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Astrocyte-specific heme oxygenase-1 hyperexpression attenuates heme-mediated oxidative injury

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In prior studies, we have observed that HO activity protects astrocytes from heme-mediated injury, but paradoxically increases neuronal injury. In this study, we tested the hypothesis that an adenovirus encoding the human *HO-1* gene driven by an enhanced glial fibrillary acidic protein promoter (Ad-GFAP-HO-1) would increase HO-1 expression selectively in astrocytes, and provide cytoprotection. Treatment with 100 MOI Ad-GFAP-HO-1 for 24 h resulted in HO-1 expression that was 6.4-fold higher in cultured primary astrocytes than in neurons. Astrocyte HO activity was increased by approximately fourfold over baseline, which was sufficient to reduce cell death after 24-h hemin exposure by 60%, as assessed by both MTT and LDH release assays. A similar reduction in cell protein oxidation, quantified by carbonyl assay, was also observed. These results suggest that HO-1 transgene expression regulated by an enhanced GFAP promoter selectively increases HO-1 expression in astrocytes, and is cytoprotective. Further investigation of this strategy in vivo is warranted.

Keywords: Cell culture; Free radical; Gene therapy; Iron; Hemoglobin toxicity; Intracerebral hemorrhage; Oxidative stress; Stroke

Introduction

A growing body of experimental evidence supports the hypothesis that extracellular hemoglobin (Hb) contributes to iron-dependent oxidative injury after intracerebral hemorrhage. Since the heme groups of Hb are sequestered in hydrophobic pockets that limit their ability to participate in free radical reactions (Hebbel and Eaton, 1989), the deleterious effect of Hb is likely mediated by heme transfer to membrane lipids and proteins (Bunn and Jandl, 1967). This process is most efficient when Hb autoxidizes to methemoglobin (Balla et al., 1993), which occurs in intracranial hematomas (Bradley, 1993). Hemin, the oxidized form of heme, is a highly reactive compound that produces an iron-dependent injury to both neurons and astrocytes in cell culture and in vivo (Huang et al., 2002; Regan et al., 2002; Goldstein et al., 2003).

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Under physiologic conditions, the cellular concentration of free heme is tightly regulated, and is generally maintained at $<10^{-9}$M (Taketani, 2005). However, it increases significantly in CNS cells exposed to micromolar concentrations of extracellular Hb or hemin (Chen-Roetling et al., 2005; Chen-Roetling and Regan, 2006), suggesting that endogenous catabolic mechanisms may be insufficient to maintain homeostasis in cells adjacent to an intracerebral hematoma. Heme breakdown is mediated by the heme oxygenases (HO), which catalyze the rate-limiting step in its conversion to bilirubin, carbon monoxide, and iron. Two HO isoforms have been characterized in the CNS to date, which are the products of separate genes, but share similar mechanisms for substrate recognition and heme degradation (Chang et al., 2005). HO-1 is normally expressed at a very low level, but is rapidly induced in astrocytes, microglia, and some neurons by extravascular Hb, hemin, and a variety of oxidants (Matz et al., 1997; Turner et al., 1998). HO-2 is constitutively expressed, predominantly by neurons (Matz et al., 1997).

Prior cell culture studies suggest that HO has considerable but opposite effects on heme-mediated injury to astrocytes and neurons. Consistent with the benefit provided by HO in ischemia and trauma models (Doré et al., 1999; Chang et al., 2003), HO-1 knockout astrocytes were more vulnerable to Hb or hemin (Chen-Roetling et al., 2005; Chen-Roetling and Regan, 2006), while increasing astrocyte expression by genetic or pharmacologic means was protective (Teng et al., 2004; Chen and Regan, 2005). Putative protective mechanisms include the antioxidant effect of bilirubin production (Doré et al., 1999), and also the conversion of a lipid soluble oxidant, hemin, to iron, which is then sequestered in ferritin (Balla et al., 1992). Paradoxically, HO appears to accelerate Hb or hemin cytotoxicity in neurons in some cell culture and in vivo models (Rogers et al., 2003; Koeppen et al., 2004; Gong et al., 2006), presumably due to iron-mediated oxidative stress (Nakamura et al., 2004). Although the molecular basis for the discrepancy between neurons and astrocytes has not been precisely defined, it may reflect the limited ability of neurons to detoxify excess iron by increasing ferritin synthesis (Wu et al., 2003).

The disparate effect of HO on heme-mediated injury to neurons and astrocytes suggests that it may be a challenging therapeutic target. Although increasing HO expression in astrocytes has a potent

and highly reproducible protective effect, nonselective approaches may also increase expression in neurons and thereby increase their vulnerability. A cell-specific approach may therefore be optimal. The glial fibrillary acidic protein (GFAP) promoter has previously been shown to direct gene expression with considerable specificity for astrocytes (Mucke et al., 1991; Brenner, 1994; Holland and Varmus, 1998; McKie et al., 1998; Morimoto et al., 2002). It is noteworthy, however, that this original GFAP promoter is relatively weak when compared with viral promoters such as the human CMV promoter (Morelli et al., 1999). A stronger driver of transgene expression may be preferable for mechanistic cell culture studies, and for in vivo investigation of the feasibility of gene therapy. Toward this end, novel second generation GFAP promoters have recently been described (de Leeuw et al., 2006). Due to redundancy of their enhancer regions, transgene expression driven by these promoters has been reported to be up to 75-fold greater than that provided by the parent GFAP promoter in the U251 glial cell line, without any loss of specificity (de Leeuw et al., 2006). However, their efficacy in cell injury models has not yet been reported. In the current study, we tested the hypothesis that transfer of the human *HO-1* gene driven by an enhanced GFAP promoter would increase HO-1 specifically in primary cultured astrocytes, and lead to HO activity levels that would be sufficient to protect against heme-mediated oxidative injury.

