Review

The Role of Osteopontin in Tumor Progression and Metastasis in Breast Cancer

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Abstract

The use of cancer biomarkers to anticipate the outlines of disease has been an emerging issue, especially as cancer treatment has made such positive steps in the last few years. Progress in the development of consistent malignancy markers is imminent because advances in genomics and bioinformatics have allowed the examination of immense amounts of data. Osteopontin is a phosphorylated glycoprotein secreted by activated macrophages, leukocytes, and activated T lymphocytes, and is present in extracellular fluids, at sites of inflammation, and in the extracellular matrix of mineralized tissues. Several physiologic roles have been attributed to osteopontin, i.e., in inflammation and immune function, in mineralized tissues, in vascular tissue, and in kidney. Osteopontin interacts with a variety of cell surface receptors, including several integrins and CD44. Binding of osteopontin to these cell surface receptors stimulates cell adhesion, migration, and specific signaling functions. Overexpression of osteopontin has been found in a variety of cancers, including breast cancer, lung cancer, colorectal cancer, stomach cancer, ovarian cancer, and melanoma. Moreover, osteopontin is present in elevated levels in the blood and plasma of some patients with metastatic cancers. Therefore, suppression of the action of osteopontin may confer significant therapeutic activity, and several strategies for bringing about this suppression have been identified. This review looks at the recent advances in understanding the possible mechanisms by which osteopontin may contribute functionally to malignancy, particularly in breast cancer. Furthermore, the measurement of osteopontin in the blood or tumors of patients with cancer, as a way of providing valuable prognostic information, will be discussed based on emerging clinical data. (Cancer Epidemiol Biomarkers Prev 2007;16(6):1–11)

Introduction

Recently, the use of cancer biomarkers to predict future patterns of disease has been an emerging issue, especially as cancer treatment has made such positive strides in the last few years (1-11). Breakthroughs in the development of reliable cancer biomarkers may be imminent because of advances in genomics and computer technology, which allow the analysis of vast quantities of data (12-17).

A biomarker is any substance, which when detected in biological samples or tissues, is associated with an increased risk of a disease. The term cancer biomarker most commonly refers to serum markers such as the prostate-specific antigen; markers for inherited mutations, such as the breast and ovarian cancer susceptibility genes *BRCA1* and *BRCA2* , and markers for somatic or noninherited mutations, which account for most cancers (18-20). Growth factors, which circulate in the blood and may contribute to the development of tumors, are under investigation as possible cancer biomarkers.

Despite promising new methods and findings, controversy abounds in this field, as a consequence, none of the biomarkers used nowadays have adequate sensitivity, specificity, and predictive value for population screening (21). Nevertheless, it is highly desirable to pursue new biomarkers suitable for population screening and early diagnosis (22).

Serum biomarkers are produced by body organs or tumors, and when detected in high amounts in the blood, can be suggestive of tumor activity. These markers are nonspecific for cancer and can be produced by normal organs as well. Most biomarkers are used infrequently for screening purposes. They are more often used to evaluate treatment effects or to assess the potential for metastatic disease in patients with established disease. In this context, osteopontin, a phosphorylated glycoprotein found in all body fluids, extracellular matrix (ECM) components, and the proteinaceous matrices of mineralized tissues (23, 24), also constitutes a possible biomarker. Osteopontin was found to be overexpressed in the tumors and serum of women with ovarian cancer and was correlated with progression (25, 26). Recent studies have shown that the overexpression of osteopontin was also related with breast cancer evolution and metastasis (23); therefore, there is a potential utility for osteopontin in monitoring disease status in patients with breast cancer.

This review aims to provide an overview of the characteristics, functions, and mechanisms of interaction of osteopontin that could be further exploited in developing its value as a breast cancer biomarker, either to provide important diagnosis information, to evaluate treatment effects, or to assess the potential for metastatic disease in patients.

The Occurrence of Osteopontin in Normal Human Tissues

In human tissues, osteopontin has been found to be produced by epithelial cells of the gastrointestinal, urinary and reproductive

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tracts, the gall bladder, pancreas, lung bronchi, lactating breast, salivary glands, and sweat ducts (27). Osteopontin was localized to the luminal surfaces in these sites, as well as in human secretions including blood, milk (28, 29), and urine (30). Overall, these findings suggested that osteopontin might have a protective role in interactions between epithelial surfaces and the external environment.

In milk, osteopontin is likely to have a physiologic role (29), as it was noticed that milk is a rich source of the protein. Cell growth, differentiation, and a high degree of tissue remodeling occurs during various stages in the mammary gland. During pregnancy and lactation, these processes ensure the establishment of the spatial relations between stromal and epithelial cells, and the organization of the latter into a branched tree of ducts and terminal alveoli. The highly metabolic active epithelial cells lining the ducts rest on a basal membrane of collagen and other ECM proteins, and signals from the ECM to the cells are important for cell differentiation and milk secretion (31). The description of expression and regulation of osteopontin mRNA in the developing mammary gland has been carefully described in mouse by several researchers (32-34). *In vitro* experiments with mammary epithelial cells transfected with osteopontin antisense mRNA show the same characteristics as transgenic epithelial cells *in vivo*. The transfected cells tested positive for matrix metalloproteinase-2/procollagenase activity, whereas the control cells did not; hence, up-regulation of matrix metalloproteinase-2 and down-regulation of osteopontin seem to be partly responsible for abnormalities in the mammary gland. Possibly, matrix metalloproteinase-2 and osteopontin compete for integrin-binding on cells, and binding of osteopontin may induce normal cell differentiation, whereas binding of matrix metalloproteinase-2 induces undesired tissue degradation (34-36).

