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In the hypoxic central nervous system, endothelial cell proliferation is followed by astrocyte activation, proliferation, and increased expression of the $\alpha 6\beta 4$ integrin and dystroglycan

Longxuan Li¹, Jennifer V. Welser¹, Paula Dore-Duffy², Gregory J. del Zoppo³, Joseph C. LaManna⁴, and Richard Milner¹

¹ Department of Molecular and Experimental Medicine, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037

² Department of Neurology, Wayne State School of Medicine, Detroit, MI

³ Department of Medicine, University of Washington, Seattle, WA

⁴ Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, Cleveland, OH

Abstract

Cerebral hypoxia induces a profound angiogenic response in the central nervous system (CNS). Using a mouse model of chronic cerebral hypoxia, we previously demonstrated that angiogenic vessels in the hypoxic CNS show marked upregulation of the extracellular matrix (ECM) protein fibronectin, along with increased expression of its major receptor, $\alpha 5\beta 1$ integrin on brain endothelial cells (BEC). As cerebral hypoxia also leads to glial activation, the aim of the current study was to define the temporal relationship between BEC responses and glial cell activation in this model of cerebral hypoxia. This revealed that BEC fibronectin/ $\alpha 5\beta 1$ integrin expression and proliferation both reached maximal level after 4 days hypoxia. Interestingly, up to 4 days hypoxia, all dividing cells were BEC, but at later time-points proliferating astrocytes were also observed. GFAP staining revealed that hypoxia induced marked astrocyte activation that reached maximal level between 7–14 days hypoxia. As newly formed cerebral capillaries require ensheathment by astrocyte end-feet in order to acquire mature brain endothelium characteristics, we next examined how expression of astrocyte end-feet adhesion molecules is regulated by hypoxia. This showed that the astrocyte adhesion receptors $\alpha 6\beta 4$ integrin and dystroglycan were both markedly upregulated, with a time-course that closely resembled astrocyte activation. Taken together, this evidence shows that cerebral hypoxia promotes first an endothelial response, in which fibronectin promotes BEC proliferation. This is then followed by an astrocyte response, involving astrocyte activation, proliferation and re-organization of astrocyte end-feet, which correlates with increased expression of astrocyte end-feet adhesion molecules.

Keywords

Hypoxia; angiogenesis; endothelial; astrocyte; activation; integrin

INTRODUCTION

During cerebral hypoxia, the CNS attempts to compensate by sprouting new capillaries, in the process called angiogenesis (Kanaan et al. 2006; LaManna et al. 1992). In order to identify molecular mechanisms important for promoting cerebral angiogenesis, our lab and others study this process in an animal model of chronic cerebral hypoxia, in which a marked angiogenic response occurs (LaManna et al. 1992; Milner et al. 2008a). The functional significance of these changes is well illustrated by the finding that animals receiving this treatment are subsequently protected against the destructive effects of cerebral ischemia, a phenomenon called ischemic pre-conditioning (Dowden and Corbett 1999; Miller et al. 2001). Considering the therapeutic potential of cerebral angiogenesis, it becomes a high priority to define the molecular mechanisms that promote the angiogenic response to hypoxia. These have yet to be fully defined, but studies have implicated hypoxic inducible factor-1 α (HIF-1 α) (Chavez et al. 2000), and the growth factors vascular endothelial growth factor (VEGF) (Kuo et al. 1999) and Angiopoietin-2 (Ang2) (Pichiule and LaManna 2002).

In light of the fundamental role of extracellular matrix (ECM) proteins in regulating angiogenesis in other systems (Stromblad and Chersesh 1996), we have focused our efforts on defining the expression profile and potential roles of fibronectin and associated integrin receptors on angiogenic blood vessels in the hypoxic CNS. Recently, we described hypoxic upregulation of fibronectin and the $\alpha 5\beta 1$ integrin on cerebral microvessels that reached its maximal level of expression after 4 days hypoxia, and declined thereafter (Milner et al. 2008a). Angiogenesis is a multi-stage process involving several key steps including: de-differentiation of mature endothelial cells, proliferation, survival, migration, capillary tube formation and finally stabilization into mature endothelium (Grant and Kleinman 1997). At an early stage of the angiogenic process, endothelial cells proliferate to increase in cell number before migrating out to form new vascular sprouts and capillary tubes. In light of our previous finding that fibronectin and $\alpha 5\beta 1$ integrin show maximal expression after 4 days hypoxia (Milner et al. 2008a), and our in vitro demonstration that fibronectin strongly promotes BEC proliferation (Wang and Milner 2006), this suggests that the strongest stimulus of BEC proliferation in the hypoxic model might occur after 4 days hypoxia.

