Functional Role of Interleukin 1β (IL-1β) in IL-1β–converting Enzyme–mediated Apoptosis

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Summary

Prointerleukin-1β (pro-IL-1β) is the only known physiologic substrate of the interleukin-1β (IL-1β)–converting enzyme (ICE), the founding member of the ICE/ced-3 cell death gene family. Since secreted mature IL-1β has been detected after apoptosis, we investigated whether this cytokine, when produced endogenously, plays a role in cell death. We found that hypoxia-induced apoptosis can be inhibited by either the IL-1 receptor antagonist (IL-1Ra) or by neutralizing antibodies to IL-1 or to its type 1 receptor. IL-1Ra also inhibits apoptosis induced by trophic factor deprivation in primary neurons, as well as by tumor necrosis factor α in fibroblasts. In addition, during the G1/S phase arrest, mature IL-1β induces apoptosis through a pathway independent of CrmA–sensitive gene activity. We also demonstrate that Ice, when expressed in COS cells, requires the coexpression of pro-IL-1β for the induction of apoptosis, which is inhibited by IL-1Ra. Interestingly, we found that mature IL-1β has antiapoptotic activity when added exogenously before the onset of hypoxia, which we found is caused in part by its ability to downregulate the IL-1 receptor. Our findings demonstrate that pro-IL-1β is a substrate of ICE relevant to cell death, and depending on the temporal cellular commitment to apoptosis, mature IL-1β may function as a positive or negative mediator of cell death.

Apoptosis, a process by which organisms eliminate unwanted cells, is executed through the activation of a tightly regulated program (1). A genetic pathway of programmed cell death was first identified in the nematode Caenorhabditis elegans. In this worm, the products of the ced-3 and ced-4 genes carry out the program of cellular suicide (2). IL-1β–converting enzyme (ICE), a cysteine protease responsible for the activation of pro-interleukin-1β (pro-IL-1β), is a mammalian homologue of CED-3 (3–5). Previous work has demonstrated that Ice overexpression induces programmed cell death, and that mature IL-1β is released during apoptosis mediated via a variety of stimuli (LPS, TNF-α, and Shigella flexneri infection) (5–8). The cowpox virus gene product CrmA, a member of the serpin family and an inhibitor of ICE, prevents apoptosis (5, 7, 9–13). Furthermore, the inhibition of apoptosis mediated by CrmA correlates with its inhibition of mature IL-1β production (7). Recent reports indicate that TNF-α–induced apoptosis is mediated through a CrmA–inhibitable pathway, suggesting the involvement of the ICE family (7, 14). The detection of mature IL-1β release during apoptosis strongly suggests that ICE is activated in cell death since in vivo, ICE is the major (if not the only) protease responsible for processing pro-IL-1β (15, 16).

While the critical role of the ICE family in cell death is well accepted, the function of mature IL-1β in apoptosis is controversial. Exogenous IL-1β has been shown to induce apoptosis in some systems (17–19) and to prevent it in others (20, 21). In this study, we establish a dual functional role for mature IL-1β in ICE-mediated apoptosis. We found that when produced endogenously (i.e., after ICE activation), IL-1β mediates cell death, but when provided exogenously, IL-1β can either stimulate or inhibit cell death. We demonstrate that if IL-1β binds to its receptor before exposure to an apoptotic stimulus, it inhibits programmed cell death (by downregulating the IL-1 receptor); in contrast, if IL-1β binds after ICE is activated, it enhances cell death. In addition, we demonstrate that Ice requires the coexpression of pro-IL-1β to induce apoptosis in COS cells.

Abbreviations used in this paper: DRG, dorsal root ganglia; HU, hydroxurea; ICE, IL-1β–converting enzyme; IL-Ra, IL-1 receptor agonist; NGF, nerve growth factor; pro-IL-1β, prointerleukin-1β.
Cell death was inhibited by blocking IL-1β from binding to its receptor, indicating that after ICE activation, COS cells required IL-1β signal transduction for the completion of the suicide program. Our results demonstrate that endogenously produced mature IL-1β plays an integral role in ICE-mediated apoptosis.

Materials and Methods

Hypoxia-induced Apoptosis Assay. HeLa and HeLa/CrmA cells were seeded in 35-mm dishes at a density of $6 \times 10^4$/dish in DMEM/10% FCS and grown overnight. The medium was then changed, and factors were added: IL-1Ra (R & D Systems, Minneapolis, MN), IL-1 antibody (Calbiochem-Novabiochem Corp., San Diego, CA), or IL-1 type 1 receptor antibody (R & D Systems). Dishes were placed in an anaerobic chamber with a BBL GasPack Plus (Becton Dickinson, Cockeysville, MD), which reduces the oxygen concentration to <100 ppm within 90 min. After 16 h, cells were removed from the chamber, immediately trypsinized, and scored for viability by trypan blue exclusion.

