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Pro-calcific responses by aortic valve interstitial cells in a novel *in vitro* model simulating dystrophic calcification.

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Summary

Etiopathogenetic mechanisms in calcific aortic valve stenosis are still poorly understood despite this being the third major cause of heart disease in western world. In prior *in vitro* cultures simulating metastatic calcification, pro-calcific effects on aortic valve interstitial cells (AVICs) resulted by adding bacterial endotoxin lipopolysaccharide (LPS) at high inorganic phosphate (Pi) levels. Here we accomplished improved *in vitro* models simulating either metastatic (Pi = 2.6 mM) or dystrophic calcification (Pi = 1.3 mM), in which LPS-stimulated bovine AVICs underwent extra-stimulation with macrophage-cytokine-containing media derived from parallel cultures of allogeneic monocyte/macrophages in turn stimulated with LPS. In dystrophic calcification-like cultures, lower calcium amount was spectrometrically assessed with parallel reduced alkaline phosphatase activity with respect to metastatic calcification-like cultures, with an about three-fold slower progression of mineralization. Hydroxyapatite crystal precipitation was ultrastructurally found to correlate with AVIC degeneration processes culminating with the formation of phthalocyanin-positive lipidic layers (PPLs) at the surface of cells and cell-derived matrix-vesicle-like bodies, acting as calcium nucleators according to a pattern mirroring those we had previously found in *in vivo* conditions. In conclusion, an *in vitro* model has been developed enabling reliable simulations of the effects exerted on AVICs by putatively pro- or anti-calcific agents.

Key words

Ectopic calcification; aortic valve stenosis; valve interstitial cell cultures; phthalocyanin reaction

Introduction

Calcific aortic valve stenosis (CAVS), albeit being the third major cause of adult heart disease in the western world and the leading indication for surgical valve replacement, is still far from being understood concerning the underlying etiopathogenetic mechanisms (Akat et al., 2009).

Consistently with metastatic calcification (MC), pro-calcific effects were found to be exerted by both bacterial endotoxin lipopolysaccharide (LPS) and high inorganic phosphate (Pi) levels, on osteoblast-like smooth muscle cells isolated from aortic wall (Tintut et al., 2002) as well as on subpopulations of bovine aortic valve interstitial

cells (AVICs) (Rattazzi et al., 2008), as indicated by increasing in calcium precipitation rate and alkaline phosphatase (ALP) activity.

Ultrastructurally, in both experimental *in vivo* calcification model (Ortolani et al., 2002a, b; 2003; 2007) and actual renal-disease-independent CAVS (Ortolani et al., in preparation), we previously found that aortic valve mineralization results from a peculiar degenerative process affecting AVICs, which is characterized by an intracellular acidic lipid release from degenerating organelles culminating in thick lipidic layer formation at the surface of cells and cell-derived matrix-vesicle-like bodies, named "phthalocyanin-positive-layers" (PPLs), acting as major apatite nucleators.

Since PPL appearance represents a mineralization-related degenerative feature shared by both the above-mentioned *in vivo* conditions, here we accomplished improved *in vitro* models reproducing the same degenerative pattern, so simulating dystrophic calcification (DC), as it occurs in CAVS; *in vitro* high calcium conditions mimicking MC were also explored.

Materials and Methods

Cell culture. Primary AVIC cultures were obtained from de-endothelized bovine aortic valve leaflets subjected to digestion with collagenase I (125 U/ml; Sigma), elastase (8 U/ml; Sigma), and protease inhibitor (0.375 mg/ml; Sigma) for 30 min at 37°C. AVICs were cultured with DMEM (Sigma) plus 10% FBS (Gibco), 1% L-glutamine, and 1% penicillin/streptomycin and were expanded up to the sixth passage. Macrophage-cytokine-containing medium was obtained collecting lympho/monocytes by Ficoll density gradient centrifugation of bovine peripheral blood. After lymphocyte removal, monocyte/macrophages were stimulated with LPS (100 ng/ml; Sigma) for 1h and cultured in DMEM complete medium for additional 12h to allow macrophage degranulation. At confluence, AVICs were incubated with 2 ml of DMEM complete medium alone (control) or with 2 ml of DMEM complete medium added with 0.5 ml of macrophage-cytokine-containing medium, LPS (100 ng/ml) plus either 2.6 mM Pi for 9 days or 1.3 mM Pi for 9, 15, 21 and 28 days.

