Caspase-1 activation of caspase-6 in human apoptotic neurons

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Received 10.1.05; revised 18.7.05; accepted 18.7.05; published online 26.8.05
Edited by B Zhivotovsky

Abstract

Active caspase-6 (Csp-6) induces cell death in primary cultures of human neurons and is abundant in the neuropathological lesions of Alzheimer's disease. However, the mode of Csp-6 activation is not known. Here, we show that the Csp-1 inhibitor, Z-YVAD-fmk specifically prevents activation of Csp-6 and cell death in human neurons. A transient increase in Csp-1-like activity and an increase in the p23Csp-1 subunit occur early after serum deprivation. Recombinant active Csp-1 (R-Csp-1) cleaves recombinant and neuronal pro-Csp-6 *in vitro* resulting in Csp-6 activity. However, R-Csp-1 does not induce cell death when microinjected in human neurons despite the inhibition of serum-deprivation induced cell death with a Csp-1 dominant negative construct. These results show that Csp-1 is an upstream positive regulator of Csp-6-mediated cell death in primary human neurons. Furthermore, these results suggest that the activation of Csp-1 must be accompanied by an apoptotic insult to induce Csp-6-mediated cell death.

Cell Death and Differentiation (2006) 13, 285–292.

doi:10.1038/sj.cdd.4401753; published online 26 August 2005

Keywords: caspase-6; caspase-1; primary human neurons; apoptosis; cell death

Abbreviations: AD, Alzheimer's disease; Csp, caspase; R-Csp, recombinant Csp; IL-1 β , interleukin 1 β

Introduction

Csp-6 is a short proarm effector Csp¹ that is rarely considered as an effector Csp of the nervous system because mice null

for Csp reveal that Csp-3, but not Csp-6, is implicated in neuronal developmental cell death.² However, we found that Csp-6, not Csp-3, is activated in serum-deprived human primary neurons.³ Microinjection of recombinant active Csp-6, but not recombinant active Csp-3, Csp-7 or Csp-8, induces TUNEL positive neuronal cell death into human primary neurons.⁴ Furthermore, active Csp-6 and Csp-6 cleaved Tau are abundant in the major neuropathological hallmarks of Alzheimer's disease (AD) brains; neuritic plaques, neurofibrillary tangles and neurofibrillary tangles.⁵ Other studies provide evidence for a primary role of Csp-6 in neuronal apoptosis. The p75 neurotrophin receptor-mediated cell death of immortalized striatal rat neurons and rat embryonic hippocampal neurons induce a higher level of Csp-6 activation than other Csp and, in hippocampal neurons, cell death is completely blocked with antisense oligonucleotides inhibiting Csp-6 expression.⁶ Human glioma cells undergoing apoptosis by dominant-negative focal adhesion kinase expression, Bcl-2/Bcl-x antisense and FTY720 immunosuppressant^{7–9} and dopaminergic neurons SN4741 exposed to oxidative stress¹⁰ show higher and earlier Csp-6 activation than other Csp. Endoplasmic reticulum stress and expression of the amyloid precursor protein-binding protein induce Csp-6 activation and apoptosis of mouse primary cultures of neurons.¹¹ Finally, epileptic seizures induce a strong activation of Csp-6 in the rat hippocampus that precedes Csp-3 activation.¹²

Despite significant evidence for a role of Csp-6 in neuronal cell death, the mode of Csp-6 activation is not clear. *In vitro*, Csp-6 is activated by Csp-3, Csp-9, Csp-1 and Csp-11, a mechanism that is consistent with the known Csp proteolytic cascade.^{13,14} *In vivo*, active Csp-3 has been shown to induce Csp-6 activation.¹⁵ On the other hand, in yeast cells, Csp-6 cannot be activated by Csp-3, Csp-8 or Csp-10¹⁵ and Csp-3-deficient breast cancer MCF7 cells undergo apoptosis via Csp-6 activation.¹⁶

In this study, we investigate potential upstream Csp activators of Csp-6 in human neurons. Using *in vitro* fluorogenic assays with Csp-specific substrates, immunoblotting of active Csp subunits, a Csp-1 dominant-negative (DN), and Csp-specific inhibitors, we find that Csp-1 induces Csp-6-mediated neuronal cell death. R-Csp-1 cleaves recombinant and neuronal pro-Csp-6 *in vitro* resulting in Csp-6 activity. However, R-Csp-1 does not induce cell death when microinjected in human neurons. These results show that Csp-1 is an upstream positive regulator of Csp-6-mediated cell death in primary human neurons. Furthermore, these results suggest that the activation of Csp-1 must be accompanied by an apoptotic insult to induce Csp-6-mediated cell death.

