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Peptide Inhibitors of the ICE Protease Family Arrest Programmed Cell Death of Motoneurons In Vivo and In Vitro

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Summary

Members of the CED-3/interleukin-1β-converting enzyme (ICE) protease family have been implicated in cell death in both invertebrates and vertebrates. In this report, we show that peptide inhibitors of ICE arrest the programmed cell death of motoneurons in vitro as a result of trophic factor deprivation and in vivo during the period of naturally occurring cell death. In addition, interdigital cells that die during development are also rescued in animals treated with ICE inhibitors. Taken together, these results provide the first evidence that ICE or an ICE-like protease plays a regulatory role not only in vertebrate motoneuron death but also in the developmentally regulated deaths of other cells in vivo.

Introduction

Programmed cell death (PCD) is a fundamental component of normal development and homeostasis in all multicellular organisms (for review, see Glucksmann, 1951; Saunders, 1966; Wyllie et al., 1980; Raff, 1992; Schwartz and Osborne, 1993). In particular, development of the CNS includes restricted periods of naturally occurring cell death (for review, see Cunningham, 1982; Cowan et al., 1984; Oppenheim, 1991). PCD has been well documented in the lumbar spinal motoneurons of the chick, where approximately 50% of the neurons produced during embryogenesis die before birth (Hamburger, 1958; Hollday and Hamburger, 1976; Oppenheim et al., 1978; Hamburger and Oppenheim, 1982). Survival of motoneurons is dependent on their interaction with muscle targets, since removal of the limb induced greater than 90% motoneuron death, whereas transplantation of a supernumerary limb increased the numbers of surviving motoneurons (Hamburger, 1958; Hollday and Hamburger, 1976; Hamburger and Oppenheim, 1982). Despite intense research, little is known about the molecular mechanisms mediating this death. One gene that is required for cell death during the development of the free living nematode Caenorhabditis elegans is *ced-3* (Ellis and Horvitz, 1986; Ellis et al., 1991). CED-3 has recently been reported to have both structural (Wilson et al., 1994; Walker et al., 1994) and functional (Yuan et al., 1993; Miura et al., 1993) homology with the mammalian interleukin-1β-converting enzyme (ICE), a novel cysteine protease with specificity for cleavage after aspartate residues (Thornberry et al., 1992). ICE is one member of a family of cysteine proteases that share a pentapeptide moity that includes the active site cysteine (Thornberry et al., 1992). A role for ICE-like proteases in mammalian cell death in vitro has been suggested by the activity of a known inhibitor of this protease family, the viral serpin CrmA, to block cell death induced either by heterologous overexpression of ICE or related proteases (Kumar et al., 1994; Fernandes-Alnemri et al., 1994) or by serum or growth factor deprivation (Miura et al., 1993; Gagliardini et al., 1994). Futhermore, involvement of an ICE-like protease has also been recently demonstrated in Fas/APO-1–mediated apoptosis (Kuida et al., 1995; Enari et al., 1995; Los et al., 1995). Thus, the identification of specific ICE-like proteases and their substrates that have a regulatory role in cell death is currently the focus of intense research. The aspartate-directed substrate specificity (Asp-ase activity) of ICE has allowed for the development of peptide inhibitors that potentially inhibit ICE proteolytic activity (Thornberry et al., 1992). These compounds mimic the aspartic acid in the P1 position of known ICE substrates and are thus active site inhibitors. As such, these compounds may also be expected to inhibit ICE family members that retain Asp-ase activity, including nedd-2 or Ich-1 and a protease like ICE (prICE) (Kumar et al., 1994; Wang et al., 1994; Lazebnik et al., 1994).

In this study, we have exogenously applied these peptide inhibitors of ICE to motoneurons lacking trophic support in vitro and shown that they block cell death. Furthermore, we have also administered these peptides to chick embryos in ovo and shown that naturally occurring motoneuron cell death and the death of the hindlimb interdigital cells is inhibited. Thus, members of the *ced-3*/ICE cysteine protease family appear to be key regulators of both vertebrate and invertebrate cell death.

Results

Peptide Inhibitors of ICE Prevent the Death of Motoneurons Cultured in the Absence of Muscle Extract

We have taken advantage of a tissue culture model system that allows isolation of a relatively pure population of motoneurons whose survival is dependent on muscle extract, a potent source of target-derived trophic support (Bloch-Gallego et al., 1991; Milligan et al., 1994). Like their in vivo counterparts, the death of motoneurons deprived of

trophic support in vitro requires new gene expression and occurs by apoptosis (Oppenheim et al., 1990; Milligan et al., 1994). Motoneurons deprived of trophic support at the time of plating become irreversibly committed to undergo cell death after 16–18 hr (Milligan et al., 1994). Figure 1 shows that treatment with peptide inhibitors of ICE during this period at concentrations known to be effective in intact cells (Thornberry et al., 1992) substantially reduces the extent of motoneuron death observed after 3 days. Administration of either reversible peptide aldehyde or irreversible peptide chloromethylketone protease inhibitors had similar inhibitory effects on motoneuron death, although the peptide aldehyde appeared to be more effective. Treatment with the ICE inhibitors had no effect on cells receiving muscle extract, indicating that they were not toxic to motoneurons at the doses tested (Figures 1A and 1B). Treatment with control peptide aldehyde or chloromethylketone inhibitors that lack aspartate in the P1 position had no survival-promoting effects, further suggesting that it is the specific inhibition of ICE or ICE-like proteases that inhibits death (Figures 1C–1E). Morphologically, cells deprived of muscle extract but treated with the peptide inhibitors of ICE had phase bright cell bodies and neurites, although the length and branching of the neurites were not as extensive as those of control cells supplied with muscle extract (Figure 2). The morphologies of inhibitor-treated motoneurons suggest that ICE family protease inhibition, though sufficient to prevent death in the absence of muscle extract, was not sufficient to promote optimal cell growth.

