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Suppression of Interleukin-1β-converting Enzyme-mediated Cell Death by Insulin-like Growth Factor*

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COS cells are resistant to cell death induced either by interleukin-1β-converting enzyme (*ICE) and ICE homolog (ICH-1_L) overexpression or by serum deprivation. COS cells deprived of serum undergo apoptosis after transfection with an ICE expression construct, but not an ICH-1_L construct. ICE-mediated apoptosis of COS cells in serum-free medium is suppressed by insulin-like growth factor (IGF)-1 and insulin. Viability of Rat-1 cell line (Rat-1/ICE) expressing low levels of ICE-LacZ fusion protein is lower than those of cell lines expressing either both Bcl-2 and ICE or mutant $ICE^{Gly \rightarrow Ser}$ during serum deprivation. Enzymatic activation and processing of ICE are observed in cells induced to die by serum deprivation, which are suppressed by IGF-1. IGF-1 or insulin suppresses ICE-mediated cell death without affecting the expression levels of Bcl-2, Bcl-x, or Bax. Taken together, these results indicate that ICE is activated by growth factor deprivation, and IGF-1 is able to suppress ICE-mediated cell death through a mechanism independent of the expression of Bcl-2, Bcl-x, or Bax.

Members of the mammalian ICE family are homologs of *Caenorhabditis elegans* programmed cell death gene product Ced-3 (1–8). Overexpression of *Ice* or *Ich-*1_L induces apoptosis of cultured cells in cell type-specific manner (2, 5). ICE is a novel cysteine protease which is inhibited by the product of *crmA* gene, a serpin encoded by cowpox virus (9–11). CrmA prevents or delays apoptosis induced by various stimuli such as tumor necrosis factor-α (TNF-α)[1] (7, 12), anti-FAS antibody (7, 13–15), and neurotrophic factor deprivation (16). Moreover, peptide inhibitors of the ICE protease family suppress death of motoneurons during development and in culture (17). Together, these observations suggest that the ICE family plays an important role in apoptosis.

Recent work has shown a correlation between the regulation of *Ice* expression or ICE processing and apoptosis. Increased *Ice* expression correlates with the onset of apoptosis induced by the loss of extracellular matrix in epithelial cells (18). DNA damage-induced interferon regulatory factor (IRF)-1 dependent T lymphocyte apoptosis is preceded by increase in *Ice* transcription, and it appears that impairment of *Ice* expression in IRF-1 (−/−) splenocytes may contribute to the suppression of DNA damage-induced apoptosis (19). Furthermore, evidence of enzymatic activation of ICE has been observed in cells induced to die by treatment of TNF-α in the presence of cyclohexamide (12). HeLa cells treated with both TNF-α and cycloheximide undergo apoptosis and secret mature interleukin-1β (IL-1β), which is mainly processed by ICE (12). These observations strongly suggest that ICE plays an important role in controlling mammalian apoptosis, and furthermore, ICE may function at a convergent step in apoptosis induced by various stimuli.

Recently, insulin-like growth factor (IGF-1) and its receptor have been implicated in playing an important role in tumorigenesis (20–22). Inhibition of tumorigenesis was observed in nude mice injected with human FO-1 melanoma cells in which the number of IGF-1 receptors was markedly reduced by antisense. Some viral oncogenes may be capable of activating IGF-1 gene expression to promote transformation. Simian virus 40 large T (SV40 T) antigen activates the IGF-1 promoter and thereby increases the secretion of IGF-1 (23). This observation may explain the lack of transformation by SV40 T antigen in IGF-1 receptor-deficient mice (24). Expression of c-Myb also increases the secretion of IGF-1 (25). In contrast, the Wilms' tumor gene, a tumor suppressor, has been reported to down-regulate the expression of the IGF-1 receptor and IGF-2 (26). These observations suggest that IGF-1 and IGF-1 receptors are important and may be essential components for cell transformation. Since increasing evidence suggests that suppression of apoptosis is a critical step in tumorigenesis, it is possible that IGF-1 could promote tumorigenesis by inhibiting apoptosis.

Because the ICE family is critical for apoptosis and the inhibition of apoptosis by growth factors, such as IGF-1, may be an important step in tumorigenesis, we investigated the potential interactions between growth factors and the ICE family. Here we show the regulation of ICE-mediated apoptosis by growth factors, which is different from the inability of ICH-1 to induce apoptosis of serum-deprived COS cells. ICE was activated in Rat-1 and COS-1 cells induced to die by serum deprivation, as evidenced by the processing of ICE protein. IGF-1 and insulin, but not EGF and FGF, suppressed ICE processing and ICE-mediated cell death.

