Sensitization to Death Receptor Cytotoxicity by Inhibition of Fas-associated Death Domain Protein (FADD)/Caspase Signaling

REQUIREMENT OF CELL CYCLE PROGRESSION*

Upon binding of their ligands, death receptors belonging to the tumor necrosis factor (TNF) receptor family initiate a signaling pathway leading to the activation of caspases and ultimately apoptosis. TNF, however, in parallel elicits survival signals, protecting many cell types from cell death that can only be induced by combined treatment with TNF and inhibitors of protein synthesis. Here, we report that in NIH3T3 cells, apoptosis in response TNF and cycloheximide is not inhibited by the broad spectrum caspase inhibitor benzylxoycarbonyl-Val-Ala-Asp-fluoromethyl ketone (zVAD.fmk). Moreover, treatment with zVAD.fmk sensitizes the cells to the cytotoxic action of TNF. Sensitization was also achieved by overexpression of a dominant-negative mutant of Fas-associated death domain protein and, to a lesser extent, by specific inhibition of caspase-8. A similar, but weaker sensitization of zVAD.fmk to treatment with the TNF-related apoptosis-inducing ligand (TRAIL) or anti-CD95 antibody was demonstrated. The unexpected cell death in response to TNF and caspase inhibition occurs despite the activation of nuclear factor-κB and c-Jun N-terminal kinases. The mode of cell death shows several signs of apoptosis including DNA fragmentation, although activation of caspase-3 was excluded. TNF/zVAD.fmk-induced cell death is preceded by an accumulation of cells in the G2/M phase of the cell cycle, indicating an important role of cell cycle progression. This hypothesis is further strengthened by the observation that arresting the cells in the G1 phase of the cell cycle inhibited TNF/zVAD.fmk-induced cell death, whereas blocking them in the G2/M phase augmented it.

The still growing family of tumor necrosis factor (TNF)

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The abbreviations used are: TNF, tumor necrosis factor; DR, death receptor; TRAIL, TNF-related apoptosis-inducing ligand; NF-κB, nuclear factor-κB; CHX, cycloheximide; FADD, Fas-associated death domain protein; zVAD.fmk, benzylxoycarbonyl-Val-Ala-Asp-fluoromethyl ketone; zLTD.fmk, benzylxoycarbonyl-Leu-Glu-Thr-Asp-fluoromethyl ketone; DAPI, 4',6-diamidine-2'-phenylindole dihydrochloride; SAPK/JNK, stress-activated protein kinases; ROS, reactive oxygen species; NAC, N-acetylcysteine; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; DN, dominant negative; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio-1-propanesulfonic acid; AFC, 7-amino-4-trifluoromethyl coumarin; FCS, fetal calf serum; PI, propidium iodide.

lignands accomplishes primarily, but not exclusively, important functions in the development and regulation of the immune system (1). Both the ligands and their receptors are combined into molecule families based on distinct sequence homologies. The family of TNF receptors is primarily defined by their sequence homology within the extracellular, ligand binding domain (1, 2). A subgroup of the TNF receptor family members is characterized by the presence of a so-called death domain within their cytoplasmic region that is responsible for the induction of programmed cell death (1–5). These death receptors (DR) include the p55 receptor for TNF (TNF-R55), the CD95 molecule (Fas, APO-1), DR3, DR6, and the two receptors for the TNF-related apoptosis-inducing ligand (TRAIL), DR4 (Apo2, TRAIL-R1) and DR5 (KILLER, TRAIL-R2). Although Fas ligand and TRAIL mainly induce apoptosis on cells expressing their receptors, TNF in addition strongly activates nuclear factor-κB (NF-κB), which is thought to regulate the expression of one or more protective genes that inhibit the cytotoxic signal (6–8). Therefore, in most cells, apoptosis is induced by TNF only in combination with inhibitors of gene expression, like cycloheximide (CHX) or actinomycin D.

