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Malachite green and phthalocyanine-silver reactions reveal acidic phospholipid involvement in calcification of porcine aortic valves in rat subdermal model

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Summary. Subdermal implant models are helpful in the study of calcification "in vivo" and for testing anticalcific treatments. After implantation of porcine aortic valve leaflets in rat subcutis, we previously found that glutaraldehyde-Cuprolinic blue reactions (GA-CB) at low pH induce favourable tissue unmasking from mineral deposits, and visualize peculiar, electrondense layers that outline the calcifying cells and matrix vesicle-like structures. The layer-forming material seemed to consist of acidic phospholipids because of its anionic nature and differential susceptibility to chemical/enzymatic extractivity. In the present investigation, pre-embedding glutaraldehyde-Malachite green (GA-MG) reactions and subsequent osmium post-fixation were compared with pre-embedding GA-CB reactions, combined with post-embedding von Kossa silver staining (GA-CB-S), to assess whether the layer-forming material is actually composed of acidic phospholipids and exhibits calcium-binding properties. After lowering standard pH, GA-MG reactions also caused sample demineralization and the appearance of pericellular osmium-MG-reactive layers comparable to CB-reactive ones. Moreover, GA-CB-S reactions showed that major silver precipitation was superimposed to the CB-reactive layers, whereas minor metal extra-precipitation occurred at three distinct, additional sites. These results demonstrate that a unique process of cell degeneration occurs in this calcification model, in which acidic phospholipids accumulate at cell surface, replacing cell membrane and acting as major apatite nucleator. However, the overall observations are consistent with the hypothesis that certain phases are common to the various types of normal and/or abnormal calcification.

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Introduction

In both native and prosthetic heart valves, the major sites for mineral nucleation are the cells and/or cell-derived matrix vesicle-like bodies (Kim and Huang, 1971; Kim, 1976; Ferrans et al., 1980; Schoen et al., 1985, 1986), the latter being known to act as hydroxyapatite nucleators in physiological calcification. Accordingly, membrane phospholipids, alone or in calcium-acidic-phospholipid-phosphate complexes, have been reported to induce apatite precipitation in cartilage (Cotmore et al., 1971; Vogel and Boyan-Salyers, 1976; Wuthier and Gore, 1977; Wu et al., 1993; Kirsch et al., 1994), bone (Shapiro, 1970; Boskey and Posner, 1976; Boyan and Boskey, 1984), and enamel (Shapiro et al., 1966; Odutuga and Prout, 1974), as well as in various soft tissues that have undergone pathological or experimental mineralization (Boskey et al., 1988), including aortic valves (Jorge-Herrero et al., 1991, 1994) and atherosclerotic aortas (Dmitrovsky and Boskey, 1985).

This is in line with the accepted concept that calcification depends on cascades of distinct molecular interactions that are partially similar to each other, when different tissues and/or conditions are compared. For example, macromolecules involved in physiological calcification, such as alkaline phosphatase, osteopontin, carboxyglutamic-acid-containing-proteins, phospholipids and proteoglycans have also been found in mineralizing vascular tissues (Luo et al., 1997; Niederhoffer et al., 1997; Proudfoot et al., 1998; Dhore et al., 2001, Engelse et al., 2001), and, specifically, in native or bioprosthetic heart valves (Kim and Huang, 1971; Kim, 1976; Jorge-Herrero et al., 1991; Levy et al., 1980, 1991; Fishbein et al., 1982; Maranto and Schoen,

1988; Shen et al., 1997).

Reactions with mixtures of Cuprolinic blue (CB) and glutaraldehyde (GA) at low salt-critical-electrolyte concentrations and acidic pH (Scott and Dorling, 1965; Scott, 1980) have recently proven appropriate for the study of mineralization occurring in aortic valves subsequent to subdermal, xenogenic implantation because they induce (i) tissue unmasking from calcium and (ii) salt-concentration-dependent appearance of peculiar, electrondense layers outlining calcifying cells and matrix vesicle-like structures (Ortolani et al., 2002a,b). Because of the cationic nature of this copper phthalocyanine, it was concluded that the procedure allows anionic molecules to be retained and visualized. In addition, resistance to digestion with hyaluronidase and susceptibility to extraction by chloroform/methanol suggested that these CB-reactive layers are formed by acidic phospholipids accumulating at cell edges and replacing cell membrane phospholipids.

To confirm this assumption, and assess whether the substance forming these pericellular layers is also involved in cell mineralization, it was necessary to compare more specific reactions aimed at phospholipid localization with others aimed at highlighting calcium-binding sites.

Malachite green (MG) is a water soluble, weakly basic diaminotriphenylmethane dye, which enables acidic lipids to be retained during fixation because of the presumed ionic attraction between the two MG tertiary amines and lipid anionic groups. Fixation with mixtures of GA and MG (GA-MG) and post-fixation with osmium tetroxide give rise to the formation of electrondense GA-MG-OsO_4 complexes. When associated with pre-embedding sample decalcification, this method permitted localization of acidic phospholipids in hard tissues such as dentin, epiphyseal cartilage and bone (Golberg and Escaig, 1987; Nefussi et al., 1992; Bonucci and Silvestrini, 1994; Silvestrini et al., 1996).

The localization of calcium-binding sites is still widely assessed using the one hundred-year-old von Kossa method (von Kossa, 1901). This reaction depends on the substitution of phosphate- or carbonate-bound calcium by silver ions and their subsequent reduction to metallic silver by reducing agents. The specificity of this reaction is still debated (Danscher, 1983; Kassem et al., 1992; Rungby et al., 1993) and there are rare reports of von Kossa reactions performed on resin semithin sections and subsequent assessment of silver localization on ultrathin sections (Danscher, 1983).

In the present investigation, a modified GA-MG incubation method was used and compared with pre-embedding GA-CB reactions, followed by post-embedding silver staining (GA-CB-S), to assess whether the peculiar material which accumulates around calcifying cells and matrix vesicles is actually formed by calcium-binding, acidic phospholipids. In fact, (i) modified GA-MG reactions induced simultaneous demineralization and visualization of MG-reactive, pericellular layers closely comparable with CB-reactive ones, and (ii) co-localization of GA-MG and GA-CB-S reactions was apparent, supporting the idea that these pericellular layers may represent calcium-acidic-phospholipid-phosphate-like complexes.

