Dual Signaling of the Fas Receptor: Initiation of Both Apoptotic and Necrotic Cell Death Pathways

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Summary

Murine L929 fibrosarcoma cells were transfected with the human Fas (APO-1/CD95) receptor, and the role of various caspases in Fas-mediated cell death was assessed. Proteolytic activation of procaspase-3 and -7 was shown by Western analysis. Acetyl-Tyr-Val-Ala-Asp-chloromethylketone and benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Ala-Asp(OMe)-fluoromethylketone, tetrapeptide inhibitors of caspase-1- and caspase-3-like proteases, respectively, failed to block Fas-induced apoptosis. Unexpectedly, the broad-spectrum caspase inhibitors benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone and benzyloxycarbonyl-Asp(OMe)-fluoromethylketone rendered the cells even more sensitive to Fas-mediated cell death, as measured after 18 h incubation. However, when the process was followed microscopically, it became clear that anti-Fas-induced apoptosis of Fas-transfected L929 cells was blocked during the first 3 h, and subsequently the cells died by necrosis. As in tumor necrosis factor (TNF)-induced necrosis, Fas treatment led to accumulation of reactive oxygen radicals, and Fas-mediated necrosis was inhibited by the oxygen radical scavenger butylated hydroxyanisole. However, in contrast to TNF, anti-Fas did not activate the nuclear factor κB under these necrotic conditions. These results demonstrate the existence of two different pathways originating from the Fas receptor, one rapidly leading to apoptosis, and, if this apoptotic pathway is blocked by caspase inhibitors, a second directing the cells to necrosis and involving oxygen radical production.

Key words: Fas antigen • apoptosis • necrosis • caspases • oxygen radicals

Depending on the cell type and the stimulus, a cell may die in either of two distinct ways: apoptosis or necrosis. Necrosis is characterized by swelling of the cell and the organelles, and results in disruption of the cell membrane and in lysis (1). Release of the cytoplasmic content leads to an inflammatory response. Apoptosis can be recognized by membrane blebbing, shrinking and condensing of the cells and their organelles, internucleosomal degradation of the DNA, and disintegration of the cell, after which the fragments are phagocytosed by neighboring cells (2, 3). Apoptosis occurs at various stages in the life cycle of multicellular organisms. From embryonic development through immunological regulation, as well as part of pathological situations, cell death by apoptosis is the most common way to eliminate superfluous, inappropriate, or harmful cells (4). However, inappropriate or defective apoptosis is the cause of many human diseases (5). Therefore, many studies aim to elucidate the apoptotic pathways and interactions between proapoptotic and antiapoptotic cellular components. Although mechanisms signaling to necrosis cannot be fully explained in molecular terms, apoptosis is generally believed to be mediated by caspase activation (6–8).

The TNF receptor type 1 (TNFR-1) and Fas (APO-1/CD95) are two cell surface receptors sharing structural and functional homology. Both are characterized by extracellular repeats of cysteine-rich domains (9), but TNFR-1 and Fas also have a common intracellular motif, called the “death domain” (DD),1 of ~80 amino acids (10, 11). These

1Abbreviations used in this paper: Ac-DEVD-amc, acetyl-Asp(OMe)-Glu(OMe)-Val-Ala-Asp(OMe)-aminomethylcoumarin; Ac-YVAD-amc, acetyl-Tyr-Val-Ala-Asp(OMe)-aminomethylcoumarin; Ac-YVAD-cmk, acetyl-Tyr-Val-Ala-Asp-chloromethylketone; BHA, butylated hydroxyanisole; DD, death domain; DR, death receptor; DHR123, dihydrorhodamine, 123; FADD, Fas-associated DD protein; L929hFas, Fas-transfected L929 (cells); MORT, mediator of receptor-induced toxicity; NF-κB, nuclear factor κB; PI, propidium iodide; TRADD, TNFR-associated DD protein; TRAIL, TNF-related apoptosis-inducing ligand; zAAD-cmk, benzyloxycarbonyl-Ala-Ala-Asp-chloromethylketone; zDEVD-fmk, benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Ala-Asp(OMe)-fluoromethylketone; zD-fmk, benzyloxycarbonyl-Asp(OMe)-fluoromethylketone; zVAD-afc, benzyloxycarbonyl-Val-Ala-Asp(OMe)-aminotrifluoromethylcoumarin; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone.
The clustered DDs of TNFR-1 recruit TNF-associated DD protein (TRADD), which then forms a docking site for Fas associated DD protein (FADD) / mediator of receptor-induced toxicity (MORT)1 (15, 16). After clustering of Fas, FADD directly associates through its own DD to that of Fas (13, 17, 18). For both TNFR-1 and Fas, FADD recruits FADD-like IL-1β-converting enzyme (FLICE)/MORT1-associated CED-3 homologue (MACH)/procaspase-8 by a homotypic interaction through their so-called “death effector domain” (19, 20). The COOH-terminal part of procaspase-8 consists of a CPP32/YAMA/caspase-3–like domain. It is presumed that recruitment of procaspase-8 in the Fas complex after Fas ligand binding results in activation of procaspase-8 (21), which in turn initiates a caspase activation cascade, eventually leading to apoptosis (22–24).

