Structure/Function Analysis of p55 Tumor Necrosis Factor Receptor and Fas-associated Death Domain

EFFECT ON NECROSIS IN L929sA CELLS*

Receptor and Fas-associated Death Domain

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Received for publication, August 8, 2000
Published, JBC Papers in Press, September 14, 2000, DOI 10.1074/jbc.M007166200

Tumor necrosis factor (TNF) induces a typical apoptotic cell death program in various cell lines by interacting with the p55 tumor necrosis factor receptor (TNF-R55). In contrast, triggering of the fibrosarcoma cell line L929sA gives rise to characteristic cellular changes resulting in necrosis. The intracellular domain of TNF-R55 can be subdivided into two parts: a membrane-proximal domain (amino acids 202–325) and a C-terminal death domain (DD) (amino acids 326–413), which has been shown to be necessary and sufficient for apoptosis. Structure/function analysis of TNF-R55-mediated necrosis in L929sA cells demonstrated that initiation of necrotic cell death, as defined by swelling of the cells, rapid membrane permeabilization, absence of nuclear condensation, absence of DNA hypoploidy, and generation of mitochondrial reactive oxygen intermediates, is also confined to the DD. The striking synergistic effect of the caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone on TNF-induced necrosis was also observed with receptors solely containing the DD. TNF-R55-mediated necrosis is not affected by the dominant negative deletion mutant of the Fas-associated death domain (FADD-(80–205)) that lacks the N-terminal death effector domain. Moreover, overexpression of FADD-(80–205) in L929sA is cytotoxic and insensitive to CrmA, while the cytotoxicity due to overexpression of the deletion mutant FADD-(1–111) lacking the DD is prevented by CrmA. These results demonstrate that the death domain of FADD can elicit an active necrotic cell death pathway.

Published, JBC Papers in Press, September 14, 2000, DOI 10.1074/jbc.M007166200

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This paper is available online at http://www.jbc.org

Published, JBC Papers in Press, September 14, 2000, DOI 10.1074/jbc.M007166200

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tivity by the caspase inhibitors zVAD-fmk or CrmA resulted in enhanced ROI formation and considerably increased the sensitivity to TNF-induced necrosis (26), suggesting a modulator role for caspases in the oxidative metabolism.

In this paper we demonstrate that the DD of TNF-R55 as such is sufficient for mediating necrotic signaling pathways of TNF. We show that the membrane-proximal region, upstream of the DD, is required neither for necrosis nor for formation of mitochondrial ROI. Furthermore, the strong sensitization of TNF-induced necrosis in the presence of caspase inhibitors is also confined to the DD. In contrast to apoptotic systems, overexpression of FADD-(80–205) lacking the DED is cytotoxic for L929a cells in a CrmA-insensitive way, while overexpression of a FADD-(1–111) mutant containing the DED is cytotoxic in a CrmA-inhibitory way. This indicates that the death domain of FADD might be implicated in TNF-R55-mediated necrosis.

MATERIALS AND METHODS

Cell Culture—The mouse fibrosarcoma cells L929a (27), the mouse fibrosarcoma cells 2T2.5 (Hans Schreiber, Chicago), and the human HeLa H21 cell line were cultured in Dulbecco modified Eagle’s medium, supplemented with 5% newborn calf serum and 5% fetal calf serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml), and L-glutamine (0.03%). Stable transfected clones of these cells were generated as described previously (28) and maintained under selection by adding 400 μg/ml G418 (Life Technologies, Inc., Paisley, United Kingdom) to the medium.

