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ORIGINAL ARTICLE

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Microcalcifications of breast cancer and atypical cystic lobules associated with infiltration of foam cells expressing osteopontin

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Abstract We studied the pattern of calcification and the expression of osteopontin protein and mRNA by the histiocytes and noninvasive carcinoma cells of 20 breast cancers and the histiocytes and atypical ductal cells of sixteen cases of atypical cystic lobules. Ten breast cancers showed low-grade cribriform carcinoma in situ containing secretory material; the remaining ten cancers displayed high-grade carcinoma in situ with central necrosis characteristic of comedo type carcinoma. Hematoxylin-eosin and Kossa staining revealed calcium hydroxyapatite calcifications in 80% of cribriform type carcinomas, 50% of comedo type carcinomas, and 56% of atypical cystic lobules. Immunohistochemical staining demonstrated osteopontin protein in intraluminal secretory material, necrotic debris, or stroma, and in histiocytes in all the Kossa-positive carcinomas and atypical cystic lobules. In situ hybridization revealed osteopontin mRNA mainly in the histiocytes and especially in those near the calcifications. In two cases, rare carcinoma cells contained osteopontin protein and mRNA. The close relation between hydroxyapatite crystals and osteopontin-producing histiocytes suggests that osteopontin plays a role in the biomineralization that occurs in certain non-invasive breast cancers and atypical cystic lobules. The differences in the morphology of the calcifications and the intraductal contents suggest that the mechanism leading to the osteopontin production might vary depending on the underlying lesion.

Keywords Breast cancer · Atypical cystic lobules · Osteopontin · Microcalcification

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Introduction

Microcalcification serves as an important indication of early breast carcinoma. One finds carcinoma in approximately 25% of non-palpable lesions biopsied because the mammogram revealed calcifications [23, 30]. Researchers have described two types of calcifications, type I and type II [9]. Type I microcalcifications appear as colorless, birefringent crystals composed of calcium oxalate (CaC_2CO_4-$2H_2O$). They most often form in apocrine cysts and less commonly in other benign conditions [13]. Type II microcalcifications, which consist of the bone mineral crystal, hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], stain with hematoxylin and the Kossa technique. They occur in both benign and malignant conditions[19, 22], although more commonly in ductal carcinoma in situ (73% of cases) [13] than in benign breast disease (31% of cases) [28]. These hydroxyapatite crystals can take two forms: a “laminar” or “secretory” appearance, or a “granular,” “amorphous,” or “necrotic” appearance [15, 19].

The mechanisms by which calcifications arise have not been clarified fully. Deposition of the crystals in a secreted mucinous or proteinaceous matrix probably creates “secretory” calcifications, whereas precipitation of hydroxyapatite in necrotic cellular debris would seem to give rise to the “granular” type [8]. Recent publications report that bone matrix proteins, which include osteopontin (OP), osteonectin, and bone sialoproteins, play a role in initiating and controlling hydroxyapatite crystallization and in regulating other aspects of mineralization [4, 16, 18]. The studies of Castronovo et al. revealed that the expression of several bone matrix proteins including OP

correlates with the presence of hydroxyapatite microcalcifications [4]. The OP gene encodes highly modified proteins of 55–65 kDa with a preserved integrin binding RGD tripeptide [1]. RGD-mediated cell adhesion and proliferation via OP is proposed to act through the integrin $\alpha_v\beta_3$ [35]. The integrin family of proteins can induce cellular responses such as cell adhesion, changes in intracellular pH, changes and oscillation in intracellular free calcium concentrations, and phosphorylation of tyrosine.

Although OP has been detected in variable but generally high percentages of primary breast cancers [2, 3], the cellular origin of the molecule remains controversial [3, 12, 14, 32, 34, 37]. Brown et al. propose that macrophages serve as the most important source of the protein and that the secreted molecule binds to tumor cells, possibly through the GRGDS cell binding domain in OP [3]. Other authors, using in situ hybridization and matched sections, have concluded that OP originates from tumor cells [12, 32, 34,37]. Sharp et al., for instance, demonstrated that OP transcripts were detected in both invasive and in situ carcinoma components and that the transcripts were not seen in surrounding stromal cells or in peritumoral macrophages [32].

