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ROLE OF OSTEOPONTIN IN CELLULAR SIGNALING AND TOXICANT INJURY*

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■ **Abstract** Osteopontin (OPN) is a glycosylated phosphoprotein found in all body fluids and in the proteinaceous matrix of mineralized tissues. It can function both as a cell attachment protein and as a cytokine, delivering signals to cells via a number of receptors including several integrins and CD44. Expression of OPN is enhanced by a variety of toxicants, especially those that activate protein kinase C. In its capacity as a signaling molecule, OPN can modify gene expression and promote the migration of monocytes/macrophages up an OPN gradient. It has both inflammatory and anti-inflammatory actions. Some experiments suggest that it may inhibit apoptosis, possibly contributing to the survival of cells in response to toxicant injury. Elevated OPN expression often correlates with malignancy and has been shown to enhance the tumorigenic and/or metastatic phenotype of the cancer cell. Recent studies have revealed that OPN plays critical roles in bone remodeling and cell-mediated immunity.

INTRODUCTION

Much of what we discuss below is based on information acquired in the past five years regarding possible functions of OPN in the mammalian organism. References to the earlier literature may be found in several recent reviews (1–5). As illustrated in Figure 1, the acidic protein is multiply phosphorylated on serine residues and lacks extensive secondary structure. Two major species differing by about 5 kDa in apparent molecular weight are often observed; they appear to differ in posttranslational modifications—glycosylation or phosphorylation. Molecular weights deduced from SDS-PAGE mobility range from 55 to 80 kDa depending

*Abbreviations: LPS, lipopolysaccharide; NOS, nitric oxide synthase; OPN, osteopontin; IL, interleukin.

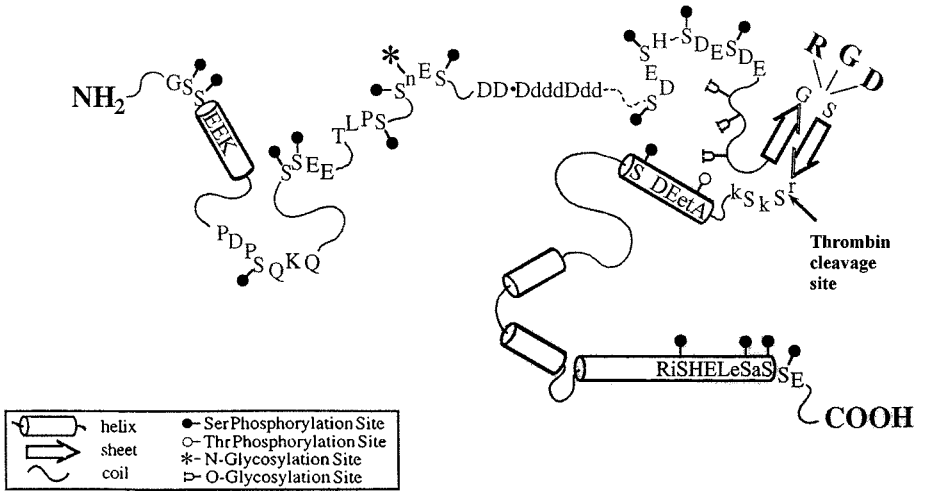

Figure 1 Schematic of the structure of the osteopontin (OPN) protein. Structural motifs are shown on the cartoon. OPN from seven species (rat, mouse, cow, pig, human, rabbit and chicken) has been sequenced: The capital letters indicate amino acids that were identical in the seven species, whereas the lower case letters indicate conserved amino acids. The signal sequence that directs secretion of the protein is not illustrated. The central aspartic acid-rich segment is believed to be important in binding to bone mineral. Immediately C-terminal to the arg-gly-asp (RGD) sequence is the conserved thrombin cleavage site (adapted from Reference 5 with permission of the authors and publisher).

upon the electrophoretic conditions. OPN is encoded by a single gene, much of which is highly conserved in vertebrates. The gene generally gives rise to a single mRNA species, although occasional splice variants are observed. The promoter is responsive to many different transcription factors, with the consequence that transcription is subject to multiple controls (6). In appropriately stimulated cells the mRNA encoding this secreted protein is substantially increased in abundance, facilitating its easy detection in differential screening strategies. Because OPN can interact with several ubiquitously expressed receptors, presumably stimulating downstream signal transduction pathways, the nature of the signal OPN delivers to the cell is complex and dependent upon the cellular context.

OSTEOPONTIN RECEPTORS AND CELL SIGNALING

Integrins

OPN can bind both to extracellular matrix components, notably collagen (7), and to cell surface receptors. Based on protein sequence information, early research into cell surface receptors focused on arg-gly-asp (RGD)-dependent interactions

of OPN, and these efforts led to the identification of $\alpha_v\beta_3$, $\alpha_v\beta_1$, and $\alpha_v\beta_5$ integrins as OPN receptors (8, 9). In addition, non-RGD-dependent interactions have since been described. The leu-pro-val (LPV)–containing domain of OPN is a site of interaction with $\alpha_4\beta_1$ (10), and a cryptic, ser-val-val-tyr-glu-leu-arg (SVVYGLR) domain that interacts with $\alpha_9\beta_1$ following exposure by thrombin cleavage of intact human OPN has been identified (11, 12). The conservation of the thrombin cleavage site in all the OPN sequences that have been determined suggests that cleavage is important in some necessary physiological process. Bennett's group discovered that for some cells of hematopoietic origin cleavage of OPN by thrombin enhanced its ability to promote adhesion, confirming earlier reports that for some cell types thrombin-cleaved OPN supported adhesion, spreading, and migration better than the intact molecule (13). Adhesion of platelets or B lymphocytes, but not epithelial or mesenchymal cells, to intact OPN required agonist (ADP or 12-O-tetradecanoyl phorbol-13-acetate, respectively) stimulation—likely to activate the $\alpha_v\beta_3$ integrin.

CD44v

Certain variants of the hyaluronin receptor CD44 have also been shown to be receptors for OPN. In one study a splice variant of CD44 associated with tumor cells (CD44v7–v10) and expressed by fibroblasts was found to facilitate adhesion and migration to OPN (14). However, this interaction may be restricted to specific isoforms of CD44 because a second study using COS cells and purified fusion proteins failed to reveal OPN interactions with the most common CD44 isoforms (CD44H, CD44E, CD44v3, or CD44v3, v8–v10) (15). Uede and colleagues showed not only that V6 or V7 (encoded in exons 11 and 12) had to be present in CD44v to bind OPN but also that non-RGD-dependent interactions of the $\beta1$ integrin with at least two separate regions of OPN were also required for the CD44-OPN interaction (16). The authors suggested that specific CD44 variants and $\beta1$-containing integrins bind cooperatively to OPN.

