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Short Communication

Integrin $\alpha_v\beta_3$ Is Expressed in Selected Microvessels after Focal Cerebral Ischemia

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The endothelial and smooth muscle integrin $\alpha_v\beta_3$, a receptor for vitronectin and fibrinogen, participates in angiogenesis associated with wound healing and tumorigenicity. The microvascular expression of $\alpha_v\beta_3$ and fibrin during experimental middle cerebral artery occlusion and reperfusion in a nonhuman primate model was examined by computer-assisted video imaging microscopy. No microvascular expression of $\alpha_v\beta_3$ was seen in the control subjects (n = 3) or the non-ischemic basal ganglia of subjects undergoing 2-hour MCA:O (middle cerebral artery occlusion) or 3-hour occlusion with 1-hour (n = 3), 4-hour (n = 3), and 24-hour (n = 3) reperfusion. In the ischemic territory, $\alpha_v\beta_3$ appeared initially at 2 hours of middle cerebral artery occlusion. Up-regulation of $\alpha_v\beta_3$ was confined to the media of 30.0- to 50.0-μm-diameter arterioles in the ischemic core and correlated significantly with fibrin deposition in those vessels ($P < 0.0005$). Integrin $\alpha_v\beta_3$ and its ligand fibrinogen appear in a subpopulation of microvessels after focal cerebral ischemia. (Am J Pathol 1996, 149:37–44)

An uncharacterized aspect of the microvascular responses to focal cerebral ischemia are the coordinate endothelial and vascular wall processes that occur during inflammation and angiogenesis. Integrins play a prominent role in selected vascular cellular adhesive processes and intercellular communication, but their appearance in cerebrovascular ischemia has only recently been demonstrated. Focal cerebral ischemia/reperfusion (I/R) involves adhesive interactions between polymorphonuclear leukocytes mediated by the β_2 integrin CD18[1] and microvascular endothelial cells.[2–4] In addition, the platelet integrin $\alpha_{IIb}\beta_3$ is exposed during ischemia and subsequent reperfusion consistent with known fibrin-dependent platelet activation in a well defined primate model of cerebral artery occlusion/reperfusion (MCA:O/R).[2,5,6] Both $\alpha_{IIb}\beta_3$ expression and intramicrovascular fibrin formation significantly increase in a time-dependent manner during reperfusion.[6,7] These interactions contribute to early post-I/R microvascular occlusion events manifest as microvascular no-reflow.[2] They also contribute to later events within a 24-hour envelope, in an as yet incompletely defined sequence of inflammatory processes.[8] Neovascularization appears to be a very late phenomenon (7 days) during cerebral infarction.[9,10] Unexplored are the participation of cerebral microvascular intimal and basal lamina structures in remodeling that may lead to angiogenesis and neovascularization.

The integrin $\alpha_v\beta_3$, expressed on endothelial cells and smooth muscle cells in noncerebral vessels, is a major adhesion receptor[11] for the Arg-Gly-Asp-containing proteins fibrinogen, vitronectin, von Willebrand factor,[12] thrombospondin,[13] osteopontin (OPN)[14] fibronectin,[15] and laminin.[16] Integrin $\alpha_v\beta_3$,

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defined by the monoclonal antibody (MAb) LM609, is important in angiogenesis associated with tumor development and wound repair.[17,18] Both may be abrogated by blockade of $\alpha_v\beta_3$ as shown recently by exposure of tumor explants or tumor-necrosis-factor-α-impregnated patches to LM609 in the chick chorioallantoic membrane system.[18]

In the present immunohistochemical study, we demonstrate the up-regulation of $\alpha_v\beta_3$ in a subpopulation of downstream lenticulostriatal microvessels, which are complemented by microvessel-associated fibrin deposition after MCA:O/R in the awake nonhuman primate. $\alpha_v\beta_3$ expression may reflect a response to fibrin formation and/or cytokine release as the first stage in vascular reorganization after focal cerebral ischemia.

