Biologic Determinants of Dystrophic Calcification and Osteocalcin Deposition in Glutaraldehyde-Preserved Porcine Aortic Valve Leaflets Implanted Subcutaneously in Rats

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Bioprosthetic cardiac valve calcification is a frequent complication after long-term valve replacement. In this study the authors sought to examine the biologic determinants of this type of dystrophic calcification using subcutaneous implants of glutaraldehyde-preserved porcine aortic valve leaflets (GPVs) in rats. GPVs and clinical valvular bioprostheses were prepared identically. Retrieved implants were examined for calcification and the deposition of osteocalcin (OC), a vitamin K-dependent, bone-derived protein, that is found in other dystrophic and ectopic calcifications. GPVs implanted in 3-week-old rats calcified progressively (GPV Ca^{2+}, 122.9 ± 6.0 μg/mg) after 21 days, with mineral deposition occurring in a morphologic pattern comparable to that noted in clinical retrievals. Calcified GPVs accumulated osteocalcin (OC, 183.4 ± 19.4 ng/mg); Nonpreserved porcine aortic leaflet implants did not calcify (Ca^{2+} + 5.6 ± 1.0 μg/mg). Millipore diffusion chamber (0.45-μ pore size enclosed GPV implants accumulated calcium and adsorbed osteocalcin despite the absence of attached host cells. GPVs implanted for 21 days in 8-month-old rats calcified less (GPV Ca^{2+}, 22.4 ± 5.0 μg/mg) than did GPVs implanted in 3-week-old rats (see above). High-dose warfarin therapy (80 mg/kg) did not alter GPV calcification (GPV Ca^{2+}, 39.6 ± 2.9 μg/mg) in 72-hour subcutaneous implants in 3-week-old male rats, compared with control rats (GPV Ca^{2+}, 40.8 ± 4.8 μg/mg). (Am J Pathol 1983, 113:143–155)

IT IS ESTIMATED that stent-mounted glutaraldehyde-preserved porcine aortic valves (GPVs) have been used in over 500,000 cardiac valve replacements since 1971.[1-12] Late calcific degeneration of GPV occurs commonly in long-term valve replacements and often results in bioprosthesis failure.[3-19] Bioprosthesis calcification is accelerated in children and young adults compared to older patients.[9-13] The principal pathologic abnormalities in clinical GPV calcification are collagen fibril calcification[17] and mineral deposition in association with devitalized porcine connective tissue cells.[17,19] The bone protein, osteocalcin, and other proteins containing the vitamin K-dependent calcium-binding amino acid, γ-carboxyglutamic acid (Gla) are present in clinical GPV calcifications[20,21]; no Gla proteins occur in nonmineralized GPV. Experimental GPV calcification of subcutaneous implants demonstrates morphologic and biochemical abnormalities comparable to those noted in

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clinical specimens,[22] including the deposition of Gla-containing proteins in the mineralized leaflets.[22]

The objective of the present study was to examine several determinants of GPV calcification and accompanying osteocalcin deposition in subcutaneous GPV implants in rats. The specific issues addressed in these experiments included the morphologic and biochemical sequence of events; the importance of glutaraldehyde pretreatment; the role of host-cell attachment; the importance of host age; and the effects of vitamin K antagonism.

Materials and Methods

Materials

Analytic grade glutaraldehyde (50% aqueous) was obtained from Eastman Kodak (Rochester, NY). Sodium monobasic phosphate and sodium chloride were obtained from Malinkrodt Chemicals (St. Louis, Mo). Purified rat osteocalcin and rabbit-derived antiserum to rat osteocalcin were generously donated by Drs. Caren M. Gundberg and Peter V. Hauschka of Children's Hospital Medical Center, Boston, Massachusetts.

GPV Preparation

Porcine hearts obtained from the Connecticut Packing Company, Hartford, Connecticut, were transported to the laboratory on ice. Aortic valve leaflets were dissected and rinsed in cold sterile saline and placed in 0.625% glutaraldehyde buffered with sodium phosphate (0.05 M), 0.140 M NaCl, pH 7.40. After 24 hours the valve cusps were transferred to 0.2% glutaraldehyde in the same buffer. The valve leaflets were stored in this solution at room temperature for a minimum of 4 weeks.[1,2]

Experimental Animals

Sprague–Dawley-derived rats (CD strain) were obtained from Charles River Laboratories (Burlington, Mass). Three-week-old male weanlings (60–80 g) were used for most studies. Studies on age effects included 8-month-old male rats. The rats were fed Lab Chow (Ralston-Purina, St. Louis, Mo).

Surgical Procedures

Ether-anesthetized animals had two to four subcutaneous pouches separated by at least 2 cm dissected in the midabdominal wall. An entire valve cusp was implanted into each pocket. The wounds were closed with surgical staples. The valves were retrieved at sacrifice under ether anesthesia, and blood sampling was done by percutaneous cardiac puncture.

Millipore Diffusion Chambers

In some experiments, in order to permit free diffusion of extracellular fluid into the chamber but block host-cell contact with the GPV enclosures, GPVs were enclosed in Millipore diffusion chambers (Millipore, Bedford, Mass), which consisted of 13-mm lucite rings, sealed with 0.45-μ pore size Millipore-Millex (mixed cellulose–acetate, cellulose nitrate) membranes.

Warfarin Therapy

Sodium warfarin (Coumadin, Endo Laboratories, Garden City, NY) was administered subcutaneously to selected animals at a dose of 80 mg/kg at the time of surgery and 24 hours after subcutaneous GPV implants. After 72 hours the animals were anesthetized, blood sampling was performed, and the leaflets were retrieved. One-stage prothrombin times (Sigma Chemical, St. Louis, Mo) were determined with the use of normal rat plasma as a control.

Methods of Analysis: Morphologic

Samples removed for morphologic evaluation were fixed immediately in 0.1 M cacodylate-buffered 2.5% glutaraldehyde, 2% paraformaldehyde, pH 7.2.[23] Portions of the specimens were dehydrated in graded concentrations of ethanol and embedded in JB-4 glycol methacrylate medium (Polysciences, Warrington, Pa). Sections 2–3 μ thick were cut with glass knives and stained by hematoxylin and eosin (H&E), and von Kossa stain for the demonstration of calcium phosphates. Additional specimens containing the GPV plus the implant bed obtained in some studies were fixed 10% neutral buffered formalin, embedded in both paraffin and methacrylate, sectioned at 6 μ, and stained as above. Additionally, some sections were stained with alizarin red.

