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LETTERS

Using scanning electron microscopy to study mineral deposits in breast tissues

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Abstract

Thin sections of breast tissues from patients with possible tumors also contained closely associated mineral deposits. Using scanning electron and backscattered electron microscopy (SEM-BSE) energy and wavelength dispersive chemical analyses, we found the deposits ranged from <1 μm to 50 μm in size and were composed predominantly of calcium phosphate. Semi-quantitative chemical determinations indicate that the mineral exhibits different Ca/P ratios depending on the site of deposition. This high-resolution methodology to chemically analyze precipitates in human tissues is a novel approach that may provide some new insights into mineral deposition in soft tissues. Application of the technique to establish compositional biases in a wide variety of tissues seems warrented. Specifically, additional data on mineral deposits in breast tissues could contribute toward the understanding of the localized changes and to the development of disease at this site.

Introduction

Mammograms, or transmission X-radiographic images of the breast, are a non-invasive technique used for detecting sites of potential tumors of the breast. In many cases micron-sized mineral deposits are also detected in the mammogram. The clinical diagnosis depends on discriminating affected tissues from normal tissues and a small sample in a suspected tumor area is excized (biopsy). The standard way to evaluate the possibility of specific types of breast disease is through examination of this biopsy sample (Busing et al. 1981; Millis et al. 1976; Symonds et al. 1992; Spencer et al. 1994). Employing optical microscopy on thin (6 μm thick) sections of the excized tissues a histological examination is made to identify the cells, cell products, and any tissue characteristics that could be useful in the diagnosis and treatment of the patient. The significance of mineral deposits in tissues not normally mineralized and their potential use as indicators of changes or disease in the tissues are not yet fully explored nor understood.

Calcium-containing mineral deposits have been described as part of many different tissues in the human body. Calcium phosphate mineral material is the normal constituent of bones and teeth, but also has been identified as a part of the pathologic deposits in arteries (arteriosclerosis), and in other sites such as bursa (bursitis) and the breast. The mineral materials are usually excized from the tissue, employing not only physical but chemical extraction methods, to make an accurate identification. However, the methods used to isolate the inorganic probably alter the composition of the mineral phase (Skinner et al. 1972) and, most significantly, it is the histologic context, the surrounding cells, and extracellular materials that have the greatest significance in whatever mineral is deposited. By examining the standard tissue sections as prepared in most pathology laboratory situations by SEM-BSE microscopy, we can image and study the mineral deposits in situ, and integrate the mineral occurrence with the cell and tissue data reported by pathologists. In addition, we can utilize the energy and wavelength dispersive X-ray analysis available on the instrument to determine the chemical composition of the mineral materials.

As investigators particularly interested in biominerals and specifically calcium phosphate deposition in human tissues, we obtained samples of breast tissues for analysis that showed micron-sized mineral deposits. We demonstrate below that the composition of the deposits, the calcium to phosphorous ratio (Ca/P), varies depending on the site of deposition.

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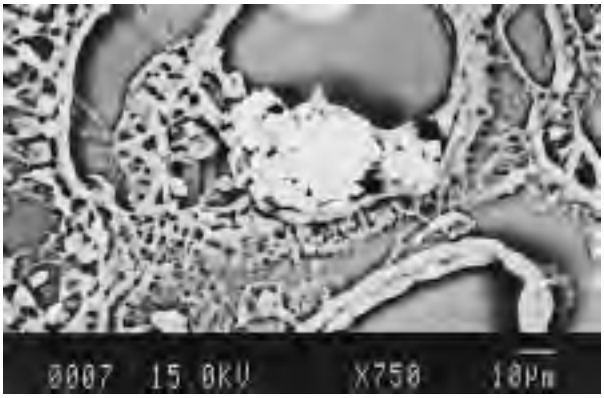

FIGURE 1. SEM-BSE photo of calcium phosphate deposits, the dense white aggregate at the center, in tissues affected by sclerosing adenosis (benign). Histological section, 6 mm thick, of breast tissue.

MATERIALS AND METHODS

A variety of changes, including mineral deposits, were observed in over 150 breast biopsies, both benign and malignant that were collected between January 1, 1994 and June 4, 1994 at Yale-New Haven Hospital (Poggi et al., in preparation). Five samples with clearly defined histological context from optical evaluation were selected for the present high-resolution microprobe analysis. These samples are from patients who had no known comorbidities or other diseases that could predispose the formation of mineral deposits in soft tissues.

