institute,Inc., Osaka, Japan) (8, 12) and/or a CPP32-specific inhibitor, Ac-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO ; Peptide Institute,Inc.) (3) were also added at a concentration of 500 μM. After 20 hours, supernatants were harvested and then 100 μl of medium and 10 μl of Alamar Blue (Biosource International, Camarillo, CA) were added and mixed well. After 2 hours, fluorescence was measured on FluoroskanII (Labsystems, Helsinki, Finland) using an excitation wavelength of 544 nm and emission wavelength of 590 nm. Percent cytotoxicity was calculated as follows : $(1 - \text{test wells} / \text{medium alone}) \times 100$.

Cytokine analyses. Concentrations of IL-1β and IL-8 in the culture supernatants were measured by using ELISA kits (Genzyme, Cambridge, MA) according to the manufacturer's instructions. The IL-1β ELISA used is specific for mature IL-1β and does not detect pro-IL-1β.

Enzyme activity assay. RA synovial cells (5×10^4 cells/well) were allowed to attach overnight in a 24-well plate. On the following day, CH-11 or control IgM (1 μg/ml) was added and incubated for the indicated periods. After brief trypsinization, collected cells were lysed in a lysis buffer (0.5% Nonidet P-40, 0.5 mM EDTA, 150 mM NaCl, 50 mM Tris, pH7.5). The lysates were centrifuged at 15,000 $\times$ *g* for 10 min., and the supernatants were collected. Fifty μl aliquots of the extracts were incubated with fluorogenic peptide substrates YVAD-MCA (YVAD-4-methyl-coumaryl-7-amide) specific for ICE (8, 12, 13) or DEVD-MCA specific for CPP32 (3, 13) (Peptide Institute, Inc.) at final concentration of 10 μM in 200 μl reaction buffer (20 mM HEPES, pH7.5, 0.1M NaCl, 5mM DTT) for 2 hours, and the fluorescence of released AMC (7-amino-4-methyl-coumarin) was measured on FluoroskanII using an excitation wavelength of 355 nm and emission wavelength of 460 nm. One units/ml was defined as the enzyme activity that liberates 10 μmol/ml of AMC during 2 hours.

RESULTS

Effect of ICE or CPP32 inhibitor on Fas-mediated apoptosis of RA synovial cells. As we previously demonstrated (1), ligation of the Fas antigen on cultured RA synovial cells by an anti-Fas mAb led to apoptotic cell death in 60-80 % of the cells (Figure 1). This cytotoxicity induced by anti-Fas mAb was strongly inhibited by a CPP32-specific inhibitor (DEVD-CHO), but not by an ICE-specific inhibitor (YVAD-CHO). No further inhibition was observed with the combination of both YVAD-CHO and DEVD-CHO as compared to DEVD-CHO alone. This indicates that CPP32, but not ICE, is the predominant mediator of the Fas-mediated apoptosis in RA synovial cells.

Activation of ICE and CPP32 by anti-Fas stimulation. It has been reported that triggering of Fas rapidly activated the ICE activity in a human B-lymphoblastoid cell line (10). We then examined the enzymatic activities of ICE and CPP32 in the cell lysates of anti-Fas-stimulated RA synovial cells by using fluorogenic peptide substrates, YVAD-MCA for ICE and DEVD-MCA for CPP32. As shown in Figure 2, a striking increase in activity was observed at 4 hours after the anti-Fas stimulation and this activity was decreased at 8 hours. In contrast, no increase in ICE activity was observed. This indicates that CPP32, but not ICE, is specifically activated in RA synovial cells upon ligation of Fas.

IL-1β release in response to Fas ligation. To further explore the participation of ICE in Fas-mediated apoptosis, we examined the release of mature IL-1β from anti-Fas-stimulated RA synoviocytes. As estimated by reverse transcription-polymerase chain reaction (RT-PCR), all three RA synovial cells tested (KM, FA, and CB) constitutively expressed IL-1β mRNA (data not shown), suggesting that they expressed pro-IL-1β. However, no spontaneous or anti-Fas-induced secretion of mature IL-1β into the supernatant was detectable (Table 1). This