Sodek, Zohar, and coworkers (5, 17, 18) have described an intracellular form of OPN that forms a complex with CD44 and ERM (ezrin/radixin/moesin) proteins in a perimembranous location in several cell types, including fibroblasts and macrophages. The colocalization of OPN with CD44 and ERM proteins was particularly prominent at the leading edge of migrating cells. How the normally secreted OPN was sorted into this cellular site was not evident; it did not appear to have been taken up from the medium. The motility and the ability of both OPN-null and CD44-null (knockout) mouse fibroblasts to adhere to hyaluronin and to migrate were impaired, consistent with the importance of this complex in cell motility.

Our understanding of the signaling pathways (see Figure 2) activated by the interaction of OPN with its specific cell surface receptors is in its formative stages. Findlay's group demonstrated that phosphorylation of OPN by casein kinase 2, but not by protein kinase C, enhanced the adhesion of osteoclast-like cells; in contrast, osteoblasts attached equally well to both phosphorylated and unphosphorylated OPN (19). Integrins, albeit different ones, including an alternatively spliced $\beta3$

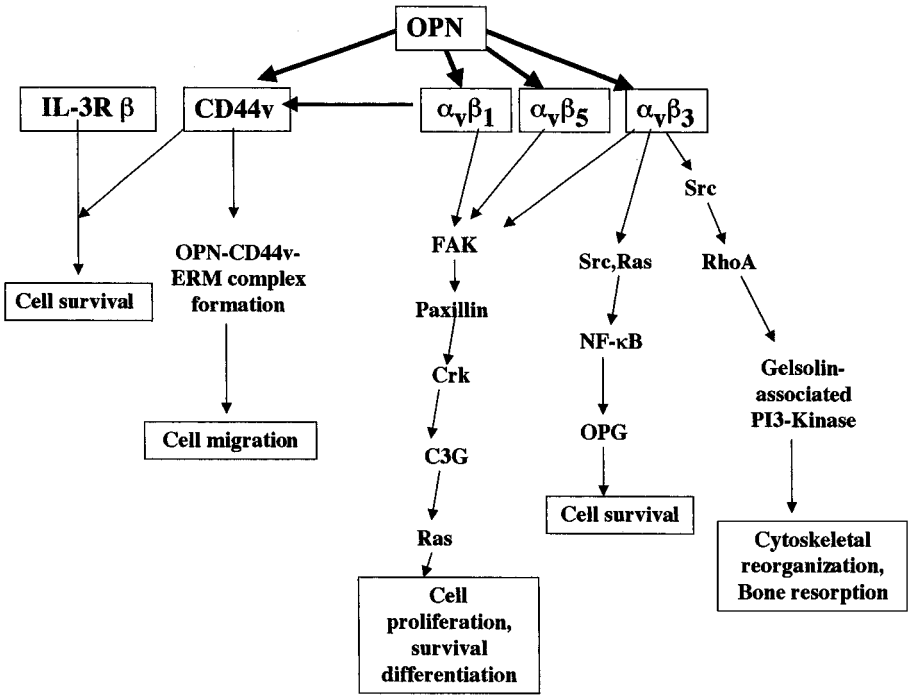

Figure 2 Signal transduction pathways that may be activated by OPN. See text for details.

integrin, appeared to be required by the two cell types. Interpretation of various experimental observations has been complicated by whether or not OPN and/or its cleavage products were delivered in a soluble or immobilized form; soluble peptides are well known to act also as inhibitors of integrin function by blocking integrin clustering. Also, how a cell responds to an OPN signal depends upon the context in which the signal is delivered.

Signaling via OPN as a Soluble Cytokine

OPN has recently been shown to serve an important role as a regulator of immune cell function. In peritoneal macrophages it induces expression of IL-12 and suppresses production of IL-10 following induction by LPS or IL-4 (20). The pathways leading to these effects have not yet been elucidated, but it has been suggested that changes in IL-12 expression result from OPN-CD44 interactions, whereas the effects on IL-10 are mediated through the $\alpha_v\beta_3$ integrin. In T-cells, OPN acts as a costimulatory molecule, amplifying the CD3-mediated proliferative response (21).

Signaling events consequent upon OPN engagement of integrin receptors have been described in many cell types including fibroblasts, osteoclasts, osteoblasts,

melanoma cells, and endothelial cells. Figure 2 presents a synopsis of some of the known or suspected signaling intermediates. OPN signaling through the $\alpha_v\beta_3$ integrin in osteoclast-like cells has been intensively studied because this interaction is a potent stimulator of osteoclast adhesion and resorption of bone (22). An investigation of the early (within minutes) consequences of occupancy of the chicken osteoclast $\alpha_v\beta_3$ receptor by soluble OPN and RGD peptides revealed that an immediate reduction in cytosolic $[Ca^{+2}]_i$ was dependent upon calmodulin and resulted in decreased osteoclast binding to bone particles and decreased bone resorption (23). In contrast, a second study reported a transient increase in $[Ca^{+2}]_i$ following stimulation of human tumor-derived osteoclast-like cells with soluble RGD-containing peptides, including OPN and bone sialoprotein (24). However, in this study the receptor mediating the stimulatory effect was not determined. In osteoclast-like cells derived from a giant cell tumor the soluble $\alpha_v\beta_3$ ligands vitronectin, OPN, echistatin, fibronectin, anti-$\alpha_v\beta_3$ antibody LM609, and RGD peptides produced a transient increase in $[Ca^{+2}]_i$ (25).

In addition to changes in intracellular calcium, Hruska's group has shown that the OPN/$\alpha_v\beta_3$ interaction stimulates gelsolin-associated phosphatidylinositol 3-hydroxyl kinase (PI 3-kinase) in avian osteoclasts, leading to increased levels of gelsolin-bound phosphatidylinositol 3,4-P2 and phosphatidylinositol 3, 4, 5-P3. These changes directly stimulated uncapping of barbed-end actin and actin filament formation; both were dependent on c-Src (26–28). Podosomes are structures in osteoclasts that are analogous to focal adhesions in that they represent sites of association with the extracellular matrix but are more short-lived and stimulate cell motility. They contain an F-actin core and various actin-binding proteins including gelsolin, which is not found in focal adhesions. In the absence of gelsolin, podosomes do not form, leading to reduced osteoclast motility and deficits in bone resorption (29). Rho-A stimulates podosome formation and bone-resorbing activity in osteoclasts, mimicking the action of OPN/$\alpha_v\beta_3$ signaling (30).

