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Short Communication

Occult Deposition of Eosinophil Peroxidase in a Subset of Human Breast Carcinomas

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Degranulation of eosinophils has been observed in a variety of human tumors and in other diseases but has not been previously described in breast cancer. To determine whether eosinophil degranulation also occurs in breast carcinomas, we performed immunohistological studies on cryostat sections obtained from 26 breast cancer biopsies and from 2 benign breast tissues using a monoclonal antibody specific for human eosinophil peroxidase (EPO). For control purposes, the tissues were also immunostained with a mouse IgG1 negative control antibody and with monoclonal mouse anti-human myeloperoxidase. Of the 26 breast cancer specimens, 14 (53%) had extensive, unsuspected deposition of EPO that was located primarily in the connective tissue stroma around and within the tumor. Only 3 of the breast cancer cases had no immunohistochemical evidence of EPO. Thus, 23 of 26 cases of breast cancer (88%) had EPO deposits detectable within or around the tumor. By contrast, none of the benign breast tissues had similar deposits of EPO, and substantial extracellular myeloperoxidase deposition was detectable in only 3 cases of breast cancer. From these studies we conclude that there is eosinophil degranulation and extensive occult deposition of EPO in a major subset of human breast cancers. (Am J Pathol 1996, 148:701–706)

A wide variety of human cancers and diseases are often infiltrated by degranulating eosinophils that release eosinophil peroxidase (EPO) and major basic protein into the stroma.[1–14] The degranulation of eosinophils in these disorders is generally undetectable in tissue sections that have been routinely stained with hematoxylin and eosin and requires special procedures such as immunohistochemistry to demonstrate.[7,8,10–14]

Although human and rodent breast cancers have long been known to contain abundant peroxidase activity,[15,16] the origin of the peroxidase has not yet been definitively established. Moreover, the possible involvement of eosinophils in human breast cancers has not been extensively studied. Therefore, to determine whether EPO accounts for the high peroxidase activity that has been reported in some human breast carcinomas, we performed immunohistological studies on cryostat sections obtained from 26 breast cancer biopsies and 2 benign breast tissues using a murine monoclonal antibody that is highly specific for human EPO.[7,14] The results of the immunohistochemical studies were then compared with the presence of myeloperoxidase and cytochemically detectable peroxidase activity in corresponding additional cryostat sections. Here we report the novel and unexpected finding that a high proportion of human breast cancers contained extensive, occult deposition of EPO.

Materials and Methods

Reagents

An IgG murine monoclonal antibody called SF25.5 that binds to human EPO was derived as described

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elsewhere.[7] This antibody is highly specific for EPO[7] and does not bind to any other cell types, to myeloperoxidase, or to normal breast tissues when radiolabeled and administered to human subjects.[17] Also included in this study was a mouse monoclonal IgG negative control antibody (Dako Corp., Carpinteria, CA) diluted to the same working concentration as the anti-EPO antibody (10 μg/ml PBS with 1% fetal calf serum). For comparative purposes, a monoclonal mouse anti-human myeloperoxidase antibody (DAKO-MPO, MPO-7) was used in the immunohistochemical staining procedure at the same concentration as the other antibodies.

Tissue Specimens

In this study, we examined all of the cryopreserved, primary breast carcinomas that were available in the University of California, Irvine, Cancer Center Human Tumor Bank (Orange, CA). The study set consisted of 25 cases of ductal adenocarcinoma of the breast (well to poorly differentiated) and 1 case of lobular carcinoma. All of these cases were well characterized by routine light microscopy and by other special studies as appropriate. Two cryopreserved specimens of benign breast tissue, obtained from patients with breast carcinomas in other sites, were used as controls. This study was approved by the Institutional Human Subjects Review Board at the University of California, Irvine.

Immunohistology and Cytochemistry

Breast tissues were frozen in Histo-Prep embedding media (Fisher Chemical, Fair Lawn, NJ), sectioned at 2 to 6 μm thickness on a microtome, air dried on a glass slide, and fixed in acetone. The cryostat sections were then subjected to two separate assays. The first assay was a cytochemical procedure for detecting peroxidase activity (EPO, myeloperoxidase, and lactoperoxidase) using a chromogenic substrate, aminoethyl carbazole, and hematoxylin counterstain.[14] For optimal sensitivity, this procedure needed to be performed within 10 minutes after tissue sectioning.

