Inhibition of Caspases Increases the Sensitivity of L929 Cells to Necrosis Mediated by Tumor Necrosis Factor

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**Summary**

Murine L929 fibrosarcoma cells treated with tumor necrosis factor (TNF) rapidly die in a necrotic way, due to excessive formation of reactive oxygen intermediates. We investigated the role of caspases in the necrotic cell death pathway. When the cytokine response modifier A (CrmA), a serpin-like caspase inhibitor of viral origin, was stably overexpressed in L929 cells, the latter became 1,000-fold more sensitive to TNF-mediated cell death. In addition, TNF sensitization was also observed when the cells were pretreated with Ac-YVAD-cmk or zDEVD-fmk, which inhibits caspase-1– and caspase-3–like proteases, respectively. zVAD-fmk and zD-fmk, two broad-spectrum inhibitors of caspases, also rendered the cells more sensitive, since the half-maximal dose for TNF-mediated necrosis decreased by a factor of 1,000. The presence of zVAD-fmk also resulted in a more rapid increase of TNF-mediated production of oxygen radicals. zVAD-fmk–dependent sensitization of TNF cytotoxicity could be completely inhibited by the oxygen radical scavenger butylated hydroxyanisole. These results indicate an involvement of caspases in protection against TNF-induced formation of oxygen radicals and necrosis.

**Materials and Methods**

**Cells.** L929 murine fibrosarcoma cells and HeLa H21 cervix carcinoma cells were cultured in DMEM supplemented with 5% newborn bovine serum and 5% FCS, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and L-glutamine (0.03%). KYM rhabdomyosarcoma and PC60R55R75 murine T cell hybridoma cells were cultured in RPMI 1640, supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and L-glutamine (0.03%), and additionally 2-mercaptoethanol (5 × 10⁻⁵ M) and sodium pyruvate (1 mM) for PC 60R55R75 cells.

**Cytokines, Antibodies, and Reagents.** Recombinant murine TNF was produced in our laboratory and was purified to at least 99% homogeneity. The specific activity was 1.4 × 10⁸ IU/mg, as determined in a standardized cytotoxicity assay on L929 cells. Actinomycin D, butylated hydroxyanisole (BHA); diethylmaleate (DEM), H₂O₂, and tert-butyl hydroperoxide (tBuOOH) were purchased from Sigma Chemical Co. (St. Louis, MO). Monochlorobimane was supplied by Molecular Probes (Eugene, OR). Dihydrorhodamine 123 (DHR123; Molecular Probes) was prepared as a 5 mM stock solution in DMSO and was used at 1 mM. PI was dissolved at 3 mM in PBS and was used at 30 μM.

Abbreviations used in this paper: BHA, butylated hydroxyanisole; CrmA, cytokine response modifier A; DD, death domain; DEM, diethylmaleate; DHR123, dihydrorhodamine 123; N, nuclear factor; PI, propidium iodide; tBuOOH, tert-butyl hydroperoxide.
The caspase peptide inhibitors zDEVD-fmk, zVAD-fmk, and zD-fmk were purchased from Enzyme Systems Products (Dublin, CA). Ac-YVAD-cmk and zAAD-cmk were supplied by Calbiochem-Novabiochem International (San Diego, CA). Anti-cytokine response modifier A (CrmA) antibodies were provided by D. Pickup (Durham, NC).

Plasmids. Cowpox CrmA cDNA (a gift from D. Pickup, Durham, NC) was inserted as an EcoRI fragment into the EcoRI site of pcAGGS (16). pSV2neo, which contains the neomycin-resistant gene under control of the SV40 early promoter, was used as a selection marker (17).

Cytotoxicity assay. Cells were seeded on day −1 at 2 × 10⁴ cells/well in a 96-well plate. The next day, inhibitors and TNF were added at the given concentrations. Typically, the cells were incubated with TNF or H₂O₂ for 18 h, and cell viability was assessed using staining with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide as previously described (18). The percentage of cell survival was calculated as follows: (A₅₉₅/₆₅₅ treated cells − A₅₉₅/₆₅₅ medium)/(A₅₉₅/₆₅₅ untreated cells − A₅₉₅/₆₅₅ medium) × 100.

