Expression of Bone Sialoprotein, a Bone Matrix Protein, in Human Breast Cancer

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Abstract

Micronodifications are often associated with human mammary lesions, particularly with breast carcinomas. To date, the molecular mechanism that leads to the deposition of hydroxyapatite in the mammary tissue has not been elucidated. Bone sialoprotein (BSP) is a glycoprotein the expression of which coincides with the appearance of the first hydroxyapatite crystals during bone development. In this study, we report the observation that BSP, a bone matrix protein, is expressed in human mammary cancer cells. Using an immunoperoxidase technique, we studied the expression of BSP in 79 breast lesions, including 28 benign and 51 malignant specimens. Two polyclonal antibodies, one directed against intact human BSP and the other against a synthetic peptide of BSP (residues 277–294), were used and gave identical results. Normal mammary glands expressed undetectable or barely detectable amounts of BSP, and the majority of the benign lesions examined were generally unstained (0) or weakly stained (1+). Most of the breast carcinoma specimens (around 87%) showed a significant increase (P = 0.0001) in BSP expression. Breast carcinomas with micronodifications had the highest immunoreactivity (2+ or 3+) to BSP antibodies. This is the first demonstration that BSP expression is significantly increased in breast cancer. Expression of BSP by breast cancer cells could play a major role in the deposition of micronodifications and in the preferred bone homing of breast cancer cells.

Introduction

Micronodifications are a common radiological and histological finding in human breast carcinomas (1). However, they are not restricted to malignant breast diseases but can be also detected in benign breast lesions. Pathologists have described two types of micronodifications: (a) type 1, due to calcium oxalate and are found mainly in benign lesions; (b) type 2, composed of hydroxyapatite and seen in either benign or malignant breast lesions (2, 3). Hydroxyapatite is the basic calcium phosphate found in mature bones and teeth (4). The mineralization of these tissues occurs by deposition of carbonated hydroxyapatite crystals in an extracellular matrix consisting of type I collagen and a variety of NCPs (5). The NCPs, composing about 10% of the organic bone matrix, are proteoglycans, glycoproteins, and γ-carboxyglutamic acid-containing proteins (5). Among these, bone sialoprotein, osteonectin, and osteopontin are three glycoproteins that have recently been the focus of intense investigation because they may play an important role in the initiation and regulation of mineralization in bone tissue.

BSP is a phosphorylated sialoprotein very rich in sialic acid (15% of total carbohydrate) (6) that contains several clusters of up to 10 consecutive glutamic acid residues (7). These negatively charged domains, together with the sialic acid residues and tyrosine sulfate groups, are presumably responsible for the strong interaction of BSP with hydroxyapatite. An analysis of BSP mRNA and protein distribution shows that BSP is relatively restricted in expression compared to other noncollagenous bone matrix proteins like osteonectin or osteopontin (5). BSP has been found in mature osteoblasts, in osteoclasts, and in hypertrophic chondrocytes (8). However, BSP, which is primarily a product of cells producing mineralized matrix can, under certain circumstances, be produced by human decidua and trophoblast cells (9). To date, no study has reported the detection of BSP in cancer. It is interesting to note that expression of other NCPs such as osteonectin and osteopontin have been detected in a variety of malignant lesions (10, 11). In this study, we investigate the possibility that BSP could be expressed in human breast cancer cells.

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. Nadj (Jackson Memorial Hospital, Miami, FL). The human tissues examined included: 14 fibroadenomas; 14 fibrocystic dysplasias; 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. Paraffin sections of placental membranes containing trophoblast tissue were used as positive control. Pathological reports were available for each specimen.

