Identification of Mineralized Elastic Fibers on Wet Samples by SEM

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ABSTRACT A method is described that could be of potential use for the rapid ultrastructural identification of abnormal and fragmented elastic fibers in very small wet samples of dermal biopsies from patients affected by Pseudoxanthoma elasticum (PXE). Moreover, the method, which consists of the use of sealed capsules transparent to electrons, allows the rapid and accurate localization and detection of mineralized areas in PXE patients and of their ion composition by X-ray microanalysis. This methodology could be of great help in any tissue disorder, especially in connective tissue disorders, characterized by structural alterations associated with ion precipitation. *Microsc. Res. Tech. 67:296–299, 2005.*

INTRODUCTION

Pseudoxanthoma elasticum (PXE) is a genetic disorder affecting the skin, the eye, and the cardiovascular system. The gene responsible for PXE encodes for the protein MRP6, a member of the ABC family of membrane transporters, whose physiological function is still unknown (Bergen et al., 2000; Le Saux et al., 2000; Ringpfeil et al., 2000). The prevalence of PXE has been recently estimated to be 1:75,000 (Neldner and Struk, 2002).

Although rare, the number of new cases is continuously increasing because of widespread knowledge of the disorder, and therefore technical improvements in diagnostic procedures may be of great help for the recognition of early signs of the disease.

Clinical manifestations of PXE are in fact progressive with time and consist of skin papules and cutaneous laxity mainly on neck, axillae, groin, and flexural areas, of angioid streaks and recurrent hemorrhages in the retina, and of cardiovascular involvement resembling age-associated alterations (Neldner and Struk, 2002). The great majority of these alterations can be attributed to degeneration of the elastic fibers that undergo progressive mineralization and fragmentation (Martinez-Hernandez and Huffer, 1974; Ross et al., 1978). In a series of studies, abnormalities have been described affecting several extracellular matrix components such as collagen and proteoglycans (Pasquali-Ronchetti et al., 1981, 1986; Tiozzo-Costa et al., 1988), as well as fibroblast behavior in vitro (Quaglino et al., 2000); however, elastic fiber mineralization is considered the parameter of choice for diagnostic purposes on skin biopsies.

To date, light and electron microscope analyses on skin biopsies take time and are rather expensive. Moreover, by light microscopy, mineral precipitates can be visualized after Von Kossa staining, which requires rather large skin samples and rather big mineral precipitates to be seen.

A method that is rapid and of high sensitivity is described, which can be applied to PXE as well to other disorders with connective tissue alterations and mineral precipitation, and requires very small tissue samples.

MATERIALS AND METHODS

Samples

Skin samples were obtained after informed consent from four adult PXE patients during plastic surgery or by punch biopsies. Tissue samples as small as 1 mm^3 were used.

Morphology

Skin specimens underwent the following treatments: (i) washes in cacodylate buffer, (ii) washes in cacodylate buffer and overnight fixation in 2% paraformaldehyde in cacodylate buffer, (iii) washes in cacodylate buffer, overnight fixation in 2% paraformaldehyde in cacodylate buffer and 1-h staining in 1% uranyl acetate in water after fixation. In some cases, frozen samples stored at $-80^{\circ}C$ were also used.

Samples were then placed in the Quantomix X320 sealed specimen capsules separated from the vacuum in the microscope chamber by an impermeable, electron-transparent membrane (Gileadi and Sabban, 2003).

Samples were observed in a standard scanning electron microscope (Philips XL-30 and XL-40) equipped with a back scattered electron detector (BSE).