Materials and Methods

Animals

Twelve adolescent male baboons (*Papio anubis/cynocephalus*) weighing 8.0 to 11.4 kg were utilized for the MCA:O(/R) studies, and three separate primates weighing 9.2 to 25.0 kg served as controls. Tissues employed here were from subjects included in studies previously reported.[5,6]

The procedures used throughout this study were approved by the institutional Animal Research Committee and were performed in accordance with standards published by the National Research Council (The Guide for the Care and Use of Laboratory Animals), the National Institutes of Health Policy on Humane Care and Use of Laboratory Animals, and the United States Department of Agriculture Animal Welfare Act. In compliance with these standards, every effort was made to assure that the subjects were free of pain or discomfort.

Experimental Ischemia

Preparation of the nonhuman primate model of MCA:O/R has been described in detail elsewhere.[2,5,6,19] All animals were allowed a 7-day procedure-free period after surgical implantation of the MCA balloon[19] before entry into the experimental protocol. The subjects were all clinically free of infection or apparent inflammation and had normal hematological studies and neurological function before MCA:O.[20]

In this time course study, three animals underwent MCA:O for 2 hours, and 9 animals underwent MCA:O for 3 hours and subsequent reperfusion for 1 hour (n = 3), 4 hours (n = 3), or 24 hours (n = 3). A separate group of three animals did not undergo any model preparation procedure and served as an unoperated control group. The experimental paradigm previously reported for this awake model required inflation of the eccentric MCA balloon for 2 or 3 hours with subsequent reperfusion.[2,5–7,19]

Each experiment was terminated by transcardiac pressure perfusion of the cranial structures with chilled isosmotic nonfixative perfusion flush solution at between 180 and 220 torr with 700 to 800 ml/min flow for 4 minutes under pentothal Na^+ anesthesia (15 mg/kg infusion) and mechanical ventilation.[5,6] Each brain was then excised *en bloc* from the cranium and immersed in ice. Tissue blocks (1.0 cm × 1.0 cm × 0.2 to 0.5 cm) from symmetrically located sites of both basal ganglia, and the temporal and parietal cortices were embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN), frozen in 2-methylbutane/dry ice, and stored at −80°C until sectioning.

Antibodies

Well characterized antibodies (monoclonal or polyclonal) were used for the immunohistochemical studies. The MAb CLB HEC-75 against the endothelial cell CD31 (PECAM-1) was provided by J. van Mourik (Central Laboratorium van de Bloedtransfusiediest, Amsterdam, The Netherlands)[21] or was obtained commercially (DAKO-CD31, JC/70; Dako Corp., Carpinteria, CA). The murine anti-human fibrin MAb MH-1, which recognizes an epitope unique to the intact human fibrin monomer or fibrin degradation products was the kind gift of American Biogenetic Sciences (Notre Dame, IN).[6] Laminin was identified with the rabbit anti-human polyclonal antibody LAM89 (Chemicon, Temecula, CA), and smooth muscle α-actin was identified with a murine anti-human MAb (1A4; Sigma Chemical Co., St. Louis, MO). To study the response of the vascular integrin $\alpha_v\beta_3$ to focal cerebral I/R, murine MAbs against the human α_v integrin subunit (LM142), the β_1 integrin subunit (P4C10), $\alpha_v\beta_3$ (LM609), and integrin $\alpha_v\beta_5$ (P3G2) provided by D. Cheresh (The Scripps Research Institute) were employed.[18] LM609 was also obtained from Chemicon.

Immunohistochemistry

Consecutive 10-μm cryostat sections from matched regions of the ischemic (right) and normal non-ischemic (left) basal ganglia separate from those previously reported[5,6] were prepared for immunohistochemistry. The regional specimens from each

