Osteopontin and its Integrin Receptor $\alpha_\nu \beta_3$ Are Upregulated During Formation of the Glial Scar After Focal Stroke

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Background and Purpose—Microglia and astrocytes in the peri-infarct region are activated in response to focal stroke. A critical function of activated glia is formation of a protective barrier that ultimately forms a new glial-limiting membrane. Osteopontin, a provisional matrix protein expressed during wound healing, is induced after focal stroke. The present study was performed to determine the spatial and temporal expression of osteopontin and its integrin receptor $\alpha_\nu \beta_3$, during formation of the peri-infarct gliotic barrier and subsequent formation of a new glial-limiting membrane.

Methods—Spontaneously hypertensive rats ($n=19$) were subjected to permanent occlusion of the middle cerebral artery and killed 3, 6, and 24 hours and 2, 5, and 15 days after occlusion. The spatial and temporal expression of osteopontin mRNA was determined by in situ hybridization, and that of osteopontin ligand and its integrin receptor $\alpha_\nu \beta_3$ was determined by immunohistochemistry.

Results—Osteopontin mRNA was expressed de novo in the peri-infarct region from 3 to 48 hours; by 5 days osteopontin mRNA expression was restricted to the infarct. Osteopontin protein was expressed by peri-infarct microglia/macrophages at 24 hours and by microglia/macrophages at 48 hours in the infarct. Integrin receptor $\alpha_\nu \beta_3$ was expressed in peri-infarct astrocytes at 5 and 15 days.

Conclusions—Early microglial/macrophage expression of osteopontin mRNA defines the borders and final infarct area at 24 hours. At 5 days osteopontin ligand is at a distance from the peri-infarct astrocytes expressing integrin receptor $\alpha_\nu \beta_3$. By 15 days astrocytes expressing integrin receptor $\alpha_\nu \beta_3$ are localized in an osteopontin-rich region concomitant with formation of the new glial-limiting membrane. The de novo expression and interaction of osteopontin ligand with its receptor integrin $\alpha_\nu \beta_3$ suggest a role in wound healing after focal stroke. (Stroke. 1998;29:1698-1707.)

Key Words: astrocyte ■ cerebral ischemia ■ infarcts ■ macrophages ■ microglia ■ neuroglia

G lial scar formation in the peri-infarct region after focal ischemia has been characterized as a negative event that prevents regeneration of the injured nervous system. After focal ischemia, glial cells initiate a classic wound-healing response with formation of a barrier between the injured and healthy tissue. The barrier is a cellular and molecular response with formation of a barrier between the injured and healthy tissue.

Formation of the glial scar involves the transformation of quiescent astrocytes and microglia to their activated state in the peri-infarct region. Activated astrocytes undergo hypertrophy as they extend their processes to encompass the developing infarct, while activated microglia directly adjacent to the injured cells transform into ameboid phagocytic cells, responsible for clearing the necrotic zone of cellular debris. The hypertrophy, hyperplasia, and migration that typify cell activation require cells to change their interaction with the extracellular matrix concomitant with rearrangements of the cytoskeletal network.

Integrins are a family of transmembrane receptors that couple intracellular cytoskeletal elements with extracellular matrix molecules. After injury to the brain, integrins and the extracellular matrix molecules are upregulated as part of the wound healing process. Chondroitin sulfate proteoglycans and tenascin are upregulated in astrocytes in the region adjacent to an injury.

Two integrin receptors, $\alpha_\nu \beta_3$ and $\alpha_\nu \beta_6$, have been suggested to play a role in vascular integrity and remodeling after focal ischemia within the infarcted area. In particular, integrin $\alpha_\nu \beta_3$ has been demonstrated to be upregulated concomitant with an increase in one of its ligands, fibrinogen. Integrin receptor $\alpha_\nu \beta_6$ can also interact with other blood vessel–associated proteins containing the arginine-glycine-aspartate (RGD) motif such as vitronectin, von Willebrand factor, thrombospondin, fibronectin, laminin, and osteopontin. The expression of these integrins...
and extracellular matrix molecules has been studied in the context of vascular remodeling and wound healing, but little is known regarding their potential role in nonvascular tissue remodeling associated with wound healing in the brain.