EXPERIMENTAL PROCEDURES

Plasmid Construction—pβactM10T was constructed by inserting a 169-base pair *Bgl*II DNA fragment encoding T7 epitope tag into the *Bam*HI site of pβactM10, in which the *lacZ* gene was deleted from pβactM10Z (2). The T7 epitope tag fragment was obtained by polymerase chain reaction using pET-21a(+) (Novagen, Madison, WI) as template and $T7_1$ and $T7_2$ as primers. The primer sequences are as follows: $T7_1$, GAAGATCTGGCTAGCATGACTGGTGGACA; $T7_2$, GAAGATCT-

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[1] The abbreviations used are: TNF, tumor necrosis factor; ICE, interleukin-1β converting enzyme; ICH-1_L, ICE homolog; IGF-1, insulin-like growth factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; bFGF, basic fibroblast growth factor; LacZ, β-galactosidase; IRF, interferon regulatory factor; IL, interleukin; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

GCTAGTTATTGCTCAGCGGTG. The plasmid expressing human pro-interleukin 1-β (pRc/CMV-hIL-1β) was constructed by inserting a human pro-IL-1β cDNA into *Hin*dIII/*Not*I sites of pRc/CMV (Invitrogen, San Diego, CA). Construction of pJ485, pactβgal, and pβactH37Z plasmids was described previously (2, 5).

Cell Lines and Cell Culture—Rat-1 and COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc.) with 10% fetal calf serum (FCS). Stable Rat-1 cell lines that overexpress *crmA* (Rat-1/CrmA) and *bcl-2* (Rat-1/Bcl-2) were described previously (2). Stable Rat-1 cell lines expressing either mouse *Ice* (Rat-1/ICE) or mutant *Ice* containing a glycine to serine mutation at the active site of ICE (Rat-1/mICE$^{Gly\rightarrow Ser}$) were established by transfecting the cells with either pβactM10Z or pJ485. The stable Rat-1/Bcl-2 cell line expressing mouse *Ice* (Rat-1/Bcl-2/ICE) was established by transfecting Rat-1/Bcl-2 cells with pβactM10Z. Stable clones were selected by using 600 μg/ml of G418. G418-resistant single colonies were cloned and examined for the expression levels of ICE using Western blot analysis.

Serum Deprivation-induced Cell Death and β-Galactosidase Assays—Rat-1 and COS-1 cells were subcultured one day before serum deprivation, washed three times with DMEM, and incubated with a serum-free defined medium containing DMEM and 0.1% low mitogenic serum (Sigma: CPSR-2). COS-1 cells were transfected with either pβactM10Z or pβactH37Z using lipofectamine according to a protocol from Life Technologies, Inc. and maintained in DMEM supplemented with 10% FCS for 4–6 h, after which they were washed three times with DMEM. Growth factors (IGF-1 and PDGF: R & D (Minneapolis, MN), insulin, EGF, and FGF; Sigma) are added to the defined medium, and the viable cells were counted using 0.4% trypan blue staining.

To detect the expression of chimeric *lacZ* genes in transfected cells, cells were fixed with 1% glutaraldehyde for 10 min, rinsed three times with phosphate-buffered saline, and stained in X-gal buffer (0.5 mg/ml 5-bromo-4-chloro-3-indolyl β-galactoside, 3 mM $K_3Fe(CN)_6$-^3H_2O, 3 mM $K_4Fe(CN)_6$-^3H_2O, 1 mM $MgCl_2$, 10 mM KCl, 0.1% Triton X-100 in 0.1 M sodium phosphate buffer (pH 7.5)) at 37 °C for 3 h.

Antibodies—Rabbit anti-ICE antibody was generated by using peptide corresponding to RGEKQGVVLLKD of the mouse ICE p20 subunit by Research Genetics (Huntsville, AL) and purified by the peptide affinity chromatography. T7 tag antibody was obtained from Novagen (Madison, WI) and human IL-1β antibody was from Calbiochem. β-Galactosidase and Bcl-2 antibodies were from Cappel (Durham, NC) and DAKO (Carpinteria, CA), respectively. Bax and Bcl-X antibodies are from Santa Crutz Biotech (Santa Crutz, CA) and C. B. Thompson.