In addition to BEC, other cell types in the CNS also mount responses to hypoxia, including glial cells. Astrocytes show a marked activation response to cerebral hypoxia that includes cell hypertrophy and proliferation (Garnier et al. 2001; Wakita et al. 1994). Interestingly, mounting evidence suggests that astrocytes and microglia may regulate endothelial cell function and angiogenesis through the release of cytokines and growth factors (Lehrman et al. 1998; Morganti-Kossmann et al. 1992; Wang et al. 1995). In light of this, the goal of the current study was to define the temporal relationship between BEC responses (fibronectin/ $\alpha 5\beta 1$ integrin expression and proliferation) and glial cell responses (activation, proliferation and adhesion molecule expression) in a mouse model of chronic cerebral hypoxia.

MATERIALS AND METHODS

Animals

The studies described have been reviewed and approved by The Scripps Research Institute Institutional Animal Care and Use Committee. All animals were maintained under pathogen-free conditions in the closed breeding colony of The Scripps Research Institute (TSRI).

Hypobaric Hypoxia

C57Bl/6J mice, 8–10 weeks of age, housed 4 to a cage, were placed into a hypobaric chamber maintained at a pressure of 300 Torr, (0.4 ATM, equivalent to 8% normobaric

oxygen) for periods up to 14 days. Littermate controls were kept in the same room in a similar chamber under similar conditions except that they were kept at normobaric pressure for the duration of the experiment. Every few days, the chamber was returned briefly to normobaric pressure for cage cleaning and food and water replacement as needed.

Immunofluorescent studies and antibodies

Immunofluorescent (IF) studies were performed as previously described (Milner et al. 2008a) on 10µm thick frozen sagittal sections of cold saline-perfused brains taken from C57/Bl6J mice subject to either normoxia (control) or hypobaric hypoxia for 1, 2, 4, 7 or 14 days. The following monoclonal antibodies were obtained from BD Pharmingen (La Jolla, CA): rat monoclonal antibodies reactive for the $\alpha 5$ integrin subunit (clone 5H10-27), $\alpha 6$ integrin (clone GoH3), $\beta 4$ integrin (clone 346-11A), Mac-1 ($\alpha M\beta 2$ integrin, clone M1/70), CD31 (PECAM-1) (clone MEC13.3), clone MECA-32. The rat monoclonal antibody against the microglial antigen F4/80 was obtained from Serotec, and the mouse monoclonal antibody reactive for β -dystroglycan (clone 43DAG/8D5) was obtained from Novocastra (Newcastle, United Kingdom). The Cy-3 conjugated mouse monoclonal against GFAP (clone G-A-5) was obtained from Sigma. Rabbit polyclonal antibodies against the following proteins were used in this study: fibronectin (Sigma), laminin (Sigma), Ki67 (Vector Laboratories, Burlingame, CA), IBA-1 (WAKO), ZO-1 and claudin-5 (Zymed Laboratories, San Francisco, CA), β -actin (Neomarker, Fremont, CA). Goat anti-mouse albumin was obtained from Bethyl Laboratories (Montgomery, TX). The Cy3-conjugated secondary antibodies against rat IgG, mouse IgG and rabbit IgG were all obtained from Jackson ImmunoResearch (West Grove, PA).

For the dual-color IF studies of Ki67 and CD31, tissue sections were incubated with the Ki67 antibody overnight at 4°C, washed then incubated with the Cy3-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch) for 30 minutes at 37°C, washed, then incubated with the anti-CD31 rat monoclonal antibody for one hour at 37°C, washed, then incubated with the AlexaFluor 488-conjugated anti-rat secondary antibody (Invitrogen, Carlsbad, CA) for 30 minutes at 37°C, and finally washed thoroughly and mounted. Dual-color fluorescent studies of Ki67 and GFAP were performed in a similar manner, though using an Alexafluor 488-conjugated secondary anti-rabbit (Invitrogen) to identify Ki67-positive cells and a Cy3-conjugated anti-GFAP mouse monoclonal antibody (Sigma) to detect astrocytes.

Quantification of the number of blood vessels positive for the different antigens was performed by capturing images with a X20 objective on a Zeiss Axio Observer A1 microscope and Zeiss Axiocam MRC digital camera. These images were used to determine the number of positive events within this field of view. For each antigen, three brain sections were taken from each animal, and matched between animals so that the approximate position of sections used was equivalent between different experimental conditions. Multiple images were taken of each section, and quantified by eye for the number of positive events per field of view. Each experiment was performed with three different animals per condition, and the results expressed as the mean $\pm$ SEM of the number of antigen-positive events per field of view. Statistical significance was assessed by using Student's t test, in which $p < 0.05$ was defined as statistically significant.