Apoptosis is a physiological process that is essential for the development and homeostasis of every multi-cellular organism (for review, see Refs. 9–11). A critical event during apoptosis is the activation of proteases belonging to the caspase family (12). Caspases are cysteine proteases with a unique substrate cleavage site after an aspartic acid residue (13). The role of these proteases in cell suicide is to disable critical homeostatic and repair processes and to cleave key structural components, resulting in the systematic and orderly disassembly of the dying cell. In the last few years, it has become clear that members of the caspase family are the main effectors of the apoptotic signaling pathway triggered by ligands of the TNF family (for review, see Refs. 4, 5, and 14–16). The adapter protein FADD (Fas-associated death domain protein; MORT1) and, subsequently, caspase-8 (FLICE, Mach) are recruited to TNF-R55 and CD95 after ligand binding and thereby start a cascade of caspases finally leading to cell death. The involvement of FADD/caspase-8 signaling in TRAIL-induced apoptosis is controversially discussed and may occur in a cell type-specific manner (4). Although in many cells strong activation of caspase-8 is sufficient to induce cell death, in select cell types an amplification mechanism is necessary for the execution of apoptosis in response to DR-triggering. Moderate activation of caspase-8 via the generation of a proapoptotic form of the Bel-2-related protein Bid, leads to the release of cytochrome c from the mitochondria, which in complex with APAF-1 activates caspase-9, thereby enhancing the activation of the caspase cascade (17).

Using in vitro models, it has become clear that FADD not only is essential for the induction of cell death in response to most
death receptors, but is moreover critically involved in embryonic development and activation-induced proliferation of peripheral T-cells (18–21). In addition, it has been suggested that FADD promotes proliferation of pro-T cells and functions as a tumor suppressor (22). Finally, it has recently been reported that caspases are activated in primary T-cells after anti-CD3 stimulation and that this activation is necessary for the proliferative response (23, 24), thereby linking FADD/caspase signaling to growth stimulation.

Apoptosis induced by DR-triggering has been shown to be blocked by overexpressing dominant negative forms of FADD (25) or caspase-8 (26) or by inhibiting either caspases (27) or, in some cells, mitochondrial cytochrome c release (28). Here, we report that NIH3T3 cells unexpectedly are not protected by the broad spectrum caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) from apoptosis induced by TNF and protein synthesis blocker CHX. Moreover, we show that NIH3T3 cells are sensitized to TNF and related death-inducing ligands by inhibition of FADD/caspase signaling. We provide evidence that cell cycle progression is required for this cytotoxicity, leading to an accumulation of cells in the G2/M phase of the cell cycle, from which they do not further advance but instead die by a cell death, showing several features of apoptosis despite the lack of caspase-3 activity.

**EXPERIMENTAL PROCEDURES**

**Reagents and Expression Vectors—**Highly purified human TNF-α (3 × 10⁷ units/mg) was generously provided by Dr. G. Adolf (Bender GmbH, Vienna, Austria). Recombinant human TRAIL was obtained from Biomer. The caspase inhibitor zVAD.fmk was purchased from Bachem, benzyloxycarbonyl-Leu-Glu-Thr-Asp-fluoromethylketone (zLETD.fmk) was purchased from Biotrend, cycloheximide, thymidine, nucodazol, and G418 were purchased from Sigma. The anti-gonadotropin-CD95 antibodies were obtained from Pharmingen (anti-murine-CD95, J02, anti-human-CD95, CH-11). The expression constructs for dominant-negative FADD (FADD-DN) and caspase-8 (caspase-8 (1–436), FLICE-DN) were generously provided by D. Goeddel and are described elsewhere (25, 29). The vector containing the neo gene (BMGNeo) has been published (30).

**Cell Culture and Generation of Stable Transfectants—**NIH3T3 and HeLa cells were cultured in a flutont solution in Dulbecco’s modified Eagle’s medium without HEPES supplemented with 10% fetal calf serum, 10 mM glutamine, and 50 µg/ml of each of streptomycin and penicillin. To generate stable transfectants, NIH3T3 cells were seeded at a density of 1 × 10⁶ cells/10-cm dish and transfected with 1 µg of BMG-neo plasmid in combination with 5 µg of FADD-DN or 5 µg of FLICE-DN using the LipofectAMINE reagent (Life Technologies, Inc.) according to the instructions of the manufacturer. After 48 h, cells were trypsinized and harvested, and after an additional 24 h, treated with 500 µg/ml G418 to select for stable transfectants. Colonies were picked after they had reached a diameter of 1 cm and further cultivated without G418. Expression of FADD-DN or FLICE-DN was verified by immunoblotting using anti-FLAG (Eastman Kodak Co.) or anti-caspase-8 (Santa Cruz) antibody.

