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Lysosomal rupture, necroapoptotic interactions and potential crosstalk between cysteine proteases in neurons shortly after focal ischemia

Münire Kilinc [a,1], Yasemin Gürsoy-Özdemir [b,1], Günfer Gürer [b,2], Sefik Evren Erdener [b], Esra Erdemli [c], Alp Can [c], Turgay Dalkara [b,*]

[a] Department of Neurology, Faculty of Medicine, Baskent University, Ankara, Turkey
[b] Department of Neurology, Faculty of Medicine, and Institute of Neurological Sciences and Psychiatry, Hacettepe University, Ankara, Turkey
[c] Department of Histology and Embryology, School of Medicine, Ankara University, Ankara, Turkey

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ABSTRACT

Ischemic cell death is a complex process and the initial distinction between apoptosis and necrosis appears to be an oversimplification. We previously reported that in ischemic neurons with disrupted plasmalemma, apoptotic mechanisms were also active. In the present study, we investigated cellular co-localization of another necrotic feature, lysosomal rupture, with apoptotic mechanisms in the mouse brain and assessed the potential interactions between cysteine proteases. The lysosomal enzymes were spilled into the cytoplasm 1–4 h after ischemia/reperfusion, suggesting that lysosomal membrane integrity was rapidly lost, as occurs in necrosis. The same neurons also exhibited caspase-3 and Bid cleavage, and cytochrome-c release. Caspase-3 activity preceded cathepsin-B leakage in most neurons, and declined by 12 h, while lysosomal leakage continued to increase. Concurrent inhibition of cathepsin-B and caspase-3 provided significantly better neuroprotection than obtained with separate use of each inhibitor. These data suggest that necrotic and apoptotic mechanisms may act both in concert as well as independently within the same cell beginning at the onset of ischemia to ensure the demise of damaged neurons. Therefore, combined inhibition of cysteine proteases may abrogate potential shifts between alternative death pathways and improve the success of stroke treatments.

Introduction

The discovery of apoptosis caused an explosion of interest in cell death mechanisms with the hope of preventing neuronal loss in CNS diseases. However, it was soon realized that cell death processes were more complex than originally thought (Nicotera et al., 1999; Syntichaki and Tavernarakis, 2003). Several studies have shown that the differences between necrosis and apoptosis are not obvious in many human pathological states, nor are they in several experimental models (Martin et al., 1998; Uysal et al., 2003; Yamashima, 2004).

Brain infarction was traditionally considered to be a classic example of necrosis. Nevertheless, several morphological and biochemical features observed during apoptosis were also documented in ischemic brains (Asahi et al., 1997; Namura et al., 1998; Onteniente et al., 2003; Velier et al., 1999; Yamashima, 2004). Genetic or pharmacological manipulation of anti- or pro-apoptotic cascades was shown to significantly change the brain's resistance to ischemia (Chan, 2004; Martinou et al., 1994). It was therefore concluded that less severely, but nonetheless irreparably damaged ischemic neurons were dismantled with apoptotic mechanisms, whereas severely damaged cells died by swelling and necrosis. However, electron microscopic studies indicated that ischemic neuronal death was characterized by a mixture of apoptotic and necrotic ultrastructural changes (Martin et al., 1998; van Lookeren Campagne and Gill, 1996). Supporting these observations, caspase activity as well as TUNEL-positive nuclei displaying apoptotic morphology have been co-localized within neurons that lost their membrane integrity after focal ischemia (Unal-Cevik et al., 2004). Consequently, it has been proposed that apoptotic and necrotic death pathways may operate concurrently in ischemic cells depending on the severity of cellular injury. In the latter study, neurons were examined 6–72 h after ischemia. However, caspase activation and release of lysosomal enzymes occur within a few hours following ischemia (Benchoua et al., 2001, 2004; Namura et al., 1998). Therefore, it remains to be elucidated whether or not necrotic and apoptotic mechanisms may be concomitantly triggered within neurons soon after ischemia. This point is important because concomitant activation of apoptotic as well as necrotic proteases in the same cell would necessitate combined inhibition of cathepsins and caspases to avoid potential shifts between the two systems and increase the success of neuroprotection (Leist

* Corresponding author. Department of Neurology, Hacettepe University Hospitals, Ankara 06100, Turkey. Fax: +90 312 3093451.
E-mail address: tdalkara@hacettepe.edu.tr (T. Dalkara).
[1] Equally contributed.
[2] Deceased.