Ca quantification: scraped AVICs and their respective medium were collected into distinct TFM vessels and treated with 1 ml of 65% nitric acid (suprapur; Merck) and 0.5 ml of 37% hydrogen peroxide. After suitable irradiation using the High Performance Microwave Digestion Unit MLS 1200 mega (Milestone), samples were diluted in 50 ml of ultrapure water (Elgastat). Spectrometric ICP-MS measurements were performed using a Spectromass 2000 Type MSDIA10B (Spectro).

ALP activity: after medium removal, AVICs were treated with 500 μl of 1% Triton-X 100 in 0.9% sodium chloride for 1 h at +4°C. After centrifugation, 30 μl of supernatant were added to 1 ml of 10 mM p-nitrophenyl phosphate (Chema Diagnostica). Measurements were performed using a Cary 50 BIO spectrophotometer (Varian) reading the absorbance at 405 nm. Statistical significance of both Ca and ALP estimations was defined using Student's t test ($P < 0.05$).

Ultrastructural analysis: cultured AVICs were fixed with 0.05% Cuprolinic blue (E.M. Sciences) in 25 mM sodium acetate buffer containing 0.05 M $MgCl_2$ and 2.5% glutaraldehyde, pH 4.8, over-night at room temperature under agitation; post-fixed with 2% OsO_4; dehydrated in graded ethanols; and embedded in Epon 812 resin.

Thin sections contrasted with uranyl acetate and lead citrate were observed and recorded using a Philips CM/12 STEM electron microscope.

Results

After 9-day-long incubation, addition of (i) LPS, (ii) macrophage-cytokine-containing medium derived from cultures of allogenic monocyte/macrophages in turn stimulated with LPS, and (iii) Pi caused an increase in both calcium (Ca) amount and ALP activity, compared to control cultures (figs. 1A,B). In addition, Ca amount was more than three-fold higher, while an about 30% increasing was found for ALP activity, for MC-like cultures as compared with DC-like ones.

On assessing the time-course for DC-like cultures, a linear time-dependent increase in Ca amount was detected. A linear increase was also found for ALP activity, but with the maximum at 21 days and a dramatic drop (> 50%) at 28 days. Both Ca amount and ALP activity measured for DC-like cultures at the 28th day were comparable with those for MC-like cultures at the 9th day.

In parallel cultures in which stimulation with conditioned medium derived from macrophage cultures was omitted, a similar trend was found but with very lower values for equal incubation times (not shown).

On ultrastructural examination after pre-embedding reaction with phthalocyanin Cuprolinic blue (CB), the cells derived from DC-like cultures showed sequential time-dependent degenerative features consisting in (i) appearance of CB-reactive dense bodies (fig. 2A), (ii) disgregation of organelles and release/spreading of CB-reactive material throughout the cytoplasm (fig. 2B), and (iii) centrifugal clustering of this material (fig. 2C), that culminated with the formation of blebbing PPL-layers (fig. 2D), often superimposed by hydroxyapatite needle-like crystals (fig. 2D, inset). All the above features, including PPLs, were already present in MC-like cultured cells at the 9th incubation day (not shown).

Discussion

The present results confirm that Pi and LPS promote calcification-related processes in cultures of vascular-tissue-derived cells. In the DC-like system, the progression of the calcific process occurred with an about three-fold slower progression with respect to MC-like system, but was destined to reach comparable calcific outcomes as shown by the similar Ca amounts found at 28 days and 9 days, respectively, as well as by the effective hydroxyapatite precipitation after the longest incubation time.

The lower Ca amounts measured when omitting macrophage-culture-derived medium indicate a role for macrophage-derived cytokines as indirect factors triggering soft tissue mineralization, consistently with the occurrence of inflammatory processes as etiopathological co-factor.

Also in this *in vitro* system, early hydroxyapatite nucleator was found to be represented by PPL-like material resulting from a peculiar AVIC degenerative process that mirrors those in both subdermal model and actual CAVS (figs. 2E,F). Such a shared cell degeneration pattern suggests valve calcification takes place according to

a process other than ossification, questioning a concept that is more and more gaining ground (Akat et al., 2009). Rather, ossification should be considered as an epiphenomenon, possibly involving AVIC subpopulations.

