Differential Signaling to Apoptotic and Necrotic Cell Death by Fas-associated Death Domain Protein FADD*

Tom Vanden Berghe‡, Geert van Loo¶, Xavier Saelens§, Maria van Gurp, Greet Brouckaert, Michael Kalai$, Wim Declercq** and Peter Vandenabeele‡‡

From the Molecular Signalling and Cell Death Unit, Department of Molecular Biomedical Research, the Flanders Interuniversity Institute for Biotechnology (VIB) and Gent University, Technologiepark 927, B-9052 Zwijnaarde, Belgium

Two general pathways for cell death have been defined, apoptosis and necrosis. Previous studies in Jurkat cells have demonstrated that the Fas-associated death domain (FADD) is required for Fas-mediated signaling to apoptosis and necrosis. Here we developed L929rTA cell lines that allow Tet-on inducible expression and FK506-binding protein (FKBP)-mediated dimerization of FADD, FADD-death effector domain (FADD-DED), or FADD-death domain (FADD-DD). We show that expression and dimerization of FADD leads to necrosis. However, pretreatment of the cells with the Hsp90 inhibitor geldanamycin, which leads to proteasome-mediated degradation of receptor interacting protein 1 (RIP1), reverts FKBP-FADD-induced necrosis to apoptosis. Expression and dimerization of FADD-DD mediates necrotic cell death. We found that FADD-DD is able to bind RIP1, another protein necessary for Fas-mediated necrosis. Expression and dimerization of FADD-DD initiates apoptosis. Remarkably, in the presence of caspase inhibitors, FADD-DD mediates necrotic cell death. Communal necrosis studies revealed that FADD-DD in the absence of procaspase-8 CA is also capable of recruiting RIP1. However, when procaspase-8 CA and RIP1 are expressed simultaneously, FADD-DD preferentially recruits procaspase-8 CA.

Depending on the cell type, cellular context, or stimulus, a cell may die by apoptosis or necrosis (1). Apoptosis is characterized by membrane blebbing, shrinking and condensing of the cell and its organelles, and internucleosomal DNA degradation. Finally, the cell disintegrates, and apoptotic bodies are phagocytosed by professional phagocytes or neighboring cells, in most cases without any detrimental effect on the surrounding tissue (2, 3). Apoptosis is generally considered a process that depends on a family of cysteine proteases called caspases (4). Necrosis is characterized by swelling of the cell and its organelles and results in disruption of the cell membrane and cell lysis, often accompanied by inflammation due to release of cellular contents (1, 5). Although apoptosis and necrosis have clearly distinguished morphological and biochemical features, they can be initiated by tumor necrosis factor (TNF)1 or by Fas, depending on the cellular context. In L929rAhFas cells, TNF signals to necrotic cell death, whereas anti-Fas treatment of the same cells leads to apoptosis (6). Although pretreatment with the pan-caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp-(OMe)-fluoromethyl ketone (zVAD-fmk) inhibits anti-Fas-induced apoptosis, the cells nevertheless die necrotically (6). Recently we showed that TNFR1-induced necrosis is reverted to apoptosis by pretreatment with the Hsp90 inhibitor geldanamycin, which leads to the proteasome-dependent degradation of Hsp90 client proteins such as receptor-interacting protein 1 (RIP1) (7). Thus, both death domain-containing receptors, TNFR1 and Fas, can initiate apoptotic and necrotic cell death depending on the cellular context.

The Fas-associated death domain protein (FADD), which is recruited to the receptor through its carboxyl-terminal "death domain" (DD), is a key adaptor protein in death receptor-induced apoptosis (8, 9). The amino-terminal "death effector domain" (DED) of FADD interacts with a homologous DED within the prodomain of caspase-8 (10, 11), providing a platform for caspase-8 activation and consecutive downstream caspase activation either directly or through the cleavage of Bid and mitochondrial cytochrome c release (12, 13). The role of FADD in TNF receptor 1- and Fas-induced necrosis is still not defined. In FADD-deficient Jurkat cells, TNFR1-induced apoptosis is blocked, and the cells die via a necrotic pathway. However, both cell death pathways are blocked when these cells are exposed to Fas ligand (14, 15). This suggests that TNFR1-induced necrosis can occur independently of FADD, whereas Fas-induced necrosis cannot. In a previous study we showed by transient transfection experiments that overexpression of FADD-DD (amino acids 79–208) lacking the intact DED (8) is cytotoxic to L929A cells in a CrmA-insensitive way, whereas overexpression of FADD-DED (amino acids 1–111) containing the DED is toxic in a CrmA inhibitory way (16). In another study using a caspase-8-deficient Jurkat cell line, we showed that FADD is involved in signaling to necrotic cell death (17, 18). Here, we used stably

