Abstract—Osteopontin (OPN) is a multifunctional molecule highly expressed in chronic inflammatory and autoimmune diseases, and it is specifically localized in and around inflammatory cells. OPN is a secreted adhesive molecule, and it is thought to aid in the recruitment of monocytes-macrophages and to regulate cytokine production in macrophages, dendritic cells, and T-cells. OPN has been classified as T-helper 1 cytokine and thus believed to exacerbate inflammation in several chronic inflammatory diseases, including atherosclerosis. Besides proinflammatory functions, physiologically OPN is a potent inhibitor of mineralization, it prevents ectopic calcium deposits and is a potent inducible inhibitor of vascular calcification. Clinically, OPN plasma levels have been found associated with various inflammatory diseases, including cardiovascular burden. It is thus imperative to dissect the OPN proinflammatory and anticalcific functions. OPN recruitment functions of inflammatory cells are thought to be mediated through its adhesive domains, especially the arginine-glycine-aspartate (RGD) sequence that interacts with several integrin heterodimers. However, the integrin receptors and intracellular pathways mediating OPN effects on immune cells are not well established. Furthermore, several studies show that OPN is cleaved by at least 2 classes of proteases: thrombin and matrix-metalloproteases (MMPs). Most importantly, at least in vitro, fragments generated by cleavage not only maintain OPN adhesive functions but also expose new active domains that may impart new activities. The role for OPN proteolytic fragments in vivo is almost completely unexplored. We believe that further knowledge of the effects of OPN fragments on cell responses might help in designing therapeutics targeting inflammatory and cardiovascular diseases. (Arterioscler Thromb Vasc Biol. 2007;27:2302-2309.)

Key Words: osteopontin ■ osteopontin proteolytic processing ■ inflammation ■ atherosclerosis

OPN is an aspartic acid-rich, N-linked glycosylated protein that may be highly phosphorylated on serines and threonines depending on the cell type.1-2 Functionally, OPN is a secreted protein with important roles in normal physiological as well pathological processes. During development it is expressed during gastrulation in the notochord and the embryonic /maternal interface,3 and later in regions of cartilage condensation and bone formation,4,5 and a number of epithelial tissues.5 However, despite OPN expression during the embryonic phase, OPN−/− mice develop normally and are fertile.6 In the adult, OPN protein is generally restricted to bones, kidneys and the epithelial linings.2

Clinically, OPN plasma levels are elevated in many diseases characterized by chronic inflammation, including Crohn disease,7 several types of cancer,8-10 autoimmune diseases,11,12 and obesity.13 Importantly, OPN plasma levels are significantly associated with the presence and the extent of cardiovascular disease independently of traditional risk factors.14 Plasma OPN levels are also higher in patients with restenosis, and it is an independent risk factor for restenosis.15 Furthermore, Ohmori et al determined that OPN levels were higher in patients presenting signs of vascular calcification than those without and correlated with the number of segments affected.14 Finally, very recently Golledge et al have shown that serum and tissue concentrations of OPN are associated with human abdominal aortic aneurysm.16 These results suggest that OPN plasma levels in patients affected with cardiovascular disease may reflect the extent of atherosclerosis and that OPN may play a role in plaque formation and vascular disease progression.

OPN Role in Biomineralization

Several studies, including in OPN−/− mice, demonstrate that one major physiological function of OPN is the control of biomineralization. OPN−/− mice have impaired bone resorption leading to increased trabecular bone with age and in response to parathyroid hormone, ovariectomy or mechanical unloading.17,18 OPN−/− bones are hypermineralized and more fragile than those from wild-type mice,19,20 indicating that OPN plays a role in regulating apatite crystal size and growth.
most likely by its ability to directly bind to specific apatite crystal faces.\textsuperscript{21} In addition, OPN appears to modulate osteoclast differentiation as well as bone resorption through the ligation of the $\alpha_\beta_3$ integrin.\textsuperscript{22}

OPN is also highly expressed in the kidney and is found at high levels in urine. Uropontin, the urinary form of OPN, suppresses growth and aggregation of calcium oxalate crystals, thus blocking the binding of the crystals to renal epithelial cells. As a consequence, OPN mice show increased susceptibility to oxalate-overload induced stone formation.\textsuperscript{23,24} More recent studies also indicate that OPN plays a major role in the regulation of ectopic calcification and pathological mineralization of the vasculature, which will be described in greater detail below.\textsuperscript{25–27}