The immunohistochemical procedure to detect the presence of EPO and myeloperoxidase in tissue sections used an avidin-biotin glucose oxidase detection procedure (Vector Laboratories, Burlingame, CA) to detect bound primary antibody and was followed by a nuclear fast red counterstain.[7,14] This detection method was selected to avoid interference by endogenous peroxidase activity that was present within the tissues.

The negative controls for each case consisted of tissue sections that were incubated without the primary antibody or with the irrelevant mouse monoclonal antibody at the same concentration as SF25.5. A positive control was also included in each staining run and consisted of a cytopreparation of purified human eosinophils[18] incubated with the SF25.5 antibody.

Each stained slide was reviewed by two observers. For purposes of tabulation, EPO and peroxidase activity in each slide were classified as extensive (present in every 250× microscopic field), present (detectable in the tissue but not present in every microscopic field), or absent (none visible). In addition, the microanatomic location (stromal, within tumors; within blood vessels, etc.) of the EPO and peroxidase activity were noted in each case.

Results

Deletion of the primary antibody or substitution with the negative control antibody in all cases resulted in no background staining of the tissues (Figure 1a), and there was also no staining of the normal breast tissues that were incubated with the antibody to EPO. The results of the other immunohistological studies are presented in Table 1.

Extensive deposition of EPO was present in 13 of 25 cases of ductal adenocarcinoma and in the single case of lobular carcinoma that was available for study. The EPO was most often deposited near small blood vessels at the interface between tumor and adjacent normal, fatty tissue (Figure 1b) and in the connective tissue stroma between nests of tumor cells (Figure 1c). In the latter location, the EPO deposits often assumed a fibrillar, ameboid appearance that seemed to follow connective tissue fibers.

The most extensive EPO deposits nearly encircled the nests of tumor cells but did not extend into the tumor nests themselves (Figure 2a). In two of our cases, the EPO deposition was primarily present within ducts composed of malignant cells (Figure 2b) and spared adjacent normal ducts. An especially noteworthy finding was the frequent presence of free granules of EPO in the connective tissue stroma of breast cancers in the absence of identifiable, intact eosinophils (Figure 2c).

Myeloperoxidase deposits in 23 of the breast cancers were confined to a few intact neutrophils widely scattered throughout the tumor. In 3 of the cases, however, there was extensive extracellular deposition of myeloperoxidase primarily located within necrotic and acutely inflamed portions of the tumor.