Signaling via OPN as an Immobilized Cell Adhesion Protein

The interaction of surface-bound OPN with the $\alpha_v\beta_3$ integrin has been shown to activate the NF-κB pathway in endothelial cells and to inhibit apoptosis in these cells (31). NF-κB is a major regulator of inflammation in endothelial cells and may further be involved in the angiogenesis that typically accompanies wound healing via effects on endothelial cell survival. OPN/$\alpha_v\beta_3$ activation of NF-κB in endothelial cells required both active Src and Ras because dominant negative constructs of these genes inhibited the ability of OPN to induce NF-κB. The protective effect of OPN on endothelial cells was dependent, in part, on the NF-κB-dependent induction of the soluble tumor necrosis factor receptor family member, osteoprotegerin (32). The activation of NF-κB points to an important role for OPN in regulating inflammatory processes.

OPN Stimulation of Cell Survival and/or Proliferation

OPN can enhance cell survival and modulate phosphorylation of intracellular signal transduction intermediates such as focal adhesion kinase, paxillin, and Src (33). Lin and colleagues have shown that in a synergistic interaction with GM-CSF, OPN stimulates the survival and growth of both the proB cell line Ba/F3 and IL-3-dependent mouse bone marrow cells via an interaction with CD44 (34). They concluded that the antiapoptotic activities of IL-3 and GM-CSF observed in some cell types was dependent upon a signal generated by OPN's interaction with CD44. The survival pathway activated in BA/F3 cells by OPN did not involve activation of NF-κB, unlike the situation in endothelial cells. OPN is able to bind to insulin-like growth factor–binding protein-5, and this enhances the ability of this protein to stimulate proliferation of rat smooth muscle cells, presumably by making IGF-1 more available to the cells (35). Because OPN accumulates in atherosclerotic lesions, this action of OPN may promote the growth of the plaque.

INFLAMMATORY FUNCTIONS OF OPN

OPN is routinely found at sites of inflammation caused by disease or tissue injury, for example by a toxicant. OPN protein levels are dramatically upregulated within, and in proximity to, activated T cells and cells of the monocyte/macrophage lineage in a number of chronic inflammatory diseases. Indeed, OPN was cloned by Cantor and colleagues as Eta-1, early T-cell activation gene 1 (36). These finding have suggested a role for OPN in modulating the inflammatory process, for example by stimulating macrophage infiltration. OPN also stimulates polyclonal B cell activation and may contribute to the pathogenesis of autoimmune disease (37–39).

Monocyte/Macrophage Migration

A major feature of the inflammatory response is the accumulation of white blood cells at the site of infection or injury. Once there, white blood cells disinfect, debride, and stimulate healing through promotion of angiogenesis and tissue regeneration. Several studies suggest that OPN facilitates the earliest aspects of this process as a regulator of monocyte/macrophage infiltration. For example, ischemic episodes in the brain result in increased OPN expression, first in the microglia and then in infiltrating astroglia and macrophages (40, 41). OPN is expressed by stellate cells and by activated macrophages, including Kupffer cells, in necrotic areas of rat livers subjected to carbon tetrachloride intoxication (42). It was proposed that OPN contributes to the further infiltration of Kupffer cells and hepatic macrophages into the necrotic areas.

Using a rat subcutaneous injection model, Giachelli and colleagues demonstrated that neutralizing antibodies to OPN could block macrophage infiltration in response to the bacterial chemotactic peptide, formylmethionine-leucinephenylalanine (43). Renal interstitial macrophage accumulations were substantially decreased by antibodies to OPN in mice following ureteral obstruction (44) and in rats

suffering from experimental crescentic glomerulonephritis (45). Studies in mice with a targeted deletion in OPN support these findings. Acute macrophage influx in obstructed kidneys was three- to fivefold lower in OPN-null mice compared with wild-type mice (44), and the number of macrophages responding to chemical carcinogen-induced squamous cell carcinoma was decreased in OPN-null mice compared with wild-type mice (46). However, the role of OPN in macrophage infiltration may depend on context: Peritoneal instillation of *Mycobacterium bovis* bacillus into OPN-null mice elicited greater cellular exudates than in wild-type mice (47), whereas similar levels of macrophages were observed in wild-type and OPN-null mice during incisional wound healing (48).

Monocyte/Macrophage Differentiation

Numerous correlative studies and in vitro findings support the contention that OPN may modulate the inflammatory process. Thus, it has been shown to control macrophage adhesion (43, 47), migration (43, 49, 50), generation of nitric oxide (51, 52), cytokine release (20, 47), differentiation (46), and phagocytosis (53). OPN is a monocytic differentiation marker, and its expression increases as monocytes differentiate (54). In one study OPN-producing tumors were able to inhibit macrophage activation (as measured by increased mannose receptor expression) when compared with tumors deficient in OPN (46). These data suggest that OPN may control the monocyte/macrophage activation state.

The ability of OPN to inhibit cytokine (LPS + γ-interferon)–stimulated macrophage NO production (by preventing induction of NOS2 transcription) is also consistent with a regulatory role of OPN on macrophage activation state, and indicates a potential anti-inflammatory function (51, 52). Scott et al (55) showed that OPN suppresses the induction of NOS2 by LPS in rat vascular tissue. Evidence suggests that in the heart OPN might mediate the suppression of NOS2 expression by glucocorticoids, which induce OPN in cardiac myocytes and microvascular endothelial cells (56). Because high levels of NO can impair myocyte function, OPN may attenuate damage to the heart in some circumstances. The ability of OPN to suppress the induction of NOS2 by LPS and γ-interferon in RAW264.7 cells appears to be a function of their state of differentiation and the substrate on which they are growing (57). As illustrated in Figure 3, cells grown on a collagen matrix, and to a lesser extent chondroitin sulfate, are refractory to the action of OPN, compared with cells adherent to plastic or laminin. The ability of OPN to modulate cell behavior at picomolar concentrations implicates a receptor with very high affinity for the protein, whereas the sometimes biphasic response of the cell as a function of OPN concentration (not so evident in Figure 3) hints at the complexity of the signals generated (51, 52).

