Caspase-independent Cell Killing by Fas-associated Protein with Death Domain

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Abstract. The binding of Fas ligand to Fas recruits caspase 8 to Fas via an adaptor, FADD/MORT1, and activates a caspase cascade leading to apoptosis. Here, we describe a human Jurkat-derived cell line (JB-6) that is deficient in caspase 8. This cell line was resistant to the apoptosis triggered by Fas engagement. However, the multimerization of Fas-associated protein with death domain, through the use of a dimerizing system, killed the JB-6 cells. This killing process was not accompanied by the activation of caspases or DNA fragmentation. The dying cells showed neither condensation nor fragmentation of cells and nuclei, but the cells and nuclei swelled in a manner similar to that seen in necrosis. These results suggested that Fas-associated protein with death domain can kill the cells via two pathways, one mediated by caspases and another that does not involve them.

Key words: apoptosis • caspase • Fas • Fas-associated protein with death domain • necrosis

Two categories of cell death have been defined: apoptosis and necrosis (Kerr et al., 1972). The apoptotic process is accompanied by condensation and fragmentation of cells and nuclei, as well as by degradation of the chromosomal DNA into nucleosomal units (Wyllie 1980; Wyllie et al., 1980). The necrotic process is characterized by swelling and disintegration of the cells and nuclei. Most apoptotic processes are mediated by a family of cysteine proteases called caspases (Cohen, 1997; Nicholson and Thornberry, 1997). Caspases are activated by various apoptotic stimuli and cleave cellular substrates such as poly(ADP-ribose) polymerase (PARP),1 lamin, actin, p27, and inhibitor of caspase-activated DNase, which is responsible to cause morphological changes of nuclei, and degradation of chromosomal DNA. The molecular mechanism of the necrotic cell death process, however, is not well understood.

Fas ligand (FasL) is a member of the tumor necrosis factor (TNF) family. By binding to Fas, its receptor, FasL induces apoptosis (Baker and Reddy, 1996; Nagata, 1997). This apoptotic process proceeds by the binding of an adaptor molecule, Fas-associated protein with death domain (FADD), to the death domain of the Fas cytoplasmic region. This binding causes recruitment of pro-caspase 8 to the Fas receptor to establish the death-inducing signaling complex (DISC) (Kischkel et al., 1995). Pro-caspase 8 is processed at the DISC to the mature active enzyme, and activates other caspases, such as caspase 3, in the downstream of the caspase cascade. In this report, we describe a Jurkat-derived cell line that does not express caspase 8. This cell line was resistant to Fas-induced cell death. However, multimerization of FADD in this cell line killed the cells. This killing process proceeded without caspase activation, and was accompanied neither by apoptotic morphological changes of the cells nor by DNA fragmentation. An analysis of the dying cells under the electron microscopy indicated that this process was accompanied by necrotic morphological changes.

Materials and Methods

Establishment of Stable Transformants

Plasmid MF2FE carries a DNA fragment coding for the FK506 binding protein (FKBP)-Fas chimeric molecule that is consisting of the cytoplasmic region of Fas, two tandem repeats of FKBP12, the myristilation-targeting peptide, and the influenza hemagglutinin (HA) epitope (see Fig. 2.
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Requirement of Caspase 8 for Fas-induced Apoptosis

During establishment of Jurkat cell transformants overexpressing Bel-2, we obtained a clone (JB-6) that was completely resistant to Fas-induced cell death (Fig. 1 A). This cell line, JB-6, expressed Fas as abundantly as the parental
Figure 2. Killing of cells by oligomerization of Fas and FADD. (A) A schematic representation of the chimeric molecules, FKBP-Fas and FKBP-FADD. FKBP, Fas, and FADD represent a domain of human FKBP12 from the amino acid position from 2 to 107, the cytoplasmic domain of Fas (residues 179–319), and the entire coding region of FADD, respectively. Myr and HA indicate the myristilation targeting peptide and influenza hemagglutinin epitope, respectively. (B) Expression of the chimeric molecules, FADD, and caspases in Jurkat-derived cell lines. JF-1 and -19 clones are Jurkat cells transformed with FKBP-Fas, while JM-84 and -94 are Jurkat cells transformed with FKBP-FADD. The JB-6 cells were also transformed with FKBP-Fas (BF-2 and BF-4) or FKBP-FADD (BM-5 and BM-79). The cytosolic extracts (derived from $2.5 \times 10^5$ cells/lane) prepared from the indicated cells were separated on SDS-PAGE and analyzed by Western blotting with antibodies against HA, FADD, caspase 8, and caspase 3. Arrows indicate the positions of respective molecules. (C) Effect of FK1012 on the cell viability. Cells were incubated at 37°C for 6 h with the indicated concentrations of FK1012, and the cell viability was determined by the WST-1 method. Results are expressed as percentage of that obtained without FK1012. The experiments were done in triplicate, and the average values are shown with the standard deviation indicated by bars. (D) Effect of FK1012 on the $^3$H-thymidine incorporation. The indicated transformant clones ($3 \times 10^6$ cells/100 μl) were incubated at 37°C for 20 h in the presence of 0.5 μM FK1012. The cells were then pulsed with $^3$H-thymidine, and the radioactivity incorporated into the cells was determined as described in Materials and Methods. Results are expressed as percentage of that obtained without FK1012. The experiments were done in triplicate, and the average values are shown with the standard deviation indicated by bars.
Jurkat cells (Fig. 1 B); however, it expressed very little, if any, caspase 8 (Fig. 1 B). Other signaling molecules, such as FADD and caspase 3, were expressed in JB-6 cells as abundantly as in Jurkat cells. The expression level of caspase 10 (Mch 4, FLICE 2), which is another caspase containing the death effector domain (Fernandes-Alnemri et al., 1996; Vincenz and Dixit, 1997), was also comparable between Jurkat and JB-6 cells (data not shown). These results suggested that the resistance of this cell line to Fas-induced apoptosis was likely due to the lack of caspase 8. Accordingly, when an expression vector for human caspase 8 was introduced into the JB-6 cell line, the transformants (BC clones) regained the sensitivity to Fas-induced apoptosis (Fig. 1, A and B).