In bone, osteopontin is produced by the matrix-synthesizing osteoblasts at the mineralization front and by bone-resorbing osteoclasts (37-39). The osteopontin in bone is mainly localized to the cement lines and surfaces of mature bone trabeculae (40). Osteopontin preferentially accumulates at cell-matrix and matrix-matrix interfacial structures in bone. Hence, osteopontin has multiple presumed functions, including the attachment of osteogenic cells to the bone matrix, control of mineralization (41-43), coupling of bone formation, and resorption (44, 45). Moreover, osteopontin is expected to add physical strength to the ECM, as it can be cross-linked by transglutaminase to various matrix proteins, including collagen (46).

Besides epithelial and bone cells, osteopontin is also produced by activated macrophages and lymphocytes (47-51), as well as kidney tubule cells, arterial endothelial and smooth muscle cells, cells of the inner ear (38), fibroblastic cells in embryonic stroma, and in wound healing sites (52).

The Structure of Osteopontin

Osteopontin was identified, together with bone sialoprotein, as a major sialoprotein in the mineral ECM of bone (53-56). The name "osteopontin" was introduced to reflect the potential of the bone protein to serve as a bridge between cells and hydroxyapatite through RGD (arginine-glycine-aspartate motif) and polyaspartic acid motifs discovered in the primary sequence of the protein (39). However, the same gene product was identified as a putative lymphokine produced by activated lymphocytes and macrophages and called *Eta-1* (early T lymphocyte activation gene 1; ref. 47); and thus, a more general pattern of expression for osteopontin emerged. Accordingly, secreted phosphoprotein I was introduced as an alternative name, to reflect the broader functional role of this protein, and in some genomic contexts, represents its "official" name (57, 58). Nevertheless, the name osteopontin has largely been retained, in keeping with the nomenclature used for the human gene (59).

The amino acid sequence of osteopontin is nowadays available for several species, i.e., rat (39), mouse (60), human (61), pig (62), rabbit (63), and cow (64). The referenced mammalian osteopontin sequences are identical in ~33% of the residues, and in addition, many similar amino acids are conserved between the sequences. Identical residues are scattered in clusters. More specifically, the larger clusters are located in the hydrophobic leader sequence (the first 16 residues), in a potential site for N-linked glycosylation, and in several sites for O-linked glycosylation and phosphorylation. A stretch of consecutive aspartic acid residues was also found in all species, as well as a cell attachment RGD motif almost immediately followed by a thrombin cleavage site.

Generally, osteopontin is extremely hydrophilic with a low isoelectric point (3.5) and displays an unusual amino acid composition with 42 serine, 48 aspartic acid, and 27 glutamic acid residues, together constituting almost half the residues in human osteopontin (298 residues; refs. 65, 66). It is important to notice that 27 out of the 42 serine residues are phosphorylated (67).

When purified and isolated in solution, osteopontin was found to be flexible along its entire length and to have no significant regions that persist in a single structural environment for more than a few milliseconds (68). That a protein is completely flexible in solution does not mean that it will always remain so. Portions of this protein that strongly interact with other proteins (such as factor H and the $\alpha_v\beta_3$ or other integrin structures and CD44 for osteopontin) will almost certainly adopt specific structures in relation to their binding partners (68).

The conservation among mammalian species of certain residues is presumably indicative of the functions osteopontin performs (38). For instance, osteopontin has been found to play a role in bone mineralization both due to its characteristic amino acid composition and interaction with integrin receptors on cells lining these surfaces (39, 69). The RGD motif that is particularly exposed in the osteopontin molecule represents a major, although not unique, binding ligand for the family of integrin receptors, and was found to be involved in cell attachment, cell migration, and intracellular signaling (70).

Osteopontin Gene Expression and Regulation

Genes encompassed within a 600 kb region on human chromosome 4 encode several noncollagenous bone and dentin proteins (Fig. 1). They include osteopontin, bone sialoprotein,

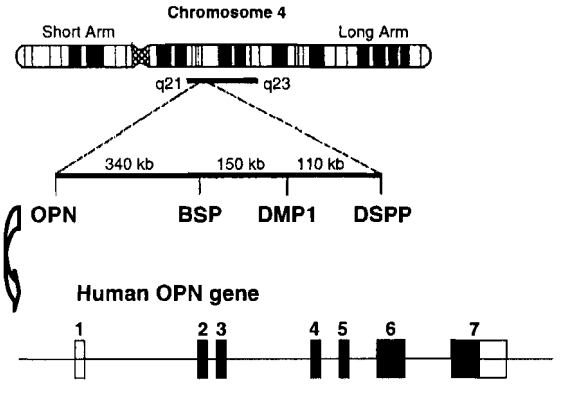

Figure 1. Chromosomal location of the SIBLING genes and gene structure of human osteopontin. The gene location of human osteopontin has been mapped to the long arm of chromosome 4, close to the bone sialoprotein (*BSP*), dentin matrix protein I (*DMPI*), and dentin sialophosphoprotein (*DSPP*) genes. Exons are boxed; *filled boxes*, coding regions; *open boxes*, untranslated regions. Adapted from refs. (73, 200, 201).

dentin matrix protein I, and dentin sialophosphoprotein, all of which have been categorized as members of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family–related proteins (68). The four proteins are somewhat similar being secreted, sialylated, phosphorylated, and acidic in nature. The SIBLING family is the result of duplication and a subsequent divergent evolution of a single ancient gene (68). The primary sequences of the proteins, however, do not show homology and these are therefore not the basis for calling them a related family (68). Rather, the exon-intron boundaries and the similar properties of the individual exons define the SIBLING family.