To determine whether the inhibitor-treated neurons remained viable but were irreversibly damaged, inhibitor-treated motoneurons were cultured an additional 3 days, either in the continued absence of muscle extract or following supplementation with muscle extract. After 6 days in culture, motoneurons that received only peptide inhibitors on day 1 were sickly or dead (Figure 3 and Figure 4D). In contrast, motoneurons treated with peptide inhibitors and subsequently supplemented on day 3 with muscle extract continued to survive, had extensive neurite outgrowth, and appeared as healthy as the motoneurons that were continuously supplied with muscle extract (Figure 3 and Figure 4E). Thus, motoneurons rescued for 3 days by ICE inhibitors remain capable of responding to trophic factor(s) present in muscle extract. These results suggest that an ICE-

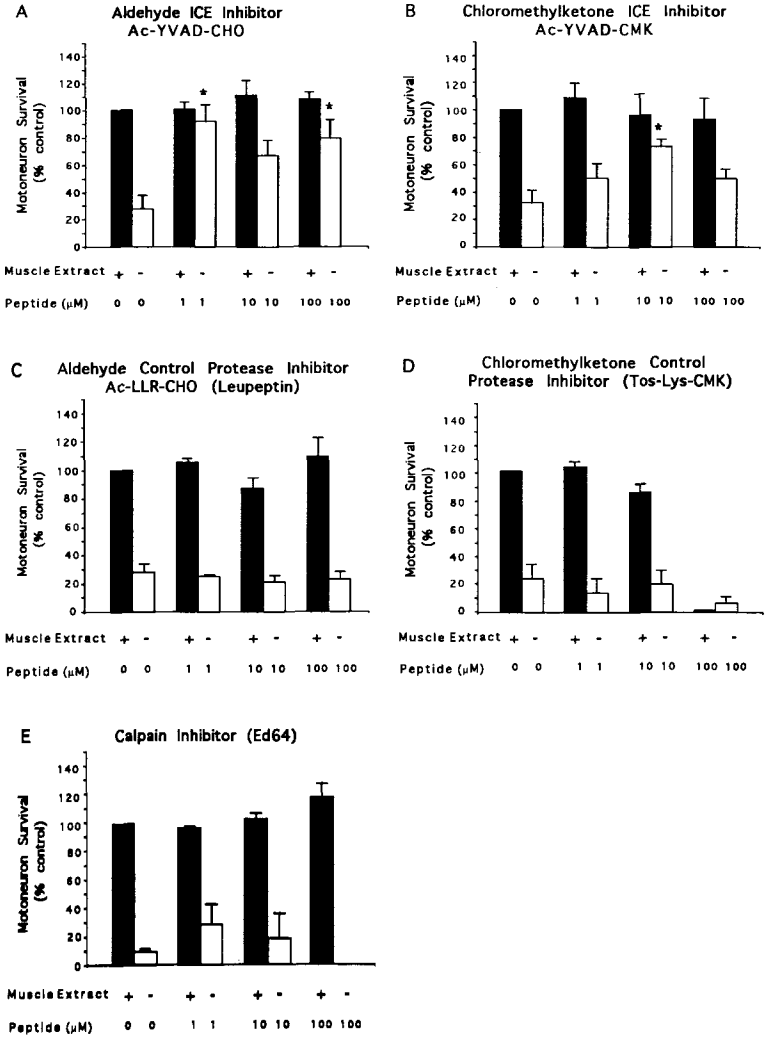

Figure 1. Peptide Inhibitors of ICE Arrest Motoneuron Death in the Absence of Muscle Extract In Vitro

Motoneurons were plated with or without muscle extract and treated with an aldehyde peptide inhibitor of ICE (Ac-YVAD-CHO; A), a chloromethylketone peptide inhibitor of ICE (Ac-YVAD-CMK; B), leupeptin, a control peptide aldehyde protease inhibitor (Ac-LLR-CHO; C), a control chloromethylketone protease inhibitor (Tos-Lys-CMK; D), or the membrane permeable calpain inhibitor Ed64 (E). After 3 days in culture, surviving motoneurons were detected with the SC1 monoclonal antibody and counted. Only the peptide inhibitors of ICE had survival-promoting activity. Results are expressed as percent of control, where control represents cultures supplied with muscle extract at plating ($n = 3$ individual experiments; two coverslips per condition per experiment; mean ± SEM). A nonparametric Mann-Whitney U test was performed to determine statistically significant differences compared with cells receiving no muscle extract and no peptide treatment. Asterisk indicates $p \leq .05$.

