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Characterization of Mice Deficient in Interleukin-1β Converting Enzyme

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Abstract Interleukin-1β converting enzyme (ICE) processes the inactive proIL-1β to the proinflammatory mature IL-1β. ICE belongs to a family of cysteine proteases that have been implicated in apoptosis. To address the biological functions of ICE, we generated ICE-deficient mice through gene targeting technology. ICE-deficient mice developed normally, appeared healthy, and were fertile. Peritoneal macrophages from ICE-deficient mice underwent apoptosis normally upon ATP treatment. Thymocytes from young ICE-deficient mice also underwent apoptosis when triggered by dexamethasone, gamma irradiation, or aging. ICE-deficient mice had a major defect in the production of mature IL-1β and had impaired IL-1α production on LPS stimulation *in vitro* and *in vivo*. ICE-deficient mice were resistant to LPS-induced endotoxic shock. J. Cell. Biochem. 64:27–32. © 1997 Wiley-Liss, Inc.

Key words: interleukin-1β converting enzyme; gene targeting; apoptosis; IL-1β; IL-1α; inflammation

Interleukin-1β (IL-1β)-converting enzyme (ICE) is the cysteine protease that cleaves biologically inactive proIL-1β to generate the proinflammatory mature IL-1β [Black et al., 1988; Kostura et al., 1989]. ICE cleavage requires an Asp in the P1 position of its substrates [Howard et al., 1991; Sleath et al., 1990]. ICE is synthesized in monocytic cells as an inactive 45 kDa precursor that is proteolytically processed to generate an active enzyme composed of 10 kDa and 20 kDa polypeptides [Miller et al., 1993; Thornberry et al., 1992].

IL-1β is a pluripotent cytokine that is implicated in the pathophysiology of various diseases, including rheumatoid arthritis, septic shock, inflammatory bowel disease, and insulin-dependent diabetes mellitus [Dinarello and Wolff, 1993]. IL-1β is synthesized as a 34 kDa inactive precursor without a conventional signal sequence. It requires cleavage by ICE to generate the 17 kDa bioactive, secreted form of the cytokine [Black et al., 1988; Kostura, et al., 1989]. This requirement has been demonstrated in cell culture and ex vivo, but is not well studied in animals [Cerretti et al., 1992; Thornberry et al., 1992; Young et al., 1988]. A second form of IL-1, IL-1α, binds the same receptors as IL-1β and shares its proinflammatory activities [Dinarello and Wolff, 1993]. ICE had not been known to play a role in regulating the production of IL-1α [Howard et al., 1991].

The finding that ICE is a mammalian homolog of the product of the essential cell death gene, *ced-3*, of C. elegans [Yuan et al., 1993] has led to the identification of a novel ICE/ced-3 cysteine protease family which has been implicated in apoptosis or programmed cell death. Over expression of ICE or its homologs in cells results in apoptosis [Faucheu et al., 1995; Fernandes et al., 1994; Fernandes et al., 1995; Kamens et al., 1995; Kumar et al., 1994; Miura et al., 1993; Tewari et al., 1995; Wang et al., 1994]. The serpin CrmA, an ICE inhibitor encoded by the cowpox virus, and tetrapeptide (Ac-YVAD) ICE inhibitors can protect cells from death in certain systems [Enari et al., 1995; Los et al., 1995; Tewari and Dixit, 1995; Wang et al., 1994].

To address the role of ICE in IL-1 production, in inflammation, as well as in apoptosis, we generated mice that have a null mutation in the ICE gene [Li et al., 1995]. This was achieved through gene targeting in embryonic stem (ES) cells by homologous recombination [Capecchi, 1989].

Generation of ICE-Deficient Mice

The murine ICE gene is a single copy gene with 10 exons (Casano et al., 1994). Exon 6 contains Cys-284 and Gln-282, amino acids in-

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volved in catalysis and P1 substrate specificity, respectively (Walker et al., 1994; Wilson et al., 1994). We generated a replacement-type targeting vector that deleted 31 base pairs from exon 6 and put the rest of the ICE coding sequence out of reading frame. This targeting vector was used to disrupt the murine ICE gene in D3 ES cells through double selection with neomycin and gancyclovir. Of 600 clones screened by Southern analysis, one clone had undergone homologous recombination. Cells of this mutant ES-clone were injected into blastocysts from C57BL/6 mice to generate chimeric mice. Heterozygous mice for the ICE null mutation were generated through mating chimeras with C57BL/6 mice. Heterozygous animals were mated, and approximately 24% (89/369) of the animals were homozygous for the null allele as determined by Southern blot analysis of tail DNA.

