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Requirement of an ICE/CED-3 protease for Fas/APO-1-mediated apoptosis

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THE Fas/APO-1 receptor is one of the major regulators of apoptosis[1–7]. We report here that Fas/APO-1-mediated apoptosis requires the activation of a new class of cysteine proteases, including interleukin-1β-converting enzyme (ICE)[8–10], which are homologous to the product of the *Caenorhabditis elegans* cell-death gene *ced*-3 (refs 11, 12). Triggering of Fas/APO-1 rapidly stimulated the proteolytic activity of ICE. Overexpression of ICE, achieved by electroporation and microinjection, strongly potentiated Fas/APO-1-mediated cell death. In addition, inhibition of ICE activity by protease inhibitors, as well as by transient expression of the pox virus-derived serpin inhibitor CrmA or an antisense ICE construct, substantially suppressed Fas/APO-1-triggered cell death. We conclude that activation of ICE or an ICE-related protease is a critical event in Fas/APO-1-mediated cell death.

The signal transduction pathway elicited by Fas/APO-1 is almost completely unknown. Initiation of apoptosis may involve a new class of cysteine proteases, including the product of the *C. elegans* cell-death gene *ced-3*, mammalian interleukin-1β-converting enzyme (ICE) and the related proteases Nedd-2/Ich-1, prICE and CPP-32 (refs 11–17). Overexpression of CED-3, ICE or Nedd-2/Ich-1 in Rat-1 fibroblasts has been shown to result in apoptotic cell death[12,15]. We therefore investigated whether Fas/APO-1-mediated apoptosis involved an ICE-related proteolytic activity. In L929-APO-1 cells[18] or B-lymphoblastoid SKW 6.4 cells, apoptosis triggered by the agonistic monoclonal antibody anti-APO-1 was strongly inhibited by the ICE inhibitor YVAD-CHO, a tetrapeptide aldehyde ($K_i = 0.76$ nM)[8] (Fig. 1a). Inhibition was also observed with the protease inhibitor dichloroisocoumarin, but other serine protease inhibitors, such as

PMSF and leupeptin, calpain inhibitors and the cysteine protease inhibitor E-64 were not effective (data not shown). In addition, ICE-like proteolytic activity was readily induced by Fas/APO-1 ligation (Fig. 1b). The fluorogenic ICE substrate DABCYL-YVADAP-EDANS, which contains the cleavage site of the interleukin-1β precursor[19], was cleaved after treatment of permeabilized cells with anti-APO-1, but no effects were detected using classical cysteine or serine protease-specific substrates (Fig. 1b, c).

To explore further the participation of ICE in Fas/APO-1-mediated apoptosis, ICE was overexpressed using several techniques. First, murine ICE complementary DNA was microinjected into nuclei of L929-APO-1 cells. After treatment with anti-APO-1, apoptotic cells could be recognized as round-shaped cells revealing membrane blebbing and cytoplasmic condensation. When cells microinjected with ICE cDNA were treated with a suboptimal dose of anti-APO-1, a nearly threefold increase in the number of apoptotic cells was detected compared with cells microinjected with the empty vector alone (Fig. 2a). In contrast, microinjection of vaccinia virus-derived *crmA* cDNA, the product of which inhibits ICE activity by forming a serpin-like pseudosubstrate[20–22], significantly suppressed anti-APO-1-induced cell death. These observations suggested the involvement of ICE or an ICE-related protease in the Fas/APO-1 signalling pathway. Although ICE is the only protease known to be inhibited by CrmA, it is possible that a related protease with similar substrate specificity was inhibited by CrmA. To investigate more specifically the role of ICE, we further included an antisense ICE construct. ICE, *crmA* and antisense ICE cDNAs were overexpressed by electroporation, which resulted in transfection efficiencies of more than 80% as assessed by reporter gene plasmids. Figure 2b shows that apoptosis was increased by transient expression of ICE after anti-APO-1 treatment, whereas apoptosis induced in their *crmA*- or antisense-ICE-transfected counterparts was reduced. The effects on apoptosis were further evaluated in L929-APO-1 cells after cotransfection with the *lacZ* gene as a marker of gene expression (Fig. 3). In comparison with cells transfected with the vector control, the percentage of round apoptotic cells out of the total number of blue-stained cells was substantially increased in ICE cDNA-transfected cells after anti-APO-1 treatment. As in the previous experiments, no significant difference in cell viability of L929-APO-1 cells was observed without Fas/APO-1 activation. This is in apparent contrast to other cell types undergoing apoptosis by overexpression of ICE alone[12,15]. In line with the previous data, transient overexpression of CrmA or antisense-ICE resulted in an inhibition of anti-APO-1-induced apoptosis of ∼50% (Fig. 3g).

