Calcification of Human Valve Interstitial Cells is Dependent on Alkaline Phosphatase Activity

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Background and aim of the study: **The calcification of heart valves is associated with valve degeneration and failure, but the mechanisms involved are poorly understood. The presence of lamellar bone has been demonstrated in calcified aortic valves. Since osseous calcification is closely associated with alkaline phosphatase (ALP) activity, it was hypothesized that ALP activity might be implicated in the calcification of isolated leaflet interstitial cells (ICs).**

Methods: **Human valve leaflet ICs were isolated from transplant-explanted hearts at the time of transplantation (n = 12).**

Results: **Isolated leaflet ICs expressed the fibroblast-specific antigen (100% of cells) and smooth muscle (SM) α-actin (70-80% of cells), but osteoblastic markers were not expressed. Cultured ICs did not calcify spontaneously, however when the growth medium was supplemented with β-glycerophosphate (an organic phosphate) it induced the formation of calcified nodules that expressed osteonectin and ALP, but not SM α-actin. β-Glycerophosphate-induced calcification of ICs showed a time-dependent effect on the calcium content of treated cells over a 14-day period. ALP activity was considerably increased in β-glycerophosphate-treated ICs, and this correlated with the calcium content (r = 0.5:p = 0.01). Levamisol (an ALP inhibitor) inhibited the β-glycerophosphate-induced calcification process, as well as the expression of osteoblastic differentiation markers.**

Conclusion: **Isolated and cultured leaflet ICs did not calcify spontaneously, though organic phosphate induced the formation of calcified nodules that expressed osteoblastic markers. The calcification of isolated ICs was seen to be dependent on ALP activity.**

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The calcification of heart valves is a common occurrence in different valve diseases, with calcified aortic disease being by far the most prevalent heart valve pathology in the industrialized world (1). Despite the high prevalence of heart valve calcification, little is known of the cellular and molecular mechanisms involved in the calcification process. The ectopic calcification of vascular structures is viewed as an active cellular process (2). Studies have shown that mature lamellar bone is often encountered in calcified heart valves, suggesting a phenomenon analogous to the ossification process (3,4). The calcification of bone has been shown to be closely associated with the expression of alkaline phosphatase (ALP) (5). Thus, in the present study, it was hypothesized that organic phosphate-induced calcification of isolated human interstitial cells (ICs) would be dependent on ALP activity.

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Materials and methods

Leaflet tissues

Leaflet tissues were obtained from 12 aortic valves retrieved from explanted hearts during transplantation. The leaflets were harvested and transported to the laboratory in ice-cold saline solution for immediate cell isolation. Approval to conduct the study was granted by the Laval Hospital Ethical Committee.

Leaflet IC isolation and immunohistological studies on cultured cells

The tissue samples were minced and digested with collagenase (Gibco, Burlington, ON, USA) at 37ºC for 30 min. The cells were washed three times in phosphate-buffered saline (PBS), resuspended, and cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, Oakville ON, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1 mM pyruvate (Gibco). The tissue culture was placed in an humidified incubator maintained at 37ºC with 5% CO_2. The growth medium was changed every

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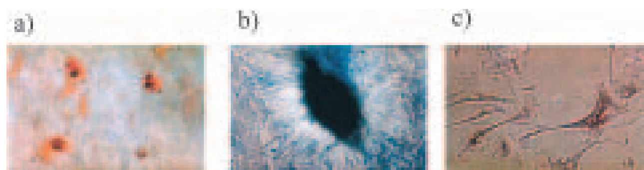

Figure 1: a) Alizarin red staining; (b) alkaline phosphatase activity; and (c) expression of osteonectin in cells treated with the calcifying medium.

