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BRAIN RESEARCH

Research Report

Angiogenesis as a predictive marker of neurological outcome following hypoxia–ischemia

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ARTICLE INFO

Article history:
Accepted 25 June 2007
Available online 6 August 2007

Keywords:
CD31
Clomethiazole
L-NAME
NOS
Inflammation

ABSTRACT

Cerebral ischemia induces angiogenesis within and around infarcted tissue. The protection of existing and growth of new blood vessels may contribute to a more favorable outcome. The present study assessed whether angiogenesis can be used as a marker for neurodegeneration/neuroprotection in a model of hypoxia–ischemia (HI). Increased CD31 immunoreactivity 7 days post-HI indicated increased angiogenesis compared to controls ($P<0.001$). Treatment with the $GABA_A$ receptor modulator, clomethiazole (CMZ; 414 mg/kg/day), normalized the level of angiogenesis compared to HI + saline ($P<0.001$). Conversely, the non-selective nitric oxide synthase (NOS) inhibitor, L-NAME (5 mg/kg/day), markedly decreased angiogenesis compared to controls ($P<0.001$). Circulating plasma levels of IL-1α, IL-1β and GM-CSF were significantly elevated post-HI. CMZ treatment attenuated these increases while also stimulating IL-10 levels. L-NAME treatment did not alter IL-1α or IL-1β levels, but decreased endogenous IL-10 levels and exacerbated the ischemic lesion ($P<0.001$). CMZ treatment has been shown to increase NOS levels, while L-NAME halted the HI-induced increase in NOS activity ($P<0.001$). We conclude that angiogenesis can be used as a marker of neurodegeneration/neuroprotection for cerebral HI and is correlated to NOS activity and circulating inflammatory mediators.

1. Introduction

The process of angiogenesis begins with the proliferation and migration of endothelial cells to the site of injury, initiating the sprouting of new capillaries from pre-existing blood vessels. This remodeling process involves the interaction of the endothelium with the extracellular matrix and the endothelial expression of integrins and matrix metalloproteinases. In addition, functional maturation of these newly formed capillaries requires the survival and differentiation of endothelial

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Abbreviations: BSA, bovine serum albumin; CBF, cerebral blood flow; CMZ, clomethiazole; EGCG, epigallocatechin gallate; eNOS, endothelial nitric oxide synthase; HI, hypoxia–ischemia; L-NAME, N^{ω}-nitro-L-arginine methylester; NO, nitric oxide; NOS, nitric oxide synthase; PECAM-1/CD31, platelet endothelial cell adhesion molecule-1; PND, postnatal day; TTC, 2,3,5-triphenyltetrazolium chloride; TBS, Tris-buffered saline

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doi:10.1016/j.brainres.2007.06.100

cells. Nitric oxide (NO) derived from endothelial nitric oxide synthase (eNOS) plays an integral part in each of these stages and is a potent mediator of angiogenesis (Appleton, 2003). Given that newly formed microvessels can improve tissue perfusion within the ischemic penumbra, thereby promoting the recovery of neurological function following ischemia (Zhang et al., 2002), increased angiogenesis could be a valuable therapeutic option for treating cerebral ischemia. Angiogenesis has been shown to peak between 3 and 7 days post-cerebral insult with the maturation still continuing until day 21 (Hayashi et al., 2003). A correlation between angiogenesis and stroke has been shown based upon observations of decreased morbidity and mortality rates in stroke patients displaying increased cerebral blood flow (CBF) and microvessel density (Krupinski et al., 1994). From this, it is suggested that promotion of angiogenesis in patients at risk of, or suffering from a stroke, maybe of benefit.

Endothelial cells constitutively express a number of different antigens, and specific labeling of these antigens is regularly used for their detection. One such antigen is platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31), which has been shown to aid in the adhesion of leucocytes to the endothelium in order for diapedesis to occur (Bevilacqua, 1993). CD31 is a 130-kDa member of the immunoglobulin super-family and is expressed in large amounts on endothelial cells at the cell-to-intracellular junction (Newman, 1997). CD31 has been shown to be important in numerous biological processes, among others, vascular development (Baldwin et al., 1994) and angiogenesis (Zhou et al., 1999). For instance, treating animals with blocking anti-CD31 antibodies has been shown to inhibit tumor-induced angiogenesis (Zhou et al., 1999). Furthermore, we have shown that CD31 is a reliable marker for VEGF-induced angiogenesis during chronic inflammation (Appleton et al., 1996). However, the role of CD31 during cerebral ischemia-induced inflammation has not been elucidated and characterized.