Besides occurring in ductal carcinomas in situ, calcifications of the "secretory" type also form in a lesion that we refer to as "atypical cystic lobule" [25] and others have called "low-grade clinging carcinoma of flat type" [6], "columnar alteration with prominent apical snouts and secretions" [10], "hypersecretory hyperplasia with atypia" [26], and "atypical cystic duct" [21,36]. This alteration represents a proliferation of luminal cells showing low-grade cytological atypia without architectural atypia. The cells often possess apical snouts, and secretory material frequently accumulates in the glandular lumina. This lesion frequently merges with fully established ductal carcinoma in situ of the micropapillary/cribriform type. On the basis of these morphological [10, 25, 26,36], immunohistochemical [21,25], genetic [24], and clinical studies [7], certain authors have suggested that atypical cystic lobules and related lesions represent early stages in the formation of certain types of low-grade ductal carcinoma in situ.

We undertook the following study to correlate the expression of OP with the presence of calcifications in breast cancers and atypical cystic lobules.

Materials and methods

Case selection and classification

Using computer-generated lists of specimens in the departments of pathology of the Massachusetts General Hospital and the Gunma University Hospital, we retrieved cases showing predominantly (nine cases) or exclusively (11 cases) noninvasive ductal carcinoma or atypical cystic lobules. We based the diagnosis of ductal carcinoma in situ on conventional criteria [29] and then subdivided the carcinomas into two groups. Cribriform (Cr) carcinomas show low-grade features, form cribriform or micropapillary architectural patterns, and contain eosinophilic secretions within neoplastic glands. Comedo (Co) carcinomas display intermediate or high grade cytological characteristics and central ("comedo") necrosis of the malignant ductal cells. We used our previously published criteria to make the diagnosis of atypical cystic lobule [25]. The study group comprises ten cases of Cr type carcinoma, ten cases of Co type carcinoma, and 16 cases of ACL.

Sections from 17 of the 20 carcinoma cases contained apparently normal ducts and lobules, which we used for our study of normal tissues and benign changes.

Conventional microscopical examination

Using the original hematoxylin and eosin stained sections, we examined the noninvasive portions of the study cases and recorded the type and location of calcifications, and the presence of foam cells.

Immunohistochemical staining and Kossa staining

Using formalin-fixed and paraffin-embedded sections, immunohistochemical staining for keratin, Kp-1, and OP was performed. Five micrometer paraffin sections were deparaffinized with xylene, soaked through an alcohol series, and washed in phosphate-buffered saline (PBS) (pH 7.4). Following antigen retrieval by means of microwave heating in citrate buffer, the sections were incubated for 1 h with normal goat serum and then reacted with the primary antibodies overnight at 4°C. After the incubation with the secondary antibody for 1 h at room temperature, the conventional avidin-biotin-peroxidase complex method was performed at room temperature using the Vectastain ABC kit (Vector Laboratories Inc., Burlingame, Calif.). Slides were immersed in diaminobenzidine tetrahydrochloride solution, then lightly counterstained with hematoxylin. Immunostaining for Kp1 required pronase predigestion. The antibodies and their dilutions were: monoclonal anti-OP (10A16, IBL, Japan; 1:200), monoclonal anti-keratin (KL-1, Immunotech, France; 1:5), and monoclonal anti-Kp1 (CD68, DAKO, Kyoto, Japan; 1:50). We interpreted brown discoloration of the cytoplasm as a positive result. For negative controls of the OP staining, we stained duplicate sections from each case using OP absorbed primary anti-OP antibody.

Kossa staining was performed according to the usual protocol using formalin fixed and paraffin embedded sections. They were reacted in 5% $AgNO_3$ under sunlight.

In situ hybridization

Sections 4 μm thick were cut from paraffin blocks and mounted on silane coated glass slides. They were then rinsed in ethanol, rehydrated with diethyl pyrocarbinate treatment, and rinsed in 0.1 M PBS. They were treated with 5 μg/ml proteinase K in PBS at 37°C for 30 min to eliminate protein, rinsed in PBS, and refixed in 4% paraformaldehyde in PBS for 5 min. They were rinsed in PBS, and basic protein was removed by incubation in 0.2 M HCl at room temperature for 15 min. After rinsing in PBS, they were rinsed in 0.1 M triethanolamine for 1 min at room temperature then in 0.25% acetic acid in 0.1 M triethanolamine for 10 min. The sections were rinsed in PBS, deparaffinized in xylene, dehydrated in an ethanol series, and air-dried for the hybridization.

