

Effects of metallic ions and diphosphonates on inhibition of pericardial bioprosthetic tissue calcification and associated alkaline phosphatase activity

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This study focused on the association of extrinsic alkaline phosphatase (AP) activity with both early and advanced calcification of glutaraldehyde-pretreated bovine pericardial bioprosthetic (GPBP) tissue, and the inhibition of both calcification and AP activity by pre-incubation in diphosphonates (sodium-ethanehydroxydiphosphonate [NaEHDP], aminopropanehydroxydiphosphonate [APD]) and metallic salts (FeCl_3 , $\text{Ga}(\text{NO}_3)_3$, AlCl_3). GPBP specimens were implanted subcutaneously in 3 wk old male rats after pre-incubation. Following explantation of the tissue at 72 h and 21 d, calcification was assessed morphologically by light microscopy and chemically by atomic adsorption spectroscopy for calcium content and by molybdate complexation for phosphorus. AP activity was characterized by enzymatic hydrolysis of paranitrophenyl phosphate and by histochemical studies. In both control and pretreated groups, AP levels were greater in 72 h explants than 21 d retrievals, which demonstrated extensive calcification in control explants. All pre-incubations that resulted in inhibition of calcification after 21 d, except for APD, were associated with 72 h AP content which was lower than control specimens. The typical time of initiation of calcification was 72 h, as determined by previous studies with this model system. Covalently bound APD inhibited calcification. Increased AP activity in the APD group may be due to the toxicity of this agent with resultant acute inflammation, or other incompletely understood effects of diphosphonates on calcification and AP. Furthermore, EHDP and Ga^{3+} incubations were also associated with decreased GPBP AP at 72 h compared to control, but were not effective for inhibiting calcification after 21 d. We concluded that inhibition of peak GPBP AP activity is not necessarily associated with the prevention of GPBP mineralization.

Keywords: *Bovine pericardium, calcification, alkaline phosphatase*

Received 5 August 1992; accepted 21 October 1992

Bioprosthetic heart valves fabricated from either glutaraldehyde-pretreated porcine aortic valve cusps or bovine pericardium (GPBP) are often used to replace deteriorating human cardiac valves. The primary reason for the clinical failure of porcine aortic valve bioprostheses is dystrophic calcification¹⁻³. Calcification also contributes to the failure of bovine pericardial valves. Bioprosthetic heart valve calcification occurs as a multifactorial process, in which an interaction of host factors (youth), implant factors (glutaraldehyde fixation) and implant factors (stress-strain) are crucial¹⁻⁶. Calcification of clinical bioprosthetic valves can be simulated either by

large animal circulatory models or by subdermal implants in rodents⁴⁻⁶. The initial calcification event in the rat subdermal model is the mineral deposition in devitalized cells intrinsic to the bioprosthetic tissue within 48-72 h, followed later by collagen mineralization.

Matrix vesicles (MV) present in the extracellular matrix of mineralizing tissues are considered to initiate deposition of hydroxyapatite mineral in endochondral bone formation and calcific diseases⁷. An enrichment of alkaline phosphatase (AP) within the MVs suggests an important role for this enzyme in the initiation of calcification^{8,9}; following nucleation, AP activity declines during progressive bone mineralization¹⁰. However, some studies have shown that AP catalytic activity is not

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absolutely required for MV calcification¹⁰. The fact that the enzyme was found distributed widely in tissues that normally do not undergo mineralization has raised questions as to the specificity of the relationship between AP and calcification¹¹.