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and Jaattela, 2001; Rami, 2003; Stoka et al., 2001; Syntichaki and Tavernarakis, 2003; Yamashima, 2000).

We studied the leakage of cathepsin-B or lysosome-associated membrane protein-1 (LAMP-1) into the cytoplasm as a marker of lysosomal rupture. Cathepsin-B is mainly located within lysosomes, and LAMP-1 is a lysosomal membrane protein (Evans and Aguilera, 2003; Mort and Buttle, 1997; Turk et al., 2001; Yamashima, 2000). The appearance of active forms of caspase-3-p20 and Bid (truncated Bid, tBid) along with release of cytochrome-c from mitochondria was used as a marker of apoptotic activation. Although the above immunohistochemical markers had consistently been shown by several laboratories to specifically detect these proteins in the ischemic tissue (e.g. (Yamashima et al., 2003)), we also confirmed our findings with electron microscopy (EM) and caspase-3-p20 immunogold labeling.

Several lines of evidence suggest that lysosomal rupture takes place within hours following focal ischemia (Benchoua et al., 2004; Seyfried et al., 1997). However, its relation to apoptotic mechanisms in ischemic neurons has not been investigated, although the independent role of each pathway in ischemic cell death has been studied by several groups, and a crosstalk between the apoptotic pathways and cathepsin-B has been proposed (Hill et al., 1997; Leist and Jaattela, 2001; Lo et al., 2003; Namura et al., 1998; Rami, 2003; Stoka et al., 2001; Yamashima, 2000, 2004). In this study, we assessed the interactions between these pathways at the cellular level. We chose early time points after 1 h of middle cerebral artery (MCA) occlusion because, neurons do not rapidly degenerate and provide a window of opportunity to assess the interactions between cysteine proteases.

Materials and methods

Middle cerebral artery occlusion

Animal housing, care, and application of experimental procedures were done in accordance with institutional guidelines (ethical approval number: 2002/20-2). Swiss albino mice (28–34 g) fasted overnight with free access to water before the experiment and were anesthetized with chloral hydrate (400 mg/kg, IP) for the duration of ischemia and 30 min of reperfusion. To minimize variability in the insult severity, the regional cerebral blood flow (rCBF) was monitored (see below), the body temperature was maintained at $37.0 \pm 0.2^{\circ}$ C until the mice recovered from anesthesia, and only animals with arterial blood pressure within a physiological range were included in the study. Proximal occlusion of the right MCA was performed with a filament as previously described (Unal-Cevik et al., 2004). Briefly, a nylon filament (8-0) was inserted into the common carotid through a small incision proximal to the bifurcation and advanced in the internal carotid artery up to the origin of MCA (10 mm from the bifurcation). The distal 3-mm of 8-0 filament was coated with silicon. A flexible probe (PF-318 of PeriFlux PF 2B, Perimed) was placed over the skull (2 mm posterior, 6 mm lateral to bregma), away from large pial vessels, to monitor the rCBF by laser-Doppler flowmetry. After obtaining a stable 10-min epoch of pre-ischemic rCBF, the MCA was occluded and rCBF was monitored for the duration of ischemia (typically dropped to 10–20% of baseline) and for the first 5–10 min of reperfusion until observing that the rCBF has recovered. Reperfusion was accomplished by pulling the filament back. Sixty-four mice were subjected to 1 h of proximal MCA occlusion and 1 h ($n=14$), 4 h ($n=40$) and 12 h ($n=10$) of reperfusion.