Osteopontin is encoded by a single copy gene located on the human chromosome 4 (71), the mouse chromosome 5 (57), and the pig chromosome 8 (72). The gene structure of human osteopontin is presented in Fig. 1, and a similar structure with seven exons and six introns is seen in the genes encoding osteopontin from other species. Codon triplets are not interrupted by introns, and consequently, exon skipping will not affect the codon triplets in the remaining exons. The human gene sequence spans ~9 kb and the open reading frame consists of 942 nucleotides from the start codon (in exon 2) to the stop codon (in exon 7; ref. 73).

The predicted molecular weight of a protein translated from human osteopontin mRNA is 35 kDa (39). The 5′-untranslated region includes exon 1, which starts with a transcription initiation site (AGC), whereas the 3′-untranslated region consists of the last part of exon 7, which includes three potential polyadenylation attachment signals (AATAA; refs. 61, 71, 73). Exon 2 encodes the signal peptide and the first two amino acids in the mature protein; exons 3 and 5, the two characteristic Ser-Ser-Glu-Glu phosphorylation sequences; exon 4, the two transglutaminase-reactive glutamine residues; and exon 6, the aspartic acid–rich sequence. Exon 7 is the largest exon encoding approximately half of the proteins including the RGD motif and the central thrombin cleavage site.

The expression of osteopontin is induced by many factors, e.g., tumor promoters and chemical agents, acting on specific cell types and through different signaling pathways. In most of the studies reported, control is exerted on the level of transcription (70). The promoter sequence of osteopontin provides clues for understanding the molecular basis of transcriptional regulation. Analyses of the osteopontin promoters have uncovered many potential sites for transcription factor interactions (72, 73), and a still increasing number of transcription factors have been shown to be directly implicated in osteopontin transcription. Among others, these include progesterone, glucocorticoids, 1α25-dihydroxyvitamin D_3, and basic helix-loop-helix proteins, such as activator protein-1. Activator protein-1, for example, interacts with a highly conserved enhancer-like element present in many viral and cellular genes, including the osteopontin gene. Collectively, these genes are controlled by the Fos and Jun family of oncoproteins, and consequently, osteopontin is believed to be an effector of activated oncogenes functioning to facilitate tumor growth and metastasis (70).

The murine osteopontin promoter sequence contains a *ras*-activated enhancer, which is believed to be partly responsible for the increased transcription of osteopontin observed in *ras*-activated cell lines (74). Induction of osteopontin transcription and tumorigenic transformation by 12-*O*-tetradecanoylphorbol-13-acetate in a mouse epidermal cell line (75) suggests the existence of 12-*O*-tetradecanoylphorbol-13-acetate–responsive elements in the osteopontin gene as well. As for viral regulation of osteopontin, v-Src, which is a viral oncogene produced by the Rous sarcoma virus, is known to stimulate the activity of the osteopontin promoter in mice (76).

The active metabolite of vitamin D, 1α25-dihydroxyvitamin D_3, also regulates osteopontin expression in mouse epidermal cells. However, the induction of osteopontin synthesis and secretion is not correlated with the transformation of the cells as in the case of 12-*O*-tetradecanoylphorbol-13-acetate stimulation (75). In addition, vitamin D also influences osteopontin levels in osteoblasts, and several vitamin D response elements have been identified in the mouse, chicken, pig, and human osteopontin genes. Moreover, it has been shown that vitamin D not only regulates osteopontin at the transcriptional level, but also seems to modulate the phosphorylation state of osteopontin because vitamin D–stimulated osteoblasts secrete a nonphosphorylated form of the protein (77).

Table 1. Osteopontin receptors and receptor-binding motifs

Receptor	Name	Motif	Reference
Integrin	$\alpha_v\beta_3$	RGD	(44)
	$\alpha_v\beta_1$	RGD	(202)
	$\alpha_v\beta_5$	RGD	(202)
	$\alpha_5\beta_1$	RGD	(203)
	$\alpha_8\beta_1$	Unknown	(85)
	$\alpha_4\beta_1$	SVVYGLR	(204)
	$\alpha_9\beta_1$	SVVYGLR (NH_2-terminal thrombin cleaved fragment)	(93)
Nonintegrin	CD44 (v6-10)	Non-RGD	(97-99)

NOTE: RGD, Arg-Gly-Asp motif; SVVYGLR, Ser-Val-Val-Try-Gly-Leu-Arg motif; CD44 (v6-10), CD44 variants, isoforms 6-10. Except for $\alpha_9\beta_1$, all integrins bind the uncleaved osteopontin and an NH_2-terminal thrombin-cleaved fragment, which contain the RGD and SVVYGLR sequences. Integrin $\alpha_9\beta_1$ only binds the NH_2-terminal thrombin-cleaved fragment.

Osteopontin Metabolism and Receptors

As described above, osteopontin is expressed by a variety of cells and is involved in various processes mediated by receptor interactions (38). Osteopontin is regarded as a molecule that mediates cell-matrix and cell-cell communication, and in many cases, this communication results in the adhesion or targeted migration of cells (78). The interaction between osteopontin and cells is mediated by specific receptor-binding motifs in the osteopontin sequence and receptors on the cell surface (Table 1). Like other proteins in the ECM (such as collagen, fibronectin, vitronectin, laminin, and others) osteopontin exists both as an immobilized ECM molecule in mineralized tissues and as a cytokine in body fluids containing the RGD sequence, which facilitates RGD-dependent interactions with integrin receptors and mediates cell attachment/signaling (38, 79). RGD-independent interactions with both integrin and non-integrin receptors have also been shown (70).