Oligomerization of FADD Kills the Cells Lacking Caspase 8

It is assumed that the engagement of Fas causes oligomerization of FADD, which leads to activation of caspase 8 (Boldin et al., 1996; Muzio et al., 1996). To examine whether the oligomerization of FADD causes cell death, two copies of FKBP (FK506-binding protein) were joined to the death domain of human Fas (Itoh and Nagata, 1993) or to full-length human FADD (Boldin et al., 1995; Chinnaian et al., 1995; Fig. 2 A), and introduced into Jurkat cells or JB-6 cells. As shown in Fig. 2 B, the Jurkat-derived transformant clones JF-1 and -19, and the JB-6-derived BF-2 and -4 clones all expressed FKBP-Fas of 44 kD,
The transformants were then treated for 6 h with increasing concentrations of FK1012, which should induce FKBP dimerization (Spencer et al., 1993, 1996), and thus oligomerization of FKBP-Fas or FKBP-FADD. As shown in Fig. 2C, FK1012 did not show any toxicity to the parental Jurkat or JB-6 cells at a concentration of 0.5 μM. When Jurkat cell transformants expressing FKBP-Fas or FKBP-FADD were treated with FK1012, viability (as assayed by the WST-1 method) was lost, indicating that the oligomerized FKBP-Fas or FKBP-FADD could kill the cells. The JB-6 transformants expressing FKBP-Fas were resistant to the FK1012-induced cell death, and >65% of the cells were still alive after treatment with 0.5 μM of FK1012 for 6 h. These results were consistent with the observation that JB-6 cells were resistant to the anti–Fas antibody-induced cell death as described above (Fig. 1A). On the other hand, the JB-6 transformants expressing FKBP-FADD were efficiently killed by FK1012 (Fig. 2C). The sensitivity of these cells to FK1012 was comparable to that found with Jurkat cell transformants expressing FKBP-FADD. The cell viability was also assayed by 3H-thymidine uptake. As shown in Fig. 2D, the cells were incubated for 20 h with 0.5 μM of FK1012, and then pulsed for 4 h with 3H-thymidine. The Jurkat cell transformants (JF and JM clones) expressing FKBP-Fas or FKBP-FADD lost the ability to incorporate thymidine. The JB-6 transformants expressing FKBP-Fas (BF clones) survived after treatment with FK1012, while the BM clones expressing FKBP-FADD were efficiently killed by the same treatment.

**Necrotic Death Caused by FADD**

The above results indicated that the killing of cells by oligomerization of Fas requires caspase 8. However, FADD, which mediates the Fas signal to caspase 8, can kill cells without caspase 8. To examine the molecular mechanism behind the FADD-induced cell death, activation of caspase 3 and DNA fragmentation were analyzed in Jurkat and JB-6 transformant clones. As shown in Fig. 3, A and D, when the Jurkat cell transformants were treated with FK1012, the proforms of caspases 8 and 3 disappeared within 6 h. This processing of caspases was accompanied by a strong activation of caspase 3–like protease, the cleavage of PARP and lamin B1, and the degradation of chromosomal DNA (Fig. 3). On the other hand, the JB-6 cell transformants expressing FKBP-Fas did not show any proteolysis of the nuclear proteins lamin B1 and PARP, nor any DNA fragmentation, as predicted by their unresponsiveness to FK1012 treatment. The transformants expressing FKBP-FADD also showed neither caspase 3 activation nor DNA degradation by the treatment with FK1012, despite massive cell death. These results indicated that oligomerization of FADD can kill the cells without activating caspases or causing DNA fragmentation. The dying cells were then examined using transmission electron microscopy. As shown in Fig. 4, death of Jurkat cells induced by
oligomerization of FKBP-Fas or FKBP-FADD was accompanied by apoptotic morphological changes such as chromatin condensation, cell shrinkage, and apoptotic bodies. On the other hand, the caspase 8–deficient BM cells dying as a result of FADD oligomerization showed neither condensation of cells nor fragmentation of chromatin. Rather, the cells swelled without forming apoptotic bodies. These results indicated that FADD can kill cells without inducing apoptosis in BM cells.

