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MORPHOLOGICAL OBSERVATIONS OF MINERALIZING PERICARDIUM CARDIAC GRAFTS

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Abstract

Pericardial patch grafts were implanted in the hearts of young sheep for periods ranging from two to 120 days. Explants seven to 21 days old revealed the formation of a "pseudoneointima" (PNI) on the blood contacting surface of the pericardium. The PNI was more heavily mineralized than the pericardium. Mineralization was most intense on the blood contacting surface of the PNI and on the chamber surface of the pericardium. After three weeks of implantation, the PNI was much thinner and was organized into a thin fibrous capsule without any signs of mineralization. In the pericardium, mineral deposits were seen in fibroblasts. Moreover, cell-related mineralization was evident prior to calcification of the surrounding collagen matrix.

KEY WORDS: Pericardium, Pseudoneointima, Light Microscopy, Scanning Electron Microscopy, X-ray Microanalysis Transmission Electron Microscopy and Mineralization

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Introduction

A major problem associated with the use of pericardial bioprosthetic implants is the development of mineral deposits within the graft.[3] In a previous publication,[4] we described an experimental cardiovascular implant model to study this phenomenon. Specifically, our model consisted of bovine pericardial patch grafts implanted in atrial and ventricular myocardial windows of young sheep. This working model displayed mineralization as early as one week after implantation and represented a relatively inexpensive means to study mechanisms of mineralization in cardiovascular prostheses.

We have already reported that mineralization occurs in a pseudoneointima (PNI). And, it was also seen associated with the pericardium. We report here additional observations which increase our understanding of the mineral deposition process.

Materials and Methods

Glutaraldehyde fixed patch grafts of bovine pericardium were implanted in myocardial windows in young (3 to 4 months old) sheep.[4] Clinical quality processed pericardium was supplied by Shiley Laboratories of Irvine, California. The grafts consisted of pericardium mounted on flanged epoxy rings (Fig. 1a) that were surgically implanted in both atrial and ventricular walls using inflow venous occlusion.[4] A total of 86 explants were retrieved for study ranging in implantation duration from two to 120 days. Twenty-three of these, implanted for two to 120 days, were examined microscopically.

Patch grafts were removed from the sheep after induction of anesthesia and anticoagulation with heparin. The graft was surgically exposed, the sheep were exsanguinated, and the graft, with surrounding myocardium, was excised. Ex-

plants were rinsed in ice cold physiological saline, photographed, fixed in formalin-glutaraldehyde solution[5] and radiographed to locate grossly visible areas of mineralization.[4] Adjacent longitudinal sections (Fig. 1b, XYZ) were removed for light microscopy, scanning electron microscopy and transmission electron microscopy. The histological sections were routinely stained with hematoxylin and eosin and the v. Kossa method. The sections for SEM study were freeze-dried, mounted on SEM stubs with silver paint and coated with a thin layer of silver. SEM studies were also performed on 5-10 μm thick sections cut from the histological block. The sections were cleared of paraffin using xylene and air-dried between two glass slides. Sections for TEM studies were processed by conventional methodology.[6] The instruments used were a JEOL 35C scanning electron microscope (operated at 25 kV) with a Kevex 7000 energy dispersive X-ray analyzer (EDX), a JEOL 100 B transmission electron microscope, and a JEOL 100 CX analytical STEM with a PGT system III EDX.

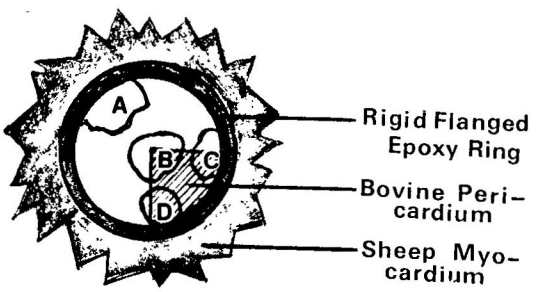


Figure 1a: Diagram of pericardium explant showing the relationship of bovine pericardium to sheep myocardium. The shaded portion, B,C,D, represents the lesion distribution of PNI overlying the pericardium.

