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Alan C. Nelson
Whitaker College

Frederick J. Schoen
Harvard Medical School

Robert J. Levy
Harvard Medical School

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SCANNING ELECTRON MICROSCOPY METHODOLOGY FOR STUDY OF THE PATHOPHYSIOLOGY OF CALCIFICATION IN BIOPROSTHETIC HEART VALVES

Alan C. Nelson,* Frederick J. Schoen,[1] and Robert J. Levy[2]

Department of Nuclear Engineering, Whitaker College, M.I.T., Cambridge MA 02139
[1]Pathology Departments, Brigham & Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, MA 02115
[2]Departments of Cardiology & Pediatrics, Children's Hospital, Harvard Medical School, 300 Longwood Ave., Boston, MA 02139

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Abstract

Scanning electron microscope (SEM) morphologic analysis combined with energy dispersive characteristic X-ray (EDX) microprobe analysis provides insight into the mechanisms associated with disease-related crystal formation in biological materials. SEM and EDX were employed in analyzing specimens which were embedded in standard fashion in glycolmethacrylate (JB-4). The specimen surfaces under electron microscope investigation resulted from microtomy used in the preparation of reference light microscope histological sections; thus histology served as a direct reference for the SEM and EDX analyses.

The particular application of these methods was in the study of bioprosthetic heart valve calcification, largely responsible for clinical failure of these heart valve substitutes. To simulate the clinically observed mineralization processes, glutaraldehyde-pretreated porcine heart valve leaflets were implanted subcutaneously in rats and subsequently removed at various time intervals from 1 to 56 days. Also, to address the hypothesis that the calcification process generates crystalline materials analogous to those in bone, EDX data obtained from pure hydroxyapatite were compared with the embedded tissue results. Further, EDX results were compared with data obtained by chemical analysis of the bulk specimens to assess the validity of the electron microscope technique.

KEY WORDS: Calcification, Heart Valve, Bioprosthesis, Porcine Valve, Characteristic X-ray, Microprobe, Block Preparation

*Address for correspondence:
Alan C. Nelson , Department of Nuclear Engineering, Massachusetts Institute of Technology, 77 Mass. Ave. E25-330, Cambridge, MA 02139 Phone No.: (617) 253-5799

Introduction

Within the last decade approximately half a million glutaraldehyde-pretreated porcine aortic valves were used as cardiac valve replacements in humans (Angell et al., 1982; Cohn et al., 1981; Lakier et al., 1980). The long-term performance of these bioprostheses has been impaired by the formation of calcific deposits (Schoen and Levy, 1984; Ferrans et al., 1980). Investigations of the mechanisms and time-course of calcific mineral deposition in bioprosthetic valve material have been pursued to better understand this phenomenon (Levy et al., 1983; Schoen et al., 1984).

A model system was developed in the rat to study the biochemical and morphological progression of the calcific degeneration of glutaraldehyde-pretreated porcine heart valve leaflets (Levy et al., 1983). Leaflet tissue subcutaneously implanted was surgically removed at various times post-implantation and was prepared for histology by dehydration and embedding in glycolmethacrylate (JB-4). The block-mounted specimens were analyzed directly on the microtome cut surface employing SEM and EDX. A conventional hematoxylin and eosin (H and E) stained section cut from the block for detailed morphologic analysis (Schoen et al, 1984) served as a reference to help locate the major valve tissue regions: ventricularis, spongiosa, and fibrosa (Ferrans et al., 1978). The spongiosa and fibrosa were examined individually under combined SEM and EDX; the method permits correlation of data obtained by electron microscope methods with data obtained by light microscope histology.

Materials and Methods

Surgery, specimen retrieval, and chemical analysis were done according to previously described methods (Levy et al., 1983). A portion of each retrieved valve leaflet was fixed immediately in Karnovsky's fixative (Karnovsky, 1965), then dehydrated in graded ethanol and embedded in glycolmethacrylate. Sections 2-3 μm thick were cut with a glass knife microtome and stained with H and E and with von Kossa stain, which forms silver deposits on calcium phosphates. These histology sections served as light microscope references for the location of specific tissue

regions to be analyzed under SEM and EDX; in particular, the spongiosa and fibrosa.