Recent studies from our laboratory have demonstrated specific and transient induced expression of the provisional matrix protein osteopontin after focal stroke.\(^\text{18}\) Osteopontin, an integrin ligand, is an acidic, secreted phosphoprotein containing an arginine-glycine-aspartate (RGD) motif that interacts with the integrin \(\alpha\beta_3\) to promote cell migration.\(^\text{19}\) Osteopontin and integrin \(\alpha\beta_3\) are expressed during repair of myocardial necrosis\(^\text{20}\) and restenosis.\(^\text{21}\) Functionally, osteopontin has chemotactic activity for smooth muscle cells\(^\text{22}\) regulating cell adhesion and migration\(^\text{23}\) by interacting with three different integrin receptors: \(\alpha\beta_1\), \(\alpha\beta_3\), and \(\alpha\beta_5\). Cell adhesion is mediated by integrin receptors \(\alpha\beta_1\) and \(\alpha\beta_3\).\(^\text{24}\) While integrin receptor \(\alpha\beta_3\) mediates cell migration.\(^\text{25}\) Our studies demonstrated that osteopontin mRNA was maximally expressed at 5 days after ischemia by ED1+ macrophages after focal stroke. Furthermore, in a migration assay osteopontin had the capacity to induce directed migration of astrocytes.\(^\text{18}\)

In the present study we extend our investigation of osteopontin expression induced by ischemic brain injury. The objective of the present study was (1) to define the spatial and temporal expression kinetics of osteopontin mRNA and protein after ischemic injury; (2) to identify the phenotype of cells expressing osteopontin ligand and integrin receptor \(\alpha\beta_3\); and (3) to determine the timing of receptor-ligand interaction as a first step in exploring potential functional aspects of osteopontin and integrin receptor \(\alpha\beta_3\). Our results regarding the spatial and temporal expression of osteopontin by microglia and invading monocytes and of integrin receptor \(\alpha\beta_3\) by astrocytes demonstrate a potential role for osteopontin and \(\alpha\beta_3\) in the formation of the peri-infarct glial scar and reestablishment of the glial limits after focal stroke.

**Materials and Methods**

**Middle Cerebral Artery Occlusion and Tissue Preparation**

Adult male spontaneously hypertensive rats (weight, 250 to 350 g) were anesthetized with sodium pentobarbital (60 mg/kg IP). This rat strain was chosen for the low variability and consistency of infarct between animals. Permanent occlusion was chosen because it represents a model of infarct that most closely resembles focal stroke in humans. Animals were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals, NIH publication 85–23, revised 1985. Procedures in which laboratory animals were used were approved by the Institutional Animal Care and Use Committee of SmithKline Beecham Pharmaceuticals.

Focal ischemia was produced by occluding the middle cerebral artery (MCA) as previously described.\(^\text{26}\) After right craniotomy and removal of the dura mater, the MCA was permanently occluded and cut dorsal to the lateral olfactory tract at the level of the inferior cerebral vein by means of electrocoagulation (Force 2 Electrosurgical Generator, Valley Laboratory Inc). During and after surgery, the rat’s body temperature was maintained at 37°C. In sham-operated rats the dura was opened over the MCA, but the artery was not occluded. After permanent MCA occlusion, rats were killed at 3 (n=3), 6 (n=3), and 24 (n=3) hours and 2 (n=3), 5 (n=3), and 15 (n=2) days; sham rats (n=2) were killed 2 days after surgery. Naive control rats (n=2) were also examined. Rats were overdosed with sodium pentobarbital and perfused through the aorta with 50 mmol/L Dulbecco’s PBS containing 2% paraformaldehyde for 15 minutes. The brain was then removed and postfixed in PBS containing 2% paraformaldehyde for 4 days at 4°C. Brains were cryoprotected in 20% sucrose in PBS at 4°C, then frozen in OCT (Tissuetek, Miles Inc) and stored at −70°C until sectioned.

**In Vitro Transcription and In Situ Hybridization**

The methods followed for transcription reactions and in situ hybridization were as previously published.\(^\text{27}\) \(^{32}\)P-UTP-labeled riboprobes were synthesized from linearized plasmids containing the entire rat osteopontin cDNA from base pairs 1 to 1435. The cloning of the rat osteopontin has been described previously.\(^\text{26}\)\(^\text{27}\)\(^\text{28}\) Transcription reactions were performed at 37°C for 90 minutes in 15 μL of 40 mmol/L Tris-HCl, pH 7.5, with 6 mmol/L MgCl\(_2\); 2 mmol/L spermidine; 0.5 mmol/L each ATP, CTP, GTP; 2 mmol/L dithiothreitol; 1 μg linearized template; 1 μL T7 or T3 polymerase; and 25 μCi \(^{32}\)P-UTP (2000 Ci/mmol; New England Nuclear). The DNA template was removed by a 10-minute incubation at 37°C with RNase-free DNase (1 U/μL). Synthesized cRNA was separated from unincorporated nucleotides by ethanol precipitation and resuspended in 10 mmol/L Tris, 1 mmol/L EDTA, pH 8.