Macrophage Phagocytosis

A role for OPN in phagocytic processes has also been suggested by its high level of expression in actively phagocytosing macrophages (58). Wound healing studies in

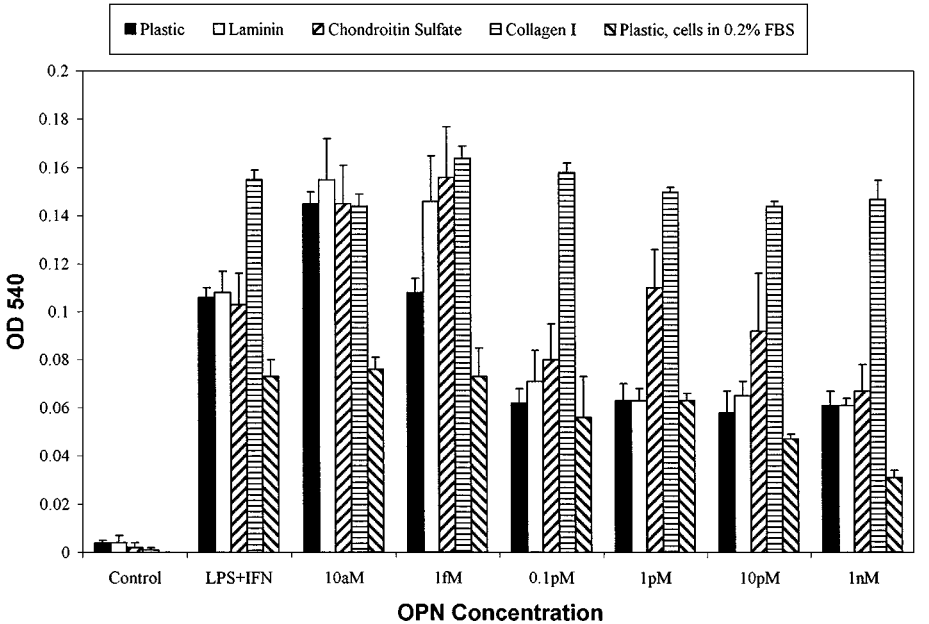

Figure 3 The presence of extracellular matrix molecules modulates the ability of OPN to inhibit the induction of NOS2, and consequent NO synthesis, by LPS and γ-interferon. The wells of a 24-well plate were precoated with the indicated material as described by Tian et al (57). Each well contained 7.5×10^4 RAW264.7 macrophage-like cells in 0.5 ml of medium containing 1% fetal bovine serum. The effectiveness with which OPN, added at the indicated concentration 1 h before LPS and γ-interferon, could inhibit NO production by the induced cells was assessed at 42 h. NO was detected using the Griess reagent and measured at OD_{540}. All measurements were in triplicate.

OPN-null mice are consistent with a role for OPN in phagocytic processes because incisional wounds made in OPN-null mice showed more residual debris and less matrix organization, as well as an alteration in collagen fibrillogenesis, compared with wounds made in wild-type mice (48).

Cell-Mediated Immunity and Granuloma Formation

In addition to acute inflammation, recent studies suggest a role for OPN in delayed-type and chronic inflammation initiated by T cell–mediated immunity. In a rat model of crescentic antiglomerular basement membrane glomerulonephritis, intraperitoneal application of an anti-OPN antibody to rats led to a significant (54%) reduction in skin swelling and leukocyte infiltration in the delayed-type hypersensitivity response compared with rats treated with control IgG following challenge with the immunizing rabbit IgG antigen (45). A similar effect was observed in the OPN-null mouse using a *Herpes simplex* virus sensitization and foot-pad rechallenge assay (20).

Although granulomatous diseases are considered manifestations of cell-mediated immunity, the role of OPN in this type of inflammation is uncertain. In the Ashkar study (20) granulomatous inflammation was induced by subcutaneous injection of polyvinylpyrrolidone (PVP) (also collagen and latex). Granulomatous inflammation in response to this treatment was decreased greatly in OPN-null mice compared with wild-type, and correlated with decreased expression of interleukin 12 and γ-interferon, two major regulators of cell-mediated immunity. In a study by Nau and colleagues, OPN-null mice exhibited attenuated host resistance and increased granuloma formation in response to infection with the intracellular pathogen *Mycobacterium bovis* bacillus (59). In this case, lack of OPN greatly diminished the ability of the mice to clear the mycobacterium, an effect also observed for systemic *Listeria monocytogenes* infection (20). In a cardiomyopathic hamster model, immunization of the animal with bovine serum albumin induced OPN expression and granuloma formation in the lungs, lymph nodes, and heart (60). These investigators showed that OPN contributed functionally to the formation of granulomas in the lungs of normal hamsters by introducing into the lung via the trachea an adenovirus vector expressing mouse OPN and observing pulmonary granuloma formation; a control vector did not induce granulomas.

OPN AND THE VASCULATURE

A function for OPN in vascular injury was suggested following the discovery that mRNA levels are upregulated in vascular smooth muscle cells following balloon angioplasty (61). Although its expression in normal arteries is very low and its role in the normal vascular system remains unclear, OPN has been implicated in a number of vascular pathologies, including atherosclerosis and restenosis (62). In these lesions OPN accumulates in calcified deposits and is synthesized by macrophages and to a lesser extent by endothelial and smooth muscle cells. Based on its ability to promote smooth muscle cell migration and regulate inflammation, OPN has been proposed to contribute to the etiology of the these vascular lesions as well as to the repair of vascular injury (3). Neutralizing antibodies directed against OPN inhibited neointimal formation following de-endothelialization of rat carotid arteries (63). Parrish & Ramos observed that vascular injury caused by allylamine was accompanied by enhanced thrombin cleavage of OPN and increased aortic smooth muscle proliferation (64). Because an antibody to the α_v integrin reduced the proliferative response, the authors suggested that thrombin-cleaved OPN was responsible for the increased smooth muscle cell proliferation.

OPN expression in cultured rat aortic smooth muscle cells is increased by glucose in a dose-dependent and protein kinase C–dependent manner (65). On the basis of this observation, the authors suggested that OPN could play a role in the development of diabetic vascular complications. The presence of OPN in calcified native and bioprosthetic heart valves suggests that OPN is a regulator of vascular calcification in general. In this vein, OPN is a potent inhibitor of calcification of smooth muscle cell cultures (66), and OPN-null mice exhibit enhanced

calcification of subcutaneously implanted bioprosthetic heart valve material when compared with wild-type mice (SA Steitz, MD McKee, L Liaw, CM Giachelli, manuscript submitted). In the latter study, OPN appeared to regulate the ability of the host to resorb ectopic calcification by promoting the development of carbonic anhydrase II–positive, macrophage-derived cells and acidification of the implants. Thus, much like its role in bone, OPN probably serves both to regulate bioapatite crystal growth through physical interactions, as well as to regulate host cell resorptive mechanisms via receptor-mediated interactions.

