Modifications enhance the apoptosis-inducing activity of FADD

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Abstract

The ability to enhance apoptosis-inducing activity in specific cells, despite the presence of cellular antiapoptotic proteins, would allow the removal of target cells from a cell population. Here, we show that modification of Fas-associated protein with death domain (FADD) by fusing the tandem death effector domains (DED) of FADD to the E protein of λ phage, a head coat protein with self-assembly activity, greatly increases the apoptosis-inducing activity of FADD in both adherent NIH3T3 and HEK293 cells. Induction of apoptosis in cell lines that stably express modified FADD (2DEDplusE) resulted in rapid blebbing, and most cells detached from the flask within 5 h. In contrast, following induction of apoptosis, it took over 24 h for the cells expressing unmodified FADD to exhibit these signs. The cells expressing the modified FADD underwent apoptosis through the typical apoptosis cascade via activation of caspase-3, and apoptosis was inhibited by a caspase inhibitor (i.e., z-VAD-fmk). Theoretically, as our adhesive stable cell lines undergo apoptosis rapidly and in synchrony following mifepristone- or tetracycline-controlled production of a single apoptosis protein without affecting any other cellular pathways, they provide excellent model systems in which to analyze the phenomenon of apoptosis in adhesive cell lines, in particular, blebbing and detachment. [Mol Cancer Ther 2007;6(6):1793–803]

Introduction

As abnormal regulation of apoptosis is believed to be a direct cause of many diseases, there has been much research aimed at understanding the mechanisms by which apoptosis occurs (1). Cultured cells of various origins have been used to analyze apoptosis in mammals; thus, it is known that the susceptibility of different cell types to apoptosis varies. The method used to induce apoptosis must be appropriate for a given cell line. For effective induction of apoptosis in cultured cells by an apoptosis-related protein, excessive overproduction of the protein, serum starvation, or treatment with cycloheximide may be necessary (2, 3). Moreover, long times (24–48 h) are often required for apoptosis to be completed. The susceptibility of cultured cells to apoptosis is similar to that exhibited in vivo by cells of the tissue from which the cell line was derived. Cell lines derived from T or B cells have a strong propensity to undergo apoptosis, which is consistent with their role in vivo, as most unnecessary T and B cells are selected by apoptosis. Cells from most other tissues only die when damage occurs (e.g., apoptosis in virus infection and necrosis in injury), or if they have reached a particular developmental stage. This suggests that commonly used cell lines may be resistant to cell death due to the innate features of the cells from which they were derived, in addition to the absence of receptors for apoptosis. Moreover, inhibitor of apoptosis protein gene amplification and protein overexpression have been identified in several human cancers, suggesting a means by which cancer cells escape apoptosis during tumorigenesis and become resistant to chemotherapy and radiation treatments (4).

We aim to develop a technique whereby we can remove desirable or unnecessary cells in vivo, such as cancer cells or infected cells, by inducing apoptosis specifically in the target cell population. Such technology could have many applications not only in medical therapy but also in basic biological research, including the production of mice that lack a particular cell type. Many cellular antiapoptotic proteins have been identified (5, 6), and it is likely that more will be found. We therefore believe that enhancement of apoptosis-inducing activity will be required to achieve complete apoptosis of a particular cell population, particularly as production of an apoptosis-related protein in vivo would be relatively low even when expressed under the control of a strong promoter (7–10).

Fas/APO-1/CD95 is a member of the tumor necrosis factor receptor superfamily. Binding of the Fas ligand induces trimerization of Fas in the target cell membrane. Activation of Fas causes the recruitment of Fas-associated protein with death domain (FADD) via interactions between the death domain of Fas and FADD (11). Procaspase-8 binds to Fas-bound FADD via interactions between the death effector domains (DED) of FADD and procaspase-8, leading to the activation and processing of caspase-8 (12). This complex is called the death-inducing signaling complex (13). The concentration of caspase-8 determines which of two paths are taken towards...
apoptosis. If caspase-8 levels are high, it directly cleaves caspase-3. Caspase-3 inactivates the inhibitor of caspase-activatable DNase, and caspase-activatable DNase is then free to enter the nucleus and cleave DNA into lengths of ~180 bp (14). If caspase-8 concentration is low, it truncates BID into tBID, a form of BID that is capable of releasing cytochrome c from mitochondria. Cytochrome c interacts with proteins such as Apaf-1, dATP, and procaspase-9 to produce active caspase-9 (15). Caspase-9 then cleaves caspase-3, which activates caspase-activatable DNase in the same way as it does in the first pathway. Fas-induced apoptosis can be effectively blocked at several stages. Apoptosis inhibitors Bel-2 and Bcl-XL block apoptosis through the BID-mediated pathway but not the direct (caspase cascade) pathway (6, 16, 17). Another group of inhibitors, known as FLIPs, contains two DEDs capable of binding to the Fas-FADD complex (5, 18). In this way, they prevent the recruitment of caspase-8 and inhibit both apoptosis pathways.

