Responses of Endothelial Cell and Astrocyte Matrix-Integrin Receptors to Ischemia Mimic Those Observed in the Neurovascular Unit

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Background and Purpose—Apposition of endothelial cells and astrocyte foot processes to the basal lamina matrix is postulated to underlie the cerebral microvessel permeability barrier. Focal cerebral ischemia induces rapid loss of select matrix-binding integrins from both cell compartments in the nonhuman primate. This study is the first to examine the conditions underlying integrin loss from these cell-types during ischemia in vitro and their relation to the changes in vivo.

Methods—The impact of normoxia or standardized oxygen-glucose deprivation on integrin expression by murine primary cerebral endothelial cells and astrocytes grown on matrix substrates (collagen IV, laminin, and perlecan) of the basal lamina were quantitatively assessed by flow cytometry.

Results—Endothelial cell expression of the β1 and α5 subunits significantly increased on all matrix ligands, whereas astrocytes displayed modest significant decreases in α5 and α6 subunits. Oxygen-glucose deprivation produced a further significant increase in subunit β1 expression by both cell types, but a clear decrease in both α1 and α6 subunits by murine astrocytes.

Conclusions—Ischemia in vitro significantly increased endothelial cell β1 expression, which is consistent with the increase in B1 transcription by microvessels peripheral to the ischemic core. The loss of α1 and α6 integrins from murine astrocytes is identical to that seen in the nonhuman primate in vivo. These findings establish both isolated murine cerebral endothelial cells and astrocytes as potential integrin response cognates of microvascular cells of the neurovascular unit in primates, and allow determination of the mechanisms of their changes to ischemia. (Stroke. 2008; 39:191-197.)

Key Words: astrocytes ■ endothelial cells ■ integrins ■ ischemic stroke ■ matrix
sels plus their known roles in cell viability suggest that these postischemic alterations may be relevant to edema formation, detachment of astrocyte end-feet during focal ischemia, and hemorrhagic transformation. But, identifying the mechanisms underlying the ischemia-induced integrin-matrix responses requires isolation of the cell components.

To establish identity between in vitro cell culture responses and brain tissue responses, and to elucidate the mechanisms regulating integrin expression, we examined how integrin expression by endothelial cells and astrocytes is managed, first by ECM proteins present in the basal lamina of cerebral vessels, then by standardized ischemia in vitro (oxygen glucose deprivation [OGD]). The tested hypothesis states that: (1) expression of select integrins by endothelial cells and astrocyte end-feet within cerebral microvessels of the (primate) neurovascular unit can be recapitulated in vitro, (2) the response to OGD coincides with the impact of focal ischemia on the cells in vivo, and (3) these responses are conserved between the 2 species. These experiments are intended as the basis for investigating the mechanisms of interactions between endothelial cells and astrocytes within the neurovascular unit of the primate cerebrum.25

Materials and Methods

Animals

The studies described here 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).

Cell Culture

Mouse primary brain endothelial cells were prepared as previously described,28 according to the method of Sapatino,29 with some modifications. Brains of adult C57BL6 mice (2 to 3 months old; The Scripps Research Institute, and Jackson Laboratories) were removed, cleaned of meninges and external blood vessels, then finely chopped, and dissociated for 1 hour in an enzymatic solution containing 30 mg/mL collagenase (GIBCO), and 0.05% trypsin (Sigma). Cells were collected by centrifugation at 150 g for 10 minutes (in order to separate the myelin, retained at the top of the tube, from the vascular tubes and other cells at the bottom of the tube). The collected vascular tubes and cells were resuspended and filtered through a 40-μm cell strainer (Falcon) to separate the vascular tubes from single cells. The tubes were then washed and centrifuged in MEM-HEPES before being resuspended in endothelial cell growth media consisting of Hams F12, supplemented with 10% FCS, heparin, ascorbic acid, L-glutamine (all from Sigma) and endothelial cell growth supplement (Upstate Cell Signaling Solutions, Lake Placid, NY). The endothelial cells were then added to 6-well plates, previously coated for 24 hours with type I collagen (Sigma), and grown in a humidified incubator at 37°C and 5% CO2. Assessed by live immunostaining for the antibody reactive for the integrin αv (clone 42), α5 (5H10-27 [MFR5]), or α6 (clone G0H3), and the isotype control antibodies, rat anti-KLH (A110-2), mouse anti-TNP (clone 49.2), and hamster anti-TNP (clone A19-3), were from BD Pharimingen (La Jolla, Calif). A monoclonal antibody reactive for the β1 integrin subunit (clone MB1.2) was obtained from Chemicon (Temecula, Calif). The phycocerythrin (PE)-conjugated secondary antibodies were also obtained from BD Pharimingen.