Results

Inhibition of Csp-1-like activity prevents serum-deprivation-induced Csp-6 activation and neuronal cell death

To determine if an upstream Csp activates Csp-6, serum-deprived neurons were incubated with 5 μ M of various

fmk-conjugated Csp inhibitors and tested for Csp-6 activation (Figure 1a). The Csp-1 Z-YVAD-fmk inhibitor is as efficient in preventing Csp-6 VEIDase activity as the general Csp inhibitor, Boc-D-fmk and Csp-6 Z-VEID-fmk and Z-IETD-fmk inhibitors. In contrast, the Csp-2- and Csp-9-like Z-VDAVD-fmk and Z-LEHD-fmk inhibitors, do not prevent serum-deprivation-induced VEIDase activity. Similarly, Z-YVAD-fmk, but not Z-VDAVD-fmk or Z-LEHD-fmk, prevents neuronal cell death in serum-deprived neurons to the same extent as Z-VEID-fmk and Z-IETD-fmk (Figure 1b). The potential involvement of Csp-3 was previously ruled out.³ Furthermore, the active p20 subunit of Csp-6 is not produced in Z-YVAD-fmk-treated serum-deprived neurons (Figure 1c). To determine if the YVAD inhibitor has a nonspecific effect on active Csp-6, its activity was verified at various concentrations on R-Csp-6 in an *in vitro* assay. We compared the activity of Ac-YVAD-CHO and Z-YVAD-fmk since these can have differential effects *in vitro* and *in vivo*. The Ac-YVAD-CHO inhibitor shows a dose-specific inhibition of R-Csp-1, as expected, but also inhibits slightly but not significantly the R-Csp-6 activity. The Z-YVAD-fmk has a similar effect on R-Csp-1 but the highest concentration of 10 μ M also

significantly inhibits R-Csp-6 activity. The esterification of the Z-YVAD-fmk used in these assays prevents a direct comparison with the activity of the inhibitor in cells since it is de-esterified upon entering the cells. Therefore, we also performed the *in vitro* assay with de-esterified Z-YVAD-fmk. The de-esterified Z-YVAD-fmk does not inhibit R-Csp-6 activity at low concentrations but inhibits partially at 1 μ M and almost completely at 10 μ M concentrations. We then determined the amount of Z-YVAD-fmk inside the treated neurons based on standard curves (correlation coefficients of 0.95–0.98) of de-esterified Z-YVAD-fmk on R-Csp-1 activity. We find that there is $0.50 \mu\text{M} \pm 0.15$ Z-YVAD-fmk remaining in cells after 1.5 h and $0.088 \mu\text{M} \pm 0.01$ Z-YVAD-fmk after 24 h. These amounts are under the level of inhibitor that would significantly and directly inhibit Csp-6. These results suggest that Csp-1 activity is involved in the conversion of pro-Csp-6 into active Csp-6 in serum-deprived neurons and contributes to serum-deprivation-mediated neuronal cell death. In contrast, the other two initiator Csp, Csp-2 and Csp-9, are unlikely to be involved in Csp-6 activation since their inhibitors do not prevent Csp-6-mediated cell death by serum deprivation.

Figure 1 Csp-1 inhibitor, but not Csp-2, -3, or -9 inhibitors, prevents activation of Csp-6 and cell death in serum-deprived human neurons. (a) Csp-6 activity in serum-deprived neurons treated with 5 μ M caspase inhibitors. Data represent the mean and S.E.M. of three independent experiments. *Indicates statistically significant inhibition of Csp-6 activity with these inhibitors at $P < 0.05$ relative to control (Ctl). (b) TUNEL-positive neuronal cell death in serum-deprived neurons treated without Ctl or with various Csp inhibitors. The + serum (+S) represents neurons kept under normal culture conditions. Data represent the mean and S.D. of four independent experiments * $P < 0.02$ indicates a statistical difference from serum-deprived neurons without Csp inhibitors. (c) A 10630 antisera immunoblot of the active p20 subunit of Csp-6 in serum-deprived (–S) neurons treated with (+ YVAD) or without (–YVAD) Z-YVAD-fmk Csp-1 specific-inhibitor. (d) Percent inhibition of R-Csp activity in an *in vitro* assay of R-Csp-1 or R-Csp-6 with Ac-YVAD-CHO, Z-YVAD-fmk and de-esterified Z-YVAD-fmk (Z-YVAD-fmk^{DE}). Data represents the mean and S.E.M. of three independent experiments. * $P < 0.001$ compared with no inhibitor