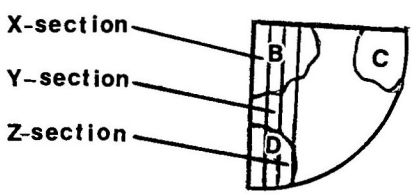
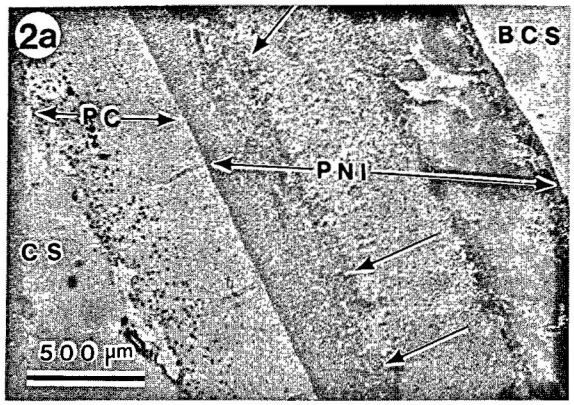


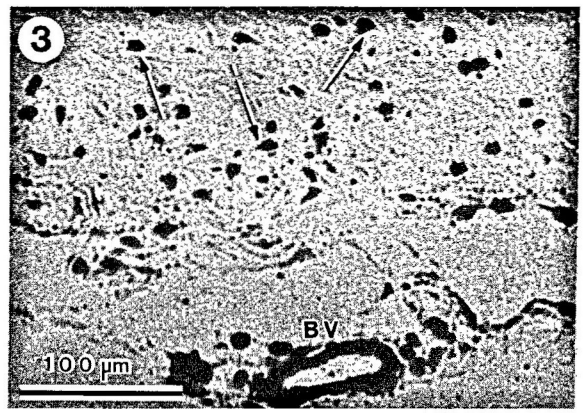
Figure 1b: Schematic enlargement of the shaded portion, shown in Figure 1a. Adjacent sections were selected for light microscopy (X), scanning electron microscopy (Y) and transmission electron microscopy (Z).

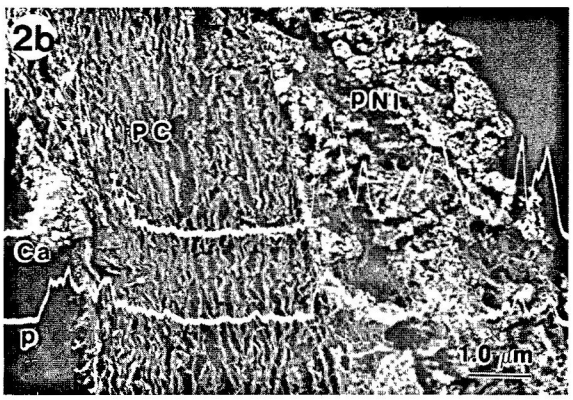
Results

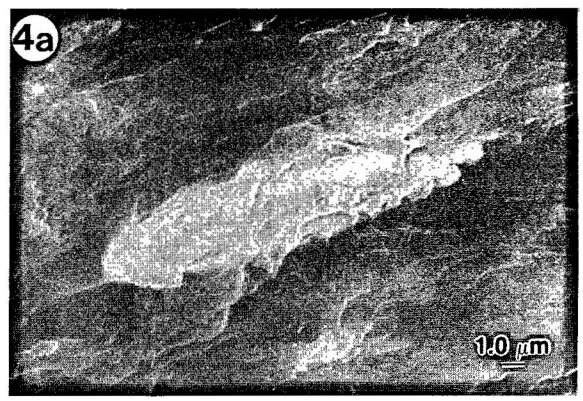
For implantation periods ranging between 7 to 21 days, mineralization was much more intense in the PNI than in the pericardium. Furthermore, as seen on the histological section stained with v. Kossa (Fig. 2a; one week implantation), the deposits of mineral were most intense on the blood contacting surface (BCS) of the PNI and the chamber surface (CS) of the pericardium (PC). As can be seen in Fig. 2a the mineral appeared to be deposited in bands or layers of mineral. A much finer band of mineral (arrows) was sometimes noted in the PNI near the PNI-pericardium interface and as mineralization progresses, the two mineral bands in the PNI coalesce . EDX analysis performed on the 5 to 10 μm thick sections cut from the histological block confirmed the presence of calcium and phosphorous in regions which stained positive with v. Kossa. This is seen in Fig. 2b as calcium (Ca) and phosphorous (p) line scans superimposed on a back-scattered electron image (BEI). Fig. 2c confirms the location of the bands with a calcium map of the two layers of mineralization in the PNI and pericardium.