**OPN Role in Tissue Remodeling**

OPN acts both as a matricellular protein, thereby facilitating adhesion and migration, as well as a soluble cytokine.\textsuperscript{28} Several in vitro studies indicate that OPN induces adhesion, migration, and survival of several cell types, including smooth muscle cells, endothelial cells, renal epithelial cells, and inflammatory cells.\textsuperscript{29–34} In vivo studies of OPN in disease and injury have suggested an important function for this molecule in inflammation and tissue remodeling.\textsuperscript{1,2} In particular, OPN is de novo expressed in cells participating in renal and cardiovascular system of remodeling and repair processes.\textsuperscript{25,35–39}

In addition to being expressed in specific tissues, OPN is highly expressed in inflammatory cells associated with many diseases including cancer,\textsuperscript{40} arterial restenosis,\textsuperscript{41} native and bioprosthetic valve stenosis,\textsuperscript{42,43} renal tubulointerstitial fibrosis,\textsuperscript{36} myocardial infarction,\textsuperscript{44} stroke,\textsuperscript{45} wound healing,\textsuperscript{6,44} and in a number of granulomatous diseases including sarcoidosis, foreign body granulomas, and tuberculosis.\textsuperscript{49–51} Specific effects on inflammatory cells and the modulation of inflammation will be discussed in more detail below.

**OPN Structure and Proteolytic Processing**

OPN contains several cell interacting domains as well as protease cleavage sites that may be important in regulating its activity (Figure 1). Cell interacting domains do not require phosphorylation (because recombinant bacterial proteins re-

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*Figure 1.* A, OPN structural features. The cell adhesive domains are indicated in color. The adhesive domain amino acids sequences as well as the known specific integrin receptors for each are also shown in color. The calcium binding domains are shown in gray, and other matrix binding domains are in black. Also shown are known phosphorylation sites. B, Amino acid sequence of human OPN. The adhesive sequences are shown in color. Arrows indicate known cleavage sites. Thrombin cleavage site in blue and MMP cleavage sites in orange.
The in vivo relevance of OPN fragments is unclear and largely unexplored. To date, only 3 studies have addressed the role of the cryptic SVVYGLR sequence in vivo. In a model of rheumatoid arthritis, an antibody specifically neutralizing only the SLAYGLR domain of mouse OPN (homologous to the SVVYGLR of human OPN) greatly reduced proliferation of synovial cells, leading to bone erosion and inflammatory cell infiltration in the arthritic joints. These findings correlated with increased expression of the integrin α4 and α5 in monocytes isolated from arthritic mice compared with monocytes isolated from normal mice.70 The highly inflammatory environment of arthritic joints is likely rich in thrombin and MMPs, thus it is plausible that new bioactive OPN fragments could be generated. Another very recent study showed that the SVVYGLR sequence induces promMP9 expression in isolated vascular smooth muscle cells and in diabetic mouse aortas.71 Finally, the SVVYGLR peptide has been shown to induce angiogenesis of in vitro and in vivo.72,73 These data highlight the potential role of the OPN SVVYGLR sequence in signaling, and further understanding of the regulatory events initiated by the different OPN fragments might foster the development of strategies to reduce inflammatory diseases and preserve vascular structure and function.

OPN also interacts with structural components of the extracellular matrix. OPN contains 2 conserved N-terminal domains with heparin binding homology that are likely to regulate its binding to heparan sulfate proteoglycans. Furthermore, studies by Yokosaki et al indicate that the MMP N-terminal fragment lacking the lysine and arginine residues is also unable to interact with αv and further that the αv binding is dependent on the tyrosine residue.54,61 Are there pathophysiological functions for OPN fragments? Several in vitro studies have demonstrated that the N-terminal fragments generated both by thrombin cleavage and MMP cleavage induced enhanced adhesion when compared with the full length molecule. This appears to be attributable mostly to increased activity of the RGD site, perhaps an indication of conformational change resulting in higher affinity binding.53,62 In addition, the thrombin cleaved fragment also increases haptotaxis.62 The SVVYGLR cryptic domain exposed after thrombin cleavage is also able to induce adhesion and migration through the αv and α5 integrins,53 although at least the α3-dependent functions appear to be lost in the N-terminal MMP generated fragment.54,61 The C-terminal fragment of OPN generated by thrombin and MMP cleavage does not contain any integrin adhesive domains, it does not mediate adhesion when presented in immobilized form to cells, and it appears to suppress OPN mediated adhesion and migration in monocyte-derived cells.53,63–64

