Osteopontin

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SUMMARY

Osteopontin (OPN) is a protein that is widely expressed and will likely have multiple biological functions based on structural modifications of the protein and the local environments in which it is expressed. Evidence is accumulating that OPN plays a role in cells derived from monocytes, including macrophages and osteoclasts. Presently there is strong evidence that OPN can activate the migration and adhesion of many cells *in vitro* and, therefore, may contribute to the accumulation of cells at sites of inflammation *in vivo*. The possibility exists that OPN does more than simply mediate the accumulation of cells; studies are needed that evaluate activating or inhibitory signals induced by OPN. In addition, more studies are required to understand OPN's function in normal and disease states.

BACKGROUND

Discovery

Osteopontin (OPN) is a protein that is widely expressed *in vivo* (Denhardt and Guo, 1993) and it has been associated with immune/inflammatory responses (Uede *et al*., 1997). The protein may have multiple functions because it is expressed in disparate tissues such as kidney (Kohri *et al*., 1993), bone (Oldberg *et al*., 1986), breast milk (Senger *et al*., 1989), and epithelial surfaces (Brown *et al*., 1992). Within the inflammatory/immune system, OPN protein or its transcript has been identified in T cells (Patarca *et al*., 1989), macrophages (Miyazaki *et al*., 1990), and NK cells (Pollack *et al*., 1994). Because of findings in OPN null mice and in pathologic specimens, experiments have only recently begun that study OPN's role as a cytokine.

Alternative names

OPN has had several names, reflecting the fact that it has been identified in multiple systems. The protein was first described in the culture supernatants of transformed cell lines and was termed secreted phosphoprotein 1 (spp1) (Senger *et al*., 1979). The name bone sialoprotein II was applied after the protein was isolated from rat bone (Franzen and Heinegard, 1985). After cloning the cDNA for this protein, the same group identified an integrin-binding RGD sequence (Oldberg *et al*., 1986). They found that osteosarcoma cells bound to surfaces coated with OPN and reported that the protein adhered tightly to hydroxyapatite (Oldberg *et al*., 1986). The investigators proposed the name OPN because this cell-derived component of the osteoid matrix could form a bridge between cells and bone (Oldberg *et al*., 1986). Finally, the protein was found to be expressed in T lymphocytes early after activation, leading to the name early T lymphocyte activation 1 (Eta-1) (Patarca *et al*., 1989). Ultimately, it became clear that Eta-1 and OPN were identical proteins (Patarca *et al*., 1993).

Structure

OPN is structurally complex. It is a secreted phosphoprotein composed of approximately 300 amino acids, depending on the species, with multiple post-translational modifications including glycosylation and phosphorylation. In addition, a thrombin-cleavage site that is present within the protein is thought to be important in activating the biological properties of

OPN (Senger *et al*., 1994). Although it is a secreted, soluble protein, it is possible for OPN to be immobilized by crosslinking to the extracellular matrix (Beninati *et al*., 1994). To date, the protein has been shown to be a ligand for integrin receptors and for CD44. Whether these receptors are responsible for the inflammatory properties of OPN or whether additional receptors are involved remains unclear.

Main activities and pathophysiological roles

The most well-defined biologic activities of OPN are adhesion and migration. Multiple cell types have been shown to adhere to OPN (Liaw *et al*., 1994; Senger *et al*., 1994; Nasu *et al*., 1995). The RGD integrin-binding motif appears to be important for this process. Relevant for the immune/inflammatory systems, OPN can activate the migration of macrophages (Giachelli *et al*., 1998; Singh *et al*., 1990) as well as smooth muscle cells (Liaw *et al*., 1994).

OPN has been identified in many pathologic states. OPN can bind calcium (Chen *et al*., 1992) and is associated with calcified atherosclerotic and neoplastic lesions (Hirota *et al*., 1993, 1995). The protein has been linked to various other inflammatory processes including the granulomas of *tuberculosis* (Nau *et al*., 1997) and *sarcoidosis* (Carlson *et al*., 1997). OPN is expressed in macrophages after myocardial injury (Murry *et al*., 1994) and in macrophages during skin wound healing (Liaw *et al*., 1998). Finally, the protein is not only associated with transformed cells (Craig *et al*., 1989; Senger *et al*., 1979), but expression of antisense OPN reduces the tumorigenicity and the metastatic potential of transformed cells (Behrend *et al*., 1994).