Research has demonstrated AP activity in glutaraldehyde-pretreated porcine aortic valve tissue¹² and GPBP¹³ before and after glutaraldehyde fixation. However, after fixation AP activity is only detectable by histochemistry, and is not extractable using the established butanol extraction procedure¹³. Furthermore, subdermal implant studies have demonstrated peak extractable AP levels in GPBP after 72 h implantation, coinciding with the onset of calcification¹³. AP activity declined thereafter, while bulk mineral increased¹³. In previous studies, metallic salt pre-incubations, which included either ferric chloride or aluminium chloride, have been shown to inhibit calcification of GPBP tissues in the rat subdermal model^{14, 15}. This inhibition was associated with reduced AP activity after 21 d¹³. These metallic inhibitors have also been shown to be localized to the site at which AP is present¹⁵. This is probably due to the high affinity of these cations for membrane-associated phosphorus-rich sites, which have also been shown to be the loci of the initial calcific deposits. Furthermore, diphosphonates are potent calcification inhibitors¹⁶⁻¹⁸. Their mechanism of action appears to be the marked affinity of these compounds for crystalline calcium phosphates^{19, 20}. In addition, diphosphonates also influence cellular metabolism; dichloromethanediphosphonate and sodium ethanedihydroxydiphosphonate increased the AP activity of cultured rat calvaria cells²¹, and *in vivo*, chickens treated with these diphosphonates demonstrated inhibition of bone mineralization associated with increased AP activity²².

The present study was carried out to understand the effects of diphosphonates and metallic salts on both calcification and AP activity during dystrophic mineralization of rat subdermal implants of GPBP. The goals of this study were to: (1) document the effects of various inhibitors of GPBP calcification on early (72 h) and late (21 d) mineralization and AP accumulation in rat subdermal implants; (2) to examine further the morphologic distribution of AP with either progression or inhibition of GPBP mineralization; and (3) to assess the effects of possible calcification inhibitors on the Ca-P mineral phase, as indicated by Ca/P ratios.

MATERIALS AND METHODS

Materials

Electron microscopy-grade glutaraldehyde was purchased as an 8% aqueous solution (Polyscience, Warrington, PA, USA). The buffer used for cross-linking and incubation of the drugs included 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES, Sigma Chemical, St Louis, MO, USA) with 0.1 M NaCl (EM Science, Cherry Hill, NJ, USA). Ferric chloride hexahydrate (reagent grade) and the phosphate standard were obtained from Sigma Chemical, St Louis, MO, USA. Gallium nitrate (99%) and ammonium molybdate (VI) were obtained from Aldrich (Milwaukee, WI, USA). Aluminium chloride

was obtained from Mallinckrodt (Paris, KY, USA). Aminopropanehydroxydiphosphonate (APD) was donated by Dr James Benedict (Intermedics Orthopedics Inc., Wheatridge, CO, USA). Sodium ethanedihydroxydiphosphonate (NaEHDP) was donated by Procter and Gamble (Cincinnati, OH, USA). L-ascorbic acid was purchased from Fisher Chemical (FairLawn, NJ, USA). 2-amino-2-methyl-1-propanol, sec-butanol, *p*-nitrophenyl phosphate (pnpp) and *p*-nitrophenol (pnp) were also obtained from Sigma.

Methods

In vitro incubations

Fresh mature bovine parietal pericardium was obtained from the slaughter house (P. Dunbar, Milan, MI, USA) and immediately cross-linked for 24 h in 0.6% glutaraldehyde at pH 7.4 (50 mM HEPES buffer) at 4°C, then stored for 2-4 wk in 0.2% glutaraldehyde in the same buffer at 4°C, as described previously^{5, 6, 23}. GPBP was cut into 1 cm diameter pieces and rinsed free of glutaraldehyde.

Following fixation and storage, the GPBP pieces were pre-incubated for 24 h at room temperature in either APD, or NaEHDP both at concentrations of 0.1 M, buffered to pH 7.4 plus NaCl (0.1 M). GPBP pieces were also pre-incubated in several metallic salt solutions at pH 3 (0.001 M HCl, NaCl 0.1 M) including AlCl₃ (0.1 M), Ga(NO₃)₃ (0.1 M), FeCl₃ (both 0.1 M and 10⁻⁴ M). Control specimens of GPBP were pre-incubated in either pH 7.4 (50 mM HEPES) or pH 3(0.001 M HCl) solutions (0.1 M NaCl) for 24 h at room temperature.

Implant and retrieval methods

After pre-incubation in the various solutions, the GPBP specimens were washed with sterile saline, and then implanted subdermally in male, weanling rats (50-60 g, CD, Sprague-Dawley, Charles River Laboratories, Burlington, MA, USA) according to methods described previously^{5, 6, 15, 18, 23}. The rats were anaesthetized by an i.m. injection of ketamine hydrochloride and xylazine. Ten GPBP specimens were implanted subdermally per rat (six in the abdominal wall and four in the dorsal wall) (five rats per group study). At 72 h, five pieces (from the abdominal implant site) were removed under anaesthesia. After 21 d, the rats were killed by CO₂ asphyxiation and the remaining samples retrieved.