Although addition of staurosporine or etoposide is often used as a general method for inducing apoptosis, they are inhibitors of protein kinase and topoisomerase II, respectively (19, 20). These are very strong inducers of apoptosis but never act only at apoptosis cascades (e.g., caspase cascade) and are sure to affect many pathways in cellular function in addition to apoptosis. We have made some stable cell lines to analyze the enhanced apoptotic-inducing activity of modified FADD proteins. Considering that in these adhesive stable cell lines apoptosis may be induced rapidly and synchronously by expression of just one protein in the apoptosis cascade, they are an excellent model system in which to analyze the phenomenon of apoptosis, in particular, blebbing and detachment of cells. We have analyzed in detail a system in which the induction of apoptosis is enhanced by modification of FADD. This has been done in detail for a number of reasons: (a) to confirm that our modifications to FADD enhance apoptosis through the apoptotic cascade; (b) to examine whether stable cell lines expressing modified FADD are suitable to use for analysis of the phenomenon of apoptosis in adhesive cells, in particular, to study blebbing and detachment of cells; (c) to evaluate whether our system is useful as an assay system for screening a variety of antiapoptotic proteins, in particular, large proteins that have not been screened extensively by other groups.

Materials and Methods

Construction of Plasmids Expressing Original FADD and Two Modified FADD

Two pairs of oligos (5′-CCCATGGACCATTCTGGTG3′ and 5′-GGAGATCTGATGTCGATGTACACAACCGCC-3′, 5′-AGATCTGCAGGTGCATTGA3′ and 5′-TCAGGGTTTCTGAGGAAGA-3′) were used for PCR amplification of the FADD coding sequence from the genomic DNA of R1 embryonic stem cells (which originated from the F1 offspring of 129X1/SvJ and 129S1/Sv mice). The resulting two PCR products were digested with BglII, ligated to construct a full-length FADD open reading frame, and cloned into the pGEM-T Easy Vector (Promega). For construction of the expression vector pGene5CM-VEGFP, we combined the 3.4-kb NaeI DNA fragment of pGene/VS-HisB and the 1.6-kbp Asel-MluI DNA fragment of pEGFP-C3 (Clontech). For construction of pGeneEGFP-FADD, the full-length FADD in pGEM-T Easy was digested with NcoI and SalI and ligated into the SpeI/NotI site of pGene5CM-VEGFP. We used an upstream primer (5′-GGAGATCTGATGTCGATGTACACAACCGCC-3′) and a downstream primer (5′-CCGTCGACTTACCAGTGATTTGAGGACACCGACACCGCC-3′) to amplify the head coat protein (i.e., E protein) from λ phage clind 1 ts857 Sam7 DNA (Invitrogen Corp.). Although full-length FADD is 205 amino acids, 2DED2DD was a fusion protein composed of amino acids 1 to 96, 1 to 205, and 95 to 205 of FADD. Another fusion protein, 2DEDplusE was composed of two repeats of amino acids 1 to 96 from FADD and the full-length E protein from λ bacteriophage. These two modified FADDs were ligated into the SpeI/NotI site of pGene5CM-VEGFP to construct the plasmids pGeneEGFP-2DEDD2DD and pGeneEGFP-2DEDplusE, respectively.

The plasmid pT-Rex-EGFP-FRT-DEST was constructed from PT-Rex-DEST30 (Invitrogen), pEF5/FRT/V5-DEST (Invitrogen), and pEGFP-C3 (Clontech) and was kindly provided by Dr. Nakajima (DNA sequences are available upon request). The expression plasmid allows production of a foreign protein under the control of a tetracycline repressor system and constitutively produces enhanced green fluorescent protein (EGFP) from a cytomegalovirus (CMV) promoter. For construction of pTrex-FADD, pTREx-2DEDD2DD, and pTREx-2DEDplusE, respectively.

Measuring Cell Death Activity by Transient Transfection of Original and Modified FADD

Geneswitch-3T3 cells, originally from the mouse NIH3T3 cell line, were obtained from Invitrogen. The Geneswitch-3T3 cells were grown in DMEM supplemented with 10% fetal bovine serum (Invitrogen). Expression plasmids encoding unmodified or modified FADD were transfected into Geneswitch-3T3 cells using FuGene 6 Transfection Reagent (Roche) in poly-D-lysine–coated glass-bottomed dishes (MatTek Corp.). Twenty-four hours after transfection, EGFP signal was observed by fluorescence microscopy (Olympus IX71) with a UPlanFI ×10/0.30 Ph lens, and nine fields of cells per dish were recorded using a CCD camera (Hamamatsu ORCA-ER) under fixed conditions (typically 1 s, no gain). The number of cells with fluorescent EGFP signals was counted. Mifepristone was then added into the medium to a final concentration of 10 nmol/L. Twenty-four hours after mifepristone addition, the fluorescent signal of the cells was again observed and recorded with the CCD camera under the same conditions. The
number of fluorescent cells following treatment was calculated as a ratio to the number of fluorescent cells counted before treatment. Three independent experiments were done. Representative results are given.

**Generation of Stable Expression Cell Lines**

Geneswitch-3T3 cells were transfected with 11 μg of expression plasmid pGeneEGFP-2DEDplusE or empty vector pGene5CMVEGFP with 1.1 μg pMC1nepolyA (Stratagene) using FuGene 6 transfection reagent. The transformants were grown in DMEM containing 10% fetal bovine serum with 50 μg/mL Hygromycin B and 100 μg/mL neomycin. Forty neomycin-resistant colonies that expressed EGFP were isolated and expanded. After subculture of the stable cell line, we added mifepristone to a final concentration of 10 nmol/L and incubated the cells at 37°C in a CO2 incubator to examine expression of the gene of interest. Just a few of the hygromycin-resistant clones were used for the various experiments, but the other clones also gave similar results when tested.