GENE AND GENE REGULATION

Accession numbers

Rat cDNA: M14656
Mouse cDNA: J04806, X14882
Human cDNA: X13694, J04765, M83248

Sequence

The genomic structure of OPN has been thoroughly investigated. There appears to be only one copy of the OPN gene in the mouse (Craig *et al*., 1989) and human (Young *et al*., 1990) genomes. Genomic clones for mouse and human OPN were published soon after the cDNA sequence was determined (Miyazaki *et al*., 1990; Hijiya *et al*., 1994). The murine OPN gene was cloned from a murine liver library in which six exons and five introns were described (Miyazaki *et al*., 1990). The untranslated sequence of exon 1 corresponded to the 5′ untranslated sequence of the Eta-1 cDNA (Patarca *et al*., 1989). However, the first intron identified by Miyazaki *et al*. conflicted with the structure of the human gene recovered by the same research group from a human liver library (Hijiya *et al*., 1994). The human gene contained an additional exon located 1083 bp 5′ upstream of what was identified as exon 1 on the mouse (Hijiya *et al*., 1994). This apparent discrepancy was resolved by Craig and Denhardt when they isolated another OPN genomic clone from a murine embryo genomic library (Craig and Denhardt, 1991). This isolate contained an exon 1 homologous to that which had been identified in the human gene (Craig and Denhardt, 1991). A study of promoter site(s) in the murine OPN gene was conducted by primer extension analysis (Zhang *et al*., 1992) and confirmed the existence of one promoter site at exon 1 described by Craig and Denhardt (1991). OPN mRNA could not be identified that utilized the exon 1 proposed by Miyazaki *et al*. (Zhang *et al*., 1992; Behrend *et al*., 1993). Behrend and colleagues have suggested that the cDNA isolated by Miyazaki *et al*. and by Patarca *et al*. represents an incompletely processed nuclear transcript (Behrend *et al*., 1993).

Chromosome location

Chromosomal localization has been performed for the murine and the human OPN genes. The mouse OPN gene was localized to chromosome 5 by a linkage analysis of inbred strains of mice, an estimated 0.00 cM from the Ric locus (see Pathophysiological roles in normal humans and disease states and diagnostic utility) (Fet *et al*., 1989). Young and colleagues performed chromosomal mapping of the human OPN gene using human/rodent hybrid cell DNAs demonstrating a single copy gene localized to chromosome 4 (Young *et al*., 1990). They carried their analysis further by performing *in situ* hybridization on human metaphase chromosome spreads and localized the OPN gene to 4q13 (Young *et al*., 1990). This chromosomal analysis is interesting because mouse chromosome 5 is homologous to the entire short arm and the proximal end of the long arm of human chromosome 4 (Lalley *et al*., 1988). This localization is also interesting because it places OPN in the same chromosomal location characteristically associated with human chemokines such as IL-8 (Modi *et al*., 1990; Sherry and Cerami, 1991).

Regulatory sites and corresponding transcription factors

Hijiya and others analyzed the promoter region 5′ to the human OPN gene (Hijiya *et al*., 1994). A similar analysis was performed for the murine gene (Miyazaki *et al*., 1990), but is excluded here because of the confusion surrounding the identity of exon 1. **Table 1** summarizes the sequence analysis performed by Hijiya *et al*. in which a number of potential regulatory motifs were identified (Hijiya *et al*., 1994). Of note, a number of NF-IL6 sites and one IRF-1 site were identified, suggesting the regulation of OPN expression by immune and inflammatory responses.

In addition to transcription control regions upstream of the OPN gene, regulatory elements have been described in the first intron (Botquin *et al*., 1998). OPN is expressed in the inner cell mass and the hypoblast of the developing murine embryo. The transcription factors Oct-4 and Sox-2 bind specific sequences in the first intron of the OPN gene. A novel palindromic Oct recognition element contributes to the transcriptional activation of OPN and is antagonized by Sox-2 binding (Botquin *et al*., 1998).

Table 1 Regulatory elements in the 5′ region upstream of human OPN

Site	Number of elements[a]
AP-1	1
AP-2	1
AP-3	1
CF-1	3
CTCF	4
E2A	2
E2BP	1
E4TF1	1
EtS-1	1
GATA-1	4
IRF-1	1
myb	2
NF-IL6	4
Oct-1	1
PEA-3	2
PPAR	1
SIF	1
SP-1	2
TCF-1	11
VDR-like	2

[a] Summarized from Hijiya *et al*. (1994).

Cells and tissues that express the gene

After OPN was first identified in the culture supernatants of transformed cell lines, protein was subsequently purified from a number of different systems and the gene was cloned (**Table 2**). The murine cDNA, known as 2ar, was cloned from an epidermal cell line after treatment with phorbol esters (Smith and Denhardt, 1987). The gene is also induced in normal mouse epidermis after phorbol ester treatment and was found to be identical to secreted phosphoprotein 1 (spp1) and rat OPN (Craig *et al*., 1989).

OPN transcription is developmentally regulated and can be found in calvaria, placenta, decidua, and kidney (Nomura *et al*., 1988). Additional studies of OPN expression in adult mice have shown that the transcript is detectable in kidney, ovary, lung, brain, and skin (Craig and Denhardt, 1991). Pregnant or lactating female mice have detectable OPN transcript in their deciduum and placenta and increased expression in the uterus, skin, and fatty tissue (Craig and Denhardt, 1991).