Cytokines, Antibodies, and Reagents—Recombinant murine TNF was produced in our laboratory and was purified to at least 99% homogeneity. The specific activity amounted to 2.2 × 10^10 IU/mg, as determined in a standardized cytotoxicity assay on L929a cells. htr1 and htr9 are agonistic mouse monoclonal antibodies directed against the extracellular domain of the p55 human tumor necrosis factor receptor (hTNF-R55) and was generously provided by Dr. M. Brockhaus (Hoffmann-La Roche, Basel, Switzerland) (27). The agonistic anti-human Fas antibody, clone 2R2, was purchased from Cell Diagnostica (Munster, Germany). BHA (Sigma) was dissolved in ethanol and used at 100 μM. zVAD-fmk (Enzyme Systems Products, Dublin, CA) was dissolved in ethanol and used at 25 μM. Propidium iodide (PI; Sigma) was prepared in phosphate-buffered saline (3 mM) and was used at 30 μM. Dihydrorhodamine 123 (DHR123; Molecular Probes, Eugene, OR) was dissolved at 5 mM in dimethyl sulfoxide and was used at 1 μM.

Plasmids—Constitutive expression of hTNF-R55 and various mutants thereof were obtained by cloning the cDNA after the early SV40 promoter in pSV255 as described previously (29). pSVneo, containing the neomycin resistance gene, was used as a selection vector. Mutants were generated by standard cloning procedures and subsequently verified by DNA sequencing. For transient transfection assays, the different variants were cloned into pCDMA (Invitrogen, Carlsbad, CA). The expression vectors for CrmA (cDNA was a gift of D. Pickup, Durham, NC) and human Fas (the cDNA was a gift from S. Nagata, Department of Genetics, Osaka University Medical School, Suita, Japan) have been described previously (30). Mouse RIP and procaspase-8 (31) were cloned via reverse transcription-polymerase chain reaction and introduced into the mammalian expression vector pCDNA1 and pCDNA3 (Invitrogen, Carlsbad, CA), respectively. The human FADD and TRADD genes were also picked up by reverse transcription-polymerase chain reaction and cloned into pCDNA1. FADD-(80–205), encoding a DED-deficient molecule and reported as a dominant negative molecule in many systems, was cloned into pCDNA1 and pCDNA3 (Invitrogen, Carlsbad, CA). The expression of the FADD-(1–111) mutant containing the DED was cytotoxic in a CrmA-inhibitory way. This indicates that the death domain of FADD might be implicated in TNF-R55-mediated necrosis.

RESULTS

The DD of hTNF-R55 Is Required for Induction of Necrosis—L929a cells were stably transfected with cDNAs encoding different hTNF-R55 variants (Fig. 1A) and a pSVneo selection plasmid. After selection, several clones were screened for plasma membrane expression of hTNF-R55 and the different mutants. FACs analysis revealed constitutive cell membrane expression of full-length hTNF-R55, of the deletion mutants hR55203–304, hR55327–426, and hR55243–383, as well as of the hR55-L351A mutant mimicking the DD-inactivating lpr mutation originally found in Fas (Fig. 1, A and B). Specific triggering of the membrane-associated hTNF-R55 mutant was achieved by treatment of the cells with the agonistic antibody htr1. The L929a transfecants expressing these two receptor variants, containing an intact DD (clone A, hTNF-R55 and hR55203–304, 304, displayed htr1-dependent death (Fig. 2A). In contrast, oligomerization of hR55327–426, hR55-L351A, or hR55243–383 with htr1 revealed the inability of TNF-R55 variants lacking the death domain to trigger cell death (Fig. 2A). Next, we excluded the possibility of having selected for TNF-resistant L929a cell clones (27). Therefore, we treated these murine cells with human TNF, which interacts both with human and murine TNF-R55, and with agonistic anti-murine TNF-R55 polyclonal antibodies. We found that triggering of endogenous TNF-R55 was still cytotoxic (data not shown).
Microscopic evaluation of treated cells revealed that both hTNF-R55 and hR55\(\Delta 203-304\) induced characteristic necrotic swelling of the cell, resulting in loss of membrane integrity and finally cell lysis (Fig. 2B). Staining with PI further demonstrated the absence of nuclear condensation (Fig. 2B). Triggering of hR55\(\Delta 327-426\), hR55\(\Delta 243-383\), or hR55-L351A did not result in cell death or in any morphological changes (data not shown). Hence, the DD of TNF-R55 is required and sufficient for TNF-R55-mediated necrosis.