Csp-1-like activity occurs within 30 min of serum deprivation in human neurons

To confirm which upstream Csp activates Csp-6 in serum-deprived neurons, Csp activity was measured using Csp-specific fluorogenic peptide substrates. We chose to study very early times of serum deprivation in order to identify the first Csp activated after serum deprivation. Csp-6-like VEIDase activity is significantly increased within 1.5 h of serum deprivation whereas the more common effector, Csp-3, is not activated (Figure 2a). A small transient increase of Csp-1-like YVADase activity occurs at 30 min of serum deprivation; this activity returns to normal at 1 h of serum deprivation (Figure 1a). In contrast, there is no increase in Csp-9-like LEHDase activity within 1.5 h and Csp-2-like VDVADase activity unexpectedly decreases by 50% at 1 and 1.5 h of serum deprivation. Western blot analysis confirms the activation of Csp-6 since the pro-Csp6 decreases while the p20 subunit of Csp-6 increases with time of serum deprivation (Figure 2b). As previously observed, pro-Csp-3 levels remain constant (Figure 2b)³ and since there is no increased DEVDase activity, we can also rule out the activation of effector Csp-7. Initiator pro-Csp-9 levels remain normal (relative to β -actin levels) and pro-Csp-2 levels decrease considerably, consistent with the observed decrease in VDVADase activity (Figure 2a and c). The biological significance of the diminished pro-Csp-2 levels and VDVA-Dase activity is not clear at this time. A more complete analysis of Csp-1 reveals that the large p23 subunit of Csp-1 increases within 30 min of serum deprivation (Figure 2d). The lower levels of Csp-1 and p23Csp-1 subunit between 24 and 96 h reflect a decreased loading of protein as observed with β -actin immunostaining. The elevated levels of Csp-1 subunit at 1–3 h while the activity returns to normal at 1 and 1.5 h of serum deprivation (Figure 2a) indicate that, as described before, the active subunit may be rapidly negatively regulated upon activation, rapidly secreted or degraded.¹⁷ No active subunits of Csp-2, -3 or -9 appear with time of serum deprivation. The absence of Csp-9 active subunits does not necessarily indicate inactivity since Csp-9 can be active by dimerization.¹⁸ Similarly, active subunits can degrade rapidly or aggregate in insoluble form¹⁹ and remain undetected. However, these data are consistent with the lack of increased activity of Csp-2, -3 or -9 in serum-deprived neurons (Figure 2a). These data indicate that Csp-1, although only transiently activated, is the only Csp activated before Csp-6 in serum-deprived neurons.

Csp-1 activates Csp-6 *in vitro*

To investigate if Csp-1 can directly activate Csp-6, R-Csp-1 was incubated *in vitro* with recombinant pro-Csp-6. Recombinant active Csp-1 generates the p20 subunit of Csp-6 in a dose-dependent manner (Figure 3a). The anti-p20Csp-6 antisera does not cross-react with the R-Csp-1 (Figure 3b). The 20 kDa size of the Csp-6 fragment is confirmed in a less overloaded Western blot (Figure 3c). Similarly, the catalytic mutant proCsp6 is also cleaved by recombinant active Csp-1 confirming that the cleavage of pro-Csp-6 into its p20 subunit is not the result of self-activation (Figure 3c). Furthermore, in