Western Blot Analysis

Western blot analysis was used to determine levels of the tight junction proteins ZO-1 and claudin-5 in brain lysates, as previously described (Milner et al. 2007). Within each brain sample, levels of these proteins were first normalised to the level of β -actin, and then expressed as the fold-increase over the level present within the brain of normoxic animals.

RESULTS

Expression of fibronectin, $\alpha 5\beta 1$ integrin and MECA-32 in angiogenic cerebral vessels peaks after 4 days hypoxia

In a previous study we demonstrated that cerebral hypoxia promotes a strong induction of vascular expression of fibronectin, and its receptor, $\alpha 5\beta 1$ integrin, and MECA-32, a marker of immature brain endothelium (Milner et al. 2008a). The highest level of expression of all three proteins was attained after 4 days hypoxia, and decreased thereafter. In the current study, we performed a more complete analysis, to determine whether expression of fibronectin, $\alpha 5\beta 1$ integrin and MECA-32 reach peak level even before 4 days hypoxia. Six groups of mice were studied: normoxia (control), and mice subject to hypoxia (8% O_2) for 1, 2, 4, 7 or 14 days ($n=3$ animals in each group). Protein expression was examined by immunofluorescence (IF), and the number of positive events quantified in two areas of the CNS, the brainstem and cerebral cortex. Consistent with previous observations (Kanaan et al. 2006; LaManna et al. 1992), hypoxia induced a strong angiogenic response. 14 days hypoxia increased the blood vessel density, as measured by the number of CD31-positive vessels per field of view, from 63.8 ± 3.2 to 97.0 ± 6.2 in the brainstem ($p<0.05$) and from 58.1 ± 4.1 to 87.9 ± 6.4 in the cerebral cortex ($p<0.05$) (Figure 1A). Quantification of vessels positive for fibronectin, $\alpha 5$ integrin, and MECA-32 revealed that vascular expression of all three markers increased in response to hypoxia, with the maximal level of expression attained after 4 days hypoxia, before declining at later time-points (Figure 1B-D). Compared to normoxic conditions, 4 days hypoxia increased the number of fibronectin-positive vessels per field of view, from 41.4 ± 2.2 to 78.2 ± 7.2 in the brainstem ($p<0.05$) and from 37.7 ± 4.2 to 74.0 ± 7.2 in the cerebral cortex ($p<0.05$), and increased the number of $\alpha 5$ integrin-positive vessels per field of view, from 24.4 ± 0.6 to 58.2 ± 5.4 in the brainstem ($p<0.01$) and from 23.0 ± 3.1 to 53.9 ± 3.8 in the cerebral cortex ($p<0.01$). In addition, compared with a total absence of MECA-32 expression under normoxic conditions, 4 days hypoxia induced expression of MECA-32, to 18.2 ± 2.7 MECA-32-positive vessels per field of view in the brainstem ($p<0.01$) and 25.9 ± 2.9 positive vessels in the cerebral cortex ($p<0.005$).

BEC proliferation is an early event in hypoxic-induced cerebral angiogenesis

While it is clear that cerebral hypoxia leads to a pronounced angiogenic response (Kanaan et al. 2006; LaManna et al. 1992), what has not been directly examined to date is the influence of hypoxia on BEC proliferation in the angiogenic vessels. To examine this important aspect of angiogenesis, we performed IF studies using the cell proliferation marker Ki67. In the normoxic adult CNS, Ki67-positive cells were seen only in the well-described neurogenic areas of the adult CNS, which include the sub-ventricular zone (SVZ), rostral migratory stream (RMS) and the dentate gyrus of the hippocampus. Other areas of the brain contained very few if any Ki67-positive cells (Figure 2). After 1 or 2 days hypoxia, an occasional Ki67-positive cell was seen. However, following 4 days hypoxia, many Ki67-positive cells were detected throughout the CNS parenchyma. Quantification revealed that the number of Ki67 cells reached a maximum level after 4 days hypoxia, and declined thereafter. Compared to normoxic conditions, four days hypoxia increased the number of Ki67-positive cells per field of view, from 0.1 ± 0.1 to 14.9 ± 1.9 in the brainstem ($p<0.01$) and from 0.5 ± 0.1 to 12.2 ± 1.8 in the cerebral cortex ($p<0.01$). To determine the identity of the proliferating cells, we performed dual-IF with the endothelial-specific marker CD31, and Ki67 (Figure 3). This showed that after 4 days hypoxia, the vast majority of Ki67-positive cells were endothelial cells ($98.5 \pm 1.5\%$ and $97.5 \pm 2.5\%$ in the cerebral cortex and brainstem respectively). Interestingly, this revealed that groups of Ki67-positive cells often displayed a linear arrangement (Figure 3A, 4-day time-point), suggesting that cohorts of neighboring endothelial cells proliferate first, before producing angiogenic sprouts (see arrow in Figure 3A), and migrating out to form new capillaries.