**zVAD-fmk Increases DD-mediated Necrosis**—Recently, we demonstrated that overexpression of CrmA, which is a specific inhibitor of caspase-1 and caspase-8 (33), strongly increased TNF-induced necrosis in L929sA cells, instead of blocking it. A similar observation was made when cells were pretreated with zVAD-fmk (26). To elucidate the mechanism of zVAD-fmk-induced synergy, the different TNF-R55 mutants were triggered in the presence of this caspase inhibitor. As shown in Fig. 3A, htr1-induced necrosis by clustering hTNF-R55 was 100-fold sensitized in the presence of zVAD-fmk. In contrast, the necrotic inactive mutants hR55-L351A, hR55\(\Delta 327-426\), or hR55\(\Delta 243-383\) remained insensitive to htr1 treatment, even in the presence of zVAD-fmk (Fig. 3, B, D, and E). However, these clones retained the ability to respond to a combined treatment of human TNF and zVAD-fmk, indicating that the endogenous zVAD-fmk-sensitive pathway(s) were still intact in these cells (data not shown). Necrotic cell death induced by hR55\(\Delta 203-304\), on the other hand, was enhanced as strongly by zVAD-fmk as the full-length receptor (Fig. 3C). This demonstrates that the synergistic action of caspase inhibitors to necrotic cell death occurs independently of the membrane-proximal region of hTNF-R55.

**Induction of DD Necrosis Is Accompanied by ROI Production**—The production of mitochondrial ROI by TNF has been shown to be crucial for necrotic cell death of L929sA cells (24). Nevertheless, it is still not clear which signaling pathways originating from TNF-R55 are involved in the formation of ROI. When hTNF-R55 was triggered by htr1, the generation of ROI could be detected by the accumulation of oxidized DHR123 in PI-negative cells (Fig. 4A). Simultaneously, necrotic cell death was monitored by the uptake of PI (Fig. 4B). After 3 h of incubation, about 50% of the cells were dead, whereas the remaining plasma membrane-intact cells produced twice as much ROI. Aggregation of hR55\(\Delta 203-304\) resulted in a delayed cell death, as reported previously (34). However, the extent of ROI production in PI-negative cells at 50% cell death was exactly the same as with full-length receptor. Treatment of cells expressing hR55\(\Delta 327-426\) or hR55-L351A did not result in the production of ROI (data not shown). Thus the DD alone is sufficient to generate a full oxidative response.

To examine whether an increase in ROI is required for ne-
compared with living cells (light arrows). No membrane blebbing, characteristic for apoptosis, is observed. The strong cytotoxic effect of FADD-(80–205), that lacks the DED and is not able to recruit procaspase-8, prompted us to distinguish whether FADD is at the bifurcation of necrotic or apoptotic cell death in L929a cells. Therefore, we tested whether cell death by transient overexpression of human TNF-R55 mutants, TRADD, FADD, and RIP was affected by cotransfection with the caspase-8 inhibitor CrmA. Clearly, overexpression of CrmA is not able to block TNF-R55-, hR55-link-326–426-, TRADD-, and RIP-induced cell death in L929a cells (Fig. 8A). Furthermore, also FADD-induced cell death is insensitive to CrmA-mediated inactivation of caspases. This indicates that FADD-induced cell death occurs despite the presence of CrmA. To elaborate further on the ability of FADD to induce cell death in the presence of a caspase-8 inhibitor, we tested the influence of CrmA overexpression on the cytotoxicity by FADD mutants that either lacked the DED domain or the DD domain, hR55(-203–304) showed a typical pattern of diploidy and tetraploidy, without indication of internucleosomal degradation of DNA (Fig. 2B). PI uptake was hardly visible in the picture of the transectant just started to die as PI uptake was hardly visible, confirming that cell swelling preceded loss of cellular membrane integrity.

