The Role of Osteopontin in Recovery from Hind Limb Ischemia

Craig L. Duvall, Daiana Weiss, Scott T. Robinson, Fadi M.F. Alameddine, Robert E. Guldberg, W. Robert Taylor

Objective—Osteopontin (OPN) is a highly phosphorylated extracellular matrix glycoprotein that is involved in a diversity of biological processes. In the vascular wall, OPN is produced by monocytes/macrophages, endothelial cells, and smooth muscle cells, and it is thought to mediate adhesion, migration, and survival of these cell types. In this study, we hypothesized that OPN plays a critical role in recovery from limb ischemia.

Methods and Results—We induced hind limb ischemia in wild-type and OPN\(^{−/−}\) mice. OPN\(^{−/−}\) mice exhibited significantly delayed recovery of ischemic foot perfusion as determined by LDPI, impaired collateral vessel formation as measured using micro-CT, and diminished functional capacity of the ischemic limb. In the aortic ring assay, normal endothelial cell sprouting was found in OPN\(^{−/−}\) mice. However, OPN\(^{−/−}\) peritoneal monocytes/macrophages were found to possess significantly reduced migration in response to chemoattraction.

Conclusions—This study provides evidence that a definitive biological role exists for OPN during ischemic limb revascularization, and we have suggested that this may be driven by impaired monocyte/macrophage migration in OPN\(^{−/−}\) mice. These findings provide the first in vivo evidence that OPN may be a key regulator in postnatal vascular growth. (Arterioscler Thromb Vasc Biol. 2008;28:290-295)

Key Words: osteopontin ■ hind limb ischemia ■ angiogenesis ■ arteriogenesis ■ collateral vessels

The development of functional collateral blood vessels into ischemic tissue requires a precise interplay between numerous cell types, growth factors, and cues provided by the extracellular environment. To improve therapeutic angiogenesis regimens, each of these areas must be better understood individually and, more importantly, in the context of their interactions with one another. Although its full complement of functions is not fully elucidated, the extracellular matrix (ECM) is believed to provide the structural scaffolding that maintains the organization of vascular cells into blood vessels and also to initiate signals that stimulate specific cellular events such as survival, proliferation, and migration.

Osteopontin (OPN) is a unique component of the ECM that may have an important role in control of vascular growth. OPN is synthesized as a 34-kDa protein and functions in a variety of biological processes including inflammation, immunity, wound repair, tumorigenesis, cell adhesion, cell migration, and bone mineralization and remodeling. OPN has been classified as a “matricellular protein,” a categorization for nonfibrillar, bioactive ECM proteins that are thought to mediate cellular functions by providing a functional link between cell surface receptors, structural ECM molecules, and cytokines, growth factors, and proteases.\(^1\) OPN interacts with as many as 7 different integrins through its RGD or more novel SVVYGLR binding sequences.\(^2-7\) Also, an intracellular form of OPN that localizes to the cell membrane where it binds to the cell surface glycoprotein CD44 has been found to be an integral component in cell migration.\(^8,9\)

Previous research has shown that OPN is important for normal arterial physiology,\(^10\) and it has been found to be produced by monocytes/macrophages, endothelial cells (ECs), and smooth muscle cells (SMCs).\(^11\) Moreover, vascular cell interactions with OPN mediated through cell surface integrins regulate multiple cellular functions that are potentially important to angiogenesis and arteriogenesis. In this context, OPN signaling through the \(\alpha_\beta\) integrin has been found to mediate EC survival in a process that involves the NF-\(\kappa\)B signaling pathway.\(^12\) Also, the angiogenic growth factor VEGF has been shown to trigger an increase in \(\alpha_\beta\) expression, OPN expression, and thrombin cleavage of OPN, which provides a cooperative mechanism that enhances EC migration.\(^13\) In addition, OPN expression is induced by stimulation with FGF, and this cross-talk may provide a mechanism for recruitment of monocytes and amplification of FGF-induced angiogenesis.\(^14\) OPN also mediates several processes relevant to arteriogenic collateral vessel formation...
including adhesion and migration of both macrophages and SMCs.15,16 There have also been a less substantial number of in vivo studies that have implicated OPN in postnatal neovascularization. For example, OPN mRNA has been shown to be locally upregulated at sites of ischemia-induced retinal neovascularization in mice.17 and OPN has been found to correlate with progression of the angiogenesis-related processes tumorigeneration and metastasis.18 In addition, we have recently reported a role for OPN in angiogenesis and healing after bone fracture.19