Flp-In T-REx-293 cells (Invitrogen) were cotransfected with expression vector (pTREx-FADD, pTREx-2DED2DD, and pTREx-2DEDplusE) and pOG44, which encoded Flp recombinase, using FuGene 6 (Roche). Because the Flp-In T-REX-293 cells contain a single integrated Flp recombination target (FRT) site, all of the hygromycin-resistant clones should be isogenic. However, some clones with β-galactosidase activity were detected in the pooled polyclonal cells, although the manufacturer’s instructions were followed. To obtain truly isogenic stable cell lines, the hygromycin-resistant clones were picked and expanded to generate individual monoclonal cell lines. We verified that the expression construct had integrated into the FRT site by testing each clone for lack of β-galactosidase activity. After subculture of the stable cell line, we added tetracycline to a final concentration of 1 μg/mL and incubated the cells at 37°C in a CO2 incubator to induce expression of the gene of interest. One of the hygromycin-resistant clones was used for the experiments, but the other clones also gave similar results.

**Analysis by Fluorescence Microscopy**

The cells were plated in appropriate medium on poly-L-lysine–coated glass-bottomed dishes. Twenty-four hours after subculture, mifepristone was added. At the indicated time, the cells were washed with PBS and fixed with 4% paraformaldehyde in 0.1 mol/L phosphate buffer and 3.4% sucrose for 30 min at room temperature. The cells were then incubated with blocking solution (10% fetal bovine serum with 50 μg/mL Hygromycin B and 100 μg/mL neomycin, 0.1% Triton X-100, 0.1% sodium azide and 10% goat serum in PBS overnight at 4°C) and then incubated with 1:2,000 horseradish peroxidase–conjugated anti-rabbit or anti-mouse IgG antibody (Promega) in 1% bovine serum albumin/PBS, respectively. After washing, the bands were detected using the ECL plus amido gel and blotted onto a nitrocellulose transfer membrane (PROTRAN, Schleicher & Schuell) using a semi-dry system (ATTO Co.). Membranes were blocked with 1% blocking reagent (Beringer) in PBS and then incubated with 1:1,000 anti–cleaved caspase-3 (Asp175; 5A1) rabbit monoclonal antibody (Cell Signaling Technology) or 1:5,000 anti-actin (Ab-5) mouse monoclonal antibody (BD Biosciences) according to the manufacturer’s instructions and visualized with AQUA-C imaging software (Compix, Inc.). Typically, 5 × 104 cells were plated per poly-L-lysine–coated glass-bottomed 35-mm dish. Twenty-four hours after subculture, mifepristone was added, and the cells were then recorded continuously for 10-min intervals.

**Caspase-3 Assay**

Cells (1 × 106) were plated in an appropriate medium. The next day, before the indicated time, tetracycline was tested. Representative results are given.
Modified FADD Strongly Induces Apoptosis

Results
Expression of Modified FADD Triggers Cell Death

To produce a trigger protein with strong apoptosis-inducing activity by modification, we attempted to assemble procaspase-8 because procaspase-8 contains a weak proteinase activity even before it is processed and can self-activate to form caspase-8. The DED of FADD binds to the DED of procaspase-8. Two FADD DEDs were fused to the E protein of λ phage, which is a head coat protein with self-assembly activity (22, 23), to form 2DEDplusE (Fig. 1A). The idea was that when the 2DEDplusE protein molecules are produced in a cell, they should assemble automatically as the E protein should self-associate, and each DED should bind to procaspase-8. The assembled procaspase-8 should then be converted to the active form and induce a strong apoptosis signal. The activity of this modified factor was tested by transiently expressing them in cultured cells and counting the number of cells that died. To prevent constitutive production of the apoptosis-inducing factors in transfected cells, we used an inducible mammalian expression system (i.e., the GeneSwitch system), which has extremely low levels of leaky expression. Unmodified FADD and 2DEDplusE expression was controlled by an inducible promoter under the control of mifepristone, and EGFP expression was driven by a constitutive CMV promoter. As EGFP was constitutively expressed, it was able to be used to estimate cell number and therefore cell survival. As transient expression of the modified factors was done to compare apoptosis-inducing activity, it was necessary to establish whether a low number of GFP-positive cells resulted from cell death following apoptosis induction or from low transfection efficiency. GeneSwitch-3T3 cells that express the GeneSwitch regulatory protein were transfected with the plasmids. Twenty-four hours after transfection, the number of GFP-positive cells was counted using the aid of a fluorescence microscope. Mifepristone was then added to the medium to induce expression of the modified proteins. At the indicated time after adding mifepristone, the number of GFP-positive cells was counted again. Figure 1B shows the number of cells that survive per GFP-positive transfecant. The number of GFP-positive control cells, which had been transfected with empty expression vector, was approximately the same before and after mifepristone treatment. The increase in the number of GFP-positive cells was due to accumulation of EGFP during the additional 24 h. When FADD protein was expressed, ~75% of the transfecants had died 24 h after induction. In contrast, all transfecants expressing 2DEDplusE protein were dead 24 h after induction. It is clear from this experiment that expression of the 2DEDplusE protein induced stronger apoptosis activity than unmodified FADD. We also constructed another modified FADD using two DEDs and two death domains as shown in Fig. 1A. The 2DED2DD induced medium apoptosis activity (data not shown).