Inhibition of 125I-IL-1β Binding by IL-1Ra. HeLa cells (10⁴) were added in 10-cm dishes, and after an overnight incubation, IL-1Ra was added for 2 h at 37°C. After the addition of BSA (1 mg/ml) to the medium, the cells were incubated at 4°C for 15 min, and then 125I-IL-1β (100 ng/ml) was added at 4°C for 1 h. For detection of 125I-IL-1β binding, cells were treated with 50 mM glycine-HCl, pH 2.6, for 1 min and quantitated by γ counting.

Neuronal Trophic Factor Deprivation Assay. Postnatal day 1 mouse dorsal root ganglia (DRG) neurons were isolated, dissociated with trypsin for 1 h at 37°C, and plated in an eight-chamber slide coated with poly-l-lysine/laminin (Sigma Immunochemicals, St. Louis, MO). Wells were seeded at a density of ~1,000 neurons/well (eight wells per mouse). Neurons were cultured in Ham's F-12 media supplemented with 20% FCS (Biowhittaker, Walkersville, MD), nerve growth factor (NGF) 200 ng/ml; Sigma), brain-derived neurotrophic factor (100 ng/ml; Preptech, Rocky Hill, NJ), glutamine (2 mM), and penicillin/streptomycin. The medium was replaced daily with either trophic factor containing medium (TF(+)=20% FCS and NGF [200 ng/ml]) or trophic factor-deficient medium (TF(-)=serum and NGF-free medium in the presence of a saturating concentration of mouse NGF mAb (100 ng/ml; Boehringer Mannheim, Indianapolis, IN), and IL-1Ra (100 ng/ml, unless otherwise indicated in the text). Healthy neurons were counted using a phase contrast microscope 24 and 48 h after the initial media change.

Growth Arrest/Cytokine-induced Apoptosis Assay. L929, HeLa, and HeLa/CrmA cells were seeded (2 × 10⁴ cells/well) in 24-well plates and grown overnight in DMEM with 10% FCS. After 12 h, the wells were washed three times with serum-free DMEM, and hydroxyurea (HU) 2.5 mM; Sigma) was added to HeLa and HeLa/CrmA cells (22). After 5 h, IL-1Ra (40 ng/ml) was added to the appropriate wells, and 1 h later, either TNF-α or mature IL-1β (generous gift from Dr. Lee Gehrike, Massachusetts Institute of Technology, Cambridge, MA) was added. 24 h later, IL-1Ra was again added to the appropriate wells, and cell death was evaluated by trypan blue exclusion 60 h after the initial addition of HU. Each condition was tested independently three times in duplicate, and 200 cells were counted per well. For nuclear morphology determination, cells were grown on two-well slides, fixed in 4% paraformaldehyde, and incubated with Hoechst dye No. 33258 (10 μg/ml; Sigma).

Results

Endogenously Produced Mature IL-1β Plays a Role in a CrmA-inhibitable Apoptosis Pathway Induced by Hypoxia. Recently, BCL-2 and p53 have been implicated in hypoxia-mediated apoptosis (23-25). To investigate if the ICE family is involved in hypoxia-induced apoptosis, we tested whether CrmA could inhibit this process. Survival of HeLa cells cultured for 16 h under hypoxic conditions was 10.1%, compared with 69.0% survival of HeLa cells that stably express CrmA (HeLa/CrmA; Fig. 1 a). Thus, CrmA-inhibitable members of the ICE family play an important role in hypoxia-induced apoptosis. To address whether endogenously produced mature IL-1β plays a role in hypoxia-induced cell death, we used several methods to prevent IL-1 from binding to its receptor. We used the IL-1Ra (a naturally occurring cytokine that binds to the IL-1 receptor, blocking IL-1-mediated signal transduction) (26, 27), an anti-IL-1 polyclonal neutralizing antibody, and an anti-IL-1 type 1 receptor neutralizing mAb (the type 1 receptor mediates IL-1 signal transduction). Each of these reagents inhibited hypoxia-induced cell death, suggesting that hypoxia activates an ICE-like, CrmA-inhibitable pathway, and that endogenously produced mature IL-1β plays a role in hypoxia-induced cell death by binding to the IL-1 type

COS Cell Transfections. COS cells were plated (2 × 10⁴) in six-well plates in DMEM with 10% FCS. After 12 h, the wells were washed with serum-free and antibiotic-free medium, and were transfected using lipofectamine with either lac-Z, β actin-lacZ (1 μg), or pro-IL-1β (0.5 μg) for 3 h. The medium was then replaced with DMEM/10% FCS. IL-1Ra (40 ng/ml) was then added to the appropriate wells, and after 1 h, IL-1β (100 ng/ml) was added. X-gal staining was performed 36 h after the transfection, and the percentage of round blue (dead) cells was scored (5).