SEM and EDX were performed directly on the cut face of the embedded specimen block. To minimize outgassing of the tissue block in the SEM high vacuum, blocks were pre-pumped for 1 hour at 0.01 Torr. To improve electrical conduction, a trail of colloidal silver was painted from the top of the tissue block down the side to join a cup of crushed aluminum foil which supported the electrically grounded specimen in the SEM stage. A copper transmission electron microscope (TEM) grid was attached to the top surface silver colloid to serve as a reference for EDX calibration. A very thin layer of gold, insufficient to obscure the calcium and phosphorous EDX peaks being studied, was sputter coated onto the specimen block to suppress SEM beam-induced charging. Optimal coating was obtained using a sputter coater (Polaron Instruments, Doylestown, PA) with two 5-second pulses at 20 mA current and 2.4 kV. This gold coating had a greenish transparent sheen which served as a visual indication of proper coating thickness.

The specimen block was loaded in the SEM (ISI-DS130, Mountain View, CA) and pumped for 10 minutes at 10^{-6} Torr before the microscope was operated. X-ray analysis was performed at 80 μA and 15 kV; these settings further suppressed charging. SEM morphological data were collected in the secondary electron mode with a Robinson backscatter detector. A secondary electron image was compared with the standard histology section, since with SEM alone it was difficult to distinguish precise boundaries of spongiosa and fibrosa.

Backscatter images crudely showed the location of calcific deposits as relatively high density areas, and since these occur mainly in the spongiosa, backscatter was helpful in distinguishing between spongiosa and fibrosa layers. After mapping the tissue spongiosa and fibrosa under SEM, EDX spectra from each tissue layer were collected.

The EDX analysis was performed using a TN-2000 system (Tracor-Northern, Middleton, Wis.) programmed to identify Ca, P, Al, and Cu peaks, to subtract a background spectrum from the spectrum of interest and to print out the values of the appropriate peaks. Calibration of the TN-2000 was checked before and after each analysis using Al and Cu K_α peaks. Subsequent Ca and P data were normalized against the Cu Kα peak from the copper grid on each sample to account for slight variations in the SEM set-up parameters and in SEM emission currents. In this fashion, data from different specimens taken at different times could be compared.

The background X-ray spectrum was taken from a region on the specimen block surface which did not contain tissue. This background spectrum, due mainly to the gold coating and Bremsstrahlung radiation, was stored in the TN-2000 memory to be subtracted from the actual tissue spectrum, leaving the required Ca and P peaks. For each valve leaflet, several areas of spongiosa (100 μm x 100 μm) were analyzed with each pair of readings separated by about 1000 μm. At each site, the X-ray spectrum was accumulated, stripped of background, normalized, and printed. Each spectrum was collected on an energy scale from 0 to 10.24 keV, and the particular peaks of interest are Ca ($K\alpha$ = 3.7 keV) and P ($K\alpha$ = 2.0 keV). Data were collected over 60 seconds in every case. The energy dispersive characteristic X-ray analysis was performed for post-implantation time periods of 1, 3, 7, 14, 21, 28 and 56 days; at least four specimens were analyzed at each time point with at least five measurements of each data point. All data were normalized using the copper grid technique.

$$\text{Normalized Ca(P) counts} = \frac{\text{Net recorded Ca(P) counts} \times 200{,}000}{\text{Net recorded Cu counts from grid}}$$

Results

To illustrate the morphology of the calcification process, combined SEM secondary electron and backscatter micrographs were made from each specimen block surface at each post-implantation time, and these micrographs were compared with histology of the same specimens. Figures 1 and 2 compare light microscope histology with associated SEM micrographs to illustrate the direct correlation of features by the two methods of analysis. The secondary electron component provided good topology while the backscatter component showed brightening, which corresponds to relatively high density calcific deposits.