Biochemical analysis

Explants were rinsed with copious volumes of saline and deionized water, freeze-dried, minced and subjected to acid hydrolysis as described previously^{5, 23}. Aliquots of the hydrolysates were analysed for calcium by atomic absorption and for phosphorus using a molybdate complexation²⁴. AP enzymatic activity was quantitated as described previously¹². Briefly, butanol extracts of 5-6 mg GPBP samples were assayed for hydrolysis of pnpp normalized by Lowry protein content²⁵ (pH 10.4 and 37°C at a wave length of 405 nm using a Lambda 3B spectrophotometer (Perkin-Elmer, Oak Brook, IL, USA).

Morphologic analysis

A representative sample of each GPBP explant was placed in Karnovsky's fixative²⁶ for subsequent morpho-

Table 1 Inhibition of glutaraldehyde pretreated bovine pericardium (GPBP) calcification and alkaline phosphatase (AP) activity: rat subdermal implant results

Pretreatment	72 h Explants				21 d Explants			
	Ca*	P*	AP**	Ca/P***	Ca*	P*	AP**	Ca/P****
Control (pH 7.4)	2.2 ± 0.20(4)	0.7 ± 0.10(4)	317.30 ± 41.4(7)	2.7	90.0 ± 5.9(4)	45.20 ± 2.3(4)	78.2 ± 15.2(4)	1.5
APD (0.1 M)	2.1 ± 0.60(5)	1.4 ± 0.60(5)	576.60 ± 85.0(12)†	1.1	3.4 ± 0.6(5)†	1.50 ± 0.2(5)	101.7 ± 20.0(9)	1.7
NaEHDP (0.1 M)	1.5 ± 0.06(5)	0.4 ± 0.04(5)	165.60 ± 14.3(13)†	3.3	89.3 ± 8.7(5)	84.20 ± 24.2(5)	52.4 ± 6.9(10)	0.8
Control (pH 3)	3.2 ± 1.40(2)	0.6 ± 0.30(2)	249.60 ± 49.2(9)	4.3	95.6 ± 25.9(3)	52.00 ± 13.6(3)	42.9 ± 5.8(6)	1.4
FeCl ₃ (0.1 M)	4.8 ± 0.30(5)	2.9 ± 0.10(5)	185.90 ± 34.1(13)	1.2	19.0 ± 3.0(5)†	12.30 ± 2.2(5)	157.6 ± 76.6(10)	1.2
FeCl ₃ (10 ⁻⁴ M)	2.3 ± 0.50(5)	1.04 ± 0.20(5)	490.10 ± 145.3(14)	1.7	93.3 ± 14.2(5)	50.60 ± 8.1(5)	87.4 ± 7.7(10)†	1.4
Ga(NO ₃) ₃ (0.1 M)	5.2 ± 0.80(5)	2.3 ± 0.30(5)	217.00 ± 34.6(12)	1.7	98.0 ± 5.34(5)	102.30 ± 29.2(5)	103.6 ± 40.0(10)	0.7
AlCl ₃ (0.1 M)	9.8 ± 0.60(5)	7.4 ± 0.40(5)	93.07 ± 8.5(12)†	1.7	9.3 ± 0.5(5)†	25.26 ± 4.3(5)	33.7 ± 2.6(10)	0.3

Mean ± SEM.

* µg/mg of dried tissue.

** nm paranitrophenyl phosphate/min/mg protein.

*** Molar ratio.

† P < 0.05 compared with controls.

() Number in parentheses is the number of analyses.

logical analysis. For histochemical demonstration of AP, a separate GPBP explant sample from each drug group were frozen with dry ice in several drops of OCT medium (Miles Inc., Elkhart, IN, USA) for subsequent cryomicrotomy and AP staining assessment, using the conventional method²⁷. Sections of GPBP (10 µm thick) were cut with a cryomicrotome, mounted on glass slides, and dried at room temperature. The sections were incubated at 37°C for 30 min in a medium for AP modified from Gomori²⁷ containing 50 ml of 5 mM MgCl₂ in distilled water, 1 ml Naphtol AS-MX phosphate in pH 10.5 buffer (Sigma), and 25 mg Fast Red TR or Fast Blue RR (Sigma) as described previously¹².