subject were chosen to provide at least three blocks per time point. Sections were fixed with methanol for 3 minutes at 4°C, immersed in 100 μmol/L glycine in phosphate-buffered saline (PBS; 100 mmol/L Na_2HPO_4 and 140 mmol/L NaCl adjusted to pH 7.4) for 10 minutes, rinsed with PBS wash solution, and subsequently incubated with Blotto to reduce nonspecific binding.[6,22] Each section was incubated with primary antibody (50 μl) for 120 minutes at 37°C, washed, then incubated with biotinylated horse anti-mouse IgG (1:400 in reagent diluent; Vector Laboratories, Burlingame, CA) at 37°C for 30 minutes. The peroxidase signal was developed with 3-amino-9-ethyl carbazole (AEC kit, Biomeda Corp., Foster City, CA) as described.[5,6] All sections were counterstained with Mayer's hematoxylin (Biomeda) for 1 minute, blued in saturated sodium bicarbonate solution, and mounted. The following controls were routinely performed: 1) deletion of the primary antibody, 2) deletion of the secondary antibody, and 3) the use of TIB115, a murine MAb against the SV40 large T viral antibody as an irrelevant primary antibody.

To identify the relationship between fibrin deposition and $\alpha_v\beta_3$ expression in the same vessels, sections were double stained with MAb MH-1 (signaled by fluorescein-isothiocyanate-conjugated anti-mouse IgG from American Biogenetic Sciences) and a second primary MAb LM609 (signaled with biotinylated horse anti-mouse IgG). Co-localization of vascular fibrin (fluorescein-isothiocyanate-associated MH-1 at 540 nm) and LM609 immunoreactivity was assessed by one observer not aware of the source, timing, or localization of the specimen. Microvessels were classified as to the presence or absence of fibrin deposition and $\alpha_v\beta_3$ expression. Separately, localization of LM609 to laminin and smooth muscle α-actin was defined in selected microvessels by laser confocal microscopy (LSM Invert 410, Karl Zeiss, Oberkochen, Germany).

Quantitation of Microvascular Outcomes

Qualitative assessment of the relative peroxidase signal associated with the integrin subunits α_v and β_1 and the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on identifiable microvessels was graded by one observer blinded to specimen designation as follows: −, negative; +, light; ++, moderate; +++, moderate-strong; ++++, very strong.[5] The absolute number and the minimal transverse diameters of microvessels containing CD31, fibrin, or $\alpha_v\beta_3$ antigen from the target basal ganglia from non-overlapping images (×400) in a standard 1000-field matrix (66.4 mm^2) were determined with the aid of a computerized video imaging system.[2] The number of vessels <100 μm in diameter stained by the MAb CD31 was regarded as the total number of microvessels per field.[5,6,23] The vascular distribution of $\alpha_v\beta_3$ or fibrin are presented as relative fraction of the total or as the fraction of microvessels displaying $\alpha_v\beta_3$ or fibrin to those displaying CD31.

Neuron alterations were assessed according to a modification of the scheme of Eke et al[24] as previously used.[5,6] Type IV neuron silhouettes were located in regions of variable degrees of visible tissue disturbance; tissue peripheral to those regions uniformly had type II and type III neuron damage.

Statistics

All values are expressed as the mean or as the mean ± SD. Data from at least three matched blocks per time point were analyzed. Comparisons among the time points used the one-way analysis of variance followed by Tukey's multiple comparison method, whereas the fibrin and $\alpha_v\beta_3$ double-stain comparisons used the χ^2 test. Significance was set at $2P < 0.05$ for all comparisons.

Results

Single-vessel occlusion by reversible vascular compression in the awake nonhuman primate is a suitable model to explore the responses of the microvasculature to focal cerebral ischemia.[5,8] A significant reduction in neurological score from 100 to 41.4 ± 14.7 by 1 hour after occlusion confirmed the persistence of hemiparesis and sensorimotor deficits in both MCA:O and MCA:O/R cohorts. Other characteristics (eg, baseline blood cell counts and neurological score) were not different among the cohorts (data not shown). Microvascular events associated with ischemia were confined to the corpus striatum and temporal cortex ipsilateral to the MCA occlusion.

