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Tumor necrosis factor-induced apoptosis is mediated by a CrmA-sensitive cell death pathway

(programmed cell death/tumor necrosis factor α/interleukin 1β-converting enzyme)

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ABSTRACT **We report here that the activation of the interleukin 1β (IL-1β)-converting enzyme (ICE) family is likely to be one of the crucial events of tumor necrosis factor (TNF) cytotoxicity. The cowpox virus CrmA protein, a member of the serpin superfamily, inhibits the enzymatic activity of ICE and ICE-mediated apoptosis. HeLa cells overexpressing *crmA* are resistant to apoptosis induced by *Ice* but not by *Ich-1*, another member of the *Ice/ced-3* family of genes. We found that the CrmA-expressing HeLa cells are resistant to TNF-α/cycloheximide (CHX)-induced apoptosis. Induction of apoptosis in HeLa cells by TNF-α/CHX is associated with secretion of mature IL-1β, suggesting that an IL-1β-processing enzyme, most likely ICE itself, is activated by TNF-α/CHX stimulation. These results suggest that one or more members of the ICE family sensitive to CrmA inhibition are activated and play a critical role in apoptosis induced by TNF.**

Interleukin 1β-converting enzyme (ICE), a novel cysteine protease, is homologous to the *Caenorhabditis elegans* cell death gene product CED-3 (1). ICE was identified as the enzyme responsible for cleaving prointerleukin 1β (pro-IL-1β) to generate mature biologically active IL-1β (2, 3). The sequence Lys-Pro-Xaa_4-Gln-Ala-Cys-Arg-Gly, encompassing the active-site cysteine, and the amino acid residues that form the P1 carboxylate-binding pocket are entirely conserved in CED-3 and ICE. The structural similarity between ICE and CED-3 suggests functional conservation of both proteins. Emerging evidence suggests that members of the *Ice/ced-3* gene family play an important role in controlling programmed cell death (apoptosis) (4–7). Expression of *Ice* and *Ich-1(Nedd-2)*, a new member of the *Ice/ced-3* family, induces programmed cell death in a variety of cell lines, which is inhibited by expression of *bcl-2*, a mammalian protooncogene that can suppress apoptosis. The death of chicken dorsal root ganglia neurons induced by trophic factor deprivation in culture can be suppressed by microinjection of an expression construct of *crmA*, a cowpox virus gene encoding a serpin that is a specific and potent inhibitor of ICE but a weak inhibitor of $ICH\text{-}1_L$ (4–6, 8, 9). Expression of *crmA* also protects Rat-1 cells from apoptosis induced by serum removal (6). These results suggest that members of the ICE family sensitive to CrmA inhibition play important roles in controlling cell death induced by a variety of stimuli.

Tumor necrosis factor α (TNF-α) is a pleiotrophic tumoricidal cytokine (10). TNF-α affects the growth, differentiation, and function of a multitude of cell types and is a mediator of inflammation and cellular immune responses (11–14). One of the striking functions of TNF-α is to induce apoptosis in transformed cells (15). In the case of nontransformed cells, TNF-α can also induce apoptosis in the presence of metabolic inhibitors (10). Here we report that TNF-α activates the activity of the ICE family in HeLa cells and that TNF-α-induced apoptosis is suppressed by CrmA. Our results suggest that TNF-α activates the ICE/CED-3-mediated cell death pathway.

MATERIALS AND METHODS

Cells and Tissue Culture. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% (vol/vol) fetal bovine serum. HeLa cells were transfected with the pHD1.2 *crmA* expression vector (5) by calcium phosphate precipitation. Two days after transfection, 600 $\mu g \cdot ml^{-1}$ of G418 (GIBCO) was added for selection. Resistant colonies were cloned by limiting dilution. To test the dosage response of HeLa and HeLa/CrmA cells to TNF-α treatment, cells were seeded in DMEM plus 10% (vol/vol) fetal calf serum in a 24-well plate at a density of 4×10^4 cells per well. After an overnight incubation, the cells were washed twice with serum-free DMEM. Drugs were then added to a total volume of 0.2 ml of serum-free DMEM and incubated for 24 hr. Cells were then trypsinized, and dead cells were scored on a hemocytometer by trypan blue exclusion (Sigma). At least 200 cells were scored per well. Each concentration was tested in duplicate each time in three separate experiments.