Two distinct tissue sites with mineral deposits are described as ''stromal'' and ''ductal'' based on their histologic location. Stroma is a general term for the connective tissue materials or matrix normally produced by breast cells. Ducts are tubes within the tissues that are the conduits for the secretion of milk. In the large study, stromal mineral deposits were found (1) in fibroadenomas, a benign proliferation of stroma that usually occurs in younger women; (2) at sites that had scar tissue, an aggregation composed of collagen (a fibrous protein) as a result of a previous biopsy; or (3) in areas of stroma surrounding invasive ductal carcinoma. Ductal mineral deposits were found at sites of sclerosing adenosis, a benign condition characterized by increased deposition of fibrous collagen, and at sites where cancerous cells were identified (intraductal carcinoma), but had not yet invaded the surrounding stromal tissues.

The standard methods of preparation for histological examination of tissue in the Yale Pathology Department were employed with modifications as follows: Blocks of tissue were fixed in 10% zinc-formalin solution, dehydrated with serial ethanol solutions, cleared with xylene, and infiltrated with parafin. Sections of 6 μm thickness were cut with a microtome and mounted on a 22 × 60 mm Thermanox (NUNC) plastic cover slip. Some of the larger mineral deposits fractured during cutting. Figure 1 illustrates the deposition of calcium phosphate in ducts affected by sclerosing adenosis. The sections were deparafinized using xylene (to obviate any volatilization of parafin under the high-energy beam of the microprobe) and carbon coated. The samples were not polished.

Polarizing light microscopy and a JEOL JXA-8600 electron microprobe equipped with one energy dispersive spectrometer (EDS) and four wavelength dispersive spectrometers (WDS) and KEVEX software were used to examine the sections at the Department of Geology and Geophysics, Yale University. An accelerating voltage of 15 KeV and 10 nA current was used for most of the investigations. These settings were chosen to avoid overheating the fragile samples and to minimize tissue destruction. BSE images at magnifications from 55–3000× recorded the morphologies of the tissue and the mineral deposits. The chemical determinations were made using WDS, off peak background corrections, ZAF matrix corrections, and counting times of 20 s using a beam diameter of 1 μm. The following standards were used: fluorapatite for Ca, P, and F; orthoclase for K; rhodonite for Mn; enstatite glass for Mg; grunerite for Fe. Between 7 and 14 replicate analyses on the mineral material in each of the five samples were collected and corrected for matrix and background contributions to achieve semiquantitative results.

The results are semi-quantitative because the specimens were not polished, the mineral deposits have porous texture, and are of exceedingly fine grain size. We reasoned that each of the elements in our analyses would be similarly affected by these sample drawbacks and by calculating ratios of elements, such as Ca/P for the apatitic material, we would obtain a reasonable approximation of the relative proportions of these elements.

RESULTS

The results presented in Table 1 illustrate the potential of using the microprobe to examine mineral materials in human tissue sections, and in particular for determining the chemical range of calcium phosphate deposits.

Both calcium oxalate and calcium phosphate materials were identified in the tissues. The oxalate materials were of relatively large size (5–20 μm) and exhibited distinctive crystal morphology and optical character of weddellite [$Ca(C_2O_4)\cdot 2\ H_2O$] under polarized light (Fig. 2). The EDS spectral analysis on our instrument, which is incapable of detecting C or O, exhibited major and virtually only Ca on the oxalate crystallites (see below for other elements detected). This result is distinct from the more fine-grained mineral deposits that showed Ca and P. The occurrence of weddellite in breast tissues has been documented and discussed by other researchers (Frappart et al. 1984; Radi 1989; Going et al. 1990; Gonzalas et al. 1991).