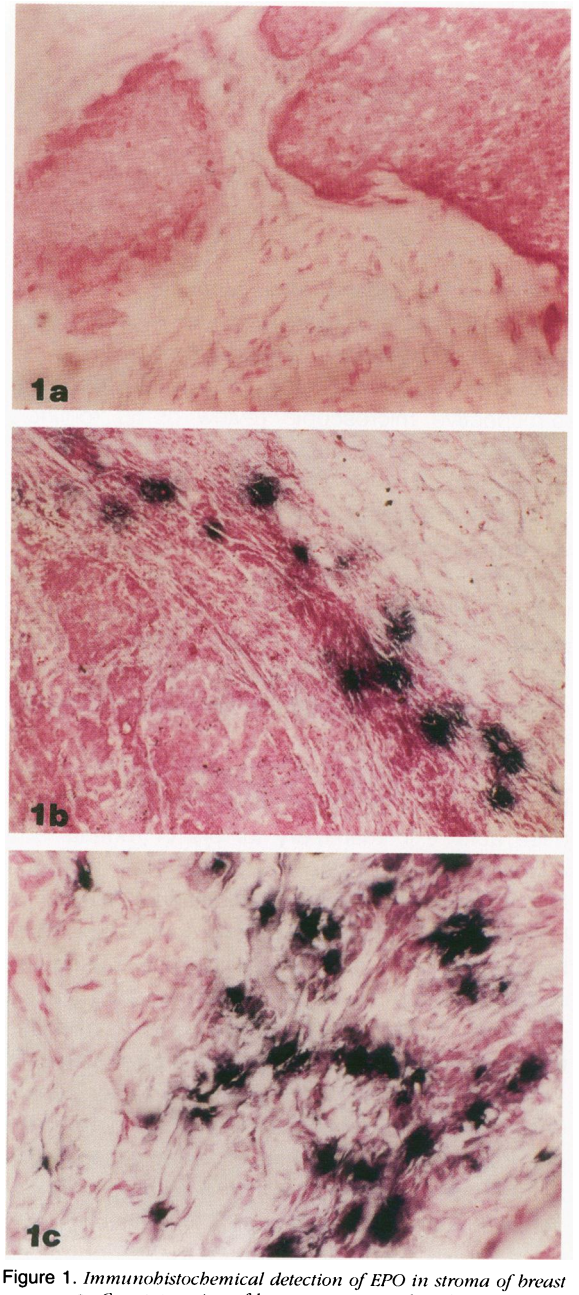

Figure 1. *Immunohistochemical detection of EPO in stroma of breast cancers.* **a:** *Cryostat section of breast cancer incubated with the negative control monoclonal antibody. There was no background staining.* **b:** *EPO deposits are seen as dark blue staining at the interface between tumor and adjacent normal fatty tissue.* **c:** *The connective tissue stroma within breast cancers contained abundant deposits of EPO in a fibrillar or ameboid pattern. Nuclear fast red counterstain and nitroblue terazolium substrate; original magnifications, ×250 (***a** *and* **b**) *and ×400 (***c**).

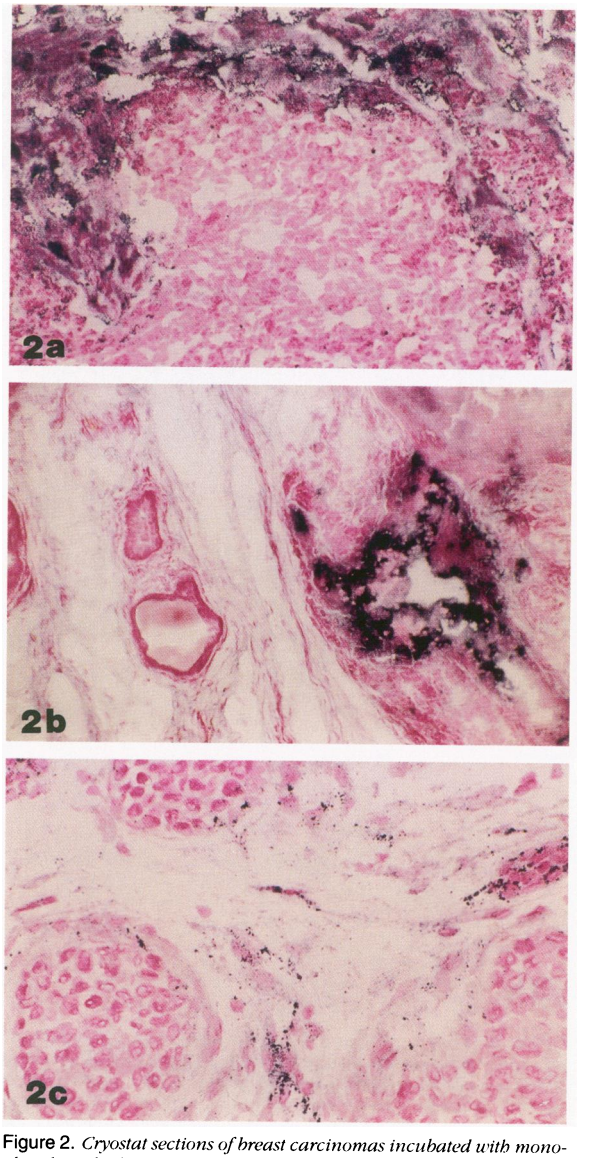

Figure 2. *Cryostat sections of breast carcinomas incubated with monoclonal antibody specific for EPO.* **a:** *Abundant deposits of EPO nearly encircle a nest of tumor cells.* **b:** *Densely staining EPO activity was sometimes present within ducts composed of malignant cells but was not detectable in adjacent normal tissue.* **c:** *Granular deposits of EPO were detectable in the stroma between tumor cells, even in the absence of intact eosinophils. Original magnifications, ×250 (***a** *and* **c**) *and ×100 (***b**).