ROLE OF OPN IN THE KIDNEY IN RESPONSE TO ISCHEMIC INSULT OR TOXICANT INJURY

Toxicants often have their most severe impact on lung, liver, and kidney function, organs involved in detoxification of xenobiotics. Thus, they are also the organs most susceptible to toxicant injury, one manifestation of which is macrophage infiltration and cytokine release (67). Although OPN is among the cytokines expressed at the site of inflammation, the importance of its presence remains elusive. As discussed above, two known functions are to attract macrophages and to inhibit NOS2 induction in response to endotoxin and LPS.

OPN expression in the kidney tubular epithelium is known to be enhanced in response to various chemical or physical insults, and this increased expression is often associated with the infiltration of mononuclear cells (68). Also, renal ischemia/reperfusion results in increased OPN expression, although with different kinetics in different tubule segments (69). In an interesting in vivo study antisense oligodeoxynucleotides targeted to the OPN mRNA were injected into Goodpasture syndrome rats (70). This treatment preserved renal plasma flow while attenuating monocyte infiltration and OPN expression in the tubular epithelium. Agents that have been reported to enhance OPN expression in the stressed kidney include insulin-like growth factor I (71), angiotensin II (61, 72), endothelin-1 (73), and IL-1 (74).

There appears to be a delicate balance between the pro-inflammatory and anti-inflammatory actions of OPN. On the one hand, the ability of OPN to attract macrophages may exacerbate tissue injury as the consequence of the release of inflammatory mediators by the macrophages. As discussed above, anti-OPN antibodies ameliorate the development and progression of antiglomerular basement membrane nephritis in rats, consistent with the idea that OPN accelerates the glomerular and interstitial macrophage accumulation and accompanying inflammation (45). On the other hand, OPN may attenuate tissue injury by, for example, inhibiting the production of NO by activated macrophages (52).

Studies in an OPN-Null Mouse Model

To shed light on the role OPN has in the kidney's response to injury, several groups have investigated injury/disease progression in an OPN-null mouse model.

Goligorsky's group (75) reported that ischemic injury was more pronounced in the absence of OPN than in its presence. They further showed that in cell culture OPN reduced the extent of cell death resulting from exposure of proximal tubule cells to hypoxia, confirming an earlier report (33). A second study using a ureter ligation model, which resulted in hydronephrosis and elevated OPN expression, found that the kidneys of OPN-null mice exhibited more interstitial and tubular apoptotic cell death than did the kidneys of wild-type controls; however, the progression of the disease was similar in the presence or absence of OPN (44). The data suggested not only that OPN mediated macrophage influx and interstitial fibrosis but also that OPN aided in the survival of the stressed cells.

In contrast to these studies that implicated OPN in the response of the kidney to particular insults, Wüthrich and colleagues detected no significant difference between the response of wild-type and OPN-null mice to nephritis induced by antiglomerular basement membrane antibodies (76). Glomerular crescent formation, tubulointerstitial infiltration, and the formation of thrombi in the glomerular capillaries were similar in the two genotypes. They also found no deficiency either in delayed-type hypersensitivity or in the humoral response to rabbit IgG. Reconciling these seeming contradictions will require additional information. On the one hand, there is the possibility of compensatory mechanisms in the knockout mouse; on the other hand, species differences and unknown antibody cross-reactions could complicate the picture in wild-type animals.

Active components of the complement cascade appear to contribute to tubulointerstitial disease, at least in some model systems (77). Fisher and his colleagues have shown that OPN (and bone sialoprotein) can bridge one of the inhibitors of complement-mediated cell lysis, Factor H, either to CD44 or to $\alpha_v\beta_3$ (78). The receptor-OPN-Factor H complex then disrupts the alternate pathway of the complement cascade and protects the cells from lysis. However, OPN must bind first to the cell receptor and then to Factor H for the pathway to be disrupted. When Factor H binds to OPN first the receptor binding sites are completely masked and Factor H cannot be bridged to the cell surface, leading to cell lysis. This result and the observation that a variety of antisera to OPN cannot significantly immunoprecipitate OPN once Factor H is bound, suggests that OPN's actions may be limited to an autocrine/paracine mode. The abundance of Factor H (0.5 mg/ml) in plasma ensures that OPN will be rapidly bound and likely inactivated.

ROLE OF OPN IN STRESS-INDUCED BONE REMODELING

OPN protein comprises about 2% of the noncollagenous protein bone (1, 5, 79). In situ hybridization studies revealed that OPN mRNA was present at high levels in both osteoclasts and osteoblasts, at variable levels in osteocytes and some chondrocytes, and at low levels in quiescent lining cells. McKee & Nanci have established that OPN protein is largely found in the lamina limitans and cement line interfaces

in mineralized tissues, and that it coats newly synthesized bone, or bone fragments, possibly to make the bone more suitable for bone cell attachment or macrophage phagocytosis (80). It also lines the osteocyte cavity and the canaliculi and may function there to suppress mineralization (81). In bone, OPN has properties of an extracellular matrix protein, promoting osteoblast and osteoclast attachment via the $\alpha_v\beta_3$ integrin; attachment of osteoclasts, but not osteoblasts, has been reported to require that the protein be appropriately phosphorylated (19). It also serves as an attachment factor for endothelial cells, inducing in them expression of the soluble TNF receptor osteoprotegerin, which can enhance endothelial cell survival (32).

Bone Modeling and Remodeling

The formation of mineralized tissues (bone, teeth) during development is known as modeling; the restructuring of bone in response to stress or strain is known as remodeling. Both modeling and remodeling are under the control of a variety of hormones and cytokines whose mechanisms of action are poorly understood. Recent studies have suggested that OPN is an important mediator of bone remodeling, which occurs in response to changes in physical or hormonal stimuli; examples include loss of bone mineral when bones are not stressed (disuse osteoporosis) or after a reduction in estrogen levels (postmenopausal osteoporosis). Ovariectomy has been reported to increase osteoclastogenesis and osteoblastogenesis in cultured bone marrow and to elevate the number of osteocytes expressing OPN, particularly in those bones that tend to be resorbed (82). Yamate and coworkers reported (*a*) that OPN expression by osteoclast and osteoblast progenitors in murine bone marrow increased after ovariectomy and (*b*) that OPN stimulated the interaction between osteoclast progenitors and stromal/osteoblastic support cells that generated tartrate-resistant acid phosphatase–positive cells (83).