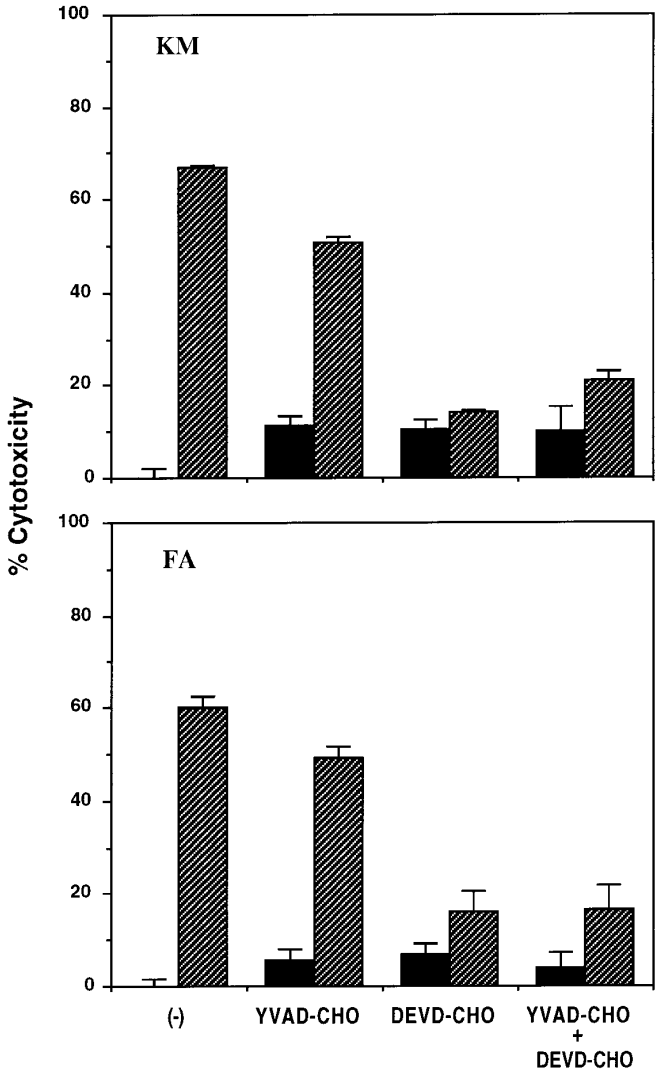

FIG. 1. Inhibition of Fas-mediated apoptosis by ICE and/or CPP32 inhibitors. RA synovial cells derived from two different patients (KM and FA) were cultured with or without 1 μg/ml of anti-Fas antibody (CH-11) (hatched bars) or control IgM (closed bars) in the presence or absence of ICE (YVAD-CHO) and/or CPP32 (DEVD-CHO) inhibitors (500 μM each) for 20 hours. Residual viable cells were measured by the Alamr Blue method. Percent cytotoxicity was calculated as described in Materials and Methods. The data represent means ± SD of triplicate samples. Similar results were obtained in three independent experiments.

further indicates no significant contribution of ICE to Fas-mediated cellular responses in RA synoviocytes.

IL-8 secretion in response to Fas ligation. It has been reported that the ligation of Fas by anti-Fas mAb led to IL-8 release from a human colon epithelial HT-29 cells (14), which represented another function mediated by Fas than apoptosis. Therefore, we also examined the IL-8 secretion from the anti-Fas-stimulated RA synovial cells. Although RA synoviocytes secreted substantial amounts of IL-8 spontaneously, anti-Fas stimulation greatly enhanced the IL-8 secretion (Table 1). We next examined whether this IL-8 secretion is accompanied with the CPP32-mediated apoptosis. As indicated in Figure 3, the CPP32 inhibitor did not inhibit

