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Viral caspase inhibitor p35, but not crmA, is neuroprotective in the ischemic penumbra following experimental stroke

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Abstract

Apoptosis, a predominant cause of neuronal death after stroke, can be executed in a caspase-dependent or apoptosis inducing factor (AIF)-dependent manner. Herpes Simplex Virus (HSV) vectors expressing caspase inhibitors p35 and crmA have been shown to be neuroprotective against various excitotoxic insults. Here we further evaluated the possible neuroprotective role of p35 and crmA in a rat stroke model. Overexpression of p35, but not crmA, significantly increased neuronal survival. Results of double immunofluorescence staining indicate that compared with neurons infected with crmA or control vectors, p35-infected neurons had less active caspase-3 expression, cytosolic cytochrome c and nuclear AIF translocation.

Keywords

cerebral ischemia; apoptosis; caspase inhibitor; crmA; p35; gene therapy; stroke

Introduction

Both caspase-dependent and –independent apoptotic pathways contribute to ischemic damage after stroke (Graham and Chen, 2001, Plesnila et al., 2001, Cao et al., 2003, Zhao et al., 2004, Zhang et al., 2005, Niimura et al., 2006). In the caspase-dependent pathway, caspase 8 activation causes mitochondria injury and cytochrome c release (Yin et al., 2002), which then triggers caspase 9 and caspase 3 activation (Li et al., 1997). In the caspase-independent pathway, apoptosis inducing factor (AIF) translocates from the mitochondria to the nucleus, where it causes DNA fragmentation (Susin et al., 1999). Upregulation of active caspase-3, 8 and 9 has been detected in vulnerable neurons after cerebral ischemia (Ferrer and Planas, 2003), and caspase-3 knock out mice have smaller infarctions (Le et al., 2002), suggesting such components of the classical apoptotic pathways play critical roles for neuronal death after stroke. Caspase-1 also contributes to ischemic damage, although it worsens ischemic damage through its ability to enhance the inflammatory response (Jander et al., 2002). In addition, many synthetic caspase inhibitors have been shown to be neuroprotective in injury models, including stroke (Bump et al., 1995, Fink et al., 1998, Zhao et al., 2005b).

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In this study, we address whether the use of Herpes simplex virus (HSV)-mediated gene therapy to overexpress caspase inhibitors can reduce ischemic damage in a stroke model caused by permanent distal MCA occlusion. Viral-based gene delivery systems are powerful tools for understanding the mechanisms of ischemic neuronal death and survival after stroke (Sapolsky and Steinberg, 1999, Harvey et al., 2003, Zhao et al., 2006b). Our laboratories have demonstrated that HSV-mediated gene therapy with Bcl-2, HSP72 or glucose transporter, among other transgenes, protects against ischemic neuronal loss in global and focal ischemic models (Lawrence et al., 1995, Yenari et al., 1998, Zhao et al., 2004). Despite the fact that conventional caspase inhibitors composed of synthesized peptides have been shown to attenuate ischemic damage after stroke (Rabuffetti et al., 2000, Plesnila et al., 2001), there are several reasons for us to employ gene therapy with virally derived caspase inhibitors to treat stroke in this study.

First, virally-derived caspase inhibitors are more physiological compared with synthesized peptides, and they are homologs of some mammalian genes; thus gene therapy using such virally-derived caspase inhibitors may provide insights into the action of the endogenous genes and clarify the underlying mechanisms (Roy et al., 2001, Roy et al., 2002). We intended to compare the neuroprotective effects of two well known caspase inhibitors, baculoviral p35 and cowpox virus crmA. P35 is a pan caspase inhibitor, which inhibits caspases 1, 3, 6, 7, 8, and 10 while crmA selectively inhibits caspases 1 and 8 (Bump et al., 1995, Zhou et al., 1997). Using the HSV vector system and primary neuronal cultures, we have demonstrated the neuroprotective ability of p35 and crmA after excitotoxic stimuli (Roy et al., 2001, Roy et al., 2002, Roy and Sapolsky, 2003). In this study we further compare the protective effects of p35 and crmA. Second, a single administration of synthesized peptides may provide only transient inhibition. In contrast to administered peptides, HSV-mediated gene delivery enables sustained gene expression. In addition, HSV vectors preferentially infect neurons (Yenari et al., 2001), enabling us to preferentially track the protective effect of caspase inhibitors in neurons. Lastly, there is cross talk between caspase-dependent and AIF apoptotic pathways. We therefore further studied the possible modulatory effects of these viral caspase inhibitors on apoptosis, focusing on the activation of caspase 3, cytosolic translocation of cytochrome c and nuclear translocation of AIF.