Under normal conditions, Fas triggering has so far only been reported to result in cell death by apoptosis. But depending on the cell line, activation of the TNF-1–signaling pathway leads either to apoptotic or to necrotic cell death (25, 26). Mere trimerization of the TNF-1 DD is sufficient for signaling to necrosis of L929 cells (14). The same intracellular domain mediates both TNF-mediated necrosis and apoptosis. In contrast to apoptosis, TNF-mediated necrosis is not dependent on caspase activity in L929 cells. On the contrary, inhibition of caspases sensitizes the cells to TNF cytotoxicity, and this sensitization is correlated with a higher production of reactive oxygen radicals (27). It has been shown previously that TNF-induced necrosis in L929 cells is preceded by mitochondrial radical production, and scavenging of these radicals by agents such as butylated hydroxyanisole (BHA) blocks TNF cytotoxicity (28). There is much evidence that reactive oxygen radicals are the key mediators of necrotic cell death in L929 cells.

In this paper we show that when the apoptotic pathway induced by Fas triggering in L929 cells is blocked by a caspase inhibitor, the cells die in a necrotic way, which involves oxygen radicals.

**Materials and Methods**

Cells. L929 cells and their TNF-resistant derivatives L929r1 (29) were cultured in DMEM, supplemented with 5% newborn bovine serum and 5% FBS, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and l-glutamine (0.03%).

Abs, Cytokines, and Reagents. R recombinant murine TNF was produced in Escherichia coli and purified to 99% homogeneity (30). The specific activity was 1.4 × 10^8 IU/mg as determined in a standardized cytotoxicity assay on L929 cells. Anti–human Fas Abs (agonistic Abs clone CH-11; immunodetection Abs clone UB-2) were purchased from ImmunoTech (Marseille, France). Dihydrorhodamine 123 (DHR123; DHR123; Molecular Probes, Inc., Eugene, OR) was prepared as a 5 mM stock solution in DM SO and used at 1 μM. Propidium iodide (PI; Becton Dickinson, Franklin Lakes, NJ) was dissolved at 3 mM in PBS and used at 30 μM. BHA was purchased from Sigma Chemical Co. (St. Louis, MO) and prepared as a 500 mM stock solution in ethanol.

The caspase inhibitor benzyloxy carbonyl–Asp(O Me)–Glu(OMe)–Val–Asp(OMe)–fluoromethylketone (ZDEVD-fmk), benzyloxy carbonyl–Val–Ala–Asp(OMe)–fluoromethylketone (ZVAD–fmk), and benzyloxy carbonyl–Asp(OMe)–fluoromethylketone (Z–fmk) were purchased from Enzyme Systems Products, Inc. (Livermore, CA). Acetyl–Tyr–Val–Ala–Asp–chloromethylketone (Ac–YVAD–cmk) and benzyloxy carbonyl–Ala–Ala–Asp–chloromethyl ketone (ZAAS-cmk) were supplied by Calbiochem-Novabiochem International (San Diego, CA).

Anticytokine response modifier A Abs were provided by Dr. D. Pickup (Duke University Medical Center, Durham, NC). Polyclonal Abs against recombinant murine caspases were prepared by the Centre d’Economie R urale (Laboratoire d’Hormonologie Animale, M arloie, Belgium).

Plasmids and Transfections. Human Fas cDNA was provided by Dr. S. Nagata (O saka Bioscience Institute, Osaka, Japan), and was inserted as an X hol–X ba fragment in pfEF–BOS (31). pH Y TW, containing the hygromycin resistance gene, was used as a selection vector.