Representative specimens fixed as above were post-fixed in osmium tetroxide, dehydrated in graded ethanol, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEOL-JSM-100 transmission electron microscope (JEOL, Nutley, NJ). Scanning electron microscopy and elemental analysis was done with the use of a Tracor Northern-2000 (Tracor Instruments, Middleton, Wis) energy dispersive spectrometer on JB-4 embedded specimens using a multiprobed com-

puterized ISI DS-130 (ISI, Santa Clara, Calif) scanning electron microscope.

Methods of Analysis: Biochemical

Tissue Preparation

Retrieved valve leaflets were rinsed in sterile saline, washed free of salts with distilled water, freeze-dried, and pulverized to a fine powder with a Spex liquid nitrogen freezer mill.[20] Samples for analysis were dried to constant weight in a vacuum dessicator.

Mineral Analysis

Calcium was determined by atomic absorption spectroscopy using a Perkin Elmer Model 603 on aliquots of 6 N HCl hydrolysates of liquid-nitrogen–milled valve leaflets, which were diluted in acidified lanthanide solutions.[20,24,25] Serum calcium measurements were performed on appropriate dilutions in acidified lanthanide solutions with the same instrument. Phosphorus content of retrieved tissue specimens was determined on aliquots of the 6 N HCl hydrolysates prepared as described above, according to the methodology of Chen.[26] Serum phosphorous analyses were performed on trichloroacetic acid-precipitated serum aliquots, also using the methods of Chen.[26]

X-ray diffraction analysis was carried out on powdered tissue specimens.[24] Samples were analyzed for x-ray diffraction powder patterns in a Debye-Scherer camera with a 57.3 mm radius. The crystal type was determined from measurement of diffraction line position, and through diffraction line comparisons with a hydroxyapatite standard, and a pattern of rat bone powder, both kindly supplied by L. Bonar of Children's Hospital Medical Center, Boston, Massachusetts.

Amino Acid Analysis

Alkaline hydrolysates of GPV specimens using 2 N KOH were prepared according to established methods in order to determine Gla levels by automated amino acid analysis.[27,28] Amino acid analysis sensitivity was 10 pmol with less than 2% variation on triplicate samples.[28]

Osteocalcin Radioimmunoassay

Osteocalcin was quantitated by radioimmunoassay procedures established in our laboratories.[21] Exhaustive EDTA extractions (0.5 M EDTA, pH 6.9) of powdered GPV specimens were appropriately diluted and analyzed by competitive binding immunoassays[21] with an antiserum developed in rabbits to rat osteocalcin using an assay label of ^{125}I-rat osteocalcin. Serum osteocalcin levels were obtained on dilutions of rat serums according to established methods.[21] Triplicate assays demonstrated 8% or less variation, and the sensitivity of the assay system was 0.5 ng.

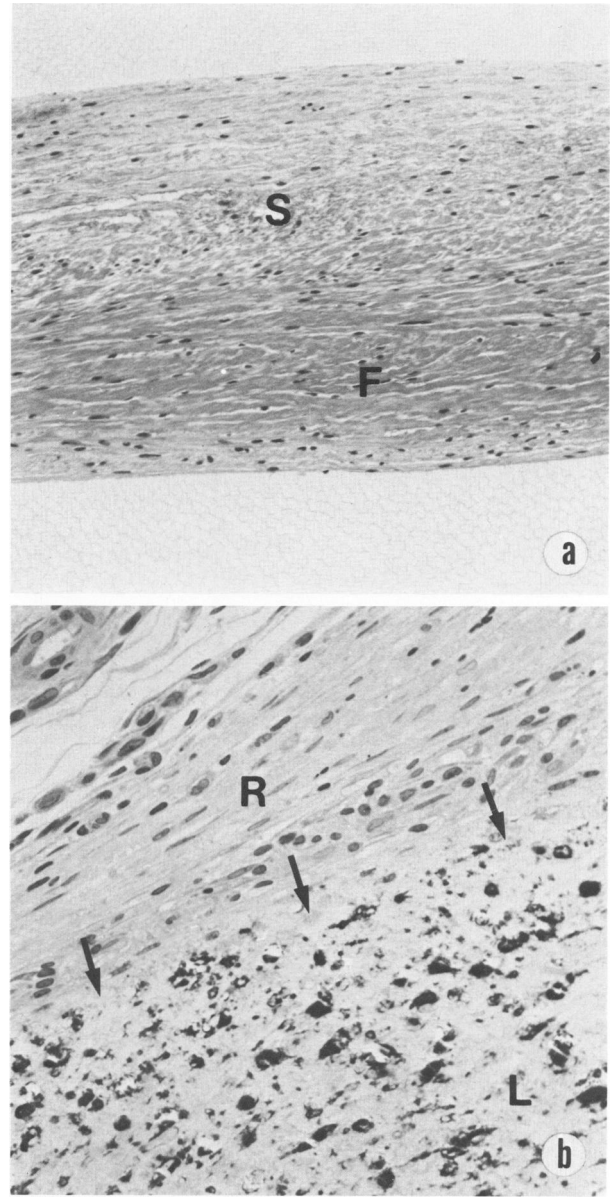


Figure 1 — a — Light micrograph of an unimplanted, glutaraldehyde-preserved porcine aortic leaflet (*GPV*). Fibrosa (*F*) and spongiosa (S) are identified (× 150) **b** — GPV with surrounding tissue reaction after implantation subcutaneously for 21 days. The junction of the leaflet with host tissue reaction is denoted by the *arrows*. Calcification is prominent in the leaflet (*L*), and a mononuclear inflammatory response (*R*) is notable at its surface. There is neither host cellular invasion into the leaflet nor calcification external to the leaflet. There is no local response to the calcific deposits. (H&E, × 375) (With a photographic reduction of 10%)