Materials and Methods

Generation of amplicon plasmids and HSV vectors

The cloning of p35 and crmA plasmids have been documented previously (Roy et al., 2001). The DNAs were cloned individually into the bicistronic expression plasmid, p α 4 α 22 β gal. The control plasmid used in the study was p α 4 α 22 β gal, a monocistronic plasmid containing only the β -gal gene (Lawrence et al., 1995). We have shown previously that transfection efficiency of the monocistronic vector is comparable with bicistronic vectors (Yenari et al., 1998, Yenari et al., 2001, Zhao et al., 2003).

Viral vector production has been described previously in detail (Ho, 1994). Plasmid titers of p35, crmA and control vector ranged from 5.5×10^6 to 7.5×10^6 particles/ml.

Vector delivery & focal cerebral ischemia

All animal protocols were approved by the Stanford University Administrative Panel on Laboratory Animal Care. In this study, a focal ischemia model generated by bilateral common carotid artery (CCA) occlusion plus permanent distal middle cerebral artery occlusion (MCAO) was employed (Chen et al., 1986). As we previously demonstrated, this model generates well-defined and highly reproducible cortical infarction (Zhao et al., 2005a, Zhao et al., 2006a). This enabled us to deliver the HSV vectors into a pre-assumed ischemic margin even before

ischemia onset. In this study, thirty-six male Sprague-Dawley rats (Charles River, Hollister, CA) weighing 280–320 g were used. P35, crmA and control vectors, 2.5µl each, were delivered bilaterally (n=12 rats/each group) at the following coordinates: AP –2 mm and ML ±2.8 mm, relative to bregma. At 12 to 16h after vector injection, rats underwent permanent middle cerebral artery occlusion (MCAO) combined with 1h occlusion of bilateral common carotid artery (CCA) as described previously (Zhao et al., 2004). Briefly, isolation of both CCAs was followed by small craniectomy at the junction of zygomatic arch and squamous bone. With aneurysm clips, both CCAs were clipped and distal MCA was coagulated and cut. The clips were released 1h after coagulation of MCA.

X-gal staining and cell counts

The bipromoter vector system employed in this study co-expresses both transgene and reporter gene in a similar pattern, as described previously (Lawrence et al., 1996, McLaughlin et al., 2000, Yenari et al., 2001). The reporter gene driven by the β -gal transcriptional unit demonstrates more than 98% covariance in expression with the transgene (Fink et al., 1997). In addition, we have shown that this HSV vector is neurotropic (Yenari et al., 2001). We have verified gene expression of p35 and crmA by RT-PCR in vitro (Roy et al., 2001). However, HSV based gene therapy does not change the overall infarction due to its limited transfection efficiency (Yenari et al., 2001, Zhao et al., 2003). Therefore, the protective effect of gene therapy is detected by counting the X-gal-positive neurons and compared with the number of infected neurons in the non-ischemic cortex. The same criteria from previous studies for cell counting was employed: only those X-gal positive cells in the cortex which showed neuronal morphology with extended processes and cell body diameter roughly from 15–25 µm were counted (Yenari et al., 2001, Zhao et al., 2003). Rats were sacrificed 48h after MCAO. After transcardial perfusion with PBS followed by 4% paraformaldehyde (PFA), brains were postfixed using 4% PFA with 20% sucrose solution for 24h and prepared for cryostat sectioning. Sections of 30µm were collected and every third section was stained with X-gal (5'-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; Molecular Probes, Eugene, OR) to visualize control, p35 and crmA transduction. Slides were counterstained with cresyl violet to visualize infarct and cellular morphology. The number of infected X-gal positive neurons in the ischemic cortex was counted from 10 consecutive adjacent slides, divided by the number in the contralateral non-ischemic cortex and expressed as a percentage (Yenari et al., 1998, Yenari et al., 2001, Zhao et al., 2004).