Cytotoxicity Assays. Cells were seeded on day –1 at 2 × 10^4 cells/well in 96-well plates. The next day, inhibitors and anti-Fas (clone CH–11) were added at the given concentrations. Typically, cells were incubated with anti-Fas for 18 h, and cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide staining as described previously (32). The percentage of cell survival was calculated as follows: (A untreated medium)/(A treated medium) × 100.

Measurement of Fas Expression, Oxygen Radical Accumulation, Cell Death, and DNA Histograms by Flow Fluorometry. For FACS analysis, cells were kept in suspension by seeding them the day before in uncoated 24-well tissue culture plates (Sarstedt, Inc., Newton, NC).

To visualize membrane expression of Fas, cells were stained with anti-Fas Abs (clone UB–2) and FITC–conjugated anti–mouse IgG (Harlan Sera-Lab Ltd., Loughborough, Leicestershire, UK), and analyzed on a FACScalibur® flow fluorometer (Becton Dickinson) equipped with a 488 nm argon ion laser. FITC fluorescence was detected at 525 nm.

To measure oxygen radical production, DHR123 was added at the same time as anti–Fas to suspension cultures, and samples were taken at different time points. PI fluorescence was detected at 610 nm and served as the measure for the percentage of loss of membrane integrity to quantify cell death. RFL23 fluorescence, as a result of DHR123 oxidation, was analyzed on intact PI-negative cells and detected at 525 nm. Relative RFL23 fluorescence is defined as the ratio of emitted fluorescence at a given time point to initial fluorescence for the same condition. DNA histograms were obtained by subjecting the samples to one freeze–thaw cycle in the presence of PI, and analyzing PI fluorescence of the cells as described above.

Confocal Microscopy. L929 cells were seeded on the day before measurements on Lab-Tek chambered coverslips (N unc, Inc., Naperville, IL). The next day, ZVAD–fmk was added at 25 μM final concentration, followed 2 h later by the addition of anti–Fas Abs (500 ng/ml) or TNF (10,000 IU/ml). Before microscopic analysis, PI was added, and the cells were observed with a confocal microscope (model LSM 410; Carl Zeiss, Inc., Thornwood, NY), using an excitation wavelength of 488 nm and analyzing through a 610-nm long-pass emission filter.

Fluorogenic Substrate Assays for Caspase Activity. Cytosolic cell extracts were prepared by lysing the cells in a buffer containing 1% NP-40, 200 mM NaCl, 20 mM Tris·HCl, pH 7.4, 10 μg/ml...
leupeptin, aprotinin (0.27 trypsin-inhibitory U/ml), and 100 μM PM SF. Caspase-like activities were determined by incubation of cell lysate (containing 25 μg total protein) with 50 μM of the fluorogenic substrates acetyl-Tyr-Val-Ala-Asp-aminomethylcoumarin (Ac-YVAD-amc), acetyl-Asp(O Me)-Glu(O Me)-Val-Asp(O Me)-aminomethylcoumarin (Ac-DEVD-amc), or benzoyloxy carbonyl-Val-Ala-Asp(O Me)-aminotrifluoromethylcoumarin (zVAD-afc) (Peptide Institute Inc., Osaka, Japan) in 200 μl cell-free system buffer, comprising 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 PM SF, and 1 mM dithiothreitol. The release of fluorescent aminomethylcoumarin or aminotrifluoromethylcoumarin was measured for 1 h at 2-min intervals by fluorometry (Cytofluor; PerSeptive Biosystems, Cambridge, MA); data are expressed as the maximal rate of increase in fluorescence.

Western Analysis of Caspase Activation. Cells were treated with anti-Fas for 0.5 or 2 h, or were left untreated as a control; 7.5 × 10⁶ cells were then lysed in 1 ml lysis buffer (1% NP-40, 10 mM Tris pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 1 mM PM SF, 0.3 mM aprotinin, and 1 mM leupeptin). 50 μg total protein was loaded per lane on 15% SDS-polyacrylamide gels, and after separation and blotting the different caspase fragments were detected using polyclonal antisera raised against recombinant murine caspases and developed by ECL (Nycomed Amersham plc, Little Chalfont, Buckinghamshire, UK).