Figure 2 Caspase activity in serum-deprived human neurons. (a) Csp-1-, Csp-2-, Csp-3-, Csp-6-, and Csp-9-like activity measured with Ac-YVAD-AFC, Ac-VDVAD-AFC, Ac-DEVD-AFC, Ac-VEID-AFC and Ac-LEHD-AFC, respectively at various times of serum deprivation. The activity with serum deprivation was expressed relative to the activity at time 0 which was arbitrarily placed at 1. The data represent the average and S.E.M. of eight independent experiments. * $P < 0.05$. (b) Western blots of effector pro-Csp-3, pro-Csp-6, p20 subunit of Csp-6 (P20Csp-6) and β -actin in serum-deprived human neuron cultures. (c) Western blots of initiator pro-Csp-9, pro-Csp-2 and β -actin in serum-deprived human neuron cultures. (d) Western blots of pro-Csp-1 and p23 subunit of active Csp-1 (p23Csp1) and β -actin in serum-deprived human neuron cultures

neuronal protein extracts, R-Csp-1 cleaves endogenous pro-Csp-6 into its active p20 subunit and cleavage is inhibited with the Csp-1-like inhibitor, Z-YVAD-fmk (Figure 3d). VEIDase activity is generated in Csp-1-treated neuronal protein extracts or recombinant proCsp-6 (Figure 3e). In contrast, Csp-1 cleavage of the catalytically inactive Csp-6 mutant (Csp-6C/A) does not produce VEIDase activity. These results indicate that recombinant active Csp-1 can convert pro-Csp-6 into its active subunits and generate Csp-6 activity.

Figure 3 Recombinant active Csp-1 cleaves and activates pro-Csp-6 *in vitro*. (a) Western blots of recombinant pro-Csp-6 (ProCsp-6) or p20 Csp-6 subunit (p20Csp6) after incubation of recombinant pro-Csp-6 with various amounts of recombinant active Csp-1. (b) Western blot of R-Csp-1 and R-Csp-6 with anti-p20Csp-6 and re-probed with anti-Csp-1 antisera after stripping. (c) Western blot of Pro-Csp-6 or catalytically inactive Pro-Csp-6 (Csp-6C/A for the C163A mutation) incubated with or without R-Csp-1. (d) Western blot of neuronal pro-Csp-6 (Csp-6) incubated with or without R-Csp-1 in the absence or presence of Z-YVAD-fmk. (e) Csp-6 VEIDase activity (mean $\pm$ S.E.M.) measured from R-Csp-6, Csp-6 C/A and neuronal pro-Csp-6 treated with 30 units of R-Csp-1. N.D. indicates nondetectable caspase activity and N.E. indicates neuronal protein extracts. * $P < 0.02$ comparing R-Csp-1 treated with nontreated

Microinjected R-Csp-1 does not induce cell death in primary human neurons but dominant-negative Csp-1 prevents serum-deprivation-induced cell death

To assess if active Csp-1 triggers the downstream activation of Csp-6 in human neurons not submitted to an apoptotic insult, recombinant active Csp-1 was microinjected into the cytosol of human neurons at various concentrations and cell death assessed at 4 and 6 days after the injection. In contrast to previous observations with recombinant active Csp-6,⁴ recombinant active Csp-1 did not induce cell death even at the highest dose and after 6 days (Figure 4a). To further test the implication of Csp-1 in serum-deprivation-induced Csp-6 activity, neurons were transfected with a dominant-negative form of Csp-1 (Csp-1DN). The Csp-1DN, but not the control EGFP vector, inhibits apoptotic cell death in serum-deprived neurons (Figure 4b). Overall, these results confirm that activation of Csp-1 is necessary, but not sufficient, to induce serum deprivation mediated cell death in the absence of any other insult.

Discussion

Upstream activators of Csp-6 have yet to be uncovered. In this paper, we show that Csp-1 activation occurs within 30 min of

Figure 4 Microinjected active Csp-1 does not induce human neuronal cell death. (a) TUNEL-positive cell death in neurons at 4 and 6 days after microinjection with varying doses of active R-Csp-1 or 2.5 pg of R-Csp-6. Microinjections were performed in 200 neurons for each of three independent neuronal preparations. * $P < 0.001$ compared with non-R-Csp-injected neurons. (b) Percent cell death in EGFP alone (Ctl) or EGFP plus Csp-1 DN-transfected human neurons after 24 h of serum deprivation. Data represent the mean and S.E.M. of five independent experiments. * $P < 0.001$ by *t*-test

serum deprivation in primary cultures of human neurons, that the Z-YVAD-fmk Csp-1-like inhibitor prevents serum deprivation-induced Csp-6 activation and neuronal cell death, that Csp-1 DN inhibits serum deprivation induced cell death, and that R-Csp-1 cleaves and activates purified recombinant or neuronal endogenous pro-Csp-6 *in vitro*. To our knowledge, this is the first demonstration of Csp-1-mediated activation of Csp-6.