In bone, the OPN gene is expressed in osteoclasts (Dodds *et al*., 1995; Tezuka *et al*., 1992) which are believed to differentiate from blood monocytes (Reinholt *et al*., 1990). Others have demonstrated that osteoblasts can produce OPN *in vitro* (Salih *et al*., 1997). Human OPN appears to exist as two different species in bone and decidua cells (Young *et al*., 1990). This is most likely related to differential splicing, though the functional consequences of the two species are unknown (Young *et al*., 1990).

Cloned murine T lymphocytes, helper and cytolytic, and a cloned NK cell line produce the Eta-1 transcript after activation by Con A (Patarca *et al*., 1989). Cell lines with monocytic lineages express OPN mRNA (Miyazaki *et al*., 1990).

The expression of OPN in tumors has been studied where splice variants also have been described (Saitoh *et al*., 1995). The function of these alternative forms of OPN is unclear but may be related to tumorigenicity (see Pathophysiological roles in normal humans and disease states and diagnostic utility).

Table 2 OPN cDNA cloning

Species	Tissue	Name	Accession numbers	Reference
Rat	Osteosarcoma	Bone sialoprotein	M14656	Oldberg *et al*., 1986
Mouse	Epidermal cell line	2ar	J04806/X14882	Craig *et al*., 1989
		Spp-1		
Human	Osteosarcoma	Op-30	X13694	Kiefer *et al*., 1989
Human	Primary bone cell culture	OPN	J04765	Young *et al*., 1990
Human	Kidney	Uropontin	M83248	Shiraga *et al*., 1992
		Nephropontin		

PROTEIN

Accession numbers

The accession numbers of murine and human OPN are summarized in **Table 3**. Although the contents of the table are not exhaustive of the multiple OPN entries available, the first two entries in the table summarize multiple different submissions to the database grouped under a single accession number. The final entry highlights the predicted proteins from splice variants that have been identified (Saitoh *et al*., 1995). The two smaller forms, OPN-b and c, have deletions in amino acid numbers 58–71 and 32–58, respectively.

Table 3 OPN protein sequences

Species	Accession numbers	Amino acids	Reference
Mouse	129261	294	Miyazaki *et al*., 1989, 1990; Craig *et al*., 1989; Patarca *et al*., 1989; Worcester *et al*., 1992
Human	129260	314	Kiefer *et al*., 1989; Young *et al*., 1990; Shiraga *et al*., 1992; Kohri *et al*., 1993; Hijiya *et al*., 1994
Human (alternative splice)			
OPN-a	2119710	314	Saitoh *et al*., 1995
OPN-b	2119708	300	Saitoh *et al*., 1995
OPN-c	2119709	287	Saitoh *et al*., 1995

Description of protein

OPN is a secreted soluble protein that is highly acidic with numerous posttranslational modifications. The protein is 294 amino acids in length in the mouse and 314 in humans (see Table 3). There is a 16 amino acid segment of hydrophobic residues that corresponds to a secretion signal sequence. The mature protein has a mass of 44,000 Da measured by sedimentation equilibrium centrifugation (Prince *et al*., 1987). However, the estimated molecular weight on SDS-PAGE is variable and can be up to 75,000 Da (Prince *et al*., 1987; Senger *et al*., 1989). This difference has been attributed to gel characteristics (Prince *et al*., 1987; Senger *et al*., 1989).

Biochemical analyses indicate that OPN is a highly acidic protein (Patarca *et al*., 1989). Approximately 100 amino acids from the N-terminus there are stretches rich in aspartic acid residues: 11 of 12 amino acids in mouse (Craig *et al*., 1989; Miyazaki *et al*., 1990), 10 of 11 in rat (Oldberg *et al*., 1986), and 8 of 10 or 7 of 10 in human (Kiefer *et al*., 1989; Young *et al*., 1990). Overall, OPN has a pI of approximately 4.5 (Patarca *et al*., 1989).

Analyses of the amino acid sequence deduced from cDNA sequence have identified potential functional domains within OPN. First, there is an integrin-binding motif composed of GRGDS (Oldberg *et al*., 1986). This motif is highly conserved among the species studied thus far and is believed to be OPN's major domain for integrin binding. The GRGDS sequence is thought to be on a turn within a β sheet and on the surface of the molecule (Craig *et al*., 1989). The domain rich in aspartic acid residues is thought to be important for binding hydroxyapatite (Oldberg *et al*., 1986) or calcium (Patarca *et al*., 1989). A seven amino acid stretch with homology to thrombospondin may also bind calcium (Patarca *et al*., 1989). Finally, there are two potential heparin-binding sites within OPN (Prince, 1989).

Discussion of crystal structure

The crystal structure of OPN has not yet been elucidated. The extensive posttranslational modifications may make accurate determination of the crystal structure difficult.

Ribbon diagrams have been used to illustrate spatial relationships between domains of the protein (Denhardt and Guo, 1993; Uede *et al*., 1997). The predicted secondary structure includes eight α helix domains and six β sheet domains (Denhardt and Guo, 1993; Uede *et al*., 1997). Of some interest, the GRGDS motif is located just 5′ of a thrombin cleavage site (Denhardt and Guo, 1993). Thrombin treatment of OPN enhances integrin binding (see *in vitro* activities) (Senger *et al*., 1989). This enhancement may be the result of exposure of the GRGDS sequence.