Immunostaining. COS cells (1.5 × 10⁶/well) were plated in a poly-l-lysine-coated two-chamber slide and transfected after 12 h, as described above. Cells were fixed after 36 h with 4% paraformaldehyde (15 min), blocked with 1% heat-inactivated goat serum/2% BSA in PBS (2 h), and incubated with a rabbit polyclonal antibody against IL-1 (1:300; Calbiochem-Novabiochem) plus a hybridoma supernatant containing a mouse mAb raised against human ICE (12 h at 4°C). Cells were then washed 3X with PBS and incubated with a goat anti-mouse FITC-labeled antibody plus a goat anti-rabbit RITC-labeled antibody (1:200; Cappel Laboratories, Durham, NC) and Hoechst dye No. 33258 (10 mg/ml) for 45 min. Cells were rinsed three times with PBS, examined with an Axioplan microscope, and photographed with a 40× objective.

IL-1 Receptor Downregulation. IL-1β (100 ng/ml) was added as the cells were placed into the hypoxia chamber (90 min are required to reach oxygen concentrations of 100 ppm). IL-1 receptor-binding assay: HeLa cells (10⁴) were seeded in 10-cm dishes and grown overnight. Media was then exchanged containing 1 mg/ml of BSA and 100 ng/ml of IL-1β at 4°C for 1 h. After washing twice with cold medium, the cells were incubated with fresh warm medium at 37°C for 0, 30, 60, and 120 min. Cells were then treated as described above with glycine, and the radioactivity was scored.
Endogenous IL-1β Mediates Trophic Factor Deprivation–induced Sensory Neuron Apoptosis. We next investigated in a primary culture system whether endogenous IL-1β plays a role in apoptosis. DRG neurons undergo apoptosis in culture upon NGF withdrawal (28). We have previously shown that chicken DRG neuronal death induced by trophic factor deprivation is inhibited by CrmA, suggesting involvement of the ICE family (10). We tested whether endogenously produced mature IL-1β, which is produced by neurons in culture (29), plays a role in trophic factor withdrawal–mediated DRG neuronal apoptosis. Addition of IL-1Ra (100 ng/ml) to newborn mouse DRG neurons inhibited trophic factor withdrawal–induced apoptosis by 69.2 and 37.8% over 24 and 48 h, respectively (Fig. 2). Inhibition of neuronal apoptosis by IL-1Ra was dose dependent (43.5% in 24 h at a concentration of 40 ng/ml). These results suggest that endogenously produced mature IL-1β plays a role in DRG neuronal apoptosis after trophic factor withdrawal.

Secreted IL-1β Mediates TNF-α–induced Apoptosis of HeLa and L929 Cells. TNF-α induces apoptosis via a CrmA–inhibitable pathway (7, 14). In addition, we have demonstrated that mature IL-1β is secreted by TNF-α–treated cells undergoing apoptosis, suggesting ICE activation during this process (7). We tested whether secreted mature IL-1β plays a role in TNF-α–induced apoptosis of L929 cells. IL-1Ra protected L929 cells from TNF-α–induced death by up to 64.9%, suggesting that secretion and receptor binding of mature IL-1β plays a role in TNF-α–induced cell death (Fig. 3 a). In addition, HU-treated, G1/S phase–arrested HeLa cells are induced to undergo apoptosis by TNF-α (22). Under these conditions, IL-1Ra also inhibited HeLa cell death by 56.0% (Fig. 3 b). HeLa cells induced to die by TNF-α and cycloheximide were also protected by IL-1Ra, as well as by three different neutralizing IL-1 antibodies (data not shown). HeLa/CrmA cells were protected from TNF-α–induced apoptosis by 59.5%, suggesting that an ICE-like activity is involved in the cell death signaling pathway mediated by this cytokine (Fig. 3 b).