That mineralized tissues appear normal in mice unable to make OPN suggests that OPN is not required for bone formation during development (48, 84). However, Noda and colleagues (84a) discovered that the presence of OPN in bone is essential for bone remodeling induced by estrogen deprivation. After ovariectomy, the trabecular bone volume of the proximal epiphyses of the tibia, measured by microcomputed tomography (μCT), decreased by 12% in the OPN-null compared with 58% in wild-type mice. Histomorphometrical analyses of the trabecular bones also showed that the bones of the OPN-null mice were resistant to mineral loss subsequent to estrogen deprivation, thus confirming the μCT analysis. There was an approximate threefold increase in the relative number of osteoclasts attached to the bone surface in wild-type compared with OPN-null mice.

Although frank osteopetrosis has not been observed in OPN-null mice, in the control nonovariectomized mice the trabecular bone volume and the number of osteoclasts attached to bone in the OPN-null was over twice that in the wild-type. Whether this is a consequence of modeling or remodeling is not certain, but this difference in the control animals raises the possibility that OPN might be important in normal bone development also. Recent studies of bone discs (wild-type,

OPN-null) implanted subcutaneously or intramuscularly in mice (wild-type, OPN-null) have revealed an involvement of OPN in bone angiogenesis and osteoclast recruitment (85). Vascularization and resorption of the bone discs were significantly impaired in the absence of OPN.

Does OPN Mediate the Detection of Bone Strain?

Physical stimuli increase expression of OPN in adherent cells. For example, the Burger/Klein-Nulend laboratory found that mechanical stimulation by intermittent hydrostatic compression stimulated OPN synthesis by certain primary osteoblastic cultures and by MC3T3-E1 cells (86). In primary bone cells cultured without mechanical stimulation OPN expression declined dramatically. These researchers proposed that OPN serves as a stress-sensitive attachment molecule in bone cells, conveying to the cell via its cell surface receptors the extent of fluid flow. Terai et al (87) reported that mechanical stress imposed during the early stages of experimental tooth movement induced OPN expression in almost all osteocytes and some of the osteoblasts and bone lining cells, particularly on the pressure side; the increase in OPN synthesis was associated with an increase in osteoclast index, consistent with the thought that OPN was serving as a chemotactic factor for mononuclear tartrate-resistant acid phosphatase–positive osteoclast precursor cells. Increased OPN expression could also strengthen the binding of cells to both bone mineral and bone matrix.

Evidence suggests that osteoblasts form bone as long as they perceive excessive strain, whereas osteoclasts resorb bone until physiological strain is reached (88, 89). Signals from calcium homeostatic hormones and inflammatory cytokines also regulate bone metabolism. As a molecule that mediates the attachment of bone cells to the bone matrix, OPN is poised to transduce any stress that would move the cell relative to the matrix. It can bind strongly to hydroxyapatite (likely via the polyaspartate sequence that is a conserved domain in the protein), to osteocalcin (90), and to type I collagen, especially after being cross-linked by tissue transglutaminase (7). It can bind to the osteocyte via $\alpha_v\beta_1$ or $\alpha_v\beta_5$ and CD44v, all known to be expressed by the osteocyte. Evidence that integrins are involved in transducing cyclical mechanical stimulation of bone cells into changes in the cellular membrane potential was described by, among others, Salter et al (91). Mechanical tension on the receptor(s) would be transmitted to the cell's cytoskeleton, which could activate stretch-activated membrane channels on the cell surface and stimulate signaling pathways (92).

These data suggest that OPN may be a transducer of bone strain as well as a mediator of osteoclast function. It could do this physically by coupling the osteocyte to bone matrix or chemically by acting in a paracrine fashion to convey a signal from a perturbed osteocyte to the bone lining cells. OPN may also, or instead, exert its action as a cytokine by controlling the level or activity of a substance (e.g. IL-1, IL-6, TNF, NO, prostaglandin, PTH) regulating bone remodeling. Finally, OPN released by the osteocyte and delivered to the bone surface might attract osteoclast

precursor cells and promote osteoclast motility; in the marrow it could stimulate osteoclast differentiation, possibly by synergizing with a RANK/RANKL-generated signal.

Parallels Between Inflammation and Bone Remodeling

Rodan has reviewed the similarities between osteoclast activation in response to hard tissue damage and macrophage activation (i.e. inflammation) in response to soft tissue damage; for example, the same cytokines (prostaglandin E, IL-1, IL-6, TNFα) activate both (93). OPN also promotes migration of both cell types. Activated macrophages phagocytose foreign material. Activated osteoclasts attach to bone and form a lysosome-like extracellular resorption space that is enclosed in part by the cell membrane and in part by bone surface; the juxtaposed cell membrane forms what is called a "ruffled border" (94). Hydrogen ions and proteases are secreted into this extracellular lacuna to dissolve the calcium phosphate mineral and to hydrolyze bone matrix proteins respectively (95, 96).

The lifetime of the osteoclast, which dies by apoptosis, is a key determinant of bone turnover; TGFα stimulates apoptosis, whereas IL-1, TNF, mCSF, and IL-6 inhibit it (97). Osteoclasts may undergo a number of cycles of bone resorption and migration before succumbing. OPN-null osteoclasts appear to be hypomotile and to form resorption pits on dentine that are shallower than normal, apparently because of a defect in an actin-signaling pathway that is required for podosome assembly (29; M Chellaiah, N Kizer, U Alvarez, SR Rittling, DT Denhardt, and KA Hruska, in preparation).

OSTEOPONTIN FUNCTION IN TUMORIGENESIS

The connection between OPN and tumorigenesis originated in 1979 with the first description of OPN by Senger as a secreted transformation-related phosphoprotein (98, 99). In 1987 it was cloned as a tumor promoter–inducible gene under the control of protein kinase C (100). OPN is expressed at higher levels in a variety of transformed cells in culture as compared to their nontransformed counterparts (101). Because many toxic agents are also thought to be cancer-causing in mice and humans, the role of OPN in tumorigenesis is germane to its function in the response to toxicants.