The intensity of immunohistochemical staining in these 3 cases was similar to the staining seen with the EPO antibody in the same cases.

The results of the cytochemical assays for peroxidase activity correlated well with the immunohistological studies. In all cases in which EPO was detectable with the monoclonal antibody, there were also amorphous, stromal deposits of cytochemically detectable peroxidase activity (Figure 3a) along with scattered intact eosinophils. In five of the breast cancer cases, there were also large globular deposits of peroxidase activity within ducts and adjacent to nests of tumor cells (Figure 3b). These intraductal globular deposits of peroxidase did not stain with the monoclonal antibody to EPO or to myeloperoxidase and were, therefore, presumed to represent lactoperoxidase within milk fat globules.

Table 1. *Immunohistochemical Studies of Human Breast Tissues with EPO Antibody*

Diagnosis	Number of cases	Degree of EPO deposition			Location of EPO deposits		
		Extensive	Present	Absent	Stroma	Ducts	Blood vessels
Ductal carcinoma	25	13	9	3	19	2	3
Lobular carcinoma	1	1	NA	NA	1	0	0
Benign	2	0	0	2	0	0	0

NA, not applicable.

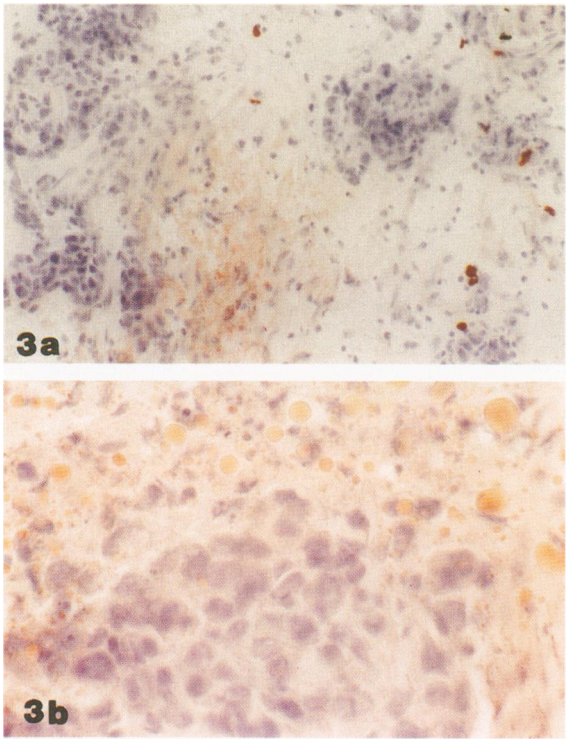

Figure 3. *Cryostat sections of breast carcinomas cytochemically stained for peroxidase activity.* a: *Amorphous and fibrillar deposits of peroxidase activity (red-orange staining) were present in the connective tissue stroma between nests of tumor cells. Also present were occasional intact eosinophils, seen here as intensely red-orange cells.* b: *In five of the breast cancer cases, there were large globular deposits of peroxidase activity adjacent to and within nests of tumor cells. These deposits did not stain with any of the monoclonal antibodies used in the study and were, therefore, presumed to represent lactoperoxidase within milk fat globules produced by the tumor cells. Hematoxylin counterstain and aminoethylcarbazole substrate; original magnifications, ×100 (a) and ×400 (b).*