Western Blot Analysis—Cells were collected either by scraping or by trypsinization and directly dissolved in protein lysis buffer containing 60 mM Tris-Cl (pH 6.8), 1% sodium dodecyl sulfate (SDS), 10% glycerol, and 0.5% β-mercaptoethanol. Proteins were separated on SDS-polyacrylamide gels by electrophoresis and transferred to Immobilon-P transfer membranes (Millipore) using multiphor II transfer apparatus (Pharmacia Biotech Inc.) for 3 h. The membranes were incubated overnight at 4 °C with TBST blocking buffer (20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 0.2% Tween-20) containing 4% nonfat dried milk and then incubated with primary antibodies (200–1000-fold dilution) in blocking buffer for 2 h. After membrane were washed three times with TBST, they were then incubated with horseradish peroxidase-linked goat anti-mouse immunoglobulin (1:1000 dilution, Southern Biotechnology, Birmingham, AL) or horseradish peroxidase-donkey anti-rabbit immunoglobulin (1:1000 dilution, Amersham Corp.) for ECL. After washing with TBST, proteins were detected using ECL Western blotting detection reagent (Amersham). For detection of β-galactosidase, the membrane treated with anti-β-galactosidase antibody was incubated with alkaline phosphatase-linked anti-rabbit immunoglobulin (1:1000 dilution, Life Technologies, Inc.) and stained with 0.1 M Tris-Cl (pH 9.5) containing 330 μg/ml nitro blue tetrazolium and 185 μg/ml 5-bromo-4-chloro-3-indolyl phosphate, 10 mM $MgCl_2$, and 0.15 M NaCl.

RESULTS

In the absence of growth factors, cells undergo apoptosis (5, 16). To investigate the effects of individual growth factors on cell death, Rat-1 cells were induced to die by serum deprivation and the effect of growth factors on suppression of serum deprivation-induced cell death was examined (Fig. 1). 50% of Rat-1 cells underwent apoptosis after 2 days of incubation in serum-free defined medium (Fig. 1, *A* and *B*, *Control*). To dissect out the effects of individual growth factors on the suppression of apoptosis, growth factors were added individually to the serum-free defined medium. Addition of IGF-1 (100 ng/ml) or

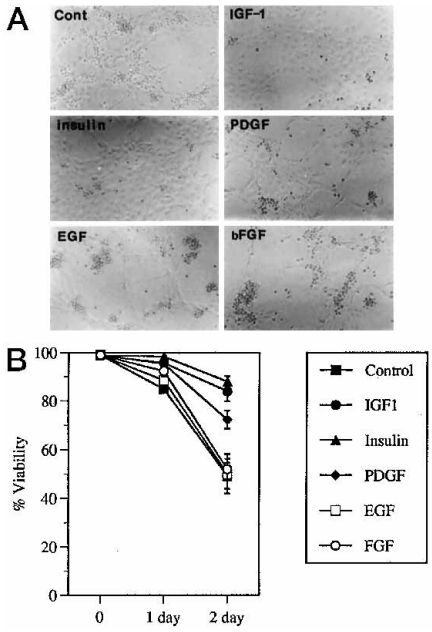

FIG. 1. **Prevention of serum deprivation induced cell death by IGF-1 and insulin.** *A*, Rat-1 cells were incubated in serum-free defined medium (*Control*) or defined medium supplemented with growth factors (either IGF-1: 100 ng/ml; insulin: 5 μg/ml; PDGF: 10 ng/ml; EGF: 10 ng/ml; or bFGF: 100 ng/ml) for 2 days and photographed using a light microscope. *B*, the viability of the same cells shown in *A* was determined by staining with 0.4% trypan blue. Each point indicates mean ± S.E. from at least three different experiments.

insulin (5 μg/ml) to the serum-free defined medium increased cell viability to 85 and 89%, respectively. While PDGF (10 ng/ml) showed weaker effects (70% viability), EGF (10 ng/ml) and bFGF (100 ng/ml) had no effects on cell survival. These results suggest that IGF-1 and insulin suppress Rat-1 cell death induced by serum deprivation.