The vascular distributions defined by CD31 in both basal ganglia in each subject within the MCA:O and MCA:O/R cohorts were not different among themselves or from those of the non-ischemic control subjects, as previously observed.[5]

In non-ischemic control subjects, integrin subunit β_1 was strongly expressed on all microvessels throughout the basal ganglia and cortices (see Table 1). By comparison, the α_v integrin subunit was weakly expressed on a few (<3.5% of total), relatively large (>10.0 μm) vessels in both basal

Table 1. *Localization of Microvessel-Associated Integrins*

			Control		α_v		β_1		$\alpha_v\beta_3$		$\alpha_v\beta_5$	
	Duration	n	Non-I/R	Post-I/R	Non-I/R	Post-I/R	Non-I/R	Post-I/R	Non-I/R	Post-I/R	Non-I/R	Post-I/R
Control		3	–	–	+[1]	+[1]	++++	++++	–	–	–	–
MCA:O	2 hours	3	–	–	+	++	++++	++++	–	+ to ++	–	–
MCA:O/R	1 hour	3	–	–	+	++	++++	++++	–	+ to ++	–	–
	4 hours	3	–	–	+	+ to +++	++++	++++	–	+ to +++	–	–
	24 hours	3	–	–	+	+ to ++	++++	++++	–	+ to +++	–	–

MCA:O/R was a 3-hour MCA occlusion with indicated periods of reperfusion. Control was done with deletion of primary antibody. Identical data were obtained with the irrelevant antibody TIB-115. –, negative; +, lightly positive; ++, moderately positive; +++, moderate-strongly positive; ++++, very strongly positive.

*Integrin subunit α_v expressed on few microvessels >10.0 μm diameter.

ganglia, but integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ were not detected on any microvessel (Table 1). Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ were not seen in the non-ischemic basal ganglia at any time during MCA:O or MCA:O/R (Table 1).

Integrin $\alpha_v\beta_3$ antigen appeared exclusively in the microvessels of the ischemic basal ganglia within the ischemic zone with uniformly severe neuronal injury (type IV) of all subjects after either MCA:O or MCA:O/R (Table 1). The relative mean fraction of microvascular $\alpha_v\beta_3$/CD31 in the ischemic basal ganglia was significantly greater than in the non-ischemic zone throughout MCA:O and MCA:O/R and remained elevated throughout reperfusion (Figure 1). Integrin $\alpha_v\beta_3$ was confined principally to vascular medial smooth muscle (data not shown), where it was found in 6.0 ± 3.9% of microvessels as early as 2 hours after MCA:O (Figure 1). Integrin subunit α_v appeared prominently in the media of microvessels of the ischemic basal ganglia of all MCA:O and MCA:O/R subjects. In contrast, $\alpha_v\beta_5$ antigen was not expressed (Table 1).

In the ischemic basal ganglia, integrin $\alpha_v\beta_3$ was expressed in a selected subset of microvessels (Figure 2), 77.7 ± 27.1% made up of 30.0- to 50.0-μm microvessels during MCA:O and MCA:O/R (Figure 3). $\alpha_v\beta_3$ was generally not associated with capillaries (0.06 ± 1.42%).

The integrin $\alpha_v\beta_3$ functions as a receptor for multiple ligands in vascular cells.[11] $\alpha_v\beta_3$ expression and fibrin deposition were found in the same microvessels, being highly correlated in all affected microvessels ($P < 0.0005$; Figure 3). A co-localization study of $\alpha_v\beta_3$ expression and fibrin deposition demonstrated that over all time points 38.2 ± 14.1% of microvessels displayed both $\alpha_v\beta_3$ and fibrin antigen, whereas 31.9 ± 12.3% lacked both. MH-1-detectable fibrin antigen was rarely (3.6 ± 2.1%) found in the absence of $\alpha_v\beta_3$ antigen (Table 2).

Discussion

Integrin-dependent cell adhesion regulates not only cell structure and morphology but also proliferation, differentiation, and gene expression and thereby intercellular communication. Recent experimental studies have associated β_2-integrin-