Discussion

In this carefully controlled immunohistochemical study, we have demonstrated that 53% of the human breast carcinomas that we studied contained strikingly abundant EPO deposits, located primarily within the connective tissue stroma of the tumor. These results were surprising because most routinely stained breast cancer specimens have very little apparent infiltration by eosinophils. The most likely explanation for this unexpected finding is that degranulating eosinophils embedded in fibrous connective tissue around and within tumors require careful microscopic examination to detect in routinely stained sections because they assume an ameboid (medusa cell) configuration.[19,20] Indeed, a retrospective microscopic review of 50 routinely fixed and stained breast cancer specimens at our facility (including the cases used in this study) revealed the frequent presence of such medusa cells when diligently sought (unpublished observations). Furthermore, our immunohistochemical studies of breast cancers often showed fibrillar, ameboid deposits of EPO that were remarkably similar to the EPO deposits that we previously described in the collagen bands of nodular sclerosis Hodgkin's disease.[7]

Our data, therefore, confirm and significantly extend previous reports describing abundant peroxidase activity in mammary tumors of rodents and humans.[15,16,21–24] Lyttle and DeSombre[15] were the first to demonstrate that a "significant number of human breast cancer specimens contain appreciable amounts of peroxidase activity." In a subsequent study, the same investigators demonstrated that rat mammary tumor peroxidase had certain similarities to estrogen-induced peroxidase in the rat uterus.[16]

The similarities between mammary tumor peroxidase and estrogen-induced uterine peroxidase prompted other researchers to seek a correlation between peroxidase activity and estrogen dependence in human breast cancer.[23,24] Elevated peroxidase activity was detected in 65 to 71% of human breast cancers, but a correlation could not be established between peroxidase activity and the presence or absence of estrogen receptors in the tumors. Interestingly, there was a moderate but statistically significant ($r = 0.37$, $P = 0.001$) correlation between peroxidase activity in breast cancers and infiltration of the tumor by lymphocytes.[23]

Although the preceding studies confirmed the presence of elevated levels of peroxidase in a major subset of mammary tumors of rodents and humans, the cellular origins of the enzyme remained to be identified. Velocity gradient centrifugation studies of mechanically dispersed rat mammary tumors by Brightwell and Tseng[22] suggested that the peroxi-

dase activity was primarily located in the non-epithelial stromal cells (eg, fibroblasts and granulocytes). The authors of that study speculated that migrating eosinophils were probably responsible for the peroxidase in the breast tumor parenchyma, but confirmatory studies were not performed.

Our study is significant, therefore, because it clearly attributes the bulk of the peroxidase activity in human mammary tumors to EPO. We base this conclusion on the relatively infrequent presence of cytochemically detectable lactoperoxidase in the tumors, the relative paucity of myeloperoxidase within the tumors, and the highly specific results of our controlled immunostaining studies with anti-EPO antibody. In addition, there was a strong relationship between the cytochemical assays of peroxidase activity and the immunohistochemical detection of EPO.

At this time, there is no evidence that eosinophils within breast cancers are responding to a specific immunological stimulus. Thus, the exact function of eosinophils in breast cancers remains uncertain. In rodents, studies of hormone-responsive tissues such as the breast and the uterus have suggested that eosinophil migration occurs in response to estrogen administration.[25–28] There is also abundant evidence that eosinophils generally infiltrate injured tissues where they play a major role in connective tissue remodeling and repair by modulating the activity of fibroblasts.[29,30]

Consequently, the degranulating eosinophils that are present in breast cancers could conceivably represent the host's immunologically nonspecific remodeling or inflammatory response to tissue damage created by the growing tumor. The abundant EPO that we observed in the connective tissue stroma of some breast cancers provides indirect support for this notion. Furthermore, eosinophil degranulation commonly occurs in inflammatory fibrotic lesions but not in noninflammatory fibrous proliferations such as dense stromal fibrosis of the breast.[13] Additional studies to investigate this intriguing possibility, therefore, are clearly warranted.

Finally, our findings may eventually have important clinical implications. The monoclonal antibody against EPO that was used in this study has already been radiolabeled and shown to localize to tumors in patients with Hodgkin's disease infiltrated by eosinophils.[17] Thus, the EPO antibody may also prove to be useful for investigational radioimmunotherapy of some breast cancer patients whose tumors contain extensive extracellular deposits of EPO.

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