Mature IL-1β alone does not induce apoptosis of most healthy proliferating cells (including HeLa and L929). To examine if IL-1β would induce cell death in G1/S phase–arrested cells, we exposed HU-treated HeLa cells to this
cytokine. Indeed, G1/S phase-arrested HeLa cells treated with exogenous IL-1β died in a dose-dependent fashion (83.7% at 100 ng/ml), which was inhibited by the IL-1Ra (Fig. 3 c). HU-arrested, mature IL-1β, and TNF-α–treated cells underwent typical apoptotic changes of cellular shrinkage, nuclear condensation, and fragmentation (Fig. 3, d–i). It is interesting that HeLa/CrmA cells were not protected against mature IL-1β as they were against TNF-α killing, suggesting that mature IL-1β induces the apoptotic cascade distal to ICE, and that in HU treated cells, this cytokine causes cell death through an ICE-independent pathway (Fig. 3 c). This indicates that CrmA is indeed blocking an ICE-like function, and that production and secretion of mature IL-1β is a downstream effector of the apoptotic TNF-α/ICE cascade. HeLa cells, however, are required to be primed (in this case with HU arrest) to establish the appropriate intracellular milieu to be sensitized to mature IL-1β–induced apoptosis. HU treatment likely mimics intracellular signals that are part of the apoptotic cascade.

**IL-1β Potentiates Apoptosis of COS Cells Induced by ICE.** Next, we asked directly whether pro-IL-1β processing is required for ICE-mediated apoptosis. For this...
Figure 5. Immunofluorescence of COS cells transiently transfected with pro-IL-113. (a and b), Ice (c and d), or pro-IL-113 plus Ice (e–g). COS cells transfected with pro-IL-113 and immunostained with an anti-human polyclonal IL-1 antibody and a secondary RITC-coupled antibody demonstrate nuclear and cytoplasmic morphologic appearance typical of healthy cells. Cells transfected with Ice and immunostained with an anti-human ICE mAb and a secondary FITC-conjugated antibody display normal cytoplasmic morphology, yet contain condensed nuclei, suggesting the initiation of apoptosis, which cannot be completed in the absence of IL-113. Coexpression of Ice plus pro-IL-113 induces typical apoptotic features (condensed nucleus and round morphology).

Purpose, we used COS cells. These cells are unusual because they are resistant to cell death induced by Ice overexpression (30). Transfection of Ice into Rat-1 cells induces 94.2% apoptosis within 24 h (30). In contrast, COS cells transiently expressing Ice-lacZ or pro-IL-113 genes for 36 h died 9 and 6.3%, respectively. However, 51% of COS cells coexpressing Ice-lacZ and pro-IL-113 died, and treatment of these double-transfected cells with IL-1Ra inhibited apoptosis. In addition, treatment of Ice-lacZ transfected cells with extracellular mature IL-113 efficiently induced cell death (Fig. 4). Exogenous mature IL-113 did not induce apoptosis in COS cells, indicating that ICE processes additional substrates for cell death, and that in COS cells, after ICE activation, IL-113 signal transduction is required for the induction of apoptosis. Dual immunofluorescence staining (with anti-ICE and anti-IL-1 antibodies) of COS cells cotransfected with Ice and pro-IL-113 indicated that only cells expressing both ICE and pro-IL-113, but not either protein alone, undergo apoptosis (Fig. 5). We consistently noticed that the nuclei of cells transfected with Ice were smaller than that of control cells (Fig. 5 c). These cells were alive, as demonstrated by their flat morphology and adherence to the plate (Fig. 5 d), suggesting that ICE initiates the apoptotic process, but requires additional factors (i.e., mature IL-113) for the complete execution of the cell death pathway.

Preincubation of Exogenous IL-113 Protects Hypoxia-mediated Cell Death by Downregulating the IL-1 Receptor. Conflicting evidence exists regarding the role of IL-113 in apoptosis. In some systems, IL-113 induces cell death, whereas in others, it inhibits cell death (17–21). IL-113 inhibition of cell death requires its addition before the apoptotic stimulus, as demonstrated both with TNF-α killing of L929 cells and with excitatory amino acid–mediated neuronal death (20, 21). We determined if exogenous mature IL-113 preincubation inhibits cell death in a system where ICE activation and mature IL-113 receptor binding are important for apoptosis. HeLa cells preincubated with exogenous IL-113 are markedly protected from hypoxia-induced cell death (10.1 vs. 58.7% survival; Fig. 6 a). To explain the inhibition of apoptosis by IL-113, we investigated whether preincubation with exogenous IL-113 in our system before exposure to
apoptotic stimuli downregulates the IL-1 receptor (34). Indeed, receptor-binding assays demonstrate that exogenous IL-1β significantly downregulates the IL-1 receptor (Fig. 6b). Downregulation of the IL-1 receptor in part explains the protective role of exogenous IL-1β, when added before the induction of apoptosis. The effect of IL-1β receptor-binding on apoptosis is dependent on whether ICE is active (enhancing cell death) or inactive (inhibiting cell death, in part by downregulating the IL-1 receptor).