Materials and methods

Aortic valve calcification was induced using the rat subdermal model (Schoen et al., 1986). Five porcine aortic roots were fixed by incubation in degassed 0.625% (w/v) glutaraldehyde in 10 mM borate buffer, pH 7.4, containing 0.9% (w/v) NaCl and with continuous stirring of the suspending solution under a nitrogen atmosphere. After 6 h, the aortic roots were re-suspended in fresh solution, treated in the same conditions for a further 18h, and then stored in 0.2% (w/v) glutaraldehyde for 2 days. Two aortic valve leaflets were then excised from each aortic root and implanted subdermally into abdominal pouches of five 3-week-old male Sprague-Dawley rats. After subcutaneous implantation for 6 weeks and subsequent retrieval, each sample was washed for 1 min once in 0.9% (w/v) NaCl and twice in de-ionized water, freeze-dried and re-hydrated with 10 mM borate buffer, pH 7.4, containing 0.9% (w/v) NaCl .

Samples were excised from the re-hydrated leaflets, suitably reduced, and subdivided into 5 lots, of which lots 1 and 2 served as routine controls for light and electron microscopy, respectively. The samples of lots 1 and 2 were fixed with 2.5% (w/v) glutaraldehyde plus 2.5% (w/v) formaldehyde in 0.1M phosphate buffer, pH 7.2, at room temperature (22-24 °C) overnight. The samples of lot 3 were immersed in 0.067 mol/L cacodylate buffer solution, pH 6.8, containing 3% glutaraldehyde and 0.1% Malachite green (Analyticals, Carlo Erba), at room temperature for 4 days. The samples of lot 4 were processed as for lot 3, but with pH adjusted to 4.8. The samples of lot 5 were immersed in 25 mM sodium acetate buffer, pH 4.8, containing 0.05% Cuprolinic Blue (Electron Microscopy Science), 0.05M $MgCl_2$ and 2.5% glutaraldehyde, at room temperature for 4 days.

The samples of lot 1 were then dehydrated in graded ethanols and embedded in paraffin for histological examination.

The samples of lots 2 to 5 were then post-fixed with 2% OsO_4 dissolved in 0.1M phosphate buffer, pH 7.2, dehydrated in graded ethanols and embedded in Araldite/Epon.

Semithin sections were conventionally stained with Toluidine blue. Several semithin sections of samples from lot 5 were subjected to the von Kossa reaction, i.e. they were mounted on glass slides, covered with a drop of 1% silver nitrate aqueous solution and placed on an 80 ºC warm plate that was exposed to sunlight for 15 min. After washing in distilled water and drying, the sections were reduced with 5% sodium thiosulfate in aqueous solution for 5 min at 80 ºC. As a control,

histological sections also underwent von Kossa reactions using the above processing, with the only variation that the reduction phase was performed at room temperature. Some of the reacted sections were then counterstained with hematoxylin and eosin.

Ultrathin sections were also achieved from semithin sections of lot 5, after the von Kossa-reaction and re-embedding. In detail, conic Beem capsules (Agar Scientific) which had been previously cut at the top were glued onto slides over single mounted sections and then filled with epon-araldite fluid. After resin-polymerization, the re-embedded sections were detached from the slides after -80 ºC freezing and subjected to ultramicrotomy.

All thin sections were mounted on formvar-coated copper grids, Slot 2x1, and contrasted with uranyl acetate and lead citrate. Observations and photographic records were made with a Philips CM12 transmission electron microscope.