Measurement of Oxygen Radical Formation and Cell Death by Flow Cytometry. DHR 123 was added at the same time as TNF to suspension cultures, obtained by seeding cells in uncoated 24-well tissue culture plates (Sarstedt, Newton, NC). Cell samples were taken at different time points and analyzed on a FACScalibur flow cytometer equipped with a 488-nm argon ion laser. PI fluorescence was detected at 610 nm and served as a measure for cell death. Rhodamine 123 fluorescence, as a result of DHR 123 oxidation, was analyzed on PI-negative cells and detected at 525 nm. Relative rhodamine 123 fluorescence is defined as the ratio between emitted fluorescence at a given time point and initial fluorescence for the same condition.

Quantitation of Free Thiol Groups in Cell Lysates. Cytosolic cell extracts were prepared by lysing the cells in a buffer containing 1% Nonidet P-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-1– or caspase-3–like activities were determined by incubation of cell lysate (containing 25 μg total protein) with 50 μM of the fluorogenic substrates Ac-YVAD-AMC or Ac-DEVD-AMC (Peptide Institute, Osaka, Japan), respectively, 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 (12). The release of fluorescent 7-amino-4-methylcoumarin was measured for 1 h at 2 min intervals by spectrofluorometry; data were expressed as the increase in fluorescence as a function of time.

Measurement of Nuclear Factor (NF)-κB Activity. Western blot analysis of transfected L929 clones. Lane 1, control L929 cells transfected with pSV2neo alone; lanes 2–4, different clones cotransfected with pcAGGS CrmA and pSV2neo. A rowhead, CrmA expression. (B) Sensitizing effect on TNF-mediated necrosis in L929 cells. ▼, control; ●, clone 2; ○, clone 5; and ▲, clone 12.

Results

Overexpression of CrmA renders L929 cells more sensitive to TNF-mediated necrosis. L929 cells were cotransfected with cDNA encoding CrmA from cowpox virus and a pSV2neo selection plasmid. Individual neomycin-resistant clones were screened for CrmA expression by Western analysis and tested for their sensitivity to TNF-mediated necrosis (Fig. 1). Cells expressing CrmA were up to 1,000 times more sensitive to TNF as compared to mock-transfected cells (LD₅₀ of ~0.05 IU/ml, as compared to ~50 IU/ml for control clones). These results suggest a protective role for CrmA-sensitive caspases against TNF-induced production of oxygen radicals.

Figure 1. CrmA expression enhances TNF-induced necrosis. (A) Western blot analysis of transfected L929 clones. Lane 1, control L929 cells transfected with pSV2neo alone; lanes 2–4, different clones cotransfected with pCAGGS CrmA and pSV2neo. A rowhead, CrmA expression.

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Blocking of Caspases by Oligopeptide Inhibitors Sensitizes L929 Cells to TNF-mediated Necrosis. L929 cells were pretreated for 2 h with various caspase inhibitors, and their sensitivity to TNF was analyzed. When the cells were pretreated with Ac-YVAD-cmk or zDEVD-fmk (100 μM), which are tetrapeptide inhibitors of caspase-1 and caspase-3 subfamily members, respectively, they became significantly more sensitive to TNF-mediated cell death (with LD$_{50}$ of 1 IU/ml as compared to 30 IU/ml for controls; Fig. 2A). When Ac-YVAD-cmk and zDEVD-fmk were combined, no additional sensitization was observed, suggesting that they act on the same pathway. Two more broad-range caspase-blocking agents are zVAD-fmk and zD-fmk. When these inhibitors were added before TNF stimulation at a concentration of 25 μM, they drastically sensitized the cells to TNF (LD$_{50}$ of 0.02 IU/ml). In contrast, zAAD-cmk, an
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Inhibition of granzyme B, did not alter TNF sensitivity, excluding nonspecific effects. Taken together, it is evident that members of the caspase family are responsible for protection against TNF-induced necrosis in L929 cells. Presumably additional caspases besides caspase-1 or caspase-3 are involved in this protective effect, as suggested by the weak sensitization by Ac-YVAD-cmk and zDEVD-fmk, compared to the strong effect of zVAD-fmk and zD-fmk.