To examine whether ICE is activated when cells are induced to die by serum deprivation and whether ICE-induced cell death can be suppressed by growth factors, we established an cell culture assay system. In contrast to Rat-1 cells, COS cells are resistant to cell death induced by *Ice* and *Ich-1*$_L$ overexpression in the presence of 10% serum (5) and can survive at least 5 days in the absence of serum. However, serum deprivation after transfection with an *Ice* construct (pβactM10Z) expressing murine ICE-LacZ fusion protein induces COS cell death (Fig. 2*A*), indicating that serum in cell culture medium suppresses ICE-induced cell death in COS cells. Serum deprivation does not enhance the ability of ICH-1$_L$ to induce COS cell death, showing different sensitivities of ICE and ICH-1$_L$ to the effects of growth factors (Fig. 2*A*). To further characterize the effect of growth factors on the suppression of ICE-induced COS cell death, COS cells were transfected with pβactM10Z and maintained in serum-free defined medium supplemented with growth factors for 2 days (Fig. 2*B*). Addition of IGF-1 (100 ng/ml) or insulin (5 μg/ml), but not EGF (10 ng/ml), to the defined medium suppressed ICE-induced COS cell death. PDGF (10 ng/ml) and FGF (100 ng/ml) showed weaker effects on ICE-induced cell death. These results suggest that ICE is activated by serum deprivation, and either IGF-1 or insulin is able to suppress ICE-induced cell death.

To further characterize the effects of IGF-1 and insulin on ICE-induced cell death, Rat-1 cell lines expressing either low levels of ICE-LacZ (Rat-1/ICE), both Bcl-2 and ICE-LacZ (Rat-1/Bcl-2/ICE), or mutant ICE-LacZ (Rat-1/mICE$^{G\rightarrow S}$), in which the active site pentapeptide QACRG had been changed into QACRS, were established. In the presence of 10% serum in the

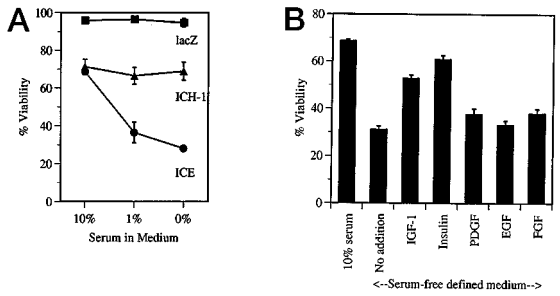

FIG. 2. **Different sensitivities of ICE and ICH-1 to growth factors.** *A*, COS-1 cells were transiently transfected with either pactβgal (*lacZ* alone), pβactM10Z (*mIce-lacZ*), or pβactH37Z (*Ich-1*$_L$*-lacZ*) plasmids and incubated with DMEM containing 10, 1, or 0% FCS. After 2 days, cells were incubated in X-gal buffer as described under "Experimental Procedures" and examined for apoptosis based on the morphology of the blue cells. *B*, prevention of ICE-induced cell death by IGF-1 and insulin. COS-1 cells are transiently transfected with pβactM10Z (*mIce-lacZ*) plasmid and maintained in serum-free medium supplemented with growth factors for 2 days. Cells were fixed and stained with X-gal buffer and examined for apoptosis. The concentrations of growth factors are same as those in Fig. 1. Data are from three independent experiments with mean ± S.E.