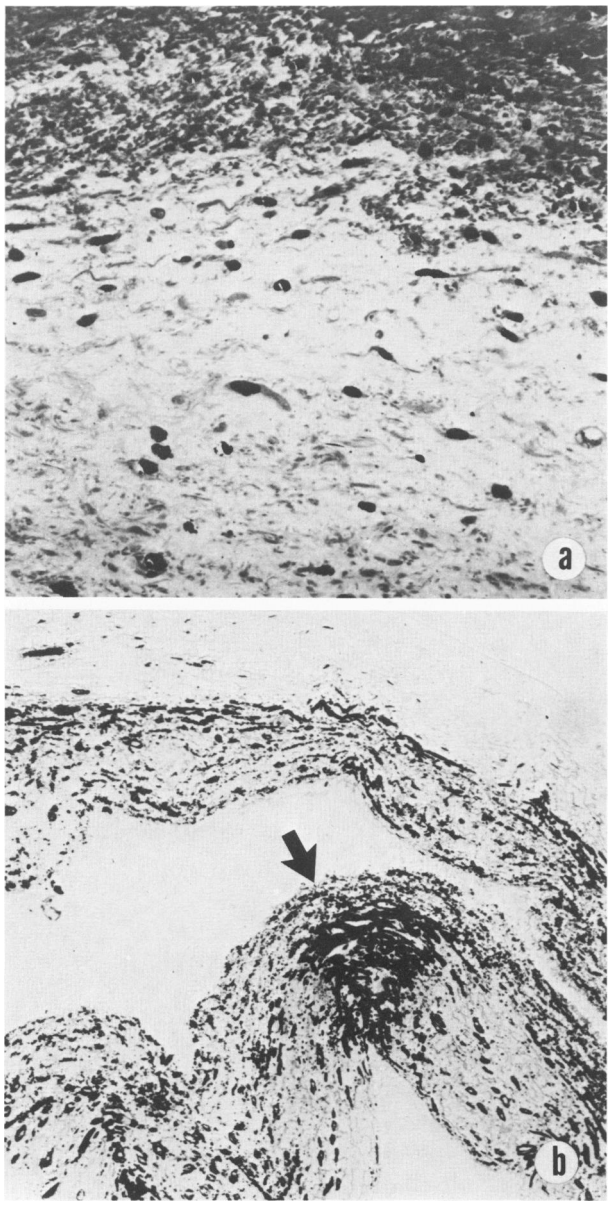


Figure 2—Light photomicrographs of (**a**) 3-day and (**b**) 7-day GPV subcutaneous implants stained with the von Kossa technique. Calcific deposits are present after 3 days, are increased at 7 days, and are accentuated in tissue folds (*arrow*). (**a**, ×375; **b**, ×60)

Data Analysis

Data are expressed as means ± standard error of the mean (SEM). The significance of differences between measurements made on control and experimental implants was assessed with the Student *t* test. Results were termed significant when $P < 0.001$.[29]

Results

Kinetics of GPV Calcification

Gross examination of retrieved specimens revealed time-dependent progressive calcification. Light microscopic examination of implants and environs demonstrated a moderate, polymorphic, predominantly mononuclear inflammatory response in the surrounding connective tissue, which, although present at all periods sampled, was most intense in the 7-day implants (Figure 1). Some mononuclear cells were adherent to almost all GPV surfaces; however, penetration of these host cells into the cuspal substance was unusual at any time period.

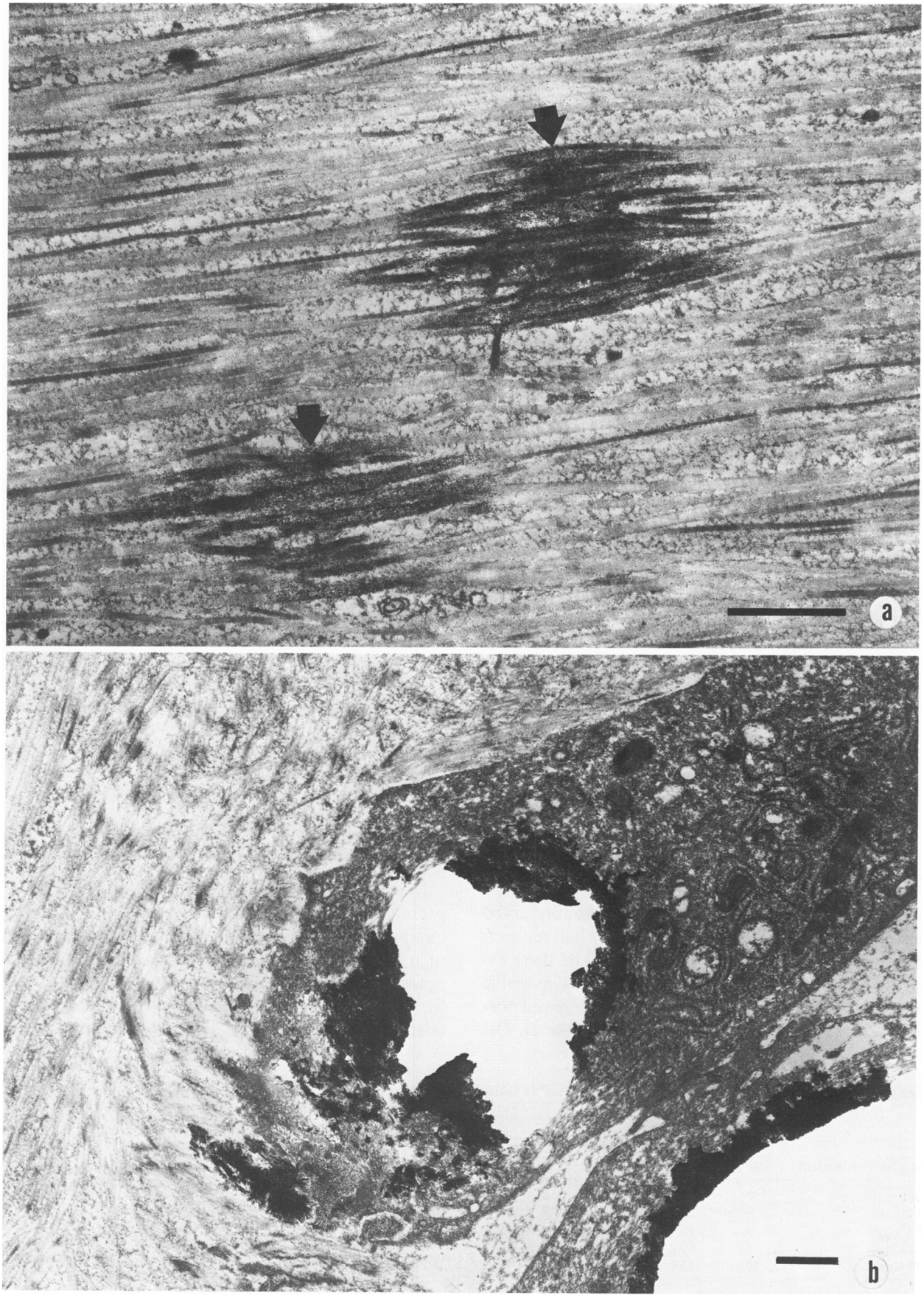
The earliest observed mineral deposits were distributed diffusely throughout the tissues involved but were most prevalent in the valvar fibrosa (Figure 2). By 21 days the pattern had reversed with later implants demonstrating greater calcification in the spongiosa than fibrosa. Host-cell reaction to calcific deposits was never noted. Accumulation of mineral was enhanced in areas of cuspal tissue folds (Figure 2), suggesting that static mechanical stress accelerated the calcification process.