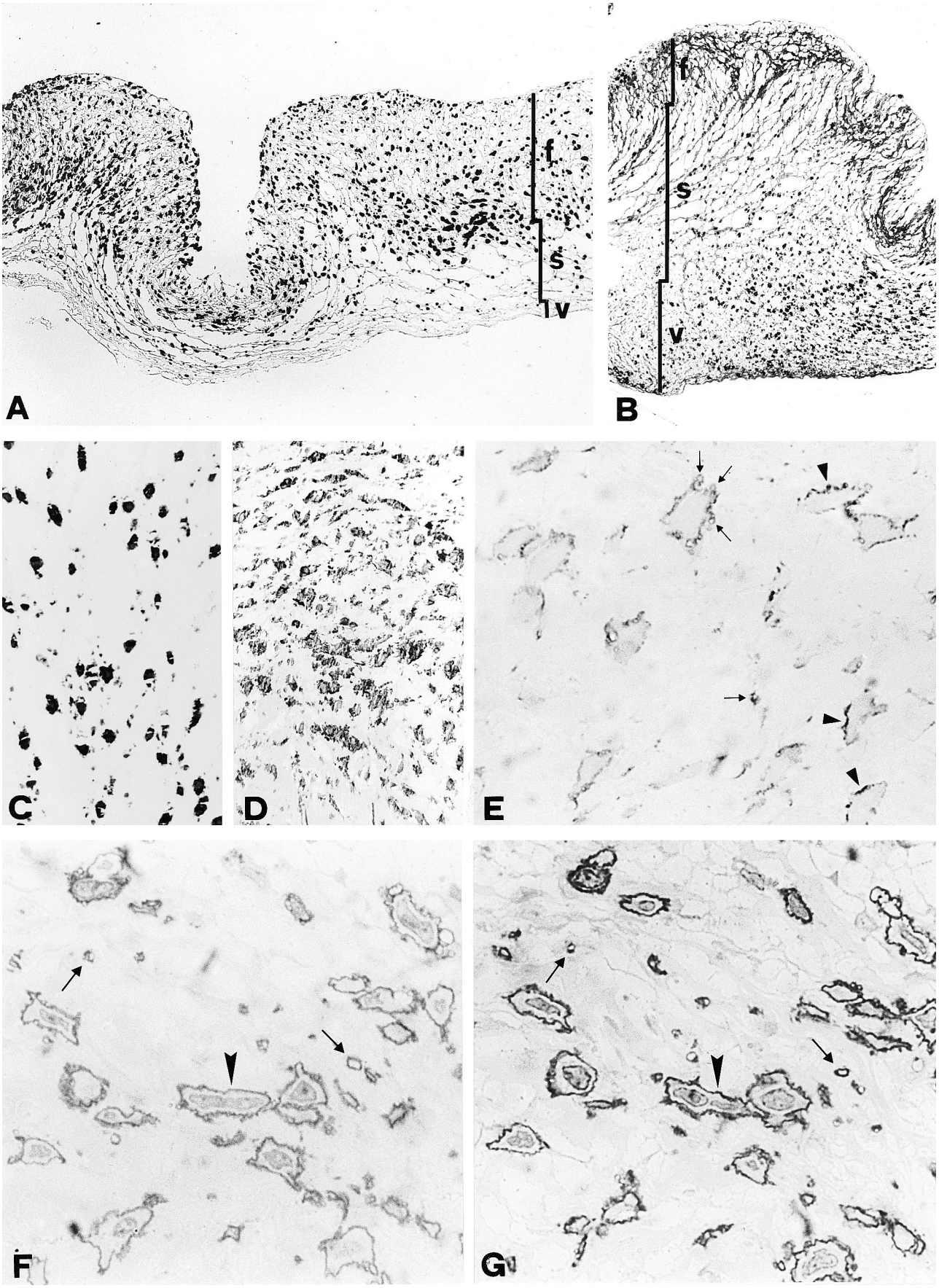
Results

The von Kossa reaction on histological sections of unreacted samples, revealed prominent metallic silver precipitation on the cells lying in all the valve leaflet layers, i.e. "tunica fibrosa", at facing Valsalva's "sinus", "tunica spongiosa", lying at an intermediate position, and "tunica ventricularis", facing the left ventricle chamber (Fig. 1A). More advanced calcification was observed to involve the extracellular matrix at topical foci, mainly along leaflet free margins and commissures (not shown). An identical pattern of silver precipitation appeared on resin semithin sections of unreacted samples (Fig. 1B, C). Smaller heterogeneous metal deposits, resembling calcium-covered cell-derived bodies, were also observed throughout the extracellular matrix.

When standard GA-MG reactions were applied, i.e. using pH 6.8, only weak decalcification was observed. The cells were thus not stainable with toluidine blue on conventional semithin sections (not shown) or they were covered by silver precipitates after the von Kossa reaction (Fig. 1D), albeit to a lesser extent than for reacted sections of unreacted samples (Fig.1C). Remarkable cell masking by mineral was also apparent on thin sections (Fig. 2A). In contrast, tissue unmasking from mineral was observed after adjusting pH to 4.8. On semithin sections not counterstained with toluidine blue, the cells were outlined by barely perceivable graish borders, which also characterized matrix-vesicle-like bodies, whereas nuclei were unstained (not shown). When the von Kossa reaction was superimposed, silver precipitation gave rise to thick, discontinuous profiles around most cells and roundish matrix-vesicle-like bodies which showed variable diameters (Fig. 1E). Thin sections showed sharp cell nuclei which contained well preserved eu- and heterochromatin, and lacked nuclear membrane (Fig. 2B-E). Altered cytoplasms contained some cytomembrane-deprived organules or organule debris, bundles of randomly distributed cytoskeletal filaments and a number of empty cytoplasmic vesicles, merging at cell surface to form larger vesicles. These structures were empty or contained some mineral deposits and protruded outside the cells, giving them peculiar humped features. These silhouettes were strongly enhanced because the cell bodies were also encircled by 40 to 70 nm-thick, electrondense layers. On more detailed observation, these layers apppeared to be formed by an intermediate, continuous sub-layer spanning about one third of the entire thickness, while the sub-layers at the outer and inner aspects were characterized by the distribution of granular/spicular material which seemed to be formed by mineral deposits (Fig. 2C). Varying quantities of similar structures were also observed within the superficial vesicles. In places, there was blebbing of the pericellular layers, giving rise to matrix-vesicle-like bodies (Fig. 2C, E). Topically, the pericellular material was observed to spread toward the neighboring extracellular matrix, enveloping either elastic fibers subjected to initial calcification (Fig. 2C, D), or electronlucent collagen fibrils, still exhibiting faint D-band patterns (Fig. 2E). Often, this centrifugal spreading involved additional amorphous material, which embedded juxtacellular collagen and elastin, connecting them with pericellular layer outer aspect.

On semithin sections of GA-CB-reacted samples, mineral unmasked cells showed evident nuclei and sharp bluish outlines, which also characterized matrix-vesicle-like bodies (Fig. 1F). On thin sections, the pericellular profiles showed a thickness comprised within a range of 40-70 nm, as for GA-MG reacted samples, but exhibiting a uniform electrondensity, instead of a trilaminar feature (not shown). After superimposing the post-embedding von Kossa reaction onto the pre-embedding GA-CB reaction, comparable features appeared, with stain changing from bluish to brown-black (Fig. 1G). On thin sections, electrondense, CB-reactive layers appeared around cells (Figs. 3A, 3C) and matrix vesicles (Fig. 3B), onto which silver precipitates with diameters ranging from 30 to 50 nm were