The SEM micrographs gave a qualitative picture of the calcification process as it progressed through time, beginning with a fairly uniform tissue evolving to a tissue containing discrete mineral deposits. To demonstrate that these high-density bright areas were in fact composed primarily of Ca and P, the EDX was used in the dot map mode to show the morphology of Ca and P on the sample surface, Figure 2c. In addition, the normalized Ca/P ratio from EDX was computed for specimens at each time interval, Figure 3. The Ca/P ratio for the long-term valve specimens approaches 1.5 in the spongiosa and 1.0 in the fibrosa. Separate EDX analysis of a powdered hydroxyapatite sample gave a Ca/P count ratio of about 1.6, which can be compared directly with the EDX Ca/P count ratios for spongiosa and fibrosa.

Further, the EDX data were compared with data obtained from biochemical analysis of the bulk material (Levy et al., 1983). The biochemical analysis measured levels of Ca and P in implanted valve material as a function of the duration of implantation. Figure 4 shows the EDX data for Ca and P in the bulk specimens. The linear trends illustrated in Figure 4 indicate a strong correlation between the site-specific chemistry measured by EDX and the bulk tissue chemistry determined by chemical analysis. The calculated linear correlation coefficients for the Ca and P data in Figure 4 are $r = 0.92$ ($P = 0.15$) and $r = 0.90$ ($P = 0.21$) respectively for spongiosa, and $r = 0.86$ ($P = 0.34$) and $r = 0.89$ ($P = 0.24$) for fibrosa. High r-values and fairly low P-values show that the data are well correlated in a linear fashion.

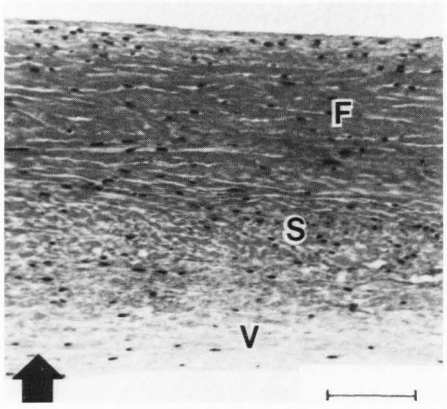

Fig. 1a. Unimplanted valve demonstrating layers of valve architecture: ventricularis (V), spongiosa (S), and fibrosa (F). The direction of blood flow is indicated by an arrow. Bar = 100μm.

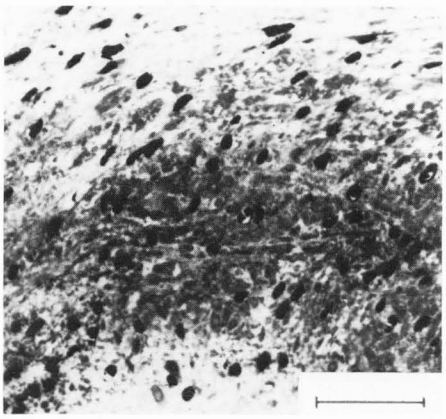

Fig. 1b. Porcine aortic valve cusp implanted for three days demonstrating small calcific deposits which are seen as black spots. Von Kossa stained for calcium phosphate. Bar = 50 μm.

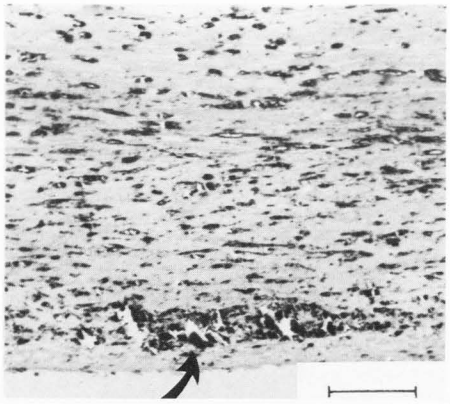

Fig. 1c. Valve cusp implanted for 21 days in same orientation as 1a. showing more extensive diffuse deposition of calcium phosphate crystals, as well as early confluence to nodules at the spongiosa/ventricularis junction (arrow). Bar = 100μm.

