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Increased Expression of Osteonectin and Osteopontin, Two Bone Matrix Proteins, in Human Breast Cancer

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Microcalcifications are a common phenomenon associated with breast cancer and are often the only mammographic sign of a malignant breast disease. Although microcalcifications are not restricted to breast cancer and can be also associated with benign lesions, it is noteworthy that they are composed exclusively of hydroxyapatite in breast carcinoma. Hydroxyapatite is the bone-associated phosphocalcic crystal the deposition of which in bone tissue requires the coordinated expression of several molecules such as osteonectin (OSN) and osteopontin (OPN), synthesized by cells of the osteoblastic lineage. In this study, we evaluated the expression of these two bone matrix proteins, using an immunoperoxidase technique and specific antibodies, in 79 breast lesions including 28 benign and 51 cancerous specimens. We found that normal mammary tissue associated with the lesions examined expressed generally undetectable or lightly detectable (0 or 1+) amounts of OSN and OPN (92 and 81%, respectively). Benign breast lesions, including fibroadenoma and fibrocystic dysplasia, were generally weakly stained (0 or 1+) with both anti-OSN and anti-OPN antibodies (96.4 and 60.7%, respectively). Interestingly, the majority of both in situ and invasive breast carcinoma lesions showed a strong expression (2+ or 3+) for OSN or OPN (74.5 and 84.3%, respectively). High expression of these two bone matrix proteins was associated with frequent microcalcification deposition in the lesion. This study is the first extensive study of OSN and OPN expression in mammary cancers. Our data suggest that OSN and OPN could play a role in the formation of ectopic microcalcifications often associated with breast cancer. It is also tempting to speculate that the expression of these two glycoproteins by breast cancer cells play a role in the preferred bone homing of breast metastases. (Am J Pathol 1995, 146:95–100)

The frequent deposition of hydroxyapatite crystals in primary breast cancers and the preferred homing of mammary metastases in the skeleton is a phenomenon that has not yet been clearly elucidated. Development of bone metastases is very frequent in breast malignant disease and is of poor prognosis. Recently, we have demonstrated that human breast cancer cells express bone sialoprotein (BSP), a bone matrix protein that plays a key role in the initiation of bone matrix mineralization.[1] The biomineralization process whereby bone matrix is laid down and mineralized is not yet completely understood. This process involves several molecules expressed by cells of the osteoblastic lineage.[2] Beside BSP, osteonectin (OSN) and osteopontin (OPN) are probably the best known bone phosphoproteins.[3,4] These glycoproteins share calcium-binding properties and hydroxyapatite affinity. It has been proposed that the steric arrangement of their phosphate groups is optimal for the binding of calcium and for the subsequent formation of apatite crystals during bone matrix mineralization.[5] Both OSN and OPN are able to bind type I collagen suggesting that they may link the mineral phase to the collagen matrix.[6,7] Interestingly, OPN contains an Arg-Gly-Asp sequence that binds to a family of cell surface receptors called integrins.[8,9] Be-

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cause of the presence of this sequence, it is probable that abnormally high expression of OPN by tumor cells has important consequences for interactions between tumor cells and the host tissue matrix.

After our initial observation that breast cancer cells express BSP, it became of interest to examine whether other bone matrix proteins such as OSN and OPN could also participate in the formation of microcalcifications associated with breast tissue lesions. We have studied the immunoreactivity of breast cancer tissue to OSN and OPN. Our data suggest that ectopic mineralization observed in breast tissue could result, at least in part, from the deposition of noncollagenous proteins involved in physiological matrix mineralization.

Materials and Methods

Tissue Specimens

Seventy-nine breast lesion specimens fixed in formalin, cut into fine sections, and embedded in paraffin were provided by Dr. M. Nadji (Jackson Memorial Hospital, Miami, FL). The human tissues examined included 14 fibroadenomas, 25 *in situ* carcinomas, and 26 infiltrating carcinomas, 11 with negative lymph nodes and 15 with positive lymph nodes. Adjacent normal tissue was examined when possible. Pathological reports were available for each specimen. Paraffin sections of placental membranes containing trophoblast tissue were used as positive control because this tissue has been previously shown to express OSN[10] and OPN[11].