Preparation of ECM Protein Substrates

Individual wells within 6-well plates even coated with solutions containing collagen I, collagen IV, fibronectin, laminin (all prepared at 10 μg/mL in PBS) or perlecain (1 μg/mL; Sigma) for 2 hours at 37°C, before use. These substrates were then washed with 2 mL PBS. Brain endothelial cells or astrocytes that had been removed from primary cultures by trypsinization were then plated onto the ECM substrates and cultured in endothelial cell growth media– or DMEM-containing 10% fetal bovine serum. After 10 days, the flasks were shaken overnight to remove the loosely attached microglia and oligodendrocyte precursors. The remaining monolayer was determined as >95% astrocytes by immunoreactivity to antibodies against glial fibrillary acidic protein.

Oxygen-Glucose Deprivation

To investigate the influence of OGD on integrin expression, endothelial cells and astrocytes were cultured on 3 ECM substrates: collagen IV, laminin, or HSPG. On reaching confluence, the cell media was changed, with half the cultures receiving normal media (high glucose, 4.5 g/L), and the other half receiving low-glucose media (1 g/L). High-glucose cultures were exposed to normoxia for 18 hours. The low-glucose cultures were exposed to OGD for 18 hours, achieved by placing the cultures into a hypoxia chamber (Billups-Rothenburg), and flushing through the chamber with 95%N2/5%CO2 for 1 hour, then sealing the chamber for the duration of the experiment. In each experiment, within the culture media, measured O2 levels (Microelectrodes Inc) dropped to <2% after 2 hours to <1% after 4 hours, and were maintained at this level for the duration of ischemia in vitro.

Cell Viability Assay

Endothelial cells and astrocytes were cultured on laminin-coated glass coverslips within 24-well plates (Nunc) for 3 days. Cells were then either maintained in normal glucose and ambient O2 levels for 18 hours, or exposed to OGD for 18 hours. Afterward the cells were incubated with 1 μg/mL propidium iodide (PI, Sigma) for 15 minutes at 37°C. The cells were then washed in PBS and fixed at −20°C for 20 minutes before labeling with Hoechst stain (Sigma) for 5 minutes, washed and mounted. The percentage of damaged cells was evaluated by calculating the number of those dead or dying (PI positive) as a proportion of the total (Hoechst positive).

Antibodies

Monoclonal antibodies reactive for the integrin subunits α1 (Hs1/29), α3 (clone 42), α5 (5H10-27 [MFR5]), or α6 (clone G0H3), and the isotype control antibodies, rat anti-KLH (A110-2), mouse anti-TNP (clone 49.2), and hamster anti-TNP (clone A19-3), were obtained from BD Pharimingen (La Jolla, Calif). A monoclonal antibody reactive for the β1 integrin subunit (clone MB1.2) was obtained from Chemicon (Temecula, Calif). The phycocerythrin (PE)-conjugated secondary antibodies were also obtained from BD Pharimingen.