Measurement of Nuclear Factor κB Activity. L929 cells carried a reporter construct consisting of a luciferase gene under control of the minimal chicken conalbumin promoter preceded by three nuclear factor κB (NF-κB) sites (33). Cells were seeded on day 2 at 2 × 10⁴/microwell. The next day, cells were pretreated with different caspase inhibitors for 2 h and stimulated with TNF. After 3 h incubation, cells were lysed according to the luciferase assay protocol of Promega, Inc. (Madison, WI); luciferin (Duchefa Biochemie, Haarlem, The Netherlands) was added, and luciferase activity was measured on a Topcount Luminometer (Packard, Meriden, CT).

Results

Generation of L929 Cells Undergoing Rapid Apoptosis after Anti-Fas Addition. L929 cells were transfected with the cDNA encoding human FasR under transcriptional control of the minimal chicken conalbumin promoter preceded by three nuclear factor κB (NF-κB) sites (33). Cells were seeded on day 1 at 2 × 10⁶/microwell. The next day, cells were pretreated with different caspase inhibitors for 2 h and stimulated with TNF. After 3 h incubation, cells were lysed according to the luciferase assay protocol of Promega, Inc. (Madison, WI); luciferin (Duchefa Biochemie, Haarlem, The Netherlands) was added, and luciferase activity was measured on a Topcount Luminometer (Packard, Meriden, CT).

Figure 1. Rapid induction of apoptosis in L929hFas cells. Cells were treated with 500 ng/ml of agonistic anti-Fas mAb in the absence (circles) or presence (triangles) of 1 μg/ml actinomycin D. The fraction of PI-positive cells was determined in a time-course flow fluorocytometric experiment. Inset, Flow fluorocytometric analysis of Fas expression on Fas-transfected cells (solid line) and mock-transfected cells (broken line).

Figure 2. Determination of caspase activity in whole cell lysates of L929hFas cells. Cells were treated with 500 ng/ml of anti-Fas Ab (A) or 10,000 IU/ml of TNF (B) in the absence of actinomycin D. The cleavage activity, expressed as maximal fluorescence increase (max. ΔFt), on Ac-DEVD-amc (filled circles), Ac-YVAD-amc (triangles), and zVAD-afc (squares) was measured as described in Materials and Methods. Before lysis, the percentage of PI-positive cells was determined by flow fluorocytometric analysis (open circles).
Fas-mediated apoptosis in L929 cells is associated with activation of caspase-3 and -7. We investigated whether anti-Fas treatment of L929Fas cells resulted in induction of caspase activity. In a first approach, cells were stimulated for several time periods with anti-Fas, and total cytoplasmic lysates were incubated with fluorogenic oligopeptide caspase substrates. No cleavage of Ac-YVAD-amc or zVAD-afc was observed. However, significant hydrolysis of Ac-DEVD-amc was detected (Fig. 2A). These results confirm that during Fas-mediated apoptosis in L929 cells, caspase-3-like proteases are rapidly activated.

In a parallel set-up, cell viability was assessed using flow fluorocytometry to determine the percentage of PI-positive cells. Maximal Ac-DEVD-amc cleaving activity roughly corresponded with the onset of loss of membrane integrity. As cell death progressed in time, Ac-DEVD-amc cleavage activity decreased rapidly. Presumably, this reflects loss of active enzyme in the dying cells. As a control, L929Fas cells were treated with TNF. TNF kills these cells by necrosis, and no caspase activity could be detected after TNF treatment (Fig. 2B).

To further identify the caspases involved in Fas-mediated apoptosis in L929Fas cells, we subjected whole cell lysates of Fas-treated cells to SDS-PAGE, followed by Western analysis using polyclonal Abs raised against recombinant murine caspases. As shown in Fig. 3, cleavage of procaspases-3 and -7 and formation of p17/p18 fragments (39) were observed after 2 h. No cleavage fragments of procaspase-1, -2, -6, -11, or -12 were detected in the same lysates. This suggests that clustering of Fas very rapidly leads to activation of procaspase-3 and -7. Most probably, activation of procaspase-8 leads directly to activation of downstream procaspase-3 and -7. Indeed, the latter caspases are direct substrates of caspase-8 as demonstrated previously in vitro (22, 23).