To analyze this cell death in detail, we isolated stable transfecants expressing 2DEDplusE protein under the control of an inducible promoter or expressing GFP alone from stable integration of empty vector DNA. GeneSwitch-3T3 cells were cotransfected with the pGene5CMVEGFP-based plasmids described above and the pMCneo1 plasmid for the neomycin resistance gene. The neo-resistant and GFP-positive colonies were isolated. Figure 2 shows the effect of mifepristone-induced 2DEDplusE expression compared with control cell lines stably transfected with empty vector (Fig. 2A). Three hours after the addition of mifepristone to cells stably transfected with 2DEDplusE, more than half of the cells had detached from the surface of the dish and exhibited cell death. On the other hand, no effect was observed in control cells that were stably transfected with empty vector. Results were comparable for four independently isolated clones (data not shown).
Cells Expressing the Modified Factor 2DEDplusE Undergo Apoptosis via the Typical Apoptosis Cascade, by Activation of Caspase-3

To confirm that the observed cell death was typical apoptosis, indirect immunofluorescence analysis using antibodies against the active form of caspase-3, which do not recognize procaspase-3, was done on stable cell lines at various times after the addition of mifepristone. As shown in Fig. 2B, signals were hardly detected until 1.5 h after induction. After 2 h, weak signals were detected in the cytoplasm. Two and a half hours after induction, in addition to weak signals from most cells, strong signals were detected in some shrunken cells. Three hours after induction, strong signals were detected in most cells. The signals from shrunken cells tended to be stronger than those of healthy-looking cells. The shrunken cells were almost detached from the glass and entering the final stages of apoptosis. There were some cells that progressed through the stages of cell death faster than the majority of cells; however, the strength of the signal from the active-form of caspase-3 was observed to be uniform in most cells. The active form of caspase-3 was not detected in control cells bearing empty vector 3 h after mifepristone addition. Figure 2C shows a high magnification image of stained cells 3 h after induction. After induction with mifepristone, healthy and well-extended cells shrunk rapidly. Each

Figure 2. Activation of caspase-3 and condensation of nuclei after induction of 2DEDplusE expression. A, the adhesive stable cell line underwent apoptosis depending on modified factor under the control of mifepristone. NIH3T3 cells stably expressing 2DEDplusE under the control of the GeneSwitch expression system were subcultured in poly-lysine-coated glass-bottomed dishes. Twenty-four hours after subculture, mifepristone was added to induce expression of 2DEDplusE protein. Without any fixation, the cells stably transfected with 2DEDplusE DNA before (c) or after (d) treatment with mifepristone were observed using phase-contrast microscopy. Cells stably transfected with empty vector DNA before (a) and after (b) mifepristone treatment. Expression of 2DEDplusE resulted in rapid induction of apoptosis. Bar, 100 μm. B, the stable cell lines expressing 2DEDplusE under control of GeneSwitch were subcultured on the surface of poly-lysine-coated glass-bottomed dishes. At the indicated times after mifepristone addition, the cells were fixed with 4% paraformaldehyde and then immunostained using anti-caspase antibodies that recognize the processed, active form of caspase-3 and Cy3-labeled secondary antibodies. The cells were observed using fluorescence microscopy. a–e, images of cells stained with anti-caspase-3/Cy3 visualized by fluorescent microscopy; f–j, phase-contrast images of the same fields of view as the respective fluorescent images. Bar, 100 μm. C, higher-magnification image of anti-caspase-3/Cy3-stained cells 3 h after addition of mifepristone (a) and the same field of view shown by phase-contrast microscopy (b). Arrowheads indicate blebbing structures that are often seen in apoptosis. Bar, 10 μm. D, cells stably transfected with 2DEDplusE stained with 4,6-diamidino-2-phenylindole 6 h after mifepristone addition (a) and the same field viewed using phase-contrast microscopy (b). Closed arrowheads indicate condensation of nuclei that is often seen in apoptosis. Open arrowheads indicate nuclei that appear normal. Bar, 10 μm.
shrinking cell body formed ~10 clustered small balls (i.e., exhibited blebbing). A strong caspase-3 signal was detected in these small balls. This suggests that the small balls contained a large amount of the active form of caspase-3. At that time, abnormal nuclei had not yet been observed using phase-contrast microscopy. Then, the small balls disappeared, the cell bodies became completely round, and the cells parted from the surface. To confirm changes in nuclei in addition to the observed changes in the cytoplasm and cell adhesion, 4',6-diamidino-2-phenylindole staining was done (Fig. 2D). Several hours after mifepristone addition, condensation of chromatin and fragmentation of nuclei were observed (Fig. 2D). Even when other types of apoptosis inducers are used on 3T3 cells, nucleosome ladders are rarely detected in DNA from 3T3 cells. The fact that only a small amount of nucleosome ladder was observed in the samples is, therefore, likely to be because 3T3 cells were used. Taken together, the data nuclei. The stage of fragmentation of nuclei tended to be significantly more advanced than that of still adherent cells. The cells and nuclei were so fragile that they were easily broken, and the strings of DNA spread following the cell fixing and washing procedure. When condensation of chromatin and fragmentation of nuclei were observed in the shrunken and floating cells, the DNA was extracted and separated by agarose gel electrophoresis. This was done to see if the DNA formed a nucleosome ladder, which is a series of DNA fragments containing multiple numbers of nucleosomes and results from the breaking of chromosomes in typical apoptosis. A small amount of nucleosome ladder was detected relative to the amount of long chromosomal DNA (data not shown). Even when other types of apoptosis inducers are used on 3T3 cells, nucleosome ladders are rarely detected in DNA from 3T3 cells. The fact that only a small amount of nucleosome ladder was observed in the samples is, therefore, likely to be because 3T3 cells were used. Taken together, the data