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¶ Supported by the Flanders Interuniversity Institute for Biotechnology.

** Supported by Gent University.

†‡ To whom correspondence should be addressed: Dept. of Molecular Biomedical Research, Technologiepark 927, B-9052 Zwijnaarde, Belgium. Tel.: 32-9-3313760; Fax: 32-9-3313609; E-mail: peter.vandenabeele@dmbr.UGent.be.

1 The abbreviations used are: TNF, tumor necrosis factor; TNFR1, 55-kDa TNF receptor; CrmA, cytokine response modifier A; DD, death domain; DED, death effector domain; FADD, Fas-associated death domain; FKBP, FK506-binding protein; PI, propidium iodide; RIP1, receptor-interacting protein 1; zVAD-fmk, benzoyloxycarbonyl-Val-Ala-Asp-(OMe)-fluoromethyl ketone; FS, forward scatter; SS, side scatter; HA, hemagglutinin.

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transfected L929rTA inducible cell lines expressing chimeric FADD molecules. FKBP represents amino acids 2-108 of human FKBP12. FADD-DD and FADD-DED contain amino acids 79–208 and 1–111 of human FADD, respectively. M and HA indicate the myristoylation targeting peptide and influenza hemagglutinin tag, respectively. B, expression of the chimeric FADD molecules in L929rTA cells after doxycycline (dox) administration. Lysates were separated on SDS-PAGE and analyzed by immunoblotting with an anti-HA antibody. C, effect of doxycycline and AP1510 on cell viability. Cell lines were incubated with doxycycline for 8 h followed by administration of AP1510 for 1–8 h at 37 °C. As a control for TNF sensitivity, the different cell lines were treated with human TNF (hTNF; 10,000 IU/ml). Cell death was determined by FACS analysis, and the loss of cell membrane integrity was determined by the PI exclusion method.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—The mouse fibrosarcoma cell line L929rTA and its derivatives were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml), and l-glutamine (0.03%). Recombinant human TNF was produced in-house (7). Propidium iodide (PI) and geldanamycin were purchased from Sigma and used at 30 and 1 μM, respectively. Doxycycline (Duchefa Biochemicals, The Netherlands) was used at 1 μg/ml. AP1510, a dimerizer of FKBP (19), kindly provided by ARIAD Pharmaceuticals Inc. (Cambridge, MA), was used at 1 μM. The pan-caspase inhibitor zVAD-fmk was from Enzyme Systems Products (Dublin, CA) and was used at 25 μM. The caspase-3-like substrate acetyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-aminomethylcoumarin was from the Peptide Institute (Osaka, Japan) and was used at 50 μM. Monoclonal antibodies to hemagglutinin (HA) epitope, poly(ADP-ribose) polymerase, and cytochrome c were from Berkeley Antibody Co. (Richmond, CA), Biomol Research Labs Inc. (Plymouth, PA), and Pharmingen (clone 7H8.2C12), respectively. Rabbit polyclonal antibodies against recombinant caspase-3 were prepared at the Centre d’Economie Rurale (Laboratoire d’Hormonologie Animale, Marloie, Belgium). Rabbit polyclonal antibodies against caspase-9 andBid were purchased from Cell Signaling (Beverly, MA) and R&D Systems Inc. (Minneapolis, MN), respectively. Anti–actin antibody was from ICN (Costa Mesa, CA). Secondary antibodies were from Amersham Biosciences. Monoclonal antibodies against HA tag were from Berkeley Antibody Co. The horseradish peroxidase-conjugated antibodies against V5 and E tag were from Invitrogen and Amersham Biosciences, respectively.