Ultrastructural analysis of 3-week implants demonstrated progressive multifocal inter- and intrafibrillar collagen calcification (nodules to 2 μ), and larger deposits (to 10 μ) associated with devitalized porcine fibroblasts and other connective-tissue cells (Figure 3). Scanning electron microscopic examination and elemental analysis by electron probe spectroscopy confirmed progressive calcification beginning in the 3-day specimens. Between 7 and 21 days, confluent nodular regions (Figure 4) became prominent.

Significant accumulation of calcium began 3 days after implantation (see Table 1). Phosphate concentration paralleled calcium levels, and the calcium phosphate ratios were consistent with a predominance of hydroxyapatite. Osteocalcin, not detectable in unimplanted GPV, was present in the mineralized GPV retrievals and increased in the implants of longer duration. Tissue osteocalcin correlated well with valvular calcium content, as shown in Figure 5.

Analyses of a separate pool of three GPVs explanted after 21 days revealed tissue Gla levels of 0.219 ± 0.03 nM/mg, with osteocalcin levels of 406.6 ± 115.5 ng/mg. Assuming three Gla residues per osteocalcin molecule (molecular weight 5600), we calculated that osteocalcin accounted for roughly

Figure 3—Transmission electron photomicrographs of a GPV implanted subcutaneously in the rat for 21 days, illustrating (**a**) typical collagen fibril calcifications (*arrows*) and (**b**) calcification of a devitalized porcine connective tissue cell. The *bar* denotes 1 μ. (With a photographic reduction of 7%)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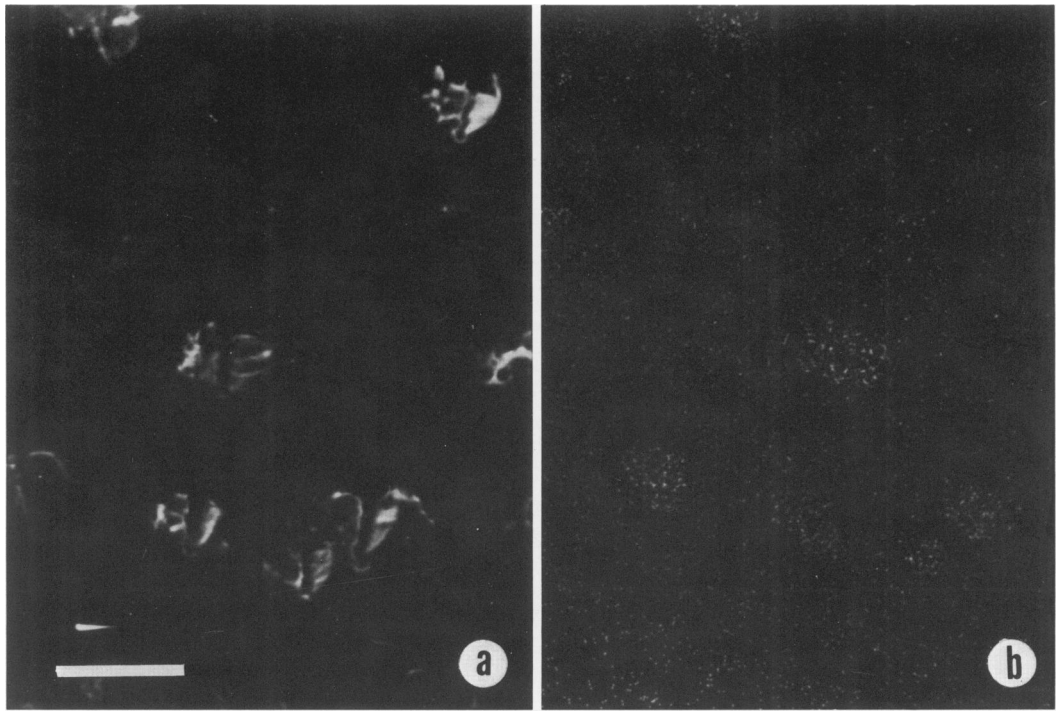

Figure 4—Scanning electron photomicrograph of a cross-section of a GPV implanted subcutaneously in the rat for 21 days, showing (**a**) diffuse apparent calcification with localized areas in devitalized cells and (**b**) a calcium pattern by x-ray dispersion energies, confirming the calcifications noted in **a**. The bar denotes 10 μ.

85% of the total Gla present in the calcified leaflets. X-ray diffraction analysis of powdered GPV material demonstrated a pattern typical of poorly crystalline hydroxyapatite (Figure 6). A comparable apatitic pattern was noted in the clinical GPV specimen analyzed and a sample of powdered rat bone.

Effect of Glutaraldehyde Pretreatment on Porcine Aortic Valve Leaflet Calcification

In contrast to the implanted GPV, nonpreserved leaflet implants were not calcified. They had an acellular necrotic center, and the outer third of these explants near either surface had an intense polymorphic inflammatory response (Figure 7). Nonpreserved porcine aortic leaflets did not mineralize (Table 2). Osteocalcin was present at high levels in calcified GPVs, while none was detectable in the unimplanted leaflets. However, low levels of osteocalcin were present in the non-glutaraldehyde-treated implants, despite their lack of detectable calcification.