Important homologies

There are several structural homologies of note in OPN. As detailed above, an RGD integrin-binding motif is within OPN like the extracellular matrix proteins fibronectin and vitronectin (Oldberg *et al*., 1986). There are also domains with homology to thrombospondin. There does not, however, appear to be significant homology with other immunologically active proteins (Patarca *et al*., 1989).

Posttranslational modifications

OPN is known to be heavily modified after translation. Isolation and analysis of purified rat bone OPN identified one phosphothreonine and 12 phosphoserine residues (Prince *et al*., 1987). Mass spectroscopy analysis of bovine milk OPN demonstrated 27 phosphoserine and one phosphothreonine residues (Sorensen *et al*., 1995). These residues were associated with recognition sequences for mammary gland casein kinase and casein kinase II (Sorensen *et al*., 1995).

Glycosylation is a prominent posttranslational modification of OPN. Sequence analysis of OPN cDNAs predicted several *O*-linked and *N*-linked glycosylation sites (Oldberg *et al*., 1986; Patarca *et al*., 1989). Rat bone OPN contains 16.6% carbohydrate distributed between one *N*-linked and 5–6 *O*-linked oligosaccharides (Prince *et al*., 1987). Among these oligosaccharides, one-third of the monosaccharide residues are sialic acid (Prince *et al*., 1987). In contrast, bovine milk OPN contains three *O*-linked and no *N*-linked oligosaccharides, even though there are three potential *N*-linked sites based on sequence analysis (Sorensen *et al*., 1995).

Although OPN is a secreted soluble protein, paradoxically it is thought to be a component of the extracellular matrix in some situations (Reinholt *et al*., 1990). The discovery that OPN is a substrate for transglutaminase provided one possible explanation for this paradox (Beninati *et al*., 1994). Transglutaminase was shown to catalyze the oligomerization of OPN and the crosslinking of OPN to fibronectin (Beninati *et al*., 1994). There are two glutamines in bovine OPN that are reactive with transglutaminase and these two residues are conserved in multiple species (Sorensen *et al*., 1994).

CELLULAR SOURCES AND TISSUE EXPRESSION

Cellular sources that produce

Limited analyses have been performed on the *in vitro* production of OPN protein from various cell sources. In addition to transformed cells (Senger *et al*., 1979), OPN protein has been identified in the culture supernatants of activated T cells (Singh *et al*., 1990).

Extensive immunohistochemical studies of normal tissue specimens have been performed. Using a rabbit antiserum raised against human breast milk OPN, the protein was found closely associated with many tissues such as lactating and nonlactating breast epithelium and luminal contents as well as the cement lines and trabecula in bone. Consistent with the results observed by immunohistochemistry, OPN has been biochemically purified from human (Senger *et al*., 1989) and bovine (Bayless *et al*., 1997) breast milk. Multiple epithelial surfaces are also positive on immunohistochemistry: (1) the gastrointestinal system (salivary gland, stomach, small and large intestine, bile duct, gallbladder, pancreas duct); (2) the urogenital system (urinary bladder epithelium, distal tubules of the kidney, testis, endocervix and endometrium, ovary, and placenta) (Brown *et al*., 1992). The fact that granulated metrial gland cells of the placenta/endometrium are derived from bone marrow and have an NK cell phenotype (Linnemeyer and Pollack, 1991) offers some explanation for the increased OPN gene expression in the pregnant uterus and deciduum (Craig and Denhardt, 1991).

Immunohistochemical studies using a monoclonal antibody that recognizes OPN have demonstrated more extensive tissue expression (Nau *et al*., 1997), though this may be in part due to the nature of the tissues being examined (see Pathophysiological roles

in normal humans and disease states and diagnostic utility).

Eliciting and inhibitory stimuli, including exogenous and endogenous modulators

Hormones and other small molecules can alter OPN expression. Consistent with the presence of steroid response elements in the promoter, topical administration of 17β-estradiol and progesterone, alone or in combination, leads to increased OPN mRNA in skin and subcutaneous adipose tissue (Craig and Denhardt, 1991). The treatment of osteosarcoma cells, osteoblasts, or fibroblasts with 1,25-dihydroxyvitamin D3 increases OPN protein production (Prince and Butler, 1987; Jin *et al*., 1990). Phorbol esters induce expression of OPN RNA in epidermal cells (Smith and Denhardt, 1987). Osteoblasts also upregulate OPN gene expression in response to mechanical stress (Toma *et al*., 1997).