Cerebral hypoxia also promotes astrocyte activation and proliferation

Interestingly, at later times of hypoxia (days 7 and 14), a smaller fraction of Ki67-positive cells co-labeled with CD31 (Figure 3A and B), suggesting that after day 4, cells other than BEC are also proliferating. As cerebral hypoxia also promotes glial activation (Garnier et al. 2001; Wakita et al. 1994), next we investigated whether cerebral hypoxia induced astrocyte or microglial activation/proliferation. Surprisingly, IF staining with the microglia-specific markers Mac-1, F4/80 and IBA-1 showed a total absence of microglial activation at all time-points (data not shown). In contrast, GFAP staining revealed that cerebral hypoxia induced a marked increase in astrocyte activation. However, while the temporal profile of endothelial cell responses to hypoxia peaked after 4 days, the intensity of the GFAP signal followed a delayed time-course, reaching a maximal level between 7–14 days hypoxia (Figure 4). This was demonstrated by an increased density of GFAP-positive cells in brainstem and cerebral cortex, and by a strongly increased GFAP signal intensity within individual cells. Compared to normoxic conditions, seven days hypoxia increased the number of GFAP-positive events per field of view, from 10.3 ± 1.0 to 67.7 ± 4.9 in the brainstem ($p < 0.001$) and from 10.0 ± 1.3 to 64.1 ± 2.3 in the cerebral cortex ($p < 0.001$). To determine whether some of the proliferating cells might be astrocytes, dual-IF with GFAP and Ki67 was performed. This revealed that after 7 and 14 days hypoxia, some of the Ki67-positive cells also stained positive for GFAP, demonstrating that cerebral hypoxia leads to astrocyte activation and proliferation (Figure 4C).

Cerebral hypoxia promotes increased expression of the tight junction proteins claudin-5 and ZO-1 in cerebral blood vessels

In the adult brain, mature capillaries have high electrical resistance and tight barrier characteristics, partly as a result of the tight junction proteins expressed between neighboring BEC, and partly due to the influence of astrocyte end-feet (Ballabh et al. 2004; del Zoppo and Milner 2006; Engelhardt 2003). To investigate whether angiogenic vessels in the hypoxic CNS display changes in vascular integrity we next examined two parameters: (i) albumin leakage through angiogenic vessels, and (ii) vascular expression of tight junction proteins. In a previous study, we used albumin leakage to document vascular breakdown in a mouse model of multiple sclerosis (MS) (Milner et al. 2007). However, using the same approach in the cerebral hypoxia model, albumin IF failed to show any deposition of albumin in the cerebral parenchyma at any of the time-points examined (not shown). Next, we examined the expression of the tight junction proteins, claudin-5 and ZO-1, both by IF and by western blotting of brain lysates (Figure 5). IF showed that 14 days hypoxia induced a marked increase in the number of vessels positive for both claudin-5 (from 43.0 ± 2.4 to 93.9 ± 7.7 in the brainstem ($p < 0.005$) and from 41.6 ± 1.9 to 81.7 ± 3.0 in the cerebral cortex ($p < 0.001$)), and ZO-1 (from 42.4 ± 2.5 to 83.1 ± 4.8 in the brainstem ($p < 0.002$) and from 40.3 ± 1.1 to 79.3 ± 1.2 in the cerebral cortex ($p < 0.001$)). Western blotting confirmed this result, revealing increased expression of both claudin-5 (increased 3.1 ± 0.6 -fold, $p < 0.02$) and ZO-1 (increased 2.6 ± 0.6 -fold, $p < 0.05$) after 14 days hypoxia. This data suggests that vessel integrity is not compromised during hypoxia-induced cerebral angiogenesis, but rather hypoxia induces a marked synthesis of tight junction proteins within BEC, to further promote vascular integrity of the BBB.

Cerebral hypoxia leads to increased expression of astrocyte end-feet adhesion molecules

The astrocyte activation and proliferation responses we observe during cerebral hypoxia are consistent with the idea that following blood vessel sprouting, astrocytes also undergo a remodeling response to match the available number of astrocyte end-feet to the increased number of cerebral vessels. Evidence suggests that specific adhesion molecules play key roles in anchoring astrocyte end-feet to the vascular basement membrane. In particular, the laminin receptors $\alpha 6 \beta 4$ integrin, and dystroglycan are highly expressed by astrocyte end-

