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Research report

Mechanisms of neurodegeneration after severe hypoxic-ischemic injury in the neonatal rat brain

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ABSTRACT

Purpose: Apoptosis is implicated in mild-moderate ischemic injury. Cell death pathways in the severely ischemic brain are not characterized. We sought to determine the role of apoptosis in the severely ischemic immature brain.

Methods: Seven-day old rats were randomly assigned to mild-moderate or severe cerebral hypoxia-ischemia (HI) group. After ligating the right common carotid artery, animals were subjected to hypoxia for 90 min in the mild-moderate HI or 180 min in the severe HI. The core and peri-infarct area were measured in H&E stained brain sections using NIS Elements software. Brain sections were processed for caspase-3, AIF and RIP3 immuno-staining. Number of positive cells were counted and compared between the two groups.

Results: The core constituted a significantly higher proportion of the ischemic lesion in the severely compared to the moderately injured brain ($P<0.04$) up to 7 days post-injury. Apoptotic cell death was significantly higher ($P<0.05$) in the core than the peri-infarct of the severe HI brain. In the peri-infarct area of severe HI, AIF-induced cell death increased over time and caspase-3 and AIF equally mediated neuronal death. Necroptosis was significantly higher ($P=0.02$) in the peri-infarct of the severe HI lesion compared to the moderate HI lesion. In males, but not in females, apoptosis was higher in moderate compared to severe HI.

Conclusions: Caspase-independent cell death plays an important role in severe ischemic injury. Injury severity, timing of intervention post-injury and sex of the animal are important determinants in designing neuroprotective intervention for the severely ischemic immature brain.

Abbreviations: AIF, Apoptosis-inducing factor; HI, Hypoxia-ischemia; GFAP, Glial fibrillary acidic protein; NF, Neurofilament

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1. Introduction

Brain cell death occurs as a continuum that ranges from necrosis to apoptosis (Carloni et al., 2007; Northington et al., 2011). Generally, two major pathways of apoptotic cell death have been identified: the intrinsic and extrinsic pathways. In the intrinsic pathway, a death stimulus causes the release of cytochrome c from the mitochondria into the cytosol, which will then bind to Apaf-1 and caspase-9 leading to the activation of caspase-3 and cell death. The extrinsic pathway involves the surface receptor Fas that activates caspase-8, which in turn activates caspase-3. The initiation of the intrinsic and extrinsic pathways is different but they both converge on caspase-3, which is known as the final "executioner" of apoptosis (Eldadah and Faden, 2000; Ferrer and Planas, 2003; Li and Yuan 2008). Although caspases have been recognized as primary mediators of apoptotic neuronal death (Northington et al., 2011; Hyman and Yuan 2012), accumulating evidence suggests that pathways independent of caspases also play a role in neuronal injury (Cregan et al., 2002; Stefanis, 2005). Apoptosis-inducing factor (AIF) is a mitochondrial inter-membrane flavorprotein that is released and trans-located to the cytoplasm and then to the nucleus in response to specific death stimuli (Daugas et al., 2000a, 2000b; Otera et al., 2005). Here, it causes chromatin condensation and DNA fragmentation (Daugas et al., 2000a, b; Cregan et al., 2002; Plesnila et al., 2004).

Clinical studies indicate that sexual dimorphism is an important interdependent risk factor for ischemic brain injury (Fullerton et al., 2003; Golomb et al., 2009; Westmacott et al., 2009). These studies reported that males had increased incidence of and worse outcomes in response to HI injury compared to females. The mechanisms of these sex-related differences of HI injury are poorly understood. Neuronal culture models of HI injury showed that cell death was predominantly via caspase-dependent pathway in the XX neurons versus AIF-dependent pathway in the XY neurons (Du et al., 2004; Sharma et al., 2011), indicating that the mechanism of cell death is innately different between sexes. Sex-specific differences in cell death were also confirmed using in vivo mouse model of neonatal HI injury (Zhu et al., 2006; Mirza et al., 2015). Therefore understanding these sexual differences in the mechanism of cell death is instrumental in designing effective neuroprotective therapies for HI injury.

Several studies from our laboratories and others exploring the mechanisms of cell death using neonatal animal models of cerebral ischemia have shown that apoptotic cell death is more prevalent in the immature than in the mature brain (Towfighi et al., 1995; Liu et al., 2004; Zhu et al., 2005). We have previously shown that AIF, in addition to caspase-3, is implicated in delayed cell death in the ischemic neonatal rat brain (Askalan et al., 2011). Most of these studies, however, are done in animal models of moderately injured ischemic brains. These animal models have been used in experimental neuroprotective strategies, some of which have shown robust efficacy against ischemic injury in the developing brain. However, with the exception of therapeutic hypothermia, none of these neuroprotective strategies have been successfully translated to clinical practice. Furthermore, while post-ischemic hypothermia has proven to be effective in newborns with mild-moderate hypoxic-ischemic encephalopathy, its protection does not extend to the severely injured neonatal brain, as shown in both animal (Nedelcu et al., 2000; Sabir et al., 2012) and human studies (Shankaran et al., 2005; Azzopardi et al., 2008).

Taken together, these studies highlight the necessity for a better understanding of the mechanisms of cell death in the severely injured brain. We therefore sought in this study to characterize the pathways that lead to cell death in the core and peri-infarct area and to understand the influence of biological sex on these pathways in the neonatal rat brain subjected to severe ischemic injury. It is only by understanding these pathways that we will be able to design neuroprotective therapies or modify existing ones (i.e. hypothermia) to be effective in protecting the severely injured developing brain.

2. Results

2.1. *The core constituted most of the ischemic lesion in the severely injured brain*

The total infarct area, the core area and peri-infarct area were measured in the moderately and severely injured brains (n=4–6 pups/time point in each group) as described in Section 4 and in Fig. 1. The core constituted a higher proportion of the ischemic lesion in the severely injured (mean percent core 96±1%) compared to the moderately injured (69±13%) brain at 4 days post-injury ($p \leq 0.04$). After 4 days, that difference disappeared and the area of the core became the same in both types of injury by 1 week post-injury and remained the same at 2 weeks post-injury.

2.2. *In severe ischemic injury, cell death was prolonged and mediated by caspase-dependent and caspase-independent pathways*

In the severely injured brain, cell death by caspase-3 and AIF pathways was significantly higher in the core compared to the peri-infarct area. Interestingly, these two pathways behaved differently over time depending on the region of the lesion. In the core, caspase-3 activity was significantly higher in day 14 compared to day 1 post-injury. In the peri-infarct area, AIF-induced cell death increased over time to reach levels of the core levels by 2 weeks postinjury (Fig. 2). These results indicate that there is a prolonged window for therapeutic intervention in the severely ischemic brain, and in order to be effective in this type of injury, neuroprotective strategies must target both caspase-3 and AIF-mediated cell death as highlighted by our subsequent set of experiments comparing the role of these two pathways in severe and moderate ischemic brain injury.

2.3. *Caspase-3 and AIF activities were differentially time-dependent in the severely and moderately ischemic brain*

In the core of moderate ischemic injury, the number of nuclear AIF positive cells peaked early followed by a sharp