two days. Immunohistological studies were conducted in 12-well plates. At near-confluence, cells were fixed with paraformaldehyde (4%), and the wells incubated with the following mouse monoclonal antibodies: SM α-actin 1:300 (Sigma), fibroblast-specific antigen 1:20 (Oncogene, Boston, MA, USA), Von Willebrand 1:50 (Neo Markers, Fremont, CA, USA), and osteonectin 1:500 (US Biological, Swampscoti, MA, USA). Cells were then incubated with a biotin-conjugated anti-mouse immunoglobulin antibody (Vector Laboratories, Burlingame, CA, USA), followed by horseradish peroxidase-conjugated streptavidin (Vector Laboratories). Biotin-conjugated antibodies were obtained from Jackson Immuno Research (Mississauga, ON, USA). The binding of these antibodies was detected by incubation with horseradish peroxidase-conjugated streptavidin and VIP substrate (Vector Laboratories).

In-vitro calcification of leaflet ICs

Leaflet ICs were cultured in DMEM containing 10% FBS, 2 mM L-glutamine and 1 mM pyruvate (Gibco). At confluence, the cells were suspended in the growth medium, and seeded in 12-well plates (2×10^4 cells/well). In some wells, β-glycerophosphate (2 mM) (calcification medium) with or without levamisol (10^{-4} M; an inhibitor of ALP) was added to the growth medium. The medium was replaced with fresh medium every two days. Leaflet ICs were cultured with or without the calcification medium for 14 days, and cytochemical studies were conducted at day 0 (before being cultured in calcification medium), 7 and 14.

Cytochemical staining of ALP and mineral deposition in cultured ICs

ALP activity was detected using the Vector Blue alkaline substrate kit (Vector Laboratories). Briefly, the growth medium was replaced with 500 μl Vector Blue phosphatase substrate and incubated for 60 min, according to the manufacturer's instructions. Mineral deposition was assessed by alizarin red staining (Sigma).

Quantification of calcium deposition

Cells were retrieved at days 0, 7 and 14, and decalcified with 0.6 M HCl for 24 h. The calcium content of the HCl supernatants was determined using the *o*-cresolphthalein complexone method (Calcium Kit; Sigma). After decalcification, the cells were washed in PBS, solubilized with 0.1 N NaOH/0.1% SDS, and the protein content was determined using a BioRad assay kit. Cell calcium levels were normalized to the cell protein content.

ALP activity in cultured ICs

Cells were washed in PBS, transferred into NP-40 (0.2%) and $MgCl_2$ (1 mM), and then sonicated. The ALP activity of sonicated cells was determined using an ALP diagnostic kit (Sigma).

Statistical analysis

Results were expressed as mean ± SEM. Data were analyzed for statistical significance by using an unpaired Student's *t*-test or a one-way ANOVA. A p-value <0.05 was considered to be statistically significant. Linear regression was used to detect any

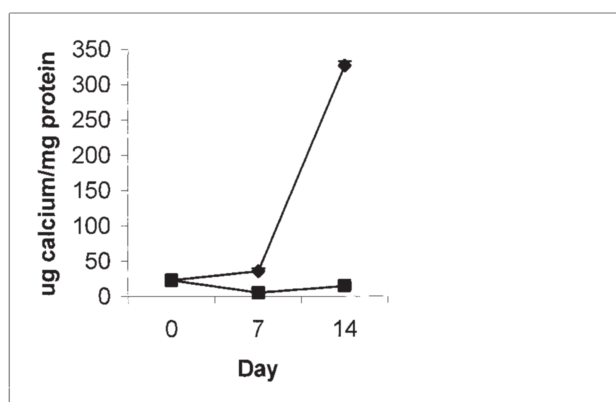

Figure 2: Calcium content at days 0, 7 and 14 in control (■) and β-glycerophosphate-treated (♦) interstitial cells (p = 0.01).