Fig. 1. A. Silver precipitation on cells in aortic valve leaflet layers "fibrosa" (f), "spongiosa" (s), and "ventricularis" (v) (top, intermediate and bottom vertical bars) after the von Kossa reaction on a a paraffin section of an unreacted sample. x 28. **B.** Similar silver precipitation pattern on a toluidine blue-counterstained resin semithin section of an unreacted sample. x 28. **C.** As in B, with counterstaining omitted x 120. **D.** Weaker silver precipitation on a von Kossa-reacted semithin section of a GA-MG-reacted sample at pH 6.8. x 120. **E.** Discontinuous silver precipitation limited to the edges of cells (arrowheads) and cell debris (arrows) on a von Kossa-reacted semithin section of a GA-MG-reacted sample at pH 4.8. x 450. **F.** Thick outlines (actually bluish-stained) shown by cells (arrowhead) and cell-debris (arrows) in semithin section of a GA-CB-reacted sample. x 450. **G.** The same pattern (actually brown-stained) on para-serial section with respect to F, also after the von Kossa reaction . x 450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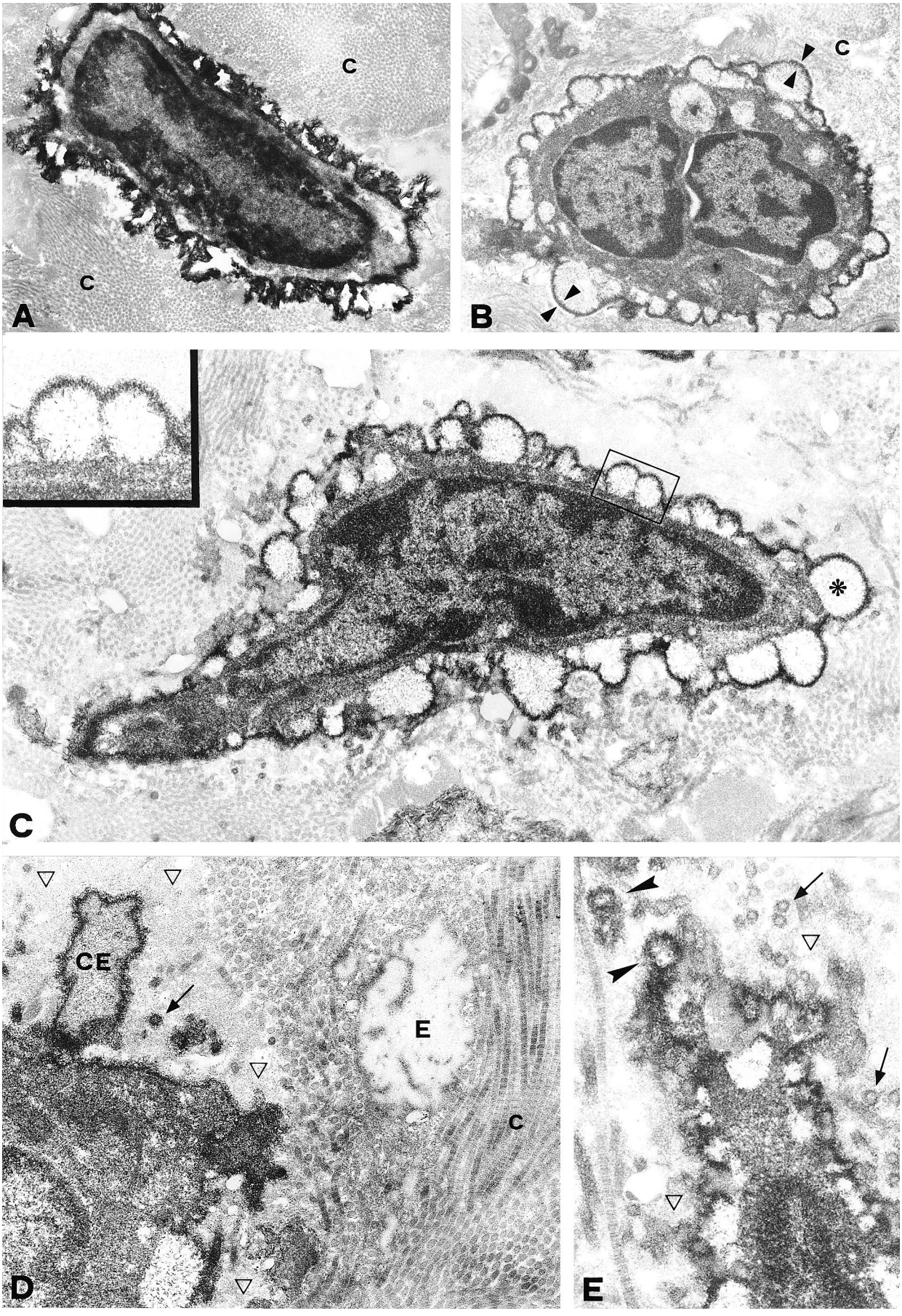
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Fig. 2. A. Weak cell decalcification shown by a thin section of a GA-MG-reacted sample at pH 6.8. x 9,000); C**:** unmineralized collagen fibrils. **B.** Mineral unmasked cell after GA-MG reaction at pH 4.8; cell humped feature is enhanced by a thick electrondense pericellular layer (opposite arrowheads). x 9,000) **C.** As in B; note a superficial vesicle undergoing pre-detaching narrowing (asterisk) and a pericellular layer three-partite structure (inset). x 16,500, inset x 42,000. **D.** As in B; pericellular electrondense material envelops an juxtacellular, calcifying elastic fiber (CE) and collagen fibrils (arrow), which differ from elastic fiber (E) and collagen fibrils (C) in unmineralized extracellular matrix; additional amorphous material (open triangles) is present x 24,500. **E** As in B; budding of small matrix-vesicle-like bodies (arrowheads) and presence of collagen fibrils enveloped by more electrondense sheaths (arrows) and/or embedded by additional, amorphous material (open triangles). x 39,000

superimposed. Further metal granules were selectively distributed in three distinct compartments. Specifically: (i) granules, ranging from 10 to 30 nm, were located on nuclear heterochromatin (Fig. 3A, C); (ii) a second class of granules with similar diameters was distributed on juxtacellular amorphous material topically spreading from the pericellular layers (Fig. 3C); and (iii) punctate silver granules with diameters of about 10 nm were scattered along the collagen fibrils, according to D-periodical patterns (Fig. 3D).

The observation of occasional rod-like CB-particles connecting adjacent collagen fibrils with orthogonal, D-periodical array revealed that few lateral glycosaminoglycan chains were still present in implanted valve extracellular matrix. However, these CB-proteoglycan-complexes appeared less electrondense than those in native valve leaflets and silver precipitate superimposition was never found on them (not shown).

