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C1-inhibitor protects against brain ischemia–reperfusion injury via inhibition of cell recruitment and inflammation

Claudio Storini,[a] Emanuela Rossi,[b] Veronica Marrella,[a] Maria Distaso,[a] Robert Veerhuis,[c] Carlo Vergani,[b] Luigi Bergamaschini,[b] and Maria-Grazia De Simoni[a,*]

[a]*Laboratory of Inflammation and Nervous System Diseases, Mario Negri Institute, via Eritrea, 62, 20157 Milan, Italy*
[b]*Department of Internal Medicine, Geriatric Unit, University of Milan, Ospedale Maggiore IRCCS, 20122 Milan, Italy*
[c]*Department of Pathology, Graduate School Neurosciences Amsterdam, Research Institute Neurosciences, Vrije Universiteit Medical Center, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands*

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Previous studies demonstrated that C1-inhibitor (C1-INH), a complement and contact-kinin systems inhibitor, is neuroprotective in cerebral ischemia. To investigate the mechanism of this action, we evaluated the expression of neurodegeneration and inflammation-related factors in mice subjected to 2-h ischemia and 2 or 46 h reperfusion. C1-INH significantly dampened the mRNA expression of the adhesion molecules *P-selectin* and *ICAM-1* induced by the ischemic insult. It significantly decreased the pro-inflammatory cytokine (*TNFα*, *IL-18*) and increased the protective cytokine (*IL-6*, *IL-10*) gene expression. C1-INH treatment prevented the decrease of *NFH* gene, a marker of cellular integrity and counteracted the increase of *pro-caspase 3*, an apoptosis index. Furthermore, C1-INH markedly inhibited the activation and/or recruitment of microglia/macrophage, as shown by immunohistochemistry. In conclusion, C1-INH exerts an anti-inflammatory and anti-apoptotic action on ischemia–reperfusion injury. Our present and past data support a major effect of C1-INH on cell recruitment from the vasculature to the ischemic site.

Keywords: Middle cerebral artery occlusion; Inflammation; C1-inhibitor; Cell infiltration; Neurodegeneration; Apoptosis

Introduction

Inflammation is one of the most relevant processes in the pathogenesis of ischemia–reperfusion brain injury (Allan and Rothwell, 2001; del Zoppo et al., 2000; Iadecola and Alexander, 2001). Soon after ischemia, several inflammatory cascades are initiated. Vascular endothelial cells become rapidly activated leading to the rapid up-regulation of adhesion molecules such as ICAM-1 and P-selectin. Consistent with the important role of these molecules in brain ischemia–reperfusion damage, ischemic infarction is reduced in ICAM-1 knockout mice and is exacerbated in mice overexpressing P-selectin (Connolly et al., 1996; Frijns and Kappelle, 2002; Pantoni et al., 1998).

Resident cellular populations, namely astrocytes and microglia, rapidly turn their phenotype into an activated state, and infiltration of recruited inflammatory cells, such as neutrophils and macrophages, from blood vessels is soon initiated (Frijns and Kappelle, 2002; Touzani et al., 1999). All these activated cells are triggered to produce an array of inflammatory molecules. These include pro-inflammatory cytokines that contribute to the pathogenesis causing exacerbation of brain injury, and other cytokines that possess anti-inflammatory properties and may mediate neuroprotection and tissue repair. Accordingly, indirect evidence and direct studies demonstrated a strict correlation between a modulation of anti- and pro-inflammatory cytokines and the ischemic damage (Allan and Rothwell, 2001).

Major players in the post-ischemic inflammatory response are also complement and contact-kinin systems although their involvement in the pathogenesis of the brain tissue injury is not completely understood yet (D'Ambrosio et al., 2001; De Simoni et al., 2003, 2004; Wagner et al., 2002). We have previously shown that C1-inhibitor (C1-INH), a serine protease inhibitor that acts as a major inhibitor of both complement and contact-kinin systems (Caliezi et al., 2000; Davis, 1988; Kirschfink and Mollnes, 2001), markedly reduces the ischemic volume and significantly attenuates the neurological deficits in mouse cerebral ischemia (De Simoni et al., 2003, 2004). C1-INH is actually endowed with multifaceted anti-inflammatory actions (Caliezi et al., 2000; Liu et al., 2003). In addition to its effect on complement and contact-kinin systems, C1-INH has been shown to affect leukocyte adhesion by binding to selectins, an action implying inhibition of migration from the vasculature to inflammation sites (Cai and Davis, 2003).

In this study, we have investigated C1-INH effect on the early and late phase of post-ischemic inflammatory processes.

* Corresponding author. Fax: +39 02 3546277.
E-mail address: desimoni@marionegri.it (M.-G. De Simoni).
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We have considered a panel of genes activated by ischemia–reperfusion and investigated possible modulating effects of C1-INH, focusing on mechanisms involved in inflammatory cell recruitment. Specifically, we assessed the mRNA expression of the adhesion molecules *ICAM-1* and *P-selectin*, as well as that of the pro- and anti-inflammatory cytokines *interleukin(IL)-1β*, *tumor necrosis factor(TNF)α*, *IL-18*, *IL-1 receptor antagonist(Ra)*, *IL-10* and *IL-6* that are produced by activated cells. To monitor the final effect on the ischemic brain tissue, we have also determined the mRNA expression of genes related to apoptosis (*pro-caspase 3*) and neuronal integrity (*NFH*). In addition, by immunohistochemistry, we have evaluated the activation of microglia/macrophage at different time points after ischemia to determine if C1-INH would influence this cellular component of the inflammatory response.