Flow Cytometry

Cell-associated integrin expression was determined as previously described.33 Briefly, endothelial cells and astrocytes were removed from their culture plates using a cell lifter (thereby avoiding the use of enzymes such as trypsin, which cleave cell surface proteins). Cells were blocked in suspension in 5% normal goat serum in PBS for 30 minutes on ice, transferred to wells within a round-bottom 96-well plate (Nunc), and then incubated for 1 hour on ice with primary antibodies specific for individual integrin subunits (antibody concentration 5 μg/mL). Cells were then washed twice in the blocking buffer before being labeled with anti-rat-PE, anti-mouse-PE, or anti-hamster-PE (all at 1 μg/mL) for 1 hour on ice and then washed twice with blocking buffer before being resuspended in 2% formaldehyde in PBS. The fluorescent intensity of labeled cells was then analyzed on a Becton Dickinson FACScan machine, with 10 000 events measured O2 levels (Microelectrodes Inc) dropped to <2% after 2 hours to <1% after 4 hours, and were maintained at this level for the duration of ischemia in vitro.

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Expression Is ECM-Dependent

Primary endothelial cells or astrocytes were cultured on single substrates (collagen IV, fibronectin, laminin, or the HSPG perlecan) which are the major proteins present within the basal lamina of primate cerebral blood vessels (Figure 1). Collagen I, not present in the vascular basal lamina, but induced in interstitial tissues in states of remodeling, served as an “external” control. The cell surface integrin expressions of endothelial cells and astrocytes, grown for 3 days on each substrate, were quantified by flow cytometry using monoclonal antibodies specific for the integrin subunits.\(^2\)

Endothelial expression of the integrin \(\beta 1\) and \(\alpha 5\) subunits was significantly increased by collagen I, collagen IV, fibronectin, and perlecan compared with plastic (Figures 2, 3, 4, and 5). Perlecan was most stimulating. Integrin \(\alpha 6\) subunit expression was significantly increased on fibronectin, but decreased on laminin. However, \(\alpha 1\) or \(\alpha 3\) presentation was not significantly affected by any substrate.

In contrast, overall, astrocytes displayed a rather small decrease in integrin expression in response to the ECM proteins (Figures 2 and 3). In particular, astrocyte \(\alpha 5\) and \(\alpha 6\) subunit expression decreased by exposure to fibronectin and laminin, and \(\alpha 6\) expression was also reduced on collagen I and HSPG. Expression of the \(\alpha 1\) or \(\alpha 3\) subunits by astrocytes was not affected by any substrate.

Taken together, these observations demonstrate that integrin expression by vascular endothelial cells and astrocytes is differentially regulated in a cell- and ECM substrate–specific manner. Establishing the ECM-dependence of baseline integrin expression paves the way for analyzing the influence of ischemia on these events.

Integrin Expression Is Influenced by Oxygen-Glucose Deprivation

Focal cerebral ischemia induces a rapid loss of integrin expression by endothelial cells and astrocyte end-feet in vivo.\(^2,3,4\) To determine whether ischemia per se is responsible for this, the impact of standardized ischemia (OGD) on integrin expression by primary brain endothelial cell and astrocyte cultures was determined. Standardized OGD had little impact on cell viability. In both endothelial and astrocyte cultures there was only 0.8% to 1.7% increase in cell death in the OGD-treated cultures compared with normoxia across all substrates (data not shown).

Endothelial cells exposed to OGD, demonstrated significantly increased expression of the \(\alpha 5\) integrin subunit on all substrates. In contrast, astrocyte cultures showed little impact of standardized ischemia on integrin expression, with only a minor decrease on fibronectin and laminin. These results are consistent with previously published findings.\(^2,3,4\) The impact of ischemia on astrocyte surface integrin expression is currently under study.

Statistical Analyses

Each cell culture experiment was repeated 3 times (on separate cultures) and the data are expressed as mean±SD. Analysis was performed using the Student paired t test. Significance was set at \(P<0.05\).