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Figure 1. Osteopontin mRNA expression after focal stroke. By in situ hybridization osteopontin mRNA expression was constitutively expressed in the septal nucleus and in ventral brain nuclei (A, arrowheads). In sham animals osteopontin mRNA was induced at the surgery site (B, arrow). At 3 hours (C) and 6 hours (D), osteopontin mRNA was induced in cells initially at the ventromedial aspect (C, arrow) and continuing to the dorsomedial aspect (D, arrowhead) of the infarct. At 24 hours, cells expressing osteopontin mRNA were still largely confined to the peri-infarct region (E), with expression at the pial surface (arrow) and by a few cells in the infarct (arrowhead). At 48 hours (F), more cells within the infarct expressed osteopontin mRNA; by 5 days (G), the majority of cells expressing osteopontin mRNA were within the infarct, with little expression in the peri-infarct region. At 15 days (H), mRNA levels returned to those found in naive rats.
Osteopontin and Integrin \(\alpha,\beta_3\) After Focal Stroke

**Antibodies**

The following commercially available antibodies were used: polyclonal glial fibrillary acidic protein (GFAP) (DAKO Corp); monoclonal ED1 (Chemicon International Inc); and monoclonal osteopontin, MPIIIIB10, (Developmental Studies Hybridoma Bank, University of Iowa). The antisera used to identify the integrin \(\alpha,\beta_3\), monoclonal SBJ293–346, was generated according to a modification of Kohler and Milstein. BALB/c mice were immunized by routine protocol with purified human integrin receptor \(\alpha,\beta_3\). Hybridomas were generated by fusing spleen cells of a BALB/c mouse with the cell line SP20-Ag14. Positive hybridomas were selected by limited dilution to assure monoclonality of the hybridoma cell line. Supernatants from hybridomas were screened against a panel of human integrin receptors (\(\alpha,\beta_3\), \(\alpha,\beta_5\), \(\alpha,\beta_6\), and \(\alpha,\beta_2\)) by ELISA, SBJ293–346 was selected on the basis of its specificity and selectivity against integrin receptors \(\alpha,\beta_3\) and \(\alpha,\beta_6\), presumably by cross-reacting with the \(\beta_3\) subunit, which is shared by both integrin receptors. The hybridoma secreting SBJ293–346 monoclonal antibody showed neutralizing activity against \(\alpha,\beta_3\) and \(\alpha,\beta_6\) receptors in an assay in which fibrinogen was used as an integrin ligand. The SBJ293–346 monoclonal antibody showed negative activity in both binding and neutralization assays against \(\alpha,\beta_3\) and \(\alpha,\beta_1\) integrin receptors.

**Immunohistochemistry**

Tissue was brought to room temperature and hydrated in PBS. For immunoperoxidase localization, endogenous peroxidase was quenched with either 3% \(\text{H}_2\text{O}_2\) in PBS (integrin \(\beta_3\)) or 3% \(\text{H}_2\text{O}_2\) in methanol (GFAP, ED1). After the quench, tissue sections were blocked with 1% BSA in PBS, then incubated with the primary antibody overnight at 4°C. The sections were rinsed with PBS, and the primary antibody was detected with the use of the ABC Elite kit (Vector Labs), with diaminobenzidine (Sigma) as the chromogen, according to the manufacturer’s instructions. For double immunofluorescence, the primary antibodies were localized with the use of secondary antibodies conjugated to BodipyFL or Texas Red (Molecular Probes). For specificity of SBJ293–346, the antibody (25 \(\mu\text{g/mL}\)) was adsorbed with purified integrin \(\alpha,\beta_3\) (25 \(\mu\text{g/mL}\)) in 1% BSA in PBS overnight at 4°C. The adsorbed antibody was then

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**Figure 2.** Osteopontin (OPN) mRNA expression at 24 hours defines the area of infarct. Quantitative analysis of the region bounded by OPN mRNA and the region containing OPN mRNA revealed that the expression of OPN mRNA at 24 hours defines the final infarct area seen at 5 days. *P<0.05 different from 3-hour measure of peri-infarct OPN mRNA; **P<0.05 different from 3-hour measure of infarct OPN mRNA (ANOVA followed by Dunnett t test using 3-hour data as control).