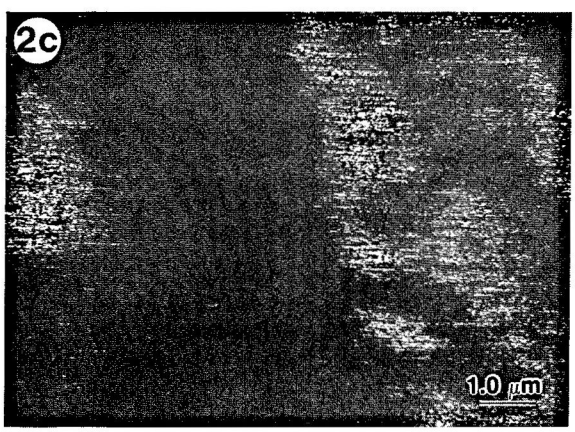
In implants older than approximately three weeks, the PNI became much less abundant. In most cases a fibrous pseudoneointima was present. Thus, the thrombotic PNI which initially contained calcium and phosphorous deposits had an intermediate or transient existence. A detailed morphological study was performed on six specimens using light and BEI imaging of which two were analyzed in more detail by TEM/EDX. As far as the pericardium is concerned those sections stained with v. Kossa show mineralized fibroblasts (arrows) and blood vessels (Fig. 3, BV). A mineralized fibroblast is noted in the BEI image (Fig. 4a) and EDX map of calcium (Fig. 4b). EDX analysis of the fibroblasts reveals the presence of calcium and phosphorous with a molar ratio of 1.5-2.0, which is suggestive of hydroxyapatite. Figure 5 shows a region of the implant close to the chamber surface. A mineralized fibroblast (F) can be seen surrounded by collagen (C) bundles. Close to the chamber side of the implant (Fig. 5, left side) higher levels of mineralization are seen. Of considerable











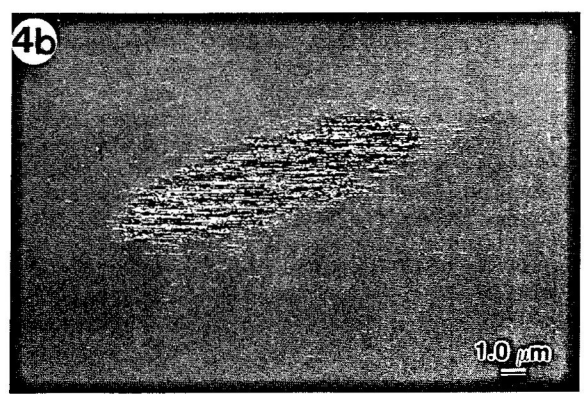


Figure 2a: Histological section of a seven day old explant stained by the v. Kossa reaction. Mineralization is most intense in the PNI and less intense in the pericardium (PC). A fainter band of mineral is seen in the PNI near the PNI - pericardium interface. Note that in both the PNI and pericardium, staining is most intense on the blood contacting surface (BCS) and toward the chamber surface (CS) of the pericardium respectively.

Figure 2b and 2c: Cross section of a two week old explant. Calcium (Ca) and phosphorous (P) line scans superimposed upon a backscattered electron image (BEI) showing two bands of mineral in the PNI and pericardium (PC) in Figure 2b, and the calcium map of the same area in Figure 2c.

Figure 3: Higher magnification of pericardium shown in Figure 2a displaying positive staining in fibroblasts (arrows) and in a blood vessel (BV).