**Generation of Cells Responsive to Inducible Dimerization of FADD**—The plasmid pUHD10–3-FKBP2-link-FADD carries the coding sequence for a myristoylation-targeting peptide from the amino terminus of v-src, two tandem repeats of FKBP12, a short linker peptide, and FADD-FADD-DD constructs to examine the type of cell death initiated after doxycycline induction and enforced FKBP-dependent dimerization.
FIG. 2. Morphological and biochemical characterization of apoptotic and necrotic L929rTA cell death induced by chimeric FKBP-FADD proteins. A, phase contrast light microscopic pictures of L929rTA cells undergoing cell death after administration of doxycycline and AP1510. B, activation of caspase-3-like DEVDase activity. Cells were incubated with doxycycline and AP1510 at 37 °C, and cell lysates were made at the indicated times after AP1510 administration. DEVDase activity was determined with 25 μg of cell lysate and 50 μM fluorogenic substrate acetyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-aminomethylcoumarin. Data are expressed as the relative increase in fluorescence as a function of time (ΔF/min). C, processing of procaspase-3 and cleavage of Bid to its truncated form (tBid) and poly(ADP-ribose) polymerase. Cells were incubated with doxycycline (dox) for 8 h followed by administration of AP1510 for 4 h at 37 °C. Lysates were separated by SDS-PAGE and immunoblotted for detection of caspase-3 (casp-3), Bid, and poly(ADP-ribose) polymerase (PARP). As a control for protein loading, an antibody for β-actin was used. D, flow cytometric detection of apoptotic hypoploid nuclei after death induction with doxycycline (8 h) and AP1510 (4 h).
(8) were amplified by PCR from human FADD cDNA (8, 9). A DNA fragment coding for a short GSGGGS linker was inserted between FKBP2 and FADD. L929rTA cells (23) were stably cotransfected with 2 μg of the plasmid pPHT, coding for hygromycin resistance, 15 μg of pTet-tTS (Clontech, Palo Alto, CA), a tetracycline-controlled transcriptional silencer (24), and 15 μg of the respective pUHD10–3-FKBP2-link-FADD expression vectors. Stably transfected cells were selected for 3 weeks in medium containing 350 units/ml hygromycin. Individual colonies were analyzed for FKBP2-link-FADD, FKBP2-link-FADD-DED, and FKBP2-link-FADD-DD expression by Western blotting using an anti-HA antibody. All hygromycin-resistant cell clones showed considerable expression of transfected FADD constructs after induction with doxycycline for at least 6 h.

Cell Death Assay and Flow Cytometry Analysis—Cells were seeded at 2 × 10^5/well in uncoated 24-well plates and incubated overnight at 37 °C in a humidified air incubator. The next day cells were treated with doxycycline, AP1510, and inhibitors as indicated. Generally, cells were treated with doxycycline for 4–24 h in combination with AP1510 (1 μM, 4–8 h after doxycycline administration). zVAD-fmk was added 2 h before AP1510 administration, whereas geldanamycin was added 18 h before AP1510 administration. Cells were analyzed on a FACS Calibur flow cytometer (BD Biosciences). Loss of cell mem-
brane integrity was determined using the PI exclusion method (excitation at 488 nm and detection at 610 nm) as reported previously (7). The percentage of cells containing hypoploid DNA was determined by PI-staining of cells after one freeze-thaw cycle to permeabilize cells, as described (25).

Western Blot Analysis—Cells were seeded at 2 x 10^4/well in 6-well plates. The next day, doxycycline and AP1510 were added to the cells as described above. Cells were washed with cold phosphate-buffered saline and lysed in caspase lysis buffer (1% Nonidet P-40, 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 0.3 mM aprotinin, and 1 mM leupeptin). For cytosolic cytochrome c detection cells were washed with cold phosphate-buffered saline and permeabilized in 0.02% digitonin dissolved in cell-free system buffer containing 10 mM Hepes, pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH₂PO₄, 0.5 mM EGTA, 2 mM MgCl₂, 5 mM pyruvate, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) and left on ice for 1 min. This treatment allows selective lysis of the outer cell membrane without affecting the organelle membranes. All lysates were cleared by centrifugation at 20,000 x g for 10 min and stored at 4°C. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell). Proteins were visualized with the chemiluminescence Renaissance reagent (PerkinElmer Life Sciences) after incubation of the membranes with primary and secondary antibodies.