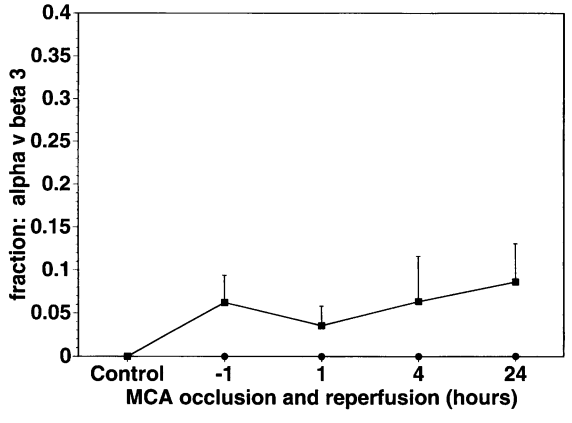

Figure 1. *Mean fraction of microvessels displaying integrin $\alpha_v\beta_3$ (per CD31) during MCA:O and MCA:O/R. ●, data from non-ischemic zones (n = 3 each); ■, from ischemia zones (n = 3 each). C, non-ischemic control; –1, 2 hours of MCA:O; 1, 4, and 24, various periods of reperfusion after 3 hours of MCA:O.*

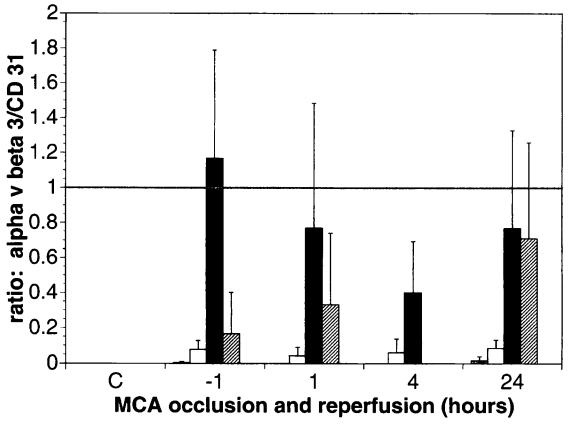

Figure 2. *Microvascular distribution of integrin $\alpha_v\beta_3$ by transverse diameter (μm) during MCA:O (2 hours) and MCA:O/R (3 hours of MCA:O with various periods of reperfusion). □, 4.0 to 7.5 μm; □, 7.5 to 30.0 μm; ■, 30.0 to 50.0 μm, ▨, 50.0 to 100.0 μm; ▨, >100.0 μm. C, non-ischemic control.*