RESULTS

The calcium and phosphorus levels, together with the AP activity data at 72 h and 21 d, are summarized in Table 1. No extractable AP activity from unimplanted GPBP was noted by enzymatic analyses. Explant GPBP AP enzymatic activity was higher in all groups after 72 h than in 21 d explants, despite lower calcium levels in all the 72 h explants compared with 21 d results. The mean calcium and phosphorus levels in the pretreated GPBP 72 h explants did not statistically differ significantly for all the drug pre-incubation groups and control groups (Table 1). Except for the APD group, all pre-incubations which resulted in inhibition of calcification after 21 d, compared to the control, also demonstrated lower AP levels than control in the 72 h explant specimens (Figure 1). Morphological assessment qualitatively confirmed these results (Figures 2 and 3). Although covalently bound APD significantly inhibited calcification in 21 d explants compared to controls (which were heavily calcified), maximal AP levels for all groups at 21 d were noted in the APD group (Figures 2 and 3). In contrast, pretreatment with NaEHDP did not inhibit GPBP calcification compared to control after 21 d, although the AP activity was slightly lower than the control in 72 h and 21 d explants.

Ga(NO₃)₃ (0.1 M) and FeCl₃ (10⁻⁴ M) pre-incubations failed to inhibit calcification. However, FeCl₃ (0.1 M) and AlCl₃ (0.1 M) pre-incubations inhibited calcification, and the latter was associated with the lowest AP levels and

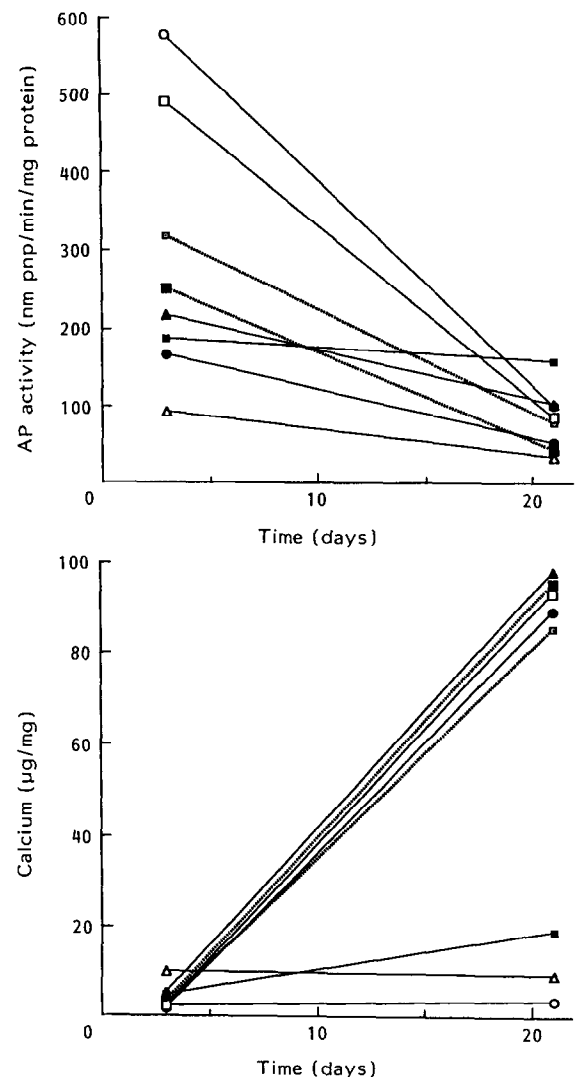


Figure 1 a, Alkaline phosphatase (AP) activity levels normalized per extractable protein content and b, calcium levels/mg dry weight of GPBP control tissues and drug-treated tissues at initiation stage of calcification (72 h) and at 21 d interval calcification. □□□□, control (Ctrl) (pH 7.4); ○—○—, aminohydroxypropanediphosphonate (APD) (0.1 M); ●—●—, NaEHDP (0.1 M); ■—■—, Ctrl (pH 3); ■—■—, FeCl₃ (0.1 M); □—□—, FeCl₃ (10⁻⁴ M); ▲—▲—, Ga(NO₃)₃ (0.1 M); △—△—, AlCl₃ (0.1 M).