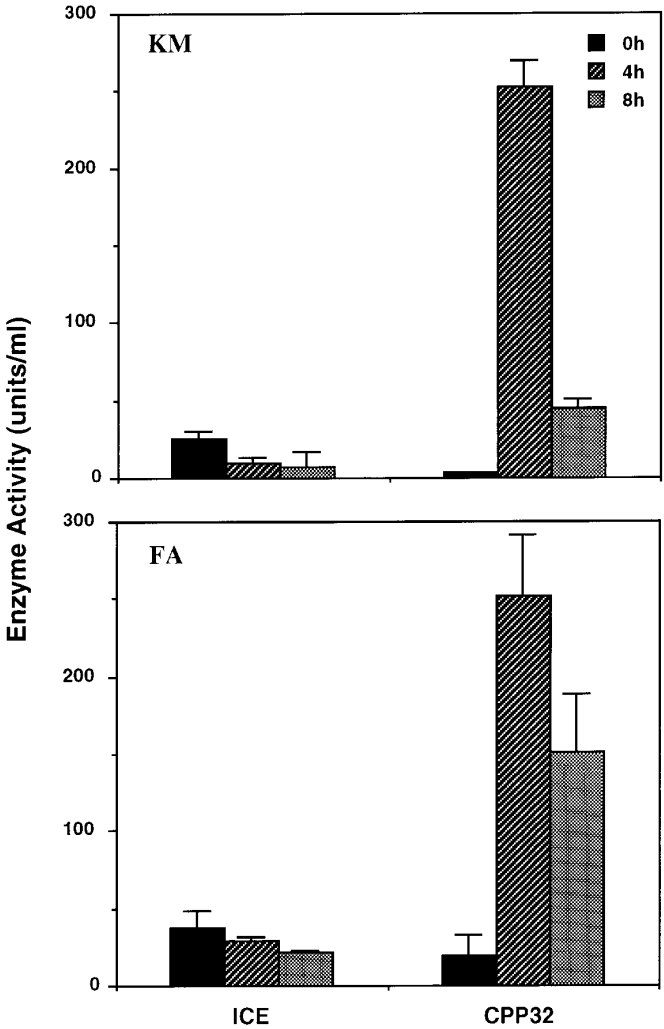

FIG. 2. Activation of ICE and CPP32 by anti-Fas stimulation. RA synovial cells derived from two different patients (KM and FA) were cultured with CH-11 (1 μg/ml) for the indicated periods. ICE and CPP32 activities in the cell lysates were measured by using YVAD-MCA or DEVD-MCA as specific substrates, respectively. The data represent means $\pm$ SD of triplicate samples. Similar results were obtained in three independent experiments.

the IL-8 secretion from the anti-Fas-stimulated RA synovial cells but rather enhanced it, which appears to result from the prevention of apoptotic cell death of anti-Fas-stimulated IL-8-producing cells. This clearly indicates that Fas-mediated stimulation of RA synovial cells leads to IL-8 secretion independently of the CPP32-mediated apoptosis.

DISCUSSION

IL-1β-converting enzyme (ICE), which proteolytically processes pro-IL-1β into biologically active mature IL-1β, has been implicated in various modes of apoptosis, including that mediated by Fas (2, 9, 10). It has been reported that IL-1β was released from apoptotic, but not necrotic, macrophages (15) and from Hela cells undergoing TNF-induced apoptosis (16), suggesting that ICE was activated in these apoptotic cells. IL-1β released from apoptotic cells is considered to play an important physiological role for the recruitment and activation of phagocytes that

TABLE 1
IL-1β and IL-8 Secretion by Anti-Fas-Stimulated Synovial Cells

	IL-1β (pg/ml)		IL-8 (pg/ml)	
Cells	IgM	CH-11	IgM	CH-11
KM	<60	<60	1385 ± 68	3957 ± 483
FA	<60	<60	96 ± 6	377 ± 75
CB	<60	<60	775 ± 66	2328 ± 284

RA synovial cells derived from three different patients (KM, FA, and CB) were cultured with CH-11 or control IgM (1 μg/ml) for 20 hours. IL-1β and IL-8 concentrations in the cell-free supernatants were measured by ELISA. The data represent means ± SD of triplicate samples.

scavenge the resulting apoptotic bodies. Furthermore, it may also act pathogenically in various pathological situations including RA. We previously found a massive apoptotic cell death in synovial tissues from RA patients in which Fas on the synovial cells may participate (1). Since ICE has been implicated in the Fas-mediated apoptosis of some cell lines (9, 10), we were interested in testing whether ICE was activated and IL-1β was released from RA synovial cells undergoing Fas-mediated apoptosis. Although the cultured RA synoviocytes constitutively expressed IL-1β mRNA and thus potentially pro-IL-1β, no ICE activity or IL-1β release was induced in the apoptotic RA synovial cells upon Fas ligation. It remains to be determined whether this resulted from no expression of pro-ICE or no activation of pro-ICE in these cells.

As demonstrated in the recently developed ICE-deficient mice, ICE appears not to be solely the predominant mediator of various modes of apoptosis, except for the Fas-mediated apoptosis of thymocytes (11, 17). A more recently identified ICE-like cystein protease, called CPP32/Yama or apopain, has been regarded as the mammalian homologue of the *C. elegans* death gene product, CED-3 (3, 18). We then examined the involvement of CPP32 in the Fas-mediated apoptosis of RA synovial cells. As indicated Figure 1 and Figure 2, CPP32 but not ICE was activated in response to Fas ligation and the anti-Fas-induced apoptosis was blocked by the CPP32 inhibitor but not by the ICE inhibitor. These results clearly indicated that CPP32 is the predominant mediator of the Fas-mediated apoptosis of RA synovial cells. It has been known that CPP32 cleaves poly(ADP-ribose) polymerase (PARP), that appears to be involved in DNA repair, genome surveillance and integrity, but not pro-IL-1β. This is consistent with our present observation that IL-1β was not released from the anti-Fas-stimulated RA synoviocytes undergoing the CPP32-mediated apoptosis. We previously demonstrated that OA synovial cells also expressed the Fas antigen but were rather resistant to the anti-Fas-induced apoptosis. It remains to be determined whether CPP32 would not be activated by the Fas ligation in OA synovial cells.