Immunoblotting (Western Blotting). Cells were lysed in SDS sample buffer (50 mM Tris·HCl, pH 6.8/100 mM dithiothreitol/2% SDS/0.1% bromphenol blue/10% glycerol), and cell lysates were subjected to SDS/15% PAGE. After the proteins were electroblotted to Immobilon nylon membrane (Millipore), the membrane was blocked with 4% nonfat milk in TBST (25 mM Tris·HCl, pH 7.5/150 mM NaCl/0.2% Tween). The membrane was incubated with the anti-CrmA antibody (5) at 5 $\mu g \cdot ml^{-1}$ for 1 hr at room temperature and then washed five times with TBST. The membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000 dilution; Amersham) for 30 min and then washed five times with TBST. CrmA protein was detected with an enhanced chemiluminescence detection kit (Amersham).

DNA Transfection. One day before transfection, cells were seeded at a density of about 2×10^5 per well in six-well dishes. For each well, 1 μg of plasmid DNA and 10 μg of lipofectamine reagent were added according to a protocol from GIBCO/BRL. Cells were incubated for 3 hr in serum-free medium containing DNA and lipofectamine; then the medium was changed to DMEM containing 10% fetal bovine serum, and incubation was continued for 24 hr. The expression of chimeric gene was detected as described (4).

Detection of IL-1β Production from HeLa Cells. HeLa cells were grown in DMEM containing 10% fetal calf serum overnight, and then the medium was changed to serum-free DMEM with or without drugs. After 24 hr, cells were scraped off and precipitated. Conditioned medium was collected,

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Abbreviations: IL-1β, interleukin 1β; ICE, IL-1β-converting enzyme; TNF, tumor necrosis factor; CHX, cycloheximide.

dialyzed against distilled water at 4°C overnight, lyophilized, and then dissolved in distilled water. Cell precipitates were extracted with the extraction buffer (20 mM Hepes adjusted to pH 7.4 with NaOH containing 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 1% Nonidet P-40, and 10 μg of phenylmethylsulfonyl fluoride, 10 μg of E64, 2 μg of pepstatin, 1 μg of leupeptin, and 0.5 μg of aprotinin per ml). Insoluble material was removed by centrifugation. Proteins were separated by SDS/15% PAGE, and IL-1β was detected by immunoblotting with an anti-human IL-1β antibody (1:300 dilution; Calbiochem) as described above.

RESULTS

Establishment of CrmA-Expressing HeLa Cells. To examine if activation of the ICE family is responsible for TNF-induced apoptosis, we established HeLa cell lines that constitutively express the cowpox virus CrmA protein. CrmA is a potent ICE inhibitor and is the only member of the serpin family with this activity (8, 9). HeLa cells were transfected with the crmA-expression vector pHD1.2 (5) and selected for G418 resistance. CrmA-expressing clones were identified by Western blotting by using an affinity-purified anti-CrmA antibody. An example of such a Western blot is shown in Fig. 1.

Overexpression of the *Ice/ced-3* Family Gene in HeLa/CrmA Cells. We tested the HeLa cell lines expressing CrmA to see if they are resistant to cell death induced by *Ice* overexpression. An expression construct of *mIce-lacZ* fusion gene (pβactM10Z) was transiently transfected (4) into control HeLa cells or HeLa cell clone 13 (HeLa/CrmA) cells, which express high levels of CrmA protein (Fig. 2); 24 hr later the cells were lightly fixed and incubated in 5-bromo-4-chloro-3-indolyl β-D-galactoside. When control HeLa cells were transfected with *mIce-lacZ*, most of the blue cells were round dead cells. In contrast, *mIce-lacZ*-induced apoptosis was efficiently prevented in HeLa cells that express CrmA. The same effect was observed with HeLa/CrmA clone 4 (data not shown). Cell death induced by overexpression of *ced-3* is poorly suppressed by CrmA (4). Recently, a third member of the *Ice/ced-3* gene family, *Ich-1/Nedd-2*, has been identified (6, 7). Overexpression of *Ich-1* induces Rat-1 and HeLa cell death efficiently, similar to that of ICE. However, cell death induced by *Ich-1* is only very weakly suppressed by overexpression of *crmA* in HeLa cells (Table 1) and Rat-1 cells (6). Thus, CrmA is an effective inhibitor of ICE but a poor inhibitor of ICH-1_L.

