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Microcalcifications Associated with Breast Cancer: An Epiphenomenon or Biologically Significant Feature of Selected Tumors?

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Radiographic mammary calcifications occur in 30–50% of breast cancers and constitute one of the most important diagnostic markers of both benign and malignant lesions of the breast. The presence of oxalate-type microcalcification appears to be a reliable criterion in favor of the benign nature of the lesion or, at most, of a lobular carcinoma *in situ*. In contrast, calcium hydroxyapatite (HA) crystals are associated with both benign and malignant breast tumors. Although the diagnostic value of microcalcifications in breast cancer is of great importance, the genesis of these calcifications is unclear. Despite numerous histological ultrastructure studies of HA deposits in breast carcinomas, to date there have been limited investigations of the potential role of these crystals in breast cancer. We review the literature examining the biological effects of HA crystals in breast cancer cell lines, specifically the mechanism of HA-induced mitogenesis and upregulation of gene expression.

KEY WORDS: microcalcifications; breast cancer; hydroxyapatite; calcium phosphate.

INTRODUCTION

In a significant proportion of breast cancer cases, the detection of microcalcifications on mammography is a unique sign indicative of the presence of a breast lesion. Since the first description of calcifications and breast cancer in 1913 the significance of microcalcifications associated with breast disease has been the subject of much debate. In this paper we review the literature in this area and present an overview of the current understanding of microcalcifications and their association with breast cancer.

MAMMOGRAPHY AND MICROCALCIFICATIONS IN BREAST CANCER

Mammographic features of various tumor types provide a reasonable estimate of the nature of the underlying pathological change *(1)*. Thus, mammography screening has radically altered the typical presentation of breast cancers, as an ever-increasing number of breast cancers are diagnosed and treated at small sizes before axillary metastases develop *(1)*. Furthermore, mammography is, as yet, practically the only diagnostic tool capable of detecting breast cancer in this pre-invasive state, with a possible

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Abbreviations used: BSP, bone sialoprotein; COX, cyclooxygenase; DCIS, ductal carcinoma *in situ*; EGF, epidermal growth factor; HA, hydroxyapatite; HFF, human foreskin fibroblasts; HMEC, human mammary epithelial cells; IL-1β, interleukin-1β; MEK, MAP kinase kinase;MMP, matrix metalloproteinase; PC, phosphocitrate; PGE_2, prostaglandin E_2; PI3-K, phosphatidylinositol 3-kinase; PKC, protein kinase C.

rate of almost 100% *(2)*. Mammographic findings and histology correlate positively, particularly when microcalcifications are observed.

The term 'calcification' is used commonly in the medical literature to refer to hard, so-called mineralized materials in various parts of the body *(3)*. Radiologically detectable microcalcifications were first described by Leborgne in 1951 *(4)*. These small deposits of calcium are usually a non-palpable finding visualized by mammography. They have a far higher attenuation compared to the surrounding tissue and absorb more radiation, thus appearing as bright spots in a mammogram. Radiographic mammary microcalcifications constitute one of the most pertinent markers of both benign and malignant lesions of the breast. Approximately 40% of mammary carcinoma present such ectopic mineralization and they are often the sole mammographic feature that indicate the presence of a tumoral lesion *(5)*. Microcalcifications are the primary indication for approximately 50% of the breast biopsies performed for non-palpable mammographic abnormalities. In addition, upto 90% of DCIS are detected in their preclinical, asymptomatic phase by mammographically visible microcalcifications *(1)*. The presence of mammographic microcalcification does not appear to depend on the age of the patient or the primary tumor size. Lymph node involvement by tumor is present in 50% of patients with mammographic microcalcification in relation to the primary tumor, but in only 24% of patients without microcalcification *(6)*. However, microcalcification in the breast is not restricted to malignant lesions but can also be associated with benign conditions such as fibroadenoma, secretory diseases, and fat necrosis.

Tabar *et al.* applied mammographic classifications of tumor type to mammograms of invasive breast cancers of size 1–14 mm *(1)*. The terminology used in this classification corresponds to the American College of Radiology's BI-RADS mammographic classification system. Classifications describe stellate (spiculated) mass with no calcifications, circular or oval lesions with no calcifications, circular lesions with non-casting type calcifications, and casting type calcifications *(1)*. Long-term survival was shown to be good for tumors ranging from 1–14 mm and for all type of classifications except for tumors with casting-type calcifications. Furthermore, the relative hazard of death from breast cancer was five times higher for tumors with casting-type calcifications than that for circular lesions without calcifications. These tumors with casting-type calcifications on mammography represent a sub-group of DCIS that has been described by Lagios and colleagues as high nuclear-grade DCIS *(7)*. Tabar's study showed that the only reliable discriminating mammographic criterion in tumors of 1–14 mm is the presence of casting-type calcifications on mammography *(1)*.

