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Research Report

Neuron death and inflammation in a rat model of intracerebral hemorrhage: Effects of delayed minocycline treatment

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ABSTRACT

After intracerebral hemorrhage (ICH), blood entry is followed by neuron death and an inflammatory response, but development of pharmacological therapies has been hampered by an inadequate understanding of the spatial and temporal relationship between neuron death and inflammation. Using a rat model of ICH, we first investigated these relationships at 6 h, and 1, 3 and 7 days. At the edge of the hematoma, no degenerating neurons were observed at 6 h; however, dying neurons were present between 1 and 3 days, with peak neuron death occurring at 1 day. This is apparently the first report of ongoing neuron death at the edge of the hematoma during a time window that is appropriate for human therapy. Neuron death was limited to the edge of the hematoma, with no degenerating neurons in the striatum surrounding the hematoma, despite robust and prolonged microglia activation. Importantly, neuron loss at the edge of the hematoma was spatially and temporally associated with accumulation and activation of microglia/macrophages. We then tested the hypothesis that treatment with the tetracycline derivative, minocycline, after the hematoma had reached a maximal size, will reduce inflammation and neuron damage. Minocycline injection (45 mg/kg i.v. at 6 h, and i.p. at 24, 48 and 72 h) failed to reduce neuron loss outside the hematoma or striatal tissue loss (assessed at 7 days), despite reducing the number of neutrophils and activated microglia/macrophages. Thus, minocycline does not appear to target the mechanisms responsible for cell death in this model of ICH.

1. Introduction

Intracerebral hemorrhage (ICH) is typically caused by spontaneous rupture of small penetrating arteries or arterioles and extravasation of blood into the brain parenchyma, which may further disrupt the blood–brain barrier and result in secondary bleeding. Despite accounting for about 15% of all strokes, the pathophysiology of ICH is not fully understood (Qureshi et al., 2001), and no drugs have been developed to reduce the damage (Rincon and Mayer, 2004). Primary injury after ICH includes physical destruction of tissue, and mass effects that compress surrounding structures including the ventricles, and increase intracranial pressure (Qureshi et al., 2001). The secondary neurological deterioration that commonly occurs has been attributed to hematoma expansion (Fujii et al., 1994; Kazui et al., 1996), edema (Gebel et al., 2002), inflammation (Castillo et al., 2002; Leira et al., 2004) and neuron death in the parenchyma surrounding the hematoma (Qureshi et al., 2003).

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Neuron necrosis and apoptosis have been reported in animal models (Gong et al., 2001; Matsushita et al., 2000; Xue and Del Bigio, 2000), and apoptotic neurons have been seen in humans, especially at the periphery of the hematoma (Qureshi et al., 2003).

Reducing secondary injury and rescuing neurons following ICH remains an attractive therapeutic goal, but a better understanding of the pathological sequence of events is needed. In both animals and humans, a prominent inflammatory response occurs, which includes activation of resident brain microglia and infiltration into the brain, first by neutrophils and then macrophages (Del Bigio et al., 1996; Gong et al., 2000). Several molecules produced by inflammatory cells have been implicated in secondary injury and neurotoxicity, including reactive oxygen species (Chan, 2001), matrix metalloproteases (Cunningham et al., 2005; Jian and Rosenberg, 2005) and tumor necrosis factor alpha (TNFα) (Barone and Feuerstein, 1999). Accordingly, there are reports that overall cell death is reduced and behavioral outcomes improved by broad spectrum immuno-suppression (Peeling et al., 2001b), free radical trapping (Peeling et al., 1998, 2001a) or TNFα inhibition (Mayne et al., 2001a,b).

Surprisingly lacking are studies of the spatial and temporal relationships between neuron death and inflammation following ICH. Much of the current thinking about therapeutic approaches is derived from studies of acute ischemic stroke, where potentially salvageable tissue (the ischemic penumbra) surrounds the irreversibly damaged core. A primary goal, then, is to prevent penumbral tissue from proceeding to infarction (Fisher and Ratan, 2003). Following an ICH, brain tissue inside the hematoma can be considered equivalent to the ischemic core, and unlikely to be salvageable. Hence, strategies aimed at reducing brain injury after ICH should focus on the tissue outside the hematoma. Felberg et al. (2002) demonstrated that neuron loss at the edge of the hematoma continues for at least 3 days after the onset of ICH. Since neutrophils and activated microglia/macrophages accumulate at the edge of the hematoma (Del Bigio et al., 1996), we hypothesized that reducing inflammation would prevent the delayed loss of neurons outside the hematoma.