Oligopeptide Caspase Inhibitors Do Not Block Fas-mediated Cell Death. Irreversible oligopeptide caspase inhibitors have been used extensively to study the role of caspases in apoptosis. Ac-YVAD-cmk and zDEVD-fmk block caspase-1- and caspase-3-like proteases, respectively, whereas zVAD-fmk and zD-fmk are broad-range caspase inhibitors (40–43). We observed that neither Ac-YVAD-cmk nor zDEVD-fmk could prevent Fas-mediated L929Fas cell death in an 18-h cytotoxicity assay, even when 100 μM inhibitor was used. Both zDEVD-fmk and Ac-YVAD-cmk entered the cells effectively. Pretreatment of the cells with zDEVD-fmk efficiently blocked cleavage of the fluorogenic substrate Ac-DEVD-amc and poly(ADP-ribose) polymerase. Moreover, Western analysis of substrate zDEVD-fmk–pre-treated cells showed an upward shift in M1, of the large subunit of caspase-3 and caspase-7, demonstrating efficient entering into the cells and binding of the inhibitor to caspases (data not shown). Pretreatment with 100 μM Ac-YVAD-cmk could inhibit by 70% the TNF-induced release of murine IL-1β by L929 cells transfected with the cDNA encoding murine pro-IL-1β, thus confirming the capability of Ac-YVAD-cmk to inhibit caspase-1-like proteases in L929 cells (data not shown). Surprisingly, both zVAD-fmk and zD-fmk increased the susceptibility of L929Fas cells to Fas-mediated cell death by 20-fold, as measured by staining with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Fig. 4). Maximal sensitization occurred already at 25 μM, which suggests that this action is specific. zAAD-cmk, a granzyme B inhibitor (44, 45), had no effect on Fas-mediated cell death. Taken together, these results...
suggest that even when activation of caspase-3 and -7 is blocked, Fas clustering still leads to death of L929hFas cells. Moreover, the broad-range caspase inhibitors sensitized the cells ~20-fold to anti-Fas. These results are reminiscent of the sensitization by caspase inhibitors observed in TNF-mediated necrosis in L929 cells (27).

zVAD-fmk and zD-fmk treatment of L929hFas cells revealed an alternative death signaling pathway originating from Fas. To study whether the increased sensitivity to Fas-mediated cell death in the presence of zVAD-fmk or zD-fmk reflected necrotic cell death, cells were examined microscopically (Fig. 5). Cells treated for 1 h with anti-Fas alone clearly showed extensive membrane blebbing. However, when zVAD-fmk was present, the cells retained the same morphology as control cells in the absence of anti-Fas. After 2 h, anti-Fas-treated cells, without caspase inhibitors or in the presence of Ac-YVAD-cmk or zDEVD-fmk, succumbed to apoptotic cell death. Quite surprisingly, cells incubated in the presence of zVAD-fmk began to exhibit an altered morphology, such as cytoplasmic swelling. After 3 h, all cells had died in a way that strikingly resembled TNF-mediated necrosis.

To further characterize this alternative Fas-mediated pathway, the nuclear morphology of TNF-induced necrosis and anti-Fas-induced cell death was compared in the presence or absence of zVAD-fmk. Nuclear morphology was revealed by PI fluorescence using confocal microscopy after 3 h of treatment (Fig. 6). When cells were treated with TNF in the absence or presence of zVAD-fmk, no chromatin condensation was observed (Fig. 6, A and B), in contrast to Fas-mediated apoptosis (Fig. 6 D). However, in the presence of zVAD-fmk, incubation with anti-Fas did not affect nuclear morphology (Fig. 6 E), as is the case for TNF-mediated necrosis. Apoptosis is also characterized by hypoploidy, reflecting the lower DNA content in apoptotic cells and cell fragments. Fig. 6, C and F, shows DNA histograms of cells treated with TNF or anti-Fas, respectively. Clearly, TNF treatment of L929 cells does not result in the formation of hypoploid particles. In contrast, triggering of Fas antigen gives rise to hypoploid particles. Pretreatment with zVAD-fmk completely prevents the appearance of a hypoploid population after anti-Fas addition, identical to the outcome of TNF treatment. Hence, when the apoptotic pathway resulting from Fas triggering is blocked, a second signaling pathway becomes apparent, which leads to necrosis and not to apoptosis.