**Cell Cycle Analysis—**Cells were collected by trypsinization, washed twice with cold PBS, and resuspended in 1 ml of freshly prepared lysis buffer (10 mM Tris/HC1, pH 8, 1 mM EDTA) and lysed by adding 250 µl of freshly prepared lysis buffer (10 mM Tris/HC1, pH 8, 1.2% SDS, 20 mM EDTA) for 5 min on ice. After centrifugation with RNaseA (100 µg/ml) for 15 min at 37°C, 300 µl of 8 M LiCl and 800 µl of isopropanol were added. After incubation for 15 min on ice, the samples were centrifuged at 14,000 rpm for 30 min at 4°C. The DNA in the supernatants was reprecipitated, and the pellets were dried and resuspended in TE, partially denatured for 10 min at 65°C, and loaded on a 1.5% agarose gel. The gel was run for 3 h, stained with ethidium bromide, and photographed.

**Measurement of Reactive Oxygen Species—**NIH3T3 cells were seeded at a density of 2 × 10⁵ cells/well, incubated with 1 µM dihydrodiam-
Caspase Inhibitor Sensitizes to Death Receptor Cytotoxicity

RESULTS

Sensitization of NIH3T3 Cells to Cytotoxicity of Death Receptors by Inhibition of FADD/Caspase Signaling—Ligands of the TNF family bind to their corresponding death receptors and induce apoptosis by activating the caspase network (4). Therefore, blocking caspases should inhibit DR-induced apoptosis. As expected, HeLa cells could be protected from apoptosis induced by TNF and CHX by the broad spectrum caspase inhibitor zVAD.fmk (Fig. 1 left panel; Ref. 27). However, while performing similar experiments with NIH3T3 cells, we found to our surprise that these cells could not be protected by zVAD.fmk but, instead, that cell death was further enhanced (Fig. 1, right panel). Moreover, although normally resistant to TNF, NIH3T3 cells were sensitized to TNF treatment, showing a significant portion of hypodiploid cells after treatment with the combination of TNF and zVAD.fmk. The pan-caspase inhibitor N-[tert-butoxycarbonyl]-Asp-fluoromethylketone displayed a similar sensitization on TNF cytotoxicity (data not shown). A moderate sensitization effect was also seen using murine C127 cells (data not shown).

Next, we analyzed whether this irregular cell death is also induced by other ligands of the TNF family. Therefore, we treated NIH3T3 cells with soluble human TRAIL and an agonistic anti-CD95 antibody, either alone or in combination with CHX or zVAD.fmk. As shown in Fig. 2, NIH3T3 cells were remarkably resistant to TRAIL and anti-CD95 antibody, whereas both stimuli induced apoptosis in combination with CHX. Costimulation with zVAD.fmk, however, sensitized the cells to both death ligands as indicated by the appearance of a significant amount of hypodiploid cells (Fig. 2). Since all three ligands that unexpectedly induced cell death when applied together with zVAD.fmk have been shown to activate the pro-apoptotic FADD/caspase-8 signaling pathway (4, 5), we tested whether it was inhibition of this pathway that led to the observed sensitization effect. As shown in Fig. 3A, treatment with an inhibitor selective for caspase-8, zLETD.fmk, did not protect NIH3T3 cells from apoptosis induced by TNF/CHX, whereas it completely blocked apoptosis in HeLa cells. Costimulation with zLETD.fmk and TNF again led to a significant amount of hypodiploid cells in NIH3T3 but not in HeLa cells. The sensitization effect of the caspase-8 inhibitor, however, was less pronounced than that of the broad spectrum caspase inhibitor zVAD.fmk (Fig. 3A). To verify that inhibition of the FADD/caspase-8 pathway sensitizes against TNF-mediated cytotoxicity, we generated NIH3T3 clones stably expressing either a dominant-negative mutant of FADD (FADD-DN) or of caspase-8 (FLICE-DN). Although a control clone transfected with the neo-expression plasmid was only marginally sensitive to treatment with TNF, TRAIL, or anti-CD95, clones overexpressing either FADD-DN (Fig. 3B) or, to a lesser extent, FLICE-DN (Fig. 3C) showed a significant portion of hypodiploid cells after stimulation with all three death ligands. The sensitization effect of zVAD.fmk treatment or overexpression of FADD-DN or FLICE-DN was most pronounced for TNF, less for TRAIL, and least for anti-CD95 antibody (Fig. 2; Fig. 3, B and C). Similar results were obtained with an independent clone for each expression construct (data not shown). Thus, we concluded that inhibition of the FADD/caspase-8 pathway leads to an unexpected sensitization effect of NIH3T3 cells to DR-induced cytotoxicity.