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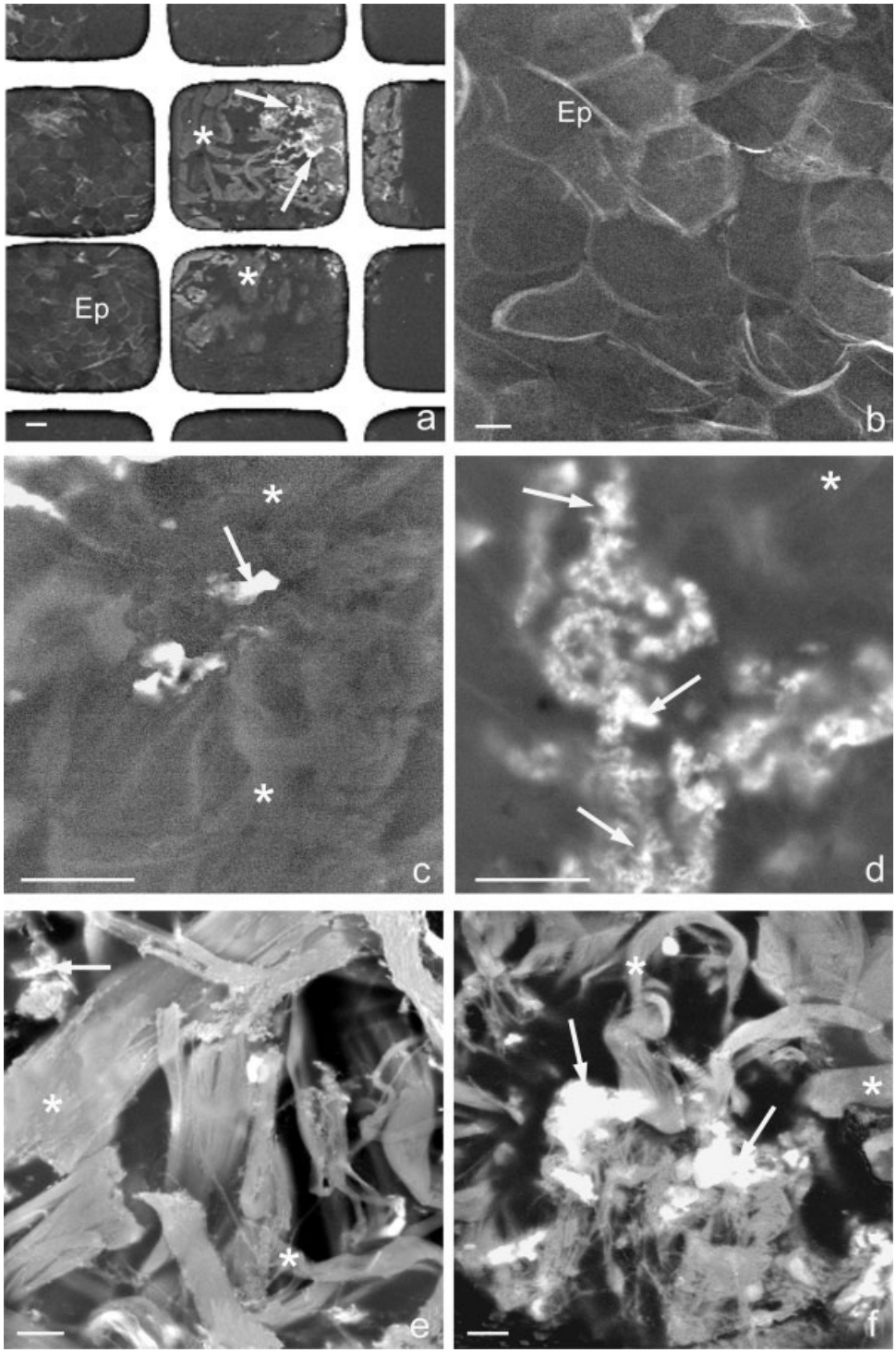

Fig. 1. Skin biopsies placed in sealed capsules and observed by conventional SEM. Samples were washed in buffer (**a–d**) or fixed and stained with uranyl acetate (**e–f**). Mineralized elastic fibers (arrows) can be clearly visualized at very low magnification. Collagen bundles (*) and epithelial cells (Ep) can also be observed in unstained samples. Bar = 100 μm (a); 20 μm (b–f).