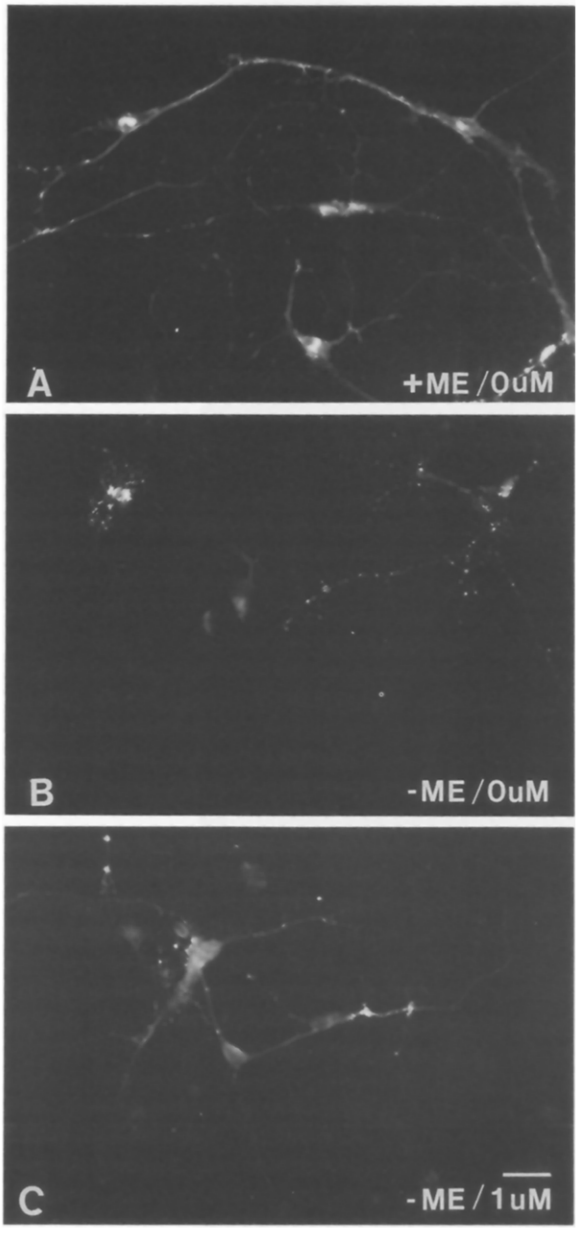

Figure 2. Photomicrographs of SC1 Immunopositive Motoneurons in Culture for 72 hr

Cells were cultured in the presence of muscle extract (A), in the absence of muscle extract (B), or in the absence of muscle extract but treated with the aldehyde peptide inhibitor of ICE (1 μM; C). Motoneurons cultured in the absence of muscle extract but treated with peptide inhibitors of ICE survive and appear to be healthy, although the extent of their neurite branching is less than that of motoneurons cultured in the presence of muscle extract. Bar, 25 μm.

like protease functions in the cell death cascade initiated by trophic factor deprivation of motoneurons in vitro. These results were obtained only with the peptide aldehyde inhibitor of ICE. Cells treated with the chloromethylketone inhibitor eventually died independent of the subsequent provision of the muscle extract (data not shown). This may be due to the irreversible nature of the protease

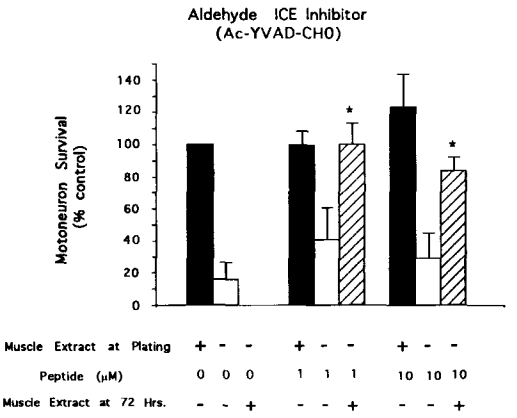

Figure 3. The Inhibition of ICE Delays the Death of Cultured Motoneurons

Cells treated as described in Figure 1. After 3 days in culture, muscle extract was added to cultures, and 3 days later (total of 6 days), surviving motoneurons were counted. Results are expressed as percent of control, where control represents cultures supplied with muscle extract at plating (n = 3 individual experiments; two coverslips per condition per experiment; mean ± SEM). A nonparametric Mann-Whitney U test was performed to determine statistically significant differences compared with cells receiving neither muscle extract nor peptide treatment. Asterisk indicates $p \leq .05$.

inhibition by the peptide chloromethylketone (Thornberry et al., 1992) or, alternatively, to a cytotoxic property of the chloromethlyketones and their metabolites relative to aldehyde inhibitors.

Administration of Peptide Inhibitors of ICE Reduces Naturally Occurring Motoneuron Cell Death in the Chick Embryo

Although administration of peptide inhibitors of ICE arrested motoneuron death in the absence of trophic support in vitro, the physiological relevance of these observations remains in question. Accordingly, we have also investigated the role of ICE-like proteases during motoneuron PCD in vivo. In the first in vivo experiment, embryos were treated with a single dose of the ICE peptide inhibitors or with control protease inhibitors on embryonic day 8 (E8), the time of maximum naturally occurring motoneuron death (Chu-Wang and Oppenheim, 1978; Clarke and Oppenheim, 1995). Embryos treated in vivo with the peptide inhibitors appeared to develop normally, and the morphology of their spinal cords was indistinguishable from controls. Approximately 15 hr later, there were significantly fewer pyknotic cells present in the lumbar spinal cord of animals treated with the peptide inhibitors of ICE as compared with animals treated with control protease inhibitors (Table 1), an effect that was dose dependent (Figure 5). By 24 hr after treatment, there was a similar reduction in the number of pyknotic cells with a corresponding increase in the number of healthy motoneurons (Table 1). The increase in healthy cells suggested there is not a morphological change in the dying neurons that would preclude their identification as pyknotic. However, whether this represents a permanent rescue of motoneurons remains to be determined.