Suppression of TNF-α-Induced Apoptosis by CrmA. We then tested the effect of CrmA on TNF-α-induced apoptosis.

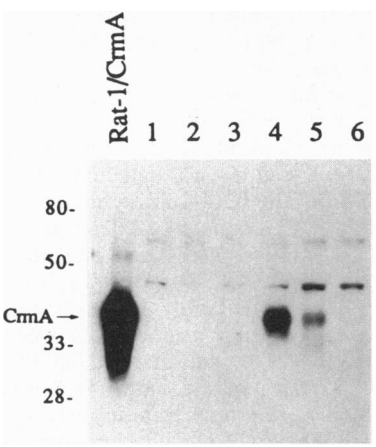

FIG. 1. Western blot analysis of HeLa cell clones expressing CrmA. Lanes: 1–6, six different G418-resistant HeLa cell clones; Rat-1/CrmA, cell lysate of a Rat-1 cell clone expressing CrmA as a positive control. Expression level of CrmA in clone 13 (not shown) is about twice that of clone 4. Sizes are shown in kDa.

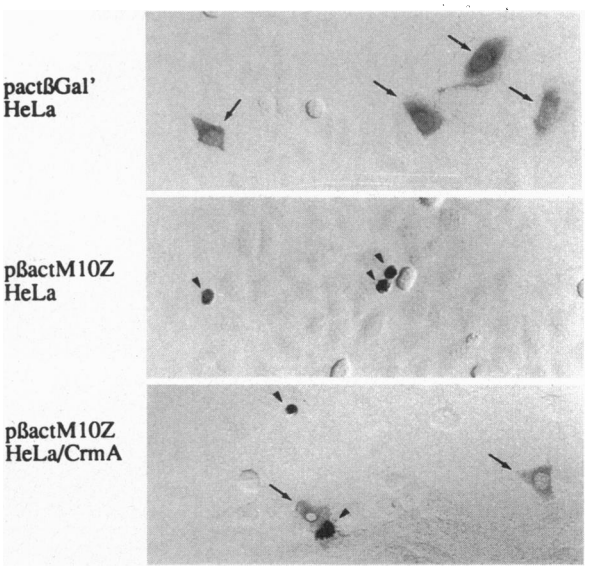

FIG. 2. Overexpression of crmA in HeLa cells suppressed ICE-induced cell death. HeLa or HeLa/CrmA cells were transiently transfected with expression constructs of pactβGal' (control) or pβactM10Z, which contains a wild-type murine Ice cDNA fused with *Escherichia coli lacZ* under the control of the chicken β-actin promoter. Expression of the control or the chimeric gene was detected as described (4). Arrows and arrowheads indicate, respectively, living cells (flat) and dying cells (round) expressing *lacZ* gene. (Bar = 50 μm.)

In the presence of cycloheximide (CHX), TNF-α efficiently induces HeLa cell death (16). Under the same conditions, the clone 13 HeLa/CrmA cells, which express high levels of CrmA protein, are highly resistant to TNF-α/CHX-mediated cell death (Fig. 3 and Table 1). The same effect was observed with clone 4 HeLa/CrmA cells, which express similar levels of CrmA (data not shown). Clone 5 HeLa/CrmA cells, which express lower levels of CrmA, are also resistant under the same condition but to a lower extent (percentage of dead cells is 30.2 ± 1.2%). We then tested the response of *crmA*-expressing HeLa cells (clone 13) to increasing amounts of TNF-α in the presence of 10 μg of CHX per ml. We found that CrmA/HeLa cells are resistant to TNF-α at concentrations ranging from 0.01 pg·ml^{-1} to 100 ng·ml^{-1} (Fig. 3 *Right*). After 24 hr of incubation in the presence of 100 ng of TNF-α and 10 μg of