Fluorometric Assay for Caspase-3-like Activity—Caspase activity was measured by incubating 25 μg of cell lysate with 50 μM fluorogenic substrate acetyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-aminomethylcoumarin (Peptide Institute, Osaka, Japan) in 150 μl of CFS buffer (10 mM HEPES-NaOH, pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH₂PO₄, 0.5 mM EGTA, 2 mM MgCl₂, 5 mM pyruvate, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol). The release of fluorescent 7-amino-4-methylcoumarin was monitored for 1 h at 37°C at 2-min time intervals in a fluorometer (CytoFluor, PerSeptive Biosystems, Cambridge, MA) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Data are expressed as the increase in fluorescence as a function of time (ΔF/min).

Coimmunoprecipitation Experiments—293T cells were seeded in 9-cm plates at 1.2 x 10⁶ cells/plate 1 day before transfection with the indicated expression vectors, viz. HA-tagged FADD, FADD-DED, and FADD-DD in FKBP-containing pCMF2E plasmid, E-tagged mouse pro-caspase-8 C347A in pCAGGS, and His/V5-tagged RIP1 in pEF1. To avoid secondary effects resulting from cytotoxicity we used a catalytically inactive form of procaspase-8 (C347A) called procaspase-8 C/A. 18 h after transfection cells were treated for 15 min with the dimerizer AP1510, washed with phosphate-buffered saline, and lysed in a buffer containing 1% Nonidet-P40, 10 mM Tris-HCl, pH 7.4, 200 mM NaCl, 5 mM EDTA, and 10% glycerol for 30 min at 4°C. Lysates (500 μl) were incubated for 1 h at 4°C with 1 μg of anti-HA antibody and then exposed to protein G-Sepharose beads prewashed with lysis buffer containing 5% bovine serum albumin. After overnight rotation at 4°C, immune complexes were eluted by boiling in 1× Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol,

Fig. 4. FKBP-FADD-induced necrosis is reverted to apoptosis by blocking Hsp-90-function. Cells expressing FKBP-FADD (A) and FKBP-FADD-DD (B) were treated with geldanamycin before oligomerization with AP1510 for 1–6 h at 37°C. Cell death was determined by FACS analysis, and the loss of cell membrane integrity was determined by means of the PI exclusion method (upper graphs). Flow cytometric detection of apoptotic hypoploid nuclei in FKBP-FADD and FKBP-FADD-DD cells after death induction with doxycycline (dox) and AP1510 in the presence of geldanamycin (middle and lower graphs). The results are representative of three different experiments.
FADD Signaling to Apoptosis and Necrosis

RESULTS

Generation of L929rTA-inducible Cells Sensitive to FADD Oligomerization—To study the role of FADD in apoptotic and necrotic cell death we established stably transfected FADD-inducible L929rTA cell lines. L929rTA cells expressing a mutant tetracycline repressor protein (22, 23) were transfected with cDNA coding for human FADD, FADD-DED (amino acids 1–111), or FADD-DD (amino acids 79–208) under the control of a minimal cytomegalovirus promoter and the tetracycline operator sequences. The addition of doxycycline, a potent tetracycline derivative, results in the binding of the transactivator to the tetracycline operator sequence, allowing transcriptional activation of the FADD constructs. In the constructs used the cDNAs of the FADD variants were fused to two copies of FKBP (FK506-binding protein), analogous to the approach used by Kawahara et al. (17), allowing conditional oligomerization using a synthetic dimerizer AP1510 (19) (Fig. 1A). To ensure flexibility of the FADD constructs, we added a six-amino acid linker/spacer (GSGGGGS) between the cognate FKBP modules and the FADD cDNAs (26). The addition of doxycycline and AP1510 induced the expression and oligomerization of the respective FADD-con- structs (Fig. 1B), leading to cell death (Fig. 1C). TNF treatment demonstrated that the different cell lines retained a comparable sensitivity to TNFR1-induced necrosis (Fig. 1D).