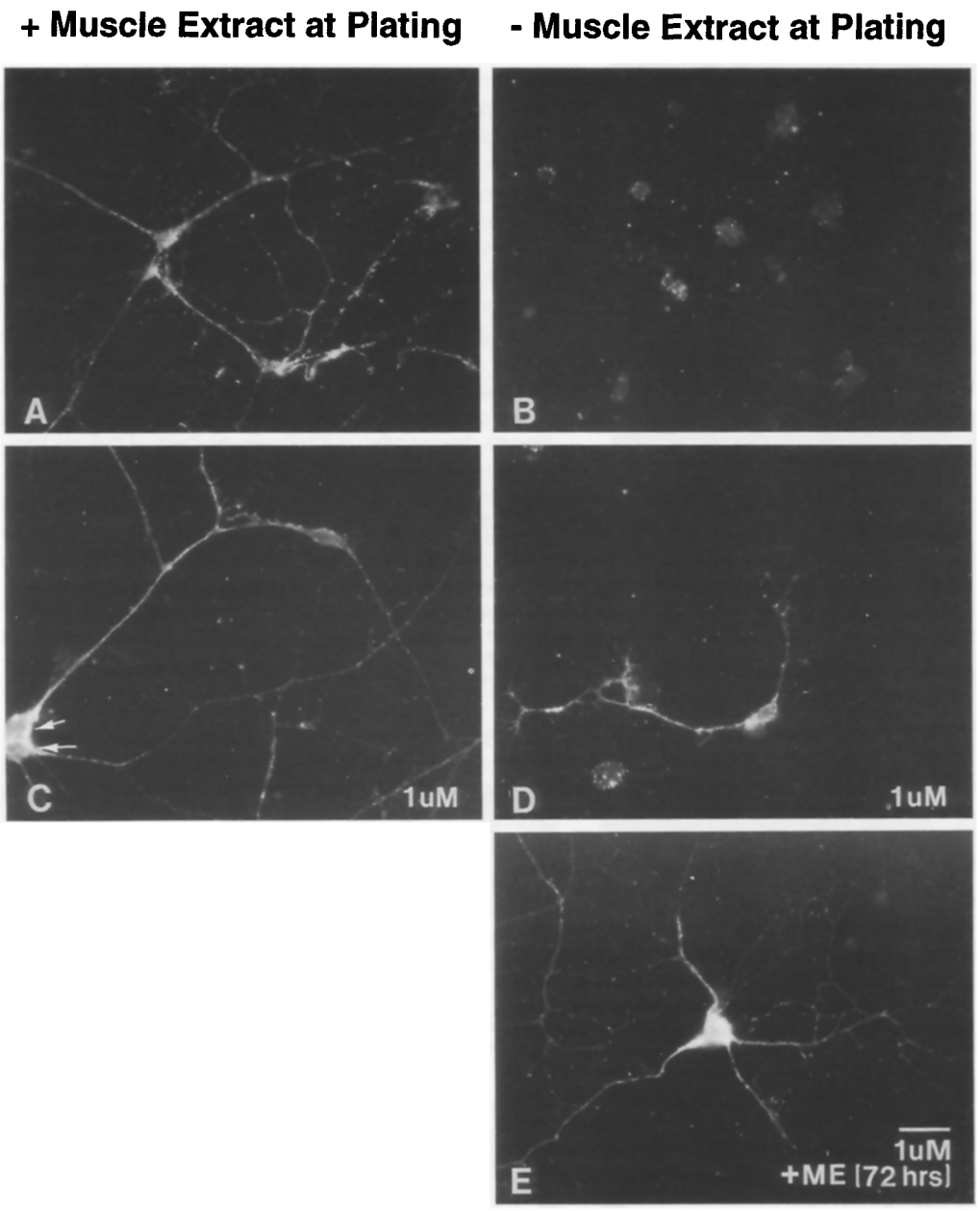

Figure 4. Morphology of Rescued Neurons In Vivo

Photomicrographs of SC1-immunopositive motoneurons cultured for 6 days with muscle extract (A), without muscle extract (B), with muscle extract and treated with the aldehyde peptide inhibitor of ICE (Ac-YVAD-CHO) every 2 hr between 14 and 24 hr in culture (C; arrows indicate two SC1 immunopositive motoneurons), without muscle extract and treated with the peptide inhibitor of ICE (D), or initially plated without muscle extract, treated with the peptide inhibitor of ICE, then treated with muscle extract at 3 days in culture (E). Bar, 25 μm.

Administration of Peptide Inhibitors of ICE Does Not Prevent All Neuronal Cell Deaths

Following limb bud removal, greater than 90% of the lumbar motoneurons die, presumably owing to a complete loss of target interaction and trophic support (Oppenheim et al., 1978). When animals that had undergone limb bud removal were subsequently treated with peptide inhibitors of ICE, motoneuron death was not prevented (Table 2). Though many examples of PCD in the developing CNS appear to be mediated by target interaction or availability of a limited supply of trophic support, there are examples in which the death of neurons is apparently target independent and possibly cell autonomous. An example of one such population is the motoneurons of the cervical spinal cord, whose survival or death is independent of presumptive target interaction or the availability of growth factors (Levi-Montalchini, 1951; H. Y., M. Tomita, N. Takahita, S. E. McKay, C. C., Q.-W. Yin, and R. W. O., unpublished data). These cells normally die between E4 and E5, with maximum loss occurring on E4.5, and they appear to die

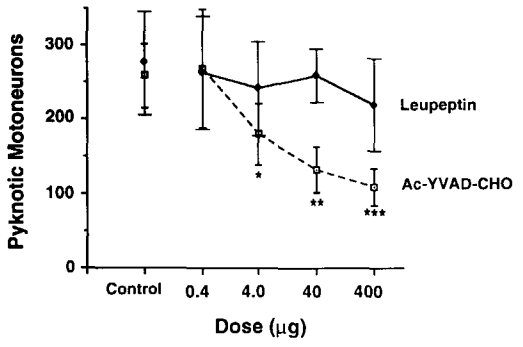

Figure 5. The Effects of the Aldehyde Peptide Inhibitor of ICE (Ac-YVAD-CHO) on Preventing Motoneuron PCD In Vivo Is Dose Dependent

Embryos were treated as described (Table 1); results represent total pyknotic cells in the lumbar spinal cord (mean ± SD; n = 5). P values compared the peptide inhibitor of ICE with leupeptin at each dose (t tests with Bonferroni correction). One asterisk, $p \leq .05$; two asterisks, $p \leq .01$; three asterisks, $p \leq .001$.

by apoptosis. When embryos were treated with inhibitors of ICE on E3 and E4, and the cervical spinal cord examined on E4.5, no increase in the number of surviving motoneurons was observed (Table 3). Another example of PCD in the developing CNS that occurs by apoptosis and appears to be independent of both targets or trophic factors is the death of undifferentiated neurons and precursor cells between E2 and E3 in the neural tube (Homma et al., 1994). When embryos are treated with ICE inhibitors on E2.5 (stage 16–17) and examined 12 hr later (stage 18), it was observed that this form of early PCD was also found to be insensitive to rescue by the peptide inhibitors (Table 3).