feet, and loss of these receptors in cerebral ischemia correlates with detachment of astrocyte processes from the vascular basement membrane and disruption of the BBB (Milner et al. 2008b; Wagner et al. 1997). To determine whether expression of these astrocyte adhesion receptors is altered during the angiogenic remodeling response to cerebral hypoxia, we performed IF with antibodies specific for the $\alpha 6$ and $\beta 4$ integrin subunits, the β subunit of the dystroglycan dimer (β DG), and laminin. This revealed that cerebral hypoxia induced a marked increase in the number of vascular structures that stained positive for laminin and its associated cell surface receptors (Figures 6 and 7). In contrast to the hypoxic induction of $\alpha 5$ integrin and fibronectin, which both peak after 4 days hypoxia, vascular expression of the $\alpha 6$ and $\beta 4$ integrins and β DG increased gradually and reached their highest level of expression at day 14, the latest time-point examined. Fourteen days hypoxia increased the number of $\alpha 6$ integrin-positive vessels per field of view, from 54.4 ± 4.7 to 99.1 ± 6.3 in the brainstem ($p < 0.01$) and from 51.1 ± 4.4 to 85.6 ± 6.6 in the cerebral cortex ($p < 0.02$), and increased the number of β DG-positive vessels per field of view, from 54.3 ± 1.7 to 98.4 ± 1.3 in the brainstem ($p < 0.001$) and from 48.6 ± 1.7 to 85.6 ± 4.2 in the cerebral cortex ($p < 0.01$). The increased expression of these astrocyte end-feet adhesion molecules implies that newly formed capillaries are quickly ensheathed by astrocyte foot processes.

In contrast to the $\alpha 6$ integrin subunit and β DG, which are expressed on all vessels, the astrocyte end-feet-specific $\beta 4$ integrin subunit was expressed on only a small subset of vessels (Figure 6), consistent with previous reports (Milner and Campbell 2006; Previtali et al. 1997; Wagner et al. 1997). As shown in Figure 7, while there were more than 50 vessels positive for laminin, $\alpha 6$ integrin or β -DG per field in the normoxic CNS, only 5 vessels per field expressed the $\beta 4$ integrin, showing that $\beta 4$ integrin is expressed on less than 10% of cerebral vessels under control conditions. However, cerebral hypoxia had the greatest impact on $\beta 4$ integrin expression. Fourteen days hypoxia increased the number of $\beta 4$ integrin-positive vessels per field of view, from 4.5 ± 0.1 to 20.2 ± 1.4 in the brainstem ($p < 0.001$) and from 4.3 ± 0.2 to 19.1 ± 1.2 in the cerebral cortex ($p < 0.001$). Thus, 14-days of hypoxia induced a 4-fold increase in the density of $\beta 4$ integrin-positive vessels. As astrocyte expression of the $\beta 4$ integrin subunit is increased with astrocyte activation (Milner and Campbell 2006; Previtali et al. 1997), we next performed dual-IF to examine the relationship between astrocyte activation and $\beta 4$ integrin expression in this cerebral hypoxia model. Our results revealed that $\beta 4$ integrin expression was always closely associated with intense areas of GFAP, supporting the idea that astrocyte activation is associated with increased $\alpha 6\beta 4$ integrin expression (Figure 8).

DISCUSSION

The aim of this study was to examine endothelial and glial cell responses to cerebral hypoxia. We found that cerebral hypoxia promoted the following events: (i) upregulation of fibronectin, and BEC expression of $\alpha 5$ integrin and MECA-32, which all peaked at 4 days hypoxia, (ii) BEC proliferation which also peaked at day 4 hypoxia, (iii) astrocyte activation which peaked between 7–14 days hypoxia, and (iv) increased expression of the astrocyte end-feet adhesion molecules $\alpha 6\beta 4$ integrin and dystroglycan, which reached the highest level at the latest time-point examined (day 14 hypoxia). These results show that cerebral hypoxia promotes first an endothelial response, involving induction of fibronectin and the $\alpha 5\beta 1$ integrin and BEC proliferation, events that peak at 4 days hypoxia. This is followed by an astrocyte response, which peaks after 7–14 days, in which astrocytes become activated, proliferate and show increased expression of the astrocyte end-feet adhesion molecules, $\alpha 6\beta 4$ integrin and dystroglycan. These results suggest that astrocytes play an integral role in the angiogenic response to cerebral hypoxia, and demonstrate the presence of regulatory mechanisms that closely match angiogenic sprouting and formation of astrocyte end-feet.