Materials and methods

Animals

Male CD1 mice (26–28 g, Charles River, Calco, Italy) were housed five per cage and kept at constant temperature (21 ± 1°C) and relative humidity (60%) with regular light/dark schedule (7 am–7 pm). Food (Altromin pellets for mice) and water were available ad libitum.

Procedures involving animals and their care were conducted conform the institutional guidelines that are in compliance with national (D.L. n.116, G.U. suppl. 40, 18 February 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358,1; Dec.12,1987; NIH Guide for the Care and Use of Laboratory Animals, U.S. National Research Council 1996).

Surgical procedure

Transient focal cerebral ischemia was achieved by middle cerebral artery occlusion (MCAO) (De Simoni et al., 2003, 2004). Anesthesia was induced by 3% isoflurane in N_2O/O_2 (70/30%) mixture and maintained by 1–1.5% isoflurane in the same mixture. As previously described, the right common carotid artery was exposed and the external carotid artery and its branches were cauterized. A 5–0 monofilament nylon suture, blunted at the tip by heat and coated in poly-L-lysine, was introduced into the internal carotid artery and advanced to the anterior cerebral artery so as to block its bifurcation into the anterior cerebral artery and the MCA. The filament was advanced until a >70% reduction of blood flow compared to preischemic baseline, measured as described below, was observed. At the end of 2-h ischemic period, blood flow was restored by carefully removing the nylon filament. During surgery, mice were maintained at constant temperature (37 ± 0.5°C) using a heating pad equipped with a rectal probe (LSI-Letica Spain).

Cerebral blood flow monitoring

To confirm the adequacy of the vascular occlusion in each animal, blood flow was measured by laser Doppler flowmetry (Transonic BLF-21) using a flexible 0.5-mm fiber-optic probe (Transonic, Type M, 0.5-mm diameter) positioned on the brain surface and secured with impression material on the skull at the following coordinates: AP = −1 mm; L = −3.5 mm (Yang et al., 1999).

Sham-operated mice

After implantation of fiberoptic probe, sham-operated mice received a midline neck incision, and the carotid sheath was exposed. The external carotid artery and its branches were isolated without being ligated or cauterized. Similar to ischemic mice, sham-operated mice were maintained at 37°C during surgery and recovery from anesthesia.

Drug treatment

The drug consisted of a purified and steam-treated lyophilisate of human C1-INH (C1 Inactivator, IMMUNO-Baxter, Pisa, Italy). Mice received an i.v. injection of 15 U (555.55 U/kg) of C1-INH or the same volume (150 μl) of saline at the beginning of the ischemic period. Fifteen U was the amount of C1-INH required to obtain 90–95% inhibition of complement hemolytic activity in mice (De Simoni et al., 2003).

Neurological deficits

Forty-eight hours after ischemia, each mouse was rated on two neurological function scales unique to the mouse (Clark et al., 1997; De Simoni et al., 2003). For both scales, mice were scored from 0 (healthy mouse) to 28. The score given represents the sum of the results of all categories for each scale. General deficit scale evaluates hair, ears, eyes, posture, spontaneous activity, epileptic behavior. Focal deficit scale evaluates body symmetry, gait, climbing on a surface held at 45°, circling behavior, front limb symmetry, compulsory circling, whisker response to a light touch. All the experiments were run by a trained investigator blinded to the experimental conditions. Data are expressed as median, range and 25th–75th percentiles since intervals between scores are not equal. Differences between ischemic mice receiving C1-INH or saline were analysed by Mann–Whitney test.

RNA extraction and RT-PCR analysis

For RT-PCR studies, we set to analyze the cortex of ischemic mice since this region surrounds the necrotic lesion, essentially located in striatum and hippocampus, and shows cell loss in a well-preserved general structure (De Simoni et al., 2003).

Total RNA was extracted from mouse cortex according to the acid guanidinium–phenol–chloroform method (De Simoni et al., 2000). For reverse transcription, total RNA (0.8 μg) was used as a substrate for single-stranded cDNA synthesis with the use of the Gene Amp RNA PCR Kit (Perkin Elmer), according to the manufacturer's instructions. An aliquot (10 μL) of cDNA synthesis mix was used for the PCR reaction in a Perkin Elmer Gene Amp 9700 System. Gene-specific oligonucleotide primers were designed on mRNA-based sequences, as in Table 1. Initial experiments were conducted to determine the optimal annealing temperature for each set of gene-specific primers and the linear phase of the product amplification (data not shown). After an initial denaturation at 95°C for 5 min, amplification was conducted through 24–30 cycles of denaturation at 94°C for 30 s, annealing at the optimal temperature (Table 1) for 30 s, and extension at 72°C for 30 s. Final extension was at 72°C for 7 min. In each PCR (RNA extraction, cDNA synthesis, and PCR), negative and positive controls were included. A portion of the PCR mixture (7 μL) was electrophoresed in a 1.5% (wt/vol) agarose gel, stained with ethidium bromide. The relative