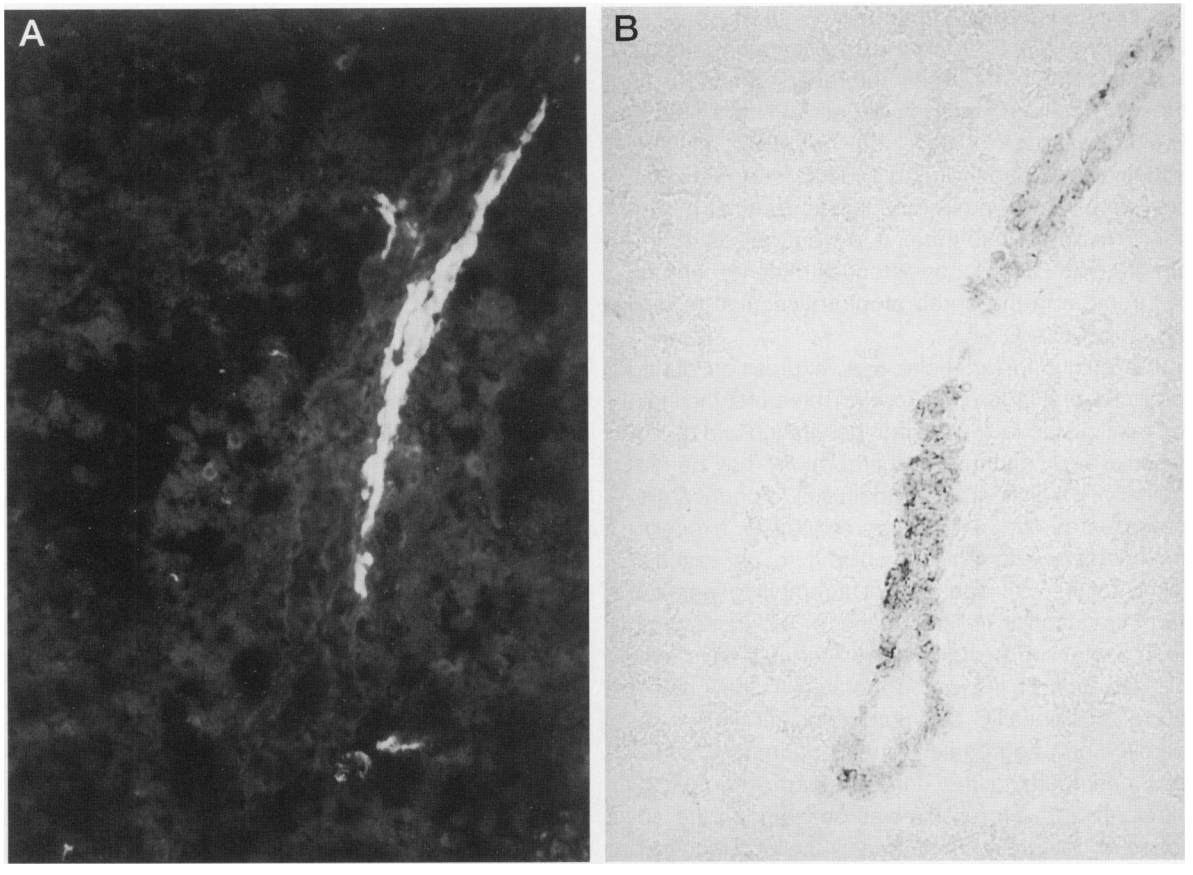

Figure 3. *Co-localization of integrin $\alpha_v\beta_3$ and fibrin in a large microvessel at 3 hours of MCA:O and 1 hour of reperfusion.* **A:** *Fibrin deposition (fluorescein isothiocyanate; MH-1) in a microvessel of 55-μm diameter.* **B:** *The presence of $\alpha_v\beta_3$ antigen (immunoperoxidase) in the same microvessel.*

mediated polymorphonuclear leukocyte-endothelial cell adhesion[3] and intravascular fibrin formation[7] with microvascular occlusion during focal cerebral I/R, but little is known about the response of the microvessels per se to ischemia. A recent survey has indicated discrete distributions of integrin α and β subunits on normal primate cerebral microvessels and astrocytes, with low levels of α_v and β_3 antigens on scattered microvessels.[25] We have demonstrated here that integrin $\alpha_v\beta_3$ is expressed on lenticulostriatal microvessels in the center of the ischemic zone as early as 2 hours after MCA:O, confined predominantly (89.8%) to the smooth muscle layer of arterioles >10 μm diameter. This antigen was significantly associated with the 30.0- to 50.0-μm-diameter microvascular subgroup at nearly all times of ischemia and reperfusion (Figure 2). The specificity of this response is supported by the coordinate increased expression of the integrin α_v subunit in the complete absence of $\alpha_v\beta_5$ expression in response to MCA:O/R in the same territory. Integrin $\alpha_v\beta_3$ is a promiscuous adhesion receptor[15,17] capable of recognizing a wide variety of adhesive proteins that can associate with vascular cells including von Willebrand factor, fibronectin, OPN, and vitro-

Table 2. *Microvascular Co-Localization of $\alpha_v\beta_3$ Expression and Fibrin Deposition*

	Duration	Detectable microvessels; $\alpha_v\beta_3$ *versus* fibrin				Significance
		+/+	+/−	−/+	−/−	
Control		0	0	0	58	
MCA:O	2 hours	39	22	4	43	$2P < 0.00001$
MCA:O/R	1 hour	29	47	1	63	$2P < 0.0005$
	4 hours	90	31	8	35	$2P < 0.0005$
	24 hours	63	50	8	33	$2P < 0.0005$
% mean total ± SD		38.2 ± 14.1	26.4 ± 7.8	3.6 ± 2.1	31.9 ± 12.3	

Numbers represent the summation of counted vessels of each group.

nectin, in addition to fibrinogen[26,27]. In the case of OPN, integrin $\alpha_v\beta_3$ appears to facilitate endothelial and smooth muscle cell migration, as $\alpha_v\beta_3$-expressing cells migrate toward an OPN gradient.[28] The appearance of $\alpha_v\beta_3$, but not $\alpha_v\beta_5$, an OPN adhesion receptor, during MCA:O suggests that one effect of ischemia is to initiate cellular migration.[29] In addition to fibrin, $\alpha_v\beta_3$ recognizes thrombin-cleaved OPN,[30] consistent with the generation of thrombin in the cerebral microvasculature during early MCA:O.[2,6]