Figure 6. Nuclear morphology and hypoploidy of Fas-mediated cell death. L929hFas cells were preincubated either without (A and D) or with 25 μM zVAD-fmk (B and E) for 2 h, and treated with 5,000 IU/ml TNF (A and B) or 500 ng/ml anti-Fas (C and D) for another 3 h. PI was added, and nuclear morphology was analyzed by confocal microscopy. Necrotic nuclei are PI-positive, but retain a normal structural appearance; apoptotic nuclei are characterized by strong condensation of chromatin. C and F show the fraction of hypoploid cell fragments measured as a function of time. Cells were preincubated without (open circles) or with (filled circles) 25 μM zVAD-fmk, and treated with 500 IU/ml TNF (C) or 500 ng/ml anti-Fas (F).
Fas-mediated cell death in the presence of zVAD-fmk or zD-fmk involves oxygen radical production. TNF necrosis in L929 cells is preceded by an enhanced production of oxygen radicals at the mitochondrial compartment (28, 46, 47). Using DHR123 and flow fluorometry, we examined whether Fas stimulation of L929 cells actually resulted in excessive oxygen radical production (Fig. 7A). Treatment with anti-Fas alone already induced enhanced radical production, rapidly disappearing when the cells lost their membrane integrity (Fig. 7B). This drop in R123 fluorescence is most probably due to mitochondrial destruction and loss of mitochondrial transmembrane potential in the rapidly dying cells. However, in the presence of zVAD-fmk, a significant rise in R123 fluorescence was observed, peaking at ~3 h.

Since scavenging of radicals by BHA blocks necrotic cell death after TNF treatment (28), we tested whether BHA could also inhibit Fas-mediated necrotic cell death. As shown in Fig. 7B, addition of BHA had no significant effect on Fas-mediated apoptosis. However, in the presence of zVAD-fmk, a strong delay was observed in the appearance of PI-positive cells, indicating that oxygen radicals are implicated in cell death induced by anti-Fas in the presence of caspase inhibitors. Apparently, no difference in PI permeability was observed between cells dying by Fas-mediated apoptosis in the absence of zVAD-fmk and by Fas-induced necrosis in the presence of zVAD-fmk. However, we observed that in the apoptotic pathway, severe membrane blebbing preceded membrane permeabilization as measured by PI staining for >1 h. Indeed, loss of membrane integrity is considered a late phenomenon in apoptosis. The mitochondrial radical production could be inhibited to a large extent by the addition of BHA (Fig. 7A). Hence, the delay in oxygen radical accumulation observed in the presence of BHA correlates with the delay in cell death, as measured by PI uptake. These results strongly support a mechanism whereby Fas signaling in the presence of caspase inhibitors leads to necrosis, much like the signaling pathway initiated after TNF treatment of L929 cells (27, 28).

Fas-mediated necrosis is not accompanied by NF-κB activation. Treatment of L929 cells with TNF not only results in necrotic cell death, but also leads to activation of NF-κB (48). We wondered whether Fas signaling in the presence of zVAD-fmk would also lead to NF-κB activation, especially since enhanced oxygen radicals have been linked with activation of NF-κB (49). To this end, L929hFas cells were transfected with a luciferase reporter gene under transcriptional control of three minimal NF-κB sites in tandem before a minimal promoter (33). As shown in Fig. 8, 2-h anti-Fas treatment of L929 cells did not result in NF-κB activation, neither in the absence (as expected) nor in the presence of zVAD-fmk, despite the high levels of induced oxygen radicals. TNF-induced NF-κB activation is shown...
as a control. These results indicate that signaling pathways leading to necrosis and to NF-κB activation are segregated, and that Fas signaling in the presence of zVAD-fmk only activates the signals leading to necrotic death. Furthermore, at least in this particular cell system, a causal relationship between oxygen radical production and activation of NF-κB cannot be established. Alternatively, addition of BHA, which effectively retards TNF-induced necrosis (27), does not influence TNF-mediated NF-κB activation (data not shown). This suggests that enhanced mitochondrial oxygen radical production is not implicated in the activation of NF-κB. Combined treatment with TNF and anti-Fas in the presence of zVAD-fmk, resulting in almost complete cell death after 2 h, does not affect the level of NF-κB activity compared with TNF plus zVAD-fmk, excluding the possibility that rapid cell death would mask Fas-mediated NF-κB activation.