The aim of the present study was to initially assess whether CD31 expression was altered following an HI-insult. In addition, the effects of the $GABA_A$ receptor modulator, clomethiazole, as a neuroprotectant and the non-selective NOS inhibitor, N^{ω}-nitro-L-arginine methylester (L-NAME), as demonstrated here to be a neurodegenerative agent, on new blood vessel formation were elucidated. As L-arginine metabolism is pivotal to both inflammation and angiogenesis, we also assessed the co-incident effects of L-NAME on NOS activity, nitrite levels and arginase activity. Recent evidence has shown that some pro-inflammatory cytokines such as TNF-α and IL-1 are pro-angiogenic, while others are anti-angiogenic (Slevin et al., 2006). Therefore, we also assessed circulating levels of pro- and anti-inflammatory cytokines (IL-1α, IL-1β, GM-CSF and IL-10), which are known to be involved in processes associated with neuroprotection/neurodegeneration.

2. Results

2.1. *Anatomical evidence of neuroprotection*

During the time course of HI and drug administration, body weight, core temperature and blood glucose levels were monitored and shown not to vary between treatment groups (data not shown).

We have previously shown that CMZ (414 mg/kg/day) is neuroprotective in this model of HI (Clarkson et al., 2005a). However, assessment of L-NAME in this model had yet to be assessed. Therefore, the extent of neuronal damage 3 days post-HI was detected using TTC staining. Assessment of neuronal infarction following HI + saline treatment revealed extensive tissue loss ipsilateral to the carotid occlusion, with no apparent anatomical damage contralaterally (Fig. 1A). Treatment with L-NAME, at approximately 5 mg/kg/day, resulted in a marked increase in infarct area compared to the HI + saline-treated controls ($P<0.001$; Fig. 1B).

2.2. *Assessment of NOS and arginase activity*

We have previously shown that treatment with CMZ following HI results in a significant increase in total NOS activity and a significant decrease in both iNOS and arginase activities (Clarkson et al., 2005a). However, assessment of the effects of L-NAME had yet to be carried out in this model. Therefore, assessment of total NOS, iNOS, arginase and tissue nitrite was carried out.

Assessment of NOS activity 3 days post-HI revealed a significant increase in both total NOS and iNOS activities in the left hemisphere from the HI + saline-treated animals compared to non-intervention controls ($P<0.05$ and $P<0.001$, respectively; Fig. 2A). Treatment with L-NAME resulted in a significant decrease in both total NOS and iNOS activities compared to HI + saline treatment ($P<0.01$ and $P<0.05$ respectively; Fig. 2A) and did not differ to non-intervention controls. Assessment of tissue nitrite levels showed an almost identical profile as the NOS data.

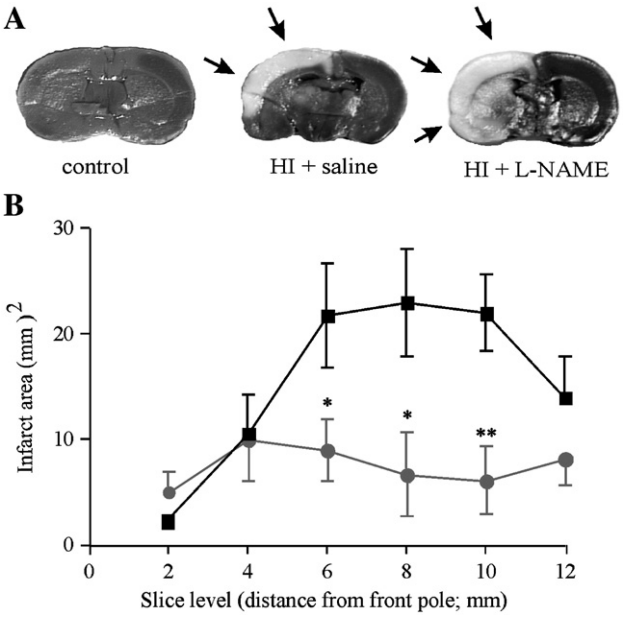

Fig. 1 – Assessment of infarct size 3 days post-HI revealed significant damage ipsilateral to the carotid ligation. L-NAME treatment (■; $n=7$) significantly exacerbated the infarct size across brain regions relative to HI + saline (●; $n=7$). Sample TTC-stained coronal sections (control, HI + saline and HI + CMZ) taken 6 mm from the frontal pole, showed gross cortical and subcortical damage as indicated by the arrows, with a significant increase in infarct size with L-NAME treatment. HI + L-NAME vs. HI + saline: $^{*}P<0.05$, $^{**}P<0.01$.