Immunohistochemistry. Bone sialoprotein was identified by the avidin-biotin peroxidase complex method (12) using two rabbit polyclonal antibodies, LF83 and LF6, kindly provided by Dr. P. Gehron Robey (Bone Research Branch, National Institute of Dental Research, NIH). LF83 was generated using a synthetic peptide of human bone sialoprotein (residues 277–294) (13), and LF6 was raised against intact denatured human BSP (14, 15). Both antibodies have been previously checked for reactivity by Western blotting and shown to react only with BSP. The specificity of these antisera was determined by blocking experiments and the antisera have been characterized for immunohistochemistry application (9, 16). Immunoperoxidase was performed using the ABC Vectastain Elite kit (Vector Laboratories) according to the supplier’s protocol. Briefly, tissue sections were deparaffinized in xylene and hydrated in phosphate-buffered saline (10 mm sodium phosphate-0.9% NaCl, pH 7.5). The blocking of endogenous peroxidase was performed with 0.3% H2O2 in methanol and the nonspecific serum-binding sites were blocked with normal goat serum (1:20). Either anti-BSP LF83 or anti-BSP LF6 at dilutions of 1:1000 and 1:200, respectively, was applied and incubated for 2 h at room temperature. Then, the tissue sections were 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 (17). Finally, sections were counterstained with hematoxylin, dehydrated, and mounted.

Evaluation of Staining. The immunohistochemically stained sections were reviewed by two independent observers. The degree of staining was evaluated using 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 if the increased expression of BSP observed in breast carcinoma compared to normal or benign breast tissue was statistically significant we used the χ2 test.

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5 The abbreviations used are: NCP, noncollagenous protein; BSP, bone sialoprotein.
Results

We used placental tissue as a positive control since it has been demonstrated previously that trophoblastic cells express BSP (9). Trophoblast tissue slides were processed with the anti-BSP antibodies, as described in "Materials and Methods," and showed positive staining. BSP was detected in syncytiotrophoblast and cytotrophoblast cells as described previously by Bianco et al. (9) (data not shown). The immunoreactivity was mainly cytoplasmic with both LF83 and LF6 antibodies. Control slides, in which the first antibody was omitted, remained negative (data not shown). To study the expression of BSP in normal and cancerous breast tissues, paraffin sections were stained for immunoreactivity to BSP. Both antibodies used gave similar immunostaining pattern with the breast lesion sections. Semi-quantitative grading of the immunohistochemical reactions observed was done according to the scale described in "Materials and Methods." Table 1 summarizes the data obtained by examining BSP expression in 79 breast lesions specimens.

In 16 of the 79 cases studied, we were able to analyze normal mammary epithelial cells adjacent to the lesions. We observed that in 75% of the cases, normal mammary epithelial cells were negative (Fig. 1A), whereas the 25% remaining cases exhibited a weak staining evaluated as 1+. We examined the expression of BSP in 28 benign lesions including 14 fibroadenomas and 14 fibrocystic dysplasias. Sixty-four percent of the fibroadenomas and 72% of the fibrocystic dysplasia lesions had no detectable level of BSP. The remaining samples were found to be 2+. None of the benign lesions in our series exhibited a strong immunostaining (3+).

Interestingly, a strong immunoreactivity to BSP was observed in breast carcinomas. The immunostaining was essentially localized to the cytoplasm of mammary cancerous cells. In 88% of breast in situ carcinomas, the staining was evaluated as 2+ or 3+ (Fig. 1E). This group included 5 in situ lobular carcinomas and 20 in situ ductal carcinomas. We did not observe a difference in the expression of BSP between these two types. The expression of BSP was also studied in 26 invasive breast carcinomas including 11 carcinomas with negative lymph nodes and 15 with positive lymph nodes. In these two groups, all the specimens demonstrated a detectable level of BSP (Fig. 1, B, C, and D). The staining was appreciated as strong (2+ or 3+) in 82% of the carcinomas with negative lymph nodes and in 93% of the carcinomas with positive lymph nodes. We found no statistically significant difference in the expression of BSP in infiltrating carcinomas with and without positive lymph nodes. The increased intensity of BSP immunoreactivity in malignant breast lesions was statistically significant when compared to the immunostaining observed in normal breast tissue ($P = 0.0001$ for both in situ and invasive carcinoma groups).