Figure 4: (a) BEI image, and (b) EDX calcium map of mineralized fibroblast at higher magnification.

interest is the presence of distinct areas of mineral within the collagen fibers (arrows). Closer scrutiny with TEM shows mineral (electron dense material; arrows) in the nucleus (N) of the fibroblast (Fig. 6); some crystals were also seen in the cytoplasm (C). This was verified by EDX analysis in an analytical STEM. A calcium map of the mineralizing cell shown in Fig. 6 confirms the location of the mineral (Fig. 7). Cellular involvement in mineralization (arrows) was also noted in the PNI, mainly in the erythrocytes (Fig. 8, SEM; Fig. 9, TEM). As far as mineralization in the pericardium is concerned positive identification of calcium phosphate mineral was made by SEM/EDX in ten out of 21 explants older than seven days. Each explant was heterogeneous and considerable difference was noted in the distribution of mineral throughout the specimen. There were no significant morphological differences between atrial and ventricular explants. Unimplanted pericardium did not reveal any PNI or mineralization by either LM, SEM/EDX or TEM.

Discussion

The major finding in this study was that the cardiovascular bioprosthesis contained loci of mineralization that were associated with cells. Moreover, in the pericardium, evaluation of three specimens indicated that mineralization of the collagen matrix was separated spatially and temporally from that of the fibroblasts. The findings that mineral is deposited in cells of both the PNI and the pericardium provides a new insight into the mechanism of mineralization of cardiac bioprosthesis.

That cells are involved with this process is consistent with the view that cells or cell-derived particles, such as matrix vesicles, initiate or control mineral deposition.[1] It is important to note that in the pericardium, mineral deposition occurs at intracellular sites. TEM studies indicate that mineral is preferentially accumulated in the nucleus; smaller amounts of apatite are seen in the cytoplasm. It is likely that mineralization of the cytoplasm follows that of the nucleus. The importance of the nucleus as an initiating site for mineral deposition is interesting as it differs from processes that occur in viable cells. Earlier studies of isolated cell organelles and whole cells indicate that calcium loading is associated with calcium granules formation in mitochondria.[2,7] An explanation for this observation is that the pericardium is fixed during tissue processing and the cells are therefore nonvital. While this process serves to maintain the structural integrity of the cells, it may also expose sites on selected macromolecules that facilitates mineral deposition. Nucleic acids have a large number of anionic groups that could bind calcium ions and thereby initiate mineral formation.

Figure 5: BEI image of a pericardium explanted after two weeks of implantation. At right, a mineralized fibroblast (F) is shown surrounded by unmineralized collagen (C). At left, i.e. towards the chamber side mineralization is easily noted (bright areas). Note specifically how islands of mineralization (arrows) are embedded within the collagen fibers.

It is also worthwhile to comment on the deposition of mineral in the collagen bundles. The possibility exists that mineral develops in collagen de novo and is distinct from that of the cells. Alternatively, mineralization of collagen is dependent on cellular apatite formation. Evidence in favor of the latter process is that mineralized collagen is never observed without the presence of mineralized cells; moreover, mineralized cells are seen in the absence of mineralized collagen. These observations provide information concerning the sequence in which mineral is deposited in cells and the extracellular matrix of the bioprostheses. Our study shows that in this system, cellular mineralization precedes extracellular mineral formation and it is likely that the presence of cells is required for the mineralization of the bioprosthesis.

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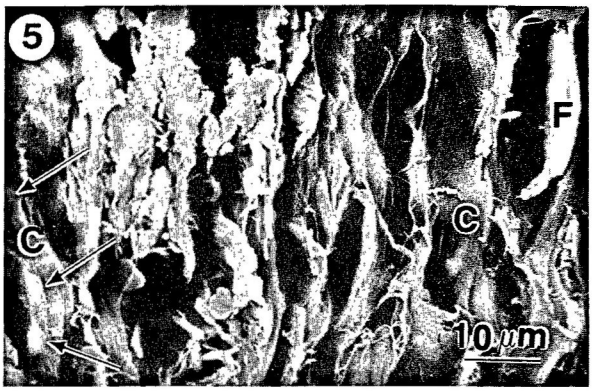