Immunohistochemistry

OSN and OPN were identified by the avidin-biotin peroxidase complex method[12] with two rabbit polyclonal antibodies, BON54 and LF19, kindly provided by Dr. L. W. Fisher (Bone Research Branch, National Institute of Dental Research, NIH). BON54 was raised against the 27–56 residue sequence of bovine bone OSN and LF19 was raised against the 1–20 sequence of human bone OPN. These antibodies have been checked for reactivity by Western blotting and shown to react with the appropriate molecules. Immunohistochemistry was performed with the ABC Vectastain Elite kit (Vector Laboratories, Burlingame, CA) according to the supplier's protocol. Briefly, tissue sections were deparaffinized in xylene and hydrated in phosphate buffered saline (10 mmol/L sodium phosphate, 0.9% NaCl solution, pH 7.5). The blocking of endogenous peroxidase was performed with 0.3% H_2O_2 in methanol and the nonspecific serum-binding sites were blocked with normal goat serum (1:20, Vector). Either anti-OSN BON54 or anti-OPN LF19, at a dilution of 1:500 and 1:200 respectively, were applied and incubated for 2 hours at room temperature. The tissue sections were then incubated with biotinylated goat anti-rabbit antibody (1:200) followed by exposure to preformed streptavidin-biotinylated horseradish peroxidase complex. Peroxidase was revealed by the 3,3′-diaminobenzidine tetrahydrochloride reaction.[13] Finally, sections were counterstained with hematoxylin, dehydrated, and mounted. Controls included omission of the primary antibody and antigen absorption tests. Blocking experiments were carried out by incubating the purified corresponding peptides in molar excess with the working dilution of the antibody and subsequent use in immunostaining.

Evaluation of Staining

The immunohistochemically stained sections were reviewed by two independent observers. The degree of staining was designated by an arbitrary semiquantitative scale: 0, negative; 1+, focal areas with sparse staining or occasional individual positive cells; 2+, at least one focus with extensive staining or numerous areas with weak to moderate staining; or 3+, extensive staining of more than 50% of the neoplastic cells.

Statistical Analysis

To determine whether the increased expression of OSN and OPN observed in breast carcinoma compared with normal or benign breast tissue was statistically significant we used the χ^2 test.

Results

Trophoblast tissue, used as a positive control, was specifically stained as expected with anti-OSN and anti-OPN antibodies (data not shown). Immunostaining was mainly cytoplasmic with both antibodies. No staining was seen in control sections without the first antibody and the immunoreactivity was completely abrogated by preincubation of the first antibody, LF19 or BON54, with the corresponding purified peptide. To study OSN and OPN expression in normal and cancerous breast tissues, 79 paraffin-embedded tissue sections were stained for immunoreactivity to either OSN or OPN antibodies. Semiquantitative grading of the immunohistochemical reactions observed was done according to an arbitrary scale described in *Materials and Methods*. Tables 1 and 2 summarize the results obtained by evaluating OSN and OPN expression in the specimens studied. Of the 79 cases studied, we were able to analyze normal mammary epi-

Table 1. *Expression of OSN in Human Breast Tissue*

Breast specimen	n*	Degree of immunoreactivity (%)			
		0	1+	2+	3+
Normal	26	23 (88.5)†	1 (3.8)	2 (7.7)	0 (0)
Fibroadenoma	14	10 (71.4)	4 (28.6)	0 (0)	0 (0)
Fibrocystic dysplasia	14	7 (50)	6 (42.8)	1 (7.2)	0 (0)
In situ carcinoma	25	2 (8)	3 (12)	12 (48)	8 (32)
Infiltrating carcinoma with negative lymph nodes	11	1 (9.1)	1 (9.1)	5 (45.4)	4 (36.4)
Infiltrating carcinoma with positive lymph nodes	15	1 (6.7)	5 (33.3)	5 (33.3)	4 (27)

* n is the number of specimens analyzed in each category.
† Numbers in parentheses indicate the percent of specimens in each category.

Table 2. *Expression of OPN in Human Breast Tissue*

Breast specimen	n*	Degree of immunoreactivity (%)			
		0	1+	2+	3+
Normal	21	10 (47.6)†	7 (33.3)	4 (19)	0 (0)
Fibroadenoma	14	4 (28.6)	3 (21.4)	4 (28.6)	3 (21.4)
Fibrocystic dysplasia	14	7 (50)	3 (21.4)	3 (21.4)	1 (7.2)
In situ carcinoma	25	1 (4)	2 (8)	8 (32)	14 (56)
Infiltrating carcinoma with negative lymph nodes	11	0 (0)	1 (9.1)	2 (18.2)	8 (72.7)
Infiltrating carcinoma with positive lymph nodes	15	0 (0)	4 (26.7)	4 (26.7)	7 (46.7)

* n is the number of specimens analyzed in each category.
† Numbers in parentheses indicate the percent of specimens in each category.