cytotic cell death, cells were incubated in the presence of the hydrophobic radical scavenger and inhibitor of oxidative phosphorylation BHA (25). Fig. 6 shows that BHA strongly delayed cell death. FADD-(80–205) induced CrmA-insensitive cell death in L929a Cells—The TNF-R55 adapter molecules TRADD and FADD have been shown to be required for TNF-R55 induced apoptosis (13). Also RIP is recruited in the TNF-R55 complex and its overexpression induces apoptotic cell death (35). In FADD-(80–205), that lacks the DED and is not able to recruit procaspase-8, prompted us to distinguish whether FADD is at the bifurcation of necrotic or apoptotic cell death in L929a cells. Therefore, we tested whether cell death by transient overexpression of human TNF-R55 mutants, TRADD, FADD, and RIP was affected by cotransfection with the caspase-8 inhibitor CrmA. Clearly, overexpression of CrmA is not able to block TNF-R55-, hR55-link-326–426-, TRADD-, and RIP-induced cell death in L929a cells (Fig. 8A). Furthermore, also FADD-induced cell death is insensitive to CrmA-mediated inactivation of caspases. This indicates that FADD-induced cell death occurs despite the presence of CrmA. To elaborate further on the ability of FADD to induce cell death in the presence of a caspase-8 inhibitor, we tested the influence of CrmA overexpression on the cytotoxicity by FADD mutants that either lacked the DED domain or the DD domain, hR55(-203–304) showed a typical pattern of diploidy and tetraploidy, without indication of internucleosomal degradation of DNA (Fig. 2B). PI uptake was hardly visible in the picture of the transectant just started to die as PI uptake was hardly visible, confirming that cell swelling preceded loss of cellular membrane integrity.

**DISCUSSION**

Both hTNF-R55 and Fas mediate apoptosis via their so-called DD motif, which allows aggregation with other DD-containing proteins (36). The important role of the TNF-R55 DD in apoptotic cell death has been demonstrated in various cell lines. In contrast, the specific receptor domains involved in TNF-R55-induced necrosis remain to be characterized. Therefore, we performed a structure/function analysis of hTNF-R55 in respect with cell killing in the fibrosarcoma cell line L929a, which dies necrotically upon exposure to TNF (1, 26, 37).

We observed that TNF-R55 molecules lacking an active DD were incapable of inducing cellular necrosis. The typical necrotic morphology seen in hR55(-203–304)-mediated cell death demonstrates that the DD as such is sufficient to generate necrosis. Besides the ability to induce characteristic morphological features of necrosis, stimulation of hTNF-R55 or hR55(-203–304) showed a typical pattern of diploidy and tetraploidy, without indication of internucleosomal degradation of DNA (Fig. 2B; data not shown). Similarly, in the transient transfection assays, the receptor hR55-link-326–426, lacking the PAN binding site (38), also induced necrosis in these cells. Hence, no secondary signal, generated by the membrane-proximal region of TNF-R55, is required for necrotic cell death in L929a cells. Fas, which only contains a short membrane-proximal region (12), normally mediates apoptosis. However, in the presence of caspase inhibitors (30) or in the absence of procaspase-8 (39), Fas-induced apoptosis is switched to necrosis. This suggests that in the absence of caspase activation a
hidden necrotic pathway becomes apparent. Thus, both the DD of hTNF-R55 and Fas seem to initiate necrosis in a caspase-independent way (30). Leist and co-workers (5) identified the cellular ATP concentration as a crucial parameter in the decision between apoptosis and necrosis. In human T cells depleted of ATP, default apoptotic triggers such as staurosporine or Fas, switched from apoptosis to necrosis, indicating that the energy homeostatic condition of the cell determines the kind of cell death process activated (5). Whether the concentration of ATP is at the decision point between TNF-induced necrosis and Fas-induced apoptosis in the same cellular context of L929sA cells is not clear yet. In the L929sA system the absence of caspases clearly facilitates necrosis (26, 30). If ATP concentration would be implicated in the decision between apoptotic (high ATP) and necrotic cell death (low ATP), one would predict that a cell death signal in the absence of caspases would favor somehow ATP depletion. Otherwise, it is also possible that depending on the trigger or the cell line used, different mechanisms, such as reactive oxygen generation and/or ATP con-

**Fig. 3.** Synergistic effect of zVAD-fmk on DD-induced necrosis. Different stably transfected cell lines expressing various hTNF-R55 mutants were treated with a serial dilution of htr1 in the absence (open symbols) or presence of 25 µM zVAD-fmk (filled symbols).