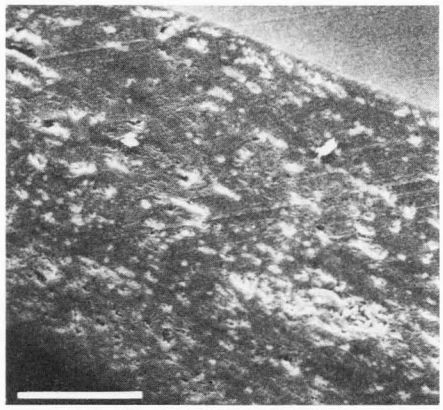

Fig. 2a. Porcine aortic valve cusp implanted for three days demonstrating calcific deposits, which are seen as bright spots, to be compared with Figure 1b. Bar = 50μm.

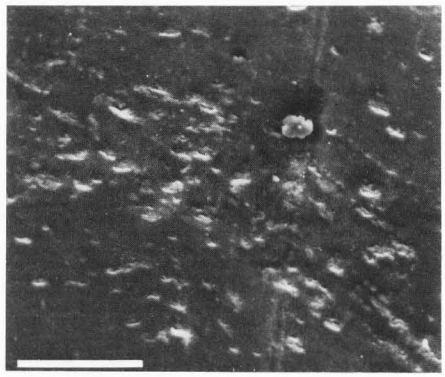

Fig. 2b. Valve cusp implanted for 21 days showing more extensive deposition of calcium phosphate crystals to be compared with Figure 1c. Bar = 50μm.

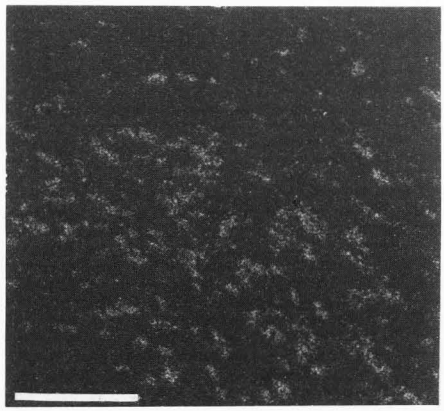

Fig. 2c. EDX dot map of calcium in 21-day cusp confirming that bright areas are composed of calcium phosphate mineral, to be compared with 2b. Bar = 50μm.

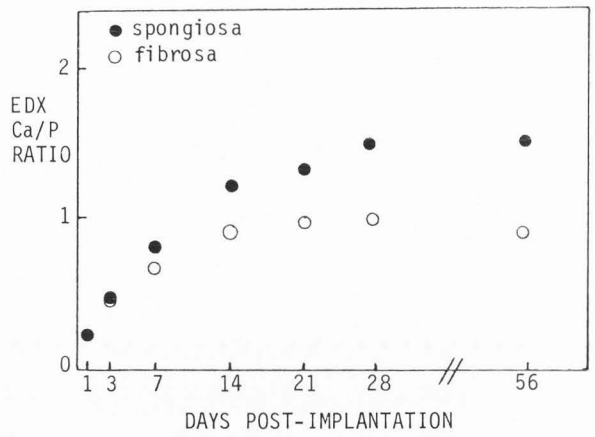

Fig. 3. The ratios of EDX counts for Ca and P in the spongiosa and fibrosa are computed for each post implantation time. After 21 days the spongiosa curve approaches a Ca/P ratio of 1.5 while the fibrosa curve approaches 1.0. Individual measurements for the EDX ratios are in units of cpm.