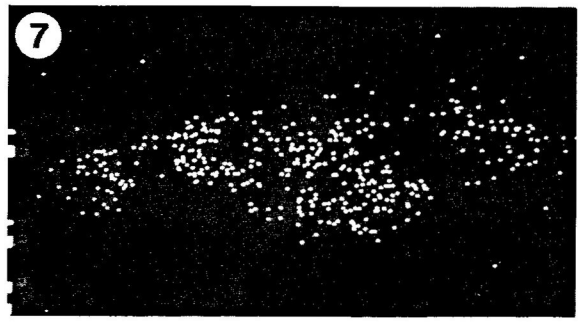

Figure 7: STEM/EDX calcium map of mineralizing cell shown in Figure 6.

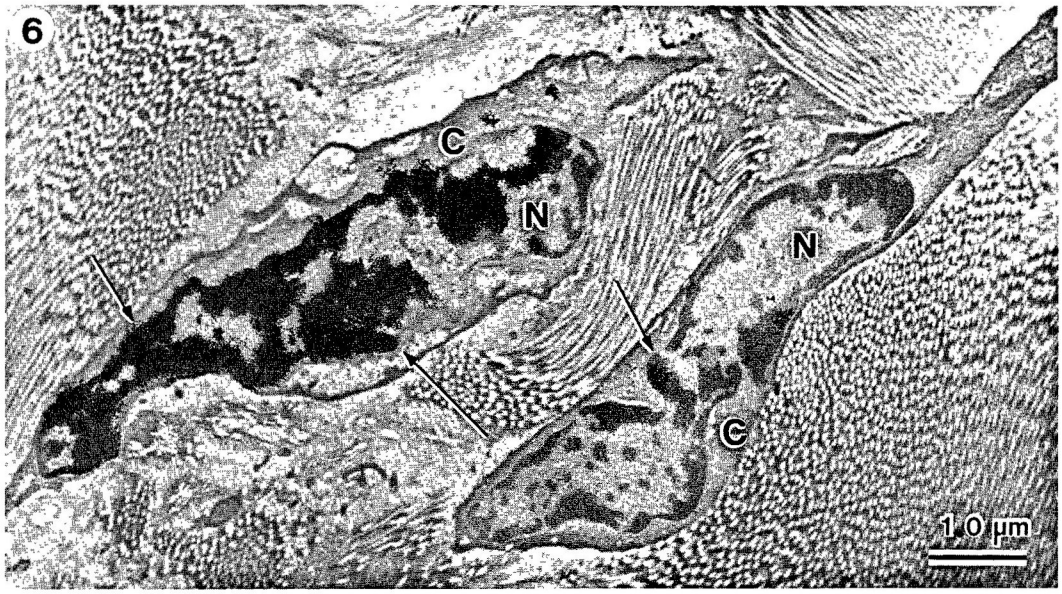

Figure 6: TEM image of partially mineralized fibroblasts in the pericardium of one week explant. Note the presence of mineral in the nucleus (N) and cytoplasm (C).

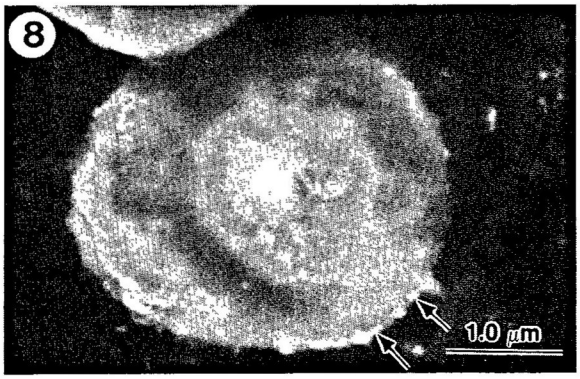

Figure 8: BEI image of an erythrocyte showing signs of mineralization. This cell was noted in a zone of mineralization in the PNI of a two week old explant.