Interdigital Cell Death Is Also Inhibited by Administration of Peptide Inhibitors of ICE

Although the effectiveness of the peptide inhibitors of ICE described here appears to be restricted to the target-dependent form of motoneuron death that is regulated by the availability of specific neurotrophic factors, we also found that these inhibitors can block certain nonneuronal PCDs. The interdigital cells of the limbs undergo PCD as a means of sculpting the digits in many vertebrates (Saunders et al., 1962). When embryos were treated with the peptide inhibitors of ICE, there was a substantial reduction in the number of pyknotic cells between the digits of the hindlimb (Figure 6). The number of pyknotic interdigital cells per section were 72 ± 18 for untreated control embryos, 34.6 ± 3 ($p < .03$) for acetyl-Tyr-Val-Ala-Asp-aldehyde (Ac-YVAD-CHO)-treated and 13.8 ± 6.1 ($p \leq .01$) for acetyl-Try-Val-Ala-Asp-chloromethylketone (Ac-YVAD-CMK)-treated embryos (mean ± SD; n = 3 for control and Ac-YVAD-CMK, n = 4 for Ac-YVAD-CHO; p values compared the peptide inhibitor of ICE with control [t tests]). These data further support the hypothesis that ICE or ICE protease family members are key components in PCD of multiple cell types.

Discussion

The results of the experiments described in this study provide the first evidence that ICE or an ICE-like protease plays an essential regulatory role in naturally occurring vertebrate cell death in vivo. Inhibition of this class of proteases with exogenous application of cell-permeable inhibitors arrests motoneuron death in vitro and, more importan-

Table 1. Pyknotic and Healthy Lumbar Motoneurons on E8 and E9

		ICE Inhibitors		Control Protease Inhibitors	
	Control	Ac-YVAD-CHO	Ac-YVAD-CMK	Leupeptin	Tos-Lys-CMK
Pyknotic lumbar motoneurons on E8	286 ± 37 (20)	146 ± 35[b] (8)	216 ± 27[c] (10)	310 ± 40 (20)	285 ± 31 (20)
Pyknotic and healthy lumbar motoneurons on E9[a]					
Pyknotic motoneurons	316 ± 47 (21)	150 ± 30[c] (6)	200 ± 33[c] (6)	297 ± 51 (20)	345 ± 39 (22)
Healthy motoneurons	13,605 ± 890 (20)	15,680 ± 684[c] (6)	16,231 ± 755[b] (7)	14,117 ± 971 (18)	13,257 ± 773 (19)

Results are expressed as mean ± SD. Multiple t tests were performed with the Bonferroni correction. P values were the same for comparisons of Ac-YVAD-CHO or Ac-YVAD-CMK with control, leupeptin, or Tos-Lys-CMK-treated animals. The number in parentheses represents the n for each group.

[a] Embryos were treated on E8 and killed on E9–0 hr (24 hr after treatment).

[b] $p \leq .001$.

[c] $p \leq .01$.

Table 2. Pyknotic and Healthy Lumbar Motoneurons on E6 Following Limb Bud Removal

	Ipsilateral		Contralateral	
	Control	Ac-YVAD-CHO	Control	Ac-YVAD-CHO
Pyknotic motoneurons	375 ± 74[a] (6)	317 ± 89[a] (6)	73 ± 16 (6)	67 ± 13 (6)
Healthy motoneurons	11,371 ± 1780[a] (6)	11,109 ± 1592[a] (6)	18,455 ± 1661 (6)	17,914 ± 1733 (6)

The aldehyde peptide inhibitor of ICE, Ac-YVAD-CHO, had no survival-promoting effect as compared with the unoperated contralateral side.

[a] $p \leq .001$, ipsilateral vs. contralateral

Table 3. Pyknotic Cervical Motoneurons on E4.5 and Pyknotic Cells in the Early Neural Tube

		ICE Inhibitors		Control Protease Inhibitor
	Control	Ac-YVAD-CHO	Ac-YVAD-CMK	Leupeptin
Pyknotic cervical neurons on E4.5	24.1 ± 3.1 (7)	25.9 ± 3.4 (6)	26.7 ± 2.2 (6)	32.5 ± 7.9 (3)
Pyknotic cells in the early neural tube				
Floor plate	0.898 ± 0.103 (6)	1.115 ± 0.246 (6)	0.872 ± 0.201 (5)	
Dorsal spinal cord	1.322 ± 0.471 (6)	1.350 ± 0.297 (6)	1.372 ± 0.235 (5)	

The peptide inhibitors of ICE, Ac-YVAD-CHO, or Ac-YVAD-CMK had no survival-promoting effects on cervical motoneurons as compared with control- or leupeptin-treated animals or on floor plate or dorsal spinal cord cells as compared with control animals. Results are expressed as mean ± SD. Multiple t tests were performed with the Bonferroni correction. The number in parentheses represents the n for each group.

tly, motoneurons and interdigital cell death in vivo. Because the agents used in this study inhibit a family of related proteases that have been both implicated in apoptosis and resemble ICE with respect to their Asp-ase activity (Lazebnik et al., 1994), the specific role of ICE, as opposed to ICE-related Asp-ases, cannot be discerned at present. This distinction awaits the discovery of inhibitors that are selective for the different members of this cysteine protease family.