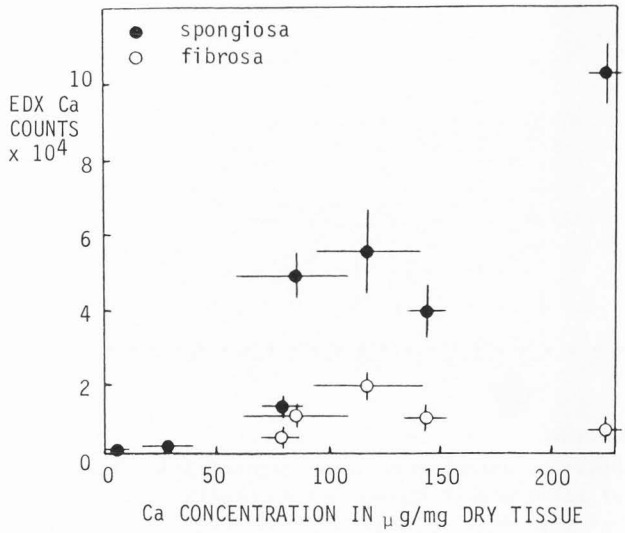

Fig. 4a. Site-specific chemistry measured by EDX for calcium plotted against bulk tissue chemistry measured by chemical analysis. These data are linearly correlated (see text). EDX in units of cpm.

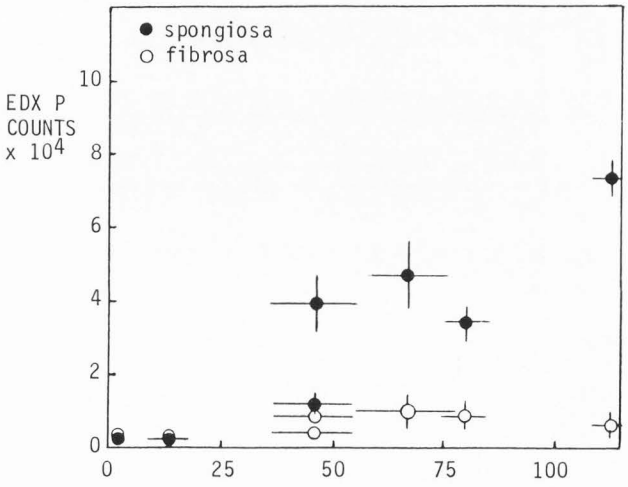

Fig. 4b. Site-specific chemistry measured by EDX for phosphorus plotted against bulk tissue chemistry measured by chemical analysis. These data are linearly correlated (see text). EDX in units of cpm.

Discussion

A general methodology has been developed which permits SEM and EDX analyses to be compared directly with histology by using the cut embedded specimen block as the object of study in the electron microscope. The technique allows the correlation of elemental distribution with surface features of the specimen. In both light microscope and electron microscope micrographs of the implanted heart valve material, regions of relatively dense matter which constituted localized mineralization were observed.

The spongiosa and fibrosa layers in the valve leaflet implants calcified to different degrees at different rates, the spongiosa being more subject to severe calcification, as noted elsewhere (Ferrans et al., 1980; Schoen et al, 1984.) EDX combined with morphology verified that the bright regions were composed of calcium and phosphate and that the EDX ratio of calcium and phosphate approached that of hydroxyapatite in the spongiosa but not in the fibrosa, Figure 3. Since the fibrosa mineralizes more slowly than spongiosa, it is possible that the Ca/P ratio might achieve a level above 1.0 over longer time periods than were studied. Accordingly, all values for the EDX Ca/P ratio less than 1.5 may represent immature crystallization of hydroxyapatite: the phenomenon of bone mineral maturation has been reported elsewhere (McLean and Urist, 1968) and can be inferred from the time-dependent increase of the Ca/P ratio demonstrated in Figure 3.

The results of the SEM and EDX analyses were highly reproducible and quantitative, in the sense that EDX X-ray peaks were normalized against a copper standard. Since all specimens were prepared in the same fashion, the trends shown in Figures 3 and 4 are representative of the actual calcification process. These figures suggest that mineralization is a time-dependent process involving mineral maturation toward a hydroxyapatite-like material and that the rate of mineral maturation and accumulation is higher in spongiosa than fibrosa. Furthermore, the site-specific chemistry of valve tissue mineralization is well correlated with histology and the bulk chemical properties of the specimens. These data provide a quantitative basis for the previously observed preponderance of spongiosa involvement in bioprosthetic heart valve calcification (Ferrans et al., 1980, and Schoen and Levy, 1984).