Effect of Host Cells on GPV Calcification—Millipore Diffusion Chamber Experiments

Millipore-diffusion-chamber-enclosed GPVs appeared grossly calcified and comparable to directly implanted specimens. The microscopic morphology of mineralization was qualitatively the same as that noted in direct implants, but the surfaces of the leaflet tissue were devoid of attached cells. Millipore-chamber-enclosed GPVs demonstrated total tissue

Table 1—The Time Course of Xenograft Valve (GPV) Calcification (Mean ± SE)

		Tissue levels			Serum levels		
Implant duration	N	Calcium (μg/mg)	Phosphorus (μg/mg)	Osteocalcin (ng/mg)	Calcium (mg/dl)	Phosphorus (mg/dl)	Osteocalcin (ng/ml)
Unimplanted	7	2.8 ± 0.7	2.5 ± 0.5	ND	–	–	–
3 Days	8	28.0 ± 7.9*	14.3 ± 3.7*	36.6 ± 11.7*	10.7 ± 1.6	5.7 ± 0.6	128 ± 20.5
7 Days	6	76.5 ± 14.1*	46.6 ± 9.1*	202.0 ± 54.4*	12.9 ± 1.0	6.2 ± 1.2	114.5 ± 17.2
14 Days	8	80.2 ± 18.0*	47.1 ± 10.8*	152.3 ± 54.6*	13.4 ± 0.7	8.7 ± 0.2	96.3 ± 7.7
21 Days	10	113.9 ± 18.0*	66.8 ± 10.7*	176.0 ± 48.9*	11.0 ± 1.1	8.0 ± 0.5	126.8 ± 29.8

* $P < 0.001$ versus unimplanted.

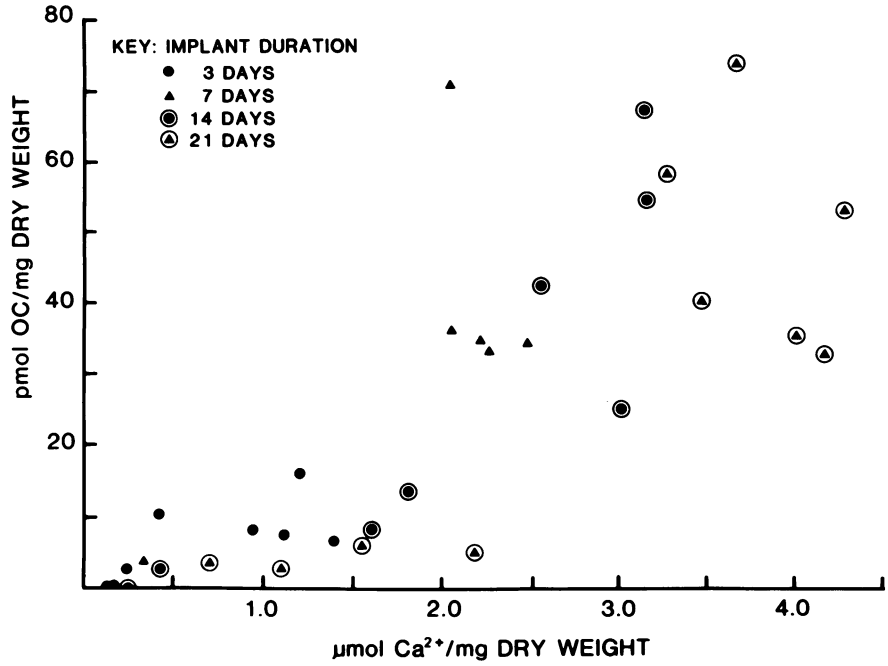

Figure 5—A plot of xenograft valve (*GPV*) osteocalcin (*OC*) in picomoles (*pmol*) per milligram versus calcium in micromoles (μmol) per milligram for rat subcutaneous GPV implants retrieved from the kinetic study (see Results; $r = 0.79$, $P < 0.001$).

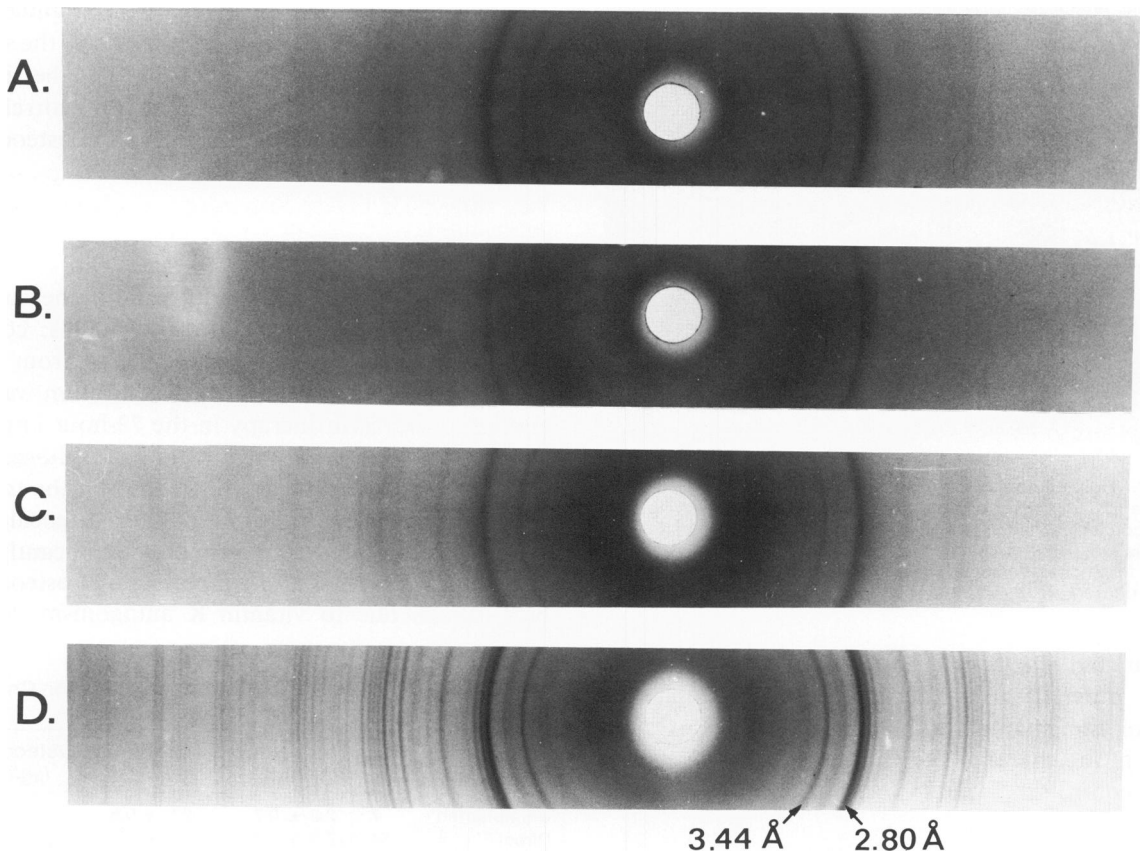

Figure 6—X-ray diffraction patterns obtained with a Debye-Scherer camera (see Materials and Methods) on powdered specimens of (**A**) calcified GPV, implanted for 21 days in a 3-week-old male rat, (**B**) calcified GPV obtained from an 18-year-old woman requiring valve replacement after 4 years, (**C**) rat femur, and (**D**) a crystalline hydroxyapatite standard. All the biologic samples show poorly crystalline hydroxyapatite by comparison to **D** and display the major diffraction lines of hydroxyapatite indicated at $d = 2.80$ Å, and $d = 3.44$ Å.