The RNA probe for OP was prepared using a digoxigenin (DIG) RNA labeling kit (Boehringer Mannheim, Mannheim, Germany). To produce DIG-labeled RNA, cloned OP DNA was transcribed in vitro with T7 or T3 RNA polymerase in the presence of DIG-UTP. Briefly, DNA in the transcription vector with phage promoter was linearized with restriction enzyme (apa I or xba I). RNA synthesis by in vitro transcription from the phage promoter was performed by incubation of T7 or T3 polymerase with ATP, CTP, GTP, UTP, and DIG-UTP for 2 h at 37°C. After determining the sensitivity of the probe to OP, T7 probes were used with the mixture solution containing 50% deionized formamide, 10 mM

Tris-HCl (pH 7.6), 200 µ/ml tRNA, 10% dextran sulfate (Pharmacia, Uppsala, Sweden), 1× Denhardt's solution, 600 mM NaCl, 0.25% SDS, and 1 mM EDTA (pH 8.0). T3 probes were used at the same dilution as the negative control.

Usually, 15 µl of the probe mixture was applied on a slide and completely sealed with a coverslip. This was followed by the OP probe at 85 °C for 5 min, and the hybridization was carried out overnight at 50°C. After hybridization, the slides were subsequently washed under high stringency conditions with 5× sodium chloride and sodium citrate solution (SSC) at 55°C for 5 min, 50% formamide in 2× SCC at 50 °C for 30 min., 2× SCC at 50°C for 20 min, and 0.2× SCC at 50°C for 40 min (1× SSC contains 0.15 M sodium chloride and 15 mM sodium citrate). After that, the sections were rinsed in buffer 1 solution containing 100 mM Tris HCl (pH 7.6) and 150 mM NaCl at room temperature for 5 min. After the sections were rinsed in 4% block Ace/buffer 1 at room temperature for 60 min and washed 3 times in Tris buffered saline, they were reacted with a peroxidase (PO) conjugated anti-DIG antibody (Dako, Denmark) at 1:500 dilution at room temperature for 60 min. For the ISH signal amplification, the digoxigeninated tyramide was applied to the section at room temperature for 20 min and subsequently reacted by PO conjugated anti-DIG antibody again. After rinsing in PBS-T, the PO was visualized with 0.01 M Tris-HCl buffer (pH 7.4) containing 0.05% 3–3′-diaminobenzidine tetrahydrochloride and 0.015% H_2O_2 for 5 min. Finally, the sections were lightly counterstained with hematoxylin.

Results

Calcifications

Nonneoplastic tissue

The normal breast tissue did not contain calcifications nor did it show a reaction with Kossa staining. Rare microcysts indicating fibrocystic changes harbored type II calcifications of the "secretory" type, which stained with the Kossa technique.

Noninvasive carcinomas

Of 10 cases of Cr type carcinoma, calcifications were observed by hematoxylin and eosin staining in the noninvasive components of 8 cases (80%); black deposits were observed after Kossa staining in all 8 cases (1). All these calcifications displayed the characteristics of "secretory" calcifications. Of 10 cases of Co type carcinoma, calcifications were observed following hematoxylin and eosin staining in the noninvasive components of 5 cases (50%), and black deposits were observed in the central necrotic area of all 5 cases after Kossa staining (2B). These calcifications were of the "granular" or "necrotic" type.

Atypical cystic lobules

Of 16 cases of ACLs, calcifications were observed in 9 (56%), and black deposits were observed following Kossa staining in all 9. All calcifications were of the "secretory" type.