The effect of cytokines on OPN gene expression and protein production has been examined in several systems. Osteoblasts and fibroblasts increase OPN mRNA after exposure to TNFα, and IL-1 (Jin *et al*., 1990). TGFβ induces the expression of OPN mRNA in osteosarcoma cells (Noda *et al*., 1988) and in vascular smooth muscle cells (Giachelli *et al*., 1993). Cardiac myocytes increase OPN mRNA after exposure to IL-1β and IFNγ (Singh *et al*., 1995). NK cells increase OPN gene expression after activation with IL-2 (Pollack *et al*., 1994). NF-IL6 and IRF-1 elements are present in the 5′ region of the promoter (Hijiya *et al*., 1994). The expression of OPN mRNA in macrophages increases after infection by mycobacteria (Nau *et al*., 1997). This is of some interest because NF-IL6 regulatory elements are involved in IL-1 and TNFα expression when monocytes are exposed to lipoarabinomannan from *Mycobacterium tuberculosis* (Zhang and Rom, 1993).

LPS can alter OPN gene expression in the kidney (Madsen *et al*., 1997). Whereas OPN transcription is constitutive in the descending thin limb and the papillary surface, LPS induced expression in the distal tubules (Madsen *et al*., 1997). LPS also increases OPN mRNA in a mouse osteoblastic cell line and primary osteoblast-like cells (Jin *et al*., 1990).

Growth factors also modulate the expression of OPN. Basic fibroblast growth factor enhances OPN message in vascular smooth muscle cells (Giachelli *et al*., 1993). PDGF and EGF, alone or in combination, induce OPN expression in quiescent Swiss 3T3 cells (Smith and Denhardt, 1987).

RECEPTOR UTILIZATION

Studies into OPN receptor utilization have focused on integrins and CD44.

IN VITRO ACTIVITIES

In vitro findings

The identification of a GRGDS integrin-binding motif within OPN (Oldberg *et al*., 1986) generated much interest in OPN as an activator of cell adhesion. A summary of several studies is presented in **Table 4**. In addition to characterizing the integrin receptors involved, several groups have tested the functional importance of the RGD motif. Altering the motif from RGD to RGE in recombinant rabbit OPN eliminates the protein's ability to promote adhesion or haptotaxis of P388D1 macrophages (Nasu *et al*., 1995). A deletion of the RGD motif or an RGD to RGE mutation abolishes the ability of recombinant murine OPN to promote adhesion and migration of fibroblast cells (Xuan *et al*., 1995).

In addition to adhesion, several investigators have studied OPN's effect on cell migration. Several studies are listed in Table 4. OPN can trigger cell migration in a modified Boyden chamber assay for chemotaxis. Nasu *et al*. varied this slightly by examining haptotaxis (Nasu *et al*., 1995). In these experiments, OPN was dried on to the filters of a Boyden chamber apparatus before cells were added. OPN caused migration of the macrophage-like cell line P388D1 only when it was precoated on the filters, not when it was in fluid phase (Nasu *et al*., 1995).

Several reports have shown an inhibitory effect of OPN on the induction of nitric oxide synthase type 2 (NOS2) *in vitro*. OPN appears to reduce nitric oxide synthesis and NOS2 mRNA in several cell types treated with LPS and IFNγ (Hwang *et al*., 1994; Rollo and Denhardt, 1996; Rollo *et al*., 1996; Singh *et al*., 1995). These intriguing findings appear at odds with many of the proinflammatory effects of OPN (see *In vivo* biological activities of ligands in animal models). One possible explanation for the conflicting results is the source of the OPN used in the experiments. OPN is heavily modified after translation and alterations of these modifications can have a significant impact on receptor binding (Shanmugam *et al*., 1997). Ultimately, more experiments *in vitro* and *in vivo* are required to fully understand OPN's pro- and anti-inflammatory properties.

Finally, OPN's ability to bind Ca^{2+} has been studied (Chen *et al*., 1992). OPN purified from rat

Table 4 Summary of OPN *in vitro* activities

Activity	Cell type	Reference
Adhesion	Carcinoma cells	
	ras-3T3, mammary	Xuan *et al*., 1994
	Fibrosarcoma, bladder	Senger *et al*., 1994
	Vascular smooth muscle and endothelial cells	Liaw *et al*., 1994
	Embryonic kidney	Hu *et al*., 1995b
	Fetal lung fibroblasts	Senger *et al*., 1994
	Macrophage-like cells (P388D1)	Nasu *et al*., 1995
	Platelets	Bennett *et al*., 1997
	B lymphocytes	
Migration	Endothelial cells (chemotaxis)	Senger *et al*., 1996
	Vascular smooth muscle cells (chemotaxis)	Liaw *et al*., 1994
	Macrophage-like cells (P388D1) (haptotaxis[a])	Nasu *et al*., 1995
Antagonize NOS2	Macrophage-like (RAW264.7)	Rollo *et al*., 1996
	Peritoneal macrophages	Rollo and Denhardt, 1996
	Cardiac myocytes	Singh *et al*., 1995
	Kidney epithelial cells	Hwang *et al*., 1994
Calcium binding	NA[b]	Chen *et al*., 1992

[a] Haptotaxis defined as 'the ability of cells to detect and move up gradients of substratum adhesiveness' (Nasu *et al*., 1995).
[b] Not applicable.

calvaria was shown to bind $^{45}Ca^{2+}$ in an *in vitro* dot blot assay. Under conditions with excess $^{45}Ca^{2+}$, OPN was found to bind 50 atoms of Ca^{2+} per molecule of protein (Chen *et al*., 1992). This value is significantly higher than what might be expected from the two Ca^{2+}-binding sites predicted by the protein sequence (Patarca *et al*., 1989). Chen and colleagues attributed the high Ca^{2+}-binding capacity to an electrostatic mode of binding rather than specific binding sequences (Chen *et al*., 1992).