Fas-mediated necrosis does not involve endogenous TNFR. Fas antigen was originally described as a TNFR-associated protein (50). Because Fas-mediated necrosis in L929hFas cells, in the presence of zVAD-fmk, showed characteristics identical to TNF-mediated necrosis, we wondered whether during stimulation of the abundantly expressed Fas with agonistic Abs, TNFR-1 molecules could be cointernalized or cotrapped, and in this way would initiate the signaling pathway leading to necrosis. The fact that necrosis in L929 cells after triggering of Fas was not accompanied by activation of NF-κB already suggested that this was not the case. To further exclude any kind of association and subsequent cross talk between both receptors, we expressed human Fas in L929r1 cells, which are TNFR-1-resistant derivatives characterized by the total absence of TNFRs on the cell surface (29). Several clones were isolated, many of which showed Fas expression levels similar to L929hFas. Fig. 9 shows dose–response curves of L929hFas, compared with an L929r1.hFas clone expressing similar levels of cell-surface Fas. When Fas was triggered, the TNFR-1-resistant cells rapidly died in an apoptotic way, very much like TNF-sensitive L929hFas cells. In the presence of zVAD-fmk, both cell types died by necrosis, as was clear from microscopic examination of cellular and nuclear morphology, and exhibited an increased sensitivity to Fas triggering. Taken together, these results clearly indicate that Fas triggering in the presence of zVAD-fmk leads to necrosis independently of TNFR-1. Conclusively, the signaling leading to necrosis can be directly activated either by TNFR-1 or by Fas, the latter occurring when the pathway to apoptosis is blocked by broad-spectrum caspase inhibitors.

Discussion

The DD-containing TNFR-1 and Fas-R have been widely studied to unravel the mechanism of apoptosis induction. Regarding Fas-induced apoptosis, the role of caspases has been documented by the use of caspase inhibitors, by identification of caspase fragments, indicative of their activation, and by detection of Fas-induced caspase activity based on cleavage of specific substrates both in vivo and in cell lysates (20–22, 51–55). But in addition, other members of the TNFR family, such as WSL-1/death receptor (DR)3/APO-3 (56–58), TNF-related apoptosis-inducing ligand (TRAIL)-R1/DR4/APO-2 (59), and TRAIL-R2/DR5/TRAIL receptor inducer of cell killing (TRick)2 (60–62), contain a DD in their intracellular part, and the cell death pathway of TRAIL-R2 seems to be linked to caspase-10b (61). L929 cells are killed easily by TNF treatment; the mechanism involves enhanced production of reactive oxygen radicals by the mitochondria, which leads to cell death by necrosis (28, 46, 47). Clustering of only the DD derived from TNFR-1 is sufficient to initiate cell death in L929 cells (14).

In this study, we wanted to determine whether, in L929 cells expressing Fas, caspases are activated in the Fas-induced pathway leading to apoptosis. Using fluorogenic peptide substrates for caspase activity in cell lysates, we found proteolytic activity only on the tetrapeptide caspase substrate Ac-DEVD-amc, which correlated very well with the onset of apoptotic cell death. By Western analysis, only procaspase-3 and -7 were found to be activated after Fas triggering. However, as both caspases are inhibited efficiently by zDEVD-fmk (41, 63), and Fas-mediated apoptosis in L929 cells is not, it seems that these proteases are not indispensable for the regulation of apoptosis in L929 cells, but merely play an executing role in the late stage of apoptosis by cleaving proteins that are not absolutely required for signaling to apoptosis (7). Like zDEVD-fmk, Ac-YVAD-cmk, an inhibitor of caspase-1 (and -4/11; references 43 and 53), failed to block Fas-induced apoptosis in L929 cells.

When broad-spectrum pseudosubstrate caspase inhibitors, such as zVAD-fmk and zD-fmk, were added to the cells before a challenge with anti-Fas Abs, the cells still...
died. However, closer inspection of the type of cell death revealed that at sufficiently high concentrations of inhibitors, apoptosis was indeed inhibited, but now the cells underwent rapid necrotic cell death, as characterized by morphological criteria, such as swelling of the cells, the absence of nuclear condensation, and the appearance of hypoploid particles. Furthermore, the generation of oxygen radicals was identified as the effective mechanism to necrosis. Therefore, it seems that at least in L929 cells, two different pathways emerge from the Fas-R: one is caspase-dependent and leads to apoptosis; the other is oxygen radical-mediated, becomes evident in the presence of caspase inhibitors, and results in necrotic cell death.