Immunofluorescent staining & laser-scanning confocal microscopy

We have previously shown that activated caspase-3, cytosolic cytochrome c release and nuclear AIF translocation all appear as early as a few hours after stroke and remain until 48 hours after stroke, and gene therapy of Bcl-2 could influence these apoptotic events (Zhao et al., 2003, Zhao et al., 2004). To minimize the number of rats used in this study, brain tissues used for X-gal staining were also utilized for immunostaining. In each group (n=5), 7–10 slices adjacent to a positive X-gal stained section were washed in PBS for 3×10 minutes and then blocked in PBS containing 5% donkey serum (Sigma, St. Louis, MO) and 0.3% triton X-100 for 2 hours at room temperature. Two primary antibodies were mixed in the blocking solution and applied onto the slides, and incubated at 4°C overnight as described (Zhao et al., 2003, Zhao et al., 2004). Negative controls, in which primary antibodies were omitted, were run in parallel. For caspase 3 and β -gal double staining, rabbit anti-activated caspase 3 polyclonal antibody (PAB CM1; 1:200; Pharmingen, San Diego, CA) and mouse anti- β -galactosidase antibody (1:200; Sigma) were used. For cytochrome c and β -gal double staining, mouse anti-cytochrome c antibody (1:200; Pharmingen) and rabbit anti- β -galactosidase antibody (1:200; MP Biomedicals, Irvine, CA) were used. For AIF and β -gal double staining, goat anti-AIF antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti- β -galactosidase antibody (1:200) were used. After being washed with PBS, the sections were incubated in the mixed

secondary antibodies for 2 h at room temperature. For detection of caspase 3 and β -gal, Cy-3 conjugated donkey anti-mouse (1:200; Jackson ImmunoResearch, West Grove, PA) and FITC-conjugated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch) were used. For detection of cytochrome c and β -gal, Cy-3 conjugated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch) and FITC-conjugated donkey anti-mouse (1:200; Jackson ImmunoResearch) were used. For detection of AIF and β -gal, biotinylated anti-goat IgG (1:100; Vector laboratories, Burlingame, CA) and Cy-3 conjugated donkey anti-rabbit IgG (1:100) were used. For AIF staining, after the secondary antibody incubation, FITC-conjugated streptavidin (1:100; Molecular Probes) was added and incubated for 1h at room temperature. After washing in PBS, all slices were stained by 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) for 2 minutes and mounted and examined under a LSM 510 confocal laser scanning microscope (Carl Zeiss, Thornwood, NY). Percentages of neurons which express active caspase-3, cytochrome c or AIF among total β -gal positive neurons were calculated.

Statistical analysis

Data are presented as mean $\pm$ SEM. Differences between groups were determined by one way ANOVA followed by a Tukey post hoc test. Tests were considered statistically significant if $p < 0.05$.

Results

Viral caspase inhibitor p35, but not crmA, significantly protects against ischemic neuronal death

Viral vectors were delivered successfully into the ischemic margin (Figure 1A). The numbers of transfected surviving cells at 48 h infected with control vector in the ischemic cortex and the number of those in the non-ischemic cortex are 57 ± 7 and 122 ± 13 , respectively; with p35 vectors, they are 278 ± 69 and 332 ± 40 , respectively; with crmA, they are 220 ± 62 and 310 ± 53 , respectively (mean $\pm$ SEM). The percentages of neuronal survival at 48 h after stroke in animals treated with viral vectors of p35, crmA and control vector were $80.7 \pm 11.1\%$, $61.0 \pm 7.3\%$ and $49.5 \pm 5.3\%$, respectively (mean $\pm$ SEM). p35, but not crmA, significantly increased neuronal survival compared to control vector ($p < 0.05$; ANOVA, Tukey post hoc test; Figure 1B).