Integrins are transmembrane, dimeric proteins consisting of α and β subunits. There are multiple forms of both subunits and each heterodimer can bind a wide variety of ligands with which a cell may come in contact. Ligand binding to integrins could induce clustering and activation of the focal adhesion complex, which includes a number of regulatory and structural proteins, such as focal adhesion kinase, Src, and cytoskeletal proteins. There is evidence that activation of different components of the focal adhesion complex could in turn activate a number of different signal transduction pathways, affecting cellular properties including adhesion, migration, proliferation, and survival (80).

Osteopontin has been shown to interact with a number of different integrins via the RGD sequence, including $\alpha_v\beta_3$, $\alpha_v\beta_1$ and $\alpha_v\beta_5$ (38, 81, 82). More recently, additional integrins have been found to interact with osteopontin, including $\alpha_4\beta_1$ (83), $\alpha_9\beta_1$ (84), and $\alpha_8\beta_1$ (85).

The best characterized osteopontin receptor is the $\alpha_v\beta_3$ integrin, which facilitates RGD-mediated osteopontin adhesion

to osteoclasts (44, 81), smooth muscle cells (49, 86), and tumor cells (87, 88). Close to the RGD sequence, a site for thrombin cleavage is conserved in all known osteopontin species (67, 89, 90). The susceptibility of osteopontin to thrombin cleavage opens the possibility that osteopontin may be cleaved during the course of blood coagulation, and in tissues and fluids exhibiting thrombin-like proteolytic activity. Interestingly, several studies have provided information suggesting that thrombin-cleaved osteopontins exist side-by-side with the full-length protein *in vivo* (38). It seems to be an important characteristic of the protein because cleavage products have been observed in rat plasma and rat tumors (28), human milk (89), and pig bone (54). Fragments of osteopontin originating from either unknown or other proteolytic activities have also been identified in human milk (67) and in human uterus (91). Functionally, fragments of osteopontin produced by thrombin cleavage amplify the effects of the full-length protein (92). For example, a variety of human cell lines exhibit more extensive cell attachment and spreading on thrombin-cleaved osteopontin compared with uncleaved osteopontin (92).

The receptor on the cells mediating the attachment is the $\alpha_v\beta_3$ integrin, introducing this receptor as a major functional receptor for thrombin-cleaved osteopontin. The cleavage of osteopontin may change the conformation of the molecule, more specifically, the conformation of the sequence around the RGD motif, and thereby affect the binding to the $\alpha_v\beta_3$ integrin by allowing greater accessibility to the receptor (92). The RGD motif is contained in the NH_2-terminal fragment of thrombin-cleaved osteopontin and it is this fragment that promotes a stronger response (ref. 87; Fig. 2).

Likewise, the SVVYGLR sequence is located in the NH_2-terminal fragment (Fig. 2), and when osteopontin is cleaved by thrombin, this sequence and the RGD motif are exposed. Consequently, RGD- and SVVYGLR-binding integrins are likely to compete for osteopontin binding, however, the consequences of this competition have not been fully explored (93).

The CD44 family includes multiple protein isoforms, encoded by a single gene and generated by alternative splicing, and several of the isoforms have been shown to be overexpressed in malignant cells (94-96). CD44 is a major cell surface receptor for hyaluronate, and various forms of CD44 could also bind to osteopontin (97, 98).

Tumor cells are stimulated to spread following this interaction, however, this phenotype also seems to involve the β_1 integrin subunit (98). Osteopontin and CD44 interactions inhibit the expression of interleukin-10 by macrophages (50) and are possibly involved in the formation of metastases (99, 100).

Osteopontin and Malignancy

Several studies have described a link between osteopontin and cancer in the past years. The first evidence of this link was reported by Senger and coworkers, who described a transformation-specific secreted phosphoprotein produced by a number of transformed cell lines in culture (89, 101, 102). Afterwards, similar properties were attributed to a protein isolated from bone that was named osteopontin (55, 56, 103). This protein was sequenced by Prince (103) and several structural features, such as the presence of the RGD motif, were elucidated and discussed providing evidence for some of its functions. Subsequently, Smith and Denhardt (104) cloned a cDNA named "2ar" which was inducible by tumor promoter treatment of murine JB6 epidermal cells. Ultimately, Craig et al. (105) showed that the bone-derived osteopontin was the same as the protein encoded by the 2ar clone.

The association of osteopontin with malignancy was also supported by the fact that a *ras*-transformation of nontumorigenic NIH 3T3 cells was shown to confer them a metastatic ability (106). Moreover, osteopontin gene expression was found to be induced in these cells, then pointing to a direct relationship between osteopontin expression and the acquisition of the metastatic phenotype by the cells (107). A novel *ras*-activated enhancer was later identified in the osteopontin promoter (74).

Furthermore, increasing levels of osteopontin expression have also been found in multistage carcinogenesis in mouse skin (108). Taken together, these experimental studies strongly suggest that osteopontin may play a role in tumor progression and metastasis.

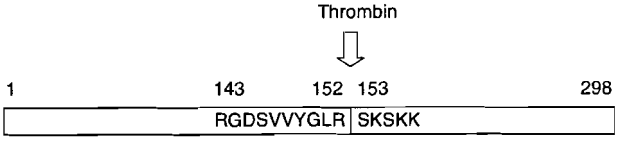

Figure 2. Thrombin cleavage of human osteopontin. Thrombin cleavage at Arg^{152}-Ser^{153} generates an NH_2-terminal fragment with the RGD and SVVYGLR sequences at the carboxyl-terminal. Both of these motifs are recognized by integrin receptors as described in the text.