These data indicate that ICE plays a role in the induction of apoptosis mediated by Fas/APO-1. Although it cannot be excluded that other ICE-related proteases may also be involved, the antisense experiments suggest that ICE is important. Because ICE is structurally and functionally related to the nematode

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FIG. 1 Involvement of an ICE-related protease activity in Fas/APO-1-induced apoptosis. a, Dose-dependent inhibition of Fas/APO-1-triggered apoptosis by the tetrapeptide ICE inhibitor YVAD-CHO L929-APO-1 (open circles) and SKW 6.4 cells (filled circles) were cultured as described[23,24], made permeable by a short hypotonic shock and then incubated with the indicated concentrations of the inhibitor for 30 min. Cells were treated in medium containing actinomycin D with 1 µg ml⁻¹ anti-APO-1 for an additional 4 hours (L929-APO-1 cells, 400 ng ml⁻¹ anti-APO-1; SKW 6.4 cells, 50 ng ml⁻¹ anti-APO-1). Apoptosis was assessed by propidium iodide uptake (2.5 µg ml⁻¹) and fluorescence-activated cell sorting (FACS) analysis. Data of specific cell death (cell death in the presence of anti-APO-1 minus cell death in the absence of anti-APO-1) were obtained from triplicate experiments. Spontaneous cell death in the absence of anti-APO-1 was less than 10%. Similar results were obtained by measuring DNA fragmentation with the dye Hoechst 33342 (Molecular Probes, Eugene, OR)[25]. b, Stimulation of ICE-like proteolytic activity by anti-APO-1. L929-APO-1 and SKW 6.4 cells were cultured at 7×10^4 in 35-mm plates in medium containing actinomycin D and either left untreated or treated with anti-APO-1 (1 µg ml⁻¹) for the indicated time points. Cells were made permeable 10 min before collection by using 0.05% digitonin and incubated with 20 µM of the fluorogenic ICE substrate DABC-YL-YVADAP-EDANS[19] (Bachem, Bubendorf, Switzerland). Cells were collected with a rubber policeman and analysed by FACS analysis using an excitation wavelength of 360 nm and emission wavelength of
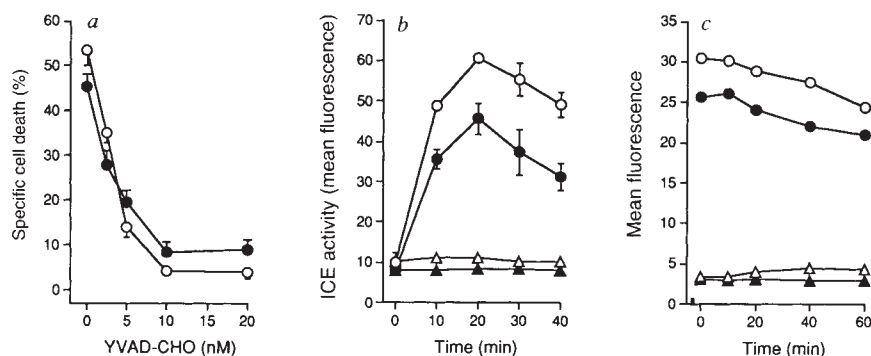
488 nm. Open circles and triangles represent L929-APO-1 cells plus/minus anti-APO-1, respectively; filled circles and triangles represent SKW 6.4 cells plus/minus anti-APO-1, respectively. c, Effect of anti-APO-1 on other protease activities: L929-APO-1 and SKW 6.4 cells were treated as described in a and incubated with the serine (trypsin-like) protease substrate Bz-Val-Gly-Arg-AMC (open triangles, L929-APO-1 cells; filled triangles, SKW 6.4 cells) or the cysteine protease-specific substrate (CBZ-Phe-Arg)₂-R110 (open circles, L929-APO-1 cells; filled circles, SKW 6.4 cells). Cleavage of the substrates was measured by FACS analysis using excitation and emission wavelengths of 366 nm and 460 nm, respectively, for Bz-Val-Gly-Arg-AMC (30 µM; Bachem) and 488 nm and 530 nm for (CBZ-Phe-Arg)₂-R110 (30 µM; Molecular Probes).