TYPES OF MICROCALCIFICATIONS

Analysis of microcalcifications by electron microscopy, microprobe analysis, and X-ray diffraction has revealed two distinct forms of microcalcifications in breast disease on the basis of their appearance and chemical composition (Table I) *(8)*. Type 1, crystalline macroscopic microcalcifications are generally found well within the mammary parenchyma. Frappart *et al.* examined microcalcifications extracted from 25 fresh specimens which were previously located by radiography. Their findings revealed that on light microscopy type I, microcalcifications appear amber in color and are partially transparent; they are also birefringent under polarized light. On scanning electron microscopy they form pyramids or dipyramids. They have relatively planar surfaces, sometimes showing resorption images. As type I

Table I. Properties of Type I and II Calcifications

	Type I	Type II
Chemical composition	Calcium oxalate $CaC_2O_4{\cdot}2H_2O$	Hydroxyapatite $Ca_{10}(PO_4){\cdot}6H_2O$
Color	Amber	Grey, white
Light microscopy	Partially transparent	Opaque
Polarized light	Birefringent	Non-birefringent
Stains with haematoxylin	No	Yes (purple)
Lesions generally found in association with	Predominately benign	Benign & malignant

microcalcifications are refractory to staining with routine dyes, they can be overlooked during histological examination. They are, however receptive to staining with alizarin red S in an alkaline solution *(9)*. On transmission electron microscopy they present a homogeneous structure, with well-defined limits. Analysis by microprobe revealed a calcium peak. Further investigation by X-ray differentiation of the monocrystals demonstrated that weddellite, a particular form of calcium oxalate ($CaC_2O_4 \cdot 2H_2O$) is involved.

This study also found that type II macroscopic microcalcifications are ovoid or fusiform, grey-white, opaque to luminous rays, and nonbirefringent under polarized light. These noncrystalline type II microcalcifications are easily detected and stain with hematoxylin *(9)*. Scanning electron microscopy showed that their surfaces are usually irregular, and they sometimes appear to be composed of tiny spheres or oolites. On microprobe analysis two peaks were observed: one corresponding to calcium and the other to phosphorous. These calcifications are composed of calcium phosphate, the most characteristic form of which is hydroxyapatite [$Ca_{10}(PO_4) \cdot 6H_2O$], in the form of needles arranged in rosettes by transmission electron microscopy.

OXALATE VERSUS HYDROXYAPATITE

Occasional investigations in the literature have examined the relationship between the histological appearance of the tumor (malignant or benign) and the chemical nature of the deposit. Busing *et al.* examined 11 specimens of breast tumors with extensive calcification and reported that oxalate in the form of weddellite was found only in proliferating but not in invasive diseases of the breast *(10)*. In contrast, apatite crystals were found only in invasive malignant tumors *(10)*. In 1977, Barth and his colleagues investigated a case of proliferative mastopathy and found oxalate *(11)*. Hassler *et al.* reported on three invasive carcinomas in which calcium apatite was found *(12)*. Furthermore, in 1975 Ahmed *et al.* found calcium apatite in cases of infiltrative carcinoma, although this study failed to specify the number of tumors investigated *(13)*. Tornos *et al.* demonstrated that when calcium oxalate was found at all in patients with breast malignancy, it was found only in association with benign portions of breast parenchyma *(14)*. Busing *et al.* concluded that oxalate calcifications are associated with proliferating but non-invasive diseases of the breast, for example, proliferative mastopathy or lobular carcinoma *in situ* (LCIS), but calcium phosphate in the crystalline form of apatite is correlated to invasive malignant tumors, e.g. undifferentiated ductal carcinoma *(10)*. Studies specifically examining the pathology associated with hydroxyapatite calcifications are few however, it is widely accepted that hydroxyapatite is also commonly seen with a variety of benign conditions such as sclerosing adenosis.

A study performed by Going *et al.*, examined weddellite calcifications obtained from breast tissue by localization biopsy in 18 women *(15)*. The authors concurred with the general consensus, that weddellite deposition is a feature of benign rather than malignant processes within the breast. This assertion was supported in this case by the observation that none of the 18 cases had invasive carcinoma. In the cases showing atypical lobular hyperplasia and LCSI, the crystals were only identified in parenchymal units, which did not show these changes *(15)*.