The Role of Osteopontin in Cancer

The roles of osteopontin in many of its diverse physiologic settings have been discussed in a number of recent reviews. For example, the role of osteopontin in inflammation and immune function have been reviewed by Weber and Cantor (78), Uede et al. (109), and Giachelli and Steitz (45), in mineralized tissues by Denhardt and Noda (70), and in vascular tissue by Ramos (110). A functional role for osteopontin in tumor progression and malignancy has been claimed by several researchers. Multiple and complex mechanisms are involved in the role of osteopontin in cancer, including interactions with cell surface receptors, growth factor/receptor pathways, and proteases. The interactions of osteopontin with various cell surface receptors could induce the activation of various signal transduction pathways, resulting in changes in the expression of a series of genes, the proteins of which contribute to altered cell behavior, including migration and invasion. These effects of osteopontin likely vary between cell types, depending for example on which integrins are expressed and which signal pathways can be activated. There is compelling evidence that soluble osteopontin could, in a variety of situations, help cells survive an otherwise lethal insult. Remarkably, this survival signaling is mediated by receptors that are generally considered to be receptors for ECM components. Denhardt and coworkers suggest that osteopontin delivers an antiapoptotic "ECM-like" signal via multiple ligand-receptor interactions to cells, both adherent and nonadherent (79).

Cell Surface Receptors and Osteopontin. A variety of integrins have been found to be expressed by tumor cells and, depending on the degree of the tumor differentiation, some integrins may be up-regulated or down-regulated (111). The overexpression of integrins is thought to cause constitutive activation of signaling pathways leading to increased growth of tumor cells (112-114).

Particularly, the $\alpha_v\beta_3$ integrin has been related to some aspects of malignancy and metastasis (112, 115, 116). Recently, a highly tumorigenic, metastatic breast cancer cell line (MDA-MB-435) was found to use the $\alpha_v\beta_3$ integrin for migration towards osteopontin, whereas two nonmetastatic breast cancer cell lines (21PT and 21NT) were found to use $\alpha_v\beta_1$, and $\alpha_v\beta_5$ integrins (117). Additionally, a coordinated regulation of

osteopontin and $\alpha_v\beta_3$ integrin has been found in some tissues (86, 118). The expression of $\alpha_v\beta_3$ integrin has also been linked to breast cancer progression through an interaction with protein kinase C-α activity. Using highly metastatic MCF-7 human breast cancer cells transfected to overexpress protein kinase C-α, Carey and coworkers (119) found that $\alpha_v\beta_3$ expression was modulated by increased protein kinase C-α activity. The same cells were used to investigate the integrin-mediated suppression of apoptosis (120) and it was found that tumor cells capable of binding osteopontin via the $\alpha_v\beta_3$ integrin may have a survival advantage.

As mentioned previously, osteopontin could also bind nonintegrin cell surface receptors, such as CD44 (38, 97, 98, 100, 121). The osteopontin and CD44 interactions could as well be mediated through integrins, as there is some evidence that osteopontin binding by CD44 variants and β1-containing integrins could cooperate to promote cell spreading and migration (98). Fujisaki and colleagues (122) proposed a mechanism for the adhesion and migration of colorectal carcinoma cells that describes CD44 induction of integrin expression, and function by both a direct pathway and also via hepatocyte growth factor (HGF) and its receptor (c-Met). This may represent an alternate pathway through which CD44 signals to activate integrin function, aiding in the adhesion and migration of tumor cells.

Growth Factor Receptor Pathways and Osteopontin. Osteopontin transcription may be activated by the *ras* oncogene (74), and plays a key role in neoplastic transformation, metastasis (24), and cancer progression (123). Osteopontin is usually absent or expressed at a low level in normal tissues but is up-regulated in certain preneoplastic and neoplastic epithelia (28, 123, 124), including that of the breast (125). Transfection of an expression vector for osteopontin induces malignant transformation and induction of metastasis in a benign rat mammary epithelial cell line (126), whereas transfection of osteopontin antisense cDNA inhibits these processes in a cell line already overexpressing osteopontin (127, 128). These results suggest that osteopontin overexpression may represent a key molecular event in tumor progression and metastasis, particularly that of the breast. Unlike many proto-oncogenes activated by a gain of function mutation, osteopontin is not typically mutated during stepwise tumorigenesis (24). Instead, various responsive elements in its promoter regulate osteopontin expression for its diverse physiologic roles (129-132), and it is presumably these elements that allow the overexpression of osteopontin in certain cancers.

Interactions of osteopontin with growth factor receptor pathways may influence tumor cell behavior. Tumor cell migration can be influenced by HGF and its receptor, Met. In Webb and coworkers' study (133), HGF and Met receptor signaling was associated with a transformed phenotype in *ras*-transformed NIH 3T3 cells. Furthermore, the HGF pathway was recently reported to affect the adhesion and invasion of cancer cells (134).

The correlation between osteopontin and the HGF/Met pathway was further explored in breast cancer models, and a synergistic relationship between osteopontin and HGF in inducing cell migration was found (117). Additionally, integrin-mediated induction of cell migration in response to osteopontin was accompanied by an initial increase in Met kinase activity, followed by an increase in Met mRNA and protein expression levels (117).

Cell Proteases and Osteopontin. Several studies have described the interactions between integrins and other membrane receptors, such as urokinase-type plasminogen activator (uPA) and its receptor (uPAR), which have been implicated in tumor metastasis. Proteolytic enzymes are believed to contribute to metastasis and tumor growth in several ways (135-138), i.e., via degradation of ECM components and facilitating migration and invasion, or by activation of other proteases.