FADD-DED-mediated Apoptosis versus FADD-DD-mediated Necrosis in L929rTA—To examine which type of cell death is induced through the oligomerization of full-length FADD and FADD deletion mutants, the corresponding L929rTA cells were treated with doxycycline and AP1510, and morphology was examined by light microscopy. FADD and FADD-DD oligomerization induced the cytoplasmic swelling typical of necrosis, whereas FADD-DED oligomerization induced a typical apoptotic morphology characterized by membrane blebbing and the formation of apoptotic bodies (Fig. 2A). Next, we analyzed the enzymatic activity and proteolytic processing of caspase-3. When treated with doxycycline and AP1510, caspase-3 enzymatic activity was detected in L929rTA cells expressing FKBP-FADD-DED but not in cells expressing full-length FKBP-FADD or the FKBP-FADD-DD mutant (Fig. 2B). Moreover, proteolysis of the 32-kDa procaspase-3 and generation of the 17-kDa fragment typical of the active caspase were observed in FKBP-FADD-DED cells but not in FKBP-FADD and FKBP-FADD-DD cells (Fig. 2C), although massive cell death occurred in all of these cell lines (Fig. 1C). This proteolytic activation of caspase-3 in cells expressing FKBP-FADD-DED was accompanied by the cleavage of the 23-kDa Bcl-2-family protein Bid to its truncated 15-kDa form and the cleavage of the caspase-3 substrate poly(ADP-ribose) polymerase, as demonstrated by the loss of the 110-kDa full-length protein (Fig. 2C). Moreover, appearance of hypoploid nuclei, a parameter for apoptotic cell death (27), was only observed after FKBP-FADD-DED induction and oligomerization (Fig. 2D). These results indicate that induction and oligomerization of FKBP-FADD-DED induce a caspase-dependent apoptosis, whereas induction and oligomerization of FKBP-FADD and FKBP-FADD-DD result in a caspase-independent necrotic cell death.

Blocking of Caspases by zVAD-fmk Shifts FADD-DED-mediated Apoptosis to Necrosis—Changes in cellular morphology and granularity of dying cells were also evaluated by flow fluorocytometry (Fig. 3A). Necrosis correlates with a small decrease in the forward-scatter (FS) and an increase in the side-scatter (SS) of the PI-positive population (red dots) compared with control cells. Apoptosis is associated with a decrease in both parameters (7, 28). The FS/SS profile of PI-positive FKBP-FADD and FKBP-FADD-DD cells after doxycycline and AP1510 treatment was reminiscent of necrotic cell death, whereas induction and oligomerization of FKBP-FADD-DED resulted in decreased FS and SS, typical for apoptosis (Fig. 3A). These flow fluorocytometric analyses are in accord with the light microscopic morphology of the cells. L929sAhFas cells die by necrosis when Fas-induced apoptotic death was blocked using caspase inhibitors (6). Interestingly, induction and oligomerization of FKBP-FADD-DED in the presence of the broad-spectrum caspase inhibitor zVAD-fmk also resulted in a switch from apoptosis to necrosis, as demonstrated by the forward-scatter and side-scatter pattern of the PI-positive population (red dots) (Fig. 3A) and by the absence of a hypoploid DNA pattern (Fig. 3B). Thus, FADD-DED kills cells via an apoptotic pathway, but if caspases are blocked the cells respond by a caspase-independent necrotic pathway.

FADD-induced Necrosis Reverts to Apoptosis by Blocking Hsp90 Function—Recently, we showed that pretreatment with the Hsp90 inhibitor geldanamycin leads to degradation of Hsp90 client proteins such as RIP1 and reverts TNFR1-in-duced necrosis to apoptosis (7). A similar switch was observed after induction and oligomerization of FKBP-FADD in the pres-
ence of geldanamycin, illustrated by the appearance of a hypoploid DNA pattern (Fig. 4A). Necrosis induced by FKBP-FADD-DD oligomerization was moderately delayed by geldanamycin, but no shift to apoptosis was observed (Fig. 4B). These results clearly demonstrate that depending on the cellular context, FADD can induce necrotic or apoptotic cell death, whereas FADD-DD only signals to necrosis.