Immunohistochemistry

After ischemia/reperfusion, mice were anesthetized with overdose of chloral hydrate and perfused transcardially with heparinised saline followed by 4% paraformaldehyde. Brains were post-fixed in 4% formaldehyde for 24 h and were then cryoprotected in 20 and 30% sucrose. Sections (20 μm thick) passing through the anterior commissure were kept frozen at -20° C or were stored in phosphate-buffered saline (PBS) as free-floating sections at $+4^{\circ}$ C until use. Cell death was evaluated using antibodies against caspase-3-p20 (1:100, Cell Signaling, USA), tBid (1:100, Biosource International, USA) cytochrome-c (1:200, Santa Cruz Biotechnology, USA), LAMP-1(1:200, Abcam, UK) and cathepsin-B (1:200, Upstate Biotechnology, USA). Neurons and astrocytes were labeled with antibodies against NeuN (1:200, Chemicon, USA) and glial fibrillary acidic protein (GFAP, 1:200, Sigma, USA), respectively. A tyramide amplification method was used for caspase-3-p20 and tBid immunofluorescence according to the manufacturer's recommendations (Molecular Probes, USA). Sections were first blocked with goat serum and then permeabilized with Triton-X and incubated with primary antibodies at 37° C for 90 min, followed by an incubation with secondary antibodies (1:200) for 90 min. Fluorescein isothiocyanate (FITC) goat anti-mouse IgG, Cy2 goat anti-rabbit IgG, Cy3 goat anti-rabbit IgG, and Cy3 goat anti-mouse IgG (all from Jackson ImmunoResearch, Europe) were used as appropriate secondary antibodies. Primary antibody omission and incubation with either blocking solution or PBS were performed to test the specificity of each immunoreactivity. Immunostained sections were mounted in glycerol/PBS medium containing 25 mg/mL sodium azide. Bright-field and fluorescent examination was performed with a Nikon Eclipse E600 microscope using appropriate filter sets. Specimens were further examined with a Zeiss LSM-510 confocal laser-scanning microscope. Single optical sections (2048×2048 pixel) were collected. Digitized images were pseudo-colored according to their original fluorochromes.

Fluoro-Jade C (FJC) staining

To specifically label degenerating neurons, Fluoro-Jade® C staining was performed as previously described (Schmued et al., 2005): 35-μm-thick free-floating sections were mounted on poly-L-lysine coated glass slides. The slides were then air dried for 30 min at 50° C. They were first immersed in 80% ethanol solution containing 1% NaOH for 5 min and rinsed sequentially in 70% ethanol for 2 min, in distilled water for 2 min, and then incubated in 0.06% potassium permanganate solution for 10 min. After rinsing in distilled water for 1–2 min, the slides were transferred to 0.0001% solution of FJC (Millipore, USA) dissolved in 0.1% acetic acid for 10 min. Finally they were rinsed through three changes of distilled water. The slides were then air dried at 50° C for 5 min, cleared in xylene and coverslipped with DPX (Sigma, USA) mounting media. Fluorescent images were acquired with Nikon Eclipse E600 microscope at 200x magnification.

Cell counting and statistics

Cells positively labeled for cathepsin-B, cytochrome-c, tBid, caspase-3-p20 or FJC were counted manually on randomly taken micrographs (×200) from the frontal, parietal and preoptic core areas of coronal sections passing through the anterior commissure, and were expressed as percentage of the total number of neurons counted on each section except FJC counts, which were given as mean ± SE number of positive cells per mm^2. The mean ratio for each time point was calculated by averaging the percent values of coronal sections obtained from 4 to 8 animals except tBid 1 h time point, for which only 2 mice were used (Table 1). One coronal section from each animal was counted unless any of the 3 areas of interest could not be evaluated for technical reasons. In that case, the corresponding area was counted on the adjacent section.

Neurons displaying cathepsin-B leakage along with apoptotic activities were also present in the periphery of the MCA area although they were more prevalent in the core. However, identification of the inner and outer borders of the perifocal (penumbral) area was