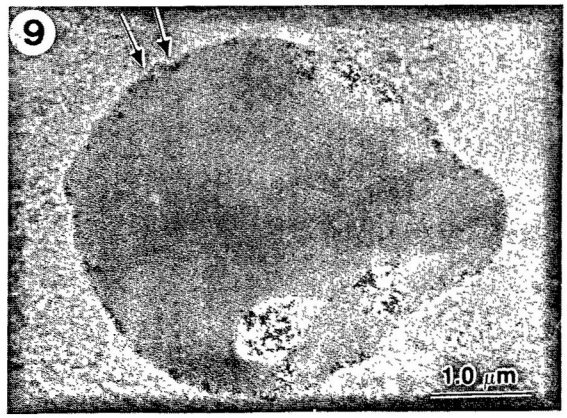

Figure 9. TEM image of an erythrocyte in the same PNI as that referred to in Figure 8.

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Discussion with Reviewers

A.C. Nelson: What evidence can the authors cite to support their claim that pseudoneointima (PNI) arises on the implant within three weeks then diminishes? What happens to the PNI associated mineralization during the diminishing phase?
Authors: Of 23 explants analyzed with SEM/EDX 13 ranged from 21 days to 120 days and these contained little or no PNI, nine were seven to 21 days old and all contained an obvious PNI and one was only two days old and did not display a PNI. Where no PNI was present there was obviously no mineralization to be observed. An "intermediate" diminishing phase was not found to study the mechanism by which the PNI is resorbed after the 21 days of implantation.
A.C. Nelson: Would the authors provide more information on the morphological and mineralization studies pertaining to implants between 21 days and 120 days? I would like to better understand the dynamic evolution of mineralization in the pericardial tissue.
Authors: Between 21 and 120 days no PNI was noted and mineralization was noted in the pericardium of about half of the explants. Due to the problems arising from the heterogeneous distribution of mineral throughout the explant, no concept of the dynamic evolution of the mineralization process in the pericardium could be formulated.

A.C. Nelson: Can the authors assess whether bioprosthetic heart valves fabricated from pericardium are likely to lead to better clinical results than porcine valve bioprostheses?
Authors: We are not presently able to answer this very important question since we have not run parallel studies on grafts derived from porcine valves.

K.A. Rosenbaur: According to the method described by v. Kossa, one should use ethanol-fixed material. Did you fix the explants in ethanol or formalin-glutaraldehyde solution? If you used formalin-glutaraldehyde what was the duration of fixation?
Authors: The specimens were not fixed in ethanol but instead in the formalin glutaraldehyde mixture for at least two hours or more. However, it was gradually dehydrated in ethanol from 70% to 100% absolute alcohol for at least one hour at room temperature.
K.A. Rosenbaur: Did you observe calcium phosphate precipitates in mitochondria?
Authors: We have not yet observed mitochondria in pericardial fibroblasts. This work is still in progress.
K.A. Rosenbaur: Is mineralization observed in the pseudoneointima a form of degeneration, called "calcific degeneration"?
Authors: We would believe that the term "calcific degeneration" may be appropriate for the mineralization observed in the PNI.

M. Ashraf: In the injured cells, the mitochondria generally accumulate enormous Ca^{++}. In this study, only nuclei show Ca^{++} deposits. Can the authors explain the role of nuclei in ions accumulation.
Authors: Since DNA has been shown to have an affinity for Ca^{++} ions, it is perhaps not surprising to see mineralization occurring in the nuclei of fibroblasts.

J.M. Riddle: Why is the mineral deposited in the PNI in layers and as discrete specks on the CS of the PC (See Fig. 2a)?
Authors: The specks of mineralization visible most heavily on the chamber side of the pericardium also collectively form a layer of mineral.
J.M. Riddle: Why and how do cells serve as a nidus for ectopic calcification?
Authors: The erythrocytes in the PNI which are seen to mineralize are believed to be dead and it is apparently the phospholipid containing membrane which seems to serve as a nidus for mineralization. In the fibroblast, the DNA acts as the initiator for mineralization.