Figure 3. Inhibition of modified FADD, 2DEDplusE-mediated apoptosis by inhibitors of caspase. Cells stably transfected with 2DEDplusE were subcultured in poly-D-lysine–coated glass-bottomed dishes. Twenty-four hours after subculture, mifepristone was added to the medium. The cells were treated with z-VAD-fmk or Ac-DEVD-CHO 2 h before the addition of mifepristone. The cells were fixed at the indicated times and immunostained with anti-caspase-3 primary antibody/Cy3-labeled secondary antibody and observed using fluorescence microscopy. a–c, images of the control experiment without any inhibitor; d–f, images of anti-caspase-3/Cy3-stained cells that had been treated with z-VAD-fmk, which can inhibit all types of caspase. g–i, images of anti-caspase-3/Cy3-stained cells that had been treated with Ac-DEVD-CHO, which mainly inhibits caspase-3 and caspase-7. Bar, 50 μm.

Figure 4. Time course of the induction of 2DEDplusE expression and activation of caspase-3. A, Northern hybridization analysis was done using total RNA from cells stably transfected with 2DEDplusE (lanes 1–6) or empty vector (lanes 7–12) at 0, 1, 2, 2.5, 3, 3.5, and 24 h (lanes 1–6 and 7–12, respectively) after mifepristone addition. Numbers on the right side of the gel refer to the positions of the RNA molecular weight markers (Novagen). B, immunoblot analysis was done using extracts of 2DEDplusE (lanes 8–14) and empty vector (lane 1–7) stably transfected cells at 0, 1, 2, 2.5, 3, 3.5, and 4 h (lanes 1–7 and 8–14, respectively) after mifepristone addition. Caspase-3 was detected using anti-caspase-3 antibody and the ECL plus Western blotting detection system. Numbers on the right side of the gel indicate the positions of the protein molecular weight markers (Amersham).
indicate that 2DEDplusE protein induces apoptosis via activation of caspase-3.

**Blocking the Effects of Modified FADD by Caspase Inhibitors**

Next, to determine whether apoptosis induced by the 2DEDplusE protein can be inhibited by caspase inhibitors, experiments using two caspase inhibitors were done. z-VAD-fmk, which can inhibit all known caspases, was added to the cell medium 2 h before induction (24). After addition of mifepristone to induce expression of 2DEDplusE protein, at the times indicated in Fig. 3, the state of the cells was observed, and caspase-3 staining was done. No change in the state of the inhibitor-treated cells was observed during the first 3.5 h, although dramatic changes were observed without inhibitor. Moreover, no cleaved caspase-3 was detected, and no activation of caspase-3 was observed (Fig. 3). z-VAD-fmk was able to completely inhibit the apoptosis-inducing properties of 2DEDplusE. This result shows that 2DEDplusE activates caspase-8 via the mechanism that we expected, and that there are no unexpected artificial effects caused by 2DEDplusE. Ac-DEVD-CHO, which can specifically inhibit caspase-3 and caspase-7, was added to the cell medium 2 h before induction (25). Inhibition was exhibited, although it was not complete. The progression of apoptosis in these cells was weak and slow compared with the cells without inhibitor. In addition, 3 h after induction, a few strong signals were observed in shrunken cells. Four hours after induction, ~30% of cells had executed apoptosis and had detached from the surface and were floating in the medium. This partial inhibition by Ac-DEVD-CHO suggests that another cascade that does not involve caspase-3 is involved in apoptosis execution. The 2DEDplusE protein binds procaspase-8 and activates caspase-8, and the activated caspase-8 mainly processes procaspase-3 immediately and induces the caspase-8/caspase-3 cascade directly. Besides the main cascade, however, activated caspase-8 processes and activates BID protein. Through activation of BID, the apoptosis signal was amplified in mitochondria and then transmitted to the caspase-9–mediated cascade, and then apoptosis was executed. We also confirmed that the unprocessed BID was decreased based on the induction of 2DEDplusE protein as shown by immunoblot analysis and anti-BID antibodies (data not shown). This indicates that the 2DEDplusE protein will activate not only the caspase-8/caspase-3 cascade but also the caspase-9 cascade. When caspase-8 activity was blocked with z-VAD-fmk during the initial stages of apoptosis induction by the 2DEDplusE protein, all reactions of apoptosis after caspase-8 activation were blocked.