**Fig. 4.** Production of ROI by the clustered DD of hTNF-R55. A, increase in mean rhodamine 123 fluorescence intensity (ΔFI) induced by 100 ng/ml htr1 in cells expressing hTNF-R55 (○) or hR55Δ203–304 (□). Fluorescence intensity was measured in 3000 PI-negative cells at the times indicated. B, parallel measurement of the percentage of PI-positive, dead cells induced by htr1 in cells expressing hTNF-R55 (○) or hR55Δ203–304 (□).

**Fig. 5.** Butylated hydroxyanisole inhibits DD-mediated necrosis. Cells were treated with 100 ng/ml htr1 (○), 100 ng/ml htr1 + 25 µM zVAD-fmk (□), 100 ng/ml htr1 + 50 µM BHA (△), or 100 ng/ml htr1 + zVAD-fmk + BHA (○). zVAD-fmk and BHA were added simultaneously as htr1. Cell death was monitored as the percentage of PI-positive cells at a given time. A, hTNF-R55-expressing cells; B, hR55Δ203–304-expressing cells.
Apoptosis by TNF-R55 is the result of ligand-induced formation of a death-inducing signaling complex leading to procaspase-8 activation (19). In this receptosome complex, TRADD is recruited by the oligomerized DDs of TNF-R55 (13–15). Next, overexpression studies showed that TRADD serves as a docking molecule for FADD. Dominant negative FADD, FADD-(80–205), prevents TNF-induced procaspase-8 activation (20). We were unable to demonstrate any caspase activation in L929sA cells after TNF stimulation (30). Nevertheless, inhibition of caspase activity by CrmA or zVAD-fmk strongly enhanced the TNF-induced necrotic process (30), which suggests that TNF might activate nondetectable levels of caspase activity. To examine whether procaspase-8 recruitment is implicated in necrotic cell death, we evaluated the effect of the dominant negative mutant of FADD-(80–205) (13, 16, 17) in L929sA cells. Unexpectedly, transient overexpression of FADD-(80–205) was already highly cytotoxic in L929sA cells. Moreover, in several attempts we were not able to generate stable transfectants of L929sA cells overexpressing FADD-(80–205) due to strong counter selection. Cytotoxicity enhancing effects of the dominant negative mutant of FADD has also been reported for TNF-induced necrosis in NIH3T3 cells in the presence of caspase inhibitors or protein synthesis inhibitors (40). However, in this particular system, FADD-(80–205) on itself was not cytotoxic, indicating that L929sA cells might be more prone to necrotic cell death. It was also found that absence of caspase-8 favors FADD-induced necrosis in Jurkat cells (33). Recently, it was also reported that FADD-(80–205) or a caspase-8-specific inhibitor sensitizes TNF-induced cell death in NIH3T3 cells (41). A similar mechanism might occur during TNF-induced necrosis in L929sA cells. Inefficient recruitment of FADD and/or procaspase-8 in the TNF-R55 DISC would result in low levels of caspase-8 facilitating necrotic signaling. In contrast, efficient recruitment of procaspase-8 in the Fas DISC complex in the same cells allows apoptotic signaling (30). The molecular mechanism for this inefficient recruitment and/or activation of caspases by TNF-R55 in L929sA cells is not clear, but it is not due to absence of endogenous TRADD. The strong synergism of CrmA or zVAD-fmk on TNF-induced necrosis (26), the observation that absence of caspases favors necrotic cell death (30, 39, 41) and that FADD dominant negative mutants facilitate TNF-mediated cell death (40, 41), suggest that caspases might be implicated in anti-necrotic mechanisms (4, 26, 30). In this paper we demonstrate that the dichotomy between necrotic and apoptotic cell death might be situated at FADD, viz. FADD-(1–111) containing the intact death effector domain would initiate CrmA-inhibited cell death (caspase-dependent apoptosis), while FADD-(80–205) consist-
RIP-induced cell death in L929sA cells. CrmA blocks cell death with 300 ng of B L929sA cells (induced by FADD-(1–111) but not by FADD-(80–205) in L929sA cells. Cell survival was calculated as described under “Material and Methods.”