Discussion

This investigation concerned soft tissue calcification in a model, heterologous subdermal implantation, which is believed to be a reliable test for studying calcification "in vivo" and hence the most predictive method for testing anticalcification treatments (Schoen et al., 1985, 1986; Bernacca et al., 1992; Mako and Vesely, 1997). One major aim was to gain evidence that cell surface modifications occurring during calcification include the accumulation of acidic phospholipids and that these lipid molecules are involved in the pericellular precipitation of mineral.

On performing GA-MG reaction, the standard acidic environment was too weak to induce sufficient tissue demineralization. Conversely, and consistently with the results previously achieved for GA-CB reactions (Ortolani et al., 2002a,b), comparable patterns were visualized after pH adjustment from 6.8 to 4.8, in that cells and matrix vesicles were unmasked from the mineral and appeared to be outlined by typical electrondense layers. Subtle differences were observable between CB-reactive and MG-reactive layers because the former showed uniform electrondensity whereas the latter were subdivided into three sub-layers. This might depend on the fact that some of the constituent anionic molecules would be more prone to extraction after interaction with MG than with CB, possibly because of lower MG specificity. Alternatively, these pericellular layers might contain additional non-lipidic anions, for example lipid-associated, calcium-binding proteins, which would be reactive for CB but not MG. It must also be considered that the granular/spicular structures forming outer and inner sub-layers of MG-reactive layers may represent either residual calcium deposits, i.e. the result of incomplete demineralization,or crystal ghosts, i.e. the result of retaining of pre-existing mineral-associated organic phase which might have acted as a core for mineral precipitation, thus mimicking their shape once demineralized (Bonucci et al., 1988).

Taking into account that GA-MG reactions and subsequent osmium post-fixation cause phospholipid retention and staining, including acidic ones (Golberg and Escaig, 1987; Nefussi et al., 1992; Bonucci and Silvestrini, 1994; Silvestrini et al., 1996), and co-localize with GA-CB reactions, which are selective for polyanions (Scott and Dorling, 1965; Scott, 1980), it must be deduced that acidic phospholipids are a major component of these electrondense layers. In addition, the co-localization of GA-MG and GA-CB-S reactions strongly suggests that acidic phospholipids act as calcium-binding molecules, or are closely associated with calcium-binding proteins. Taken together, these data suggest that the electrondense layers surrounding cells and matrix vesicles may represent an analogue of the calcium-acidic-phospholipid complexes.

The prominent lipid clustering observed at cell surfaces is an unusual process which accounts for a unique modality of cell degradation. However, the observed formation of cellular blebs and matrix vesicles fits with the observation reported for "in vitro" glutaraldehyde-treated porcine aortic fibroblasts, which also correlated with calcium and inorganic phosphate ion influx (Kim et al., 1999). Increases in cytosolic calcium and phosphate ion concentrations have also been claimed to occur in blebs and matrix vesicles formed by apoptotic and/or oncotic cells, as reviewed for physiological calcification and in a number of dystrophic calcinoses (Kim, 1995). This supports the idea that all types of tissue mineralization follow the same pathway, including calcium ion influx into the cells and subsequent formation of cellular degradation products. The observed changes could be ascribed to a similar mechanism where, although it is a distinctive step quantitatively, the massive accumulation of acidic phospholipids at cell surfaces is comparable to the process of phosphatidylserine exposure on the outer aspect of plasma membrane occurring in apoptotic cells (Martin et al., 1995).

Concerning the additional silver precipitation, it was not random but involved three distinct compartments