Large aggregates of calcium phosphates can be located in tissues using basophillic stain techniques and optical examination (Anastassiades et al. 1984; Bouropoulou et al. 1984). From approximately 150 tissue sections examined optically in the survey of breast biopsys, over

TABLE 1. Analyses of the Ca/P composition and the mineral content (wt%) of mineral precipitates in breast tissue samples

Stromal					Ductal				
Type	No.*	Ca/P	Range†	Average	Type	No.*	Ca/P	Range†	Average
Fibroadenoma‡	14	2.26 ± 0.17	30–88	⟨78.1⟩	Sclerosing Adenosis‡	5	2.38 ± 0.16	64–79	⟨72.6⟩
Fibroadenoma§	5	2.20 ± 0.10	75–88	⟨81.0⟩	Sclerosing Adenosis§	3	2.40 ± 0.09	75–79	⟨76.7⟩
Scar‡	10	1.86 ± 0.26	56–83	⟨65.6⟩					
Scar§	2	1.785 ± 0.01	79–83	⟨80.85⟩					
Adjacent carcinoma‡	8	2.05 ± 0.12	46–82	⟨58.8⟩	Intraductal carcinoma‡	9	2.44 ± 0.26	40–87	⟨80.7⟩
Adjacent carcinoma§	3	1.95 ± 0.11	77–82	⟨79.4⟩	Intraductal carcinoma§	5	2.38 ± 0.23	77–87	⟨80.7⟩
Stromal Average Ca/P ⟨1.98⟩					Ductal Average Ca/P ⟨2.39⟩				

Note: The error for Ca/P is the mean value with a confidence interval of 95%. ⟨ ⟩ denotes average.
* Number of replicate analyses.
† Range of mineral wt%.
‡ All replicate analyses.
§ Analyses with total mineral wt% >75%.

95% showed calcium phosphate mineral deposits. The mineral appeared as compound aggregates of spherultites (Fig. 1) or as individual spherules whose size ranged from <1 to 4 μm in diameter (Fig. 3). The smaller, isolated and disseminated material that we observed using SEM/BSE would likely go undetected even when magnification of 450× (usual highest optical magnification employed in pathological examinations) and polarized light were used because the calcium phosphate mineral has virtually no birefringence. A bimodal distribution of microspherules (Fig. 3) were easily visualized within a fibroadenoma using the higher magnifications afforded by SEM.

Table 1 shows the Ca/P weight ratios of the calcium phosphate mineral deposits calculated from the wavelength dispersive analysis. The Ca/P results range from 1.76 to 2.48. Because of the roughness of the surface of these non-polished samples, the composite nature of some aggregates (see discussion), or the small size of individual spherulites, we expected low energy spectral emissions and were concerned about how to evaluate these calculated results. The table is divided into stromal and ductal sets. The first line of data for each sample presents the numerical averaged values for all determinations on a particular tissue section sample, and the second line presents only those analyses with >75 wt% for the combined Ca and P (as oxides) analyzed. The second line is considered more reliable for comparison. The cutoff of 75 wt% was deliberately chosen to compare these calcium phosphate composition materials with those determined on normal bone tissues (see discussion). Note that EDS reconnaissance performed on these tissue sections was sufficiently sensitive to detect both Zn within the soft tissues surrounding the mineral deposits from the formalin preservative solution, and Al if the sections had been stained with hematoxylin/eosin, part of the usual preparative procedures for standard histological examination.

The results (Table 1) show high variability. Replicate diversity could be expected on the stromal microcalcifications as some of the spherulites were <1 μm in di-

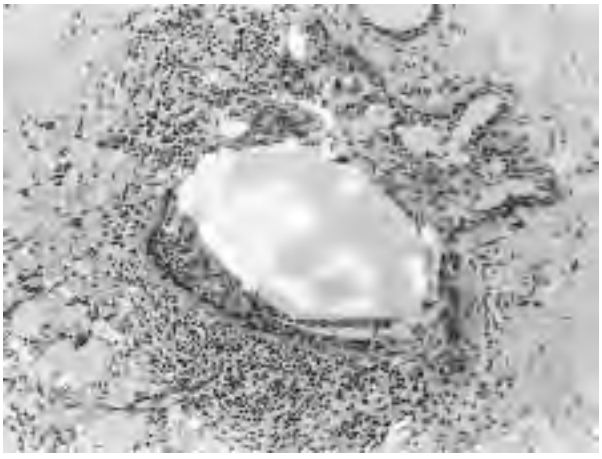

FIGURE 2. Optical microscopy photo under crossed polars of calcium oxalate deposits in a duct. Crystallites range from 1 to 60 μm. Magnification 40×. Histological section, 6 mm thick, of breast tissue.