This finding may help identify upstream regulators of Csp-6 in human conditions. There are at least four pathological conditions in which Csp-6 is known to be highly activated. First, abundant Csp-6 activity is observed in dystrophic neurites and neurofibrillary tangles of AD.⁵ Zhu *et al.*²⁰ show a 2.5-fold increase of pro-Csp-1 in AD medial temporal tissues compared to controls and Csp-1 mRNA is increased in AD.²¹ The most recognized function of Csp-1 is the conversion of pro-interleukin-1 β (IL-1 β) into mature IL-1 β thereby eliciting an inflammatory response (reviewed by Friedlander²²). IL-1 β is increased in AD but mostly associated with microglial activation suggesting a role for Csp-1 in inflammation.^{20,23–25} On the other hand, significant evidence from studies using Csp-1 inhibitors, a Csp-1 DN construct and Csp-1 null cells indicates that Csp-1 can be involved in cell death.^{26–29} The increase of Csp-1 in AD raises the possibility that Csp-1 also activates Csp-6 *in vivo*. Unfortunately, despite serious attempts, we were unable to show the active subunits of

Csp-1 in AD tissues. Therefore, the implication of Csp-1 in Csp-6-mediated cell death in AD is uncertain at this time. Second, high levels of active Csp-6 are present in human fetal and adult ischemia.⁵ There are no reports of active Csp-1 in human cerebral ischemia but in animal models, Csp-1 is activated and inhibition of Csp-1 protects against cerebral ischemia.^{30,31} Therefore, it is possible that the activation of Csp-1 in ischemia leads to the activation of Csp-6. Third, activation of Csp-6 occurs before Csp-3 in a rat kainic acid seizure model.^{12,32} The active subunit of Csp-1 has also been detected in human temporal lobe epilepsy.³³ Lastly, active Csp-6 is most important in anoikis of the gastrointestinal lining epithelial cells.³⁴ However, in this situation, Csp-1 remains as a proenzyme and thus is probably not responsible for the activation of Csp-6.³⁴

Our results suggest that Csp-1 activation of Csp-6 and induction of cell death requires additional factors since the microinjection of recombinant active Csp-1 cannot induce cell death, as opposed to the active Csp-6. Positive and negative regulators of Csp-1 have been identified (reviewed by Maritoni and Tschopp³⁵). However, these factors are usually involved in the activation of R-Csp-1 and not in enhanced activity. The ASC, apoptosis-associated speck-like protein containing a Csp recruitment domain (CARD), Ipaf1, and Receptor interacting protein 2 (Rip2), also known as Rick or Cardiak, are three proteins that interact with and activate Csp-1. Over expression of Rip2 usually occurs in LPS-activated macrophages resulting in activation of Csp-1 to promote the inflammatory response via the production of IL-1 β . It has recently been demonstrated that Rip2 expression is also induced in oxygen/glucose-deprived neurons.²⁹ ASC is not predominantly expressed in neurons³⁶ but Ipaf1 mRNA has been shown in the brain.³⁷ These adaptor proteins activate Csp-1 through interaction and oligomerization of pro-Csp-1. Therefore, these proteins should not alter the already active Csp-1 injected in these human neurons. The possibility that the injected active Csp-1 is inefficient due to rapid turnover is unlikely because we have previously shown that microinjected active Csp remain detectable in the human neurons for 24 h⁴ and very high non physiological doses of R-Csp-1 fail to induce cell death. The possibility that Csp-1 inhibitors exist remains a viable alternative. There are several identified inhibitors of Csp-1 but all inhibit the activation and not the activated Csp-1. Therefore, it is unlikely that any of these is the inhibitor that prevents Csp-1-mediated cell death in the microinjected cells. However, an inhibitor with a mechanism similar to inhibitor of apoptosis proteins (IAPs) would be possible. IAPs prevent Csp-3, -7 and -9 activity and this inhibition is abolished by the release of mitochondrial IAP inhibitors, Smac/Diablo or Omi/HtrA2, during an apoptotic insult.³⁸ While these are not good inhibitors of Csp-1, similar IAPs and mechanisms could exist to allow active Csp-1 to activate Csp-6 in apoptotic conditions. These results may explain the controversy over whether Csp-1 is an apoptotic or an inflammatory Csp. Under inflammatory conditions, the active Csp-1 may function as an IL-1 β converting enzyme with minimal Csp-6 activating activity while, in apoptosis, a co-activator or release of IAP-like inhibitors of Csp-1 would allow Csp-1 to activate most of the Csp-6 subsequently resulting in neurodegeneration and cell death.⁵