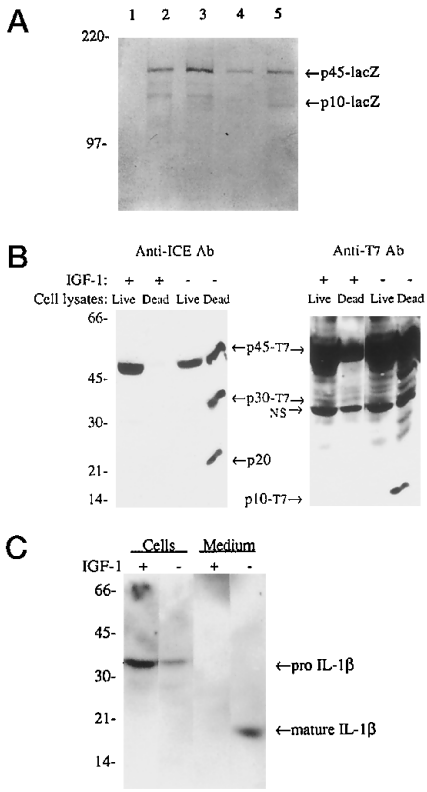

FIG. 3. **Western blot analysis showing processing and activity of ICE-fusion protein in dying Rat-1/ICE and COS cells.** *A*, processing of ICE-LacZ fusion protein in Rat-1/ICE cells. Cell lysates were prepared from live cells attached to tissue culture plate (*lanes 1–4*) and dead cells detached into cell culture medium (*lane 5*) as described under "Experimental Procedures" and electrophoresed on 8% SDS-polyacrylamide gel. Anti-β-galactosidase antibody was used to detect the ICE-LacZ fusion protein. The molecular sizes in kilodaltons are indicated at the *left*. The different sizes of ICE-LacZ fusion proteins, p45-LacZ and p10-LacZ, in live and in dead Rat-1/ICE cells, respectively, are indicated by the *arrowheads* to the *right*. *Lane 1*, Rat-1; *lane 2*, Rat-1/ICE; *lane 3*, Rat-1/mICE$^{Gly\rightarrow Ser}$; *lane 4*, Rat-1/Bcl-2/ICE; *lane 5*, Rat-1/ICE (*Dead*). *B*, processing of ICE in dying COS cells. COS cells are transiently transfected with pβactM10T and incubated in serum-free defined medium without or with IGF-1 (100 ng/ml) for 2 days. Live cells (*Live*) attached to and dead cells (*Dead*) detached from the cell culture plate were separately collected and analyzed on 12% SDS-polyacrylamide gel. Western blot analysis was performed using anti-ICE antibody (*left panel*) or anti-T7 tag antibody (*right panel*). p45-T7, p30-T7, p20, and p10-T7 indicated by *arrows* are ICE detected by anti-T7 tag and/or ICE antibodies. *NS* indicates nonspecific protein recognized by anti-T7 tag antibody. *C*, suppression of ICE activity by IGF-1. COS cells were co-transfected by pβactM10T and pRc/CMV-hIL-1β (2:1 ratio) plasmids and maintained in the defined medium without or with IGF-1 (100 ng/ml) for 2 days. The hIL-1β was detected by Western blot analysis (13.5% SDS-polyacrylamide gel) using anti hIL-1β antibody from the cell lysate or from the defined medium which has been concentrated after dialysis in distilled water.

cell culture medium, cells transfected with either pβactM10Z (*ICE-lacZ*) or pJ485 (mutant *ICE*$^{Gly\rightarrow Ser}$*-lacZ*) were selected with G418 and individual clones were examined for the expression levels of ICE-LacZ fusion proteins by Western blot analysis using anti-β-galactosidase antibody (Fig. 3*A*). Expression level of ICE-LacZ fusion protein in the Rat-1/ICE cells is at least 2–3-fold lower than mutant ICE$^{Gly\rightarrow Ser}$-LacZ fusion protein of Rat-1/ICE$^{Gly\rightarrow Ser}$ cells (Fig. 3*A*, *lane 2* and *3*) in which mutant ICE$^{Gly\rightarrow Ser}$ does not induce cell death and is almost similar to that of ICE-LacZ protein in Rat-1/Bcl-2/ICE cells in which Bcl-2 inhibits cell death inducing activity of ICE (Fig. 3*A*, *lane 4*). Expression level of LacZ protein in Rat-1/ICE, Rat-1/mICE$^{Gly\rightarrow Ser}$, and Rat-1/Bcl-2/ICE cells was also examined by immunostaining using anti-β-galactosidase antibody and is similar to that of Western blot analysis (data not shown).

Using the cell lines described above, the effects of growth factors on ICE-induced cell death were examined (Table I). In the presence of 10% serum, the viability of Rat-1 cells expressing low levels of ICE was similar to that of control Rat-1 cells (96% *versus* 98%, Table I). After serum deprivation, the viability of Rat-1/ICE cells was 72%, which is lower than those of Rat-1 cells (93%), Rat-1/Bcl-2/ICE cells (98%), and Rat-1/mICE$^{Gly\rightarrow Ser}$ cells (91%), showing that Rat-1 cells expressing ICE are more sensitive to cell death induced by serum deprivation, as observed in COS-1 cells transfected with pβactM10Z. This result suggests that ICE activity is suppressed by serum and that ICE is activated upon serum removal. The effects of individual growth factors on the ICE-mediated cell death in the Rat-1/ICE cells were examined (Table II). IGF-1 and insulin were added to the defined medium and examined for the effects on the cell death. Quantitative analysis shows that addition of increasing amounts of either IGF-1 or insulin to the serum-free medium increased the viability of Rat-1/ICE cells from 72 to 94% at 0.8 nM IGF-1 and 91% at 0.34 μM insulin, respectively. These results indicate that ICE-mediated cell death triggered by serum deprivation is suppressed by IGF-1 or insulin, consistent with the results observed in COS-1 cells (Fig. 2*B*).