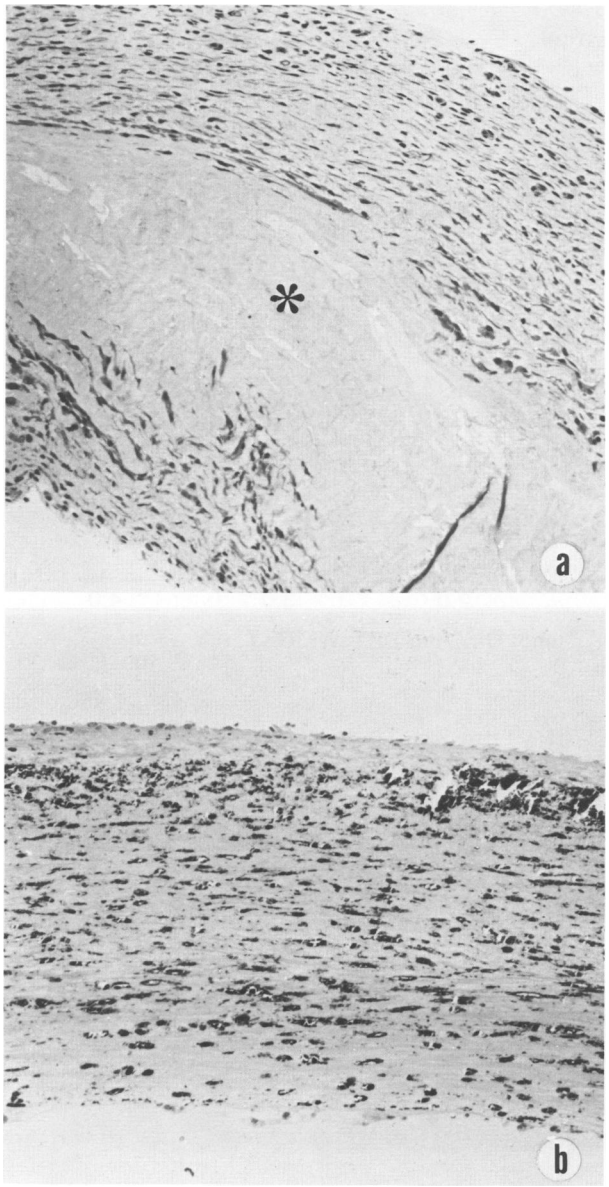

Figure 7—Light photomicrographs of cross-sections of porcine aortic valve leaflets implanted subcutaneously for 21 days. **a**—A nonpreserved porcine aortic cusp, with central necrosis (*), and peripheral inflammation, but without calcification. **b**—GPV implanted subcutaneously for 21 days, demonstrating diffuse calcification. (H&E, ×150) (With a photographic reduction of 10%)

calcium and phosphorus content at levels not significantly different from those of directly implanted GPV or hemichamber implants (see Table 3). Osteocalcin in the chamber GPVs was comparable to that in the control implants.

Effects of Host Age on GPV Calcification

GPVs explanted from the younger animals appeared grossly calcified, compared with the less affected leaflets removed from the older animals. Although GPVs from the younger animals demonstrated more extensive calcification by light microscopy, there were no qualitative morphologic differences in the general pattern of calcification between implants retrieved from young and old recipients.

Tissue calcium and phosphorus in GPVs obtained from 8-month-old rats were significantly less than in valves explanted from 3-week-old rats (Table 4). Osteocalcin was present in the valves obtained from the 8-month-old animals at much lower levels than noted in the GPVs retrieved from the young animals. Although serum calcium levels were similar in both age groups, the phosphorus levels were significantly higher in the young animals. Furthermore, the serum levels of osteocalcin were significantly higher in the younger animals. However, there was no correlation between tissue calcium, phosphorus, or osteocalcin with serum phosphorus levels.

Table 2—Glutaraldehyde-Preserved (GPV) and Nonpreserved Leaflets: Subcutaneous Calcification (Mean ± SE)

Type of valve	n	Calcium (μg/mg)	Phosphorus (μg/mg)	Osteocalcin (ng/mg)
Unimplanted	7	2.8 ± 0.7	2.5 ± 0.5	–
Implanted Nonpreserved	14	5.6 ± 1.0	9.4 ± 1.0	4.3 ± 2.5
Implanted GPV	54	122.9 ± 6.0*	64.1 ± 3.3*	183.4 ± 19.4*

* $P < 0.001$ versus unimplanted and fresh.

Effect of Warfarin Therapy

One-stage prothrombin times were immeasurably prolonged in the warfarin-treated animals; control animals had prothrombin times ranging from 12 to 14 seconds. Nevertheless, GPV calcification was not altered by warfarin therapy in the 72-hour implants studied (Table 5). GPV calcium and phosphorus contents were comparably increased in both the treated and control retrievals; however, GPV osteocalcin in the warfarin-treated retrievals was significantly less than control, reflecting a suppression of osteocalcin biosynthesis due to vitamin K antagonism. Serum

Table 3—Xenograft Valve (GPV), Millipore Diffusion Chamber Experiments (Mean ± SE)

Implant type	n	Calcium (μg/mg)	Phosphorus (μg/mg)	Osteocalcin (μg/mg)
Unimplanted	7	2.8 ± 0.7	2.5 ± 0.5	–
Direct	54	122.9 ± 6.0*	64.1 ± 3.3*	183.4 ± 19.4*
Hemichamber	11	195.9 ± 38.8*	145.9 ± 31.6	403.2 ± 53.4*
Millipore-enclosed	29	142.1 ± 12.9*	74.2 ± 6.7*	225.8 ± 27.4*

* $P < 0.001$ versus unimplanted GPV.