Caspases that have been reported to be inhibited by zVAD-fmk are caspase-1 (42, 43), -8 (20, 21), -3, -4, -11, and very weakly caspase-7 (43). The use of the inhibitors does not allow the pinpointing of caspases involved in the cell death pathways in L929 cells. Moreover, not only blocking of a caspase activity but also amplification of oxygen radical production is necessary to reveal the necrotic Fas pathway. In a previous study, we have shown that TNF-driven radical production in L929 cells is indeed strongly enhanced by zVAD-fmk and zD-fmk, and, much more weakly, by Ac-YVAD-cmk and zDEVD-fmk (27). Hence, an enhanced oxygen radical production, which is negatively regulated by zVAD-fmk- and zD-fmk-sensitive proteases, seems to make the difference between apoptotic and necrotic outcome of L929 cell death. Further studies are required to elucidate the relation between caspase inhibition and enhanced radical production.

Necrosis after either TNF or Fas triggering in the presence of caspase inhibitors involves the production of mitochondrial oxygen radicals, which can be shown, for example, by the oxidation of DHR 123 and the inhibition of necrosis by the radical scavenger BHA (28). This means that TNF- and Fas-mediated necrosis converge upstream of the mitochondria. On the other hand, Fas-mediated necrosis is not accompanied by activation of NF-kB. Moreover, the broad-spectrum inhibitors zVAD-fmk and zD-fmk even potently sensitize the necrotic pathway (27). Hence, to obtain triggering of Fas antigen resulting in mitochondrial-dependent necrosis, both inhibition of the caspase-mediated apoptotic pathway and amplification of the radical-dependent necrotic pathway are necessary. Recently, Scaffidi and co-workers (66) described the existence of two Fas-signaling pathways depending on cell type. Type I cells are characterized by rapid and massive recruitment of FADD and procaspase-8 into the death-inducing signaling complex, followed 30 min later by activation of caspase-3, which eventually leads to apoptotic cell death.

Figure 10. Schematic overview of postulated mechanisms for Fas-mediated cell death. In the absence of inhibitors, the procaspase activation cascade is triggered, including caspases-3 and -7, and the cells die very rapidly by apoptosis (type I). However, when signaling to apoptosis is blocked by caspase inhibitors, secondary signaling components initiated by still unknown Fas- or TNFR-1-associated components, induce the mitochondria to excessive oxygen radical production (type III). The mechanism of this enhanced oxygen radical production by the addition of zVAD-fmk is still unclear. One may consider three levels at which a putative caspase-X might operate: (a) inhibition of signaling to the mitochondria; (b) inhibition of reactive oxygen intermediates (ROI) scavenging; and (c) removal of damaged mitochondria (reference 27).
Alternatively, type II cells showed a reduced formation of the death-inducing signaling complex and delayed activation of caspase-3 and -8. However, both cell types displayed similar kinetics in the loss of mitochondrial transmembrane potential and cell death. Overexpression of Bcl-2 or Bcl-x<sub>s</sub> could prevent this reduction of mitochondrial transmembrane potential in both type I and II cells, but could rescue only type II cells from cell death. Hence, in type II cells, activation of caspases and subsequent cell death depend on mitochondria, whereas type I signaling is mediated directly by caspase-8 and -3, the reduction of mitochondrial transmembrane potential being a secondary phenomenon. In view of this model, Fas-mediated apoptosis in L929 cells can be regarded as type I signaling, involving rapid activation of the caspase cascade. Fas-triggered necrosis, in the presence of caspase inhibitors, and TNF-induced necrosis may represent an alternate form of signaling to cell death relying on mitochondrial alterations, the latter resulting in enhanced oxygen radical production rather than activation of caspases. A schematic overview of the proposed type I and type III signaling components is given in Fig. 10.

The therapeutic use of caspase inhibitors for escaping unwanted apoptosis is an attractive possibility. For instance, caspases have already been assigned a role in amyotrophic lateral sclerosis (67), Alzheimer’s disease (68), and Huntington’s disease (69, 70). Furthermore, neuronal damage after hypoxic-ischemic insult (71) or fulminant liver destruction after anti-Fas injection (72) could be diminished or prevented successfully by the use of peptide caspase inhibitors. However, the results presented in this paper could restrict the therapeutic use of caspase inhibitors in that they do not necessarily guarantee blockage of cell death in general. In other words, necrotic cell death could at least in some cell types become evident. Furthermore, cells dying by necrosis, instead of apoptosis, may cause an inflammatory response. At present, it is not clear which components of the biochemical machinery make a cell more prone to succumb to necrosis.

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