Sensitization of TNF-induced Necrosis by Peptide Caspase Inhibitors Is Abrogated by BHA. Death of L929 cells after incubation with TNF follows excessive production of oxygen radicals in the mitochondria, and scavenging of these radicals by some antioxidants, such as BHA, protects the cells (5). When the effect of peptide caspase inhibitors on TNF-induced necrosis of L929 cells was analyzed in the presence of BHA, their sensitizing effect was completely abrogated in the case of zDEVD-fmk or Ac-YVAD-cmk, and to a great extent, when zVAD-fmk or zD-fmk were used (Fig. 2 B). This indicates that sensitization by caspase inhibitors enhances oxygen radical-dependent cytotoxicity.

Enhanced Cytotoxicity in the Presence of zVAD-fmk Is Correlated with Increased Oxygen Radical Accumulation. Oxygen radical accumulation was fluorimetrically measured using DHR123 oxidation as a specific marker. Since rhodamine 123 fluorescence was measured in cells with intact membranes (PI-negative), the influence of PI fluorescence could be ruled out. As shown in Fig. 3 A, incubation of L929 cells with TNF resulted in a small but significant increase of oxygen radicals, which could be blocked by BHA. However, when the cells were pretreated with zVAD-fmk, oxygen radical levels raised up to 10-fold after 6 h of treatment with TNF. Again, BHA (100 μM) could strongly inhibit this radical accumulation. zVAD-fmk alone had no effect on radical production after 6 h. Fig. 3 B shows cell killing of the same samples, as measured by PI uptake due to loss of cell membrane integrity, demonstrating the correlation between oxygen radical accumulation and cell death.

Increased Oxygen Radical Accumulation After TNF + zVAD Treatment Is the Result of Higher Radical Production Rather than an Impaired Scavenging System. In the case of TNF-mediated radical production in L929 cells, it was previously shown that excess radicals are scavenged by the mitochondrial glutathione system (5). As the increased levels of oxygen radicals after TNF + zVAD-fmk treatment may result either from an enhanced production of radicals or an impaired mitochondrial glutathione system, we analyzed cellular thiol concentrations using monochlorobimane fluorescence after treatment with zVAD-fmk in the presence or absence of TNF. However, no significant decrease in fluorescence could be observed (Fig. 4 A). This suggests that the sensitizing effect of zVAD-fmk on TNF-mediated oxygen radical production is not the result of depleted thiol pools, such as mitochondrial glutathione.

Therefore, we tested whether zVAD-fmk had any effect on the accumulation of radicals induced by the addition of exogenous H$_2$O$_2$ or tBuOOH, which cause lipid peroxidation in the cells. As shown in Fig. 4 B, zVAD-fmk did not alter radical accumulation. Again, this indicates that the signaling pathway to radical formation, rather than the scavenging capacity of the cells, is affected by caspase inhibition.

TNF Treatment Does Not Result in Detectable Caspase Activity in L929 Cells. To study whether caspase activity oc-

Figure 4. Effect of zVAD-fmk on radical scavenging in L929 cells. (A) zVAD-fmk does not alter free thiol concentrations. Cells were treated with zVAD-fmk (25 μM) for 4 h [zVAD-fmk (-4)] or 2 h [zVAD-fmk (-2)] before TNF addition, or with DEM 3 h after TNF addition. Open bars, without TNF; filled bars, 1,000 IU/ml TNF. (B) Effect of zVAD-fmk on H$_2$O$_2$- or tBuOOH-induced oxygen radical production (relative DHR123 fluorescence as compared to untreated cells). ●, H$_2$O$_2$ (50 μM); ○, H$_2$O$_2$ + zVAD-fmk (25 μM); ▼, tBuOOH (100 μM); ▲, tBuOOH + zVAD-fmk; and ■, zVAD-fmk alone.
curs after TNF treatment of L929 cells, lysates were prepared after several incubation periods. Caspase-3– and caspase-1–like activities were determined with the substrates Ac-DEVD-AMC and Ac-YVAD-AMC, respectively. As shown in Table 1, no significant 7-amino-4-methylcoumarin release was detected in L929 lysates. PC60R55R75 cells, which die in an apoptotic mode after TNF treatment (21), were used as a control. After 4 h, DEVD cleavage activity began to appear, peaking at $\sim$6 h. These results suggest that caspase activity is correlated with apoptotic and not with necrotic cell death. Furthermore, the sensitization by caspase inhibitors apparently is due to inhibition of low, constitutive levels of caspases.