Table 4—The Effect of Age on Bioprosthetic Valve Leaflet Calcification (Mean ± SE)

		Tissue levels			Serum levels		
Type	n	Calcium (μg/mg)	Phosphorus (μg/mg)	Osteocalcin (ng/mg)	Calcium (mg/dl)	Phosphorus (mg/dl)	Osteocalcin (ng/ml)
Unimplanted	7	2.8 ± 0.7	2.5 ± 0.5	ND	–	–	–
3-week-old rat explants	54	122.9 ± 6.0*	64.1 ± 0.1*	183.4 ± 19.4*	11.4 ± 1.0	7.5 ± 0.4	37.9 ± 3.3
8-month-old rat explants	33	22.4 ± 5.0†	8.0 ± 1.9†	16.6 ± 3.9†	11.0 ± 0.6	5.5 ± 0.2†	15.1 ± 0.9†

* $P < 0.001$ versus unimplanted.
† $P < 0.001$ versus 3-week-old rat data.

levels of osteocalcin did not differ significantly between treated and control animals.

Discussion

Characterization of the Rat Subcutaneous Model

In the present study we examined some important aspects of the pathophysiology of the dystrophic calcification of GPVs and the associated deposition of the bone protein osteocalcin through the use of subcutaneous implants in rats. GPVs implanted in these studies were prepared in the same way as those implanted in clinical porcine bioprosthetic heart valves,[1,2] except that they were not mounted on stent supports. The morphology of the subcutaneous GPV calcification was comparable to that noted in clinical circulatory retrievals. Subcutaneous GPV implants calcified progressively and contained hydroxyapatite as the principal calcium-phosphate mineral phase.

The morphologic observations of the present study are in agreement with previously reported analyses of clinical[14-19] and experimental[22,30-32] GPV calcifications. Light microscopic examination of the rat subcutaneous lesions showed progressive calcification predominantly, but not exclusively, in the valvular spongiosa. The ultrastructural demonstration of GPV calcification associated with collagen fibrils and devitalized porcine connective tissue cells agrees with previously published clinical[16,19] and experimental results.[22,32] While the lesions produced with subcutaneous GPV implants in rabbits[22] were morphologically comparable to those in the present study, the rat system is preferable because of its accelerated calcification (3 weeks in rats compared with 6 months in rabbits).

The biochemical results of the present study agree with previous clinical[20,21] and experimental work[22,30] on GPV calcification. Although it has not been possible to document the kinetics of the progression of calcification in clinical cases, experimental work on bovine[30] and sheep[32] GPV circulatory implants and rabbit subcutaneous GPV implants has shown time-dependent increases in GPV calcium. The present study emphasizes the utility of the rat subcutaneous implant method in the study of dystrophic calcification of GPV.

Osteocalcin and Gla-Containing Proteins

The vitamin K-dependent blood coagulation factors were the first proteins discovered to contain the calcium-binding amino acid Gla.[33,34] Gla is biosynthesized in these proteins in a vitamin K-dependent, posttranslational, enzymatic carboxylation of specific glutamic acid residues.[33,34] Vitamin K antagonism, with drugs such as warfarin, inhibits Gla synthesis, resulting in a loss of calcium binding and anticoagulation.[34]

A number of extrahepatic Gla-containing proteins are associated with normal[27,28] and pathologic mineralization.[20-22,24,25] Osteocalcin, the principal Gla-containing protein of bone,[27,35,36] also occurs in pathologic cardiovascular calcifications.[21,30] Although osteocalcin is the most abundant noncollagenous protein found in bone,[35-37] the biologic and pathologic functions of this protein are incompletely understood. Recent work by Price and his colleagues indi-

Table 5—The Effect of Warfarin Therapy on Xenograft Valve (GPV) Calcification (Mean ± SE)

Specimen	N	GPV calcium (μg/mg)	GPV phosphorus (μg/mg)	GPV osteocalcin (ng/mg)	Serum osteocalcin (ng/ml)
Unimplanted	7	2.8 ± 0.7	2.5 ± 0.5	–	–
Warfarinized rat explants (72 hours)	12	39.6 ± 2.9	23.4 ± 2.2	15.3 ± 1.2*	126.7 ± 12.8
Control rat explants (72 hours)	8	40.8 ± 4.8	22.3 ± 2.6	69.0 ± 13.7†	91.5 ± 0.7

* $P < 0.001$, compared with control.
† $P < 0.001$ compared with warfarin-treated explants.

cates that long-term (8 months) warfarin administration to rats results in hypermineralization of long bones with decreased biosynthesis of osteocalcin, suggesting that osteocalcin may limit physiologic mineralization.[38]

The role of osteocalcin and vitamin K-dependent processes in calcification of cardiovascular biomaterials has been studied experimentally with calf circulatory implants of both left-ventricular assist devices (LVAD) with blood-contacting surfaces fabricated from smooth or fibrillar polyurethane,[39,40] and porcine bioprosthetic heart valves.[30] Pierce found that LVAD mineralization was dramatically reduced in calves receiving warfarin anticoagulant therapy, compared with a control group that did not receive warfarin.[39] Pierce hypothesized that this effect was due to inhibition of Gla-protein synthesis by the vitamin K antagonist warfarin.[39] However, Lian[40] reported a series of LVAD calf implants in which warfarin anticoagulation affected neither the extensive calcification nor the deposition of Gla proteins, including osteocalcin. The reasons for the discrepancy between these two studies are not clear, but may in part be explained by differences in the polyurethane formulation used in the specific LVAD bladders.[39,40] The pathophysiology of LVAD calcification has been incompletely described[39,40]; nevertheless, it is clear that, unlike GPV calcification, neither collagen calcification nor mineralization of intrinsic cells occurs, indicating probable important mechanistic differences between the two processes. It is possible, however, that mineralization of the surface thrombotic accumulation on the LVAD pumping bladder involves both blood-borne cells and Gla-containing coagulation proteins, unlike GPV mineralization. This is further suggested by the results of a study[30] on GPV valve replacements in calves on warfarin anticoagulant therapy; retrieved valves calcified and accumulated osteocalcin in spite of warfarin therapy, a result that agrees with the findings of the present study. This implies that there may be differences in Gla-protein function depending upon the specific mechanism of pathologic calcification involved, and this could explain in part the lack of effect of warfarin on GPV mineralization.