**Time Course of Transcriptional Induction of 2DEDplusE and Activation of Caspase-3**

Next, we did a time course experiment in which apoptosis was observed in cells following mifepristone addition. The Northern blotting results in Fig. 4A show that 2DEDplusE mRNA levels had increased after 1 h and reached maximum levels after 2 h. It is worthy to note that 2DEDplusE mRNA is present at moderate levels even after mifepristone induction, and that this result strongly suggests that even a moderate amount of 2DEDplusE mRNA can induce strong apoptosis activity. The amount of the active form of caspase-3 was assessed by Western blot analysis. Although no signal was
detected 1 h after induction or without mifepristone, a weak signal was detected after 2 h, and the strength of the signal rapidly reached a maximum level after 2.5 h and remained at a high level until the 4-h time point. The active form of caspase-3 was detected in control cells transfected with empty vector, even after mifepristone addition. Two minor bands corresponding to ~50 and 30 kDa were observed in the Western blots of 2DEDplusE-expressing cells. The intensities of these two bands were greatest in samples taken 2.5 and 4 h after mifepristone treatment, respectively. Because these bands are dependent on mifepristone treatment and are not detected in control cells, they may be procaspase-3. It is possible that they are other members of the caspase family. However, although a detailed analysis has not yet been done, the anti-caspase-3 antibodies should not cross-react with other proteins. Similar results were obtained using independent isolated clones expressing 2DEDplusE and independent control cells (data not shown).

**The Modified Factor 2DEDplusE Induced Rapid and Synchronous Blebbing and Detachment**

Figure 5 shows a series of phase-contrast images of mifepristone-treated adhesive 2DEDplusE-expressing cells undergoing apoptosis. In summary, at the indicated time after induction, the extended and attached cells exhibited blebbing (i.e., they shrunk into ~10 small balls). The shrunk cells then became compact and round. The rounded cells remained on the surface of the dish if it was not shaken. Following this, the rounded cells detached from the bottom of dish and disappeared from the field of view. The first shrunk cell was observed after 2.5 h. In the image taken after 2.5 h, rounded cells that were undergoing cell division were observed, but these were easily distinguished from rounded cells undergoing apoptosis. Five hours after induction, most cells had entered apoptosis and shrunk. Less time was taken for cells to appear round after induction of apoptosis by 2DEDplusE than for cells treated with a typical strong apoptosis inducer (i.e., etoposide). When the parent GeneSwitch-3T3 cells were treated with etoposide and recorded with a time-lapse system, the same amount of apoptosis induction was observed over 12 h (data not shown). This rapid execution of apoptosis is also evidence that the 2DEDplusE protein has enhanced apoptosis-inducing activity relative to FADD. In contrast, in control cells treated with mifepristone, the only rounded cells observed were undergoing cell division, not apoptosis. On average, the number of small balls attached to shrunk cells as a result of apoptosis (blebbing) was 14.

**Modified FADD Effectively Induces Apoptosis not only in NIH3T3 but also in HEK293 Cells**

Finally, we determined whether the enhanced apoptotic activity of modified FADD that was observed in NIH3T3 cells was also effective in inducing apoptosis in another cell line, the human embryonic kidney 293 (HEK293) cells. The Flp-In T-Rex 293 cell line stably expresses the Tet repressor, and the cells contain a single integrated FRT site in the genome. Flp recombinase mediates insertion of the expression construct carrying a FRT site into the genome at the integrated FRT site through site-specific DNA
recombination. The advantage of this system is that it allowed us to make direct comparison of the apoptotic activity of modified proteins, as there is no variation in plasmid copy number in the cells, even if they are stably transfected. Figure 6A shows time courses of the stable cell lines expressing the three different proteins after tetracycline addition. When expression of unmodified FADD was induced in a stable cell line by the addition of tetracycline, few, if any, apoptotic cells were observed. Twenty-five hours after induction, ~10% of cells were affected and detached from the surface of the dish. In contrast, when 2DEDplusE expression was induced, after 3 h, ~80% of the cells were affected and had detached from the bottom of the dish, whereas no significant effect was observed 2 h after tetracycline addition. After 4 h, most of the cells had detached completely. No cells adhered to the bottom of the dish. The apoptosis-inducing activity of the 2DED2DD protein is stronger than that of unmodified FADD but weaker than that of the 2DEDplusE protein. No apoptotic cell death was observed in the stable cell line expressing 2DED2DD protein 3 h after the addition of tetracycline. Five hours after induction, ~40% of cells were affected and had detached, and 6 h after induction, only ~10% of cells still adhered to the bottom of the dish. These results also were consistent with the results of caspase-3/7 assay. At the indicated times after addition of tetracycline, caspase-3/7 activity was measured by a caspase-Glo 3/7 assay as shown in Fig. 6B. Interestingly, 25 h after induction of 2DED2DD expression, the number of adherent cells increased. It is possible that this increase was due to reattachment of cells or proliferation of surviving cells. However, in stable cell lines expressing 2DEDplusE, the number of adherent cells detected did not increase. This suggests that the 2DEDplusE-induced apoptosis was complete. Taken together, the data indicate that the modified FADD (2DEDplusE) has enhanced apoptotic activity compared with unmodified FADD in at least two types of cell lines (NIH3T3 and HEK293), which are used as standard cell lines in the field of cellular biology but are known to be quite resistant to the induction of apoptosis. Additionally, when we tested the activities of these modified factors by transient transfection into other human cell lines (HeLa cells, which originate from cervical cancer; Saos2 cells, which originate from osteosarcoma; and HepG2 cells, which originate from hepatocellular carcinoma), we obtained almost the same results as with NIH3T3 and HEK293 cells (data not shown). Moreover, in a caspase-Glo 3/7 assay based on the induction of 2DEDplusE, we observed a significant increase in caspase-3 activity in the three cell lines (HEK293, NIH3T3, and HeLa cells) we tested.