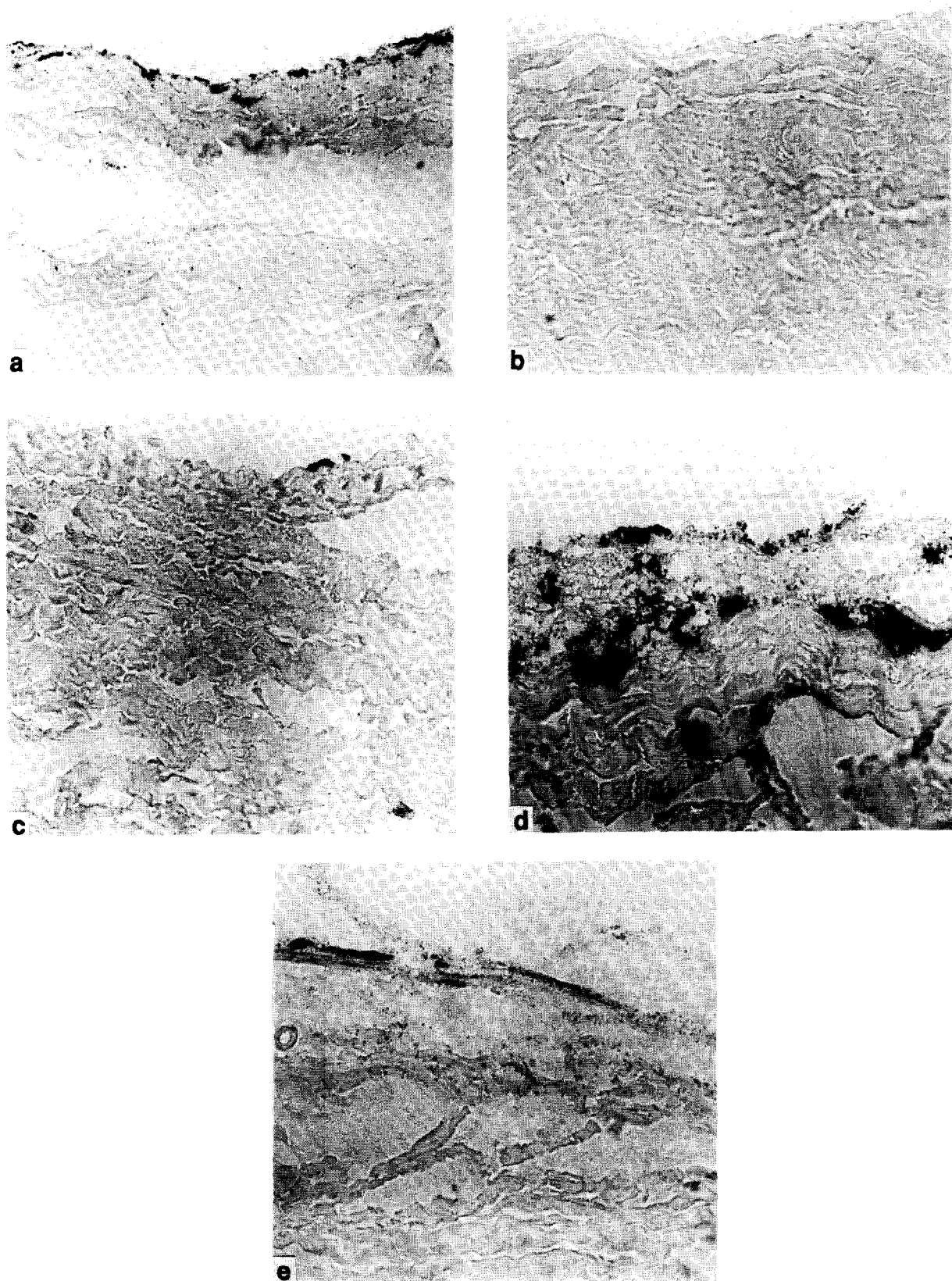


Figure 2 Effects of pretreatment in AlCl_3 and aminodiphosphonate (APD) on histochemical staining of alkaline phosphatase (AP) accumulation in bovine pericardium implanted subcutaneously in rats. **a**, and **b**, Control (untreated) bovine pericardium at 3 and 21 d, respectively. **c**, AlCl_3 pre-incubated bovine pericardium following 3 d implantation. **d** and **e**, APD pretreated tissue at 3 and 21 d, respectively. AP (dark reaction product) is evident in the control bioprosthetic tissue, **a**, **b**, at 3 d and is comparatively reduced at 21 d. AP activity is scarcely evident in 72 h AlCl_3 explants, **c**. However, AP activity is enhanced compared to controls at both 3 and 21 d, **d**, **e**, in the APD-pretreated tissue. Original magnification $\times 230$.