Though these results and the results of previous reports (Yuan et al., 1993; Miura et al., 1993; Gagliardini et al., 1994; Kumar et al., 1994; Lazebnik et al., 1994; Wang et al., 1994) suggest an involvement of ICE or an ICE-like cysteine protease in cell death, the specific protease that is mediating cell death has yet to be identified or characterized. Whereas overexpression of ICE in vitro appears to induce cell death (Miura et al., 1993), it remains undetermined if ICE itself is the physiological mediator of this cell loss. For example, although transgenic mice lacking ICE display impaired interleukin-1β and interleukin-1α production, few other deficits were observed (Li et al., 1995; Kuida et al., 1995). In fact, apoptosis occurred normally in ICE-

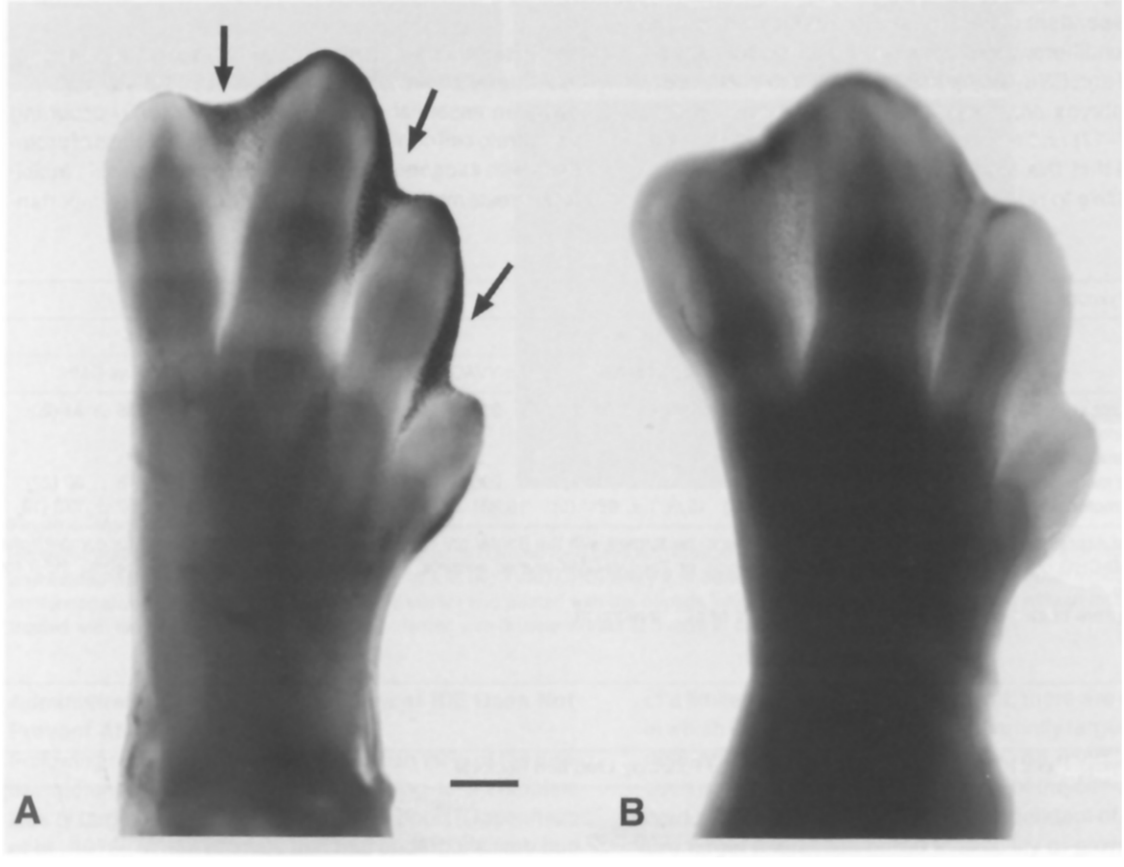

Figure 6. Arrest of Interdigital Death In Vivo

Foot pads of chick embryos on E7.5 (stage 32) vitally stained with neutral red following treatment with vehicle control solution (A) or a peptide inhibitor of ICE (Ac-YVAD-CMK, B). Note the presence of many stained phagocytes (dark profiles) containing the debris of dying interdigital cells in (A) (arrows) and their relative absence in (B). Bar, 0.5 mm.

deficient mouse thymocytes and macrophages (Li et al., 1995), although thymocytes from ICE-deficient mice fail to undergo apoptosis when stimulated with the Fas antibody (Kuida et al., 1995).

To date, several members of this family of cysteine proteases have been identified and implicated in cell death, including nedd-2/Ich-1 and cpp-32 (Fernandes-Alnemri et al., 1994; Kumar et al., 1994; Wang et al., 1994). The conserved feature of this family of proteases is the active site pentapeptide QACRG. Since the peptides used in this study serve to inactivate this catalytic pocket in ICE (Thornberry et al., 1992), it is conceivable that they would also inhibit, to varying degrees, other members of this family possessing this pentapeptide domain. In support of this supposition, these peptides have been demonstrated to be effective in inhibiting another member of the family, a protease resembling ICE (prICE), although at 1000-fold less potency (Lazebnik et al., 1994). The role of specific family members is undefined at present, and the identification of those regulating cell death is an area of intense research. Several highly conserved putative chick ICE homologs have been cloned and are currently being studied to determine their potential role in cell death (Milligan and Schwartz, unpublished data).