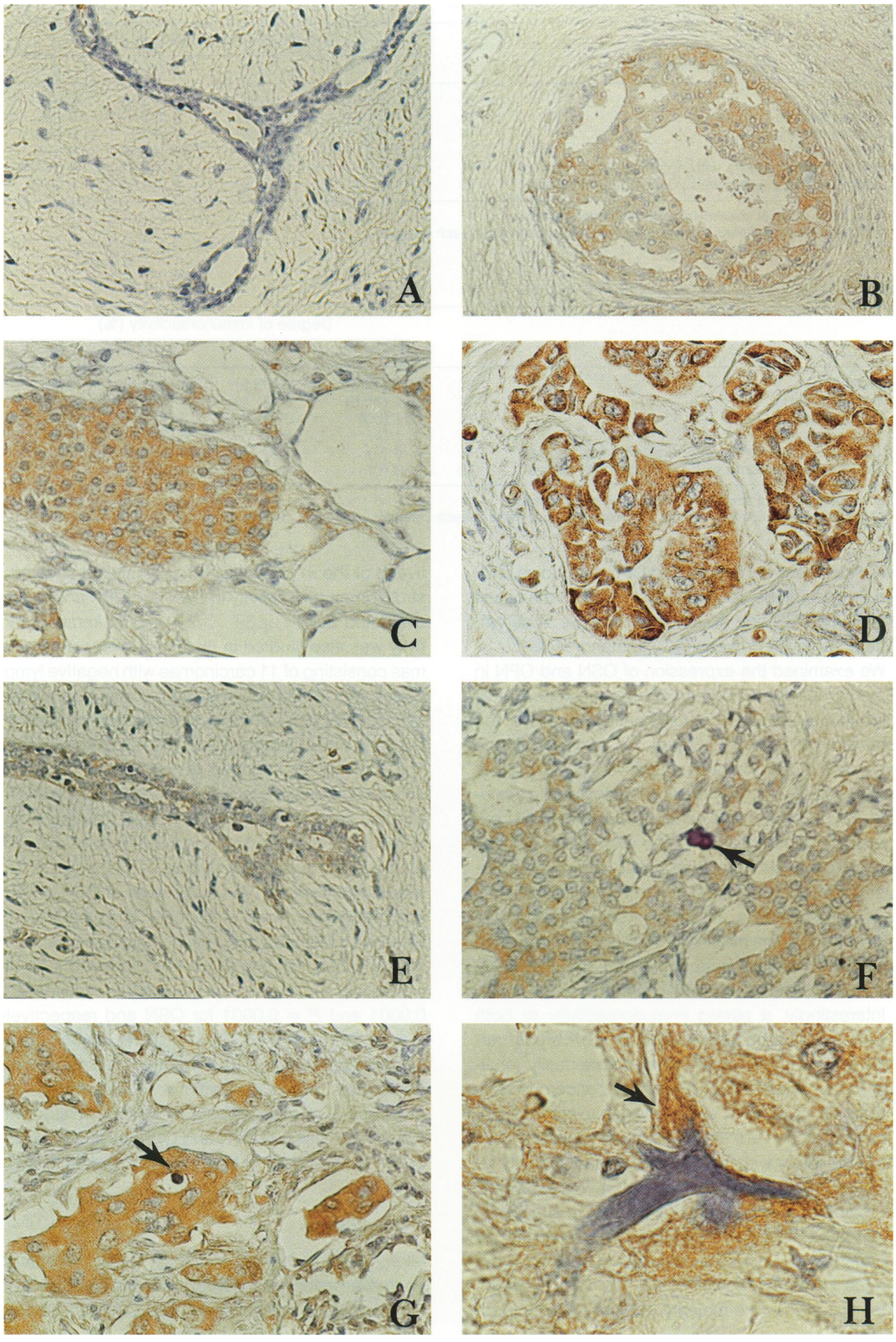
thelial cell reactivity to OSN and OPN antibodies in 26 and 21 specimens, respectively. In most of the cases (92.3% for OSN and 80.9% for OPN), we observed undetectable (0) or weak (1+) immunoreactivity.