TNF-mediated Apoptosis in HeLa H21 and KYM Cells Is Inhibited by Caspase Inhibitors. To test whether only TNF-mediated necrosis was enhanced by inhibition of caspases, the effect of zVAD-fmk was also analyzed in HeLa H21 and KYM cells, which respond to TNF treatment by dying in an apoptotic way. When these cells were pretreated with zVAD-fmk for 2 h before TNF addition, complete protection against TNF was observed, even at 40,000 IU/ml TNF (Fig. 5). These results indicate that the antagonistic role of caspases is specific for TNF-induced reactive oxygen formation leading to necrosis.

Caspase Inhibitors Do Not Enhance NF-κB Activation. Treatment of L929 cells with TNF also results in activation of the transcription factor NF-κB (22). Using a reporter construct consisting of two NF-κB sites and a minimal promoter linked to the luciferase gene, we checked whether NF-κB activation was affected by caspase inhibitors. Table 2 shows relative luciferase activities after 3 h, as compared to untreated cells. In contrast to the 1,000-fold sensitization of TNF-mediated necrosis, the presence of caspase inhibitors does not influence TNF-dependent activation of NF-κB.

At 500 IU/ml TNF, luciferase activity in the presence of zVAD-fmk was even lower than in cells treated with TNF alone. However, as revealed by microscopic analysis, cells were already dying at that point. We conclude that the higher sensitivity to the cytotoxic activity of TNF on L929 cells in the presence of caspase inhibitors is not correlated with altered NF-κB activation.

Discussion

In this study, we investigated the role of caspases in TNF-mediated necrosis. First, we used the cowpox CrmA gene product as an inhibitor of a number of caspases. Surprisingly, expression of CrmA in L929 cells rendered them

<table>
<thead>
<tr>
<th>TNF treatment (10,000 IU/ml)</th>
<th>L929 cells</th>
<th>PC60R55R75</th>
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<tbody>
<tr>
<td></td>
<td>Ac-YVAD-AMC</td>
<td>Ac-DEVD-AMC</td>
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<td>(h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>26</td>
<td>10</td>
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<td>24</td>
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Values given are maximal fluorescence releases (arbitrary U/min) measured during a 1-h incubation period with the substrates indicated.

*Decrease due to cell death.

Figure 5. Inhibitory effect of zVAD-fmk on TNF-mediated apoptosis in HeLa H21 cells (● and ○; 1 μg/ml actinomycin D added) and KYM cells (▼ and ◀). Open symbols, TNF only; closed symbols, 25 μM zVAD-fmk added 2 h before TNF treatment.
far more sensitive to TNF as compared to control cells not expressing CrmA. Furthermore, blocking of caspases by peptide inhibitors sensitized the cells to TNF-induced cytotoxicity. zDEVD-fmk and Ac-YVAD-cmk had moderate sensitizing activity, whereas zVAD-fmk and zD-fmk strongly potentiated TNF-mediated necrosis. In the latter case, the concentration of TNF required for half-maximal cytotoxicity decreased 1,000-fold. zDEVD-fmk and Ac-YVAD-cmk have a different specificity pattern, and when they were combined, they could not synergize with each other, suggesting the possibility that they inhibit consecutively acting caspases. TNF sensitization induced by zVAD-fmk was accompanied by an enhanced production of oxygen radicals, as measured by DHR123 oxidation. Scavenging oxygen radicals by BHA completely abrogated the sensitizing effect of zVAD-fmk completely. The results shown here indicate a protective role for a low level of constitutively active caspase(s) in this mode of cell death.