Our results suggest that members of the ICE family are critical mediators in the cell death of multiple cell types. Inhibition of ICE or an ICE-like cysteine protease appears to arrest the cell death process; however, the in vitro data in this study suggest that inhibition alone is not sufficient to permanently rescue the cells. This may be due to the potentially short half-lives of these peptide inhibitors that would therefore only provide temporary inhibition of the cell death process. In the absence of subsequent trophic support, these cells undergo cell death. Furthermore, we failed to observe a survival-promoting effect by these inhibitors on motoneuron cell death that results from limb bud removal. Although we cannot exclude the possibility that inappropriate dosage or timing of peptide administration was responsible, we consider this unlikely since the peptides were administered during the time of death. Alternatively, these results suggest that the cell death mechanism activated by target deprivation may differ from that employed by motoneurons undergoing naturally occurring cell death. In support of this hypothesis, though muscle extract contains a factor(s) that rescues dying motoneurons in both paradigms, treatment with another agent, dibutryl cyclic GMP, has also been shown to rescue motoneurons from normal but not limb removal–induced death (Douglas and Ribchester, 1986). In limb bud removal animals, the motoneurons presumably never have a source of target-derived trophic support, whereas motoneurons in normal embryos treated with the peptide inhibitors may be able to survive on limited amounts of trophic support once the putative ICE pathway of cell death is blocked.

If we consider this together with the data from the culture experiments, inhibition of ICE family members appears to arrest death, but in the absence of other survival signals (possibly from other trophic sources) the cells, nonetheless, subsequently die. Furthermore, we also did not observe a survival-promoting effect on early cervival motoneuron cell death or the cell death in the early neural tube, populations that are both thought to die by means independent of target interaction (Homma et al., 1994; H. Y., M. Tomita, N. Takahita, S. E. McKay, C. C., Q.-W. Yin, and R. W. O., unpublished data). These results again suggest that although inhibition of ICE arrests the cell death cascade, other signals and pathways may be required subsequently to promote survival over time. Alternatively, the molecular mechanism of PCD may differ among cell populations on depending whether neurons have been exposed to or require trophic support. A similar situation exists for the anti-apoptotic proto-oncogene Bcl-2, in which ectopically expressed protein has different survival-promoting effects on different populations of sensory neurons following growth factor withdrawal in vitro (Allsopp et al., 1993). In this case, sensory neurons deprived of neurotrophins are rescued by Bcl-2, whereas parasympathetic ciliary neurons deprived of ciliary neurotrophic factor are not.

Administration of exogenous cell-permeable inhibitors of ICE to prevent motoneuron cell death as a result of trophic factor deprivation in vitro and naturally occurring motoneuron cell death in vivo suggests that acute treatment with inhibitors of the ICE protease family arrests motoneuron death. In pathological situations, this could allow time for the cells to reorganize and recover, thereby opening up the potential for therapeutic strategies involving motoneuron death such as occurs following spinal cord injuries or in amytrophic lateral sclerosis. By constrast, the lack of effect of these inhibitors on certain other types of PCD in the nervous system indicates that there are likely alternative pathways of neuronal cell death that have yet to be revealed.

Experimental Procedures

Methods for Motoneuron Cultures

Spinal cords from E5 chicks were dissected in cold PBS (pH 7.4), incubated in trypsin (0.25% in PBS; Gibco), and the tissue dissociated by passing it several times through a 1.0 ml pipette tip. Cells were layered onto a 6.8% metrizamide (Serva) cushion and centrifuged at 500 g. The cell layer at the interface, containing predominantly motoneurons, was collected. Motoneurons were plated onto 12 mm glass coverslips (Fisher) that were initially coated with polyornithine (1 μg/ml; Sigma), washed extensively with dH_2O, and subsequently coated with laminin (20 μg/ml; Gibco). A culture medium containing Leibovitz's L15 media (Gibco) supplemented with sodium bicarbonate (625 μg/ml), glucose (20 mM), progesterone (2×10^{-8} M; Sigma), sodium selenite (3×10^{-8} M; Sigma), conalbumin (0.1 mg/ml; Sigma), putrescine (10^{-4} M; Sigma), insulin (5 mg/ml; Sigma), and penicillin–streptomycin (Gibco) was used. Unless otherwise noted, 1 ml of complete media, with or without muscle extract (20 μg/ml; prepared as previously described; Oppenheim et al., 1988) was added to the tissue culture wells that contained a coverslip seeded with cells (1×10^4 cells per coverslip). We have previously shown that motoneurons in culture become committed to die approximately 16 hr after culture in the absence of muscle extract (Milligan et al., 1994). For these experiments, motoneurons were treated with control protease inhibitors or peptide inhibitors of ICE (Thornberry et al., 1992) every 2 hr between 14 hr and 24 hr in culture (the time when cells in the absence of muscle extract are dying). Since little is known about the half-life of the peptide inhibitors of ICE, these were added as described. A single dose of peptide at initial plating of the cells or during this time proved to be ineffective at promoting survival of motoneurons in the

absence of muscle extract. Treatment with the peptide inhibitor was accomplished by adding the appropriate concentration of peptide to the cells so that the final concentration in the well would be as indicated in the figures; no more than 0.5% of the total volume of media was added at any time. Aldehyde peptide inhibitors were diluted in dH_2O, and chloromethylketone inhibitors were diluted in DMSO. After a total of 3 days in culture, cells were incubated with the monoclonal antibody SC1 (1:5 of supernatant in PBS; Tanaka et al., 1991) for 1.5 hr at 37°C, washed with PBS, fixed with 10% formaldehyde in PBS, and subsequently incubated with an FITC-labeled goat anti-mouse IgG secondary antibody (1:50 diluted in PBS; Fisher). After extensive washes with PBS, the cells were incubated with the fluorescent DNA intercalating dye 4',6-diamidino-2-phenylindole (1:100,000 in PBS; Sigma) and mounted with the aqueous mounting media Gel-Mount (Biomeda). Surviving motoneurons were counted in 5 predetermined 40× objective fields. For a motoneuron to be considered viable, its cell body must be present in the field of view, exhibit uniform SC1 immunoreactivity on its surface membrane, and possess a uniform, noncondensed DAPI stained nucleus. In control cultures (with MEX), 20–30 cells were counted per field.