In summary, our study shows that Csp-1 activates Csp-6 in serum-deprived human neurons and results in neuronal cell death. Our experimental results also suggest that Csp-1 requires an additional factor to induce Csp-6-mediated cell death in human neurons. These data may explain the difference between the inflammatory and the apoptotic Csp-1 response.

Materials and Methods

Reagents and antibodies

Purified R-Csp-1, Ac-YVAD-CHO, Boc-D-fmk, Z-YVAD-fmk, Z-DEVD-fmk, Z-VEID-fmk, Z-IETD-fmk, Z-LEHD-fmk, Ac-YVAD-AFC, Ac-VDVAD-AFC, Ac-DEVD-AFC, Ac-VEID-AFC, Ac-LEHD-AFC were purchased from BioMol (Philadelphia, PA, USA). The anti-Csp-1 polyclonal antibody recognizing full-length and the p23 fragment of Csp-1 was from Upstate (Lake Placid, NY, USA), the anti-Csp-2 Ab1 polyclonal antibody recognizing full-length and the p10 fragment of Csp-2 and the anti-Csp-6 polyclonal antibody recognizing full length and the p20 fragment of Csp-6 were from NeoMarkers (Fremont, CA, USA). The 10630 antiactive Csp-6 p20 antibody was made in our laboratory,⁵ the anti-Csp-3 polyclonal antibody recognizing the full length and the p17 subunit of Csp-3 was a gift from Dr. Nicholson (Merck Frost, Pointe-Claire, Quebec, Canada), the anti-Csp-9 polyclonal antibody recognizing the full-length and its p20 subunit was from Pharmingen (San Diego, CA, USA), and the anti- β -actin antibody was from Sigma (St Louis, MI, USA).

Human primary neuronal culture

Human primary neurons were cultured as described previously.³⁹ The McGill Institutional Review Board approved the protocol. Briefly, 12–16-week-old fetal brains were dissociated with trypsin, treated with deoxyribonuclease I, filtered through 130 and 70 μ m nylon mesh and plated on poly-lysine-coated coverslips or dishes at a density of 3×10^6 cells/ml. In general, the cultures contain ~90% neurons and 10% astrocytes. Microglial cells are eliminated with the treatment of the antimitotic agent, fluorodeoxyuridine. The experiments were conducted after 10 days of neuronal culture.

Determination of Csp activity

Human primary neurons were serum-deprived for 0, 0.5, 1 and 1.5 h. At different time points, proteins were extracted with an ice-cold cell lysis buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA and complete mini, EDTA-free protease inhibitor cocktail ((Roche, Basel, Switzerland) freshly added). The cell lysate was spun at $13\,000 \times g$ for 5 min at 4°C to remove any detergent insoluble proteins. The supernatant was collected and total protein concentration was quantified with the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Samples were maintained at –80°C until assayed or assayed immediately. Csp activity was measured by mixing 10 μ g of neuronal protein extract with 50 μ g/ml Ac-YVAD-AFC for Csp-1-like, Ac-VDVAD-AFC for Csp-2-like, Ac-DEVD-AFC for Csp-3-like, Ac-VEID-AFC for Csp-6-like and Ac-LEHD-AFC for Csp-9-like activities in Csp reaction buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, 1 mM EDTA, 10% sucrose). The time-dependent release of AFC was detected with a Bio-Rad Fluoromark fluorometer (Hercules, CA, USA) at an excitation wavelength of 390 nm and an emission

wavelength of 538 nm. Measurements were read every 2 min for 1 h and released moles of AFC calculated from a standard curve of AFC.