Based on these observations, it is feasible that OPN is functionally relevant in the setting of collateral vessel growth in limb ischemia, but the role of OPN in this context has not been previously determined. In the present study, we hypothesized that, in the mouse model of hind limb ischemia, OPN deficiency reduces vascular growth, delays reperfusion of ischemic tissues, and impairs recovery of limb functionality. To test this hypothesis, we used in vivo as well as in vitro cellular assays in wild-type and OPN−/− mice to determine the contribution of OPN to the development of functional collateral blood flow in the setting of ischemia.

Materials and Methods

Animals

Male wild-type C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Osteopontin-deficient mice were originally received from Dr Lucy Liaw of the Maine Medical Center (Portland, ME), and they were subsequently backcrossed 10 generations onto the C57BL/6 background. All protocols were approved by the Institutional Animal Care and Use Committee and done in accordance with the federal guidelines on the principles for the care and use of animals in research.

Hind Limb Ischemia Model

Animals were anesthetized by intraperitoneal injection of xylazine (10 mg/kg) and ketamine (80 mg/kg). Using aseptic technique, the right superficial femoral artery and vein were ligated proximal to the caudally branching deep femoral artery, and a second ligation was performed just proximal to the branching of the tibial arteries. The length of the artery and vein was excised between the 2 ligation points, leaving the femoral nerve intact. The skin was closed and the animals were allowed to recover.

Laser Doppler Perfusion Imaging

Laser Doppler perfusion imaging (LDPI) was completed 3, 7, and 14 days after surgery to assess perfusion to the paw of the ischemic limb (n=11 to 14 for each genotype at each time point). Mice were anesthetized with xylazine (10 mg/kg) and ketamine (80 mg/kg) and scanned with a LDPI system (PIM II Laser Doppler Perfusion Imager). The footpad was chosen as the region of interest, and average perfusion (88%) and the OPN knockouts possessing a persistently lower at around 40%. At 14 days, the difference was significantly lower at around 40%. At 14 days, the difference was significantly lower at around 40%. At 14 days, the difference was significantly lower at around 40%. At 14 days, the difference was significantly lower at around 40%. At 14 days, the difference was significantly lower at around 40%. At 14 days, the difference was significantly lower at around 40%. At 14 days, the difference was significantly lower at around 40%. At 14 days, the difference was significantly lower at around 40%.

Swim Endurance Test

Experiments measuring swim endurance were completed as a physiological test of ischemic limb functional recovery. Based on the method reported by Matsumoto and authors, we built a current pool that stimulates forced swimming.21 The water temperature was maintained at 35°C and flow rates of 5 L/min were generated. Before surgery, mice were trained for 5 minutes per day for 7 days to acclimate the animals to swimming. At 5 days after surgery, maximal exercise capacity was measured as the amount of time the animal could swim against the current before experiencing 5 consecutive seconds of submersion (wild-type n=8, OPN−/− n=8).

Aortic Ring Vascular Sprouting Assay

The mouse aortic ring assay was used as previously described.22 Briefly, the mid-thoracic portion of the aorta was dissected from the mouse, cut into 1-mm sections, and embedded in gels made from rat tail type I collagen (Serva Chemical). Aortic explants were fed MCDB-131 media containing 2.5% autologous serum and imaged at 4, 6, 8, and 10 days. The number of vascular sprouts and length of the largest sprout from each aorta were measured.