In conclusion, an *in vitro* model has been developed reproducing cell degeneration features as it occurs in *in vivo* conditions, so enabling significant simulations of the effects exerted on AVICs by putatively pro- or anti-calcific agents in future investigations.

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Figures

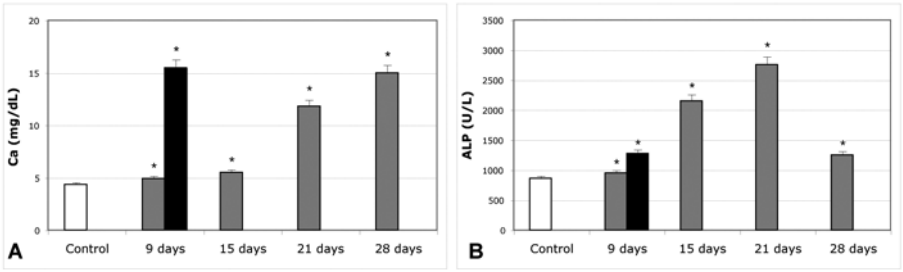

Fig. 1 - A: spectrometric estimation of Ca amounts for control cultures (white column), metastatic calcification-like cultures (black column), and dystrophic calcification-like cultures (grey columns) at different incubation times. B: spectrophotometric estimation of ALP activity as in (A). (*P < 0.05).

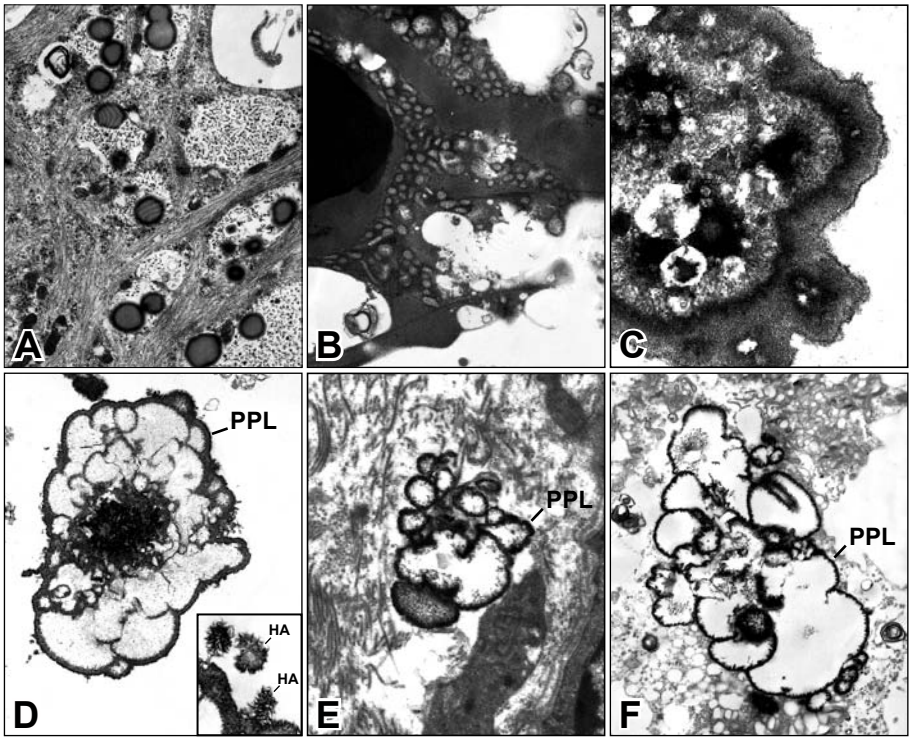

Fig. 2 - A-D: *In vitro* calcification. Cuprolinic blue reactivity and hydroxyapatite crystals in cells or cell debris after 9 d (A), 15 d (B), 21 d (C), and 28 d (D) DC-like cultures. E, F: *In vivo* calcification, for comparison. Cuprolinic blue reactivity in aortic valve interstitial cells in the subdermal model (E) and in actual calcific aortic valve stenosis (F). PPL: phthalocyanin-positive-layer; HA: hydroxyapatite crystals. Magnifications: 7,000x (A,B); 15,000x (C); 6,000x (D-F); 11,000x (D, inset).