**Figure 3.** Osteopontin expression is restricted to microglia/macrophages. By combined immunohistochemistry and in situ hybridization, osteopontin mRNA (green grains) was colocalized to cells expressing ED1, a microglia marker (A, arrow). Adjacent to an ED1+ cell expressing osteopontin mRNA is an ED1+ cell that does not (A, arrowhead). Osteopontin mRNA (B, arrow, green grains) did not colocalize with GFAP+ astrocytes (B, arrowheads). Bar =20 \(\mu\text{m}\) for A and B.
incubated with the tissue as described for the aforementioned nonadsorbed antibodies.

**Combined In Situ Hybridization and Immunohistochemistry**

Tissue preparation, in vitro transcription, and combined in situ hybridization and immunohistochemistry were performed as described above. Tissue sections (12 μm) were incubated with the ED1 or GFAP antibody overnight at 4°C. Immediately after antibody detection, the tissue underwent the in situ hybridization procedure described above. Double labeling of cells was visualized with Nomarski optics and epifluorescence with cross-polarized optics to enhance visualization of grains (which appear green) colocalized with immunoreactive cells.

**Quantitative Analysis of In Situ Hybridization**

X-ray film images of osteopontin in situ hybridization results were scanned to disk with a Hewlett Packard Deskscan program. Images were quantified with the Optimas image analysis program (Optimas Corp). Two regions were measured at 3, 6, and 24 hours and 2 and 5 days after permanent MCA occlusion. These two regions included the largest measure at 24 hours corresponding to the peri-infarct area occupied by osteopontin mRNA and that bounded by but not containing osteopontin mRNA. The area (mm²) for these two regions was analyzed by ANOVA and post hoc analysis with a Dunnett t test and was considered significant at P<0.05.

**Results**

In situ hybridization was used to analyze the spatial and temporal kinetics of osteopontin mRNA expression after permanent MCA occlusion. In the naive brain tissue, constitutive expression was observed only in the septal nucleus and ventral nuclei (Figure 1A, arrowheads). With sham surgery, osteopontin was expressed at the site of the surgery (Figure 1B, arrow); expression in the septal nucleus and ventral nuclei was also noted (Figure 1B, arrowheads). Induction of osteopontin mRNA was seen 3 hours after occlusion in cells in the ventromedial peri-infarct region (Figure 1C, arrow). Six hours after occlusion, expression of osteopontin mRNA at the ventromedial aspect of the infarct had increased (Figure 1D, arrow) and now extended to the dorsomedial aspect of the infarct (Figure 1D, arrowhead). At 24 hours, robust expression was seen throughout the entire peri-infarct zone (Figure 1E, arrow). At 48 hours, occasional microglia (D, arrowhead) expressing osteopontin were seen, with the majority of osteopontin expressed by macrophages (D, arrow). By 5 days, osteopontin was seen in a perinuclear location intracellularly (E, arrows) and in the extracellular matrix (E, arrowheads). At 15 days, osteopontin expression was restricted to a thin area adjacent to the pial surface (F). No immunoperoxidase reaction was detected in the absence of primary antibody at any time point (G). Bar=100 μm for A; 20 μm for B and C; 40 μm for D and E; and 100 μm for F and G.
1E) as well as within the subarachnoid space (Figure 1E, arrow) clearly defining the infarcted area. At this time only a few cells within the infarct expressed osteopontin mRNA (Figure 1E, arrowhead). By 48 hours, osteopontin mRNA expression persisted in the peri-infarct region, and the numbers of osteopontin mRNA+ cells had increased within the infarct (Figure 1F). At 5 days, osteopontin mRNA was largely absent from the peri-infarct region, with robust expression in the infarct (Figure 1G). By 15 days after injury, osteopontin mRNA had declined to nearly basal level (Figure 1H). Quantitative analysis of the area bounded by osteopontin mRNA (at 24 and 48 hours after injury) and of the area containing osteopontin mRNA cells at 5 days after injury revealed that the area of the infarct at 5 days was defined by the cells expressing osteopontin mRNA at 24 hours (Figure 2).