Expression in Human Tumors

OPN is frequently overexpressed in human tumors. Expression has been examined in a variety of human and murine tumors, and there is controversy over the cell type within the tumor that produces OPN. Early work indicated that whereas OPN is expressed at high levels in a variety of human tumors as assessed at the level of mRNA, this expression most often was localized to macrophages infiltrating the tumor, not to the tumor cells themselves (102). Subsequent experiments have

shown that tumors can vary dramatically in the cellular localization of OPN expression. For example, by immunohistochemical analysis, OPN expression is primarily tumor-cell-associated in esophageal squamous cell carcinomas, but macrophage-associated in esophageal adenocarcinomas (103). Interestingly, in stage 1 lung tumors high-level OPN expression was associated with a worse prognosis in adenocarcinomas but not in squamous cell carcinomas (104). Finally, in gastric tumors, OPN expression correlated positively with tumor progression (105).

Association of OPN Expression with Breast Cancer

OPN expression in breast cancer and breast cancer cell lines has been particularly well studied. OPN is expressed in many breast cancers (106–111). However, the localization of OPN expression is again controversial, with some authors reporting macrophage-specific expression and others showing tumor cell specific expression or a combination of both. In several studies, increased OPN expression correlated with indicators of more aggressive disease, including microcalcifications (108), lymph-node positivity (107), or reduced disease-free survival (111). In the mouse OPN expression is regulated during mammary gland development and is elevated in mammary tumors arising in transgenic mice expressing c-*myc* and/or *v-Ha-ras* specifically in the mammary gland (112): In these tumors OPN is expressed predominantly by the tumor cells themselves, and OPN protein levels in the serum of the tumor-bearing mice is quite high, as shown in Figure 4. Experiments in humans have likewise demonstrated a correlation between OPN serum levels and tumor burden (113).

A number of different human breast cancer cell lines have been characterized that have differing metastatic abilities. OPN is not expressed in most of these cell lines (109); however, two highly metastatic cell lines do express OPN (109, 114). In a series of mouse mammary tumor cell lines, however, OPN expression did not correlate with metastatic ability (115); in a different series a correlation was observed (116). Overall, then, the expression of OPN in human breast cancers is variable, and its role in these tumors is a subject of ongoing investigation.

Functional Correlates of OPN in Tumorigenesis

Although the association of OPN expression with tumorigenesis implies that the protein is important in this process, there is a need for more direct information on the role the protein plays in the neoplastic process. There have been several approaches taken to address this question. Functional studies in which OPN expression was downregulated in transformed cells by use of antisense strategies implicated OPN as important in anchorage-independent growth, tumor growth in vivo, and experimental metastasis (4). In addition, compelling evidence from transfection experiments has demonstrated that high-level OPN expression can confer a metastatic phenotype on benign tumor cells (117, 118). These latter experiments are especially intriguing. In this work, fragmented DNA from metastatic tumor cells was transfected into benign tumor cells, and the development of metastatic

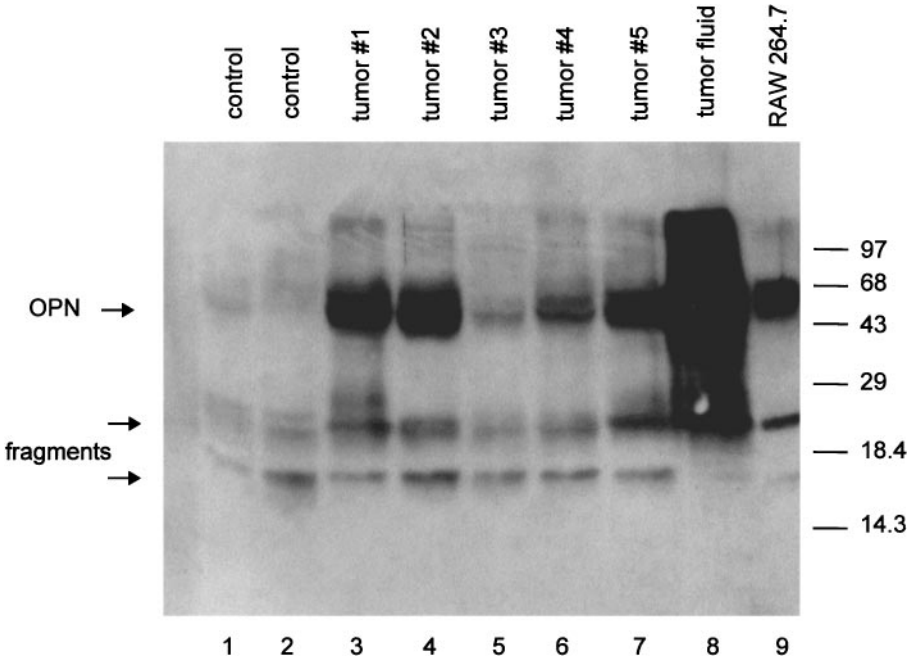

Figure 4 Osteopontin levels in sera from a series of tumor-bearing mice. Mammary tumors were developed in MMTV-*c-myc*/MMTV-*v-Ha-ras* transgenic mice (112). Blood was collected from mice at the time of sacrifice, and the OPN in 100 μl of serum was concentrated by barium citrate precipitation and separated by SDS-PAGE. OPN was identified by reaction with antibody OP-199 as described (112). Control lanes show sera from mice without tumors, lanes 3–7 show sera from different tumor-bearing mice, and lane 8 shows fluid withdrawn from a cystic tumor. Lane 9 shows conditioned medium from RAW264.7 cells, which produce high levels of OPN, as a marker. Positions of MW markers (in kDa) are shown on the right.

variants was assayed. Specific DNA sequences were identified that made the cells metastatic, and in every case the metastatic variants obtained overexpressed OPN. In some way, perhaps by binding a repressor of OPN transcription, the transfected DNA fragments, themselves noncoding, stimulated OPN expression and conversion of the benign cells to a metastatic phenotype. This effect could be reproduced by the direct transfection of OPN expression constructs, suggesting that OPN could be an effector of activated oncogenes, functioning to facilitate tumor growth and metastasis.

Recently, the availability of OPN-null mice (48, 84) has allowed experiments to be performed to test the effect of a total lack of OPN on tumorigenesis. These experiments have yielded unexpected new results, which may explain some of the controversy in the field. To date, three experimental systems have been evaluated in the OPN-null mice, yielding three quite different results. First, cell lines

derived from OPN-null mice were transformed in vitro, and tumorigenesis resulting from injection of these cells into wild-type mice was evaluated. Consistent with the results from antisense experiments, this work demonstrated clearly that OPN expression by tumor cells is required for maximal tumor growth: In every case, OPN-deficient cell lines formed tumors significantly more slowly than did comparable wild-type cells. In addition, these experiments demonstrated that OPN is dispensable for anchorage-independent growth (119). Second, spontaneous mammary tumorigenesis in MMTV-*c-myc*/MMTV-*v-Ha-ras* transgenic mice either wild-type or deficient for OPN expression was evaluated. In this experimental model, primary tumor incidence and growth rate was unaffected by a lack of OPN (120). Macrophage infiltration into the tumor was not related to OPN status, and the apoptotic rate was similar in tumors of both genotypes. Third, epidermal papilloma and carcinoma formation was evaluated in OPN-null and wild-type mice in response to topical carcinogen administration. In this model, although tumor incidence was unaffected by OPN status, tumor growth rate was substantially enhanced in the absence of OPN. Metastases resulting from these primary tumors were similarly more numerous in the OPN-null mice (46). This result was quite unexpected and may indicate that the role of OPN in tumorigenesis is complex and may vary with the specific tumor type being examined.