We then examined the effect of a broad caspase inhibitor, Z-VAD-fmk, on the cell death in Jurkat cells. As shown in Fig. 5, A and B, Z-VAD-fmk completely blocked the proteolysis of nuclear proteins and DNA fragmentation that occur during FKBP-Fas-induced cell death. Accordingly, the cell death induced by FKBP-Fas was largely prevented by Z-VAD-fmk (Fig. 5 C). In contrast, Z-VAD-fmk did not inhibit the FKBP-FADD-mediated cell death, although it inhibited proteolysis of nuclear proteins and DNA degradation. The dying Jurkat cells under electron microscope showed a necrotic morphological change (Fig. 4 E). These results suggested that FADD can kill the Jurkat cells via two pathways. One is an apoptotic pathway in which activation of a caspase cascade leads to fragmentation of chromosomal DNA, and the other is a nonapoptotic pathway that does not involve caspases, but is accompanied by necrotic morphological changes of the cells.

**Discussion**

In this report, we have shown that a derivative of human Jurkat cells that does not express caspase 8 was resistant to the apoptosis induced by the activation of endogenous Fas or oligomerization of the Fas death domain. These results indicate that caspase 8 is indispensable for the Fas-mediated apoptotic cell death process, which agrees with the recent results that embryonal fibroblasts from caspase 8 null mice are resistant to the Fas-induced apoptosis (Varfolomeev et al., 1998). On the other hand, oligomerization of FADD, an adaptor molecule that normally mediates the Fas signal to caspase 8, can kill cells that were deficient in caspase 8. This pathway does not use caspases to kill the cells, and seems to operate even in the cells (the parental Jurkat cells) that express caspase 8, because a broad caspase inhibitor could not prevent the cell death induced by oligomerization of FADD.

The Fas-induced cell death is so far thought to transduce only apoptotic signal. On the other hand, TNF was shown to transduce not only an apoptotic signal, but also a necrotic signal. In particular, treatment of mouse L929 cells with TNF produces oxidants and kills the cells by inducing necrotic morphological changes of the cells (Vercammen et al., 1998). This process was shown to be enhanced by caspase inhibitors. More recently, Vercammen et al. (1998b) established mouse L929 cells overexpressing human Fas. When these cells were treated with the agonistic anti–Fas antibody, they underwent apoptosis. But, caspase inhibitors could not prevent the cell death process, and the cells died by necrosis. This result is apparently in contrast to our observation that the Fas engagement alone cannot kill the caspase 8–deficient human Jurkat cells. However, the sensitivity of the cells to necrosis may depend on the strength of the death signal evoked by Fas or FADD, the
expression level of the downstream signal transducer for necrosis, and/or the balance between apoptotic and necrotic signals. In L929 cells, a downstream molecule(s) leading to necrosis is more abundant than in Jurkat cells, and the necrotic signal can be easily seen with the weak signal from the Fas receptor in the presence of caspase inhibitors, while its strong activation by direct oligomerization of FADD may be necessary to activate the necrotic pathway in Jurkat cells. FADD can also be activated by the TNF–TNF type I receptor system through an adaptor molecule called TRADD (TNFR1-associated death domain protein; Chinnaiyan et al., 1996; Hsu et al., 1996). It will be interesting to determine whether the TNF-induced necrosis in L929 cells is mediated by FADD or not. In addition, the TNF-induced necrosis in L929 cells can be inhibited by butylated hydroxyanisole, an anti–oxidant, suggesting an involvement of oxidants in this cell death process (Vercammen et al., 1998). Whether or not a similar oxidant(s) is activated during necrosis by oligomerization of FADD in Jurkat cells remains to be determined.

The FADD-mediated apoptosis occurs through recruitment of pro-caspase 8 to the FADD death effector domain, which leads to processing of caspase 8 and the activation of the downstream caspases, such as caspase 3, and a DNase (CAD; Nagata, 1997; Enari et al., 1998; Sakahira et al., 1998). It would be interesting to examine whether or not the same region of FADD is responsible for the necrotic signal transduction and what kinds of molecules are activated by FADD. The JB-6 cells abundantly express Bcl-2, yet oligomerization of FADD killed the JB-6 cells, suggesting that the FADD-induced necrotic cell death cannot be inhibited by Bel-2. This agrees with no inhibitory effect of Bel-2 on the TNF-induced cytotoxicity in mouse L929 cells (Vanhaesebroeck et al., 1993). In any case, the establishment of a system for studying necrosis in the absence of caspase activation will contribute to our understanding of the molecular mechanisms underlying necrosis.

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