Materials and methods

Cell cultures

Cortical primary astrocyte cultures were prepared from 1- to 2-day-old BALB/c×129/Sv mice as recently described in detail (Chen-Roetling et al., 2005). Plating medium contained minimal essential medium (MEM, Invitrogen, Carlsbad, CA), 10% equine serum (Hyclone, Logan, UT), 10% fetal bovine serum (Hyclone), 2 mM glutamine, and 10 μg/ml epidermal growth factor (EGF, Sigma-Aldrich, St. Louis, MO). Two-thirds of the medium was replaced at 5 days in vitro and twice weekly thereafter. Feeding medium was similar to plating medium, except that it lacked fetal bovine serum and EGF, and contained 10% equine serum. Mixed neuron–astrocyte cultures were prepared from fetal mice at 15- to 17-day gestational age, also as previously described (Regan et al., 2004). They were fed twice weekly with the same maintenance medium used for astrocytes until 10 days in vitro, and subsequently were fed daily. Pure neuronal cultures were plated on 24-well plates coated with 50 μg/ml poly-D-lysine, in neurobasal medium (Invitrogen) containing B27 supplement (Invitrogen). All cultures were incubated in a 5% CO_2 atmosphere at 37 °C.

Adenovirus preparation

A plasmid containing the enhanced GFAP promoter pGfa2 $(ABD)_3$-nLacZ (de Leeuw et al., 2006) was kindly provided by Dr. Michael Brenner (University of Alabama, Birmingham). Shuttle vector pCMV-HO-1 was previously provided by Dr. Lee-Young Chau (Academia Sinica, Taiwan, ROC). Both constructs were used to prepare the shuttle adenoviral vector pDUAL:GFAP-HO-1. The GFAP promoter of pGfa2$(ABD)_3$-nLacZ was excised by digestion with *Bgl*II on the 5′ end and *Bam*HI on the 3′ end, and the *HO-1* gene open reading frame was excised by digestion with *Bam*HI on the 5′ end and *Hin*dIII on 3′ end from pCMV-HO-1. Both fragments were introduced to adenoviral shuttle vector pDUAL-CCM (Vector Biolabs, Philadelphia, PA) creating pDUAL-GFAP-HO-1. This construct was sequenced and transfected to primary cultured HO-1 knockout astrocytes for expression evaluation. Sequence analysis demonstrated promoter—insert orientation and insert identity to the human HO-1 sequence, accession numberNM_002133, using the Chromas sequences analyzing software and NCBI-The Basic Local Alignment Search Tool (BLAST).

*Swa*I endonuclease was used to release the insert from shuttle pDUAL-GFAP-HO-1 and ligate it directly into the viral plasmid vector (pAd-VEC). Adenoviral plasmid clones pAd-VEC-GFAP-HO-1 were checked for expression before virus preparation by transfecting HO-1 knockout astrocytes (5 days in vitro, 1 μg DNA per well) using Lipofectamine plus reagent in serum-free medium (OptiMEM, Invitrogen). HO-1 expression was assessed via immunoblotting as described below. The viral construct was also verified using sequencing primers and restrictional analysis. For viral packaging in HEK293 cells, pAd-VEC-GFAP-HO-1 was transfected in linear form (digested with Pac-I), to produce Ad-GFAP-HO-1. After propagation and harvesting, titer was quantified by cytopathic effect assay.

Adenovirus encoding human HO-1 controlled by the CMV promoter (Ad-CMV-HO-1, Juan et al., 2001) was provided as a gift by Dr. Lee-Young Chau. Adenoviral infection of astrocytes was accomplished in medium similar to feeding medium, except that it contained 3.3% equine serum. In a prior study using this culture system, treatment with 100 MOI of serotype 5 adenovirus in this medium resulted in transfection of approximately 80% of astrocytes (Teng et al., 2004); similar efficacy has been observed for cultured neurons (unpublished observations).

Immunoblotting

Cells were homogenized in cold cell lysis buffer (210 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EDTA, 0.1% sodium dodecyl sulfate) and sonicated. After protein assay (Pierce, Rockford, IL), 20–25 μg samples of total proteins were separated on a 15% polyacrylamide gel (Bio Rad, Laboratories, Hercules, CA) and transferred to a polyvinylidene difluoride Imobilon-P transfer membrane filter (Millipore, Billerica, MA) using a semi-dry transfer apparatus. HO-1 protein expression was detected with 1:5000 dilution of rabbit anti-HO-1 antibody and HO-2 protein expression was detected with 1:2000 dilution of rabbit anti-HO-2 antibody (incubation period of 16 h at 4 °C, Stressgen Biotechnologies Corp., San Diego, CA), followed by 1-h incubation with goat anti-rabbit IgG (1:20,000, Pierce, Rockford, IL). Immunoreactive proteins were visualized using Super Signal West Femto Reagent (Pierce) and Kodak Gel Logic 2200.

Cytotoxicity assays

Cultures prepared from wild-type mice were washed free of growth medium and virus, and were placed into medium consisting of MEM with 10 mM glucose (MEM10). Hemin (Sigma-Aldrich) was diluted in this medium to final concentrations that were based on previously determined concentration–toxicity relationships in these cultures (Regan et al., 2004; Chen-Roetling et al., 2005). Plates were then incubated for 24 h, with minimal disturbance.

Cell death was quantified by measuring lactate dehydrogenase (LDH) activity in the serum-free culture medium, as previously described (Regan and Choi, 1994). This assay correlates well with