p35, but not crmA, significantly blocks both caspase-dependent and AIF-dependent apoptotic pathways in the ischemic penumbra

We detected the effects of p35 and crmA on expression of active caspase 3, cytosolic cytochrome c release and nuclear AIF translocation 48h after onset of ischemia. We previously demonstrated that active caspase-3, cytosolic cytochrome c and nuclear or cytosolic AIF was detected by immunostaining only in the ischemic region, and this was confirmed by Western blotting of subcellular fractionations (Zhao et al., 2003, Zhao et al., 2004). Although cytochrome c and AIF are normally expressed in the mitochondria, antibodies may not be able to penetrate through the mitochondrial membrane after fixation (Fujimura et al., 1998); thus immunostaining for cytochrome c and AIF in the non-ischemic hemisphere is not detectable using our techniques (Zhao et al., 2003, Zhao et al., 2004). Consistent with our previous reports (Zhao et al., 2003, Zhao et al., 2004), activated caspase 3 (Figure 2A), cytosolic cytochrome c (Figure 3A) and nuclear AIF translocation (Figure 4A) were detected in the region of the ischemic margin, while no positive staining was detected in the non-ischemic hemisphere. Moreover, double staining indicated that less β -gal positive neurons co-localized with cytochrome c, AIF and active caspase 3 staining in the ischemic cortex treated with p35 when compared to control or crmA vector. However, crmA also showed a trend towards blocking active caspase 3 expression, cytochrome c and AIF release, compared with control vector (i.e., crmA was significantly protective versus control vector when analyzed across all three of the

endpoints, namely cytochrome c, caspase 3 and AIF, but showed only trends towards protection for each endpoint individually). The number of cells expressing activated caspase 3, expressed as a percentage of total β -gal positive cells in the animals treated with control, p35 and crmA vectors were 15.0 ± 2.5 , 0.97 ± 0.49 and $8.1 \pm 2.7\%$, respectively (Figure 2B). This suggested that p35 significantly attenuated caspase 3 activation ($p < 0.05$; ANOVA, Tukey post hoc test). Similarly, p35 also significantly inhibited cytochrome c and AIF translocation. The percentages of cells with cytochrome c release among total β -gal positive cells were 19.0 ± 3.1 , 2.8 ± 0.75 and $15.7 \pm 2.9\%$, respectively, for ischemic brains infected with control, p35 and crmA vectors (Figure 3B). Percentages of nuclear AIF translocation in neurons infected with control, p35 and crmA vectors were 20.0 ± 3.9 , 4.8 ± 1.2 and $13.4 \pm 2.1\%$, respectively (Figure 4B).

Discussion

In this study, we demonstrate that HSV-mediated overexpression of viral caspase inhibitor p35 significantly protects against ischemic neuronal loss after stroke. In addition, we found that p35 not only inhibits caspase 3 activity, but also blocks cytosolic cytochrome c release and nuclear AIF translocation. In addition, crmA showed a trend towards attenuating ischemic neuronal loss and blocking the above apoptotic signals, as compared with the control vector.