Table 1. Prevention of apoptosis by CrmA

Treatment	HeLa	HeLa/CrmA
Transfection	% round blue cells	
pactβGal'	3.2 ± 0.8	1.1 ± 0.4
pβactM10Z	85.5 ± 3.0	46.9 ± 7.0
pβactH37Z	91.4 ± 4.9	87.5 ± 3.0
Drug	% dead cells	
Control	1.5 ± 0.3	5.5 ± 1.8
CHX	2.9 ± 0.7	3.8 ± 0.5
TNF	2.7 ± 1.2	2.8 ± 1.0
CHX + TNF	68.2 ± 1.9	9.7 ± 1.2

Cells were transfected and stained as described in the legend to Fig. 2. Plasmid pactβGal' is a control *lacZ* gene expression vector, and plasmid pβactM10Z is a murine *Ice-lacZ* chimeric gene expression vector (4). Plasmid pβactH37Z is an *Ich-1-lacZ* chimeric gene expression vector (6). The data are means ± SEM of the percentage of round blue cells among the total number of blue cells counted. To see the effects of CHX at 20 μg·ml^{-1} and TNF-α at 5 ng·ml^{-1}, cells were treated with drugs for 24 hr, and cell viabilities were measured by trypan blue dye exclusion. The data are means ± SEM of the percentage of dead cells. The data were collected from at least three independent experiments.