Peak timing of fibronectin- $\alpha 5\beta 1$ integrin expression and BEC proliferation

Our results show that during chronic cerebral hypoxia, maximal expression of fibronectin, $\alpha 5$ integrin and MECA-32, a marker of immature brain endothelium, all peak at the same time-point, 4 days hypoxia. This response is relatively slow when compared with the timing of HIF-1 α and Angiopoietin-2 induction, which peak within the first 24–48 hours in this system of cerebral hypoxia (Chavez et al. 2000; Pichiule and LaManna 2002). VEGF shows a biphasic appearance, with the first peak at 2 days, then a decline, followed by a second peak at 7 days hypoxia (Kuo et al. 1999). The first peak is contributed by pericytes, and the second peak by other cells, including astrocytes (Dore-Duffy and LaManna 2007). The timing of these events suggest that fibronectin and $\alpha 5$ integrin may be downstream targets of these growth factors, though this has yet to be directly addressed. Interestingly, BEC proliferation also peaked at 4 days hypoxia, before declining at later time-points. Whereas an earlier study, examining total DNA content of isolated microvessels, did not detect proliferation until the second week of hypoxia (Harik et al. 1995), our findings using Ki67 labeling of intact hypoxic CNS tissue showed that BEC proliferation peaks after 4 days hypoxia. This demonstrates a tight correlation between fibronectin- $\alpha 5\beta 1$ integrin expression and BEC proliferation, both temporally and spatially, strongly suggesting that the fibronectin- $\alpha 5\beta 1$ integrin axis stimulates BEC proliferation at an early stage of angiogenesis. Having shown that BEC proliferate in response to hypoxia, what has yet to be addressed is whether circulating endothelial precursor cells (EPCs) also contribute to the neovascularization. While some evidence suggests that EPCs incorporate into newly-formed blood vessels (Zhang et al. 2002), more recent studies indicate that EPCs do not incorporate into new vessels in the adult (Ziegelhoeffer et al. 2004), but rather play an indirect role by stimulating angiogenic behavior of pre-existing endothelial cells (O'Neill et al. 2005).

Hypoxic induction of astrocyte activation and remodeling

It is useful to compare the glial activation responses in this model of chronic mild hypoxia with those in focal cerebral ischemia. In an acute focal cerebral ischemic event, there is total cessation of blood flow to specific regions of the cerebral parenchyma, which results in immediate oxygen starvation and cell death. This is accompanied by a full-blown inflammatory reaction, involving microglial and astrocyte activation, and massive release and activation of protease cascades, resulting in widescale tissue destruction and glial scarring (Gehrmann et al. 1992; Schilling et al. 2003; Wakita et al. 1994; Wang et al. 1995). During this process, active proteases lead to the destruction of many cell surface adhesion molecules, including endothelial and astrocyte integrins (Milner et al. 2008b; Wagner et al. 1997), and degradation of ECM components of the vascular basement membrane (del Zoppo and Milner 2006; Kwon et al. 2009). In contrast to the destructive effect of complete ischemia, chronic mild hypoxia represents a mild sub-clinical stimulus, which triggers no deleterious clinical or pathological events (LaManna et al. 1992). There is no neuronal loss, no massive release or activation of proteases, and no breakdown of cell surface integrins or basement membrane ECM proteins. However, our results demonstrate that chronic mild hypoxia is powerful enough to promote a physiological remodeling response involving angiogenesis and astrocyte activation. Our findings demonstrate that 8% hypoxia is not a strong enough stimulus to provoke microglial activation. This is notable because microglial activation is a very rapid and marked feature of cerebral ischemia (Gehrmann et al. 1992; Schilling et al. 2003). In contrast, astrocytes mounted a delayed activation response, that peaked after 4 days hypoxia and continued to increase up to the 14-day time-point. Interestingly, the timing of this astrocyte response is similar to that observed in animal models of focal cerebral ischemia (Wakita et al. 1994). Taken together, these results suggest that astrocytes and microglia have different thresholds of stimulation, and that whereas microglia need a strong, ischemic stimulus, astrocytes activate with a milder stimulus. From the point of view of remodeling, this is consistent with the described roles of microglia and

astrocytes. Ischemic destruction of tissue requires tissue breakdown and clearance, roles typified by microglia, whereas mild hypoxia requires no such breakdown or clearance, but rather an astrocyte remodeling to match the increased number of cerebral vessels.

Evidence suggests that astrocytes can regulate angiogenesis in both a positive and negative direction. In the normoxic brain, astrocytes produce angiopoietin 1 (Ang1), which binds to the endothelial Tie2 receptor, acting to stabilize the vasculature (Dore-Duffy and LaManna 2007). In addition, activated astrocytes also produce TGF- β 1, which has angiostatic properties (Morganti-Kossmann et al. 1992). On the other hand, in the hypoxic CNS, astrocytes express HIF-1 α in response to hypoxia (Chavez et al. 2000), and have also been shown to express VEGF (Kuo et al. 1999). Astrocytes also produce Ang2, which has both pro- and anti-angiogenic actions, depending on the presence or absence of VEGF (Dore-Duffy and LaManna 2007). Thus it appears that astrocytes have a bi-modal influence on cerebral angiogenesis, depending on the extent and timing of astrocyte activation.