Among the 25 in situ carcinomas studied, 8 were associated with microcalcifications microscopically detectable on sections. Interestingly, 7 of these lesions expressed high levels of BSP (2+ or 3+). Similarly, among the 26 infiltrating carcinomas evaluated, 8 showed microcalcifications and 5 of these lesions exhibited a strong immunostaining (2+ or 3+) to BSP (Fig. 1F).

Discussion

Although mammary microcalcifications are often associated with both benign and malignant lesions of the breast, little is known of their formation. In humans, the physiological mineralization process is found in bones and teeth. Numerous noncollagenous bone matrix proteins have been identified and several have been extensively studied (5). However, none have had their precise biological role determined yet. BSP constitutes the predominant (~15%) protein of the noncollagenous proteins that have been identified in human bone (18). In this study, we looked at the expression of BSP in breast cells because its association with mineral deposition has been suggested by previous studies (19, 20). Bianco et al. (19) showed that the expression of BSP in bone is associated with the appearance of the first detectable hydroxyapatite crystals. Another study reported that the level of expression of BSP appears to be related to bone-forming activity and is particularly high at sites of de novo bone formation (20). We used two specific antibodies directed against BSP to demonstrate its expression in normal and cancerous mammary epithelial cells. One antibody was raised against a synthetic peptide from BSP and the second against denatured human BSP. Both antibodies gave the same pattern of specific immunostaining. This is the first report that shows an immunohistochemical localization of BSP in normal and cancerous breast tissue. We observed an overexpression of BSP in cancerous mammary epithelial cells. The intensity and the distribution of staining appear to be associated with the presence of microcalcifications, although the number of lesions showing microcalcifications is too small for a statistical analysis. We have not observed such an association in benign lesions probably because the microcalcifications associated with these lesions are very often formed by calcium oxalate crystals (3). This observation suggests that the deposition of microcalcifications in benign and malignant lesions obeys different molecular mechanisms. However, some malignant lesions showed an overexpression of BSP without any microscopically visible microcalcifications. We suggest that as in bone matrix, the ectopic hydroxyapatite mineralization in breast tissue probably requires the participation of other noncollagenous proteins such as osteonectin and osteopontin. However, that is not to say that microcalcifications would not have formed if the tumor had been allowed to progress. The variation observed in immunoreactivity to BSP, in normal breast tissue and benign breast lesions from undetectable (0) to detectable (2+), could be explained by variations in hormonal status at the moment of the biopsy. Indeed, it has been demonstrated that dexamethasone, which has been shown to promote the differentiation of osteoblast precursors (21) in rat calvaria and in the rat osteosarcoma cell line ROS 17/2.8 (22) increased the level of BSP mRNA. In contrast, 1,25-dihydroxyvitamin D$_3$, which is thought to suppress bone formation by blocking the differentiation of osteoblast precursors and stimulating bone resorption through the recruitment of osteoclast precursors, reduced the amount of BSP mRNA (22).

Cell-binding experiments, using plastic dishes coated with BSP, demonstrated that BSP promotes the attachment and spreading of rat osteosarcoma cells (ROS 17/2.8) (23). This cell-binding activity is
apparently mediated by an RGD motif in BSP, which is homologous to the cell-binding domain in vitronectin (7). The BSP receptor is an integrin indistinguishable from the vitronectin receptor on the surface of ROS 17/2.8 (23). BSP protein and the corresponding mRNA were localized in osteoclasts (9). This observation suggests that osteoclasts may use endogenously produced cell adhesion molecules to attach to bone surfaces. The skeleton is a very common target for breast carcinoma metastases. Considering the fact that BSP promotes the attachment and spreading of osteosarcoma cells (23), the possibility that cancerous breast cells metastasize to bone via receptors to BSP is most interesting. Indeed, a recent study has shown that breast cancer cell lines do attach to BSP (24).

Although the association of BSP and mineral is well established now, ongoing studies are needed to demonstrate the mechanism that
leads to the expression of BSP in malignant breast lesions and to elucidate its role in invasion and the metastasis process.

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