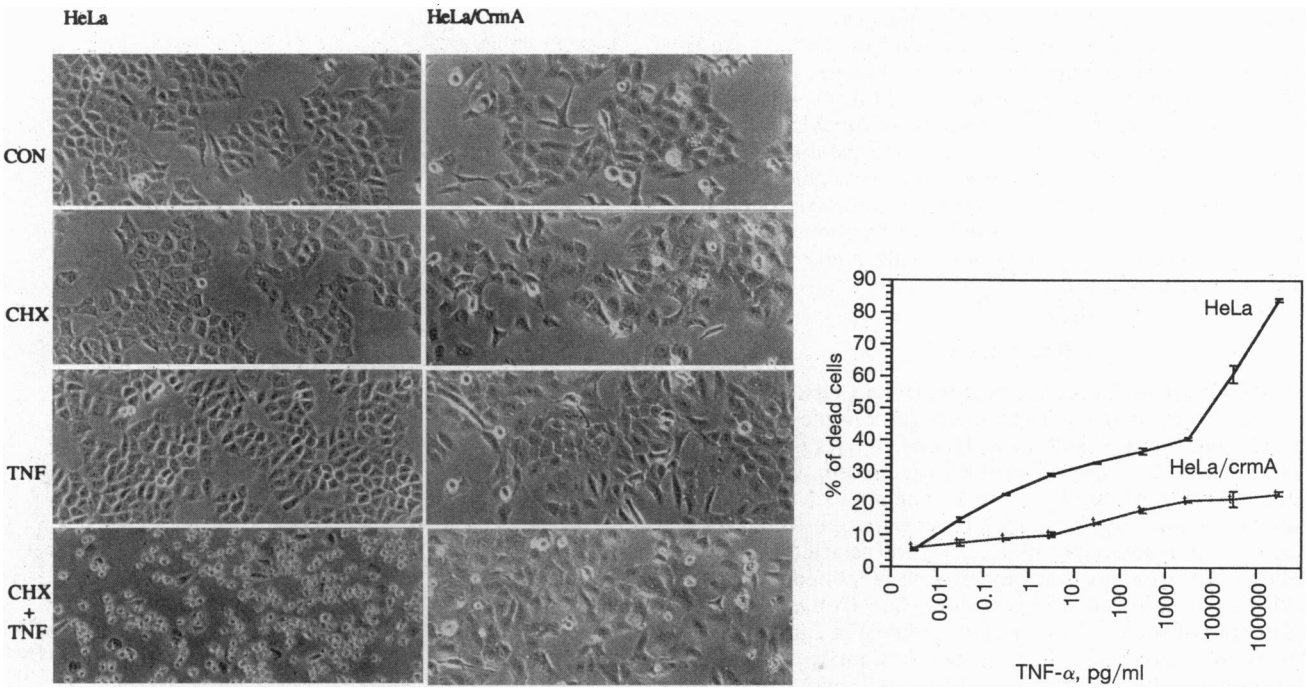

FIG. 3. TNF-induced cytotoxicity was suppressed by overexpression of CrmA. (*Left*) HeLa cells or HeLa/CrmA cells were treated with CHX (20 $\mu g \cdot ml^{-1}$; Sigma) alone, TNF-α (5 $ng \cdot ml^{-1}$; Sigma) alone, or a combination of both drugs. Cells were photographed 24 hr after drug treatment. Con, control. (Bar = 50 μm.) (*Right*) Control HeLa and HeLa/CrmA (clone 13) cells were tested for their ability to resist increasing amounts of TNF-α in the presence of 10 μg of CHX per ml; 1 μg of TNF-α has 1.1×10^5 units of activity according to R & D Systems. The results were from three experiments, with each condition done in duplicate.

CHX per ml, 83% of control HeLa cells died compared with 23% of HeLa/CrmA cells.

Activation of the ICE Family After TNF Stimulation. We have detected the expression of both *Ice* and *Ich-1* in HeLa cells (6). Since expression of *crmA* effectively prevents cell death induced by TNF-α in the presence of CHX, the cell death pathway mediated by the ICE family members sensitive to CrmA inhibition may be activated by TNF-α stimulation and may play a major role in the induction of HeLa cell death. If this is the case, TNF-α stimulation may activate endogenous IL-1β-processing activity in HeLa cells. To date, pro-IL-1β is the only known endogenous substrate of ICE. If ICE is activated after TNF-α/CHX stimulation, the endogenous 33-kDa pro-IL-1β should be processed, and the mature 17.5-kDa IL-1β should be secreted. To detect mature IL-1β, we collected conditioned media from HeLa cells with or without TNF stimulation and analyzed the processing of pro-IL-1β by Western blot (Fig. 4). Our data show that mature IL-1β was observed only after induction of apoptosis by TNF-α/CHX. HeLa/CrmA cells are much more resistant to TNF-α/CHX-induced cell death and secreted much less mature IL-1β under the same condition, which can be observed as a very faint band only after long exposure. These results strongly suggest that apoptosis induced by TNF stimulation is mediated by one or more members of the ICE family that are sensitive to CrmA inhibition and can process pro-IL-1β.

DISCUSSION

Our previous work demonstrated that overexpression of ICE induces Rat-1 cells to undergo apoptosis (4) and that expression of crmA can prevent chicken dorsal root ganglion (DRG) neurons from cell death induced by trophic factor deprivation (5). These results showed that ICE has the ability to induce cell death and that inhibition of ICE activity can prevent programmed cell death. Using pro-IL-1β processing as an indicator, we demonstrate here that mature IL-1β is produced when HeLa cells are induced to die by treatment with TNF-α and CHX. Our work has shown that the enzyme(s) that can cleave pro-IL-1β to mature IL-1β is activated in apoptosis induced by TNF-α. Since ICE is the only enzyme identified so far that can process pro-IL-1β, our data suggest that ICE is activated during apoptosis.