Table 2. NF-κB Activation Is Not Affected by Caspase Inhibitors

<table>
<thead>
<tr>
<th>TNF</th>
<th>Control</th>
<th>Ac-YVAD-cmk</th>
<th>zDEVD-fmk</th>
<th>zVAD-fmk</th>
</tr>
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<tbody>
<tr>
<td>IU/ml</td>
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<td></td>
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<tr>
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<td>1.2</td>
<td>1.1</td>
<td>1.1</td>
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<tr>
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<td>4.1</td>
<td>3.7</td>
<td>3.4</td>
<td>4.4</td>
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<tr>
<td>20</td>
<td>9.3</td>
<td>9.5</td>
<td>8.8</td>
<td>8.3</td>
</tr>
<tr>
<td>500</td>
<td>10.4</td>
<td>10.0</td>
<td>9.3</td>
<td>6.4*</td>
</tr>
</tbody>
</table>

Values are relative luciferase activities as compared to unstimulated cells. Ac-YVAD-cmk and zDEVD-fmk were used at 100 μM; zVAD-fmk was used at 25 μM.

*Decrease due to cell death.

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Figure 6. Possible mechanisms of action in caspase inhibitor-mediated sensitization of TNF-induced necrosis in L929 cells. A putative caspase (CASP-X), inhibited by CrmA or zVAD-fmk, acts as a negative regulator of mitochondrial signaling (1) or mitochondrial production of reactive oxygen intermediates (ROI; 2). Alternatively, damaging of mitochondria by ROI could impair normal functioning, resulting in an even higher radical production; normally, the cell possesses a mechanism to remove these damaged mitochondria by a process involving one or more caspases (3). Interference with this clean-up process enhances necrosis.

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may be counteracted by caspases at the level of the mitochondria themselves (Fig. 6).

A third hypothetical model is the following. Degradation of mitochondrial proteins has been documented both in physiological and pathological conditions (33). This is especially the case when membrane proteins are damaged by oxygen radicals. In mitochondria of rat liver cells, increasing the radical production results in enhanced protease activity (34). In addition, oxidative damage to intracellular proteins increases their susceptibility to proteolysis (35). Although it is known that in some of these turnover processes, mitochondrial and/or cytosolic ATP-dependent protease complexes play an important role, there is also evidence for involvement of ATP-independent proteases in mitochondrial catabolism. Possibly, caspases could be key elements in such an intracellular mitochondrial quality control system. As cells increase their production of oxygen radicals in the mitochondria after p55 TNF receptor stimulation, oxidative damage of lipids and proteins accumulates; this results in occasional failure of the electron transport system, which leads to an amplified radical production. It is conceivable that such defective mitochondria are recognized and eliminated by a specific cellular mechanism, and this is where caspases could play a role. Elimination of such deficient but oxygen radical-producing mitochondria should then be beneficial for the cell to survive the deadly TNF signal. By inhibiting cytosolic caspase activity, this "rescue mechanism" would be impaired, and hence the cells would accumulate excessive reactive oxygen-producing mitochondria and would be far more sensitive to TNF-induced necrosis. Whatever the exact mechanism is, a low activity of caspases is implied, stressing the importance of a stringent control mechanism of caspase activity in healthy cells.

Fig. 6 illustrates alternative mechanisms for possible interference by caspases in TNF-induced mitochondrial production of reactive oxygen intermediates.

The results reported here prompt us to add a cautionary note. Indeed, caspases have already been shown to be essential mediators in illness-related cell death, such as neuronal damage following hypoxic-ischemic insult (36) or fulminant liver destruction after anti-Fas injection (37), and evidence exists for the implication of caspases in amyotrophic lateral sclerosis (38) and Alzheimer's disease (39). In the first two indications, inhibition of caspases by tripeptide derivatives protects treated mice against injury and death. However, considering the 1,000-fold sensitization of TNF-induced necrotic cell death by inhibitors of caspases, one should be cautious in cases where reactive oxygen-mediated necrosis may be involved, such as neutrophil-induced endothelial cell necrosis in the systemic inflammatory response syndrome (40); liver necrosis after reperfusion, alcoholic liver disease, or hemochromatosis (iron overload) and Wilson's disease (copper overload) (41); and myocardial ischemia and reperfusion injury (42). It is not excluded that in these indications, administration of caspase inhibitors may rather have an adverse effect. Therefore, the mechanism leading to cell death should be taken into account when the use of caspase inhibitors would be considered as disease treatment.

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