ICE-Deficient Mice Had an Apparently Normal Phenotype

Apoptosis, or programmed cell death, is an essential physiological process for normal development and homeostasis [Vaux et al., 1994]. Apoptosis is also used to remove cells that have sustained genetic damage. Gross examination of ICE-deficient mice gave no overt indication of any apoptosis defect. The ICE-deficient mice developed normally, appeared healthy, and were fertile. The oldest mice at this time are 18 months of age and they still remain tumor-free. Histopathological evaluation of all major organs including spleen, lung, heart, kidney, liver, adrenal gland, brain, gastrointestinal tract, pancreas, salivary gland, thymus, and testis from 8-week-old mice showed no abnormalities. The post-lactation involution of mammary glands was also normal in female ICE-deficient mice.

Cellular and Physiologic Apoptosis Processes in ICE-Deficient Mice

To further evaluate the role of ICE in apoptosis, we studied several apoptotic processes in ICE-deficient mice. Since apoptotic pathways are involved in thymic and peripheral deletions of lymphocytes [Nagata and Suda, 1995], we investigated thymocyte and lymphocyte subsets in the ICE-deficient mice. The percentages of B lymphocytes, and $CD4^+CD8^-$, $CD4^-CD8+$, CD4+CD8+, $CD4^-CD8^-$ T lymphocytes in thymuses, spleens, and lymph nodes were comparable in ICE-deficient mice and wild-type control mice as determined by flow cytometry [Li et al. 1995]. The size and the lymphocyte counts of the above mentioned lymphoid organs were also normal. This contrasts with the MRL/lpr mice which develop lymphadenopathy and splenomegaly due to a defect in Fas-mediated apoptosis [Nagata and Suda, 1995]. The ICE-deficient mice also had normal numbers of leukocytes, erythrocytes, and platelets in the peripheral blood suggesting normal myelopoiesis.

In vitro treatment of macrophages with ATP induces apoptosis as well as IL-1β release. We studied the ability of ICE-deficient macrophages to undergo apoptosis in response to ATP. Peritoneal macrophages were treated with 5 mM ATP, and DNA fragmentation was used as a measure of apoptosis. We found that macrophages from ICE-deficient mice undergo apoptosis to a similar extent as cells from wild-type control mice.

Thymocytes undergo apoptosis *in vitro* when triggered by factors such as aging, dexamethasone, γ-irradiation, and anti-Fas antibody. We investigated apoptosis of thymocytes from young ICE-deficient mice in response to these treatments *in vitro* by determining the percent of hypodiploid cells after propidium iodide staining and flow cytometry, and by cell viability. The percent apoptotic cells from ICE-deficient and wild-type control mice was similar after 18 h culture with medium alone (aging), with 1 μM dexamethasone, and with 5 Gy gamma irradiation *in vitro*. However, as reported by Kuida et al. [1995], thymocytes from young ICE-deficient mice were resistant to Fas-induced apoptosis as measured by cell viability.

To study apoptosis in non-lymphoid tissues, we tested ICE-deficient mice in a low-dose LPS plus D-galactosamine induced shock model. In this model, mice die due to TNFα-induced hepatocyte apoptosis [Leist, et al. 1995]. We injected ICE-deficient and wild-type mice with 10 ng/mouse (n = 5 for each genotype) or 100 ng/mouse (n = 5 for each genotype) of LPS together with 20 mg of D-galactosamine/mouse intraperitoneally, and monitored the mice for survival. All ICE-deficient mice as well as wild-type mice receiving 10 ng LPS and 100 ng of LPS died within 32 h (majority within 24 h) and 24 h (majority within 12 h) after the LPS challenge, respectively. There was no significant difference in kinetics of death between the ICE-deficient mice and wild-type mice. This sug-

gested that TNFα-induced hepatocyte apoptosis occurred normally in the ICE-deficient mice.