Table 1 Calcification and osteopontin protein expression in noninvasive carcinomas and atypical cystic lobules

Lesions	Cases	Tumor cells	Extracellular material[a]	Histiocytes
Cr carcinoma	10	1 (10%)	8 (80%)	8 (80%)
Kossa + cases	8	1 (12%)	8 (100%)	8 (100%)
Kossa – cases	2	0	0	0
Co carcinoma	10	1 (10%)	8 (80%)	9 (90%)
Kossa + cases	5	1 (20%)	5 (100%)	5 (100%)
Kossa – cases	5	0	3 (60%)	4 (80%)
ACL	16	0	10 (62%)	9 (56%)
Kossa + cases	9	0	9 (100%)	8 (88%)
Kossa – cases	7	0	1 (14%)	1 (14%)

[a] Osteopontin protein was detected by immunohistochemistry in secretory material, necrotic debris, or stroma

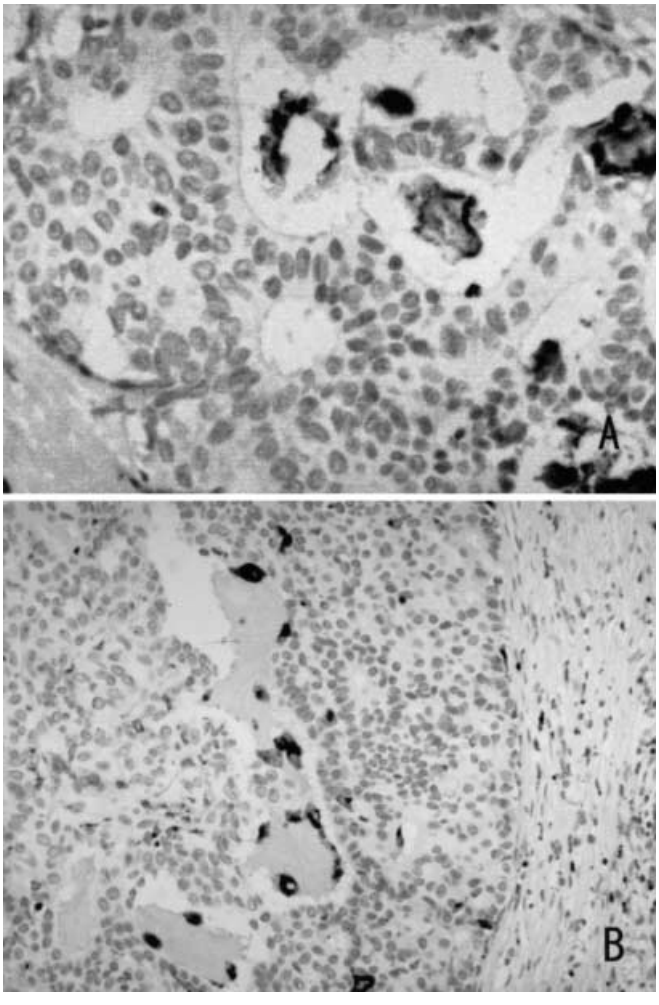

Fig. 1 Cr type carcinoma, immunohistochemical staining for osteopontin (OP, **A** ×320) and Kp-1 (**B** ×160). (OP was observed psammomatous components (**A**) and infiltrating foam cells surrounding the psammoma body were positive for Kp-1 (**B**)