We examined the expression of OSN and OPN in 28 benign lesions including 14 fibroadenomas and 14 fibrocystic dysplasias. For OSN antibody, most of the fibroadenomas (71.4%; Figure 1A) and 50% of the fibrocystic dysplasias were negative. All of the other benign specimens showed an immunostaining evaluated as 1+ or 2+ and none exhibited an OSN expression evaluated as 3+. The expression of OPN was generally stronger than OSN. Half of the fibroadenomas studied were 0 or 1+ (Figure 1E) and the other half showed a 2+ or 3+ immunoreactivity to OPN antibody. The majority (71.4%) of the fibrocystic dysplasias studied were unstained (0) or weakly stained (1+) and the remaining cases were 2+ or 3+.

Interestingly, a strong immunoreactivity to both OSN and OPN antibodies was observed in breast carcinoma. The immunostaining was essentially localized to the cytoplasm of mammary cancerous cells (Figure 1B, C, D, F, G, and H). When microcalcifications were observed, they were usually present in areas of the tumor with the highest immunoreactivity for the bone matrix proteins (Figure 1H). We examined the expression of OSN and OPN in 25 *in situ* breast carcinomas. This group included 5 lobular and 20 ductal *in situ* carcinomas. We did not observe a difference in the expression of OSN or OPN between these two types. The majority (80 and 88%, respectively) of the *in situ* carcinomas analyzed showed a strong immunoreactivity to OSN and OPN antibodies, evaluated as 2+ or 3+. OSN or OPN immunoreactivity was also studied in 26 invasive breast carcinomas consisting of 11 carcinomas with negative lymph nodes and 15 with positive lymph nodes. The expression of OSN and OPN was not significantly different between these two groups of infiltrating carcinomas. The majority of negative lymph nodes (81.8 and 90.9%) and positive lymph nodes (60 and 73.3%) infiltrating carcinomas studied showed a strong immunoreactivity evaluated as 2+ or 3+ to OSN and OPN antibodies, respectively (Figure 1C, D, and G). The association between the expression of OSN or OPN and malignancy was statistically highly significant when we compared the immunostaining observed in normal breast tissue with the one observed in *in situ* carcinoma and invasive carcinoma (respectively, $P = 0.0001$ and $P = 0.0001$ for OSN and respectively, $P = 0.0019$ and $P = 0.0003$ for OPN). Among the 51 carcinomas studied, 16 presented microscopically detectable microcalcifications. Interestingly, the presence of microcalcifications was associated with a high expression of OPN and OSN, evaluated as 2+ or 3+ in 15 and 13 of these lesions, respectively.

Discussion

Microcalcifications are the most easily detectable anomalies at the mammography exam. Nearly 40% of breast malignant lesions are associated with the pres-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ence of radiologically detectable microcalcifications.[14] Little is known about microcalcification appearance in mammary tissue but it is remarkable that they are always formed of hydroxyapatite crystals when associated with malignant breast lesions. In bone, the deposition of hydroxyapatite during physiological mineralization appears to be initiated by the expression of noncollagenous proteins. Numerous noncollagenous bone matrix proteins have been identified and several have been extensively studied.[2] However, none has had its precise biological role determined yet. Phosphoproteins, one of the major constituents of the noncollagenous proteins are considered to play an important role in the initiation and regulation of mineralization. OSN and OPN are phosphorylated glycoproteins of bone that show a high affinity for calcium and hydroxyapatite.[6,7] Using specific polyclonal antibodies directed to OSN and OPN, we demonstrated low but detectable OSN and OPN immunoreactivity in normal breast epithelial cells and in benign breast lesions. A significant increase in OSN and OPN expression was detected in both *in situ* and invasive breast carcinomas when compared with normal breast tissue and benign breast lesions. These findings are of interest in light of our recent finding that human breast cancer cells express high levels of bone sialoprotein.[1] The extensive homology found between bovine matrix OSN and murine SPARC (secreted protein acidic rich in cysteine) or BM-40 protein from murine basement membrane-producing Engelbreth-Holm-Swarm tumor, indicate that OSN could also be involved in cell proliferation and *de novo* synthesis of basement membranes observed in decidua of early pregnancy and carcinomas.[10] The significance of increased expression of OSN in cancerous breast epithelial cells is not known yet. We propose that the presence of large amounts of this bone matrix protein, postulated to play a crucial role in bone matrix assembly, could contribute to the deposition of hydroxyapatite microcalcifications in mammary lesions. Indeed, we observed a strong OSN expression, evaluated as 2+ or 3+, in the majority of the lesions presenting microcalcifications. However, the number of specimens showing microcalcifications was insufficient to make a statistical analysis. Histologically, the microcalcifications were usually associated with areas of the cancer lesion with the strongest reactivity with anti-OSN antibodies.