Intact NF-κB and SAPK/JNK Activation after TNF/zVAD.fmk Treatment—One possible explanation for the unexpected cell death induced by costimulation with TNF and zVAD.fmk would be an interference of zVAD.fmk treatment with the activation of the transcription factor NF-κB, which is thought to initiate survival signals in response to TNF (6–8). Therefore, we treated NIH3T3 cells with TNF alone or in combination with zVAD.fmk and performed electrophoretic mobility shift assays to test for NF-κB activation. As shown in Fig. 4A, we could not detect any significant difference in the activation of NF-κB in cells treated with TNF or with TNF/zVAD.fmk. The activation of kinases belonging to the mitogen-activated protein kinase family, especially the stress-activated protein kinases (SAPK/JNK), have been discussed to play a role in the
induction of cell death. Therefore, we analyzed the activation of SAPK/JNK in response to costimulation with TNF and zVAD.fmk. Again, no change in the activation level or kinetics of SAPK/JNK was observed in NIH3T3 cells treated with TNF alone or together with zVAD.fmk (Fig. 4B).

Characterization of Cell Death Induced by TNF/zVAD.fmk Stimulation—While this work was in progression two related studies were published that reported a similar sensitization effect of zVAD.fmk on TNF-induced cytotoxicity using L929 (34) or NIH3T3 and U937 cells (35), claiming that this cell death occurs without DNA fragmentation and, therefore, has to be considered as necrosis. In our experiments, however, the unexpected cell death in response to TNF and caspase inhibition was initially observed as appearance of hypodiploid cells, indicative for DNA fragmentation during apoptosis. On the other hand, apoptosis as mode of cell death seemed unlikely, since the broad spectrum inhibitor zVAD.fmk blocks the activity of all known mammalian caspases. To characterize cell death in response to TNF/zVAD.fmk stimulation in more detail, we performed several standard apoptosis assays. First, we analyzed whether caspase-8 and the executioner caspase-3 displayed any remaining activity or whether they were completely shut down. As shown in Fig. 5A, caspase-8 and, more pronounced, caspase-3 activities toward their specific fluorescent substrates were easily detected in cellular lysates from NIH3T3 cells treated with TNF/CHX, whereas the caspase activities in lysates from TNF/zVAD.fmk-stimulated cells were equivalent to that observed in lysates from untreated cells. In agreement with this result, we could not detect any cleavage products of caspase-3 indicative for its activation in a Western blot analysis using extracts from TNF/zVAD.fmk-treated cells, whereas cleaved caspase-3 was clearly seen in lysates from TNF/CHX-stimulated cells (Fig. 5B). Next, we demonstrated by agarose gel electrophoresis that NIH3T3 cells exhibit a DNA fragmentation pattern after costimulation with TNF and zVAD.fmk, whereas the caspase activities in lysates from TNF/zVAD.fmk-stimulated cells were equivalent to that observed in lysates from untreated cells. In agreement with this result, we could not detect any cleavage products of caspase-3 indicative for its activation in a Western blot analysis using extracts from TNF/zVAD.fmk-treated cells, whereas cleaved caspase-3 was clearly seen in lysates from TNF/CHX-stimulated cells (Fig. 5C). Microscopical examination of the TNF/zVAD.fmk-treated cells in comparison with TNF/CHX-treated cells by DAPI-staining revealed that both cytotoxic treatments led to similar changes in nuclear morphology, characterized by chromatin condensation with concomitant nuclear shrinkage (Fig. 5D). In contrast, the morphology of the cytoplasm differed between both stimuli. While TNF/CHX treatment led to a pronounced early rounding and detachment of the cells, TNF/zVAD.fmk stimulation induced nuclear changes before detachment. Cell swelling, however, indicative for necrotic cell death (34), was not observed (Fig. 5D). Taken together, the irregular cell death of NIH3T3 cells after ligation of death receptors with simultaneous caspase inhibition exhibited several features of apoptosis despite the lack of caspase activation.

