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BRAIN RESEARCH

Research Report

Poxvirus-derived cytokine response modifier A (CrmA) does not protect against focal cerebral ischemia in mice

Ertugrul Kilic[a,*], *Andreas Wippel*[b], *Ülkan Kilic*[a], *Peter Vogel*[b], *Mia Kim*[b], *Herman van der Putten*[c], *Christoph Wiessner*[c], *Giorgio Rovelli*[c], *Bernd W. Böttiger*[d], *Dirk M. Hermann*[a]

[a]*Department of Neurology, University Hospital Zürich, Frauenklinikstr. 26, CH-8091 Zürich, Switzerland*
[b]*Department of Anaesthesiology, University Hospital Heidelberg, D-69120 Heidelberg, Germany*
[c]*Nervous System Research, Novartis Pharma AG, CH-4002 Basel, Switzerland*
[d]*Department of Anaesthesiology, University Hospital Cologne, D-50937 Cologne, Germany*

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ABSTRACT

In ischemic stroke, cytosolic death pathways are activated in injured neurons destined to die. Neuronal injury is modulated by cell surface receptors, among which the tumor necrosis factor receptor family obtained particular interest. Cytokine response modifier A (CrmA) is a cowpox virus-derived caspase inhibitor, which interferes with the so-called death-inducing signaling complex, thereby blocking receptor-mediated apoptosis. To elucidate CrmA's therapeutic potential in ischemic stroke, we characterized a transgenic mouse line expressing CrmA under a Thy1 promoter, which we subjected to intraluminal middle cerebral artery (MCA) occlusion. Using *in situ* hybridization histochemistry and Western blots, we show that the *crmA* gene integrated into chromosome 8 of the mouse genome, CrmA being expressed in the cerebral cortex and striatum. Although robustly expressed, transgenic CrmA did not influence ischemic injury, both when relatively long-lasting (90 min) and mild (30 min) MCA occlusions were imposed. As such, neither infarct volume, brain swelling or neurological deficits following 90-min ischemia, nor disseminated neuronal injury or caspase-3 activation following 30-min ischemia were influenced by CrmA. Our data argue against a therapeutic effect of CrmA in ischemic stroke.

1. Introduction

Experimental studies during the last decade have shown that neuronal death in ischemic stroke is an active process. As such, ischemia induces mitochondrial disturbances that lead to the release of cytochrome *c* and apoptosis-inducing peptides into the cytosol, with the subsequent activation of so-called intrinsic death pathways (Schulz et al., 1999; Hata et al., 2000; Hermann et al., 2001a). Apoptotic signaling is modulated by cell surface receptors (so-called extrinsic death pathways), to

* *Corresponding author*. Fax: +41 1 255 4507.
E-mail address: ertugrul.kilic@usz.ch (E. Kilic).

Abbreviations: ABC, avidin–biotin–peroxidase complex (ABC); DAPI, 4′,6-diaminido-2-phenylidole; FITC, fluorescein isothiocyanate; LDF, Laser Doppler flow; MCA, middle cerebral artery; NGS, normal goat serum; PBS, phosphate-buffered saline; PBS–T, phosphate-buffered saline/0.3% Triton; PCR, polymerase chain reaction; PFA, paraformaldehyde; SD, standard deviation; SSC, standard saline/standard citrate; TDT, terminal deoxynucleotidyl transferase; TUNEL, terminal transferase-biotinylated dUTP nick end labeling

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which the tumor necrosis factor (TNF) receptor superfamily (e.g., CD95 [APO-1/Fas]) belongs (Ashkenazi and Dixit, 1998; Gerhardt et al., 2001; Ferrer and Planas, 2003). Extrinsic cell death pathways have been suggested to be a promising target for therapeutic interventions, as receptor signaling may be modulated very easily by pharmacological treatments.

The poxvirus-derived cytokine response modifier A (CrmA) is a broad spectrum caspase inhibitor, which potently blocks both caspase-8- and caspase-1-dependent death pathways *in vitro* and *in vivo* (Kügler et al., 1999; Roy and Sapolsky, 2003), without interfering with caspase-9-dependent signaling to major extent, at least *in vivo* (Ryan et al., 2002). As such, CrmA offers itself to study the role of receptor-mediated death signaling after focal cerebral ischemia. To elucidate its therapeutic potential, we first assessed its anti-apoptotic activity *in vitro*, investigating how CrmA influences cell injury induced by caspase-8. We then established a mouse line expressing transgenic CrmA under a Thy1 promoter. After showing that the *crmA* gene integrated into the mouse genome and was robustly expressed also in the animals' brains, we subjected CrmA-transgenic (CrmA-tg) mice to 90 or 30 min of intraluminal middle cerebral artery (MCA) occlusion, investigating CrmA's effects after stroke.