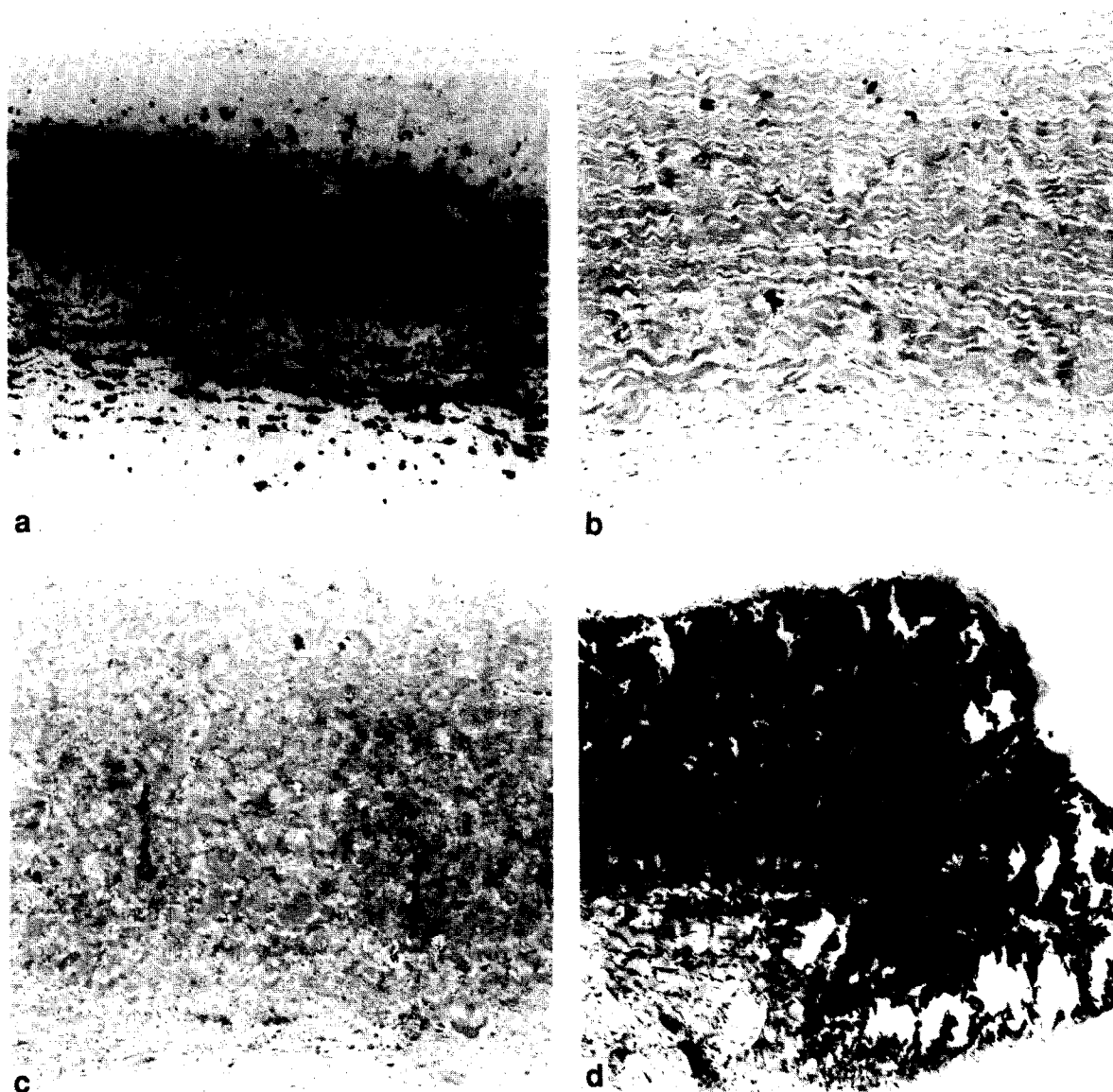


Figure 3 Light microscopy appearance of calcification of bovine pericardium implanted subcutaneously in 3 wk old rats for 21 d. **a**, Control tissue demonstrating heavy mineralization. **b**, Tissue pretreated in APD demonstrating sparse punctate calcific deposits (probably in cells). **c**, Tissue pretreated in FeCl_3 (0.1 M), demonstrating complete absence of mineralization. **d**, Tissue pretreated in gallium, demonstrating heavy mineralization. Each stained with von Kossa stain (calcium phosphate black). Original magnification $\times 120$.

decline in AP (21 d versus 72 h) of the various groups. AP activities in the Ga^{3+} group were slightly lower than controls after 72 h, and even slightly greater than controls after 21 d. Nevertheless, Ga^{3+} failed to inhibit calcification. Ga^{3+} explants demonstrated the highest P levels of all groups after 21 d. These may nucleate further Ca-P deposition.

Ca/P molar ratios of the 21 d explant groups demonstrated were consistent with the presence of calcium phosphate for the control groups and the FeCl_3 (10^{-4} M) specimens. However, the calcified $\text{Ga}(\text{NO}_3)_3$ and NaEHDP groups each demonstrated a relatively higher phosphorus content and hence lower Ca/P ratios than usually noted with hydroxyapatite deposition.

DISCUSSION

Interestingly, peak GPBP AP activities were noted on the 72 h explants regardless of pre-incubation in solutions

of diphosphonates or metallic ions. Earlier results showed that AP activity peaks in the first 72 h in control GPBP rat subdermal explants; by this time calcification is also initiated¹². However, comparisons between the various groups (control and pre-incubation) did not reveal a trend consistently linking reduced GPBP with inhibition of calcification. Thus, although