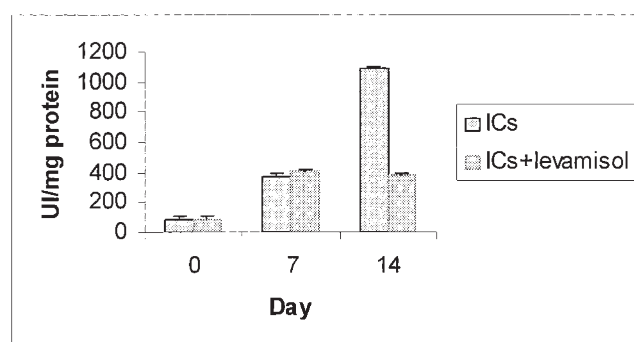

Figure 3: Alkaline phosphatase (ALP) activity in cultured interstitial cells (ICs) treated with β-glycerophosphate at days 0, 7 and 14, with and without levamisol. Significant inhibition of ALP activity was apparent at 14 days (p = 0.01).

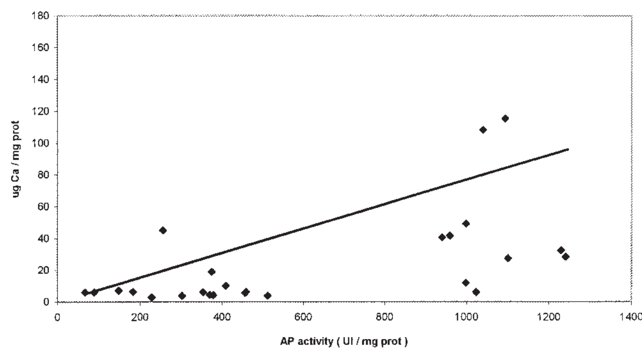

Figure 4: Linear regression analysis between alkaline phosphatase activity (ALP) and the calcium content of isolated interstitial cells (r = 0.5; p = 0.01).

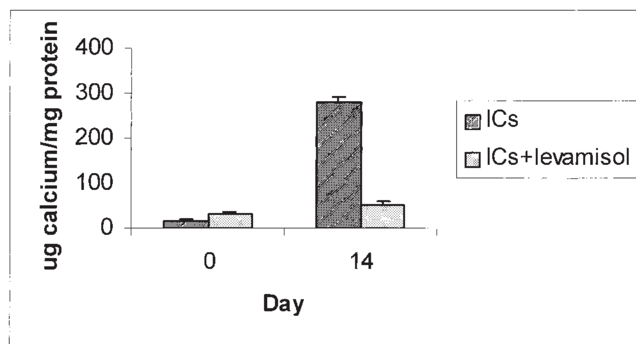

Figure 5: Calcium content of interstitial cells (ICs) at days 0 and 14, with and without levamisol addition. Levamisol treatment led to a significant reduction in calcium content of β-glycerophosphate-treated cells (p = 0.01).

correlation between ALP activity and calcium content in isolated ICs.

Results

Phenotypic characterization of the isolated and non-treated cells showed that ICs were a mixed population composed of fibroblasts and myofibroblasts. All cells expressed the fibroblast-specific antigen, but only 70-80% of cells expressed SM α-actin. However, there was no expression of osteonectin and ALP activity in those non-treated cells. Isolated ICs did not calcify spontaneously. However, when the growth medium was supplemented with β-glycerophosphate, leaflet ICs formed calcified nodules (at 14 days), as shown by alizarin red staining (Fig. 1a). Calcified nodules expressed ALP activity and osteonectin (Fig. 1b, 1c), whereas expression of SM α-actin was not observed in calcified nodules. β-Glycerophosphate induced a time-dependent effect on calcium content over 14 days, whereas control non-treated ICs maintained a basal level of calcium content over the study period (Fig. 2).

After 14 days of treatment with the calcification medium, ALP activity increased significantly (Fig. 3), and this correlated with the calcium content (r = 0.5; p = 0.01) (Fig. 4). ALP activity was significantly decreased by levamisol (Fig. 3). Levamisol was also seen to inhibit the calcification of ICs (Fig. 5) as well as osteonectin expression (data not shown).