OPN has also been reported to interact with the hyaluronic acid receptor, CD44. Although OPN does not bind to the standard CD44 isoform, CD44H,65 it was reported to interact with CD44v(705,803),(731,825)(734,803),(759,825)v(768,803),(793,825)v(803,803),(828,825)v-6 splice variants.56 Though its binding site in OPN has not yet been defined, recent data suggest that this interaction might occur with the soluble C-terminal thrombin cleaved fragment in an RGD-independent fashion.67 In addition, growing evidence suggests that OPN is a major regulator of CD44 surface expression, especially in osteoclasts.22,68,69

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of OPN facilitates proper stromal and fibrillar collagen network organization, although an indirect mechanism is also possible. These data also suggest that OPN supports the activity of phagocytic cells. Other studies in bone wound healing and in the microglia associated with the developing brain support the hypothesis that OPN is a positive regulator of phagocytic activity.83,84 These studies found that OPN-coated calcified debris were internalized by macrophages,84 and that OPN expressing microglia were active phagocytes.83 Thus, OPN appears to modulate not only inflammatory cell accumulation but also the activation state.

Besides regulating the acute phase of the inflammatory reaction, OPN may alter chronic inflammatory responses as well. Chronic inflammation is characterized by the persistence of macrophages at sites of injury and disease. Deficits in macrophage accumulation have been noted in OPN−/− mice when challenged with chronic inflammatory conditions, including atherosclerosis (see below), delayed-type hypersensitivity,80,85 granulomatous disease,51,85 anti-type II collagen antibody-induced arthritis,96 and biomaterial implantation.87,88 These data suggest that OPN may be particularly important in promoting retention of macrophages at sites of chronic inflammation. Further analysis also suggests that OPN alters other macrophage responses. Indeed, we found that OPN blocks formation of macrophage-derived foreign body giant cells in vivo and in vitro, and regulates carbonic anhydrate expression and mineral resorption by macrophages responding to ectopic mineralization.87,88 In addition, OPN control of macrophage activation state was suggested by the finding that OPN-producing tumors were able to inhibit macrophage activation (as measured by increased mannose receptor expression) when compared with tumors deficient in OPN.77 In vitro studies have determined that OPN induces macrophage secretion of interleukin (IL)-12 and dampens the secretion of IL-10, potentially contributing to the development of type-1 immunity, a hallmark of chronic inflammatory diseases.85,89 Many in vitro studies also emphasize the importance of an autocrine function for OPN in macrophages, suggesting that macrophages are both a source and target of OPN. Indeed, macrophages from OPN-null mice have several measurable deficiencies, including susceptibility to programmed cell death, which maybe responsible for the other phenotypes such as impaired macrophage accumulation88 and migration in response to MCP-1.90 OPN−/− macrophages also show lower cytotoxicity against tumor cells.91 In addition, OPN silencing in RAW 264.7 macrophage-like cells impairs their migration, sensitizes them to serum withdrawal-induced cell death, and reduces their secretion of IL-12. These features may represent impairment of RAW 264.7 differentiation from monocytes to macrophages because they correspond to decreased expression of macrophage scavenger receptor A type 1.92

Besides a clear role in macrophage biology, OPN also affects lymphocyte phenotype. OPN was cloned from activated CD4+ cells, where it is highly expressed.79 Subsequently, OPN was found expressed by lymphocytes associated with sarcoidosis, and its expression correlated with granuloma maturity. Osteopontin induced T cell chemotaxis, supported T cell adhesion, and costimulated T cell proliferation.93 OPN also induced CD40 ligand (CD40L) and IFN-gamma expression on human T cells, resulting in CD40L- and IFN-γ–dependent IL-12 production concomitantly with CD-3 stimulation. These findings suggest a functional role for osteopontin in early Th1 responses, namely regulation of T cell–dependent IL-12 production.28,94 More recently, OPN has also been implicated in regulating the survival of CD4 and CD8 T cells in experimental autoimmune encephalomyelitis, a model of multiple sclerosis, suggesting that OPN contributes to the progression of this autoimmune disease.95

Taken together, these data indicate that OPN modulates the inflammatory response at several levels, from immune cell accumulation to activation of Th1 cytokines and to cell survival, thus exacerbating the chronic inflammatory response. However, the forms of OPN, receptors, and signaling pathways involved in these processes are currently unknown.