Using a technique of combined in situ hybridization and immunohistochemistry, we determined the phenotype of the osteopontin mRNA+ cells in the peri-infarct. Osteopontin mRNA colocalized with a microglia marker, ED1 (Figure 3A, arrow); osteopontin mRNA (Figure 3B, arrow) did not colocalize with the astrocytic marker GFAP (Figure 3B, arrowheads). The majority of cells expressing osteopontin mRNA could be colocalized with ED1, but not all of the ED1+ cells expressed osteopontin mRNA (Figure 3A, arrowheads).

Osteopontin protein was first detected within cells at 24 hours after injury in the peri-infarct region (Figure 4A, arrows) extending from the pial surface to the corpus callosum and at the callosal gray matter border at the medial aspect of the infarct. Cells expressing osteopontin had a characteristic ramified microglial morphology and were seen in the parenchyma (Figure 4B, arrow) and adjacent to blood vessels (Figure 4C). By 48 hours after injury, osteopontin protein was still restricted to cells in the peri-infarct region. Of these cells at 48 hours, only occasional ramified microglia expressing osteopontin could be found in the peri-infarct region (Figure 4D, arrow). The majority of cells expressing osteopontin had transformed into macrophages by 48 hours, with osteopontin protein evident in a peri-nuclear position (Figure 4D, arrow). Five days after injury, osteopontin protein was abundantly expressed in the infarct and at the infarct/peri-infarct border. Intracellular expression in macrophages (Figure 4E, arrows) as well as in an extracellular distribution around macrophages (Figure 4E, arrowheads) was evident. Fifteen days after injury, osteopontin protein expression was predominantly extracellular and restricted to a thin rim directly adjacent to the newly formed glial-pial surface (Figure 4F). Omission of the primary antibody gave no positive immunoperoxidase reaction product (Figure 4G).

To determine the potential functional interaction between osteopontin ligand and integrin receptor $\alpha_v\beta_3$, we analyzed the expression of the $\alpha_v\beta_3$ at 5 and 15 days after injury, a time of maximal osteopontin protein expression. Earlier time points were not analyzed because the goal of the present study was to understand which cells had the potential to interact with the osteopontin localized in the extracellular matrix. By immunoperoxidase localization at 5 days after injury, expression of $\alpha_v\beta_3$ was upregulated in the gray matter adjacent to the infarct (Figure 5A) compared with the gray matter in the contralateral side (Figure 5C). The cells expressing $\alpha_v\beta_3$ appear to be hypertrophic astrocytes (Figure 5A, arrows) at the infarct/peri-infarct border (asterisks indicate...
Expression of αβ3 by apparent hypertrophic astrocytes (Figure 5B, arrows) continued through 15 days after injury as the former boundary between the peri-infarct region and infarct was replaced by a new glial limitans composed of cells expressing αβ3 (Figure 5B, asterisks). A notable difference between the 5- and 15-day expression of αβ3 was the extended astrocytic processes at 15 days (Figure 5B, arrowhead). Omission of the primary antibody gave no detectable immunoperoxidase reaction product (Figure 5D). Adsorption of the SBJ293–346 antibody with purified integrin αβ3 blocked binding of the antibody to the tissue section (Figure 6B); compare with adjacent tissue section incubated with nonadsorbed antibody in which four cells expressing αβ3 are seen (Figure 6A).

Colocalization of αβ3 and GFAP by double immunofluorescence confirmed that the integrin receptor αβ3-expressing cells were hypertrophic GFAP+ astrocytes. In the ipsilateral cortex, GFAP+ astrocytes (Figure 7A, arrows) expressed αβ3 (Figure 7B, arrows). In the contralateral cortex, GFAP+ astrocytes (Figure 7C, arrow) did not express αβ3 (Figure 7D, arrow).