The stimuli for arteriolar $\alpha_v\beta_3$ expression during focal cerebral I/R are not known. Therefore, the striking relationship between fibrin generation and microvascular $\alpha_v\beta_3$ during focal cerebral I/R may be biologically significant. Endothelial permeability increases as early as 2 hours of MCA:O, exposing the microvascular plasma column to perivascular tissue factor with consequent thrombin generation and intravascular fibrin formation.[6] The increase in fibrin-containing microvessels in the ischemic core with duration of I/R is corroborated in the present study. Additionally, polymorphonuclear leukocyte-platelet-fibrin aggregates formed during MCA:O/R[2] may contribute to the early appearance of $\alpha_v\beta_3$. Although $\alpha_v\beta_3$ expression was observed in the absence of detectable fibrin in only 3.6% of identified microvessels, it cannot be excluded that fibrin formation in nearby vessel segments (in adjacent sections), because of the three-dimensional microvascular array, did not also contribute to $\alpha_v\beta_3$ expression.

Additional stimuli may come from cytokines released from the perivascular parenchyma early during MCA:O. A recent report of tumor necrosis factor-α generation during evolving cerebral ischemia (rat) underscores the possible contribution of this and other cytokines to $\alpha_v\beta_3$ appearance.[8] The possibility that basic fibroblast growth factor induced by ischemia may stimulate $\alpha_v\beta_3$ expression in this setting has not yet been explored. Certainly, the early appearance of fibrin and $\alpha_v\beta_3$ does not preclude the possibility that $\alpha_v\beta_3$ expression is stimulated by earlier cytokine release.

A possible functional role for integrin $\alpha_v\beta_3$ in cerebral I/R is suggested by its capacity to promote vascular cell adhesion to fibrin(ogen) or von Willebrand factor.[11] Integrin $\alpha_v\beta_3$ appears on blood vessels exposed to basic fibroblast growth factor and tumor necrosis factor-α.[18] It is known that $\alpha_v\beta_3$ initiates a Ca^{2+}-dependent pathway leading to endothelial cell migration, which plays a role in the angiogenesis of inflammation, wound repair, and ontogenesis.[31] The functional importance of integrin $\alpha_v\beta_3$ to angiogenesis has been recently demonstrated by the ability of LM609 to significantly inhibit both embryonic and tumor-induced angiogenesis on chick allantoic membranes.[18,32] Angiogenesis also occurs during cerebral infarction after MCA:O. Tsutsumi et al,[9,10] using a vascular endocast technique, described arteriolar bud formation and regenerated capillaries in the infarcted tissue of the basal ganglia 7 days after MCA:O in the dog. The present experiments indicate that integrin $\alpha_v\beta_3$ expression, one potentially relevant signal for angiogenesis, appears as an early microvascular smooth muscle response to focal cerebral ischemia.

These findings may have important implications for vascular outcomes of cerebral ischemia. One relevant aspect of the present study is that the early appearance of $\alpha_v\beta_3$ in a subpopulation of arterioles during MCA:O may reflect the first stage of angiogenesis in cerebral ischemia. Arg-Gly-Asp-containing peptides, which are known to block the function of $\alpha_v\beta_3$, have been shown to inhibit both melanoma tumor invasion *in vitro* and the development of experimental metastasis in a murine melanoma model system.[33,34] Recently, it has been shown that the function-inhibiting MAb LM609 initiates vascular involution and subsequent tumor regression and new vessel formation *in vivo*.[18,32] The effect of this modulation on microvascular integrity during ischemia would be of some interest, particularly in light of the assertion that $\alpha_v\beta_3$ may trigger signaling events that mediate survival of vascular cells during vascular remodeling.[32] These findings, together with our results, suggest that integrin $\alpha_v\beta_3$ may contribute to microvascular responses to acute cerebral ischemia.

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