To examine activation of ICE, Rat-1/ICE cells were induced to undergo apoptosis by serum deprivation, stained with X-gal buffer, and viability was determined based on morphology. Rat-1 cells induced to die by *Ice-lacZ* expression are small and round blue after X-gal staining (2). Serum deprivation induced the appearance of round blue cells in Rat-1/ICE culture (Fig. 4*A*, *top*), but not in Rat-1/mICE$^{Gly\rightarrow Ser}$ and Rat-1/Bcl-2/ICE culture which are resistant to serum deprivation-induced cell death and show no color development after X-gal staining (data not shown). Addition of IGF-1 to the defined medium inhibited

TABLE I
Induction of apoptosis by serum removal

Numbers indicate viability (percent) of cells determined by staining with 0.4% trypan blue. Cells were incubated in DMEM without or with serum (10% FCS) for 1 day. Data are from two independent experiments of triplicates with mean ± S.E.

Cells	+ Serum	− Serum
Rat-1	98 ± 0	93 ± 2
Rat-1/ICE	96 ± 1	72 ± 1
Rat-1/mICE$^{Gly\rightarrow Ser}$	97 ± 2	91 ± 4
Rat-1/Bcl-2/ICE	99 ± 1	98 ± 3

TABLE II
Suppression of ICE-mediated cell death by IGF-1 and insulin

Numbers indicate viability (percent) of cells determined by staining with 0.4% trypan blue. Rat-1/ICE cells were incubated in serum-free defined medium without or with indicated amounts of growth factors for 1 day. Data are from two independent experiments of triplicates with mean ± S.E.

	+ Serum	No addition	− Serum
	96 ± 1	72 ± 1	
IGF-1			
0.023 nM			83 ± 2
0.16 nM			85 ± 0
0.8 nM			94 ± 10
Insulin			
0.014 μM			84 ± 5
0.068 μM			91 ± 1
0.34 μM			91 ± 2

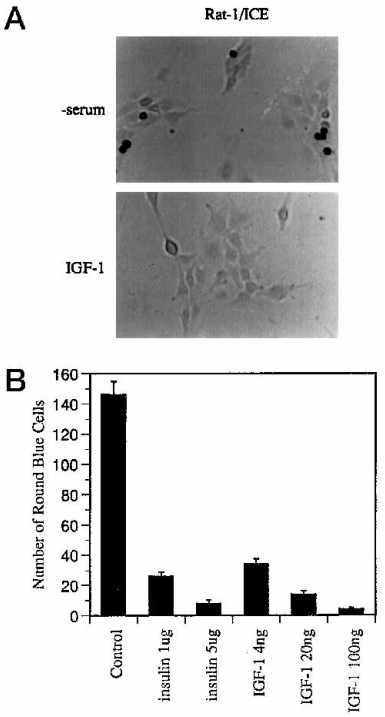

FIG. 4. **Activation and suppression of ICE.** Rat-1/ICE cells are maintained in the defined medium containing no serum or the defined medium supplemented with either IGF-1 (100 ng/ml) or insulin (5 μg/ml) for 1 day. After X-gal reaction, cells were photographed in *A*, and the round blue cells in fixed area (90 mm^2) were counted (*B*). Data are from at least two different experiments with mean ± S.E.

the appearance of round blue Rat-1/ICE cells (Fig. 4*A*, *bottom*). Rat-1/ICE cells in 10% serum are also healthy and flat and do not show color development after X-gal staining (data not shown). Western blot analysis (Fig. 3*A*, *lane 2* and *5*) and immunostaining (data not shown) using anti-β-galactosidase antibody showed that Rat-1/ICE cells contained similar amounts of ICE-LacZ fusion protein in both the absence and presence of IGF-1. These results show a correlation between appearance of round blue cells and cell death in the Rat-1/ICE cells. In Rat-1/ICE cells, the addition of increasing concentrations of IGF-1 or insulin to the defined medium significantly decreased the numbers of round blue cells by 20-fold (IGF-1: 100 ng/ml) and 13-fold (insulin: 5 μg/ml), respectively (Fig. 4*B*).