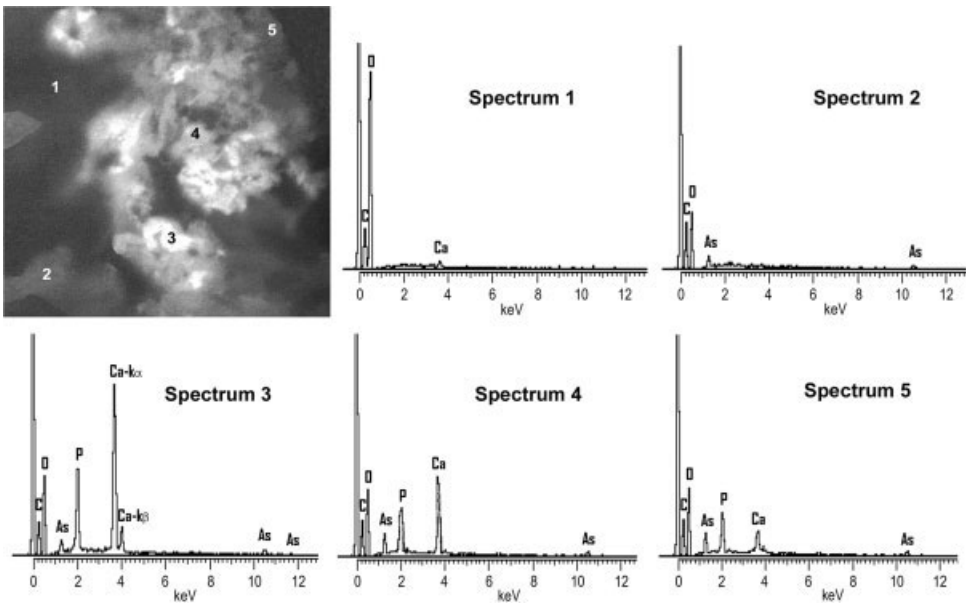

Fig. 2. X-ray microanalysis performed on different matrix components in PXE dermal biopsies. Ca, P, and As can be measured in various amounts according to the area analyzed: nonstructured extracellular matrix (1), collagen (2), highly mineralized (3), mineralized (4), and nonmineralized elastic fibers (5). Microanalysis has been performed on unfixed and unstained specimens.

Microanalysis

Microanalysis was performed on wet samples using a Philips XL-40 equipped with X-EDS (Oxford-SATW).

RESULTS

At small magnification (Fig. 1a) the mesenchymal and the epithelial components of the skin biopsy can be appreciated. The epithelium appears as a multilayer of polygonal cells attached one to the other; however, cellular details cannot be recognized, even at high magnification (Fig. 1b). Mineralized elastic fibers are clearly visible already at small magnification in unfixed and unstained samples (Fig. 1a); by contrast, in these conditions, collagen bundles appears very faint, even though their size and shape can be recognized (Fig. 1c). Elastic fibers appear as aggregates of different electron density because of their various degree of mineralization (Fig. 1d).

Fixation of samples does not improve the quality of the images; however, it has to be considered that fixed samples can be kept for longer times without alterations before structural analyses (data nor shown).

Best results, in terms of image resolution, are obtained with fixed and stained specimens, in which collagen bundles can be nicely seen (Figs 1e and 1f), also if single collagen fibrils are only rarely appreciated. Mineralized elastic fibers appear as prominent electron-dense aggregates spread among collagen bundles.

Very similar results are also obtained on samples stored for several months at –80°C. Mineralized areas are observed in stained as well as in unfixed and unstained defrosted specimens (Fig. 1d).

Microanalysis has been performed on fresh as well as on fixed and on fixed and stained samples. Differences due to sample preparation are negligible. The results of microanalytical examinations on wet unfixed samples are provided in Figure 2. Strong Ca and P signals are observed all over mineralized elastic fibers (spectra 3–5).