Peptides

Two peptide inhibitors of ICE, Ac-YVAD-CHO and Ac-YVAD-CMK, and two control peptide inhibitors, acetyl-Leu-Leu-Arg-aldehyde (Ac-LLR-CHO; leupeptin) and n-tosyl-Lys-chloromethylketone (Tos-Lys-CMK), were synthesized by Bachem Biosciences (King of Prussia, PA) and shown by thin layer chromatography and HPLC to be greater than 98% pure. Ac-YVAD-CHO is a reversible, competitive inhibitor of ICE with a reported Ki of 0.7 nM on isolated ICE (Thornberry et al., 1992). However, its peptidic nature limits cell penetration, and its reported Ki for ICE inhibition in intact monocytes is much higher (1.5 μM; Thornberry et al., 1992; Molineaux et al., 1993). Ac-YVAD-CMK is an irreversible, competitive inhibitor of ICE; its peptidic nature also limits cell penetration so micromolar concentrations are also required (Thornberry et al., 1992). Peptide aldehydes are highly selective for cysteine proteases; peptide chloromethylketones can also inhibit serine proteases, but not as effectively as they inhibit cysteine proteases. The calpain inhibitor Ed64 was purchased from Sigma.

Methods for Administration of Peptides to Examine Effects on Naturally Occurring Motoneuron Cell Death In Vivo

Although cell death of lumbar motoneurons occurs between E6 and E12, the peak period of death (i.e., the greatest number of pyknotic cells) occurs on E8 (Oppenheim et al., 1978). For these experiments, embryos were given a single administration of an agent on E8 0 hr and sacrificed 15 hr later; 400 μg was chosen since this was the most effective dose tested (see Figure 3). Ac-YVAD-CMK and Tos-Lys-CMK were administered in a solution of DMSO/BSA, whereas Ac-YVAD-CHO and leupeptin (Sigma) were in BSA alone. The solutions (50–100 μl) were dropped onto the highly vascularized chorioallantoic membrane through a window in the shell. The control groups included both DMSO and BSA or vehicle alone. Embryos were killed and staged by the Hamburger-Hamilton series (Hamburger and Hamilton, 1951). The thoraco-lumbar spinal cord was dissected, fixed in Carnoys or Bouins fixative, processed for paraffin histology, serially sectioned (10–12 mm), and stained with either thionin or hematoxylin and eosin. Healthy and pyknotic motoneurons were identified based on criteria previously described (Chu-Wang and Oppenheim, 1978; Clarke and Oppenheim, 1995) and were counted in every tenth or twentieth section through the entire lumbar enlargement. The total number of pyknotic and healthy cells was then estimated by multiplying these values by 10 or 20. Approximately 35 total sections were examined per embryo. There were on average 30 pyknotic cells in controls and 10 in the experimental high dose. All cell counts were performed blind.

Methods for Limb Bud Removal Experiments

A unilateral limb bud removal was performed on E2 as described previously (Oppenheim et al., 1978). Because induced cell death following limb removal begins on E5 (before that there is no difference in the number of motoneurons between the operated or unoperated sides), embryos were given one treatment of Ac-YVAD-CHO (40 μg) or BSA (control) at E5 0 hr and another at E5 12 hr (total peptide administered was 80 μg). Animals were killed at E6 0 hr. Methods for cell counts were the same as described above except the section thickness was 6–8 μm.

Methods for Examining Cervical Motoneuron Cell Death

Massive numbers of motoneurons in cervical spinal cord undergo PCD between E4 and E5 (peak E4.5) (Levi-Montalchini, 1951; H. Y., M. Tomita, N. Takahita, S. E. McKay, C. C., Q.-W. Yin, and R. W. O., unpublished data). Embryos were treated with 100 μg of AC-YVAD-CHO or Ac-YVAD-CMK on E3 and E4 (200 μg peptide total). Animals were killed on E4.5, fixed in Bouins, sectioned at 6–8 μm, and stained with hematoxylin and eosin. Pyknotic cells were counted in every fifth section in the ventral horn of cervical segment C10.

Methods for Cell Death in the Early Neural Tube

Embryos were treated with 50 μg of each peptide in 50 μl of vehicle at stage 16–17 (E2.5) by administration through a window in the shell directly into the amniotic sac. Approximately 12 hr later (stage 18), embryos were killed by immersion in Skoff's fixative and processed as described (Homma et al., 1994). Pyknotic cells were counted in plastic embedded serial sections (2 μm) through brachial segment 18. Pyknotic cells in the floorplate and dorsal pyknotic zones were evaluated separately. The general morphology of the embryo and the spinal cord was normal.

Methods for Limb Bud Interdigital Regions

Embryos were treated with 100 μg of peptide or vehicle in 50 μl on E6.0 and on E7.0 (total 200 μg) as described above and killed at E7.5. The footpads were placed in Bouin's fixative and processed as described above. Pyknotic cells in all interdigital regions were counted in every tenth section (6–8 μm) of serial transverse sections through the entire footpad. For vital dye staining, embryos were treated on E6.0 and E7.0 as described. On E7.5 (stage 32), animals were killed; the limbs were amputated and placed into a 0.01% solution of neutral red (Sigma) in Ringer's solution. Limbs were incubated in a 37°C water bath for 1 hr. They were then rinsed twice in cold Ringer's solution and placed into cold formal chloride fixative for approximately 20 hr.

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Note Added in Proof

Yama, a member of the CED-3/ICE protease family, has recently been cloned and is a candidate regulatory molecule in apoptosis (Cell *81*, 801–809, 1995). Once activated, purified Yama can cleave PARP to generate the 85 kDa apoptotic fragment, and Yama's activity is inhibited by CrmA.