Results

Endothelial Cell and Astrocyte \(\beta 1\) Integrin Expression Is ECM-Dependent

RNA Extraction and Quantitative Real-Time RT-PCR

Total RNA was extracted from astrocytes subjected to normoxia or OGD using the RNeasy Plus Mini kit (QIAGEN). Eluted RNA was treated with DNA-free DNase (Ambion). The RT-PCR reaction was set up according to the recommendation of the SuperScript III Platinum One-Step Quantitative RT-PCR System protocol (Invitrogen). The final reaction conditions for the RT-PCR were 200 nM per primer, 100 nM per probe, 200 \(\mu\)mol/L of each dNTP, 5 mmol/L MgSO\(_4\), and 100 ng total RNA. The PCR cycling conditions were 45 cycles of 95°C for 30 seconds and 60°C for 30 seconds. The mouse integrin \(\beta 1\) sequence was obtained from the GenBank database (NM_010578.1), for which primers and TaqMan probes were designed with primer3 software. Quantification of the amount of target in the unknown samples was accomplished using a standard curve to determine the starting concentration of the target. Gene expression was normalized through dividing the starting concentration of the target by the starting concentration of constitutively expressed \(\alpha 2\)-actin. The standard curve was generated using QPCR mouse reference total RNA (Stratagene).

Events recorded for each condition. For each experimental condition, the mean fluorescence intensity was compared with the control state and expressed as the percentage change relative to the control condition.
3 ECM substrates, but showed no changes in subunits α1, α3, or α6. Moreover, OGD induced a large significant increase in endothelial cell expression of the β1 integrin subunit on all 3 substrates (Figures 2 and 4).

However, OGD significantly reduced the expression levels of both α1 and α6 integrin subunits by astrocytes cultured on all 3 ECM substrates (Figures 2 and 4), but α3 and α5 subunit expression was not significantly affected. In contrast, ischemia induced a large significant increase in astrocyte expression of the β1 integrin subunit on laminin and HSPG. This was supported by an overall increase in subunit β1 transcription by astrocytes, that was significant when the cells were grown on plastic (Figure 5).

**Discussion**

As early as 2 hours after the onset of cerebral ischemia in the nonhuman primate, microvascular endothelial cells and astrocytes display significant loss in integrin expression.\(^{23,24}\) This is accompanied by alterations in the vascular basal lamina matrix.\(^{13,26,35}\) Because these events coincide with increased cerebrovascular permeability,\(^{36}\) loss of endothelial and astrocyte adhesion to the microvascular basal lamina may be an early pivotal event that contributes to loss of the microvascular permeability barrier. In the neurovascular unit, the proximity of astrocyte end-feet to the abluminal surface of the endothelium suggests roles in integrin signaling by the matrix in both cell compartments that support their maintenance of the permeability barrier.
To characterize these events we first examined how integrin expression by endothelial cells and astrocytes is regulated by matrix proteins present in the basal lamina, and then by ischemia caused by OGD. The major findings are that (1) whereas astrocyte integrin expression was relatively unaffected by exposure to the ECM substrate, the expressions of subunits β1 and α5 by endothelium were promoted by all ECM ligands, particularly perlecan; (2) OGD induced significant changes in integrin expression of astrocytes and endothelial cells; and (3) during OGD the murine cell responses largely recapitulated the basic changes in microvascular integrin expression observed during focal cerebral ischemia in the nonhuman primate.

It is important to establish the direct influence of basal lamina ECM proteins on cellular integrin expression levels as a primary step in understanding their contribution to potential signaling effects within endothelial cells and astrocytes in vivo. Previous studies with other cell types have shown that cellular expression of integrins can be strongly influenced by the matrix ligands on which they are cultured. Here, it is necessary to establish the baseline conditions required to grow cells on physiologically relevant basal lamina matrix substrates before investigating the influence of ischemia in vitro. Both cell- and ECM-dependence of integrin expression were observed. Expression of the β1 subunit on cultured astrocytes was relatively constant regardless of the substrate. Although several matrix proteins induced small reductions in astrocyte integrin expression, this was only consistent for the α6 subunit for cells grown on fibronectin, laminin, or HSPG. In contrast, endothelial expressions of both β1 and α5 integrin subunits were stimulated by nearly all ECM substrates, in particular HSPG.