Regulatory molecules: Inhibitors and enhancers

OPN's ability to activate adhesion can be modulated by proteolytic cleavage. Analysis of OPN derived from milk revealed a specific cleavage site for thrombin (Senger *et al*., 1989). Treatment of OPN with thrombin greatly enhances the attachment and spreading of tissue culture cells (Senger *et al*., 1994). This activity was blocked almost entirely by antibodies recognizing the $\alpha_v\beta_3$ integrin (Senger *et al*., 1994). The thrombin cleavage site of OPN is near the GRGDS integrin-binding site in multiple species (Bautista *et al*., 1994). Activation of OPN by thrombin cleavage has also been demonstrated *in vivo* where it participates in endothelial cell migration induced by VEGF (Senger *et al*., 1996).

Divalent cations, Ca^{2+}, Mg^{2+}, or Mn^{2+}, enhance cell adhesion to OPN (Hu *et al*., 1995a; Bennett *et al*., 1997; Bayless *et al*., 1998). However, adhesion via the $\alpha_v\beta_3$ integrin can be suppressed with calcium levels greater than 1–2 mM (Hu *et al*., 1995a; Bennett *et al*., 1997).

Adhesion of platelets to OPN via $\alpha_v\beta_3$ integrin requires activation by treatment with ADP (Bennett *et al*., 1997). Adhesion of B cell lines requires pretreatment of the cells with phorbol esters (Bennett *et al*., 1997).

Bioassays used

The bioactivity of OPN preparations are generally tested by adhesion mediated by the protein or by cell migration (see Table 4 for references).

IN VIVO BIOLOGICAL ACTIVITIES OF LIGANDS IN ANIMAL MODELS

Normal physiological roles

Multiple models of OPN's *in vivo* function have been developed. Using immunogold staining techniques, Reinholt and colleagues localized high concentrations of OPN in bone at the clear zone between osteoclasts and the surface of bone (Reinholt *et al*., 1990). The $\alpha_v\beta_3$ vitronectin receptor also localized to the same region. The concentration of OPN was low elsewhere in the trabecular bone and the staining of osteoblasts did not appear significantly different than controls (Reinholt *et al*., 1990). These findings suggested a model where OPN anchors osteoclasts to the surface of bone. This model can be expanded with the knowledge that increased Ca^{2+} concentration decreases adhesion to OPN (Hu *et al*., 1995a). The osteoclast would release from the bone surface after sufficient Ca^{2+} was liberated in the local microenvironment; thus, a feedback mechanism would modulate bone resorption. Consistent with this model of osteoclast adhesion, bone resorption *in vivo* can be inhibited by a peptidomimetic antagonist of the $\alpha_v\beta_3$ integrin (Engleman *et al*., 1997). Also, osteoclast adherence to and resorption of bone particles can be inhibited by soluble OPN, RGD-containing peptides, or antibodies that bind OPN or the $\alpha_v\beta_3$ integrin (Ross *et al*., 1993).

OPN has been purified from urine and is believed to be important in *renal stone formation*. Shiraga *et al*. purified a protein they named uropontin from human urine (Shiraga *et al*., 1992). N-terminal sequencing showed identity to human bone OPN though amino acid composition analysis showed some differences between the two proteins. Uropontin inhibited calcium oxalate crystal formation *in vitro* (Shiraga *et al*., 1992). The cDNA for uropontin was cloned and was found to be identical to OPN (Kohri *et al*., 1993). The protein was also purified from calcium oxalate stones (Kohri *et al*., 1993). In contrast to the *in vitro* results, expression of OPN message and protein in distal tubule cells increased when stone formation was induced by glyoxylic acid (Kohri *et al*., 1993). However, studies in mice appear to corroborate the results of Shiraga and colleagues in that OPN appears to inhibit crystal growth (Worcester *et al*., 1992). It seems likely that the normal role for OPN is to maintain the solubility of the calcium oxalate. Under conditions when stone formation is imminent and perhaps as a response to injury, OPN expression is increased. Eventually, OPN is incorporated into the stone when the Ca^{2+} and the oxalate concentrations reach a critical level. Kidney stones were not described in the two OPN knockout mice that have been generated thus far (see Knockout mouse phenotypes); this suggests that other proteins in urine also act to inhibit stone formation (Shiraga *et al*., 1992).