In the present study, osteocalcin accounted for 85% of the total Gla content and correlated with tissue calcium levels. Furthermore, the finding of osteocalcin in the GPV retrieved from Millipore enclosures implies passive absorption. Warfarin therapy, at very high dosage, failed to block the calcification in 72-hour GPV explants. However, GPV tissue osteocalcin was significantly reduced in the warfarin retrievals (Table 5), indicating that the normal biosynthesis of osteocalcin had been disrupted. Serum levels of osteocalcin were apparently affected very little by the warfarin therapy, probably because of circulating undercarboxylated osteocalcin.

It seems likely that osteocalcin is simply adsorbed onto the mineralizing GPV and has no significant function in the pathogenesis. However, the fact that osteocalcin is specifically bound to calcifying GPV may be useful in the noninvasive monitoring of pathologic progression and in devising innovative therapeutic approaches, such as utilizing monoclonal antibody-targeted drug transport.

Determinants of GPV Calcification: Host Factors

Inflammatory/Immunologic Considerations

The diffusion chamber experiments demonstrate that host-cell attachment to GPV implants is not a prerequisite for mineralization, although several clinical studies have suggested that hypersensitivity to the GPV xenograft tissue may contribute to calcification.[11,41] The results of the chamber experiments imply that the host immune response to the GPV tissue is not involved in calcification. Calcification occurred despite blockage of lymphocyte and macrophage contact with the valve. Furthermore, calcification of GPV in congenitally athymic (nude) mice has recently been demonstrated.[42]

Osteogenesis occurring in response to subcutaneously implanted materials, such as demineralized bone powder, has been well described[43-45] and, in fact, occurs in response to glutaraldehyde-cross-linked collagenous implants placed in rabbit mandibular bone defects.[45] However, cellular transformation of invading fibroblasts with resultant osteogenesis is impossible in the pathogenesis of GPV mineralization in view of the results of the Millipore chamber studies.

Age

The observed exacerbation of GPV calcification in younger animals parallels the clinical occurrence of accelerated calcification of GPV bioprostheses in younger patients.[9-13] The finding of higher serum phosphate levels[46,47] and higher serum osteocalcin levels[48] in younger animals and human subjects may be important. However, in the present study no correlation was found between individual serum osteocalcin or phosphorus levels and valvular calcium, phosphorus, or osteocalcin content. The higher serum phosphorus levels may reflect accelerated mineral and associated endocrine metabolism including enhanced parathyroid activity in the younger ani-

mals.[46,47] The age-related GPV calcification phenomenon may also reflect age-dependent aspects of vitamin D metabolism. Vitamin D metabolism is thought to be enhanced in younger animals and human subjects.[49] Vitamin D has also been recently shown to be a potent stimulus of osteocalcin biosynthesis both in cell culture[50] and *in vivo*[51] and may interact to promote age-dependent GPV calcification through its effects on osteocalcin.

Determinants of GPV Calcification: Implant Factors

Glutaraldehyde Treatment

Glutaraldehyde fixation of porcine aortic leaflets was a prerequisite for the calcification of GPV implants. No calcification of fresh porcine valve implants occurred during the 3-week implantation period, while simultaneously implanted glutaraldehyde preserved leaflets calcified severely. Glutaraldehyde is utilized in GPV bioprosthetic valve preparation as a cross-linking agent and an antiseptic.[1-3] Incubation of porcine aortic leaflets in glutaraldehyde solutions results in the formation of interfibrillar and intrafibrillar collagen cross-link compounds,[52-55] which contribute to the mechanical strength and durability of the GPVs, enabling these bioprostheses to sustain prolonged circulatory function.

The specific steps in the glutaraldehyde treatment procedures that promote the calcification are not known, but the specific types of cross-link compounds formed may be responsible. For example, the pyridinium cross-link, which is one of the major types of glutaraldehyde-induced cross-links[52-55] is directly analogous to similar compounds found in bone-derived collagen[56] and the desmosine residues of elastin.[57] The pyridinium cross-link is a quarternary amine and could theoretically cause an influx of phosphate into the intrafibrillar spaces of the collagen helices possibly leading to hydroxyapatite nucleation. Structural proteins rich in native cross-links, such as mature elastin or collagen, may promote calcification solely on the basis of the extent of cross-linking.

Stress Effects

Calcific deposits in the GPV implants were frequently enhanced in tissue bends and folds (see Figure 2a). These results are of interest because calcification of bioprosthetic GPV valve replacements is well known to be favored at hemodynamic stress points,[14-19] such as the commissural attachments to the valve stent. Although the reasons for this stress effect are not clear,[58] the integrity of the valvular stroma probably provides some protection against calcification and stress, either static or dynamic.

Clinical Implications

The results of the present study suggest pharmacologic approaches that may be of benefit in controlling GPV calcification. Since this disease process has been shown in these studies to be predominantly acellular and age-dependent, therapy with drugs such as the diphosphonates[59] may be used intermittently to prevent calcification in younger patients. The occurrence of Gla-containing proteins in the GPV calcific lesions may be clinically important in noninvasive diagnosis, and the presence of these proteins could form the basis for unique therapeutic approaches involving specific immunoadsorption of agents, which might locally diminish calcification. Nevertheless, the presence of vitamin K-dependent calcium-binding proteins including osteocalcin in the GPV calcifications and our results showing that warfarin did not affect GPV calcification suggest that novel approaches will be required for an understanding of the role of osteocalcin and other Gla-containing proteins.

GPV calcification may ultimately be alleviated by altering the GPV preparation procedures. Work by others has suggested that pretreating GPV leaflets in sodium dodecylsulfate (SDS) results in reduced calcium accumulation in circulatory and subcutaneous models.[60] Although the mechanisms of the SDS effect are unknown, we hypothesize that SDS, by extracting phospholipid membrane components, lessens the calcification noted to be associated with devitalized porcine connective tissue cells and other aspects of mineralization associated with phospholipids. Similarly, modifying the cross-links formed during the glutaraldehyde preservation procedure with agents such as sodium borohydride[57,61] might be expected to reduce calcification. Further research may establish the rationale for bioprosthetic valve preparation procedures, which would obviate calcification.

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