Flow Cytometry Determination of Circulating Monocyte Number

Flow cytometry was used to identify circulating monocytes based on low side-scatter and high CD11b expression as previously described.23

Monocyte/Macrophage Migration Assay

Cell culture transwell chemotaxis inserts (Falcon) with a pore size of 8 μm were coated overnight at 4°C with 10 μg/mL fibronectin (Sigma) and then allowed to air dry. Resident monocytes and macrophages were isolated from wild-type and OPN-deficient mice by peritoneal lavage with cold PBS. 400 000 cells were seeded onto the top of each transwell and allowed to adhere for 1 hour. Regular media or chemottractant media containing either 5% homologous serum or 100 ng/mL monocyte chemotactic protein-1 (MCP-1; R&D Systems) was added to the lower reservoirs. After incubation for 8 hours, the migrated cells were fixed and stained, and cells were counted in 3 random fields per insert using Image Pro software (Media Cybernetics).

Statistical Analysis

Data are presented as mean±SEM. ANOVA was used to identify genotype effects, and Tukey method was used for post-hoc pairwise comparisons.

Results

Laser Doppler Perfusion Imaging

LDPI was performed 3, 7, and 14 days after surgery. The acquired images illustrated a visible lag in recovery in the OPN-deficient mice (Figure 1A). Quantitative perfusion measurements further illustrated this discrepancy (Figure 1B). At 7 days, the wild-type resting perfusion values were approximately 70% of normal, whereas the OPN mice were signifi- cantly lower at around 40%. At 14 days, the difference was more pronounced, with wild types having nearly normal perfusion (88%) and the OPN knockouts possessing a persistently lower at around 40%.

Micro-CT Imaging of Collateral Vessels

Micro-CT Imaging was completed at 7 days after surgery as a quantitative, anatomic measure of collateral vessel forma-
tion. Qualitative observation of the 3-D image reconstructions of the vasculature showed decreased collateral vessel formation in the OPN-deficient mice (Figure 2A), and quantitative analysis revealed that they possessed significantly reduced vascular volume of the ischemic limb (Figure 2B).

**Swim Endurance Test**
As a physiological measure of ischemic limb recovery, exercise capacity was measured 5 days post surgery. OPN deficient mice displayed significant functional impairment of the ischemic limb as shown by a 40% decrease in maximal swim time relative to the wild types (Figure 3).

**Aortic Ring Vascular Sprouting Assay**
The aortic ring assay was used to measure EC angiogenic capacity based on the sprouting of vascular tubes into a collagen gel. Wild-type and OPN-deficient aortic rings were assayed for tube formation every 2 days from 4 to 10 days.

There was a modest but insignificant overall decrease in sprout number in OPN-deficient specimens relative to wild types, and there was no difference between genotypes for maximum sprout length (Figure 4). These data suggest that OPN deficiency did not impair the angiogenic capacity of vascular ECs.

**Flow Cytometry Determination of Circulating Monocyte Number**
Circulating monocytes were identified with flow cytometry based on low side scatter and high expression of CD11b. The number of circulating monocytes in wild-type (3.15×10⁶ cells/mL) and OPN−/− (2.96×10⁶ cells/mL) mice were found to be similar.

**Monocyte/Macrophage Migration Assay**
To determine whether a defect in monocyte/macrophage function could explain the deficiency in hind limb revascu-

Figure 1. LDPI analysis. A, Representative LDPI images display the time course of ischemic limb reperfusion in OPN−/− and wild-type mice. B, Quantitative analysis displayed a significant lag in recovery in OPN deficient mice. *P<0.001, n=11 to 14.

Figure 2. Micro-CT imaging. A, Micro-CT images illustrating decreased collateral vessel formation in OPN−/− mice. B, Quantitative analysis showed decreased vascular volume in OPN−/− animals. *P<0.05, n=7.

Figure 3. Swim endurance test. OPN−/− mice displayed decreased swim endurance 5 days after onset of limb ischemia. *P<0.01, n=8.