The amino acid sequence of CrmA protein placed it in the serpin family whose other members are all inhibitors that target serine proteases (17). Titrations of ICE with the CrmA-glutathione *S*-transferase (GST) fusion protein showed that 2.5 mol of CrmA-GST is required to inhibit 1 mol of the enzyme with a K_i of $<4 \times 10^{-12}$ M (9). Purified CrmA failed to inhibit representative members of several major families of serine proteases: trypsin, chymotrypsin, cathepsin G, pig and human elastases, thrombin, plasmin, human tissue plasminogen activator, and human urinary plasminogen activator. It also failed to inhibit papain, a cysteine protease (8). CrmA did not inhibit any of these proteases even when the ratio of CrmA to the proteases was 100:1 (wt/wt) (8). More recently, CrmA was tested for the ability to form complexes with several representatives of major protease families on native PAGE analysis: ICE, neutrophil elastase, cathepsin G, *Staphylococcus aureus* V8 proteinase and subtilisin Carlsberg. In these assays, only the ICE–CrmA complex was detected (9). Thus, CrmA is a specific inhibitor of the ICE family. Our own results showed that CrmA inhibits ICE-induced cell death much more effectively than ICH-1_L-induced cell death, suggesting that in the ICE family, CrmA prefers ICE to ICH-1_L. It is possible that other members of the ICE family that are more homologous to ICE than to ICH-1_L may also be effectively inhibited by CrmA. Although ICE is the only enzyme identified so far that can cleave pro-IL-1β to generate mature IL-1β, we cannot rule out that other members of the ICE family may be able to cleave pro-IL-1β as well. Thus, we conclude from our results that the CrmA-sensitive member(s) of the ICE family play a critical role in apoptosis induced by TNF, and at least one of them has the ability to cleave pro-IL-1β.

Mice that are deficient in ICE have been reported (18). The ICE mutant mice develop apparently normally, with the major

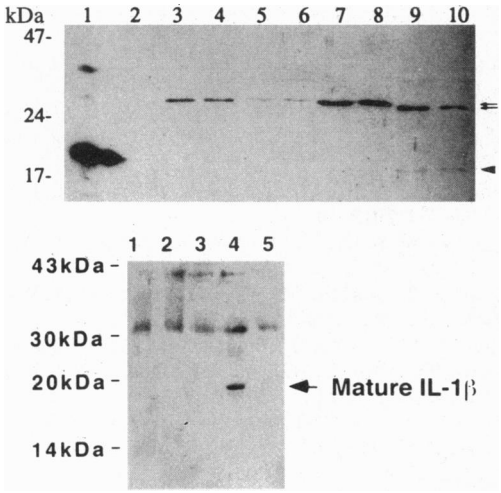

FIG. 4. Processing of IL-1β after induction of apoptosis by TNF-α/CHX in HeLa cells. (*Upper*) A Western blot of HeLa cells probed with anti-human IL-1β (Calbiochem). Lanes: 1, purified mature human IL-1β; 3–6, cell lysates (10 μg of protein per lane); 7–10, supernatant (5 μg per lane); 3 and 7, serum-free control; 4 and 8, lipopolysaccharide (LPS) (10 $\mu g \cdot ml^{-1}$; Sigma) treatment; 5 and 9, LPS (10 $\mu g \cdot ml^{-1}$), CHX (20 $\mu g \cdot ml^{-1}$), and TNF-α (5 $ng \cdot ml^{-1}$) treatment; and 6 and 10, CHX (20 $\mu g \cdot ml^{-1}$) and TNF-α (5 $ng \cdot ml^{-1}$) treatment. Cell viabilities were measured by trypan blue exclusion (97.4 ± 1.5% for the serum-free control, 97.4 ± 0.2% for LPS treatment, 56.3 ± 2.2% for TNF-α/CHX treatment). Arrows and the arrowhead indicate, respectively, pro-IL-1β and mature IL-1β. (*Lower*) A Western blot of supernatant from HeLa (lanes 1–4) or HeLa/CrmA cells (lane 5) treated with no addition (control, lane 1) or 20 μg of CHX per ml (lane 2) or 5 ng of TNF-α per ml (lane 3) or 20 μg of CHX and 5 ng of TNF-α per ml (lanes 4 and 5), probed with anti-human IL-1β (Calbiochem). The amount of protein was measured by Bio-Rad assay. Three micrograms of protein was loaded onto each lane.