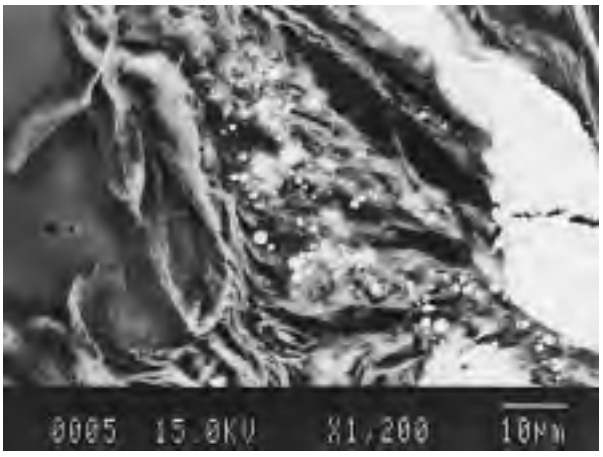

FIGURE 3. SEM-BSE photo of calcium phosphate spherules in stromal tissue (fibroadenoma). Note the bimodal size distribution of spherules in the center of the photo relative to the heavily mineralized deposit of calcium phosphate on the right. Histological section, 6 mm thick, of breast tissue.

ameter and therefore the amount of mineral in the beam was low (See Fig. 3 with the bimodal size of mineral precipitates and note that analyses on this fibroadenoma specimen give the widest range of all mineral weight percent results). On the other hand, replicate analyses on the ductal calcifications also show significant variations in Ca/P values in spite of the fact that they were aggregates of larger size. Because measurements were taken at several sites within these aggregates, the variation in the calculated Ca/P ratios suggest there are local composition variations in the mineral, that is, the mineral matter within the duct is not homogeneous.

Table 1 shows that stromal and ductal deposits have distinct calculated Ca/P ratios. The stromal microcalcifications have lower values than those of the ductal samples. The lower Ca/P value relative to ductal is reinforced when only the high total mineral values (>75% mineral) are considered. The stromal average (three different sites in three patients) is 1.98 while two ductal sites have an average value of 2.39. The differences between the sites is certainly detectable and appears reproducible. Note however, that the number of cases studied does not permit a statistical evaluation with the usual derivation of P values.

Average (and error) of fluorine determinations on these microcalcifications ranged from 0.40 ± 0.28 to 1.24 ± 0.55 wt% with the higher values measured in the stromal microcalcifications. Lower values of magnesium (range 0.12–0.51 wt%) were found in all the stromal calcium phosphates while sodium averages varied between 0.37 ± 0.21 and 0.92 ± 0.39 wt% with no obvious trend. Magnesium and potassium concentrations were negligible in all samples (average concentrations <0.05 wt%) while Fe values >0.10 wt% were measured on at least one determination of the replicate analyses for each of the five samples. The values cited here for Mg, Na, Fe, and F should be taken as indicating the presence of these elements rather than as accurate determinations because of the sample drawbacks mentioned above.

Discussion

Calcium phosphates, usually hydroxylapatite [$Ca_5(PO_4)_3(OH)$] or a carbonate-containing hydroxylapatite, are the most common Ca-bearing species in human tissues. They are present in normal as well as in benign and malignant tissue settings. To examine the biologically precipitated mineral, other potentially confounding components (biomolecules, cells, etc.) are usually eliminated by extracting and concentrating the mineral using physical and chemical methods. The mineral also may be heat treated to increase crystallinity, and thereby enable X-ray diffraction analysis to define the particular mineral species. All preparations usually show hydroxylapatite and often other phases depending on the extraction technique, the temperature of the heat treatment, and the bulk composition (Skinner 1987).

In equilibrium phase studies in the CaO-H_2O-P_2O_5 system, Skinner (1973) showed that the phase field hydroxylapatite-fluid was exceedingly restricted but had a fluid pH of 7. Small shifts in the Ca/P ratio from the hydroxylapatite ideal composition (Ca/P = 2.14) and H_2O predisposed the appearance of a second solid phase and a shift in pH. Below the Ca/P of ideal hydroxylapatite and at temperatures up to 100 °C, another calcium phosphate, such as brushite ($CaHPO_4 \cdot 2H_2O$) might appear if the precipitate was in equilibrium with its fluid. The pH was below 7. Above the ideal Ca/P for hydroxylapatite, the associated phase did not contain P. In the simple chemical system, the phase associated was $Ca(OH)_2$ and the pH about 12. The addition of other components (Na, Mg, S, CO_2, etc.), as might be expected under any natural conditions, may induce the appearance of other mineral species although apatite has a remarkably elastic crystal chemistry (McConnell 1973; Skinner 1997). The precipitation of calcium phosphates and ranges of Ca/P in biologic tissues that we outline herein are therefore not unexpected.