Determination of Csp levels

At different times of serum deprivation, human primary neurons were extracted in 1% SDS and 100 μ g of total protein was separated on a 15% polyacrylamide gel and assessed by Western blotting using conventional methods with the primary anti-Csp-1, -2, -3, -6 and -9 antibodies. Immunoreactivity was revealed with HRP-conjugated secondary antibodies and Western lightning TM reagent plus chemiluminescence (PerkinElmer, Wellesley, MA, USA).

Effect of various Csp inhibitors on serum-deprivation-induced Csp-6 activity in human primary neurons

Human primary neurons were serum-deprived 1.5 h in the presence or absence of the following Csp inhibitors: 5 μ M Boc-D-fmk for general Csp inhibition, Z-YVAD-fmk for Csp-1-like inhibition, Z-VDVAD-fmk for Csp-2-like inhibition, Z-VEID-fmk for Csp-6-like inhibition, Z-IETD-fmk for Csp-6- and 8-like inhibition and Z-LEHD-fmk for Csp-9-like inhibition. Proteins were extracted and Csp activity measured as described above with 50 μ g/ml Ac-VEID-AFC.

Effect of Csp inhibitor on recombinant active Csp-6 *in vitro*

The activity of recombinant Csp-1 and Csp-6 was assessed in the presence or absence of 0.5 nM, 1.0 nM, 100 nM, 500 nM, 1.0 μ M and 10.0 μ M of Ac-YVAD-CHO, Z-YVAD-fmk, and de-esterified Z-YVAD-fmk. Z-YVAD-fmk is esterified on the D residue. The de-esterification of Z-YVAD-fmk was performed on ice for 15 min in a solution containing 50 U/ml of porcine esterase (Sigma, St Louis, MI, USA) in cold 0.1 M borate buffer adjusted to pH 8.0 (from Calbiochem's and Sigma's suggested protocols). The esterase-treated inhibitor was diluted 1:10 prior to the Csp activity assay, which was performed as described above. De-esterification of Z-YVAD-fmk was verified by analytical HPLC on a Varian ProStar 210 system coupled to a ProStar UV-VIS detector (Varian Inc., Palo Alto, CA, USA) set at 270 nm. Analytical HPLC was performed using a C-18 column (150 $\times$ 4.60 mm), with a pore size of 5 μ m. In all, 25 μ g of untreated or esterase-treated Z-YVAD-fmk in borate buffer (as described above) was injected and eluted with a linear gradient of 0–100% acetonitrile (Fisher Scientific Canada, HPLC grade) in 0.1% TFA for 20 min, with a flow rate of 1 ml/min. De-esterification of Z-YVAD-fmk was observed as a shift in the retention time (R_t) of the compound.

Determination of cellular levels of Z-YVAD-fmk after 1.5 and 24 h incubation with 5 μ M Z-YVAD-fmk

Human primary neurons were treated for 1.5 or 24 h in presence or absence of 5 μ M Z-YVAD-fmk. After the incubation period, the cells were washed in phosphate-buffered saline and lysed in ice-cold cell lysis buffer and total protein concentration was quantified as described before. R-csp-1 activity was measured in the presence of 10 μ g of neuronal protein extract. A standard curve using known concentration of de-esterified Z-YVAD-fmk was drawn as percent inhibition over the amount of inhibitor in grams. The correlation coefficients for these standard curves were from 0.95 to 0.98. The amount of Z-YVAD-fmk present in each sample

($\sim 6 \times 10^6$ neurons) was determined from this standard curve of Z-YVAD-fmk. The molarity of Z-YVAD-FMK present in each cell was determined using the mean volume of human neurons. Since our primary human neuron cultures are composed of multiple types of neurons, and since the size of a neuron may vary from 4 to 100 μ m in diameter, we averaged the radius to 26 μ m (0.026 mm). Using the formula for the volume of a sphere ($4/3\pi r^3$), and considering that 1 m³ contains 1 l, we estimated the mean volume of our neurons to 73.6 pl.