We had previously shown that the delivery of various genes by the HSV system reduced ischemic neuronal loss after stroke. These genes include the glucose transporter Glut-1 that enhances ATP generation (Lawrence et al., 1995), Heat shock protein 70 that repairs misfolded proteins (Yenari et al., 1998), calbindin 28 that buffers an increase in intracellular calcium (Yenari et al., 2001) and several anti-oxidant genes that scavenge free radicals (Hoehn et al., 2003, Gu et al., 2004). However, caspase-dependent apoptosis is the dominant cell death mechanism in the ischemic penumbra (Wei et al., 2004). Therefore, it is intriguing to study whether the ischemic penumbra can be targeted by gene therapy, and whether gene therapy with caspase inhibitors blocks ischemic damage. We have previously shown that the HSV based vectors start to express a few hours after delivery (Fink et al., 1997, Yenari et al., 1998). In the current study, we successfully delivered vectors into the ischemic penumbra or the ischemic margin, and found that gene delivery of p35 reduces ischemic neuronal loss after stroke. In contrast, crmA overexpression showed only a trend towards protection. As we have discussed, these differential effects of p35 and crmA may lie in their different ability to inhibit caspase activity. Cowpox viral crmA physiologically inhibits only caspases 1 and 8 (Zhou et al., 1997), while baculoviral p35 protein acts as a pan-caspase inhibitor, including inhibition of caspases 1 and 8 as well as 3 and others (Bump et al., 1995). A straightforward implication is that p35's superior neuroprotective capacity following ischemia is directly due to its role as a pan-caspase inhibitor, inhibiting caspases involved in both the intrinsic and extrinsic apoptotic pathways, while crmA can only modulate caspases involved in the extrinsic pathway. Indeed, consistent with the protective effects, p35, but not crmA, significantly blocks caspase-3 activity after stroke. Plausibly related to this, prior studies have shown them to have differing effects, depending on the nature of the insult model. We have previously demonstrated that both p35 and crmA inhibit neuronal death caused by domoic acid and heat shock in primary mixed neuronal culture (Roy et al., 2001), but do not attenuate sodium cyanide-induced hypoxic damage. Interestingly, caspase activity was absent after domoic acid insult (Roy et al., 2001), suggesting the protection is not executed through blocking this classical apoptotic pathway. Instead, such caspase inhibitors reduce cell damage by attenuating ATP depletion and mitochondrial potential decreases. In addition, their neuroprotective effects differ in vivo from in vitro. While p35 reduces CA3 damage after kainic acid exposure, crmA does not. However, p35 does not attenuate damage in the dentate gyrus caused by the electron transport uncoupler, 3-acetyl pyridine (3-AP), and crmA treatment worsens such injury (Roy et al., 2002). Such differentially protective effects of p35 and crmA in vivo seem consistent with the current

findings of the differing effects of the two. Taken together, these results suggest that the protective effects of p35 and crmA depend on damage models or types of insults.

In addition to inhibiting caspase 3 activity, p35 blocks cytochrome c release and AIF translocation as well. Although caspase activation is cytochrome c-dependent in mitochondrial-mediated apoptosis, it has been demonstrated that caspase activity can form a positive feedback loop onto mitochondria and cause more cytochrome c release, thereby amplifying the apoptotic pathway (Chen et al., 2000). A recent study suggests that deficit in caspase-3 and -7 preserves mitochondrial membrane potential and blocks AIF nuclear translocation (Lakhani et al., 2006). Consistent with this, we have demonstrated that blocking caspase activity can inhibit delayed cytochrome c release in a forebrain ischemia model (Zhao et al., 2005b). In our current study, although we found that the caspase inhibitor blocks cytochrome c release and AIF nuclear translocation, whether it achieves these results by inhibiting the feed back effect of caspase on cytochrome c release or by blocking cross talk between AIF and caspase pathways needs further study.

In the ischemic cortex, the number of positive cells immunostained with active caspase-3, cytochrome c and AIF appears less in non-infected cells in brains treated with P35 vectors than in those treated with other vectors. This raises the possibility that overexpression of p35 may not only protect infected neurons but, through indirect mechanisms, protect neighboring neurons as well; we also observe evidence for such “good neighbor” effects in primary cultures (unpublished data). Whether such effect exists in vivo needs further study.

There are some limitations to this study. HSV based gene transfer can only infect individual neurons. It does not reduce overall infarct size. Therefore, it may not improve the overall neurological outcome. In the future, gene delivery in multiple sites in the ischemic cortex after focal ischemia should be performed to determine if it improves neurological function. In addition, viral vectors were injected before stroke, which is not entirely clinically relevant, since most strokes are not predictable; a future study should address whether vectors delivered post-stroke reduce neuronal loss. Moreover, it might be more appropriate in the future to deliver such genes into the hippocampal region after global ischemia to target delayed selective neuronal death and address its efficacy for preservation of brain function.

In conclusion, our study demonstrated that the viral caspase inhibitor p35 is significantly neuroprotective, while crmA only shows a trend toward neuroprotection, in the ischemic penumbra in a permanent MCAO rat model. P35 expression may block neuronal death by inhibiting both caspase and AIF dependent apoptotic pathways.