In addition, Frouge *et al.* discussed the findings of several authors who had reported an association between weddellite crystals and true neoplasia. They described 75 published cases which included 6 of LCIS, 2 of invasive carcinoma and 1 of intraductal carcinoma. The overall frequency of malignancy reported was 12.5%. In most of the studies described by Frouge *et al.*, weddellite crystals were not observed in areas of carcinoma. Frouge *et al.* also reported that polyhedral microcalcifications due to calcium oxalate were of considerable diagnostic value, since 89% of the cases were associated with benign disease *(16)*.

CAN CLUSTERING OF MICROCALCIFICATIONS REVEAL THE BENIGN OR MALIGNANT NATURE OF A TUMOR?

Literature suggests that clustering of microcalcifications is an important diagnostic criterion to distinguish between benign and malignant lesions *(17)*. When calcifications are detected on mammography, their number, morphologic appearance, size, associated findings, and distribution should be examined to characterize them *(18,19)*. An increased risk for malignancy with increasing numbers of mammographically visible microcalcifications was reported by Park *et al. (19,20)*. In contrast, Bird reported that the number of microcalcifications was of little help in analysis *(21)*. Powell *et al.* found that 84% of cancers were associated with more than 10 calcifications; they also located no cancers when fewer than five calcifica-

tions were located in a cluster *(22)*. Franceschi *et al. (23)* suggested two predictive criteria for malignancy, namely, linear/aborescent pattern and number of microcalcifications (>15 per cm^2), both of which correlate well with the findings of Park *et al (24)*. However, Park *et al*. concluded that clustering in itself is not a particularly useful diagnostic feature for differentiating malignant from benign breast microcalcifications revealed by mammography. Rather, pleomorphism of microcalcifications or associated architectural distortion, mass or increased density should be considered as more important diagnostic predictors of malignancy.

FORMATION OF CALCIFICATIONS

Although the diagnostic value of microcalcifications in breast cancer is of great importance, the genesis of these calcifications is not clear. In particular, the issue as to whether it is a sign of degeneration or of an active cell process is still unresolved. In earlier studies, breast tumors were shown to demonstrate both amorphous and crystalline deposits *(10)*. Busing *et al.* suggested that amorphous deposits conform well with the interpretation that they are of a secondary dystrophic or degenerative nature *(10,25)*. No relationship to vessels was found such as that described by Johahhessen *et al*., who showed calcifications around vessels in thyroid tumors *(26)*. Furthermore, Busing *et al.* discussed how calcium apatite might also be of a degenerative or dystrophic nature, since it seemed possible that at the beginning of the crystalline deposition there may be a gel phase *(10)*. Stegner *et al*. suggested that crystalline calcifications, in the form of oxalate, are probably the product of an active cellular process *(27)*. In addition, Ahmed proposed that luminal calcification is a consequence of an active secretory process by the tumor cells and not the result of mineralization of cellular debris and degenerate tumor cells *(27)*. Explanations in support of this secretory process include the fact that mammary epithelium is capable of concentrating calcium ions *(13)*, which are an essential component of milk. Furthermore it has been shown that the calcium ion gradient is towards the lumen along the epithelial plasma membranes and through the tight junctions *(13)*. Another factor which may enhance the secretory capacity of the carcinoma cells is a highly active plasma membranes ATPase.

Holme *et al.* also described the importance of calcium ions in cell adhesion and mediation of intracellular functions *(6)*. They indicated that disorders of calcium metabolism have frequently been mooted in malignant processes and suggested that the deposition of calcium in breast cancers might represent a biologically significant feature of selected tumors *(6)*. The authors hypothesized that an abnormality in calcium pumps or calcium regulatory proteins might result in the deposition of microcalcifications *(6)*.

Bellahcene *et al.* hypothesized that malignant mammary cells could express bone matrix proteins that would create an appropriate microenvironment to trigger hydroxyapatite formation and facilitate its interaction with bone matrix *(28)*. This hypothesis was based on the observation that human breast cancer cells are capable of inducing hydroxyapatite crystallization. Osteonectin, osteopontin and bone sialoprotein (BSP), three bone matrix proteins involved in bone matrix mineralization, are expressed in human breast cancers. BSP has been shown to initiate hydroxyapatite deposition and mediates attachment of osteoclasts to the crystals prior to their resorption *(28)*. Bellahcene *et al.* detected BSP at both the protein and the mRNA levels in human breast cancer cell lines such as MCF-7 and MDA-MB 231. These data indicate that mammary epithelial cells synthesize BSP directly rather than using uptake from the serum. Furthermore the level of BSP expression was shown to correlate with the development of bone metastases and with poor survival, which suggests that the ectopic expression of bone matrix proteins could be involved in conferring osteotropic properties to circulating metastatic breast cancer cells *(6)*.