Osteopontin, either transfected into breast cancer cell lines or added exogenously to them, was shown to increase both the invasiveness of the cells and uPA expression (139), possibly due to cell surface interactions between osteopontin-binding integrins (e.g., $\alpha_v\beta_5$) and uPA/uPAR. It has been shown that uPAR-bound uPA is required for $\alpha_v\beta_5$ integrin–mediated cell migration of human pancreatic carcinoma cells (140). Likewise, Carriero and coworkers (141) have shown that $\alpha_v\beta_5$ integrin interaction with uPAR promotes the migration of human breast cancer (MCF-7) cells. Thus, osteopontin may increase the malignant abilities of cancer cells in part via integrin-mediated induction of uPA and interactions with uPA/uPAR. On the other hand, down-regulation of uPAR has been linked to the dormancy of tumor cells *in vivo* (142). Accordingly, there is a possibility that binding and activation of specific cell surface integrins by osteopontin could take tumor cells out of dormancy by promoting interactions between integrin and uPAR.

In other cell types, osteopontin has also been found to induce the expression and activity of other proteases such as members of the metalloproteinase family, which can contribute to metastasis via multiple mechanisms (135-137, 143, 144). These studies provide evidence that osteopontin may play a critical role in tumor cell regulation of matrix proteolysis, e.g., in invasion and metastasis.

The Role of Osteopontin in Angiogenesis. Angiogenesis is the formation of new blood vessels that allows for sustained growth and metastasis of tumor cells. This complex process requires the coordinated action of growth factors and their receptors, extracellular proteins, adhesion molecules, and proteolytic enzymes (145-148).

Some studies have reported an implicated osteopontin in angiogenesis, nevertheless, many of the results remain circumstantial and further clarification on the details of this possible role are required. The association of osteopontin with this process is a consequence of its ability to bind the $\alpha_v\beta_3$ integrin, which in turn, is a marker of angiogenesis and is expressed by neovascular endothelial cells (86).

Brooks and coworkers (149, 150) found that $\alpha_v\beta_3$ integrin expression increases during angiogenesis, and that by blocking this integrin, angiogenesis can be inhibited. Moreover, a role for the $\alpha_v\beta_3$ integrin in signaling the survival and differentiation of vascular cells during angiogenesis *in vivo* was shown. Additionally, this integrin and osteopontin have been found to be significant in vascular repair and regeneration, as osteopontin can stimulate the adhesion and migration of endothelial cells, and $\alpha_v\beta_3$ and osteopontin are simultaneously up-regulated following vascular damage (86).

A role in protecting endothelial cells from apoptosis has been reported for osteopontin, possibly via the activation of nuclear factor κB (151). Furthermore, osteoprotegerin expression, a tumor necrosis factor receptor, induced by the interaction between osteopontin and $\alpha_v\beta_3$, has been found to protect endothelial cells from apoptosis (152). The increased endothelial cell survival promoted by osteopontin supports the association of osteopontin with malignancy. In addition, osteopontin contributes to angiogenesis through effects on the expression of vascular endothelial growth factors (153). Endothelial cell migration is stimulated by the cooperation between the vascular permeability factor (vascular endothelial growth factor) with osteopontin and $\alpha_v\beta_3$ integrin. Although the expression of vascular endothelial growth factor, osteopontin, and integrin $\alpha_v\beta_3$ has been related with angiogenesis in glioblastomas (154), and has been associated with poor prognosis in patients with in stage I lung adenocarcinoma (155), any clinical role for osteopontin in angiogenesis remains to be clarified.

The Role of Osteopontin on Tumor Cell Survival. Besides the possible contributions of osteopontin to the metastatic phenotype presented above, osteopontin has also been shown to exert a role in cancer by enhancing the survival of several cell types, through interactions with various host defense systems. The affected cells include tumor cells, vascular endothelial cells, or tumor-infiltrating cells of the immune system, and these effects might have conflicting influences on the malignancy and growth of a tumor. An example may be the case when osteopontin indirectly favors the survival of tumor cells via macrophage liaison. Interactions between osteopontin and $\alpha_v\beta_3$ integrins were found to affect the nitric oxide production by macrophages (72, 156). Produced by a number of different cell types, including activated macrophages and vascular endothelial cells, nitric oxide can act as a powerful signaling molecule, as well as causing localized cytotoxicity. Although nitric oxide is effective against microbial invaders and tumor cells, osteopontin was found to inhibit its synthesis (157-161), and therefore, plays an important role in tumor defenses against the immune system (156, 157). Moreover, nitric oxide has been reported to have tumor-promoting effects, however, its role in malignancy is far from being fully understood (162-164). Osteopontin production by tumor cells could promote tumor growth and metastasis by protecting them from nitric oxide (156, 158). In this way, osteopontin-producing tumor cells would be favored for growth, relative to tumor cells that did not produce osteopontin. Nevertheless, tumor cells that secrete osteopontin might promote their own destruction by attracting host inflammatory cells, such as macrophages that can be cytotoxic to the tumor cells (49, 78). Thus, the interactions of osteopontin with various aspects of host defense systems, might in some cases, lead to opposing effects on tumor growth and survival.

The Significance of Osteopontin in Human Cancer

At present, it is fully accepted that osteopontin expressed by tumor cells alters their malignant properties, specifically by affecting their ability to grow, invade, and metastasize. However, as osteopontin is known to be expressed in both normal and malignant tissues, an elucidation of its significance in human cancer is required. Recent studies suggest that osteopontin levels in the blood or tumors of patients with cancer may provide useful clinical information on patient prognoses.