**OPN Role in Vascular Disease**

**Remodeling and Biomineralization**

OPN is reexpressed in proliferating and migratory vascular cells associated with neointima formation and in inflammatory cells. In human atherosclerotic lesions, OPN is expressed in smooth muscle cells in the lesion, in angiogenic endothelial cells, and in macrophages.1,25 OPN is also re-expressed in SMC associated with human restenotic lesions.96 Consistently animal models have confirmed the role of OPN in vascular remodeling. In a rat model of vascular stenosis, OPN was expressed in intimal smooth muscle cells undergoing proliferation and migration, and smooth muscle cells intimal accumulation was inhibited by anti-OPN antibodies.38 Likewise, OPN was upregulated in regenerating endothelium following balloon denudation in rat carotid arteries.50 More recently, Isoda et al showed that forced overexpression of OPN induced thickening of the medial layer and increased neointima formation following injury. These findings also correlated with increased SMC proliferation, migration and MMP production.97 All these data indicate that during injury, OPN modulates the proliferation, migration, and accumulation of smooth muscle and endothelial cells involved in repair and remodeling processes of the vasculature.

OPN appears also to be an important regulator of vascular calcification and is associated with mineralized deposits in humans.25 In mice, OPN levels are greatly elevated in the spontaneous mineralizing arteries of MGP−/− mice and we have recently shown that OPN is major inducible inhibitor of vascular calcification.26,87 Furthermore, studies by Harney et al and Johnson et al also suggest that OPN is a component of inorganic pyrophosphate signaling and greatly synergize with it in inhibiting mineral deposition and aortic calcification.98,99 These findings suggest that OPN may be an important regulator of arterial mineral deposition under conditions of injury and disease. Vascular calcification is now recognized as a marker of atherosclerotic plaque burden as well as a major contributor to loss of arterial compliance and increased pulse pressure seen with age, diabetes, and renal insufficiency. Thus, OPN, together with other biomineralization inhibitors, may control whether calcification occurs under these pathological conditions.
Mechanistically increasing evidence suggest that phosphorylated OPN directly associates with apatite deposits and blocks crystal growth in addition to inducing RGD-mediated mineral resorption in cardiovascular tissues. Because of its beneficial anticalcific affects and ability to promote mineral resorption, OPN is currently being considered as a potential local therapeutic to limit or remove pathological mineralization in cardiovascular settings such as native valve repair and bioprosthetic valve material development.

Vascular Inflammation and Atherosclerosis

The atherosclerotic lesion is highly inflammatory and, like other chronic inflammatory diseases, is characterized by the persistence of macrophages and other immune cells. Macrophages and lipid-filled macrophages, often referred to as foam cells, are the first cells to comprise the lesion as determined in mouse animal models like the ApoE-null and the fat-fed LDLR-null mouse. More advanced lesions become complex; they are filled with smooth muscle cells and are characterized by the presence of extensive extracellular matrix and a large necrotic core filled with cholesterol clefts. In very advanced lesions, the matrix is often mineralized, and chondrocyte-like cells are associated with the mineral deposits. Nevertheless, macrophages and foam cells are persistent even in very advanced lesions. They are especially localized in the shoulder region of the plaque, suggesting an unrelenting chronic inflammatory response.

We and others have shown that OPN is highly expressed in human as well as experimental animal atherosclerotic lesions, especially associated with macrophages and foam cells. In recent years, in an attempt to define its role in atherosclerosis, 5 studies have examined the effect of OPN overexpression or deficiency in atherosclerotic lesion formation. Chiba et al examined OPN overexpression on atherosclerosis in fat-fed mice. Transgenic overexpression of OPN in lymphoid tissues via an IgG enhancer/SV40 promoter was associated with an increase in aortic lesion size. Consistently, Isoda et al showed that wide tissue overexpression of OPN in fat-fed mice resulted in fatty and mononuclear cell-rich lesion formation. In addition, these findings correlated with decreased expression of IL-10, a well-established atheroprotective cytokine, thus suggesting a possible mechanism behind OPN effects on lesion formation and progression.