In Vitro IL-1β and IL-1α Production Defect in Macrophages of ICE-Deficient Mice

To address the role of ICE in IL-1 production *in vitro*, we assessed the processing and release of IL-1β using thioglycollate-elicited macrophages. Cells were stimulated with LPS at 1 μg/ml to induce expression of proIL-1β and then treated with 5 mM ATP. IL-1β and IL-1α levels in the medium were measured by ELISA. It has been reported previously that the ATP treatment is necessary to trigger efficient processing and release of mature IL-1β and IL-1α from mouse macrophages [Hogquist et al., 1991; Perregaux and Gabel, 1994]. We confirmed this observation. In addition, we showed that a selective ICE inhibitor, the tetrapeptide aldehyde Ac-YVAD-CHO, blocked the release of IL-1β in this assay. Leupeptin, the tripeptide aldehyde LLR-CHO, was used as a specificity control and did not inhibit IL-1β release. Therefore the ATP-induced processing and release of mature IL-1β from mouse macrophages occurs via an ICE-dependent mechanism. The stimulated macrophages from ICE-deficient mice released barely detectable amounts of mature IL-1β ($<$20 pg/ml). This is in striking contrast with the levels of 2000–4000 pg/ml released from macrophages of wild-type and heterozygous mice under the same treatment. Interestingly, the release of IL-1α was also markedly reduced from the ICE-deficient macrophages to a level of about 25% of that from wild type and heterozygous cells. Our ELISA results for IL-1 release from peritoneal macrophages are similar to those obtained by Kuida et al. [1995] using splenic monocytes from ICE-deficient mice.

We also analyzed IL-1 release through *in vitro* [^{35}S]methionine labeling and immunoprecipitation. Macrophages were pulsed with [^{35}S]methionine and immunoprecipitations of IL-1α and IL-1β were done on cell lysates and cell culture supernatants. Analysis of the cell lysates showed that LPS induction of the 31 kDa IL-1α and 34 kDa IL-1β precursors was comparable in C57BL/6 and ICE-deficient macrophages. Similar results were also obtained with wild-type and heterozygous macrophages. Immunoprecipitations from the supernatants confirmed the above ELISA results for IL-1α and IL-1β. No 17 kDa mature IL-1β was detected in the medium of ICE-deficient macrophages after the 30-min ATP treatment. A trace amount of 17 kDa mature IL-1β was found in the medium of ICE-deficient cells upon additional 3 h of incubation after removal of ATP. At the 3 h time point, ICE-deficient macrophages released significantly reduced levels of both the 15 kDa mature form and the 31 kDa precursor form of IL-1α compared to wild-type and heterozygote controls.

In Vivo IL-1α and IL-1β Production Defect in ICE-Deficient Mice

To confirm the above *in vitro* observations, we treated mice with LPS and ATP and analyzed peritoneal cytokine levels [Griffiths et al. 1995]. Mice were injected intra peritoneally with LPS (1 μg) followed 2 h later with intraperitoneal ATP (5 μmoles). The peritoneal cavities were then washed after 15 min and cytokine levels in the cell-free supernatants were measured by commercial ELISAs. The IL-1β and IL-1α levels in the peritoneal washes from wild-type mice (n = 4) were 973 $\pm$ 163 pg/ml and 462 $\pm$ 78 pg/ml (mean $\pm$ SEM), respectively. Both cytokines were low or undetectable in peritoneal washes from ICE-deficient mice (n = 5). The TNFα levels were comparable in the ICE-deficient mice (179 $\pm$ 54 pg/ml) and the wild-type mice (188 $\pm$ 42 pg/ml).

We further investigated the role of ICE in IL-1 production using the high-dose LPS-induced shock model. Injection of high doses of LPS intraperitoneally into mice induces the massive systemic release of proinflammatory cytokines such as IL-1α, IL-1β, and TNFα. This leads to septic shock and results in animal death [Dinarello et al., 1993]. We administered a high dose of LPS (800 μg/mouse) to the mice, measured plasma cytokine levels in them after 4 h, and monitored their survival. The ICE-deficient mice had undetectable levels of plasma IL-1β ($<$20 pg/ml) in contrast to high IL-1β levels in wild-type mice. In addition, the plasma IL-1α levels were low or undetectable in ICE-deficient mice. Both wild-type and ICE-deficient mice had high plasma levels of TNFα ($>$550 pg/ml). All wild-type mice succumbed within 30 h after the high-dose LPS injection. However, ICE-deficient mice were highly resistant to the LPS-induced endotoxic shock. Though showing signs of endotoxemia such as raised fur and lethargy, all ICE-deficient mice survived beyond 48 h. Seventy percent of these mice were alive at 7 days.