Conclusion

SEM and EDX analysis on histological block mounted specimens has been informative in the study of mineral deposition in bioprosthetic material. The SEM/EDX method described here, combined with histological and biochemical analysis, is an important adjunct to investigations of the pathophysiology of bioprosthetic heart valve calcification. Moreover, this methodology is generally applicable to investigations involving the direct correlation of SEM data with histology.

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References

1. Angell, W. W., Angell, J. D., Kosek, J. C., (1982). "Twelve-year experience with glutaraldehyde-preserved porcine xenografts," J. Thorac. Cardiovasc. Surg. 83: 493-502.

2. Cohn, L. H., Mudge, G. H., Pratter, F., Collins, J. J. (1981). "Five to eight year follow-up of patients undergoing porcine heart valve replacement," N. E. J. Med. 304: 258-262.

3. Ferrans, V. J., Boyce, S. W., Billingham, M. E., Jones, M., Isihara, T., Roberts, W. C. (1980). "Calcific deposits in porcine bioprostheses: Structure and pathogenesis," Am. J. Cardio. 46: 732-734.

4. Ferrans, V. J., Spray, T.L., Billingham, M. E., Roberts, W. C. (1978). "Structural changes in glutaraldehyde-treated porcine heterografts used as substitute cardiac valves," Am. J. Cardiol. 41: 1159-1166.

5. Karnovsky, M. J. (1965). "A formaldehyde-glutaraldehyde fixative of high osmalality for use in electron microscopy," J. Cell Biol. 27: 137A-138A.

6. Lakier, J. B., Khaja, F., Magilligan, D. J., Goldstein, S. (1980). "Porcine xenograft valves: Long-term (60-89 month) follow-up," Circulation 62: 313-318.

7. Levy, R. J., Schoen, F. J., Levy, J. T., Nelson, A. C., Howard, S. L., Oshry, L. J. (1983). "Biologic determinants of dystrophic calcification and osteocalcin deposition in glutaraldehyde preserved porcine aortic heart valve leaflets implanted subcutaneously in rats," Am. J. Pathol. 113: 143-155.

8. McLean, F. C., Urist, M. R. (1968). Bone: Fundamentals of the Physiology of Skeletal Tissue, 3rd ed., University of Chicago Press, Chicago, 62-71.

9. Schoen, F. J., Levy, R. J. (1984). "Bioprosthetic heart valve failure: Pathology and Pathogenesis," Cardiac Morphology, Cardiol. Clinics, special issue, in press.

10. Schoen, F. J., Levy, R. J., Nelson, A. C., Bernhard, W. F., Nashef, A., Hawley, M. (1984). "Bioprosthetic heart valve failure: Pathogenesis of calcific deposits," J. Am. Coll. Cardiol. 3: 601.

Discussion with Reviewers

A. L. Boskey: Do you think that the low Ca/P ratios observed might be attributable to the presence of non-apatitic mineral phases or to high amounts of organic phosphate?
Authors: We feel that the low Ca/P ratios may suggest immature development of mineral crystals and that the spongiosa and fibrosa layers have different maturation rates, fibrosa being slower than spongiosa.

P. Frasca: Was the valve leaflet subcutaneously implanted in a particular orientation?
Authors: No, the valve leaflets were randomly oriented.

P. Frasca: Why do you think that the spongiosa mineralizes more readily than the fibrosa?
Authors: This phenomenon may be a function of cell density. The basic structure of the spongiosa and fibrosa is different, the spongiosa being more cellular while the fibrosa is more densely packed with well ordered collagen.

P. Frasca: Have you considered using a silver target (for sputter coating) instead of a gold? You would be able to coat freely and also not interfere with the P EDX peak.
Authors: We have not tried this. It is an interesting suggestion.