The Occurrence of Osteopontin in Human Tumors. The expression of osteopontin in human tumors was initially shown in several human carcinomas by Brown and coworkers (27). Considerably higher levels of osteopontin mRNA were found in all tumors screened (colon, breast, lung, stomach, endometrium, and thyroid) as compared with corresponding normal tissues. Nevertheless, two examples of benign tumors (colonic adenomas from a patient with familial polyposis and a uterine leiomyoma) showed similar osteopontin mRNA levels compared with normal tissues. Moreover, cells that were positive for osteopontin transcripts were most abundant at the advancing edge of tumors and near areas of necrosis. Supporting these findings, other studies also showed osteopontin mRNA and protein overexpression in several cancers, such as lung (165), breast (166) and esophageal cancers (167), gastric cancers (168), prostate cancers (169), and gliomas (170). Osteopontin expression in tumors has been identified by immunohistochemistry, specifically localized in the macrophages in some tumors, and in both tumor cells and macrophages in others (171).

The significance of osteopontin from different sources within a tumor is poorly understood, although prognostic studies in breast cancer (166) suggest that this may be important for the biology of the tumor. Several researchers have shown the presence of osteopontin in microcalcifications in breast tumor tissues (172, 173) and in ectopic calcification in other tumors, such as serous papillary cystadenocarcinoma of the ovary, meningiomas, papillary carcinoma of the thyroid, and pilomatricomas (174-176).

Tumor Aggressiveness and the Occurrence of Osteopontin in Primary Tumors. Osteopontin expression in some tumors that can be detected in both tumor cells and several host cells has triggered an emerging interest in its potential usefulness as a marker of tumor aggressiveness and patient prognosis.

The potential to predict a poor patient prognosis based on osteopontin overexpression was first reported by Chambers and coworkers (165). Osteopontin expression was followed both in lung tumor samples and normal tissue. The results pointed to osteopontin expression in tumor samples and negligible expression in normal tissues. The osteopontin protein was localized by immunohistochemistry to both lung tumor cells and tumor-associated macrophages. The relation of osteopontin expression in tumors and poor patient survival was found to have statistical significance. Results obtained by Shijubo and coworkers (155) also suggest that osteopontin overexpression in lung tumors might be an indicator of poor prognosis.

The association of osteopontin with breast tumor progression was studied by Tuck and coworkers (177) using samples from a patient who had bilateral mammary carcinomas of similar histology that later developed metastatic recurrence. The tumor in the right breast had spread to the lymph nodes, whereas the left-sided tumor had not. The patient later developed right-sided local recurrence followed by widespread metastatic disease. The findings from this case suggest that osteopontin, both in tumor cells and in plasma, may be a marker for tumor aggressiveness in breast cancer, and elevated levels in a primary tumor may predict for future development of metastasis. To confirm these results, the same group studied the expression of osteopontin mRNA and protein in the tumors of 154 women with lymph node–negative breast cancer (166). It was found that immunohistochemical staining for osteopontin protein was increased in the infiltrating macrophages and lymphocytes of 70% of the tumors, a proportion too high to be discriminatory in predicting patient survival. However, osteopontin staining localized specifically to the tumor cells was shown in 26% of the tumors. Osteopontin mRNA was detected in tumor cells and in inflammatory cells, indicating that both cell types could be a source for osteopontin detected within tumors. This study supports the idea that osteopontin levels within tumor cells may be a useful predictor of patient outcome in breast cancer, and also that osteopontin may play a functional role in tumor progression and aggressiveness. A correlation between osteopontin expression and an increased invasiveness or metastatic potential has also been reported in other human tumors (168, 170).

Osteopontin expression was detected in prostate cancer, being expressed by the tumor cells themselves (169, 178). Nevertheless, there is some controversy over whether osteopontin expression was associated with malignancy of the prostate carcinoma (169) because in one study, evidence was also found for the overexpression of osteopontin in benign glandular hyperplasia of the prostate (178). Likewise, in ovarian cancer, the role of osteopontin in malignancy is unclear, and although some studies point to a positive correlation of osteopontin with ovarian cancer progression (26), others show contradictory conclusions depending on the tumor-malignant potential (179). Tiniakos and coworkers found that osteopontin expression was higher in tumors of low malignant potential as compared with benign ovarian tissue; however, invasive carcinomas showed generally lower osteopontin levels (179). Liapis and coworkers (180) discussed

a possible explanation, as they found that ovarian tumors of low malignant potential underexpress the $\alpha_v\beta_3$ integrin, to which osteopontin can bind, in comparison with invasive ovarian carcinomas. Therefore, the absence of $\alpha_v\beta_3$ integrin expression in tumors of low malignant potential may account for the lack of responsiveness to the malignancy-promoting effects of osteopontin, even in the presence of higher levels of osteopontin. On the other hand, as the primary mechanism of the spreading of ovarian carcinoma is different from other cancers, it is possible that osteopontin plays different roles in cancers that spread by different routes.

The Meaning of Osteopontin in the Blood of Patients with Cancer. Recently, several studies have pointed to the potential of osteopontin to provide clinical information useful in the management of patients with breast and perhaps other cancers. Osteopontin serum levels were found to be increased 4- to 10-fold in a variety of human disseminated carcinomas, including breast, lung, and prostate (28, 181); and these higher levels were correlated with higher tumor grade.

An antigen-capture ELISA, using a combination of mouse monoclonal and rabbit polyclonal antibodies, was developed in order to assess osteopontin blood levels (182, 183). This methodology permits quantitative, rapid, and reproducible measurement of osteopontin levels in blood plasma and other fluids. Studies with women volunteers showed that osteopontin levels did not differ between premenopausal and postmenopausal women (182, 183). Moreover, it was found that although osteopontin can be hormonally regulated in some tissues (58, 184), osteopontin blood levels do not reflect hormonal changes over the menstrual cycle. After establishing the basal levels of osteopontin in healthy women, Singhal and coworkers (184) used the same assay to test for an association between elevated blood osteopontin levels and patient outcome in breast cancer. Plasma osteopontin levels were measured in patients with metastatic breast cancer, control patients (women on follow-up after treatment for primary breast cancer, with no evidence of disease), and healthy patients. Elevated plasma osteopontin was found to be associated with a shorter survival, larger numbers of sites of metastatic involvement, and poorer outcome for women with metastatic breast cancer.