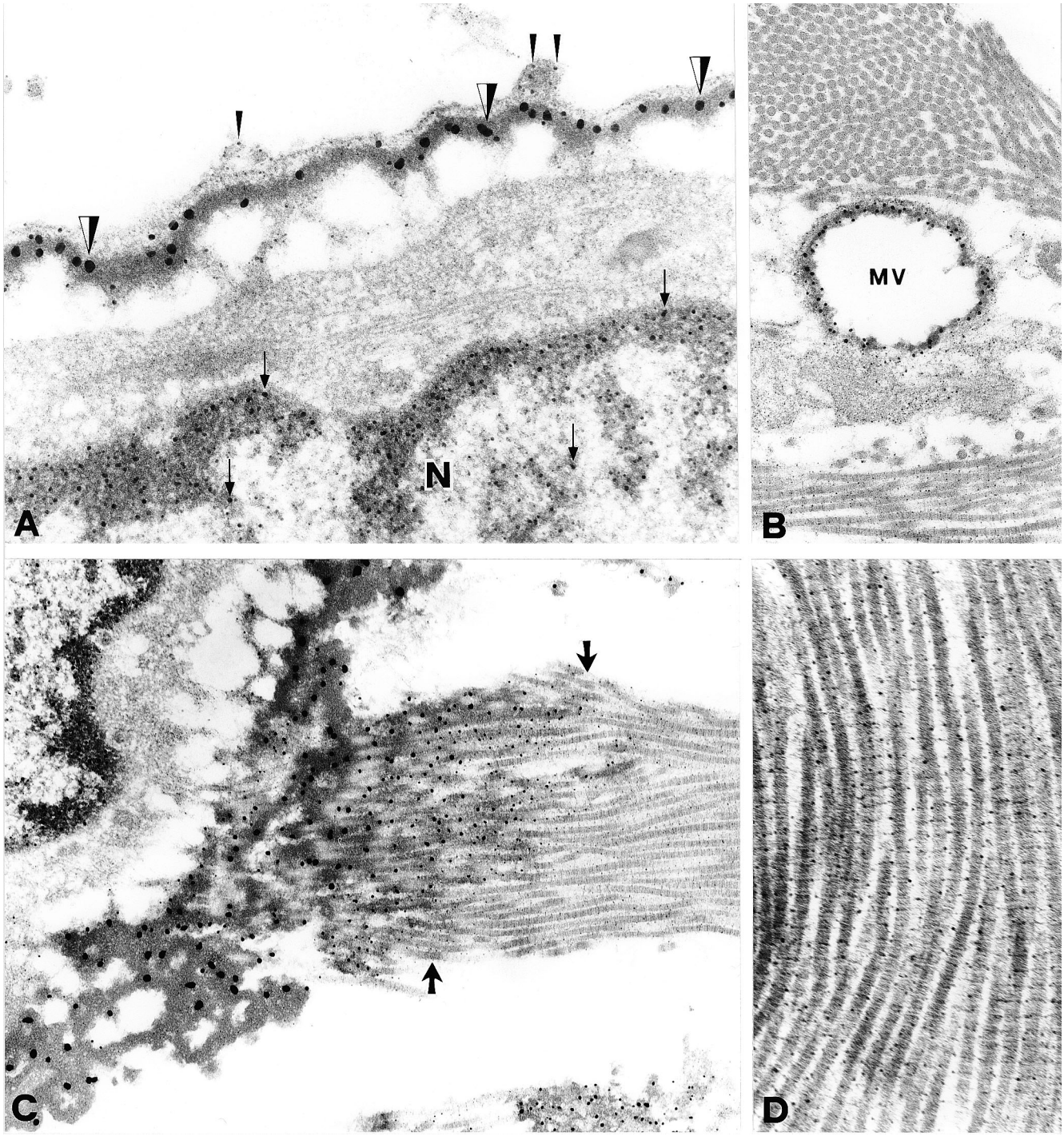

Fig. 3. Thin sections obtained from re-embedded von Kossa-reacted semithin sections of GA-CB-reacted samples. **A.** Distribution of the greatest silver precipitates (black and white arrowheads) along CB-stained pericellular layer, and two minor classes of metal granules interspersed within layer-associated amorphous material (black arrowheads) or nuclear heterochromatin (arrows). N: nucleus. x 36,000. **B.** Matrix-vesicle-like body (MV) outlined by a CB-reactive layer showing superimposed silver granules. x 31,000. **C.** As in A, plus abrupt reduction of silver granules, which become punctate where collagen fibrils are not yet embedded by amorphous material (arrows). x 28,500. **D.** D-periodical distribution of punctate silver granules along collagen fibrils (arrows). x 62,000

and was completely absent elsewhere. This means that calcium-binding sites may exist other than those present within the elecrondense pericellular layers. The different sizes exhibited by these precipitates can be explained by considering that distinct anionic molecules will bind differential amounts of silver ions, so acting as more or less effective donors of these cations for the reducing agent (e.g. sodium thiosulfate) and causing different rates in metal precipitation. Mean diameters of about 10 nm have been reported for silver granules, with 50 nm maxima (Hayat, 1993). Since these maxima were here observed for CB-reactive layers only, these structures will contain the most effective silver donors.

Silver precipitation on heterochromatin is not surprising when we consider the possible additional involvement of anionic nucleotidic chains or DNA-associated acidic proteins, as reported (Hayat, 1993), or even the presence of nuclear acidic phospholipids (Maraldi et al., 1992). Actually, the first sign of early calcification in aortic valves is intracellular calcium deposition, which has been reported to include cell nuclei (Schoen et al., 1985; Girardot et al., 1995; Ortolani et al., 2002a).

A second site of extra silver precipitation was the amorphous material lying outside the CB-reactive layers and embedding both elastic fibers and collagen fibrils. Since it is unaffected by chloroform/methanol extraction, this material was thought to be formed by proteins involved in calcification spreading from the cells to the extracellular matrix (Ortolani et al., 2002a). The present observation that it is also silver-reactive supports that assumption. In addition, this suggests that a further analogy may exist with calcification in cartilage, where more composite "nucleational cores" have been reported which are formed by calcium-acidic-phospholipid complexes and other interacting components, including annexins, alkaline phosphatase and collagens (Mc Lean et al., 1987; Genge et al., 1988; Wu et al., 1993, 1996; Kirsch et al., 1994). Actually, in both fresh and glutaraldehyde-treated bovine pericardial bioprosthetic valves, alkaline phosphatase activity was found to be associated with cell-derived phospholipid membranes, suggesting an involvement of residual cell structures in the onset of calcium deposition, according to a mechanism comparable with that occurring for cartilage matrix vesicles (Maranto and Schoen, 1988; Levy et al., 1991).

On the other hand, rat plasma proteins insudating the implanted valve leaflets cannot be ignored as an additional, exogenous source of calcium-binding macromolecules, for example carboxyglutamic-acid-containing-proteins, which have claimed to be involved in calcification of both subdermally implanted aortic valves (Fishbein et al., 1982) and pathological ones (Levy et al., 1980), as well as in that of aorta wall atherosclerotic plaques (Proudfoot et al., 1998; Dhore et al., 2001).

Regarding the third site of extra silver precipitation, the punctate precipitates observed along the collagen fibrils in D-periodical patterns, should correlate with either intrinsic fibril D-periodical structure or D-periodically associated molecules. Unfortunately, after subdermal implantation intrafibril molecular array is altered, rendering it impossible to distinguish intraperiod D-bands. It has been suggested that collagen fibril mineralization initiates with calcium deposition on phosphate groups previously bound to ε-amino groups of lysines and hydroxylysines (Bernacca et al., 1992). Although these sites are normally masked by collagen-interacting proteoglycans, most glycocaminoglycans are lost during subdermal implantation (Ortolani et al., 2002b). The fact that this metal distribution is reminiscent of anti-decorine immunogold labelling (Pringle and Dodd, 1990) could raise the question as to whether these proteoglycan cores may still be present and interact with silver ions or, alternatively, coiled dermatan-sulphate lateral chains do. However, no silver superimposition was found either on the sporadic CB-proteoglycan-complexes observed here to cross-bridge collagen fibrils, or on those in native aortic valves (Ortolani, unpublished data). Thus it seems more likely that collagen plays a role as a minor apatite nucleator, which is consistent with reported observations of sporadical apatite crystals on the collagen fibril surface in calcifying valves and the subsequent assumption that two distinct calcification mechanisms may exist, involving cells and cell-derived structures, which will act as major determinants, and collagen fibrils, which will act as minor ones (Schoen et al., 1986; Ortolani et al., 2002a).

In addition to the above considerations, the two modified GA-MG and GA-CB-S methods here employed revealed the existence of a unique cell alteration process characterized by prominent layering of calcium-binding acidic phospholipids around cells and matrix vesicles. Since this material has been found to prime mineral precipitation in subdermally calcifying aortic valves, these methods appear helpful for detecting valve propensity to calcification. This will permit greater sensitivity in testing the effectiveness of novel anticalcific treatments in bioprosthetic valve preparations.