FIG. 2 Effect of transient expression of ICE, crmA and antisense ICE cDNA on Fas/APO-1-mediated apoptosis by a, microinjection and b, electroporation.
METHODS. Microinjection: 8×10^5 L929-APO-1 cells were seeded in 60-mm tissue dishes. After overnight incubation, cell nuclei were microinjected with expression plasmids encoding murine ICE or vaccinia virus-derived CrmA. Cell injections were performed with an automatic microinjection system (Zeiss ALS) equipped with glass micropipettes which had been loaded with 1 µl of the appropriate DNA diluted to about 0.25 µg ml⁻¹ with 2.5% FITC-labelled dextran. After 20 hours of further incubation, cells were either left untreated or incubated with 0.5 µg ml⁻¹ anti-APO-1 medium containing actinomycin D. After 45 min, cells were fixed with 2.5% glutaraldehyde and inspected microscopically. Cells were regarded as apoptotic when they revealed membrane blebbing and/or a condensed cell nucleus. At least 180 cells were analysed for each condition in three independent experiments. A murine ICE cDNA was isolated by polymerase chain reaction after reverse transcription of RNA using EL4/c mRNA and oligo(dT) primers for the first-strand synthesis. A 1,322-base-pair (bp) PCR product was isolated using the primers ATCGGATCCAGCATGGCTGACAAGATCCTGAGG (plus strand) and CGGCCTCGAGCATCATCTAAGGAAGTATTGGC (minus strand) and used as a probe to screen a EL4/13 cDNA expression library cloned into pCAGGS. The pCAGGS vector was provided by J. Miyazaki and contained a CMV/β-actin promoter[26]. A 1,387-bp full-length ICE cDNA clone was isolated and its sequence confirmed by double-stranded DNA sequencing. The biological activity of the ICE cDNA product was checked after cotransfection of a pro-interleukin-1β expression plasmid (gift from J. F. DeLamarter) in COS cells. The crmA cDNA was obtained from vaccinia virus DNA after standard PCR with the primers 5'-GCGAAGCTTACACG-ACCAATATCGATTACTA-3' and 5'-CGCCATGGTTAACAATTAGTTGTCGGAG-AG-3'. The PCR product was cloned as a HindIII/KpnI fragment in pSV25S, an expression plasmid containing the simian virus 40 (SV40) early promoter. All plasmids were purified by caesium chloride density gradient centrifugation. For electroporation, L929-APO-1 cells were washed in Tris-buffered saline (TBS), resuspended at 1×10^8 cells per 0.4 ml TBS and equilibrated and transfected with 20 µg of expression plasmids using a Bio Rad electroporator (960 µFd, 230 V). After electroporation, cells were seeded at 1×10^6 cells per well in 6-well plates. Dead cells were removed after 16 h by a washing step in culture medium. Cells were treated for the indicated times with anti-APO-1 (1 µg ml⁻¹) in medium containing actinomycin D. Apoptosis was measured by
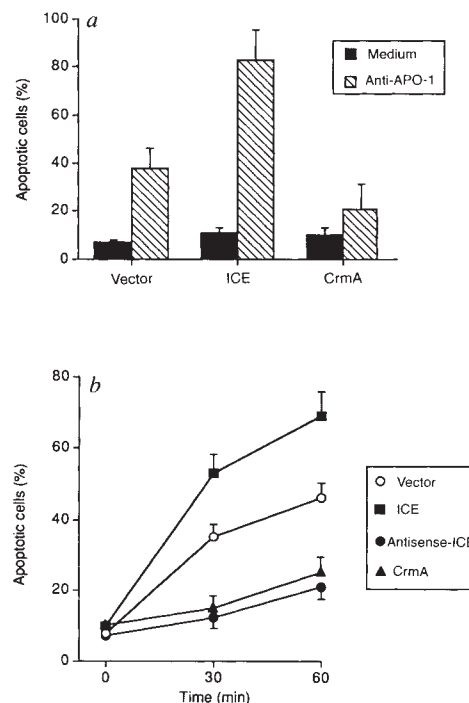
FACS analysis using Hoechst 33342 (ref. 25). Data are given as mean percentage cell death from four experiments with duplicate samples. An antisense-ICE construct was obtained after cloning a 320-bp EcoRI fragment of ICE cDNA containing 48 bp of the 5'UTR and the first 255 bp of the ICE open reading frame in reverse direction into the pCAGGS vector.
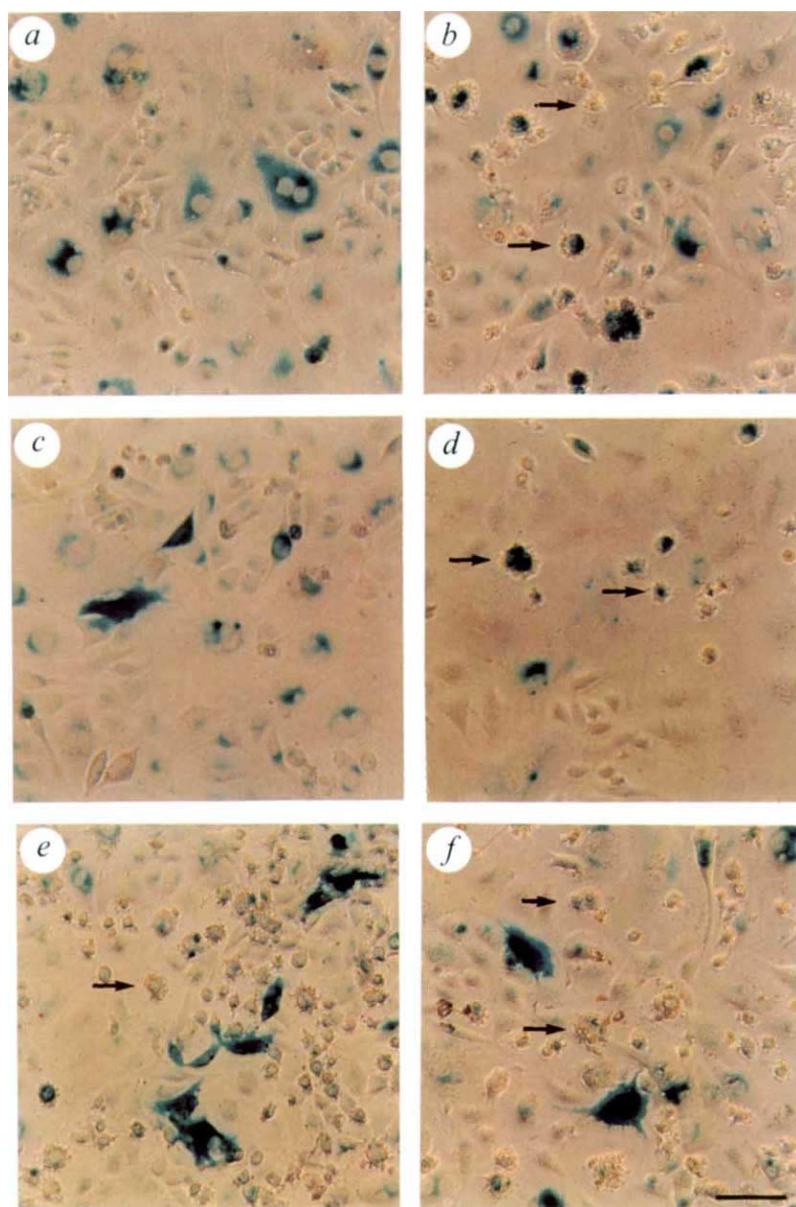
FIG. 3 X-Gal staining of L929-APO-1 cells expressing ICE, antisense ICE, *crmA* cDNA or empty vector after cotransfection with *lacZ*. L929-APO-1 cells were cotransfected with an expression vector encoding *lacZ*, together with a vector control (*a*, *b*), ICE (*c*, *d*), antisense ICE (*e*) or *crmA* cDNA (*f*). Cells were either left untreated in medium containing actinomycin D (*a*, *c*) or treated with anti-APO-1 (*b*, *d–f*) 24 h after transfection and then stained for 12 h with X-Gal solution. The scale bar represents 20 μm. Examples of apoptotic cells are indicated by arrows. *g*, Data of transfections obtained from two experiments. The values give the percentage of blue apoptotic cells out of the total number of blue cells. At least 450 transfected cells were counted for each condition. Faintly stained cells were not included.