Discussion

Transfection of certain types of cells with a construct encoding an apoptosis-related protein can induce weak or moderate amounts of apoptosis when cultured under suitable conditions. However, often more than overproduction of a protein is required, and another stimulus is needed, such as addition of an aggregating anti-Fas antibody and cycloheximide and serum starvation. Generally, it is difficult to induce apoptosis completely in most cell types, with the exception of cells with a T- or B-cell origin. This situation motivated us to make an enhanced apoptosis-inducing factor capable of inducing apoptosis when expressed in cells relatively resistant to apoptosis. We aim to develop technology that would facilitate the removal of selected target cells by apoptosis. In the present study, we have succeeded in enhancing the apoptosis-inducing activity of FADD such that apoptosis is rapidly and almost completely executed by modified FADD. FADD contains two ~90-amino-acid protein interaction modules (an NH2-terminal DED and a COOH-terminal death domain), each of which adopts a structurally similar six o-helical bundle. Interestingly, FADD does not contain an enzymatic domain. Fas ligands trimerize through the binding of Fas receptors. The death domain of FADD can bind to the death domain in the cytoplasmic domain of Fas receptors. The DED of FADD can bind the DED of procaspase-8. This complex is called death-inducing signaling complex and can activate initiator caspase-8 by autoprocessing of procaspase-8. We fused DED of FADD to E protein, which is able to self-assemble and is the coat protein of λ phage, so that the modified factor can construct artificial death-inducing signaling complex without Fas ligand and receptor. We expected that the self-assembled modified factor 2DEDplusE would bind procaspase-8. This was expected to increase the procaspase-8–activated autoprocessing activity of procaspase-8 more effectively. In fact, compared with unmodified FADD, the modified factor can induce strong apoptosis in adhesive NIH3T3 and HEK293 cells, which are known to be relatively resistant to apoptosis but are typically used in cellular studies.

There are two pieces of evidence that suggest apoptosis is enhanced. The first is based on the quantitative examination of transient expression. The ratio of cell death in cells transfected with the modified FADD 2DEDplusE 24 h after induction of expression is 100%, which is higher than in cells transfected with unmodified FADD. The other evidence is that the cells expressing 2DEDplusE rapidly underwent the morphologic changes that accompany apoptosis (i.e., blebbing and detachment) and had a higher ratio of apoptotic cells at the various time points relative to cells expressing unmodified FADD. A strong inducer of apoptosis (etoposide) takes about 12 h to result in detachment of adhesive cells. It should be emphasized that almost 100% of cells underwent apoptosis. The noted effects of 2DEDplusE on apoptosis make it suitable for use in future experiments in which we will express this protein in vivo because we predict various restrictions in the in vivo situation: low promoter strength or lower amount of protein production. Because FADD is upstream of the apoptosis cascade, we expected modification of FADD to amplify the signal more effectively than modification of the downstream caspase-3 or caspase-9. Previously, several studies have been reported in which an apoptosis-related protein has been deleted or modified into a fusion protein, such as serial deletion mutants and fusion proteins in...
which GFP is fused to a domain that regulates dimerization under the control of a chemical compound (CIDs or FK506-binding protein; refs. 8, 26). However, these studies do not mention enhancement of apoptosis-inducing activity, which probably means that activity is decreased or unchanged. Some reports of in vivo studies in recombinant mice have used toxins; however, the side effects of toxins (i.e., necrosis and inflammation) are concerning. An attempt to induce apoptosis in selected liver cells was reported by Mallet et al. (8); however, only some of the target cells in the mouse liver died following induction. We strongly believe that in vivo expression of 2DEDplusE will induce apoptosis in most cells.