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References

Bernacca G.M., Mackay T.G. and Wheatley D.J. (1992). In vitro calcification of bioprosthetic heart valves: report of a novel method and review of the biochemical factors involved. J. Heart Valve Dis. 1, 115-130.

Bonucci E. and Silvestrini G. (1994). Morphological investigation of epiphyseal cartilage after glutaraldehyde-malachite green fixation.

Bone 15, 153-160.

Bonucci E., Silvestrini G. and Di Grezia R. (1988). The ultrastructure of the organic phase associated with the inorganic substance in calcified tissues. Clin. Orthopaed. 233, 243-261.

Boskey A.L., Bullough P.G., Vigorita V. and Di Carlo E. (1988). Calcium-acidic phospholipid-phosphate complexes in human hydroxyapatite-containing pathologic deposits. Am. J. Pathol. 133, 22-29.

Boskey A.L. and Posner A.S. (1976). Extraction of a Calcium-phospholipid-phosphate complex from bone. Calcif. Tissue Res. 19, 273-283.

Boyan B.D. and Boskey A.L. (1984). Co-isolation of proteolipids and calcium-phospholipid-phosphate complex. Calcif. Tissue Int. 36, 214-218.

Cotmore J.M., Nichols G. Jr. and Wuthier R.E. (1971). Phospholipid-calcium phosphate complex: enhanced calcium migration in the presence of phosphate. Science 172, 1339-1341.

Dmitrovsky E. and Boskey A.L. (1985). Calcium-acidic phospholipid-phosphate complexes in human atherosclerotic aortas. Calcif. Tissue Int. 37, 121-125.

Danscher G. (1983). A silver method for counterstaining plastic embedded tissue. Stain Technol. 58, 365-372.

Dhore C.R., Cleutjens J.P., Lutgens E., Cleutjens K.B., Geusens P.P., Kitslaar P.J., Tordoir J.H., Spron H.M., Vermeer C. and Daemen M.J. (2001). Differential expression of bone matrix regulatory proteins in human atherosclerotic plaques. Arterioscler. Thromb. Vasc. Biol. 21, 1998-2003.

Engelse M.A., Neele J.M., Bronckers A.L., Pannekoek H. and de Vies C.J. (2001). Vascular calcification: expression pattern of the osteoblas-specific gene core binding factor alpha-1 and the protective factor matrix gla protein in human atherogenesis. Cardiovasc. Res. 52, 281-289.

Ferrans V.J., Boyce S.W., Billingham M.E., Jones M., Ishihara T. and Roberts W.C. (1980). Calcific deposits in porcine bioprostheses: Structure and pathogenesis. Am. J. Cardiol. 46, 721-734.

Fishbein M., Levy R.J., Nashef A., Ferrans V.J., Dearden L.C., Goodman A.P. and Carpentier A. (1982). Calcification of cardiac valve bioprostheses: Histologic, ultrastructural, and biochemical studies in a subcutaneous implantation model system. J. Thorac. Cardiovasc. Surg. 83, 602-609.

Genge B.R., Sauer G.R., Wu L.N., Mc Lean F.M. and Wuthier R.E. (1988). Correlation between loss of alkaline phosphatase activity and accumulation of calcium during matrix vesicle-mediated mineralization. J. Biol. Chem. 263, 18513-18519.

Girardot M.N., Torrianni M., Dillehay D., Girardot J.M. (1995). Role of glutaraldehyde in calcification of porcine heart valves: Comparing cusp and wall. J. Biomed. Mat. Res. 29, 793-801.

Goldberg M. and Escaig F. (1987). Rapid-freezing and malachite-green-acrolein-osmium tetroxyde freeze-substitution fixation improve visualization of extracellular lipids in rat incisor predentin and dentin. J. Histochem. Cytochem. 35, 427-433.

Hayat M.A. (1993). Stain and cytochemical methods. Plenum Press. New York and London. pp 318-336.

Jorge-Herrero E., Fernandez P., Gutierrez M. and Casillo-Olivares J.L. (1991). Study of the calcification of bovine pericardium. Analysis of the implication of lipids and proteoglycans. Biomaterials 12, 683-689.

Jorge-Herrero E., Fernandez P., De La Torre N., Escudero C., Garcia-Paez J.M., Bujan J. and Casillo-Olivares J.L. (1994). Inhibition of the calcification of porcine valve tissue by selective lipid removal. Biomaterials 15, 815-820.

Kassem M., Rungby J., Mosekilde L. and Erksen E.F. (1992). Ultrastructure of human osteoblasts and associated matrix in culture. Acta Pathol. Microbiol. Scand. (APMIS) 100, 490-497.

Kim K.M. (1976). Calcification of matrix vesicles in human aortic valve and aortic media. Fed. Proc. 35, 156-162.

Kim K.M. (1995). Apoptosis and calcification. Scanning Microsc. 9, 1137-1175.

Kim K.M. and Huang S. (1971). Ultrastructural study of calcification of human aortic valve. Lab. Invest. 25, 357-366.

Kim K.M., Herrera G.A. and Battarbee H.D. (1999). Role of glutaraldehyde in calcification of porcine aortic valve fibroblasts. Am. J. Pathol. 154, 843-852.

Kirsch T., Ishikawa Y., Mwale F. and Wuthier R.E. (1994). Roles of the nucleational core complex and collagens (types II and X) in calcification of growth plate cartilage matrix vesicles. J. Biol. Chem. 269, 20103-20109.

Levy R.J., Schoen F.J., Flowers W.B. and Staelin S.T. (1991). Initiation of mineralization in bioprosthetic heart valves studies of alkaline phosphatase activity and its inhibition by AlCl3 or FeCl3 preincubation. J. Biomed. Mater. Res. 25, 905-935.

Levy R.J., Zenker J.A. and Lian J.B. (1980). Vitamin K-dependent calcium-binding proteins in aortic valve calcification. J. Clin. Invest. 65, 563-566.