Khwaja and Tatton (35) report that TNF treatment with simultaneous caspase inhibition leads to the generation of re-
active oxygen species (ROS) and that pretreatment with the antioxidants glutathione ethyl ester or N-acetylcysteine (NAC) protects NIH3T3 and U937 cells from cell death. Therefore, we measured ROS production using flow cytometry. As reported by Khwaja and Tatton (35), we could demonstrate an overall increase of ROS production in NIH3T3 cells in response to TNF/zVAD.fmk treatment, which was not seen after TNF/CHX stimulation (Fig. 6B). Moreover, pretreatment with the radical scavenger butylated hydroxyanisole (BHA) protected NIH3T3 cells from cytotoxicity induced by TNF/zVAD.fmk but not by TNF/CHX (Fig. 6A). However, neither the chemically related molecule butylated hydroxytoluene (BHT; Fig. 6A) nor NAC (data not shown) were able to protect NIH3T3 from TNF/zVAD.fmk-induced cell death. Therefore, the causality of ROS production on cell death has to be questioned.

Importance of Cell Cycle Progression on TNF/zVAD.fmk-induced Cell Death—While studying the cytotoxic effect of TNF with simultaneous caspase inhibition, we noticed that quiescent cells were much more resistant than cycling cells. To investigate this effect in more detail, we used different methods to restrict cell cycle progression before stimulation with TNF and zVAD.fmk. First, we investigated the effect of plating NIH3T3 cells in varying densities. The more cells were detected in the G0/G1 phase due to a high plating density, the more resistant they were to costimulation with TNF and zVAD.fmk, whereas apoptosis in response to TNF/CHX treatment was not reduced by G0/G1 restriction (Fig. 7A). In line with this result, NIH3T3 cells kept for 3 days under low FCS conditions (0.5%) were completely protected from cytotoxicity induced by TNF or TRAIL treatment in combination with zVAD.fmk. In contrast, apoptosis induced by DR-triggering together with CHX was not significantly inhibited by serum reduction (Fig. 7B). Moreover, when arrested in the G1/S phase by double thymidine block, NIH3T3 cells were significantly less sensitive against TNF/zVAD.fmk treatment than control cells or cells treated with TNF/CHX (Fig. 7C).

To further investigate the influence of cell cycle progression, we performed time course experiments of NIH3T3 cells stimulated with TNF and zVAD.fmk and determined cell cycle distribution by flow cytometry. Before the occurrence of massive cell death, we observed a significant portion of the cells accumulating in the G2/M phase of the cell cycle (Fig. 8A). This effect was not detected using the classical apoptotic stimulus TNF/CHX (Fig. 8B). Similar but weaker results were obtained using NIH3T3 cells costimulated with TRAIL or anti-CD95 antibody in combination with zVAD.fmk (Fig. 8B). Together, these results suggested that cells have to proceed through the cell cycle and reach the G2/M phase to be sensitive to the cytotoxic effect of TNF with concomitant caspase inhibition. To
corroborate this hypothesis, we arrested NIH3T3 cells in the G2/M phase by treatment with nocodazol before stimulation with suboptimal concentrations of either TNF alone or in combination with zVAD.fmk or CHX. Cell cycle arrest in the G2/M phase was confirmed by flow cytometry (Fig. 9B). As shown in Fig. 9A, G2/M-arrested cells were preferentially sensitized to the cytotoxic effect of TNF/zVAD.fmk treatment but not to TNF/CHX stimulation.

TNF generates growth stimulatory signals in several cell types, among them fibroblasts (36). Also, anti-CD95 antibody has been shown to induce proliferation in fibroblasts (37). Taken into account that cell cycle progression is necessary for the cytotoxic effect of TNF/zVAD.fmk stimulation, we considered that growth stimulation might be a substantial difference between NIH3T3 and HeLa cells that finally leads to the observed “aberrant” cell death. Therefore, we analyzed the distribution of NIH3T3 and HeLa cells in different cell cycle phases after stimulation with TNF. As demonstrated in Fig. 10, stimulation of NIH3T3 cells with TNF led to an increase of cells in S and G2/M phases of the cell cycle, indicating a promotion of cell cycle progression by TNF. A comparable effect was seen after treatment with platelet-derived growth factor, an established growth factor for NIH3T3 cells (38). On the other hand, the cell cycle distribution of HeLa cells after TNF stimulation did not significantly differ from that observed in untreated cells (Fig. 10). Although the changes in cell cycle distribution were not pronounced, they were highly reproducible.