2. Results

2.1. *Anti-apoptotic activity of CrmA in vitro*

Anti-apoptotic activity of the *crmA* construct was demonstrated in primary mouse astroglial cells transfected with caspase-8. In cultures not transfected with *crmA*, ectopic expression of caspase-8 reduced cellular viability as a function of the amount of *caspase-8* cDNA added to the culture well (Fig. 1A). CrmA expression significantly improved cellular viability (Fig. 1B), thus demonstrating this caspase-inhibitor-exhibited anti-apoptotic activity.

2.2. *CrmA cDNA is integrated into the mouse genome and CrmA expressed in the brains of transgenic mice*

Chromosomal preparations obtained from CrmA-tg mice revealed integration of the *crmA* gene into the mouse genome (Fig. 2). Using rainbow FISH probes (CA-1601; Cambio), the *crmA* gene was localized on chromosome 8 (Fig. 2).

To evaluate whether CrmA was expressed in the brains of CrmA-tg mice on the protein level, Western blots with tissue samples obtained from the striatum, thalamus, hippocampus and cortex were prepared. These blots revealed a robust expression of the 38 kDa CrmA protein in the brains of transgenic mice that was not detectable in wt animals (Fig. 3).

2.3. *Transgenic CrmA does not influence brain hemodynamics during and after MCA occlusion*

To ensure that the severity of ischemias remained unchanged in CrmA-tg animals, laser Doppler flow (LDF) recordings were analyzed in animals submitted to focal cerebral ischemia. In both mouse lines, i.e., wt and CrmA-tg mice, MCA occlusion resulted in a decrease of LDF values above the core of the MCA territory to ~15% of pre-ischemic levels (Figs. 4A and 5A). After reperfusion, LDF values rapidly resumed baseline levels in animals subjected to 90-min MCA occlusion (Fig. 4A), whereas blood flow increased above pre-ischemic control in animals submitted to 30-min ischemia (Fig. 5A). No differences in LDF were detected between wt and CrmA-tg animals.

2.4. *CrmA does not influence ischemic brain injury in vivo*

2.4.1. *Infarct size after 90 min of MCA occlusion*

As in earlier studies (e.g., Kilic et al., 2006; Spudich et al., 2006), 90-min episodes of ischemia resulted in focal infarcts of the cerebral cortex and underlying striatum, which went along with significant brain edema in cresyl violet sections (Fig. 4B). The infarct volume, brain swelling and neurological deficit scores did not differ between wt and CrmA-tg mice (Figs. 4B–D), indicating that transgenic CrmA does not attenuate brain infarction in this model of severe focal cerebral ischemia.

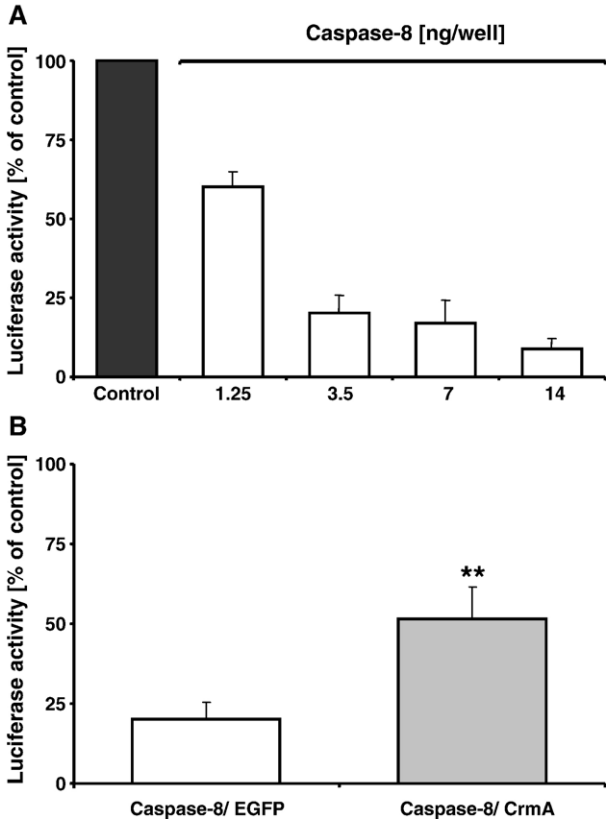

Fig. 1 – CrmA reverses the loss of cellular viability induced by caspase-8 expression. Dose-dependent loss of primary mouse glial cells by ectopic expression of caspase-8 as a function of the amount of transfected DNA (A). Viability is expressed as percentage of control, which corresponds to the luciferase activity measured in cells transfected with an EGFP expression vector without caspase-8. Co-transfection of the *crmA* construct used for the generation of the CrmA-tg mouse line potently rescues the viability of the glial cells (B), thus confirming the functionality of the CrmA protein. In B, 3.5 ng caspase-8 cDNA plasmid was added to each well. Data are means ± SD ($n = 6–7$ wells/group). $^{**}p < 0.01$ compared with wells incubated with caspase-8/EGFP.