OPN expression was also investigated in this study and we observed a low immunoreactivity in normal breast epithelial cells as previously described by Senger et al.[15] As for OSN, benign breast lesions exhibited a low expression of OPN, similar to the one detected in normal tissue, except that some fibroadenomas presented a strong immunoreactivity evaluated as 2+ or 3+. These variations could be due to the different hormonal status of the patient at the time of the biopsy. Indeed, OPN expression is modulated by steroid hormones and growth promoters.[5] Analysis of malignant breast lesions for OPN immunoreactivity revealed that it is highly expressed in both *in situ* and invasive carcinoma. Independent evidence connecting OPN expression with carcinogenesis comes from the observation that OPN is closely related or identical to a phosphoprotein secreted in elevated amounts by many mammalian transformed fibroblasts and epithelial cells.[15,16] Transformation of NIH3T3 cells with the T24-H-*ras* oncogene results in an increased OPN secretion[16] and in the acquisition of high metastatic potential.[17] The role of OPN in transformation is strongly supported by the recent demonstration that *ras*-transformed NIH3T3 cells transfected with antisense OPN showed a reduced tumorigenicity.[18] Furthermore, metastatic *ras*-transformed NIH3T3 cells adhere and spread on OPN better than their normal nontransformed counterparts.[19] Interestingly, preliminary studies have demonstrated that several breast cancer cell lines attach to bone matrix proteins such as collagen type I, BSP, and OPN in attachment assays.[20] Altogether, these observations suggest that OPN could play, beside its participation in tissue mineralization, an important role in targeting and mediating the attachment of circulating tumor cells to the areas of ossification. It has been shown that osteopontin mediates the interaction between the bone and osteoclasts and potentiates bone resorption through the integrin $\alpha_v\beta_3$.[21] The skeleton is a common site of metastasis formation in breast cancer patients. It is tempting to speculate that high expression of this protein in mammary carcinoma cells could play a role in the attachment of breast cancer cells to the bone matrix.

Figure 1. *Detection of OSN* (**A**, **B**, **C**, *and* **D**) *and OPN* (**E**, **F**, **G**, *and* **H**) *in human breast lesions. Paraffin-embedded tissue sections were immunostained with specific polyclonal antibodies and counterstained with hematoxylin as described in Materials and Methods.* **A:** *Fibroadenoma* (*staining score, 0*). **B:** In situ *carcinoma* (*staining score, 1+*). **C:** *Infiltrating carcinoma* (*staining score, 2+*). **D:** *infiltrating carcinoma* (*staining score, 3+*). **E:** *Fibroadenoma* (*staining score, 1+*). **F:** *Infiltrating carcinoma showing microcalcifications* (*arrow; staining score, 2+*). **G:** *Infiltrating carcinoma showing microcalcifications* (*arrow; staining score, 3+*). **H:** *Large microcalcifications adjacent to cancer cells with strong OPN immunoreactivity* (*arrow*) *in an infiltrating ductal carcinoma* (*staining score of the lesion, 3+*). **A**, **B**, *and* **E**, *magnification* ×*200;* **C**, **D**, **F**, *and* **G**, ×*400; and* **H**, ×*630.*

We have now demonstrated that human breast cancer cells express three bone matrix proteins: BSP, OPN, and OSP. This ectopic secretion of proteins, involved primarily in bone tissue mineralization, by mammary cancers could provide a key in the understanding of the complex process that leads to hydroxyapatite crystallization in breast cancer as well as preferred bone homing of circulating mammary metastatic cells. Elucidating the molecular bases responsible for the expression of these proteins in breast cancer could lead to effective ways to predict the tendency of a mammary tumor to metastasize in the skeleton and hopefully to impair this process.

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