AP activity and inhibition of AP may be associated with the onset and progression of GPBP calcification, the present results support the view that AP may be one of several determinants of the pathogenesis of GPBP calcification. Therefore, AP inhibition or enhancement by various inhibitors may not reveal the importance of AP in this setting.

Furthermore, previous work from our group has shown that extrinsic GPBP AP activity declines following its peak at 72 h as mineralization progresses. Comparable observations were reported in studies of the onset of bone mineralization involving endochondral MV calcification¹⁰. In the case of MVs, this decline in AP activity is not due

to proteolysis, but rather to the calcification process itself¹⁰. The direct inhibitory effect of hydroxyapatite on AP, along with the loss of metal ions from the AP active site, are thought to contribute to the decline in AP activity during MV calcification. This mechanism may also be operative in GPBP calcification.

The mechanism by which AP activity is promoted in GPBP mineralization is not yet understood. AP-active sites are activated by metal ions²⁸, such as Zn^{2+} and Mg^{2+} . The Al^{3+} , Fe^{3+} and Ga^{3+} ions probably can compete with Zn^{2+} and Mg^{2+} at the enzyme's metallo-binding sites, and thereby inhibit the phosphohydrolase activity. The Al^{3+} , Fe^{3+} and Ga^{3+} ions (10^{-1} M) in the GPBP tissues were able to inhibit AP activity, although less so with Ga^{3+} than the others. However, despite this level of enzyme inhibition by the $Ga(NO_3)_3$ pre-incubation, this pretreatment was not effective in inhibiting calcification. These results further suggest that, although AP activity may be associated with the onset and progression of GPBP mineralization, it may not be causal or essential for the pathogenesis.