Oxidative phosphorylation and concomitant oxygen radical production are required and sufficient to generate mitochondrial radical production. Also the strong synergistic effect of zVAD-fmk is confined to DD-initiated signaling components and involves enhanced receptor induced production of ROI as the radical scavenger BHA counteracts the effect of zVAD-fmk. Furthermore, reports of Khwaja (40) and Lüschen (41) demonstrated increased ROI production and protection by BHA in their cell models. Lüschen and co-workers (41) question the causality of ROI production in the observed cell death because other radical scavengers such as BHT and N-acetylcysteine could not mimic the effect of BHA. This might reflect the mechanism of action of BHA, which besides its properties as a direct oxygen radical scavenger, also possess inhibitory activities at the level of oxidative phosphorylation (25). These combined features might explain the strong anti-necrotic properties of BHA. The observation that complex I inhibitors such as antymal and complex II inhibitors such as TTFA reduce TNF-induced cell death in L929sA cells (23) underline the important contribution of the oxidative phosphorylation in the necrotic process. It is also possible that the specific inhibitory action of BHA reflects that involved ROI are formed and act in a hydrophobic environment, viz. at mitochondrial membranes, where BHA can penetrate but not most other hydrophilic, reducing agents.

How does addition of zVAD-fmk sensitize DD-mediated ROI production and consequent necrosis? This property is also shared with CrmA, since CrmA-overexpressing cells exhibited a 1000-fold sensitization to TNF-induced necrosis (26, 30). An obvious target for inhibition would be caspase-8 activation in the receptosome complex. Inhibition or very low levels of active caspase-8 might allow the formation of a more efficient necrotic DISC complex. In this respect it was shown that caspase-8 is able to proteolyze members of the receptosome complex such as RIP (45). Furthermore, inhibition of procaspase-8 recruitment by FLIP allows the conversion of a proapoptotic signal to Fas-induced proliferation in T cells (46). This demonstrates that modulation of levels of active caspase-8 might regulate the outcome of a ligand-induced signal transduction pathway.

However, our results do not exclude that zVAD-fmk and CrmA might also operate at other levels of the cell death pathway. An intriguing possibility is that zVAD-fmk- or CrmA-inhibited proteases/caspases are implicated in a surveillance system for damaged mitochondria (47). If this removal system would be blocked, accumulation of damaged mitochondria might occur, which would further increase ROI production in an autoamplifying way (4). In support of this hypothesis is the observation that zVAD-fmk synergistically enhances TNF-induced ROI production (26) and that preincubation with zVAD-fmk or overexpression of CrmA results in higher levels of spontaneous ROI production (26). Moreover, zVAD-fmk alone, in the absence of any ligand, is able to evoke some necrotic cell death in cells overexpressing Fas, hTNF-R55, or hTNF-R55Δ203–304 (data not shown). These results suggest that zVAD-fmk- or CrmA-inhibitable proteases/caspases might be implicated in the regulation of the basal oxidative metabolism.

Finally, we can conclude that TNF is able to activate directly a necrotic pathway initiated from the DD of TNF-R55. This necrotic pathway might include recruitment of FADD and is sensitized in the presence of zVAD-fmk, suggesting an antinecrotic role for caspase-8. FADD would be the point of bifurcation between apoptotic and necrotic signaling, since FADD-(1–111) initiates CrmA-inhibitory apoptosis and FADD-(80–205) initiates CrmA-insensitive necrosis. The DD of TNF-R55 is sufficient to evoke BHA-inhibitory ROI production, excluding a clear role for the membrane proximal domain in TNF-induced necrosis in L929sA cells. The observation of direct necrotic signaling by the DD of TNF-R55 and of FADD opens a new
search for the connection between a necrotic receptosome complex and the mitochondrial events such as increased ROI production.

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J. Biol. Chem. 2000, 275:37596-37603.
doi: 10.1074/jbc.M007166200 originally published online September 14, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M007166200

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