Discussion

Heart valve leaflets are composed of ICs, which regulates remodeling of the extracellular matrix. Leaflet extracellular remodeling and repair is essential to ensure valve longevity and function. ICs have been shown to be composed of a mixed population of fibroblasts and myofibroblasts (6). This was is in accordance with the present results, which confirmed the presence of both populations in the isolated cells. Fibroblasts are implicated in remodeling of the extracellular matrix; consequently, they are highly active in the synthesis of collagen, elastin, glycosaminoglycans and cytokines (7,8). Recently, the calcification of cardiovascular structures was shown to be an active cellular process conducted under the influence of pro-calcifying mediators (9,10). Therefore, ICs might be implicated in the calcification of native valves, and also perhaps in valve substitutes such as valve homografts.

Other groups have previously cloned a population of bovine vascular smooth muscle cells (VSMCs) that spontaneously calcify, and which have been named calcifying vascular cells (CVCs) (11,12). In cell culture, these CVCs express osteoblastic proteins and lose their initial phenotype, which suggests that the calcification of VSMCs is similar to the process of bone ossification. However, the results of the present studies showed that ICs do not calcify spontaneously, even under long-term culture conditions. Nevertheless, using the correct conditions and the pro-calcifying agent, β-glycerophosphate, ICs were seen to form calcified nodules after 14 days of exposure. Thus, β-glycerophosphate-induced calcification indicates that ICs are prone to calcification, at least when cultured in the correct conditions and using a calcifying medium.

It was found that ICs containing calcified nodules changed their initial phenotype towards that of an osteoblast. In addition, the calcified nodules expressed ALP activity and osteonectin, a bone glycoprotein that binds tightly to hydroxyapatite crystals and collagen type I, and is widely expressed in different calcified tissues, including atherosclerotic plaques (13). Moreover, the expression of osteonectin is essential for the maintenance of bone mass (14). Thus, it appears that osteonectin may act as a positive regulator of ossification.

ALP is an ubiquitous enzyme which is mostly

expressed in bone, liver and kidney (15), and is associated with the calcification process. Indeed, ALP has been shown to be essential for the calcification process to occur in bone and extra-osseous tissues, including VSMCs (16,17). Indeed, within these isolated ICs a positive correlation was found with ALP activity and the calcification process. Furthermore, in cultured ICs, the specific inhibition of ALP activity with levamisol caused a blockade of enzymatic activity and the ossification process. Thus, ALP activity appears to correlate with calcification and also with the expression of bone protein such as osteonectin. In VSMCs, β-glycerophosphate has been shown to induce the expression of ALP and subsequent calcification (18). ALP converts organic phosphate to inorganic phosphate (Pi), which in turn is utilized by the cells to produce calcified extracellular matrix (19). Pi has been shown to enter the VSMCs via a sodium-dependent phosphate co-transporter (15). Thus, an elevated intracellular Pi level stimulates the cells to undergo a phenotypic switch towards mineralizing cells (20). The clinical significance of organic phosphate in the calcification of heart valves remains the subject of much speculation. However, it is well documented that end-stage renal disease is associated with early valve calcification (21), and high phosphate concentrations - close to those used in the present studies (2 mM) - are usually encountered in renal dialysis patients. Elevated serum phosphate levels in these patients might be sufficient to trigger an osteoblast-like response of ICs, leading to calcification of the valve tissue. Furthermore, it has been shown in glutaraldehyde-treated tissue that calcification is initiated by cellular activity and a local increase in Pi levels (22). Cellular injury or death during either cell fixation or cryopreservation is associated with a local increase in Pi concentration that appears to derive from the hydrolysis of high-energy phosphate (23). Nonetheless, currently speculative preservation techniques of biological valves (including valve homografts) might play a role in the phosphate-induced calcification process.

In conclusion, although the mechanisms leading to calcification of heart valves remain largely unknown, accumulating data suggest that it is an active process involving a phenotypic switch of native cells toward bone-forming cells. The exact role of different pro-calcifying factors remains to be elucidated, however. A better knowledge of the mechanisms involved in the cellular and molecular processes leading to the calcification of ICs might, in time, lead to the prevention of heart valve calcification, including that of valve substitutes.

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