**DISCUSSION**

The death receptors TNF-R55, CD95, and probably TRAIL receptors signal apoptosis through the activation of a FADD/caspase-8 pathway (4, 5). Here we report an unexpected observation. Inhibition of FADD/caspase signaling renders NIH3T3 cells, normally resistant to DR-induced apoptosis, sensitive to the cytotoxic action of these death receptors. The observed aberrant cell death shows several features of apoptosis despite the lack of caspase activation. When performing kinetic analysis of NIH3T3 cells treated with death ligands and caspase inhibitors, we observed an accumulation of cells in the G2/M phase of the cell cycle before the onset of cell death. We provide evidence that cell cycle progression is necessary for the sensitization effect of caspase inhibition on DR cytotoxicity, whereas...
cell cycle arrest in the G2/M phase augments sensitization.

Cell death in NIH3T3 cells induced by combined treatment with TNF and caspase inhibitors (here termed as Tz-death) was compared with the classical apoptotic response to TNF under conditions of protein synthesis inhibition (here termed as TC apoptosis). Several differences could be defined. 1) Although both stimuli induced nuclear condensation, the overall cellular morphology of the dying cells was significantly different. 2) Tz-death occurred at TNF concentrations of as low as 0.01 ng/ml TNF, whereas TC-apoptosis required at least 1 ng/ml TNF. Interestingly, although an initial treatment for 1 h with TNF was sufficient for the induction of cell death, the caspase inhibitor zVAD.fmk had to be present for the complete incubation time. 3) A potential involvement of ROS production was demonstrated for Tz-death but not for TC-apoptosis. 4) Tz-death was preceded by an accumulation of cells in the G2/M phase of the cell cycle and required proliferation, whereas TC-apoptosis occurred independently from cell cycle progression.

While this study was under way, a similar sensitization effect of caspase inhibition on TNF cytotoxicity was published using L929 cells (34) or NIH3T3 and U937 cells (35). Both groups came to the conclusion that the mode of cell death induced by caspase inhibition and TNF was necrosis, based on the cellular morphology and the lack of DNA fragmentation in their experiments. In contrast to their results, we clearly observed DNA fragmentation in NIH3T3 cells after TNF/zVAD.fmk treatment that was indistinguishable from that seen after TNF/CHX stimulation. Nuclear morphology also showed no obvious differences between both modes of cell death. Moreover, we did not observe swelling of cells or organelles indicative of necrotic cell death (34). However, since TNF/zVAD.fmk-treated NIH3T3 cells showed several features different from true apoptotic cells after TNF/CHX stimulation, we consider the aberrant cell death to be a transitional stage between apoptosis and necrosis. It was suggested earlier that both modes of cell death represent the extremes of a continuous

FIG. 8. Transient accumulation of NIH3T3 cells in G2/M phase before death receptor/zVAD.fmk-induced cell death. A, NIH3T3 cells were left untreated or were stimulated with TNF (50 ng/ml) for 10 h or with TNF in combination with two different amounts zVAD.fmk for the indicated times. Cell cycle distribution was analyzed by PI-staining and flow cytometry. B, NIH3T3 cells were left untreated (Co) or were treated with the indicated combinations of TNF (50 ng/ml), TRAIL (1 μg/ml), anti-CD95 antibody (1 μg/ml), CHX (6 μg/ml), and zVAD.fmk (50 μM) for the indicated times and analyzed for cell cycle distribution.

FIG. 9. Augmentation of cell death induced by TNF/zVAD.fmk treatment by arresting NIH3T3 cells in the G2/M phase of the cell cycle. Either G2/M-arrested or control NIH3T3 cells were treated with suboptimal amounts of TNF/zVAD.fmk or TNF/CHX as described under “Experimental Procedures.” After 16 h of stimulation, cells were harvested and analyzed by PI-staining and flow cytometry. The percentages of hypodiploid cells from three independent determinations with the corresponding S.D. are indicated.

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2 S. Lüschen, unpublished results.
scale (39). Growing evidence is accumulating about an apoptosis-like cell death in the absence of caspase activation (40–42).