Determination of neuronal cell death

Neurons were serum-deprived for 24 h in the absence or presence of 5 μ M of the following Csp inhibitors: BOC-D-fmk for general Csp inhibition, Z-YVAD-fmk for Csp-1-like, Z-VDVAD-fmk for Csp-2-like, Z-VEID-fmk for Csp-6-like, Z-IETD-fmk for Csp-8-like, Z-LEHD-fmk for Csp-9-like. Neurons were fixed in freshly prepared 4% paraformaldehyde and 4% sucrose for 30 min at room temperature. The cells were washed with PBS, permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice. Cell death levels were assessed by TUNEL following the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN, USA) and counterstaining with Hoechst dye (0.5 μ g/ml) for 20 min. The percentage of cell death was determined by calculating the number of TUNEL-positive neurons (green fluorescence) over the total number of neurons (blue fluorescence by Hoechst staining). Results were obtained by averaging neuronal counts from 150 to 500 neurons per experiment for four independent neuron preparations.

Csp-1 cleavage and activation of recombinant pro-Csp-6 and Csp-6 C/A or neuronal pro-Csp-6

The pro-Csp-6 human cDNA construct in the pEt23b prokaryotic expression vector was a kind gift from Dr. Guy Salvesen (Burnham Institute, LaJolla, CA, USA). The catalytic mutant C163A was made with the Quick Change Site-directed Mutagenesis kit (Stratagene, LaJolla, CA, USA) using the sense 5' CAGGTCAGCTCGGGGA 3' and antisense 5' TCCCCGAGCTGCCTG 3' primers. Expression of Csp was induced with 0.05 mM IPTG for 16 h. About 5 μ g of crude *E. Coli* protein extracts were incubated with 0–100 U of purified active Csp-1 (Biomol, Philadelphia, PA, USA) in Csp-1 reaction buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10 mM DTT) for 4 h at 37°C. Western blotting was performed with anti-Csp-6 antibodies (NeoMarkers or 10630). Csp-6 activity was measured with Ac-VEID-AFC as described above. For Csp-1 activation of neuronal pro-Csp-6, 50 μ g of neuronal proteins extracted in cell lysis buffer were incubated with 100 U of purified R-Csp-1 in the absence or presence of Z-YVAD-fmk for 4 h at 37°C.

Microinjection of recombinant active Csp-1 and determination of cell death

Neurons were microinjected as described previously with 0.01, 0.25 and 2.5 pg/cell of purified active R-Csp-1 (BioMol, Philadelphia, PA, USA) and 100 μ g/ml Dextran Texas red (Cedarlane Laboratories Ltd. Ontario, Canada) in Csp-1 reaction buffer.^{4,40} In all, 100 cells on each of two cover slips were microinjected. Each experiment was repeated on three independent neuronal preparations. Cell death was measured blindly by TUNEL according to the manufacturer's protocol (*In Situ* Cell Death Detection Kit (A, P), Roche Applied Science, Laval, QC, Canada) at 4 and 6 days postmicroinjection. The percentage of cell death was calculated as the ratio of DTR-TUNEL double-positive neurons over DTR-positive cells.

Approximately 80% of cells survive the microinjection and there was no difference in percentage recovery in cells injected with or without Csp-1.

Transfection of human neurons with Csp-1 dominant-negative construct

The Csp-1 DN construct²⁶ was a kind gift from Dr. Robert Friedlander (Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA). The cDNA was transfected into human neurons at a ratio of 4 : 1 with an EGFP-expressing cDNA into the pBud vector (Invitrogen, Ontario, Canada) with a gene gun. Over 100 cells were counted in five independent neuronal preparations. Cell death was determined by visualizing condensed and fragmented chromatin with Hoescht staining. The data are expressed as the percentage of cells with condensed or fragmented chromatin over the total number of cells transfected.

Statistical evaluations

The statistical significance of different treatments was determined by ANOVA with *post hoc* tests (Statview 5.01). The difference between various treatment groups *versus* the control group was determined by Scheffé's test and $P < 0.05$ was taken as the criterion for statistical significance.

Acknowledgements

This work was supported by CIHRMOP15118, NIH NS/MH40965, VRQ and FRSQ to ALB. We thank Dr. Robert Friedlander for the kind gift of Csp-1 DN, Jennifer Hammond for technical assistance with the neuronal cultures and Guy Klaiman and Heather Turan for generating recombinant normal and catalytically inactive Csp-6, Tracy Petzke and Dr. Hinyu N Nedeve for help with the HPLC analysis of de-esterification.

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