In loss of function studies, Matsui et al observed significantly smaller atherosclerotic lesions in the aorta using oil red O staining in female ApoE−/−OPN+/− mice compared with female ApoE−/−OPN+/+ after 36 weeks of normal chow feeding. We have independently confirmed these results in the innominate artery of 40-week-old female ApoE−/−OPN+/− mice (Scatena and Giachelli, 2005 unpublished). Matsui et al also determined that while male mice showed no significant difference in lesion size, plaques were more highly calcified in ApoE−/−OPN−/− compared with Apo−/−OPN+/+ mice, consistent with a beneficial anticalcific effect of OPN. Likewise, Strom et al found decreased atherosclerotic lesion size, increased proliferating and apoptotic cells in 34-week-old ApoE/LDL receptor/OPN triple null mice compared with ApoE/LDL receptor double null mice.

On the other hand, elegant studies by Bruemmer et al examined OPN deficiency in an Angiotensin II (AII)-accelerated model of atherosclerosis and aneurysm formation in ApoE−/− mice. Three-month-old male ApoE−/− of OPN−/− littermates were treated with AII by infusion for 4 weeks. Under these conditions, ApoE−/−OPN−/− mice had less atherosclerosis than ApoE−/−OPN+/+ mice. Leukocyte/lymphocyte-derived OPN appeared to be required for OPN function because bone marrow transplants from ApoE−/−OPN−/− mice into ApoE−/− mice also led to decreased lesion size compared with mice receiving bone marrow from ApoE−/−OPN+/+ mice. Importantly, macrophage apoptosis within lesions was decreased in ApoE−/−OPN−/− compared with ApoE−/−OPN+/+ mice. The authors also confirmed the importance of OPN in leukocyte recruitment, because OPN−/− mice had decreased leukocyte accumulation in response to intraperitoneal thioglycollate treatment. Finally, ApoE−/−OPN−/− mice had less AII-induced aortic aneurysm formation and decreased MMP-2 and MMP-9 activity than ApoE−/−OPN+/+ mice, suggesting a role for OPN in MMP regulation and vessel rupture. Together, these data support the notion that OPN is an important participant in atherosclerotic plaque formation, and implicate bone marrow cells as both source and target of the proatherosclerotic effects of OPN. However, the time course of OPN functions and mechanism by which OPN promotes macrophage accumulation and plaque rupture are still unclear. It is also unknown whether OPN fragments are generated during the onset of cardiovascular disease and whether they modulate leukocytes phenotype, thereby impacting the development and progression of the atherosclerotic plaque. However, many MMPs including MMP2, 3, 7, and 9 are present in the atherosclerotic lesion and often colocalized with lesion macrophages. It is thus likely that OPN becomes fragmented in the lesion environment. Future studies that specifically address the role...
of different OPN fragments in cardiovascular disease and other chronic inflammatory disease are greatly needed.

**Conclusions**

OPN is emerging as a key regulator of immune cell biology. Most of the evidence indicates that persistence of OPN expression by immune type cells exacerbates chronic inflammatory diseases, including vascular disease. Furthermore, several in vitro and few in vivo studies show that both the thrombin and MMP proteolytically cleaved OPN fragments in general possess higher activity than the full-length form. In addition, at least the thrombin cleaved fragment also gains a new cell interacting domain (SVVYGLR). Therefore, we would like to propose that especially when associated with inflammatory processes, the secreted lower activity full-length OPN is rapidly cleaved and, thus, activated.

Much evidence also suggests that physiologically, OPN is a regulator of bone homeostasis and a natural inhibitor of soft tissue mineralization. Because of the multi-domain structure of OPN and the existence of active OPN cleaved products, it is possible that different functions might be modulated by discrete OPN domains as well as different fragments. Thus, understanding differences in the mechanisms and structure/function relationships governing anticalcific versus proinflammatory properties of OPN could help create specific therapeutics aimed at targeting these functions selectively.

**Sources of Funding**

These studies were funded by NIH grants HL62329, HL081785, and HL18645 (to C.G.) and by American Heart Association grant 025250N (to L.L.).

**Disclosures**

None.

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Arterioscler Thromb Vasc Biol. 2007;27:2302-2309; originally published online August 23, 2007;
doi: 10.1161/ATVBAHA.107.144824
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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