Figure 4. Aortic ring sprouting assay. A, Representative aortic rings illustrating a modest decrease in sprouting in OPN−/− specimens at 8 days. B, OPN−/− aortic rings displayed a trend toward reduction in number of vascular sprouts and (C) no difference in maximum sprout length over a 10-day time course. No significant differences existed between genotypes for either measurement, n=9.
discussion

previous in vitro studies have described a role for OPN in controlling survival, migration, and other functions in multiple cell types involved in postnatal vascular growth. In this study, we sought to determine the in vivo significance of OPN in angiogenesis/arteriogenesis processes. To do so, we compared wild-type and OPN⁻/⁻ mice for their capacity to form collateral vessels and for recovery of function after induction of hind limb ischemia. After ligation and excision of the femoral artery, OPN deficiency was found to significantly delay collateral vessel formation, restoration of distal limb perfusion, and recovery of the functional capacity of the ischemic limb. Because OPN has multiple functions and affects many cell types, we performed additional in vitro studies to gain insight into the potential mechanisms responsible for this delay in recovery. We found that there was no effect of OPN deficiency on ex vivo endothelial sprouting from aortic rings, suggesting that the native endothelium of the OPN⁻/⁻ mice possesses normal angiogenic capacity. In contrast, there was a substantial decrease in migration of OPN⁻/⁻ monocytes/macrophages. Considering the importance of monocytes in arteriogenic vessel growth, these data suggest that the role of OPN in regulation of collateral formation may be driven by its function in mediating monocyte/macrophage migration.

initially, we focused on the LDPI methodology to evaluate the physiological significance of OPN in reperfusion after femoral artery ligation. Using LDPI, we performed measurements on anesthetized mice and found decreased recovery of perfusion to the ischemic paw in the OPN⁻/⁻ mice at both 1 and 2 weeks after surgery. The next endpoint of our study was based on quantitative 3-D micro-CT imaging methodologies. using this technique, we are able to perform high resolution, objective, and quantitative measurements of the global vascular anatomy within the mouse hind limb. Importantly, using this methodology, we were able to detect significantly decreased total vascular volume in the ischemic limbs of the OPN⁻/⁻ mice. In addition, we measured the exercise capacity of the mice as a means for functional assessment of ischemic limb recovery. The swimming apparatus used for this study forced the mice to swim against a generated current, and, unlike treadmill tests, required that the mice equally use the normal and ischemic hind limbs. Using this system, we were able to measure a significant functional impairment of the ischemic limb in the OPN⁻/⁻ mice relative to the wild types at 5 days after surgery. This full complement of studies was essential to correlate vascular anatomy and blood flow to limb functionality, considering previous reports have indicated that measuring resting blood flow in anesthetized animals does not always accurately reflect the flow deficit in models of hind limb ischemia. taken together, these data indicate an unequivocal role for OPN in revascularization and recovery from hind limb ischemia.

EC function is generally considered to be critical for neovascularization. Therefore, to better understand our in vivo findings, we first assessed the capacity for EC tube formation in aortic explants from OPN-deficient mice. we found a modest trend toward decreased sprout number in OPN null specimens, but there were no significant differences between genotypes for sprout number or maximum sprout length. These results indicate that OPN may not play a vital role in EC migration and tube formation during angiogenesis. However, because this assay is limited to measurement of the angiogenic response of the aortic endothelium, we cannot definitively conclude that OPN does not play a role in EC function within the microvascular networks that participate in vivo neovascularization.

It is well-accepted that monocytes/macrophages localize to sites of angiogenesis and arteriogenesis and play an important role in modulation of these processes. indeed, recent studies have shown a direct functional link between circulating monocyte concentration and arteriogenic collateral vessel formation. MCP-1 is thought to be the primary stimulus.
for induction of monocyte infiltration in this setting, and MCP-1 treatment has been shown to accelerate collateralization and recovery in hind limb ischemia models in a variety of animal studies. Conversely, animals deficient in MCP-1 or CCR2-chemokine receptor, an MCP-1 receptor, have been shown to display reduced monocyte/macrophage infiltration and collateral formation.