OPN may have a protective role for humans at the interface between epithelial surfaces and the external environment (Brown *et al*., 1992). OPN was identified on the luminal surfaces of multiple epithelia and in sweat glands and ducts (Brown *et al*., 1992). There is also a significant amount of OPN in breast milk (Senger *et al*., 1989). Because certain bacteria use integrins for colonization, Brown and colleagues postulated that OPN may prevent colonization by blocking these receptors (Brown *et al*., 1992). OPN may also exert a bacteriostatic effect by limiting the amount of Ca^{2+} available to pathogens.

Species differences

To date there have been no functional comparisons between OPN protein from different species. In independent studies, OPN derived from different species was observed to have different effects (see Pharmacologic effects).

Knockout mouse phenotypes

Liaw *et al*. generated an OPN-null mouse line to assess OPN's role *in vivo* (Liaw *et al*., 1998). Although OPN is expressed during embryonic development (Nomura *et al*., 1988), mice lacking OPN grow to adulthood and are fertile. The OPN-null animals demonstrate altered wound healing with delayed debridement and abnormal collagen fibril remodeling (Liaw *et al*., 1998). The delayed debridement was attributed to altered macrophage activation. Another series of experiments using the same OPN-null line have implicated abnormal macrophage activity in increased susceptibility to a mycobacterial infection (Nau, G. J. *et al*., unpublished results).

Rittling *et al*. have generated another line of mice with a targeted disruption of the OPN gene (Rittling *et al*., 1998). In this line a high molecular weight, low abundance transcript that hybridized with OPN probes was seen on northern blot analysis. However, there was no mRNA species reminiscent of native OPN and there was no detectable OPN protein. Once again, the mutant mice are fertile and show normal development. A detailed analysis of osseous tissue revealed no abnormalities; in particular, an ultrastructural

analysis using immunogold techniques showed normal cement lines though OPN is typically abundant at this site. The authors did identify an abnormal phenotype when osteoclastogenesis was tested *in vitro*. Splenocytes or bone marrow cells from null mice yield several fold more osteoclasts than cells from wild-type animals (Rittling *et al*., 1998).

Transgenic overexpression

To date, there are no published reports on the effects of OPN overexpression in a transgenic mouse model.

Pharmacological effects

Several groups have studied the pharmacologic effects of OPN administered *in vivo*. Subcutaneous injection of OPN derived from murine T cells into mouse skin led to an inflammatory cell infiltrate within 24 hours (Singh *et al*., 1990). The cellular composition of these infiltrates had a greater than expected proportion of macrophages (Singh *et al*., 1990). Similar results were obtained with *E. coli*-derived, recombinant murine OPN injected intradermally in rats (Giachelli *et al*., 1998). In contrast, Nasu and colleagues observed an inflammatory infiltrate composed mostly of polymorphonuclear leukocytes after injecting *E. coli*-derived, recombinant rabbit OPN intradermally in rabbits (Nasu *et al*., 1995). It is unclear if the differences between these studies are related to species differences or to the source of the protein.

Consistent with the pharmacologic effects detailed above, administration of an anti-OPN antiserum appears to block inflammation. *N*-Formyl-Met-Leu-Phe (fMLP) injected intradermally in rats elicits a macrophage infiltrate in which the macrophages express OPN (Giachelli *et al*., 1998). The accumulation of macrophages at the site of fMLP was blocked by a neutralizing antiserum against OPN (Giachelli *et al*., 1998). The same neutralizing antiserum reduced inflammation in limited studies of a rat model for crescentic glomerulonephritis (Yu *et al*., 1998). Rats treated with the anti-OPN antiserum also showed diminished cutaneous delayed-type hypersensitivity reactions (Yu *et al*., 1998).

Interactions with cytokine network

As discussed earlier (see Cellular sources and tissue expression), cytokines have been shown to modulate the expression of OPN. IL-1α and TNFα increase the expression of OPN message in mouse osteoblast-like cells (Jin *et al*., 1990). TGFβ increases both OPN transcription and protein synthesis in rat osteosarcoma cells (Noda *et al*., 1988).

PATHOPHYSIOLOGICAL ROLES IN NORMAL HUMANS AND DISEASE STATES AND DIAGNOSTIC UTILITY

Normal levels and effects

The normal physiologic effects of OPN in humans are incompletely understood. Most understanding of its normal role is inferred from the observations that have been made using immunohistochemical analyses (Brown *et al*., 1992) and from studies in animals. OPN appears throughout the gastrointestinal system and secretory epithelium, including skin (Brown *et al*., 1992). Because OPN is a ligand for integrins, it may limit binding of pathogens that require surface integrins for colonization (Brown *et al*., 1992). Another possibility is that OPN may bind calcium, thereby reducing the availability of this cation to bacteria. An OPN-like protein is normally found in human urine and likely influences crystal formation (Shiraga *et al*., 1992).