To further explore which events of astrocyte biology osteopontin might be mediating, the expression of osteopontin ligand and integrin receptor αβ3 were correlated with cellular and histological changes occurring at the peri-infarct/infarct border. Colocalization of GFAP+ astrocytes, which have been demonstrated to express the integrin receptor αβ3, with osteopontin ligand expression at 5 days after injury indicated that although osteopontin (Figure 8A, arrowhead) can be demonstrated within cells adjacent to GFAP+ astrocytes (Figure 8A, arrows) in the peri-infarct region (to the left of the asterisks), extracellular osteopontin could only be detected within the infarct (Figure 8A, arrowheads to the right of the asterisks). This is in contrast to osteopontin mRNA, which is abundantly expressed in the infarct core at 5 days (Figure 1G). Ten days later, however, GFAP+ astrocytes (Figure 8B, arrows) were present within the osteopontin-rich region (Figure 8B, arrowhead) adjacent to the newly formed glial-pial boundary (Figure 8B, asterisks).

**Discussion**

Focal stroke in the spontaneously hypertensive rat produces a well-defined and characteristic infarct that is consistent between animals.2–9 The infarct is restricted to the cortical gray matter dorsal to the corpus callosum and extends to the pial surface. Development and resolution of the infarct can be mapped over a 2-week period driven by (1) activation of glia in the peri-infarct region; (2) compartmentalization of the injured cells; (3) removal of the infarcted tissue debris by phagocytes; and (4) establishment of a new glial-pial boundary. In the present study we investigated the spatial and temporal expression of the provisional matrix protein osteopontin and its integrin receptor αβ3, which are expressed by microglia and astrocytes, respectively, after injury.

Osteopontin mRNA is expressed very early after ischemia in the peri-infarct region. The timing of osteopontin gene expression as indicated by in situ hybridization is earlier than that seen by Northern blot analysis, in which osteopontin mRNA was first detected at 12 hours after ischemia.18 This methodological difference is consistent with the increased sensitivity of in situ hybridization compared with Northern blot analysis. This rapid induction of osteopontin gene expression suggests that osteopontin might function as a stress-response gene after focal ischemia. Within the promoter region of the osteopontin gene, an acute-phase response element has been identified.30 In vitro studies have shown that cells adhered to osteopontin have enhanced expression of heat shock proteins and display a greater resistance to heat shock injury.31 Furthermore, Denhardt and Chambers32 have suggested that osteopontin is a protective protein that confers cellular resistance to the damaging effects of nitric oxide and oxidative burst associated with inflammation by inhibiting induction of nitric oxide synthase.33 Though not previously considered a stress-response gene, when regarded in the context of its early gene induction pattern after injury, its specific expression in the peri-infarct region (which survives the ischemic event), and its role in cell protection, osteopontin might well be considered a stress-inducible protein with characteristics of the classic stress-response genes, such as the heat shock proteins.34

Quantitative analysis of the area bounded by osteopontin mRNA+ cells at 24 hours compared with the area occupied.
by osteopontin mRNA+ cells at 5 days after injury indicated that the areas were not significantly different (ie, were essentially equal). Analysis of the specific cell type synthesizing osteopontin demonstrated that microglia, but not astrocytes, synthesize and secrete osteopontin. Thus, the activated microglia define the infarct border as early as 24 hours. Similar conclusions were offered by Coyle,35 who found that triphenyltetrazolium chloride staining after focal stroke in the spontaneously hypertensive rat resulted in a lesion border at 24 hours that was no different than the border at 21 days. These findings indicate that the microglial activation occurs within the peri-infarct region but not the penumbra.

The early activation of microglia surrounding the infarct precedes the transformation of astrocytes from a resting to a reactive state that does not manifest fully until 2 days after injury.36,37 The osteopontin receptor integrin \( \alpha_\beta_3 \) is expressed by astrocytes at 5 and 15 days after ischemia. These time points were chosen to identify potential cell populations that could interact with the osteopontin in the extracellular matrix. At 5 days after ischemia, astrocytes expressing integrin receptor \( \alpha_\beta_3 \) are dispersed in the peri-infarct region; by 15 days these cells have reformed the glial limitans lost initially as a result of tissue injury after focal stroke. Astrocytes in vitro express \( \alpha_\beta_3 \),38 and radial glia of the developing nervous system have been reported to express integrin \( \alpha_\beta_3 \).39 Hirsch and colleagues39 suggest that this integrin might be involved in the formation and orientation of the glial fibers that extend from the ventricular zone to the cortical plate. Similarly, the astrocytes expressing \( \alpha_\beta_3 \) after focal ischemia reorganize from a stellate morphology to the more bipolar morphology characteristic of astrocytes of the glial limitans.