defect being inability to produce mature IL-1β after stimulation with lipopolysaccharide. This result suggests the redundency of the ICE family in controlling vertebrate apoptosis: multiple members of the ICE family may all contribute to the control of cell death. Alternatively, the cell death defects of the mutant ICE mice may be revealed only by counting the number of cells at appropriate places. For example, the transgenic mice expressing *bcl-2* under the control of neural-specific enolase (NSE) promoter or 3-phosphoglycerate kinase (PGK) promoter develop essentially normally, and yet upon cell counting, they contain up to 40% and 50% more neurons in the facial nucleus and the ganglion cell layer of the retina (19). *C. elegans ced-3* or *ced-4* mutants also develop and behave normally despite the fact that they contain up to 20% more cells (20). Activation of ICE has been reported in mammary epithelial cells induced to die by antibodies to β1 integrins or by overexpression of stromelysin 1, which degrades extracellular matrix (21). Such a result suggests the participation of ICE in apoptotic pathways activated by different signals.

The death of HeLa cells induced by TNF-α in the presence of cycloheximide shows DNA fragmentation, a typical feature of apoptosis (16). Expression of the E1B 19-kDa protein, a viral homolog of the Bcl-2 protein (22), by viral infection, by transient expression, or in transformed cells completely and specifically blocks the TNF-α-induced DNA fragmentation and cell death (16). Thus, TNF-α stimulation likely activates an endogenous pathway of programmed cell death.

The reasons why CHX potentiates TNF cytotoxicity in nontransformed cells is unclear. Most of the cell lines including HeLa cells, NIH 3T3 cells, and TA1 cells are not killed by TNF alone but are killed by the combined actions of TNF and CHX (23, 24). TNF-α is a pleiotrophic cytokine that may induce more than one cellular response in a single cell line. The presence of CHX may inhibit the synthesis of certain signaling molecules and, thus, potentiates the killing activity of TNF. Alternatively, CHX may simply inhibit the synthesis of a general cell survival factor(s) and, thus, allow cells to become more sensitive to TNF cytotoxicity.

HeLa cells express predominantly the p55 TNF receptor, which is thought to be responsible for cell death signaling (25, 26). The TNF p55 receptor triggers the activation of phospholipase A2, protein kinase C, sphingomyelinase, phosphatidylcholine-specific phospholipase C, and NF-κB (27, 28). In TNF p55 receptor-knockout mice, TNF-mediated induction of NF-κB is prevented in thymocytes (29). TNF p55 receptor-knockout mice are resistant to lethal doses of either lipopolysaccharides or *S. aureus* enterotoxin B, suggesting that the TNF p55 receptor mediates the pathogenesis of hepatocyte necrosis (29). One possible explanation for the resistance of HeLa/CrmA cells to TNF-α is that they might have lost the TNF-α receptor. This is highly unlikely because HeLa/CrmA cells lose the expression of *crmA* easily if propagated for more than a few weeks in culture without selection; and when they lose the expression, they become completely sensitive to TNF-α again (data not shown). In addition, three HeLa/CrmA clones tested show CrmA expression level-dependent resistance to TNF-α.

TNF-α is a major player in host inflammatory responses of mammals (6). Upon injection of endotoxin (LPS) in models of septic shock, TNF, IL-1, and IL-6 are quickly induced (30). In such conditions, the secretion of IL-1β appears to be dependent upon TNF, since passive immunization with TNF monoclonal antibodies during endotoxemia *in vivo* attenuates the appearance of IL-1β (31). Our results here suggest that TNF may play a role in activating members of the ICE family to process pro-IL-1β. Expression of mitochondrial manganese superoxide dismutase has been shown to promote the survival of tumor cells exposed to TNF (32), suggesting that generation of free radicals may play a role in cell death induced by TNF. There are several reports that TNF cytotoxicity is related to the generation of free radicals and lipid peroxides (33, 34). These observations raised the possibility that members of the ICE family may be activated directly or indirectly by free radicals.

Note: After we submitted this paper, Tewari and Dixit (35) reported that expression of CrmA inhibited TNF-α- and anti-Fas-induced cell death in MCF-7 cells.

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