ICE was isolated as an heterodimeric enzyme composed of 10-kDa (p10) and 20-kDa (p20) subunits derived from a 45-kDa precursor protein (p45) (10, 27). To examine whether ICE is processed to be activated by serum deprivation, Western blot analysis using anti-β-galactosidase antibody was performed using cell lysates prepared from cells undergoing apoptosis (Fig. 3*A*, *lane 5*). Most of the ICE-LacZ fusion protein detected in live Rat-1/ICE cells (*lane 2*) is pro-ICE-LacZ (p45-LacZ), indicated by its molecular mass (160 kDa). In contrast, after serum deprivation, a different pattern of protein bands was detected (Fig. 3*A*, *lane 5*) in which ICE is processed from p45-LacZ precursor protein to 120 kDa, consistent with releasing of p10-LacZ from ICE-LacZ fusion protein.

To further characterize the processing of ICE during cell death, COS cells were transiently transfected with pβactM10T (a p45 ICE expression plasmid containing T7 epitope at the carboxyl terminus of ICE) and induced to die by growth factor deprivation. Floating (dead) or adherent (live) cells from each cell culture plate were collected and analyzed for the processing of ICE protein by Western blot analysis using anti-ICE and anti-T7 tag antibodies (Fig. 3*B*). In the absence of IGF-1, ICE was processed into p10, p20, and 30 kDa (p30) in the floating dying cells. In contrast, ICE was not proteolytically processed in adherent live cells. This result indicates that ICE is processed into its subunits in dying cells and that the processing is suppressed by IGF-1.

One of the enzymatic functions of ICE is to process pro-IL-1β (31 kDa) into its bioactive mature IL-1β (17.5 kDa), a secreted form. To examine enzymatic activation of ICE in growth factor-deprived cells, the production of mature IL-1β was examined in COS cells (Fig. 3*C*). Because COS-1 cells do not produce IL-1β which are detectable by Western analysis, COS cells were transiently co-transfected with pβactM10T and pRc/CMV-hIL-1β (a human pro-IL-1β expression vector) and maintained in defined medium. In the presence of IGF-1, pro-IL-1β (31 kDa) was detected in the cell lysates and no mature IL-1β was detected. However, in the absence of IGF-1, lower levels of pro-IL-1β were observed in cell lysates, and mature IL-1β (17.5 kDa) was detected in the defined medium. This result shows that IGF-1 suppresses the production of mature IL-1β and suggests that IGF-1 affects the inhibition of ICE enzymatic activity.

DISCUSSION

We have reported previously that expression of *crmA* in chicken dorsal root ganglion neurons suppresses cell death induced by trophic factor removal, suggesting that ICE or ICE-like proteases play critical roles in cell death induced by trophic factor deprivation (16). Here, we show that ICE is processed to be activated during serum deprivation-induced cell death in ICE-transfected immortalized Rat-1 fibroblasts and oncogene-transformed COS-1 cells. ICE-transfected, but not ICH-1_L-transfected, COS-1 cells can be induced to die by serum removal, thus, activity of ICE might be more responsive to environmental clues than that of ICH-1.

We succeeded in establishing stable Rat-1 cell lines expressing a mouse wild type *Ice* fused with an *Escherichia coli lacZ* gene. These cell lines, however, are unstable initially; the expression of *Ice* is lost easily. After cloning, we obtained several clones that appeared to stably express *Ice*. However, we may have selected for cells which express high levels of ICE inhibitor: since these cells do not turn blue after X-gal staining when cultured in the presence of 10% serum, despite of the presence of ICE-LacZ fusion protein detected by Western analysis and immunocytochemistry. The dying Rat-1/ICE cells will turn blue when cultured in the absence of serum under which condition significant proportions of cell die. This is most likely due to the processing of ICE: x-ray crystallographic analysis of the three-dimensional structure of ICE has shown that ICE exists as a tetramer consisting of two p20 and two p10 subunits in a crystal lattice (28, 29) and an evidence that the p10 and p20 subunits of ICE associate as oligomers in transfected cells has