**FADD-DED Preferentially Recruits Procaspase-8 C/A, whereas FADD-DD Only Recruits RIP1**—To identify the initiator molecules recruited to the dimerized FKBP-FADD-DED and FKBP-FADD-DD complexes, we immunoprecipitated these artificial complexes from 293T cells transfected with the respective constructs. As expected, in cells co-expressing the catalytically inactive mutant form of procaspase-8 and FKBP-FADD-DED, dimerization of FKBP-FADD-DED led to efficient recruitment of procaspase-8 C/A (Fig. 5, lane 2), whereas FKBP-FADD-DD was incapable of recruiting procaspase-8 C/A (Fig. 5, lane 9). Conversely, we detected strong recruitment of RIP1 by dimerized FKBP-FADD-DD in cells co-expressing RIP1 and FKBP-FADD-DD (Fig. 5, lane 8). Remarkably, FKBP-FADD-DED was also capable of binding RIP1 (Fig. 5, lane 3). However, co-transfection of procaspase-8 C/A reduced the levels of FADD-DED-mediated recruitment of RIP1 to background levels (Fig. 5, lane 1). In contrast, the recruitment of RIP1 to FKBP-FADD-DD was not affected by co-transfection of procaspase-8 C/A (Fig. 5, lane 10). These results demonstrate that FKBP-FADD-DED can recruit both procaspase-8 C/A and RIP1, but binding to procaspase-8 C/A is more efficient. In contrast, FKBP-FADD-DD cannot recruit procaspase-8, explaining the inability of FKBP-FADD-DD to shift the response from necrosis to apoptosis in the presence of geldanamycin.
The role of FADD in TNFR1- and Fas-induced necrosis is controversial. In FADD-deficient Jurkat cells, TNFR1-induced apoptosis is blocked, whereas necrosis still occurs. In the same cells, both Fas-induced apoptosis and necrosis are prohibited (14, 15). This suggests that TNFR1-induced necrosis can occur independently of FADD, whereas Fas-induced necrosis requires FADD. In Fas-induced signaling, FADD seems to function as a platform to initiate both apoptosis and necrosis. In favor of a role for FADD in necrotic signaling is the finding that inducible and dimerizable FADD-DED could initiate apoptosis or necrosis, depending on the presence or absence of procaspase-8, respectively (18). In the same report, enforced dimerization of FADD-DD was not cytotoxic. However, we found that transient overexpression of FADD-DD was able to initiate necrotic cell death (16). To clarify this controversy, we used stably transfected L929rTA-inducible cell lines expressing chimeric FKBP-FADD, FKBP-FADD-DED, and FKBP-FADD-DD constructs and examined the type of cell death initiated after doxycycline induction and enforced FKBP-dependent dimerization. The different types of cell death induced by FADD, FADD-DED, and FADD-DD are illustrated in a schematic overview (Fig. 6).

Induction and dimerization of FKBP-FADD gave rise to a necrotic cell death phenotype. One could expect that overexpression and dimerization of FADD would lead to apoptosis, since FADD is thought to transmit the apoptotic signal from Fas to the caspases. Indeed, enforced dimerization of FKBP-FADD induces apoptotic cell death in HEK 293T cells (data not shown). Similarly, enforced dimerization of FADD in Jurkat cells leads to apoptotic cell death. However, in caspase-8-deficient Jurkat cells (JB6) enforced dimerization of FADD gives rise to a necrotic cell death (17). In L929 cells overexpression of TNFR1, TNFR-associated death domain (TRADD), or FADD leads to necrosis, suggesting that these cells have a pro-necrotic cellular context (16, 29, 30). Moreover, like TNFR1-mediated necrosis, FKBP-FADD-induced necrosis can revert to apoptosis by pretreating the cells with geldanamycin, a Hsp90 inhibitor (7). This suggests that depending on the cellular context, for example the presence or absence of Hsp90-binding client proteins such as RIP1, enforced dimerization of FADD can induce necrotic or apoptotic cell death. Of note, triggering of Fas in L929 cells followed by FADD recruitment clearly induces apoptotic cell death. These findings enforce the suggestion that recruitment of FADD to Fas induces a modification or conformational change in FADD, which is crucial for the apoptotic outcome (11, 31, 32).