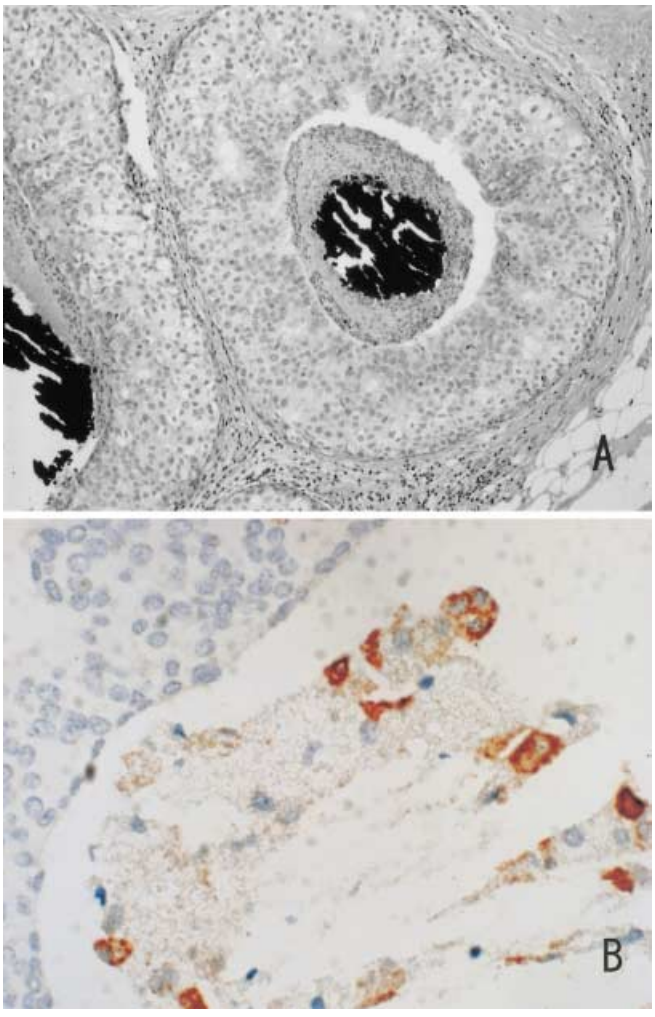

Fig. 2 Co type carcinoma, Kossa staining (**A,** ×80) and in situ hybridization (ISH) for OP mRNA (**B,** ×320). (Black deposits were observed in the center of comedo-necrosis (**A**) and infiltrating histiocytes were positive for OP mRNA by ISH (**B**)

OP expression by immunohistochemistry and in situ hybridization

Nonneoplastic tissue

Normal epithelial cells did not stain for OP protein by immunohistochemistry. We detected very small amounts of the molecule in the extracellular stroma surrounding microcysts containing "secretory" calcifications. Occasional foam cells in foci of adenosis and fibroadenosis also contained the protein. In situ hybridization did not reveal OP mRNA in any epithelial cells, but we detected a signal in a few histiocytes within the lumen of a duct in one case.

Noninvasive carcinomas

Most tumor cells failed to stain for OP protein, but we did detect it in extracellular locations and in histiocytes.

Table 2 Calcification and osteopontin mRNA expression in noninvasive carcinomas and atypical cystic lobules. *nd* not done

Lesions	Cases	Tumor cells	Histiocytes
Cr carcinoma	10	1 (10%)	8 (80%)
Kossa + cases	8	1 (12%)	8 (100%)
Kossa – cases	2	0	0
Co carcinoma	10	1 (20%)	9 (90%)
Kossa + cases	5	1 (20%)	5 (100%)
Kossa – cases	5	0	4 (80%)
ACL			
Kossa + cases[a]	6	0	5 (83%)
Kossa – cases	7	nd	nd

[a] The number of the cases is smaller than numbers in Table 1 because of technical limitations

A few cells in one carcinoma of the Cr and another of the Co type seem to express OP (1), but staining of the stroma surrounding these clusters made it difficult to feel confident that the positive reaction represented genuine staining of the cells. The secretory material within the ducts of eight of ten carcinomas of the Cr type (80%) and the necrotic debris within the ducts of five of ten carcinomas of the Co type (50%) showed a positive reaction for the protein (1). These OP positive cases included all the Kossa-positive carcinomas of both Cr and Co type (eight and five cases, respectively) (1A). Histiocytes (type B foam cells of Damiani [5]), which stained for Kp-1 and failed to stain for keratin, also demonstrated OP protein expression (1B, 2B). These OP-positive histiocytes usually abutted the secretory material in the Cr type of ductal carcinoma in situ or sat within the necrotic cellular debris in the Co type of carcinoma. A thin rim of stroma surrounding ducts harboring ductal carcinoma in situ contained OP.

In situ hybridization identified OP mRNA in histiocytes and rare tumor cells. All the Kossa-positive cases of both the Cr and Co types of carcinoma in situ contained OP mRNA positive histiocytes (2). Rare tumor cells of one carcinoma of the Co type and another carcinoma of the Cr type expressed the mRNA. We could not establish a correspondence between the OP protein positive cells and those showing OP mRNA in the two cases of carcinoma.

Atypical cystic lobules

Of 16 cases of ACL, OP protein was detected in the stroma, histiocytes, or secretory material of 10 (62%). We attempted in situ hybridization in the nine Kossa-positive cases but obtained satisfactory results in only six. Five of them (83%) demonstrated OP mRNA, which we detected only in histiocytes (3A, B; 2).

Negative Controls

All sections stained using the primary anti-OP antibody absorbed with OP failed to show a positive reaction.