Characteristic cell-specific changes in integrin expression in response to experimental ischemia, which were generally reproducible across collagen IV, laminin, and HSPG for each cell type, were observed. For astrocytes, OGD induced a broadly significant loss of the integrin subunits α1 and α6. This finding is consistent with the loss of both integrin subunits after focal cerebral ischemia in vivo, in both the ischemic core and periphery, in the nonhuman primate. In vivo, integrin α6β4, unique to astrocyte end-feet, is lost within 2 hours after middle cerebral artery occlusion. Here, the reduction in the α6 subunit (which solely partners subunit β4) exactly mirrors the loss of α6β4 in vivo. Furthermore, subunit α1 expression decreases significantly from astrocytes very early during focal ischemia in the nonhuman primate, as observed in vitro. This suggests that for the α subunits on primary astrocytes in vitro experiments ischemia represents a good model of the in vivo events and should allow investigation of the molecular mechanisms that underlie the loss of integrin expression by astrocytes.

In contrast to the reduced expression of both α1 and α6 subunits, OGD significantly increased the expression of the β1 integrin subunit on astrocytes (see Table). However, the integrin β1 subunit is lost from cerebral microvessels in the ischemic territory early after middle cerebral artery occlusion. The increased β1 expression observed here is supported by the upregulation of β1-transcription by astrocytes in vitro during OGD (Figure 5). Although there is the expectation that α subunit expression levels generally follow those of the associated β-subunits, the apparent discrepancy here is important. It can be explained by the induction of another as yet undefined integrin α subunit that matches the increased expression of the β1 subunit. Or, astrocyte expression of the α1 and α6 subunits could be more sensitive to ischemia in vitro than the β1 subunit, and therefore precede loss of the β1 subunit.

Another explanation is suggested from observation of the endothelial cell responses. On isolated primary endothelial cells, OGD induced an unexpected 2-fold increased expression of the β1 integrin subunit. Indeed, in vivo focal cerebral ischemia induces loss of all endothelial integrins including β1, except for αvβ3. But, the β1 integrin responses by endothelial cells in vitro are consistent with the previous finding of increased β1 integrin mRNA expression by microvessels within regions peripheral to the ischemic cores, but not within the core regions themselves. This suggests that brain endothelial cells (and astrocytes) can respond to ischemia by attempting to increase β1 integrin expression levels. One very important implication of this finding is that the

**Table. Summary of Responses to Ischemia**

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conditions initiated by focal ischemia effect changes in microvascular β1 integrin expression by processes which are likely post-transcriptional, as suggested by the in vitro astrocyte responses. This is now being explored.

In contrast to the generalized loss of the α integrin subunits on astrocytes, OGD also significantly increased α5 subunit expression on endothelial cells (integrin α5 transcription levels during focal ischemia in vivo have not been measured). The endogenous response of brain endothelial cells to upregulate both the α5 and β1 subunits in response to OGD is very similar to the observation that certain tumor cells can ramp up α5 integrin levels in response to hypoxia (part of the well-described Warburg effect), presumably to enable metastatic cells to attach to ECM proteins in settings of oxygen stress.49

There are some important caveats to interpreting these observations. The endothelial cell cultures in this study derived from relatively pure defined populations. It cannot be ruled-out that in vivo endothelial cell integrin antigens may disappear, not as a direct response to ischemia, but rather by processes emanating either directly from surrounding cells (astrocytes, pericytes, or microglia) or from the blood. Because microglia are present at low levels (~5% of total cells) in the astrocyte cultures but not in the endothelial cell cultures, it cannot be excluded that the differential responses of endothelial cell- and astrocyte-associated integrin changes to OGD may be due to the presence of these cells in the astrocyte, but not in the endothelial cultures. The situation in vivo is not known.

Recently, we proposed that matrix adhesion receptors could play an important role in maintaining the permeability barrier of the cerebral microvasculature.49 The findings with in vitro ischemia establish both microvascular endothelial cells and astrocytes of murine origin as platforms for examining the adhesion receptor responses of microvascular cells of the neurovascular unit within the primate striatum, and their regulations in vivo.

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Disclosures
None.

References


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