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Figure 1.

(A) Virus was delivered into the ischemic margin. A representative coronal brain section stained with cresyl/violet and X-gal from an animal treated with HSV vector. Viral vector was pre-injected into the ischemic margin (left hemisphere) and the corresponding region in the non-ischemic cortex (right hemisphere), blue color indicates transfected region. **(B)** Representative brain sections transfected with control vector, p35 and crmA in both the ischemic and non-ischemic cortex are shown. The number of X-gal positive neurons in ischemic cortex (arrows) was much higher in the brain treated with p35 compared with crmA and control vector. **(C)** Quantification of surviving X-gal positive neurons after infection with viral caspase inhibitors (p35 and crmA) and control vector. The total numbers of X-gal positive

neurons in the ischemic cortex were divided by those in the contralateral cortex and expressed as percentages (mean $\pm$ SEM). Compared to control, p35 gene therapy, but not crmA, significantly increased neuronal survival in the ischemic cortex. (* p <0.05; ANOVA).

Figure 2.

(A) Double immunofluorescence staining of β -gal and activated caspase 3 from brains survived 48 hr after stroke. Activated caspase-3 was not detected in the non-ischemic cortex (data not shown). Transfected neurons with control, p35 or crmA vectors were stained with β -gal (red) and activated caspase 3 (green). Double-labeled neurons are indicated (arrows). Transfected neurons which were not co-labeled with activated caspase 3 were also indicated (asterisks). p35 transfection resulted in fewer double-labeled neurons than control or crmA. β -gal, β -galactosidase; DAPI, 4'6-diamindino-2-phenylindole; scale bar, 20 μ m. (B) Quantification of neurons stained positively for β -gal and activated caspase 3 in brains transfected with viral caspase inhibitors (p35 and crmA) and control vectors. In the ischemic cortex, the number of neurons double-stained with both β -gal and activated caspase 3 were

divided by the total number of β -gal positive neurons and expressed as percentages (mean $\pm$ SEM). p35, but not crmA, delivery inhibited caspase 3 activation (* p <0.05; ANOVA).

Figure 3.

(A) Double immunofluorescence staining of β -gal and cytochrome c in ischemic brains 48 hr after stroke. Double-stained neurons were marked with arrows and neurons stained with β -gal only were marked with asterisks. An inset was inserted to show a magnified transfected cell with cytochrome c release. The cytochrome c was detected in the ischemic cortex but not in the contralateral cortex (data not shown), suggesting that cytochrome c release from the mitochondria into the cytosol. p35 transfection resulted in a smaller number of double-labeled neurons than control or crmA. Scale bar, 20 μ m. (B) Percentage of neurons stained positively with cytochrome c which was also positively stained with β -gal in ischemic brains treated with p35 and crmA and control vectors. p35, but not crmA, delivery inhibited cytosolic translocation of cytochrome c (* p <0.05; ANOVA).

Figure 4.

(A) Double immunofluorescence staining of β -gal and AIF in ischemic cortex 48 hr after stroke. Positive AIF staining was detected in the ischemic but not non-ischemic cortex (data not shown). After 48 hr of MCAO, most of AIF expression localized at nuclei. Double stained neurons with β -gal and AIF and β -gal only are marked with arrows and asterisks, respectively. A transfected cell with nuclear AIF staining was enlarged in an inset. p35 transfection reduced the number of double-labeled neurons than control or crmA. AIF, apoptosis-inducing factor; scale bar, 20 μ m. (B) Percentage of cells with nuclear AIF translocation among transfected neurons. Quantification of neurons stained positively for β -gal reporter and nuclear AIF after infection of viral caspase inhibitors (p35 and crmA) and control vectors. In ischemic cortex, the counts of β -gal and nuclear AIF double-labeled neurons were divided by those of β -gal

single-labeled neurons and expressed as percentages (mean $\pm$ SEM). p35, but not crmA, inhibited nuclear translocation of AIF compared with control vector (* p <0.05; ANOVA).