METHODS. The day before transfection, cells were seeded in 35-mm dishes at 7×10^4 cells and 0.5 ml culture medium. For each well, 400 ng of the *lacZ* eukaryotic expression construct CMV-β-gal (Clontech) and 1,200 ng of the ICE, antisense-ICE or *crmA* expression plasmid were used. Transfections were performed by liposome-mediated gene transfer using the DOTAP reagent for 24 h according to the instructions of the manufacturer (Boehringer Mannheim). After transfection, cells were washed in culture medium and treated with anti-APO-1 (1 μg ml^{-1}) for 45 min. To detect gene expression, cells were fixed with 1% glutaraldehyde for 5 min, rinsed once with PBS and stained for 10–12 h in X-Gal buffer containing 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM MgCl$_2$ and 1 mg ml^{-1} 5-bromo-4-chloro-3-indoxyl-β-galactoside.
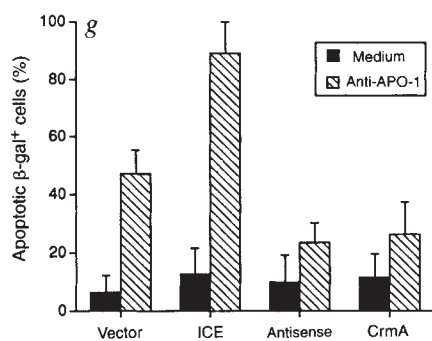
gene *ced-3*, our data imply that Fas/APO-1-mediated apoptosis follows a highly conserved signalling pathway. □

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