Biological Function of ICE

We have generated ICE-deficient mice to address the physiological functions of ICE. The ICE-deficient mice are overtly normal. They have no apparent anatomical or developmental abnormalities judged by gross examination of neonates and adults, or by histological analysis of all major organs. Hematopoietic and lymphoid cells appeared normal in number and distribution in the peripheral blood and lymphoid organs. Our oldest mice are 18 months old and we have observed no spontaneous tumors. Peritoneal macrophages from ICE-deficient mice underwent apoptosis normally upon *in vitro* ATP treatment. Thymocyte apoptosis pathways responsive to *in vitro* stimuli such as aging, dexamethasone, and gamma irradiation also appeared normal. Although thymocytes from young ICE-deficient mice showed a defect in Fas-mediated apoptosis measured by cell viability *in vitro*, the physiological significance of this observation is unclear. Analysis of these ICE-deficient mice revealed no other phenotype resembling the MRL/lpr mice that are defective in Fas-mediated apoptosis. When challenged with LPS and D-galactosamine, ICE-deficient mice were as susceptible as wild-type mice to lethality from hepatic failure secondary to TNFα-induced hepatocyte apoptosis. Our study of ICE-deficient mice suggests that ICE is not critically involved in apoptosis during development. In the adult animal, however, ICE may be required for specific pathways of apoptosis such as in Fas-mediated cytotoxicity in young thymocytes where the physiological relevance is unclear. Recent studies using specific tetrapeptide (Ac-YVAD) ICE inhibitors and the CrmA protein, a natural ICE inhibitor encoded by cowpox virus, suggest that ICE or ICE-like proteases are centrally involved in apoptosis [Enari et al., 1995; Los et al., 1995; Tewari and Dixit, 1995; Wang et al., 1994]. Our studies with ICE-deficient mice suggest that one or more of the other members of the ICE/ced-3 family rather than ICE itself may be the death protease(s) important for physiological apoptosis. Another possibility is that there are several proteases that may function redundantly in apoptosis. In this case, one would predict that mice deficient in only one of the proteases will still be capable of normal apoptosis. Only transgenic mice deficient in two or more of these proteases may reveal significant defects in apoptosis.

The ICE-deficient mice have a profound defect in IL-1 production. Studies using macrophages from ICE-deficient mice revealed an extreme but not absolute defect in the production of mature IL-1β. In addition, there is an unanticipated major reduction in release and extracellular accumulation of IL-1α. The small amount of mature IL-1β (less than 1% of control levels) released by the macrophages from mutant mice was of the correct molecular weight, suggesting that specific cleavage of pro-IL-1β may occur in the mutant cells, although at a very low level. In experiments using *in vitro* translated pro-IL-1β, we have shown that another member of the ICE protease family, ICH-2, generated mature IL-1β inefficiently [Kamens et al., 1995]. The defect in IL-1α production was unexpected, because earlier studies suggested that ICE does not cleave pro-IL-1α [Howard, et al., 1991]. In addition, we did not observe any significant inhibition of IL-1α release from peritoneal macrophages treated with the ICE inhibitor Ac-YVAD-CHO *in vitro*. Processing of pro-IL-1α is thought to be mediated by calpain [Carruth et al., 1991; Kobayashi et al., 1990]. There are several possible explanations for the reduced IL-1α release in ICE-deficient mice. Analysis of IL-1β-deficient mice has shown that production of IL-1α is normal in these animals [Zheng et al., 1995]. Therefore the lack of mature IL-1β in the ICE-deficient mice probably does not alter their IL-1α production. Notwithstanding our inability to block IL-1α release in experiments using Ac-YVAD-CHO, we favor the idea that ICE is involved directly in the processing and/or release of IL-1α. Experiments to investigate this possibility are in progress. Our *in vivo* studies using either low-dose LPS plus ATP or high-dose LPS confirmed the IL-1 production defect in ICE-deficient mice observed in *in vitro* experiments. Our results suggest that ICE is required for the dominant pathway of IL-1β production and also plays a role in IL-1α production in response to stimuli such as LPS.

The most striking result from our study of ICE-deficient mice is that they are highly resistant to the lethal effects of endotoxin. With high-dose LPS that killed all wild-type mice within 30 h, all ICE-deficient mice survived the first 48 h and 70% of them survived after 7

days. The survival of ICE-deficient mice after a high dose of LPS is similar to the protection afforded by the IL-1 receptor antagonist (IL-1ra) [Alexander et al., 1991]. The ICE-deficient mice are thus different from the IFNγ receptor-deficient and p55 TNF receptor-deficient mice, which are susceptible to high dose LPS-induced lethality [Car et al., 1994; Rothe et al., 1993]. However, since IL-1β knockout mice succumb to high-dose LPS-induced endotoxic shock [Shornick et al., 1996], the resistance of ICE-deficient mice to this model of endotoxic shock is probably due to the inhibition of LPS-induced IL-1α as well in the latter mice. Our studies suggest the therapeutic potential of ICE inhibitors in inflammatory diseases such as septic shock and inflammatory bowel diseases.

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