CRYSTAL-RELATED TISSUE DAMAGE IS NOT EXCLUSIVE TO BREAST CANCER

The potent biological effects of calcium HA crystals are well recognized in other diseases unrelated to the breast. Calcium crystals have long been implicated in the pathogenesis of a number of rheumatic syndromes, such as calcium-containing crystal deposition diseases, including pseudogout, calcific periarthritis, Milwaukee shoulder syndrome, and osteoarthritis. The concurrence of calcium-containing crystals such as basic calcium phosphate (hydroxyapatite; $Ca_{10}(PO_4)_5(OH)_2$, octacalcium phosphate, tricalcium phosphate), and calcium pyrophosphate dihydrate (CPPD; $Ca_2P_2O_7{\cdot}2H_2O$) deposition and degenerative joint disease is well established. These diseases of calcium deposition serve as some of the best-studied examples of how

calcium-regulated changes in gene expression can directly lead to pathogenic consequences *(29)*. These properties may also be relevant in breast oncology. In additional, recent work has elucidated the potential involvement of calcification in atherosclerosis. Arterial intimal calcification is a common clinical and pathological finding in patients with atherosclerosis *(30)* and the degree of intimal calcification has prognostic significance *(31,32)*. Hydroxyapatite is the major constituent of the calcific deposits seen in atherosclerotic vessels.

BIOLOGICAL EFFECTS OF CALCIUM HYDROXYAPATITE IN BREAST CANCER CELLS

Recently it has been recognized that the composition of breast calcifications may give clues to their origin and that certain compositions may be more strongly associated with malignancy *(15,16)* and adverse disease outcome *(1)*. The biological effects of calcium HA crystals on mammary cells were investigated *in vitro*, and properties of calcium HA have been observed which emphasize its pathogenic potential in breast cancer *(33,34)*. Initial experiments revealed the ability of HA to promote mitogenesis, possibly amplifying the malignant process by leading to aggravation of tumor growth *(33)*. HA was shown to increase mitogenesis in both normal (HMEC) and malignant mammary cell lines (Hs578T and MCF-7). Non-calcific, control particles of latex beads, of similar size and concentration to HA crystals, had no effect on mitogenesis. This study also showed that HA crystals stimulate mitogenesis of quiescent mammary cell lines in a concentration dependent fashion. It has been also previously shown that HA crystals stimulate mitogenesis of quiescent cultured human foreskin fibroblasts and adult articular chondrocytes in a concentration dependent fashion *(35)*. The mechanism of HA crystal-induced activation of human foreskin fibroblasts involves two processes: (1) A fast membrane associated event involving protein kinase C and mitogen activated protein kinase activation, nuclear factor-κB induction and expression of proto-oncogenes *c-fos* and *c-myc* and (2) the relatively slow endocytosis and intracellular dissolution of the HA crystals, raising intracellular calcium and causing the activation of a number of calcium-dependent processes leading to cell proliferation *(35)*.

Having established that HA crystals induce mitogenesis in mammary cells, experiments were performed which sought to elucidate the precise molecular mechanism of this induction. It was observed that direct cell–crystal contact was required for induction of mitogenesis *(34)*. When HA crystals were added, but cells were not directly in contact with HA, no induction of mitogenesis occurred. Thus, the effect was not merely the result of release of calcium into the culture medium from the addition of the HA crystals. Using the proton pump inhibitor bafilomycin A_1 to inhibit intracellular dissolution of HA crystals, the importance of intracellular crystal dissolution for the mitogenic response was also confirmed *(34)*. Treatment with bafilomycin A_1 abrogated HA-induced mitogenesis to control cell levels, suggesting that phagocytosis and intracellular crystal dissolution is required for HA-induced mitogenesis. The resultant increase in cytoplasmic calcium concentration could activate calcium-dependent pathways which result in mitogenesis.