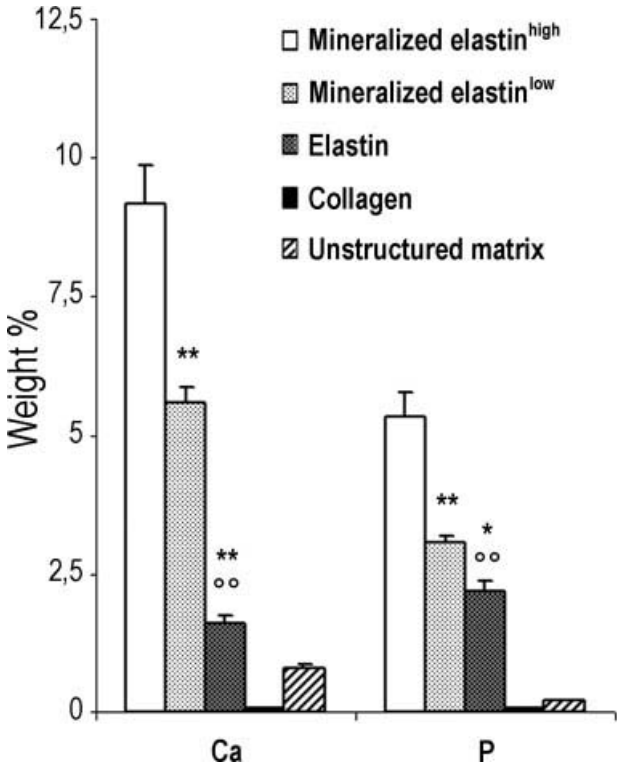

Fig. 3. Calcium and phosphorus content evaluated by X-ray microanalysis on dermal samples from PXE patients. Data have been reported as mean ± standard deviation of values measured on different specimens. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs mineralized elastin^{high}; $^{\circ\circ}P < 0.01$ vs mineralized elastin^{low}.

Interestingly, the amount of calcium and phosphorus is proportional to the degree of mineralization with the highest level in the most heavily mineralized areas of elastic fibers (Fig. 3). In these areas of elastic fibers the C/P ratio is of 1.8 ± 0.05, similar to that in bone. These same ions are also present in nonmineralized areas of

elastic fibers; however, their amount is very low, and in these regions the level of calcium is lower than that of phosphorus (spectrum 5). The As peaks are due to the Cacodylate buffer used to wash the samples and are always present in association to matrix components such as collagen and elastin, whereas they have been never detected in areas corresponding to the unstructured matrix. Furthermore, it has to be noted that Ca and P are not present on collagen bundles (spectrum 2), whereas small amounts of Ca but not P are present in the unstructured extracellular matrix (spectrum 1).

CONCLUSIONS

The device used in the present study allows the rapid identification, by conventional SEM, of mineralized elastic fibers in wet skin specimens obtained from PXE patients.

This study confirms previous observation made on samples processed for conventional electron microscopy that ion deposits are present only on elastic fibers (Martinez-Hernandez and Huffer, 1974; Walker et al., 1989).

Moreover, by X-ray microanalysis, it has been found that the amount of calcium and phosphorus are proportional to the amount of calcification and their ratio is similar to that of mineralized tissues. Ca and P are present also in nonmineralized elastic fibers; however, their ratio is inverted, suggesting that calcium and phosphorus remain entrapped within elastic fibers, but mineralization occurs only when the appropriate levels and ratio of Ca and P are reached. The observation that the accumulation of phosphorus seems to precede that of calcium underlines once more the contribution of phosphorus in the early events occurring in pathologic calcification processes (Chen and Moe, 2004).

Despite the presence of genetic tests allowing sequencing of the ABCC6 gene to find out the disease-associated mutation(s), diagnosis is still routinely performed on dermal biopsies after using specific histological stains for calcified tissues, such as the Von Kossa stain. Previous work in our laboratory demonstrated the usefulness of ultrastructural investigations by TEM to identify small mineral deposits that cannot be appreciated by light microscopy (Bacchelli et al., 1999).

The method described in this paper combines rapidity of execution with the resolution of ultrastructural examinations, as this technique offers the advantage to make a rapid, precise, and reliable demonstration of mineralization processes on untreated or on slightly fixed and stained wet samples by conventional scanning electron microscopes.

A further advantage of this technique is that it allows to observe wet samples without consistent chemical treatments such as dehydration, critical point drying, metal covering that may alter or interfere with structural organization of tissues.

Moreover, it is worth mentioning that mineral deposits are clearly observed also in defrosted specimens, suggesting that samples stored in bank collections can also be used for ultrastructural as well as microanalytical analyses. Therefore, the use of this approach could be of potential use for all investigators interested in the recognition and identification of mineralized processes in soft connective tissues.

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