Luo G., Ducy P., Mc Kee M.D., Pinero G.J., Loyer E., Behringer R.R. and Karsenty G. (1997). Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA proteins. Nature 386, 78-81.

Mako W.J. and Vesely I. (1997). In vivo and in vitro models of calcification in porcine aortic valve cusps. J. Heart Valve Dis. 6, 316-323.

Maraldi N.M., Zini N., Squarzoni S., Del Coco R., Sabatelli P. and Manzoli F.A. (1992). Intranuclear localization of phospholipids by ultrastructural cytochemistry. J. Histochem. Cytochem. 40, 1383-1392.

Maranto A.R. and Schoen F.J. (1988). Alkaline phosphatase activity of glutaraldehyde-treated bovine pericardium used in bioprosthetic cardiac valves. Circ. Res. 63, 844-848.

Martin S.J., Reutelingsperger C.P.M., Mc Gahon A.J., Rader J.A., van Schie R.C.A.A., La Face D.M. and Green D.R. (1995). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition and overexpression of Bcl-2 and Abl. J. Exp. Med. 182, 1545-1556.

Mc Lean F.M., Keller P.J., Genge B.R., Walters S.A. and Wuthier R.E. (1987). Disposition of preformed mineral in matrix vesicles. Internal localization and association with alkaline phosphatase. J. Biol. Chem. 262, 10481-10488.

Nefussi J.R., Septier D., Sautier J.M., Forest N. and Goldberg M. (1992). Localization of malachite green positive lipids in the matrix of bone nodule formed in vitro. Calcif. Tiss. Int. 50, 273-282.

Niederhoffer N., Bobryshev Y.V., Lartaud-Idjouadiene I., Giummelly P. and Atkinson J. (1997). Aortic calcification produced by vitamin D3 plus nicotine. J. Vasc. Res. 34, 386-398.

Odutuga A.A. and Prout R.E.S. (1974). Lipid analysis of human enamel and dentine. Arch. Oral. Biol. 19, 729-731.

Ortolani F., Petrelli L., Tubaro F., Spina M. and Marchini M. (2002a). Novel ultrastructural features as revealed by phthalocyanine reactions indicate cell priming for calcification in subdermally implanted aortic valves. Connect. Tissue Res. 43, 44-55.

Ortolani F., Tubaro F., Petrelli L., Gandaglia A., Spina M. and Marchini

M. (2002b). Copper retention, calcium release and ultrastructural evidence indicate specific Cuprolinic Blue uptake and peculiar modifications in mineralizing aortic valves. Histochem. J. 34, 41-50.

Pringle G.A. and Dodd C.M. (1990). Immunoelectron microscopic localization of the core protein of decorin near the d and e bands of tendon collagen fibrils by use of monoclonal antibodies. J. Histochem. Cytochem. 38, 1405-1411.

Proudfoot D., Skepper J.N., Shanahan C.M. and Weissberg P.L. (1998). Calcification of human vascular cells in vitro is correlated with high levels of matrix GLA protein and low levels of osteopontin expression. Arteroscler. Thromb. Vasc. Biol. 18, 379-388.

Rungby J., Kassem M., Eriksen E.F. and Danscher G. (1993). The von Kossa reaction for calcium deposits: silver lactate staining increases sensitivity and reduces background. Histochem. J. .25, 446-451.

Schoen F.J., Levy J.R., Nelson A.C., Bernhard W.F., Nashef A. and Hawley M. (1985). Onset and progression of experimental bioprosthetic heart valve calcification. Lab. Invest. 52, 523-532.

Schoen F.J., Tsao J.W. and Levy R.J. (1986). Calcification of bovine pericardium used in cardiac valve bioprosthesed. Implications for the mechanism of bioprosthetic tissue mineralization. Am. J. Pathol. 123, 134-145.

Shapiro I.M. (1970). The phospholipids of mineralized tissues. I. Mammalian compact bone. Calcif. Tissue Int. 5, 21-29.

Shapiro I.M., Wuthier R.E. and Irving J.T. (1966). A study of the phospholipids of bovine dental tissues. Enamel matrix and dentin. Arch. Oral. Biol. 11, 501-512.

Shen M., Marie P., Farge D., Carpentier S., De Pollak C., Hott M., Chen L., Martinet B. and Carpentier A. (1997). Osteopontin is associated with bioprosthetic heart valve calcification in humans. C.R. Acad. Sci. III 320, 49-57.

Scott J.E. (1980). Localization of proteoglycans in tendon by electron microscopy. Biochem. J. 187, 887-891.

Scott J.E. and Dorling J. (1965). Differential staining of acid glycosaminoglycans (mucopolysaccharides) by Alcian Blue in salt solutions. Histochemie 5, 221-233.

Silvestrini G., Zini N., Sabatelli P., Mocetti P., Maraldi N.M. and Bonucci E. (1996). Combined use of malachite green fixation and PLA2-gold complex technique to localize phospholipids in areas of early calcification of rat epiphyseal cartilage and bone. Bone 18,559-565.

Vogel J.J. and Boyan-Salyers B.D. (1976). Acid lipids associated with the local mechanism of calcification. A review. Clin. Orthop. 118, 230-241.

von Kossa J. (1901). Über die im organismus künstlicherzeugbaren verkalkungen. Beitr. Path. Anat. 29, 163-202.

Wu L.N., Genge B.R., Sauer G.R. and Wuthier R.E. (1996). Characterization and reconstitution of the nucleation complex for mineral formation by growth plate cartilage matrix vesicles. Connect. Tiss. Res. 35, 309-315.

Wu L.N.Y., Yoshimori T., Genge B.R., Sauer G.R., Kirsch T., Ishikawa Y. and Wuthier R.E. (1993). Characterization of the nucleational core complex resposible for mineral induction by growth plate cartilage matrix vesicles. J. Biol. Chem. 268, 25084-25094.

Wuthier R.E. and Gore S.T. (1977). Partition of inorganic ions and phospholipids in isolated cell, membrane and matrix vesicle fractions: evidence for Ca-Pi-acidic phospholipid complexes. Calcif. Tissue Res. 24, 163-171.

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