Hypoxic induction of astrocyte end-feet adhesion molecules

In the mature CNS, astrocytes play an important role in maintaining the differentiated properties of the BBB (Abbott 2002; Abbruscato and Davis 1999; Janzer and Raff 1987). Astrocyte end-feet almost completely encase the vascular basement membrane, forming a very tight interface between the endothelial and astrocyte components of the neurovascular unit. During the angiogenic remodeling that occurs in response to hypoxia, it is evident that astrocytes need to generate new end-feet to allow the naïve capillaries to mature into fully functional cerebral vessels. At all time-points of the angiogenic process, we found a close correlation between the density of blood vessels positive for laminin or the adhesion molecules, α 6 integrin and β -dystroglycan (β DG). This suggests that during angiogenesis, the sprouting of new capillaries is closely matched with the generation of new astrocyte end-feet, and is consistent with a role for laminin-mediated interactions in the later stages of angiogenesis (Li et al. 2006). While all vessels express β -DG, the β 4 integrin deserves special mention because it is expressed by only ~10% of vessels in the normoxic CNS, but exhibits strong upregulation on activated astrocytes in the hypoxic CNS, consistent with previous reports of increased expression by activated astrocytes (Milner and Campbell 2006; Previtali et al. 1997).

Our work raises two clear testable questions. First, how important is the astrocyte response in cerebral hypoxia? This could be tested by selective ablation of activated astrocytes according to the method developed by Sofroniew and colleagues (Bush et al. 1999). We predict that in the absence of an astrocyte response, newly formed angiogenic capillaries will not be covered with astrocyte end-feet and thus not mature into fully competent cerebral blood vessels. Second, what role do astrocyte end-feet adhesion molecules play in maintaining the integrity of cerebral blood vessels, and in mounting an activation/remodeling response to cerebral hypoxia? To get a handle on this question, several recent studies have selectively deleted either dystroglycan or β 1 integrins from astrocytes, and analyzed the in vivo consequences. Interestingly, loss of either dystroglycan (Moore et al. 2002) or β 1 integrins (Robel et al. 2009) from astrocytes does not appear to directly affect vascular integrity, but does result in a partial reactive gliosis. It is possible that a lack of effect on vascular integrity in either of these single knockouts could be a consequence of functional redundancy, as astrocytes express β 1 integrins, α 6 β 4 integrin and dystroglycan. In future experiments it will be interesting to examine the influence of cerebral hypoxia in these transgenic mice to examine the potential role of β 1 integrins and dystroglycan in the astrocyte remodeling response to hypoxia. The α 6 β 4 integrin may be an even more interesting candidate; it shows a much more restricted expression pattern than β 1 integrin or dystroglycan, and displays marked upregulation with astrocyte activation (Milner and Campbell 2006; Previtali et al. 1997). In light of these unique features we plan to delete this

integrin in astrocytes. Based on current knowledge, we predict that absence of the $\alpha 6 \beta 4$ integrin in astrocytes will lead to a defective activation response, disordered organization of astrocyte ECM, and reduced integrity of vascular structures.

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Figure 1.

Quantification of hypoxic-induced alterations in brain capillary density and expression of fibronectin, $\alpha 5$ integrin and MECA-32. Frozen sections of brainstem or cerebral cortex taken from mice exposed to normoxia or 1, 2, 4, 7 or 14 days hypoxia were examined for the number of structures that stained positive for CD31 (A), fibronectin (B), $\alpha 5$ integrin (C), or the MECA-32 antigen (D). Each experiment was performed with three different animals for each condition, with results expressed as the mean $\pm$ SEM of the number of capillaries positive for each antigen per field of view. Note that hypoxia increased cerebral vessel density, and induced a strong increase in vascular expression of fibronectin, $\alpha 5$ integrin, and MECA-32 in both brainstem and cerebral cortex, with the maximum level of expression of all three proteins reached after 4 days hypoxia.

Figure 2.

Examination of cell proliferation during the angiogenic response to cerebral hypoxia. Frozen brain sections of mice exposed to normoxia, or 1, 2, 4, 7 or 14 days hypoxia were examined for evidence of cell proliferation as assessed by Ki67 labeling. Panel A displays Ki67-positive cells within frozen sections of brainstem and cerebral cortex. Panel B displays the quantification, in which each experiment was performed with three different animals for each condition, and results expressed as the mean $\pm$ SEM of the number of Ki67-positive cells per field of view. Note that hypoxia induced a strong increase in the number of proliferating cells, with this effect maximal after 4 days hypoxia.

Figure 3.