Induction and dimerization of FKBP-FADD-DD also led to a necrotic cell phenotype (Fig. 6A). This is in agreement with observations that overexpression of a FADD dominant negative mutant, lacking the DED and, thus, incapable of recruiting procaspase-8, induces TNF-mediated necrosis in U937 and NIH3T3 cells (33). However, as mentioned above, Matsumura et al. (18) observed no cytotoxicity in Jurkat cells after enforced dimerization of FADD-DD. This could be due to the fact that they used an incomplete DD in their FADD-DD construct. The DD of FADD starts at amino acid position 93 (31), whereas the protein used by Matsumura started at amino acid 108, resulting in the absence of the first helix. It is conceivable that this first helix of the FADD-DD is required for recruiting RIP1 and inducing necrosis (31). Recently, a detailed analysis of the Fas/FADD-DD interaction demonstrated that an expansive surface on one side of the domain is comprised of elements in α helices 1, 2, 3, 5, and 6 (34). Pretreatment of the FKBP-FADD-DD cells with geldanamycin delayed but did not shift the cell death response from necrosis to apoptosis, since procaspase-8 could not be recruited. This observation also suggests that the artificially activated FADD-DD complex does not recruit endogenous FADD, which would initiate an apoptotic response in the presence of geldanamycin.

Recently, the receptor-interacting protein RIP1 was identified as a crucial component in the signal transduction to necrotic cell death (14). Unlike its role in the activation of NF-κB through recruitment of the IκB kinase (IKK) complex to the TNF receptor, it was shown that in Jurkat cells the kinase activity of RIP1 is required for necrotic cell death, suggesting that a RIP1 substrate may propagate the necrotic cell death pathway (14). Yeast two hybrid screening using FADD as bait demonstrated that RIP1 is able to bind FADD (35).2 The recruitment of RIP1 by FADD-DD in immunoprecipitation studies suggests that FADD-DD-mediated necrotic signaling in L929α cells occurs through RIP1.

FKBP-FADD-DD-induced cytotoxicity showed the typical morphological and biochemical features of apoptotic cell death (Fig. 6B). When caspase activation was prevented using the caspase inhibitor zVAD-fmk, apoptotic cell death induced by oligomerization of FKBP-FADD-DD was blocked but nevertheless cells died by necrosis. This suggests that the DED can also initiate necrotic cell death in the absence of active caspases. Similarly, in caspase-8-deficient Jurkat cells (JB6 cells) transfected with a chimeric FKBP-FADD construct, FKBP-FADD-DD was shown to mediate a necrotic cell death signal (17, 18). The observation that FKBP-FADD-DD was also capable of binding RIP1 confirms the pronecrotic role of FADD-DD in the presence of caspase inhibitors. However, simultaneous expression of both procaspase-8-C/A and RIP1 reduced the levels of FADD-DD-mediated recruitment of RIP1 to background levels but did not affect the extent of FADD-DD-mediated recruitment of RIP1. These results demonstrate that although FKBP-FADD-DD can recruit both procaspase-8-C/A and RIP1, the binding to procaspase-8-C/A seems to be more efficient. Moreover, in normal cellular conditions an additional level of interplay exists between caspase-8 and RIP-1. Indeed, caspase-8 is able to proteolyze and inactivate RIP1 signaling toward NF-κB activation (36–38). These results may explain why FADD-DD mediates apoptosis by recruiting and activating procaspase-8 and by preventing the NF-κB-inhibited anti-apoptotic signaling induced by RIP1. However, in the absence of caspase-8, as exemplified in caspase-8-deficient Jurkat cells (17), or if caspase activity is blocked by zVAD-fmk, RIP1 is not cleaved and may, thus, propagate necrotic cell death.

In conclusion, the present results show that apoptotic and necrotic cell death pathways can initiate at the level of the signal adapter FADD. FADD-DD very efficiently recruits RIP1, an important kinase in necrotic signaling, whereas FADD-DD preferentially recruits procaspase-8 and initiates apoptosis. However, in the presence of caspase inhibitors FADD-DD will mediate necrotic cell death by its capacity to recruit RIP1.

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Tom Vanden Berghe, Geert van Loo, Xavier Saelens, Maria van Gurp, Greet Brouckaert, Michael Kalai, Wim Declercq and Peter Vandenabeele

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