DED is a protein-interaction module similar to caspase recruitment domain and death domain. DED-containing proteins have been implicated in apoptosis regulation via interactions with DED-containing caspases (caspase-8 and caspase-10 in humans and caspase-8 in mice). In addition to DED-containing caspases, five proteins (FADD, FLIP, DEDD, DEDDD2, and PEA-15) with classic DEDs and six proteins (BAP31, BAR, DAP-3, FLASH, HIP-1, and HIPPI) with DED-like domains (pseudo-DEDs) were identified in human and mouse. A unique family of single DED-containing proteins, including DEDDD and DEDDD2, are targeted to the nucleolus (27). Overexpression of DEDD containing an NH2-terminal DED in 293T cells induced weak apoptosis (28). Overexpression of DEDD2 induced moderate apoptosis; however, a direct comparison was not made (29). The c-FLIP regulator is an enzymatically inactive relative of caspase-8 that binds to death-inducing signaling complex. Two major c-FLIP variants result from alternative mRNA splicing: a short protein with two DEDs (c-FLIPs) and a long caspase-like protein with two DEDs (c-FLIP1). The role of c-FLIPs as an inhibitor of death receptor-mediated apoptosis is well established; however, the function of c-FLIP1 remains controversial. Although overexpression of c-FLIP1 inhibits apoptosis, expression at lower levels supports caspase-8 activation and cell death. Although the c-FLIP1 containing only two DEDs works as an inhibitor of apoptosis, it is interesting that our 2DEDplusE containing two DEDs from FADD showed strong apoptosis-inducing activity. This difference can be explained by the specificity of DED for the binding partner. Because the balance of original FADD, procaspase-8, and c-FLIP in death-inducing signaling complex or cytoplasm determines the fate of cells (i.e., apoptosis or survival), we propose that the ability of modified 2DEDplusE to induce apoptosis is independent of the FADD/procaspase-8/c-FLIP balance. We are also interested in whether enhancement of apoptosis-inducing activity is observed when other types of DED from other DED-containing proteins are fused to E protein and introduced into cells. At present, we do not know if the double DED in 2DEDplusE is really effective, as we did not obtain comparative data for single DEDs fused to E protein. Our primary goal is to further enhance apoptosis-inducing activity by protein modification. We believe using a single DED should result in the same or less enhancement than using two DEDs because of some effects that result from the dimerization of two DEDs. If one takes this view, it follows that three DEDs might be worth trying. However, large recombinant proteins tend to be susceptible to attack by proteases.

To construct another type of modified FADD, in particular, a much stronger factor or specific regulator, another self-assembling protein can be used in the place of E protein. The pVIII protein, the major coat protein of bacteriophage M13, is synthesized as a preprotein (73 residues), which binds to the inner surface of the plasma membrane and, in the presence of a membrane potential, subsequently translocates as a loop structure across the membrane (30). Particles of M13 contain ~2,700 copies of the processed or mature 50-residue α-helical protein, arranged in a cylindrical sheath around the bacteriophage DNA, whereas a λ phage contains 415 copies of E protein. The pVIII protein of M13 has the advantage of a small size and may have a greater propensity to self-assemble. The mature form of pVIII may, therefore, be a good alternative to E protein for fusion to DED. Both actin and tubulin are also good candidates because they are in organized filaments in the cytoplasm and their properties, inhibitors, and stabilizers are well studied.

In the stable cell lines described in this study, expression of the transfected genes is controlled by mifepristone (RU486) or tetracycline. They are useful cell lines in which to analyze the phenomenon of apoptosis for two reasons, which are described below.

The first reason is that these stable cell lines allow apoptosis to be studied in isolation (i.e., without the undesired effects that other typical apoptosis-inducers display). For example, staurosporine, etoposide, camptothecin, and cycloheximide are also inhibitors of protein kinase, topoisomerase II, topoisomerase I, and protein synthesis, respectively. Therefore, they often affect many cellular regulation pathways in addition to the apoptosis cascade. The use of these agents, therefore, makes it difficult to distinguish whether differences in mRNA and protein levels measured after inducing apoptosis result from apoptosis or from inhibitor side effects. In particular, the stable cell lines we have used will be useful in genome-wide analysis, using DNA microarray and proteomic approaches. A cell system in which apoptosis can be studied synchronously and is dependent on the expression of only one protein is rare in adhesive cell lines if the use of other stimuli, such as aggregation anti-Fas receptor antibodies, tumor necrosis factor-α, serum starvation, or cycloheximide, are excluded. In the suspension cells (i.e., Jurkat cells), many reports have been published in which viral vectors are used to introduce genes into cells. We expect our stable cell lines to have few side effects when apoptosis is induced by tetracycline- or mifepristone-induced expression of only one protein.

The second reason that our cell lines are useful for analyzing apoptosis is that it allows synchronization of the events of apoptosis and occurs over a short period of time. This allows detailed analysis of each step of apoptosis. We can use these stable cell lines to examine the changes in
mRNA and protein levels that accompany each step of apoptosis that follows activation of caspase-8, free from side effects using DNA microarray and proteomic approaches. Moreover, because adhesive cells like NIH3T3 and HEK293 are resistant to cell death (31, 32), a detailed analysis of blebbing and cell detachment can now be undertaken (e.g., analysis using inhibitors of protein kinase and actin filaments). It should be noted that we cannot exclude the possibility that our modified FADD participates in apoptosis via unexpected mechanisms. Nonetheless, to the extent that we have examined, the downstream induction of apoptosis by our modified FADD seems normal. We have started to screen for large antiapoptosis proteins using our established stable cell lines and our ORFeome set (33) that encodes large proteins. We also believe that these synchronized stable cell lines that rapidly undergo apoptosis will be useful and effective for high-throughput screening of small compounds for identification of potential therapeutics. In line with direct application to tumor therapy, we are in the process of developing a way to use our modified FADD to specifically target tumor cells (e.g., by using tumor-specific promoters).

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Modifications enhance the apoptosis-inducing activity of FADD

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