The early proliferative stages of breast cancer are characterized by a continuous basement membrane separating the hyperplastic epithelial cells from the surrounding stroma. Pathologically, the transition from *in situ* to invasive carcinoma is usually accompanied by interruption of the basement membrane *(36)*, caused by an enhanced process of proteolysis contributing to the escape of breast cancer cells into neighboring tissues, eventually leading to the formation of distant metastases. MMPs are associated with degradation of the extracellular matrix (ECM), including the basement membrane. Numerous studies have demonstrated how inappropriate expression of MMPs can initiate a cascade of events that may represent a coordinated program leading to a phenotypic transformation in mammary epithelial cells. Morgan *et al*. found that HA caused an upregulation in the production of a variety of MMPs, including MMP-2, -9, and -13 in MCF-7 and MMP-9 in human mammary epithelial cell lines *(33)*. A correlation between expression of MMPs and the invasive phenotype of tumor cells has also been shown *(37)*, an observation which may explain why HA-induced expression of MMP-1 was observed in Hs578T cells, and not the weakly invasive MCF-7 breast adenocarcinoma cell line. HA crystals are potent inducers of MMP-1, -3 and -9 in human foreskin fibroblasts and synoviocytes *(38)*. Cheung and co-workers have shown that basic calcium phosphate crystals induce MMP-1 expression in human fibroblasts through an extracellular regulated protein kinase 1 and 2 pathway also involving *c-fos*/AP-1 and RAS signalling pathways *(39)*. The ability of HA crystals to induce

members of the MMP family at the transcriptional level may reflect a similar cascade of events in this breast cancer model.

High levels of PGE_2 are often associated with estrogen receptor-negative tumors that exhibit a high metastatic potential *(40)*. Several studies with murine mammary tumor cells indicate that PGE_2 may have a multifunctional role in controlling growth, metastasis, host immune response in breast cancer *(40)*. The cyclooxygenase (COX) enzymes (COX-1 and -2) catalyze the conversion of arachidonic acid to prostaglandins. Morgan *et al*. reported differential PGE_2 production from breast cancer cell lines MCF-7 and Hs578T, which could be increased by HA treatment *(33)*. HA crystals were found to augment PGE_2 production in Hs578T cells by almost 8-fold, but caused a modest 1.5-fold increase in MCF-7 cells. Confirmation that HA crystal stimulation upregulates COX-2 mRNA transcript levels was demonstrated in a subsequent study in which HA caused an increase in COX-2 mRNA over a range of timepoints, with maximal expression at 4–8 h, when compared with untreated controls. Treatment of both cell types with 2 mM aspirin, a general COX inhibitor, blocked the HA-induced increase in mitogenesis of the cells, bringing mitogenesis back to baseline levels. The contrasting production of PGE_2 in the cell lines MCF-7 and Hs578T in response to treatment with HA is caused by differential upstream regulation of COX-2 mRNA expression *(34)*, which has been reported in the literature to be influenced by hormone status and metastatic phenotype in human breast cancer cell lines *(41)*. These results confirm that exposure to HA crystals can cause a significant increase in PGE_2 which is due, at least in part, to direct induction of COX-2 mRNA in Hs578T cells *(33)*.

Although mainly synthesized by monocytes and macrophages, IL-1 has been found in several human tumors and may enhance tumor invasiveness, by increasing adhesion to the vascular surface *(42)*, stimulating tumor cell motility *(43)* and inducing MMP-1 and PGE_2 production *(44)*. HA was shown to induce IL-1β mRNA at 2 and 4 h in human fibroblasts *(34)*. The HA-induced IL-1β could be functioning in an autocrine loop leading to increased transcription of MMP-1 and/or COX-2, both of which are well documented to be regulated by IL-1. In addition, IL-1 has been shown to increase the stability of several mRNAs in various cell types, including MMP-1 mRNA in human fibroblasts *(45)*.

In summary, calcification is a well recognized histological and radiological feature in certain breast carcinomas. Much attention has been paid to the composition of microcalcifications in breast cancer, but very little attention has been paid to the possibility that the deposition of calcium in breast cancers might represent a biologically significant feature of selected tumors. Despite the association of microcalcifications with poorer survival in breast cancer patients, and *in vitro* data demonstrating their pathogenic potential, to date there have been limited investigations of the mechanism of calcium deposition or the potential of different mammary cell types to generate calcium mineral species. Future research in this area must focus on testing the hypothesis that mammary cells are capable of generating mineral species which reflect their biological state, providing powerful insight into the biology of the tumor microenvironment. Clarification of the potential effects of microcalcifications in breast cancer is essential to our further understanding of the pathophysiology of this devastating disease and ultimately may help resolve the unexplained prognostic association of these calcifications with benign and malignant disease of the breast.

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