Both above-mentioned reports (34, 35) favored TNF-induced ROS-generation as the direct cause of cell death, because radical scavengers like BHA or NAC could protect their cells from cytotoxicity. Our observation that the radical scavenger BHA from TNF/zVAD.fmk-induced cell death could protect NIH3T3 cells partly confirmed this hypothesis. However, neither the structurally related antioxidant BHT nor NAC were effective in our assays. Therefore, a causative role of ROS for cell death after stimulation with TNF and caspase inhibitors has to be questioned. Similar results have been obtained in previous studies in which BHA but not BHT was found to protect L929 cells from TNF-induced cell death. Therefore, these authors came to the conclusion that the inhibitory effect of BHA on TNF cytotoxicity was not due to its function as antioxidant (43).

The fact that inhibition of FADD/caspase signaling sensitizes NIH3T3 cells to DR cytotoxicity leads to the conclusion that activation of caspases accomplishes an important survival function in these cells. Initially, FADD/caspase signaling was described to be proapoptotic. However, recent evidence shows that both proteins are also involved in activation-induced proliferation of primary T cells (18–20, 22–24). It has been suggested that FADD transduces a survival signal necessary during G0-G1 transition (20). Earlier reports show that TNF-R55 and CD95 initiate growth-stimulatory signals in fibroblasts (36, 37). Here, we demonstrate that TNF induced an increased amount of cells in the S and G2/M phases of the cell cycle, implying enhanced proliferation in NIH3T3 cells but not in HeLa cells. Inhibition of FADD/caspase signaling diverts the growth stimulatory signal into the induction of cell death. TRAIL and anti-CD95 antibody displayed only a minor effect on cell cycle distribution (data not shown), which might explain the reduced cytotoxicity of these ligands when FADD/caspase signaling is blocked. In summary, we would like to propose the following hypothesis: DR-triggering induces cell cycle progression in NIH3T3 cells involving, among other signals, the FADD/caspase pathway. When this part of the signal is inhibited, cells accumulate in the G2/M phase of the cell cycle, from which they do not continue to complete the cycle but instead die by an apoptosis-like death. This would suggest an additional role for FADD/caspase signaling during the G2/M phase. Interestingly, Scaffidi et al. (44) observe that FADD is phosphorylated by a cell cycle-regulated protein kinase specifically during the G2/M phase. Since the phosphorylation apparently is not required for the proapoptotic function of FADD, the authors speculate that the phosphorylation of FADD may regulate its apoptosis-independent functions like proliferation (44). Further studies are necessary to define the exact role of FADD/caspase signaling in NIH3T3 cells.

Although overexpression of FADD-DN led to a sensitization effect to DR cytotoxicity similar to that seen with the complete inhibition of all caspases by zVAD.fmk, blocking selectively the FADD binding initiator caspase-8 displayed a reduced effect. One explanation for this observation would be that another caspase compensates for caspase-8. Candidates for such a caspase could be another FADD binding protease, caspase-10 (Mch4, FLICE-2; Ref. 45), caspase-2, which has been shown to indirectly bind to TNF-R55 and signal apoptosis (46, 47), or a yet unidentified caspase. Alternatively, inhibition of the complete caspase cascade could be required for the diminished cell death response seen with FADD-DN or the broad spectrum inhibitor zVAD.fmk.

The sensitization effect of caspase inhibitors to TNF-induced cell death might be an important issue for therapeutic approaches in the treatment of human diseases. First, a combined treatment of tumor cells with caspase inhibitors and TNF might have potential relevance for the treatment of malignancies. Future studies will identify cell types for which this treatment might be applicable. Interestingly, a transient G2/M block preceding apoptosis has been described for several anti-cancer treatments, like taxol, gamma radiation, 5-fluorouracil, and others (48–50). Although the causative role of G2/M arrest for the induction of apoptosis is controversially discussed (51), growth arrest at cell cycle checkpoints either in the G2 or in the G2/M phase appears to be a common event in many cell death
induction pathways (52). Second, if caspase inhibition is able to sensitize cells to DR-signaling in vivo, a systemic inhibition of caspases might lead as a side effect to potentially dangerous cellular destruction in otherwise healthy tissues. This is an important consideration, since the general inhibition of caspases is favored as potential new therapeutic intervention in many diseases that arise due to inappropriate apoptosis (53).

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