been provided (30). Since anti-ICE antibodies currently available to us do not recognize the endogenous activated p20 and p10 subunits of ICE, we followed the cleavage of an ICE-LacZ fusion protein indirectly using an anti-β galactosidase antibody. After induction of apoptosis by serum deprivation, we observed approximately 120-kDa protein, apparently p10-LacZ, on Western analysis, consistent with the activation of ICE. Rat-1/ICE/Bcl-2 and Rat-1/mutant ICE$^{Gly \rightarrow Ser}$ cells, which do not die in the absence of serum, do not turn blue after X-gal staining, suggesting that induction of apoptosis, but not serum removal *per se*, induces β-galactosidase activity. We hypothesize the reason that Rat-1/ICE cells in the presence of 10% serum or IGF-1 do not turn blue after X-gal staining is that ICE is in a stable complex either with itself or with certain unknown inhibitors, which might prevent the activity of β-galactosidase.

Growth factor removal from COS cells clearly induces the processing of exogenous ICE in dying cells. Two subunits of p10 and p20 and an intermediate p30 are present in dying cells, showing that ICE might be processed into an intermediate p30 and, then, into p10 and p20. p30 is slightly more active than p45 in inducing cell death (2) and, thus, may be processed into p10 and p20 more easily than that of p45. In dying cells, the biological consequence of ICE processing into its subunit p10 and p20 is the activation of ICE. When COS cells were transfected with pβactM10T and pRc/CMV-hIL-1β expression vectors and induced to die by serum removal, ICE was processed (Fig. 3*B*), and mature IL-1β was produced and secreted into the defined medium (Fig. 3*C*). In addition, the presence of IGF-1 or insulin suppressed both ICE-mediated cell death (Figs. 2 and 4) and the secretion of mature IL-1β in COS cells (Fig. 3C). These results suggest the correlation between ICE-mediated apoptosis and pro-IL-1β processing and that IGF-1 may inhibit ICE activation.

What would be the physiological relevance of Rat-1/ICE cells? Tamura *et al.* (19) showed that *Ice* is induced in splenocytes following stimulation with concanavalin A and that induction of *Ice* gene expression in these mitogen-activated mature T lymphocytes enhances the susceptibility to cell death induced by γ-radiation or other DNA-damaging chemotherapeutic agents, such as adriamycin and etoposide. In IRF-1-deficient mice, ICE is not induced after concanavalin A treatment, and mitogen-activated mature T lymphocytes are much more resistant to γ-radiation and DNA-damaging agents, suggesting that induction of ICE may be critically important for the sensitivity to radiation-induced apoptosis in these cells. In addition, expression of ICE correlated with the loss of extracellular matrix which regulates apoptosis in mammary epithelial cells (18). Rat-1/ICE cells may mimic such conditions that ICE has been induced.

IGF-1 is a regulator of cell growth and metabolism (31). What could be the downstream target molecules for the suppression activity of ICE-mediated cell death by IGF-1? IGF-1 and insulin preferentially activate cellular responses via IGF-1 and insulin receptors (31), respectively, but appear to share a common downstream signal transduction pathway so far identified (32). In addition, IGF-1 and insulin cross-react with each other receptors (31). These observations suggest that the suppression of ICE-mediated cell death by IGF-1 and insulin is likely mediated by a common pathway. Even though the mechanism responsible for the suppression of ICE-mediated apoptosis by IGF-1 remains to be elucidated, several possibilities could be considered. The ICE-LacZ fusion protein is uncleaved in healthy Rat-1/ICE cells and, conversely, is cleaved in dying cells. This is consistent with the observation that ICE processing is required for its activity (27). Thus, regulation of ICE cleavage appears to be an important step in induction of cell death. IGF-1 and insulin may function by enhancing the activity of survival factors which inhibit the activation of ICE. Bcl-2 family inhibits or delays the cell death induced by a variety of stimuli (33) and also suppresses ICE-induced cell death (5). We observed that IGF-1 and insulin do not affect the expression levels of the Bcl-2 family members examined. The levels of Bcl-2 (34), Bcl-x (35), and Bax (36) are similar in IGF-1 and insulin-treated and untreated cells on Western blot analysis (data not shown). Alternatively, inhibition of ICE activation by IGF-1 or insulin may be mediated through posttranslational modification of ICE and/or other cell survival effector molecules. Potential mechanism for suppression of ICE-mediated cell death by IGF-1 and insulin currently are under investigation.

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