Furthermore, earlier work has shown that either Al^{3+} or Fe^{3+} salts act by binding to devitalized GPBP cells, and to membrane loci that are rich in phosphorus, thereby preventing the crystallization of hydroxyapatite at these sites¹⁵. It may be argued that Fe^{3+} or Al^{3+} lowered AP levels in our studies by a critical increment, thereby reducing GPBP calcification. However, Al^{3+} , Fe^{3+} and other metal ions can inhibit mineralization by other mechanisms. Al^{3+} ions can adsorb on to hydroxyapatite crystals and severely inhibit both hydroxyapatite formation and dissolution *in vitro*^{29, 30}. Many metals can interfere with hydroxyapatite crystal growth *in vitro* by either competing with Ca^{2+} for sites in the crystal lattice or by adsorbing to the crystal surface³¹. Al^{3+} and Zn^{2+} are found to inhibit MV mineralization³². Similarly, in the GPBP subdermal rat implants, the lowest molecular weight metal ions ($Al < Fe < Ga$, according to atomic weight) showed the strongest inhibitory effects on GPBP calcium accumulation. In fact, the greater the molecular weight of the metal atom of interest, the lower degree of calcification inhibition noted. Al^{3+} , the smallest ion in relation to Fe^{3+} and Ga^{3+} was the most effective in calcification inhibition; Ga^{3+} cation which is the largest metal ion (used in this study) was unable to prevent GPBP calcification. Furthermore, the higher phosphorus content of the Ga^{3+} 21 d explants suggests that high levels of non-apatitic phospho-mineral (perhaps Ga-phosphates) may be predisposed to form after Ga^{3+} pretreatment. Therefore, these metallic ion pretreatments may be associated with unusual phospho-mineral complexes involving, perhaps, phospholipids or metallo-phosphates other than hydroxyapatite. However, the present experimental design does not permit a conclusive resolution of these issues.

APD pre-incubation inhibited GPBP calcification, but was associated with AP activity higher than controls. APD in clinical use has been associated with adverse effects related to cytotoxicity, as well as inflammatory gastrointestinal and dermatologic side effects^{33, 34}. The morphological results in the present study demonstrated an exuberant inflammatory response to the APD implants, and thus the higher level of AP may reflect enzyme activity from inflammatory cells or cells damaged due to toxic processes, rather than calcification-related events.

It would have been helpful in this regard to have quantitated the extent of inflammatory cell infiltration. However, this was beyond the scope of the present studies. Furthermore, earlier results from our group have shown that APD pre-incubation of GPBP was associated with unstable covalent binding of APD to GPBP. Thus APD is continuously leaching out of the tissue, and may thereby cause regional toxicity near the GPBP implant, which could lead to increased AP activity related to inflammation, but probably spatially dissociated and unrelated to calcification.

Conversely, NaEHDP pre-incubation, which did not inhibit calcification, resulted in a slightly lower AP activity in the 72 h explants compared to the controls. However, the net decline in AP activity for the NaEHDP explant groups was comparable to that of calcified controls (Figure 1). Furthermore, previous work from our group has demonstrated that NaEHDP rapidly diffuses out of GPBP tissue following pre-incubation, unlike Fe^{3+} , Al^{3+} and APD, which remain more closely associated with GPBP tissue³⁵. Thus, no persistent anticalcification effects occurred in the NaEHDP group, and the decline in AP activity was comparable to controls, as might be expected. In addition, elevated phosphorus levels were noted in the calcified 21 d NaEHDP explants, in which a Ca/P molar ratio was noted comparable to that of the Ga explants.

CONCLUSIONS

The peak levels of AP in rat subdermal GPBP implants were noted at 72 h in both controls and specimens pretreated with either calcification inhibitors, or related compounds which did not inhibit calcification. Thus, peak GPBP AP activity was present at 72 h, regardless of pretreatment conditions.

All inhibitors of calcification (except APD) were associated with lower 72 h AP levels than controls, including pretreatments ineffective for calcification inhibition, such as Ga^{3+} and EHDP. Thus reduced AP at 72 h is not related to inhibition of calcification.

Therefore, the results of the present experiments support the view that peak AP (72 h) is not necessarily associated with the ultimate progression GPBP calcification.

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