Analyses of extracted bone mineral shows apatitic materials with a Ca/P range from 1.95 to 2.4 (Skinner et al. 1972; LeGeros and LeGeros 1984). Low Ca/P ratios, relative to ideal hydroxyapatite are typical of the mineral precipitate in the beginning of bone formation where the amount of mineral is low, <50 wt%, and often irregularly dispersed in the volume of tissue examined (Glimcher 1984; Albright and Skinner 1987; Markel et al. 1991). This ''early'' mineral is so poorly crystallized that it has been called amorphous calcium phosphate. Similar materials have been identified in lesions in the body, such as in the intestines in patients with Crohn's disease (Roge et al. 1993), at sites of inflammation (Reid and Armstrong 1988; Skinner 1987), and in necrotic tissue or tumors where several mechanisms for apatite precipitation relative to cell death have been proposed (Anderson 1983; Millis et al. 1976).

The bulk Ca/P ratio reported here on calcium phosphate mineral materials is similar to that demonstrated by other investigators on biologic apatites in breast tissues (Faudos-Morere et al. 1988; Foschini et al. 1996). However, we believe our results demonstrate a range of mineral compositions, and that different composition calcium phosphate deposits can be identified in breast tissues depending on the site of deposition.

Glimcher (1984) estimated the size of individual calcium phosphate crystals (plates or needles) in bone tissues to have dimensions of approximately 15 Å by 500 Å. We estimate that the calcium-phosphates we describe here are probably in the same range. The deposits are porous aggregates of such crystals judging by the total weight percent of mineral materials detected in our analyses. Frappart et al. (1984) using ultra high magnification (200 000×) TEM analysis of breast microcalcifications showed that needle crystallites, presumed hydroxylapatite, were present in association with other less crystalline mineral materials. Unfortunately a Ca/P analysis was not performed and we dare not speculate on what the second precipitated mineral phase might have been. Without fur-

ther higher resolution study we cannot compare our results with these investigators.

It is our view that mineral deposits in the breast parallel other bioprecipitation situations and reflect local physical and chemical conditions. The appearance of calcium-containing mineral materials such as calcium oxalate or calcium phosphate in breast tissues associated with benign or malignant reactions are expressions of the fluid compositions in association with certain cells and tissues. Ca-rich fluids with low or absent P would be more likely to precipitate an oxalate phase while an extracellular fluid that contains Ca and P should precipitate hydroxylapatite and perhaps additional phases with the identity of the other phases dependent on the Ca/P ratio of the bulk composition.

From these initial investigations, stromal precipitates have distinctive compositions when compared to ductal precipitates and suggest that the composition of the precipitating fluids must be distinctive. The stromal deposits in our samples may represent rapid and possibly relatively recent deposits in the tissue. The scar sample we analyzed does come from a younger individual (52 years while the others were 61–66 years) who was biopsied at the same site just 6 months before. This sample epitomizes the rapidly formed, lower Ca/P ratio deposits found in other biological systems. The higher Ca/P measured on ductal microcalcifications suggest a Ca-rich fluid composition, at least at some time during deposition of the mineral materials. Ducts are the sites where more heterogeneous chemical conditions may be present, especially over time. The mineral concentration in the ducts is relatively more dense (higher total mineral deposited) but there were wide variations in Ca/P ratio on replicate analyses at these sites. A Ca/P above ideal hydroxylapatite in the experimental systems implies not only a multiphase precipitate but a pH >7. Whether or not that is the case in the ductal tissues is not known, but the fluid one might expect to find in the ducts, human breast milk, has a Ca/P ratio of 2.33 (Newton 1990).

This initial investigation shows that SEM analysis can provide coordinated morphologic and chemical results relatively easily and rapidly on the histologic sections normally utilized by pathologists to evaluate patient health or disease. The compositional variations detected in calcium phosphate mineral materials occurring in the breast tissues we examined may signal different chemical as well as biological environments. We suggest that SEM techniques can be used to investigate in situ cell and tissue reactions under different, and especially under pathologic, conditions.

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