Role in experiments of nature and disease states

Inflammation

Analyses of several pathologic conditions have provided more insight into OPN's role as a cytokine. In general, OPN is closely associated with tissue injury and the ensuing *inflammation*. Cryoinjury of rat myocardium and ischemic injury of human myocardium leads to increased OPN mRNA (Murry *et al*., 1994). In rats, the OPN message and protein are found in macrophages infiltrating the injured site (Murry *et al*., 1994). OPN expression is also associated with *coronary atherosclerosis* (Hirota *et al*., 1993; Panda *et al*., 1997). OPN is present in the plasma of patients with coronary artery *atherosclerosis*, but not in normal controls (Panda *et al*., 1997). Coronary atherectomy of atherosclerotic vessels leads to a sustained increase in plasma levels of OPN (Panda *et al*., 1997). OPN expression is increased in rat brain after a focal stroke and probably influences microglial, i.e. macrophage, infiltration (Wang *et al*., 1998). Serum levels of OPN also increase in patients with sepsis (Senger *et al*., 1988).

OPN is also associated with other, more chronic, inflammatory conditions in humans. Granulomas of *tuberculosis* and *silicosis* demonstrate high levels of tissue OPN on immunohistochemical analysis (Nau *et al*., 1997). Other granulomatous conditions, such as *sarcoidosis*, *histoplasmosis*, *temporal arteritis*, and *foreign-body reactions* also express OPN message and protein (Carlson *et al*., 1997). OPN is also increased in two murine models of *idiopathic pulmonary fibrosis* (Nakama *et al*., 1998). Finally, OPN is elevated in mice predisposed to *autoimmune disease* (Patarca *et al*., 1990). While OPN can increase antibody production from B cells (Lampe *et al*., 1991), it is unclear if the elevated serum OPN causes the autoimmune disease in the mice (Patarca *et al*., 1990).

Because OPN can activate the migration of cells, including macrophages, the protein may function as a soluble chemoattractant cytokine in the inflammatory conditions listed above. Alternatively, because OPN may be covalently linked to the extracellular matrix (Beninati *et al*., 1994), chronic OPN production and tissue accumulation may anchor macrophages that might have otherwise migrated through normal tissue.

Infection

From the standpoint of host resistance to infection, OPN has an intriguing history. Two alleles at the murine Ric locus determine the genetic resistance to a lethal rickettsial infection (Groves *et al*., 1980). OPN was subsequently mapped to the Ric locus (Fet *et al*., 1989). A comparison between a single resistant and a single susceptible allele revealed several amino acid changes, two were located in functional domains of the protein: a calcium-binding site and a heparin-binding site (Ono *et al*., 1995). Mice bearing the susceptible allele of Ric die from small inocula of the obligate intracellular pathogen *Orientia* (*Rickettsia*) *tsutsugamushi*, the causative agent of human *scrub typhus* (Groves *et al*., 1980; Patarca *et al*., 1993). Similar findings were made with group B arboviruses, though the genetic elements responsible have not been mapped as well (Goodman and Koprowski, 1962). In both cases, the defect was attributed to impaired macrophage activity (Goodman and Koprowski, 1962; Kokorin *et al*., 1976). Consistent with the increased susceptibility to a rickettsial infection, OPN-null mice show increased susceptibility to a mycobacterial infection (Nau, G.J. *et al*., unpublished results).

Analysis of the human OPN gene has also suggested that a limited number of alleles exist (Young *et al*., 1990). However, these alleles have not yet been associated with particular disease states. In addition, there have been no reports of patients deficient in OPN.

Cancer

OPN has a long association with *cancer* and tumorigenesis. As described earlier, the protein was first identified in the supernatants of tumor cells grown *in vitro* (Senger *et al*., 1979). Phorbol esters induce the expression of OPN mRNA (Craig *et al*., 1989).

A thorough study of human *carcinomas* revealed limited expression of OPN by tumor cells (Brown *et al*., 1994). OPN mRNA is expressed by human renal and endometrial tumor cells (Brown *et al*., 1994). Multiple other carcinomas showed increased expression of OPN message, but this localized to macrophages within the tumor stroma (Brown *et al*., 1994). OPN protein was identified by immunohistochemistry in both macrophages and tumor cells, especially at the 'tumor/host interface'. Gastrointestinal and breast tumor cells were negative for OPN mRNA on *in situ* hybridization analysis, suggesting that the OPN detected by immunohistochemistry was derived from the tumor stroma (Brown *et al*., 1994). Thus, OPN expression in human carcinomas generally is limited to the inflammatory macrophages within the stroma. Some carcinomas, in this case endometrial and renal, appear to make OPN independently. The fact that some tumor cells but not others express OPN was attributed to developmental origins, i.e. mesenchymal versus epithelial, of the cells (Brown *et al*., 1994).

In other instances, not only is OPN produced by tumor cells but it also correlates with the severity of *cancer*. Saitoh and colleagues found OPN expression increased with more malignant grades of human glioblastoma cell lines (Saitoh *et al*., 1995). Studies in mice have shown that reducing OPN expression in transformed fibroblasts reduces tumorigenicity and malignant potential (Behrend *et al*., 1994; Gardner *et al*., 1994).

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