Fundamentally these events require that cells change the relationship they have with the extracellular matrix from a static, fixed state to a dynamic, mobile state that allows for changes in cell attachment, shape, and locomotion. Upregulation of \( \alpha_\beta_3 \) has been reported to be essential for endothelial transformation to an angiogenic phenotype40 and for migration toward an osteopontin gradient.25 The spatial-temporal interaction of extracellular osteopontin ligand and integrin receptor \( \alpha_\beta_3 \) is at a distance at 5 days; however, by 15 days the astrocytes are localized within a matrix of osteopontin, suggesting that osteopontin may act as a chemotactic factor for astrocytes. Preliminary studies with human astrocytes demonstrate the capacity of osteopontin to induce directed migration of astrocytes.18

In addition to its chemoattractant role, ligation of integrin receptor \( \alpha_\beta_3 \) by osteopontin ligand results in the rapid production of phosphoinositides.41 Astrocytic hypertrophy and migration are dependent on GFAP, the predominant intermediate filament expressed by these cells. The assembly/disassembly of GFAP is Ca\(^{2+} \)-dependent,42 and inositol 1,4,5-trisphosphate–induced Ca\(^{2+} \) release in astrocytes is directed by the type 3 inositol 1,4,5-trisphosphate receptor.43 Thus, osteopontin ligand binding to integrin receptor \( \alpha_\beta_3 \)
could stimulate release of intracellular Ca$^{2+}$ stores, causing a subsequent reorganization of the GFAP filament network.

Ligation of integrin receptors results not only in immediate signal transduction events at the cell surface but also mediates changes in gene expression. Signaling through the fibronectin and tenascin integrin receptors upregulated synthesis of collagenase, stromelysin, gelatinase, and c-fos in fibroblasts. In endothelial cells integrin ligation promoted cell survival by suppressing p53 activity and by increasing the bcl-2/bax ratio. Although no data exist regarding integrin-mediated gene induction in astrocytes, the activation of astrocytes, their transformation to migratory cells, and subsequent acquisition of a pial astrocyte phenotype strongly suggest that a change in gene expression does occur.

Both integrin receptor $\alpha_v\beta_3$ and osteopontin interact with other ligands and receptors. Previous work analyzing the expression of integrin receptors after focal stroke demonstrated the interaction of fibrinogen with integrin receptor $\alpha_v\beta_3$ early in the ischemic event. The results presented in this study demonstrate the diverse role of integrin receptors in tissue remodeling. In addition to the role in vascular remodeling seen early after focal stroke, these findings suggest that integrin receptor $\alpha_v\beta_3$ participates in nonvascular remodeling associated with formation of a glial scar that occurs late in the resolution of the ischemic insult.

In conclusion, our data demonstrate that induction of a microglia-specific gene, osteopontin, occurs early after focal stroke. The pattern of induction in microglia is restricted to the peri-infarct region and in fact defines the final infarct area (ie, maximum size at 24 hours). Osteopontin protein is expressed by ramified microglia in the peri-infarct area at 24 hours. As the microglia transform into macrophages and the lesion develops over 5 days, macrophages in the infarcted region express osteopontin and secrete it into the extracellular matrix. The osteopontin receptor integrin $\alpha_v\beta_3$ is expressed by hypertrophic astrocytes at 5 and 15 days after stroke. These astrocytes expressing integrin receptor $\alpha_v\beta_3$ are at a distance from the osteopontin ligand in the extracellular matrix at 5 days, but by 15 days the astrocytes are localized in an osteopontin-rich region concomitant with formation of a new glial limitans. These results strongly suggest that the interaction of osteopontin ligand and integrin receptor $\alpha_v\beta_3$ may play a role in the healing of brain injury in establishing a new glial-pial boundary after stroke.

Although these data do not allow us to draw conclusions suggesting that the appearance of osteopontin mediates the expression of integrin receptor $\alpha_v\beta_3$, the data clearly demonstrate the spatial and temporal expression patterns of the two proteins, which is the first step in identifying potential receptor-ligand interactions. Further studies will be needed to address the early temporal upregulation of $\alpha_v\beta_3$ in astrocytes; the potential interaction of osteopontin with its other integrin receptors, $\alpha_v\beta_1$ and $\alpha_v\beta_2$; and the interaction of integrin receptor $\alpha_v\beta_3$ with other ligands.