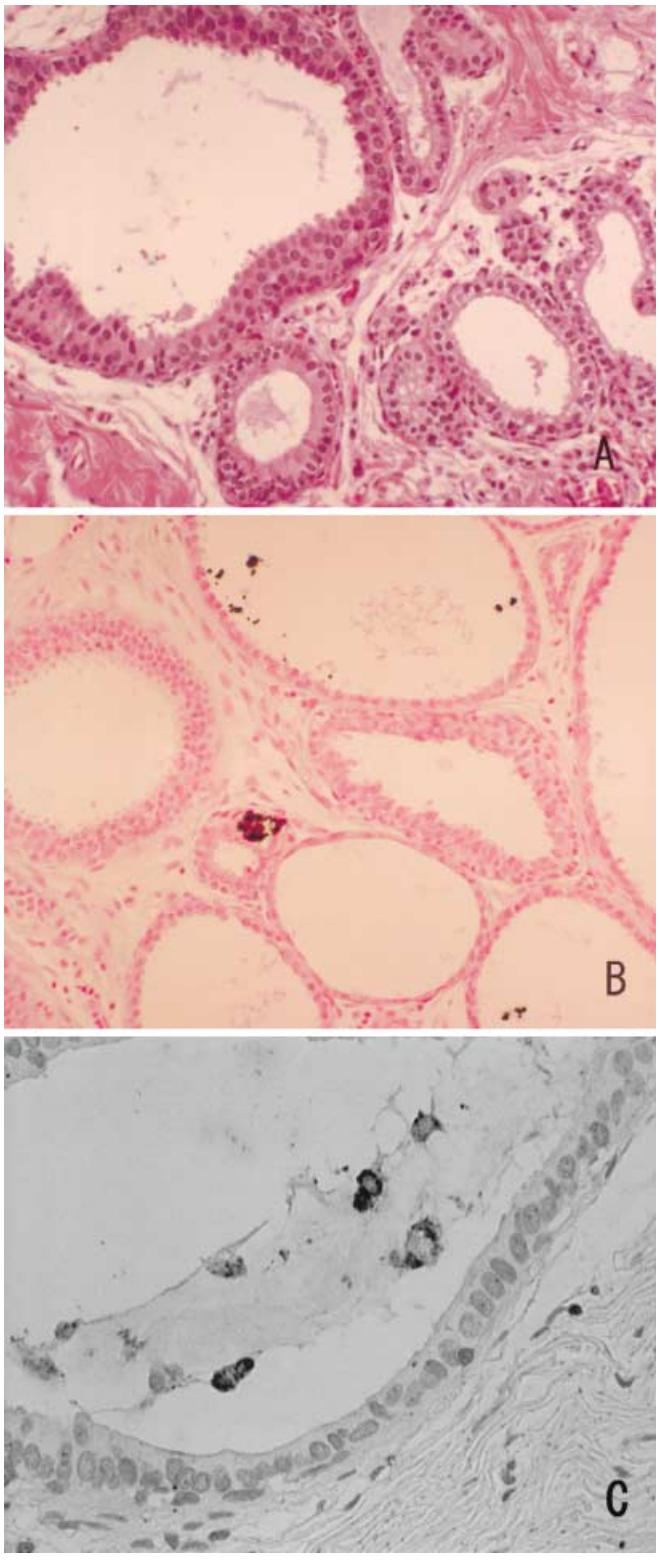

Fig. 3 Atypical cystic lobules, H&E (**A,** ×160) and Kossa stainig (**B,** ×160) and in situ hybridization (ISH) for osteopontin (OP; **C,** ×320). Atypical cystic lobules are composed of atypical luminal cells without architectural atypia (**A**). Black deposits were observed in the center of lumen (**B**). OP was observed in foam cells in the lumen (**C**)