The influence of cerebral hypoxia on endothelial cell proliferation. A. Dual-IF was performed on frozen sections of brainstem taken from mice exposed to 1, 2, 4, 7 or 14 days hypoxia using antibodies specific for CD31 (AlexaFluor-488) and Ki67 (Cy-3). Scale bar = 50 μ m, except for the lowest row (4d (HP), scale bar = 25 μ m), which is a higher power magnification of the row labeled 4d. Note that little endothelial proliferation is seen after 1 or 2 days hypoxia, but this increases rapidly after 4 days hypoxia, before declining at later time-points. Proliferating BEC are seen grouped together in a linear arrangement within a single vessel, in which an angiogenic sprout is observed (see arrow). B. Quantification of the proportion of proliferating cells that are endothelial cells at the different time-points of hypoxia. Ki67-positive cells were identified as CD31-positive or CD31-negative and the proportion of Ki67/CD31 dual-positive cells calculated. Results are expressed as the mean $\pm$ SEM of three different experiments, each performed on a different set of animals. Note that in both the brainstem and cerebral cortex, after 4 days hypoxia, almost all the Ki67-positive cells were endothelial, but at later time-points, the fraction of Ki67-positive cells that labeled with CD31 diminished, to approximately 50% after 7 days hypoxia, and further still to approximately 35% after 14 days hypoxia.

Figure 4.

The influence of cerebral hypoxia on astrocyte activation and proliferation. A. Frozen sections of brainstem or cerebral cortex taken from mice exposed to normoxia or 1, 2, 4, 7 or 14 days hypoxia were immunostained with a Cy3-conjugated monoclonal anti-GFAP antibody. Scale bar in upper two rows = 50 μ m, in lower row = 10 μ m. B. Quantification of the hypoxic induction of GFAP expression. Results are expressed as the mean $\pm$ SEM of the number of GFAP-positive events per field of view in three different experiments, each performed on a different set of animals. Note that hypoxia induced a robust increase in astrocyte GFAP expression in both brainstem and cerebral cortex, with the effect reaching maximal level between days 7–14 hypoxia. C. Identification of proliferating astrocytes within the hypoxic CNS. Dual-IF was performed on frozen sections of brainstem taken from mice exposed to 7 or 14 days hypoxia, using antibodies specific for GFAP (Cy-3) and Ki67 (AlexaFluor-488). Scale bar = 25 μ m. Note that Ki67-positive astrocytes are observed at both the 7 and 14 day time-points.

Figure 5.

Hypoxic-induced alterations in brain capillary expression of the tight junction proteins, ZO-1 and claudin-5. A. Frozen sections of brainstem taken from mice exposed to normoxia or 4 or 14 days hypoxia were immunostained with polyclonal antibodies specific for ZO-1 and claudin-5. Scale bar = 50 μ m. B and C. Quantification of this response in the brainstem and cerebral cortex. D. Western blot analysis. Note that hypoxia induced a marked increase in vascular expression of ZO-1 and claudin-5 in both brainstem and cerebral cortex, with the maximum level of expression reached at the latest time-point examined (14 days).

Figure 6.

Hypoxic-induced alterations in brain capillary expression of β -dystroglycan and β 4 integrin. Frozen sections of brainstem or cerebral cortex taken from mice exposed to normoxia or 1, 2, 4, 7 or 14 days hypoxia were immunostained with monoclonal antibodies specific for β -dystroglycan or β 4 integrin. Scale bar = 50 μ m. Note that hypoxia induced a marked increase in vascular expression of β -dystroglycan and β 4 integrin in both brainstem and cerebral cortex, with the maximum level of expression reached at the latest time-point examined (14 days).

Figure 7.

Quantification of hypoxic-induced changes in brain capillary expression of $\alpha 6$ integrin, $\beta 4$ integrin, β -dystroglycan and laminin. Frozen sections of brainstem or cerebral cortex taken from mice exposed to normoxia or 1, 2, 4, 7 or 14 days hypoxia were immunostained with antibodies specific for the $\alpha 6$ integrin (A), $\beta 4$ integrin (B), β -dystroglycan (C) or laminin (D). Results are expressed as the mean $\pm$ SEM of the number of vessels that stained positive for the different markers per field of view, and represent data from three different experiments, each performed on a different set of animals. Note that hypoxia induced a robust increase in the number of vessels positive for all 4 markers, with the effect reaching maximal level at the latest time-point examined (14 days).

Figure 8.

The relationship between astrocyte activation and $\alpha 6\beta 4$ integrin expression in the hypoxic CNS. Dual-IF was performed on frozen sections of brainstem and cerebral cortex taken from mice exposed to cerebral hypoxia for 14 days, using monoclonal antibodies specific for GFAP (Cy-3) and the $\beta 4$ integrin (Alexafluor 488). Scale bar = 50 μ m. Note that $\beta 4$ integrin expression always co-localized with cells expressing high levels of GFAP.