Fas is a typeI integral membrane protein, which belongs to the TNF receptor (TNFR) family, including TNFRI, TNFRII, and CD40 (19, 20). It has been well known that TNF-α induces the production of various cytokines by RA synovial cells (21, 22). Recently, Abreu-Martin *et al.* demonstrated that the stimulation with anti-Fas mAb as well as TNF-α induced IL-8 secretion by a human colon epithelial cell line (14). We also found that RA synovial cells secrete IL-8 upon Fas ligation (Table 1). Interestingly, this IL-8 secretion occurred independently of apoptosis since the CPP32 inhibitors efficiently inhibited the Fas-mediated apoptosis (Figure 1) but not the anti-Fas-induced IL-8 secretion (Figure 3). Therefore, the Fas-mediated signal appears to diverge to two pathways, one of which leads to the CPP32-mediated apoptosis and the other leads to the IL-8 secretion independently of apoptosis. It has been reported that TNF-

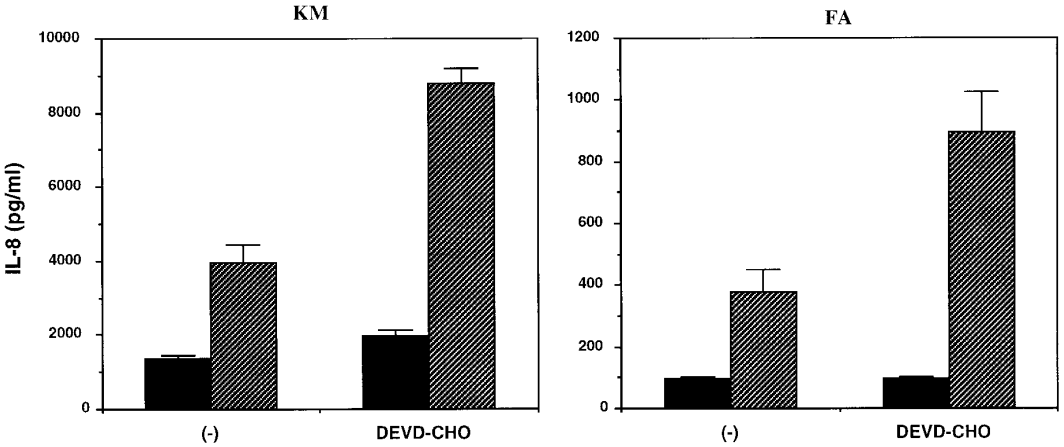

FIG. 3. Effect of CPP32 inhibitor on IL-8 secretion by anti-Fas-stimulated synovial cells. RA synovial cells derived from two different patients (KM and FA) were cultured with 1 μg/ml of CH-11 (hatched bars) or control IgM (closed bars) in the presence or absence of CPP32 inhibitor (DEVD-CHO, 500 μM) for 20 hours. IL-8 concentration in the supernatants was measured by ELISA. The data represent means ± SD of triplicate samples. Similar results were obtained in three independent experiments.

α induces IL-8 secretion by a variety of cells (23) and that CD40-mediated stimulation also enhances the IL-8 secretion by monocytes (24). TNF-α has been shown to induce the IL-8 secretion by transcriptional activation of the IL-8 gene via NF-κB activation (25, 26) and the CD40-mediated signal has been also shown to activate NF-κB (27). Although the ligation of Fas has been reported to activate NF-κB in a SV40-transformed human fibroblast cell line (28) but not in a murine fibloblast cell line (29), this remains to be determined with RA synovial cells.

As we previously discussed (1), Fas may be involved in the apoptosis of synovial cells that leads to replacement of synovial hyperplasia by fibrotic tissue and thus culminates in joint dysfunction. Apoptosis may be mainly involved in the regression of synovial cell hyperplasia in the process of RA. However, the details including the relation with clinical progress remain to be determined. Our present results suggest that Fas may be also involved in the IL-8 secretion from synovial cells. IL-8 secretion in response to the Fas ligation is not unique for RA synovial cells. In OA synovial cells, we also found that anti-Fas stimulation induced IL-8 secretion as well as in RA synoviocytes, even though they did not undergo apoptosis (data not shown). It has been shown that IL-8 is elevated in the synovium of RA patients (30, 31), which accelerates the inflammation by recruiting and activating neutrophils. Therefore, IL-8 induction may represent another pathogenic role of Fas in the progression of RA.

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