Previous work has indicated that OPN is an important mediator of macrophage adhesion and migration, and other studies have shown that OPN may be critical for macrophage infiltration into sites of injury in vivo. In addition, two recent reports have indicated that OPN plays a key role in the regulation of the hematopoietic stem cell (HSC) niche in bone marrow. In this work, we found that the number of circulating monocytes was not altered in the OPN-deficient mice indicating that monocyte production and mobilization from the bone marrow was normal in these animals. It cannot be disregarded that the HSC niche is also thought to be the source of bone marrow–derived endothelial progenitor cells (EPCs), which have been found to be a vital constituent in the response to limb ischemia. It is possible that EPC numbers, homing, or ability to incorporate into functional vasculature could be altered in the absence of OPN, and this could prove to be an important avenue for future studies.

Because of the established connection between OPN and macrophage migration, we next hypothesized that OPN could play a role in macrophage infiltration into sites of ischemic limb collateralization. To test this hypothesis, we compared macrophage/macrophage migration capacity of cells isolated from wild-type and OPN−/− mice. In these studies, we found that OPN−/− monocytes/macrophages displayed significantly reduced migration in response to chemotraction. In initial experiments, we found that wild-type cells exhibited a 3-fold stronger migratory response to homologous serum than OPN−/− cells. These results suggest that serum-derived OPN or autocrine OPN production are important for monocyte migration in this system. Similarly, MCP-1–induced migration of OPN−/− cells was significantly reduced when compared with wild-type cells, which is consistent with a previous study that found OPN to be critical for normal chemotactic response of macrophages to MCP-1 in vitro. The difference between genotypes using this OPN-free stimulus, although not as pronounced as in the serum experiments, indicates the importance of autocrine OPN stimulation and is likely attributable to the role intracellular OPN plays in formation of perimembranous complexes involved in migration.

The significance of these in vitro findings is strengthened by the fact that MCP-1 has been shown by numerous studies to play an important role in vivo in the regulation of angiogenesis/arteriogenesis, they produce growth factors and proteases that induce vascular EC and SMC proliferation and stimulate remodeling of the vascular wall to satisfy increased blood flow demands. In addition to providing paracrine stimulation of other cells types, monocytes have also been reported to tunnel through ischemic myocardium and form physical conduits that could be subsequently endothelialized and incorporated into the preexisting vasculature. Therefore, defective migratory response to chemotraction and the subsequent reduction in monocyte activity at the site of collateralization likely has significant detrimental effects on revascularization and recovery of the ischemic limb in the OPN−/− animals.

In this study, we have presented evidence suggesting that a definitive biological role exists for OPN in the vascular response to hind limb ischemia. We were able to confirm this finding using a range of anatomic, physiological, and functional measurements. We have also reported evidence that suggests that this defect could be driven by alterations in macrophage migration in the OPN−/− mice. These findings represent the first evidence that OPN may be a key regulator for ischemic limb collateral vessel growth and that OPN may be an important mediator of macrophage function in this setting.

Acknowledgments

The authors thank Dr Lucy Liaw for kindly providing OPN deficient mice and Angela Lin for assistance in development of automated methods for micro-CT image analysis.

Sources of Funding

This work was supported by NIH Grants R01AR051336, RO1HL70531, PO1HL58000, the Georgia Tech/Emory Center for the Engineering of Living Tissues (GTEC) National Science Foundation (NSF) Grant EEC-9731643, and the NSF Graduate Research Fellowship Program (awarded to C.L.D.).

Disclosures

None.

References


The Role of Osteopontin in Recovery from Hind Limb Ischemia
Craig L. Duvall, Daiana Weiss, Scott T. Robinson, Fadi M.F. Alameddine, Robert E. Guldberg
and W. Robert Taylor

Arterioscler Thromb Vasc Biol. 2008;28:290-295; originally published online November 15, 2007;
doi: 10.1161/ATVBAHA.107.158485
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://atvb.ahajournals.org/content/28/2/290

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the
Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for
which permission is being requested is located, click Request Permissions in the middle column of the Web
page under Services. Further information about this process is available in the Permissions and Rights
Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online
at:
http://atvb.ahajournals.org//subscriptions/