Potential Functions of OPN in the Processes of Tumorigenesis

The mechanisms of action of OPN in carcinogenesis and an explanation for the disparate results obtained with the OPN-null mice represent the current major questions regarding OPN and tumorigenesis.

First, it is clear from the data on wild-type and OPN-null cell lines, as well as from antisense experiments, that OPN made by tumor cells can stimulate tumor growth. In order to do this, OPN may be acting on tumor cells themselves or on associated host cells. Several lines of evidence indicate that OPN can enhance growth of cells in vitro in anchorage-independent conditions (119, 121, 122). This effect of OPN may be important in tumor growth in vivo as well. The mechanism of OPN's action to stimulate such growth, and the signaling pathways involved, have yet to be elucidated.

Because OPN stimulates the migration of a variety of cell types (11, 123–125), it may be that this function of OPN is important in its ability to intensify tumor growth. Recently, OPN has been shown to energize both migration and invasion of human breast cancer cells (111). OPN was also shown to stimulate expression of the secreted protease urokinase-type plasminogen activator by these cells, which can explain this increased invasiveness. Although invasion is commonly more associated with metastatic cell growth, this mechanism may play a role in primary tumor growth as well. Alternatively, by attracting cytotoxic macrophages to some tumors, OPN could impair tumor growth.

In addition, it is possible that OPN produced by tumors acts in a paracrine manner, affecting host cells that can contribute to tumor growth. A likely candidate

for such host cells is endothelial cells, in providing the blood supply to the tumor. OPN is known to mediate adhesion and to stimulate migration of endothelial cells (124, 126). As a ligand for the $\alpha_v\beta_3$ integrin, which is clearly critically important for endothelial cell survival and growth in vivo (127, 128), OPN as an adhesive molecule has been shown to prevent apoptosis of these cells (31). Thus, one hypothesis for OPN function in stimulating tumor growth is that it acts to promote survival of endothelial cells. However, OPN made by tumors is largely soluble—one indication of this is its accumulation to high levels in the serum of tumor-bearing mice (Figure 4). Additionally, several lines of evidence show that OPN is not associated with the extracellular matrix in tumors (SR Rittling, data not shown). Thus, OPN is unlikely to act as an adhesive ligand for endothelial cells in tumors. It remains a possibility, however, that OPN acts as a chemotactic factor for these cells.

However, these roles of OPN cannot explain the apparent suppression of tumor growth by this protein reported by Crawford and co-workers (46). A possible hypothesis to explain this observation arises from recent work exploring the role of OPN in immune cell function. Ashkar and colleagues (20) have reported that OPN is a critical cytokine regulating the type-1 immune response: It does this by upregulating the type 1 cytokine IL-12 and suppressing expression of IL-10, which in turn suppresses the type-1 response. Thus, in the absence of OPN, the balance between these cytokines is altered and the type-1 immune response is suppressed. There is a growing body of evidence that the type-1 immune response is important in the host defense against tumor antigens. For instance, IL-12 is known to mediate antitumor effects (129, 130). In addition IL-10 production by tumor cells can suppress the ability of the host to mount an immune response to a tumor isograft (131) and can suppress the response of specific T-cell subpopulations to tumor antigens (132). Taken together, these observations indicate that the effect of OPN on the host response is likely to be a positive one: OPN expression by host cells may result in an increased ability of the host to develop an immune response to a given tumor. Thus, in the absence of OPN, as in the experiments of Crawford and co-workers (46), tumor growth may in some cases be enhanced.

How can these two opposing effects of OPN be reconciled? One possibility is that there may be structural differences between OPN made by tumors and that made by host cells, such that tumor OPN may not be able to affect the host response. Alternatively, the net result of an effect of OPN may be a balance between the stimulatory and inhibitory actions on tumor growth, and which predominates may depend on other aspects of the specific tumor system in question. Thus, in experiments comparing wild-type- and OPN-deficient tumor isografts, the stimulatory effects predominate (119). In the carcinogen-induced epidermal tumor system, the inhibitory effects are paramount (46). In the mammary tumorigenesis system, these two effects are balanced, resulting in no net effect of OPN on tumor growth (120). Verifying this hypothesis and understanding the mechanisms behind it represent exciting challenges ahead for the field of OPN and tumorigenesis.

SUMMARY

OPN exhibits complex pharmacological effects, which in some experimental systems have led to differing conclusions. Some of these conflicting results may derive from the actions of OPN in the immune system, where it plays important roles in immune cell physiology, demonstrating both pro- and anti-inflammatory actions in response to tissue injury. OPN stimulates migration of macrophages and may modulate their differentiation. It appears to regulate production of certain cytokines, enhancing the production of T helper 1 cytokines. The affinity of OPN for calcium phosphate causes it to interfere with the growth of calcium-containing crystals, for example the tissue calcification following arterial injury. Atherosclerosis thus provides an example of a system in which OPN may have opposing effects, as an inhibitor of calcification and as a regulator of macrophage function. A similar situation applies in the kidney, in which some data suggest that OPN enhances tissue injury through stimulation of an inflammatory response, whereas other data reveal a protective anti-inflammatory effect. How these conflicting effects of OPN may be understood is possibly revealed through tumorigenesis studies, in which OPN produced by the tumor may stimulate the growth of the tumor, while host-derived OPN inhibits such growth; which effect predominates may be related to the precise balance between the amount, timing, and form of OPN expression during tumorigenesis. Perhaps this delicate balance also extends to and can explain the disparate results in the renal system. In bone, the situation is somewhat clearer—OPN in multiple models stimulates bone resorption, most likely by enhancing osteoclast fuction. Further work aimed at a more thorough understanding of these opposing effects of OPN will provide an exciting new chapter in our understanding of the role of OPN in response to toxicant injury.

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