Discussion

The mammalian ICE/Ced-3 family now includes at least six members: ICE, ICH-1/NEDD2, CPP32/Yama/Apopain, TX/ICErelII/ICH-2, ICErelIII, and MCH2 (4, 30, 32–39). Since this is a family of proteases whose active site cysteine residue is essential for ICE-mediated apoptosis, their proteolytic activity must be critical in mediating cell death (5). How might proteolytic cleavage by the ICE family lead to apoptosis? One possibility is that cleavage of a large number of proteins destroys the entire cellular machinery. This is unlikely because most proteins appear to remain intact when cells undergo apoptosis (40). The second possibility is that proteolytic cleavage of one critically important substrate leads to cell death. This also is unlikely because a number of proteins, including pro-IL-1β, poly-ADP ribose polymerase (PARP), U1-70 kD ribonuclear protein, and nuclear lamin are cleaved during apoptosis (7, 40–42). It is not clear (with the exception of pro-IL-1β), whether the cleavage products of these proteins mediate downstream events of cell death pathways or whether they are merely the end result of apoptosis. The third possibility is that activation of the ICE family may result in cleavage of several substrates, some being activated (mediating cell death) and others being destroyed (required for cell survival). The results reported in this study lead us to favor the last hypothesis. Our data indicate that endogenously produced mature IL-1β is directly involved in cell death and is the first identified substrate of an apoptosis-inducing gene whose product plays a direct role in mediating the apoptotic cascade.

A number of signal transduction mechanisms mediate the biological effect of IL-1β. Several of these second messengers have been implicated in apoptosis and, after ICE activation, they most likely mediate cell death after endogenous mature IL-1β receptor binding. IL-1β induces ceramide production in EL4 thymoma cells (43). IL-1β also induces apoptosis in pancreatic R1m5F cells via a pathway that is dependent on its ability to induce nitric oxide production (18). Both ceramide and nitric oxide are strong candidates for direct mediators of apoptosis (18, 44). A recent report showed that NGF deprivation of PC12 cells, which induces apoptosis, led to a substantial activation of the JNK and p38 MAP kinases (45). IL-1β has been shown to activate the JNK-p38-signaling pathway, and NGF withdrawal may induce secretion of IL-1β, which then activates the JNK-p38 pathway and cell death (46).

In light of our previous and current reported results, which point to a definite role of ICE in apoptosis, it is intriguing that ICE knockout mice are developmentally normal (15, 16). To date, the only resistance to apoptosis reported in this mouse is in anti-Fas-mediated thymocyte cell death (16). On the surface, this would seem to contradict the notion that ICE itself is important in cell death pathways. However, it is not surprising that knocking out only a single member of the ever-growing number of ICE/ced-3 homologues would not produce a striking apoptotic phenotype, considering the redundancy of such an important and terminal process as cellular suicide. Of note, mice with homozygous deletions of either Ich-1 or Ich-3 genes display grossly normal development (Bergeron, L., M. Masayuki, S. Wang, and J. Yuan, unpublished results).

Cerebral ischemia induced by middle cerebral artery occlusion has been shown to result in increased expression of IL-1β mRNA in the infarct territory (47). Strikingly, treatment with the IL-1Ra decreased the ischemia-induced infarct size by 50% (48). In addition, the brains of patients with Parkinson’s disease, Alzheimer’s disease, and Down’s syndrome have elevated levels of IL-1β (49, 50). These
findings suggest that mature IL-1β is involved in mediating neuronal cell death pathways after ischemia, as well as in neurodegenerative diseases. This might be analogous to the notion that a cell needs to be "primed," creating the appropriate intracellular milieu (in neurons with trophic factor deprivation, in HeLa cells with hypoxia or G1/S phase arrest, and in L929 cells with TNF-α) for mature IL-1β to activate the cell death program. In vivo, the "primed" cell idea may translate to an ill cell that is a burden to the organism, and in an example of cellular altruism, the ICE pathway is activated, leading to the production of mature IL-1β and culminating in cellular suicide. However, uninjured cells may be protected by the IL-1β that is produced by the dying cells, at least in part by downregulating the IL-1 receptor, hence rescuing salvageable cells. Mature IL-1β plays a pivotal role in cellular homeostasis by modulating the apoptotic cascade and activating the immune system, processes that are respectively involved in the execution and elimination of unwanted cells.

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