Acknowledgments

The authors thank Steve Trulli and Kyung Johanson for their technical expertise in preparation of the SB293–346 antibody. The MBPPII810 hybrideroma developed by Drs Solursh and Franzen was obtained from the Developmental Studies Hybridoma Bank, Department of Biological Sciences at the University of Iowa, under contract NO1-HD-7–3263.

References


In the accompanying article, Ellison and colleagues demonstrate that focal cerebral ischemia results in the early appearance of osteopontin mRNA and the late appearance of the antigen (beginning at 24 hours) together with its receptor, integrin $\alpha_v\beta_3$, in the spontaneously hypertensive rat. Integrin $\alpha_v\beta_3$ expression associated with evidence of astrocyte activation increased between 5 and 15 days, while osteopontin was apparently associated with microglial cells or macrophages. These observations are intriguing because they (1) confirm that changes in expression of selected integrins occur in the central nervous system after ischemia, (2) suggest that in vivo integrin $\alpha_v\beta_3$ antigen can appear outside blood vessels, and (3) through the appearance of osteopontin suggest the possibility of interesting receptor-ligand interactions.

The latter point is underscored by Okada et al., who showed that integrin $\alpha_v\beta_3$ was greatly upregulated very early in the myointima of noncapillary microvessels, related significantly to fibrin deposition in the same vessels, in focal brain ischemia/reperfusion in the nonhuman primate. Other recent studies have shown that selected integrins have an important role in vascular integrity and response during cerebral ischemic event. For instance, integrin $\alpha_v\beta_3$ is localized on astrocyte end-feet, where it connects with the basal lamina of extracellular matrix at the astrocyte-vessel interface. In contrast to integrin $\alpha_v\beta_3$, it was downregulated very early in focal brain ischemia/reperfusion.

Integrin $\alpha_v\beta_3$ is a promiscuous receptor in that it recognizes a wide variety of Arg-Gly-Asp (RGD)—containing ligands including fibrinogen, vitronectin, von Willebrand factor, thrombospondin, laminin, and collagen as well as osteopontin. Cell migration mediated by ligation of integrin $\alpha_v\beta_3$ to osteopontin has been demonstrated in vascular smooth muscle cells. However, other interactions associated with its vascular expression are known. A specific monoclonal antibody against integrin $\alpha_v\beta_3$ (LM609) inhibits angiogenesis in tumors and suppresses tumor growth by invasion of new vasculature. It has been demonstrated that the interaction of integrin $\alpha_v\beta_3$ with vitronectin is necessary for migration of smooth muscle cells. Recently, it has been shown that blockage of integrin $\alpha_v\beta_3$ induces apoptosis in an endothelial cell culture system.

Although osteopontin is distributed widely among epithelial structures in the body, the appearance of osteopontin in the brain has only been documented in gliomas and in this report of cerebral ischemia. The findings of Ellison et al showing that microglia and macrophages were sources of osteopontin expression in the rodent are in accord with the findings of osteopontin expression in renal ischemia. Therefore, osteopontin expression may be one of the common events after activation of macrophages by ischemic injury. This work implies that at least one aspect of osteopontin presentation occurs somewhat distant in time from the initial ischemic events.

It is intriguing to consider potential causes of upregulation of osteopontin and integrin $\alpha_v\beta_3$ in the ischemic parenchyma in these studies and whether they are related to each other in their expression. The upregulation of osteopontin mRNA expression may depend on the acute-phase responses of the promoter element of osteopontin gene, as the authors have suggested. However, the mechanism of upregulation of integrin $\alpha_v\beta_3$ in astrocytes remains to be confirmed in vivo and elucidated. Despite the relative spacial distributions of osteopontin and integrin $\alpha_v\beta_3$ expression in these studies, it is possible that both expressions result from independent stimuli or that integrin $\alpha_v\beta_3$ is related to one or another ligand. Studies of cultured endothelial cell preparations indicate that in those cells vascular endothelial growth factor induces integrin $\alpha_v\beta_3$ expression. It remains to be seen how integrin $\alpha_v\beta_3$ expression is managed in the nonvascular ischemic tissue of the rodent and its relationship to the evolution of cellular injury and gliosis.

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**References**


Osteopontin and its Integrin Receptor αᵥβ₃ Are Upregulated During Formation of the Glial Scar After Focal Stroke

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Stroke. 1998;29:1698-1707
doi: 10.1161/01.STR.29.8.1698

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/29/8/1698

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