Intracerebral hemorrhage was induced by collagenase injection into the rat striatum. Test animals were treated with minocycline, a broad-spectrum drug reported to reduce inflammation and provide neuroprotection in a variety of experimental models, including ischemic stroke, ICH, brain and spinal cord injury, multiple sclerosis and Parkinson's, Alzheimer's and Huntington's diseases (Yong et al., 2004). To ensure rapid delivery and sustained availability, minocycline was injected intravenously at 6 h, and then intraperitoneally every 24 h. The time of initial treatment was chosen to reflect the delay experienced in human ICH, and to avoid altering the development of the hematoma since, in this model, active bleeding can continue for several hours (up to 6 h in the present study). The density of dead/dying neurons was monitored at 6 h and 1, 3 and 7 days after ICH onset, and neuron loss outside the hematoma was compared with the density of inflammatory cells (neutrophils, activated microglia/macrophages). Our results show that despite reducing the number of inflammatory cells, minocycline did not rescue neurons outside the hematoma.

2. Results

2.1. *Time course of neuron death*

Before proceeding with quantitative analysis, we confirmed that several aspects of the pathology were similar to previous reports (Rincon and Mayer, 2004); i.e., the mass effect of the blood caused a shift in midline structures and compressed the ipsilateral ventricle and surrounding white matter tracts. Then, Fluoro-jade B, which is specific for degenerating neurons (Schmued and Hopkins, 2000), was used to assess neuron death at 6 h, and 1, 3 and 7 days after ICH onset (Fig. 1B), with or without minocycline treatment (Fig. 1C). Fluoro-jade-positive cells were restricted to the needle track in sham-operated animals (not shown) while no Fluoro-jade-positive cells were seen at any of the time points assessed in the undamaged contralateral striatum (Fig. 1B). At 6 h after ICH onset, no degenerating neurons were observed in the damaged, ipsilateral striatum surrounding the hematoma. By Day 1, degenerating neurons were abundant at the edge of the hematoma (85.4±13.5 cells/mm^2, n=4), but rarely seen 150 ìm further into the surrounding striatum. By Day 3, the density of Fluoro-jade-positive cells (22.3±8.9 cells/mm^2, n=6) had decreased significantly compared with Day 1 (p<0.001), and no degenerating neurons were seen on Day 7 (n=4). When minocycline treatment was begun 6 h after ICH onset, it failed to reduce the number of degenerating neurons at the edge of the hematoma (non-significant, compared with vehicle-treated controls) either on Day 1 (80.0±15.5 cells/mm^2, n=6) or Day 3 (18.9±5.8 cells/mm^2, n=6). Initially, the number of Fluoro-jade-positive cells was assessed in sections spanning the length of the rostral-caudal axis. Because there was no difference in the number of Fluoro-jade-positive cells between treatment groups, all subsequent data were calculated as the average of 4 grids in a single brain section taken at the position where the hematoma was largest. Thus, in this model of ICH, it appears that neuron death occurs mainly at the edge of the hematoma, and that degenerating neurons are present for at least 3 days.

2.2. *Temporal and spatial relationship between neuron loss and inflammatory cells*

Because inflammation has been associated with neuron death in models of stroke and other types of brain injury, we investigated the spatial–temporal relationship between neuron loss at the edge of the hematoma and activation of microglia/macrophages at 1, 3 and 7 days after ICH onset, with and without minocycline treatment (Fig. 2). At no time examined did the neuron density (NeuN-positive cells) in the striatum surrounding the hematoma (344.5±21.3 cells/mm^2, n=6) differ from the contralateral striatum (358.9±21.0 cells/mm^2, n=6); thus, we will call this region, 'intact' striatum. The only change was an apparent decrease in brightness of the individual NeuN-positive cells in the intact striatum on Days 1 and 3 after ICH onset, compared with the same region on Day 7 or with the contralateral striatum. The possibility was previously raised that NeuN antigenicity (and therefore the brightness of staining) decreases in response to metabolic