Breast Cancer Biomarkers

Regardless of the recent spectacular advances in molecular medicine, genomics, proteomics, and translational research, mortality rates for the most prevalent cancers have not been significantly reduced (14, 15, 17). Some of the best available options to combat cancer include primary prevention, earlier diagnosis, and improved therapeutic interventions. We are now witnessing the development of new drugs against cancer that are based on rational instead of empirical designs. Hopefully, some of these drugs will prove to be more effective at the clinic than older generations of medicines. In terms of primary prevention, we still don't have any robust strategies because although the major mechanisms underlying both cancer initiation and progression are well established, they are extremely complex. These processes are genetic, and epigenetic processes leading to mutations in several genes and alterations in chromosomal structure are likely accompanied by self-perpetuating changes in signal transduction pathways.

One of the best strategies to combat cancer is by early diagnosis and administration of effective treatment (185). Another approach includes close monitoring of the cancer patient after initial treatment (usually surgery) to detect early relapse, and then, additional prescribed therapy. A third valuable approach would be the stratification of patients into subgroups that respond better to different types of treatment (individualized therapy). Medical imaging and serum or tissue biomarkers are valuable tools for monitoring these patients in order to optimize clinical outcomes.

A handful of cancer biomarkers, such as prostate-specific antigen, breast and ovarian cancer susceptibility genes (*BRCA1* and *BRCA2*) for example, are currently used routinely for population screening, disease diagnosis, prognosis, monitoring of therapy, and prediction of therapeutic response. Nevertheless, it is important to notice that most of the biomarkers used nowadays haven't got adequate sensitivity, specificity, and predictive value for population screening. Biomarkers are clinically recommended mainly for monitoring the effectiveness of therapeutic interventions. Some biomarkers are also invaluable tools for the early diagnosis of cancer relapse, which may trigger additional treatments before the appearance of clinical symptoms. With current cancer biomarkers, much is left to be desired in terms of clinical applicability. We need new cancer biomarkers that will further enhance our ability to diagnose, prognose, and predict therapeutic response in many types of cancer. Because biomarkers can be analyzed relatively noninvasively and economically, it is worth investing in discovering more biomarkers in the future.

Breast cancer statistics show that >1.2 million persons will be diagnosed with breast cancer worldwide this year, according to the WHO. According to the American Cancer Society, an estimated 212,920 new cases of invasive breast cancer are expected to occur among women in the U.S. during 2006. As breast cancer is the most frequently diagnosed cancer in women, it is imperative that new alternative tumor markers be developed. Several breast cancer markers have been studied, although just a few proved to be effective (10, 11). For example, serum tumor marker levels, such as carcinoembryonic antigen and others, may reflect disease progression and recurrence, but have not proven to be sensitive for early disease detection (186). Recently, mammaglobin and maspin have been described as potential markers of early breast cancer as well as to detect occult metastasis (187-189). Estrogen and progesterone receptors have been used as markers of prognosis and predictors of response to antiestrogen therapy, and are established as a standard of care for patients with breast cancer (190-192). In addition, cell cycle markers (e.g., Ki-67; ref. 193), growth factors and receptors (e.g., HER2; ref. 9), tumor suppressor genes (e.g., p53; ref. 194), and cell adhesion molecules (e.g., E-cadherin and P-cadherin; refs. 195-198) have been studied as possible breast tumor markers. In this context, osteopontin also represents an interesting alternative as a breast cancer marker. Using a cDNA microarray system, it was found that osteopontin was overexpressed in the tumors and serum of women with a recent diagnosis of ovarian cancer (25). Additionally, Kim et al. (26) showed that osteopontin concentrations in plasma were higher in patients with ovarian cancer as compared with healthy controls, or women with benign ovarian disease or other gynecologic cancers, thus an association between levels of osteopontin and ovarian cancer suggest that future research assessing its clinical usefulness would be worthwhile (199).

Recent studies have shown that osteopontin overexpression is also related with breast cancer evolution and metastasis (23); therefore, there is a potential utility of osteopontin in monitoring the disease status of patients with breast cancer.

Conclusions

A host of interesting advances in molecular medicine, genomics, and proteomics have led to the discovery of several potential tumor markers. This review focuses on the functional roles and clinical significance of osteopontin in cancer and metastasis, and its potential as a biomarker. Osteopontin seems to be more than just a marker of malignancy because this protein may play a functional role in malignant-gene

expression and/or cancer cell behavior. Multiple and complex mechanisms are involved in the role of osteopontin in cancer, including interactions with cell surface receptors, growth factor/receptor pathways, and proteases; therefore, much remains to be learned about these mechanisms and the functional contributions of osteopontin produced by different cell types in order to establish appropriate antiosteopontin therapeutic strategies. Several possible therapeutic approaches to interfere with the malignancy-enhancing effects of osteopontin, thereby reducing tumor cell growth and metastasis, are being developed. As discussed, one of the best strategies to combat cancer is by early diagnosis and administration of effective treatment. In spite of the number of cancer biomarkers currently used routinely, their usefulness still remains limited due to the lack of adequate sensitivity, specificity, and predictive value for population screening. Additional clinical trials are required to validate the use of biomarkers in order to establish their efficacy and enhance our ability to diagnose, prognose, and predict therapeutic responses. Some studies have established an association between elevated osteopontin levels in patients' tumors or blood with a poor diagnosis; hence, it could represent a tumor marker for use in the breast cancer arena waiting to be fully exploited.

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