Discussion

The results of our study agree with the general belief that OP plays a role in the biomineralization that occurs in breast cancers. Like Hirota et al. [14] we found that histiocytic foam cells (macrophages) represent the major site of production of OP; we detected it within malignant cells in only a small percentage of the carcinomas. This low frequency of expression of OP protein and mRNA by carcinoma cells conflicts with the results of several groups who report that breast cancer cells contain the protein [2, 3, 32], the mRNA [12], or both [37]. We cannot offer a ready explanation for this discrepancy, but we would point out that the published studies do not present a coherent body of data. For example, the staining protocol used by Bellahcene and Castronovo [2] produced moderate staining in about 20% of samples of normal breast tissue and slight staining in an additional 33%. The failure of Sharp et al. [32] to detect OP protein or mRNA in macrophages likewise raises questions about the authors' techniques, for histiocytes have consistently shown expression of both the protein and its mRNA [3, 14, 37]. By using formalin-fixed tissue, microwave heating, and a monoclonal primary antibody, our methods most closely match those employed by Tuck et al. [37]. Moreover, our demonstration of OP protein and mRNA in histiocytes and our failure to detect either molecule in normal breast tissue agree with their observations [37]. Unlike these authors, who observed OP protein and mRNA in about 25% of invasive breast cancers, we did not detect these molecules in the noninvasive components of most carcinomas. Nine of our cases did contain small amounts of invasive carcinoma, however, and we detected OP protein or mRNA in occasional invasive cells. They number so few that we cannot draw conclusions from these observations, but they suggest to us that this difference in the type of cells studied might explain the difference between our observations and those of Tuck et al. [37].

Whatever the source of the OP, the evidence suggests that the molecule plays a role in the calcium hydroxyapatite deposition that takes place in certain breast cancers. The mechanisms leading to expression of the OP remain undefined, however, and so does its role in the mineralization process. Our observations suggest that different mechanisms account for the OP accumulation seen in different types of ductal carcinomas in situ. For example, the low-grade (Cr) carcinomas in situ that we studied contain eosinophilic secretions and the "secretory" type of hydroxyapatite calcifications. Milk, the physiological secretion of the breast, contains abundant OP [27, 31], so the presence of this protein in these carcinomas might reflect an aberration of the normal process of OP production that occurs during lactation. Researchers have not identified the function of the OP present in milk, but they have shown that the protein inhibits hydroxyapatite crystal growth [18, 17], that the inhibition of calcification requires phosphorylation of the molecule [20], and that milk contains the highly phosphorylated form of OP [33]. One might therefore suggest that OP helps to

prevent the growth and precipitation of hydroxyapatite crystals from this calcium, phosphate, and protein-rich fluid [18] and that the OP present in low-grade ductal carcinomas in situ might have the same effect.

The atypical cystic lobules in our study group show the same "secretory" type of hydroxyapatite calcifications and the same pattern of OP expression as most low-grade (Cr type) ductal carcinomas in situ. The epithelial cells do not contain either the protein or the mRNA; however, extracellular material next to the calcifications stains for the former, and histiocytes contain both molecules. Based on these findings, we conclude that the calcium hydroxyapatite crystals in atypical cystic lobules form through the same mechanism as the calcifications seen in low-grade Cr type of ductal carcinoma in situ.

In the high-grade (Co) carcinoma in situ, on the other hand, the OP expression would seem to represent a component of the inflammatory response to cellular damage. One observes upregulation of the protein in many types of tissue injury and at sites of pathological soft tissue calcifications, such as atherosclerotic plaques, bioprosthetic cardiac valves, and dental plaque [11]. The presence of the molecule in the necrotic material of high-grade ductal carcinoma in situ probably reflects the same phenomenon. Macrophages represent the most important source of OP found in atherosclerotic plaques and calcified valves [38], and our data indicate that these same cells synthesize the majority of the OP seen in the necrotic debris of comedocarcinomas. Studies suggest that OP inhibits matrix mineralization at sites of ectopic calcification [38] and promotes macrophage resorption of dystrophic calcification [11]. Our observation that four of the nine OP containing comedocarcinomas in our study group lack calcifications seems consistent with the belief that OP functions to limit the growth of hydroxyapatite crystals and may thereby protect against dystrophic calcification.

In summary, we detected OP in most cases of both low-grade cribriform and high-grade comedo-type ductal carcinoma in situ; macrophages seem to be the predominant source of the protein. The difference in the type and extent of calcifications in these two types of noninvasive carcinoma suggests that different mechanisms underlie OP expression in the two lesions. Atypical cystic lobules also contain OP expressing histiocytes and calcifications similar to those seen in low-grade ductal carcinomas, and this similarity suggests a link between the two lesions. Our observations also seem consistent with the developing belief that OP functions to inhibit the process of dystrophic calcification.

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