Aortic calcified particles modulate valvular endothelial and interstitial cells

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1. Introduction

Calcification is a widespread phenomenon involved in several cardiovascular diseases such as calcific aortic valve disease (CAVD) and atherosclerosis [1]. CAVD is a slowly progressive disorder of dysregulated calcium deposition, very common in the elderly. It is found in 26% of the population over the age of 65 [2,3], 35% between 75 and 84 years of age, and up to 50% of those over 85 [4,5]. CAVD ranges from mild valve stenosis to severe calcification with impaired leaflet motion, termed aortic stenosis.

The pathological pathways involved in the initiation, progression, and end stage of CAVD remain largely unknown. The mechanisms of valve calcification in heart valves appear to be similar but not identical to those responsible for atherosclerosis [6]. This might be, at least in part, due to the specific nature and responses of valve interstitial and endothelial cells to different stimuli. Recent studies suggest that underlying mechanisms of CAVD are initiated at the endothelium, leading to inflammation [7,8]. Activation of inflammatory pathways is believed to promote cardiovascular calcification by the transdifferentiation of quiescent valvular interstitial cells (VICs) into osteoblast-like VICs, forming calcific lesions which accumulate calcium phosphate mineral [7,9,10]. Mineral deposits have been shown in human valves [10,11], and we recently demonstrated that calcium phosphate particles, composed of highly crystalline hydroxyapatite associated with 100% of calcific lesions and between 83% and 100% of noncalcified regions, are present in patients with CAVD [10,12]. These particles were also detected in 80% of noncalcified aortic valve tissue from patients that had calcific lesions in another part of their cardiovascular system. An analysis of particle size showed a trend for increasing particle diameter with increasing disease severity [10]. These particles were also found on 46% of aortic valves in which the cardiovascular system was apparently entirely free from calcific lesions. Calcified particles from valves, coronary arteries, and aortae had the same unique architecture, composition, and crystallinity [10]. Spherical and semispherical deposits together with...
lamellar crystals have also been shown in calcified valves [13]. Immunolabeling of aortic valve tissues showed strong positive labelling for early osteoblastic transcription factors RUNX2 and Sp7 expression in cells near the spherical particles but no osteocalcin. Additionally, RUNX2 and Sp7 expression was observed in apparently healthy tissue with the presence of calcified particles. It is hypothesized that these particles are the first mineralized structure formed and may play a fundamental role in calcific lesion formation and ultimately calcification.

The goal of the present study is to investigate the effects of these calcium phosphate particles on human valvular endothelial cells (VECs) and VICS. The calcium phosphate particles were isolated from human donor aortae, and several concentrations of particles were studied in direct contact with a monolayer of VECs and VICS. Quantification of the particles was assessed by turbidity [14]; scanning electron microscopy (SEM) was used for characterization of the particles; and cellular behavior was studied with cell viability, proliferation, and differentiation. It is hypothesized that these particles will stimulate a shift in phenotype of the cells into an activated and osteogenic phenotype that may progress the calcification process.

1.1. Ethical statement

All human studies have been approved by the Brompton and Harfield trust ethics committee and Oxford Hospital. These studies have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All donors gave their written informed consent prior to their inclusion in the study.

2. Methods and materials

Human healthy aortae and calcified aortae were used for isolation of the particles. We have ascertained that these particles, whether isolated from valves or aortae, are identical in nature and healthy and diseased aortae were more readily available. Nine healthy aortae (mean age, 42.1 years; range, 4 days–65 years; S.D.=21.5; 6 females and 3 males) were obtained from heart valve donors, whose valves were judged unsuitable for clinical use or from explanted hearts of cardiac transplant recipients. These valves were harvested by the Royal Brompton Valve Bank from donors with no history of cardiovascular disease, but were determined unsuitable for clinical use for valve replacement surgery. Reasons for rejection ranged from tears/fenestrations in the valve cusps, donor having traveled to an endemic region, donor having recent body tattoos, signs of minor levels of calcification in the aortic wall or coronary ostia (but not in valves), structural damage during excision, or the date for their use had expired.

The normal aortae were obtained from patients free from cardiovascular and valvular complications based on history and macroscopic and microscopic evaluation. These were unused aortae from healthy heart donors, most of whom died from a cerebral hemorrhage due to head trauma with no underlying diseases. The aortic tissue was taken from the leaflets or coronary ostia. Calcium aortic tissues, n=5, were from heart donors or explanted hearts at the time of cardiac transplantation whose aortae showed visible evidence of calcification (mean age, 66.3 years; range, 54–78 years; S.D.=9.3; 2 females and 3 males). For cell isolation, healthy aortic heart valves, n=7, were used (mean age, 48 years; range, 18–59 years; S.D.=15.0; 2 females and 5 males). These were also obtained from patients free from cardiovascular and valvular complications based on history and macroscopic and microscopic evaluation. These were unused valves mostly due to fenestrations from healthy heart donors, most of whom died from a cerebral hemorrhage due to head trauma with no underlying diseases. The valves and aortae were not matched from donors. Cell culture of VICS [15] and VECs [16] was as previously described. Detailed methods can be found in the supplementary file.

3. Results

3.1. Quantification of calcified particles

Absorbance values for the two time points from all samples showed good reproducibility (Fig. 1A and B). Particles from the healthy donors (n=9) had a significantly lower median absorbance compared to the diseased donors (n=5), P=0.04 (Fig. 1C). To determine effects of calcified particles from both the healthy and diseased donors, an upper absorbance of 1.0 and a lower absorbance of 0.25 (by diluting the particles) were subsequently used as a high and low dose. Higher doses were deemed unsuitable considering the ratio of cells to particles and resulted in cell toxicity and death.

3.2. Scanning electron microscopy showed major diversity within the particles

A lot of variability in shape was seen in each sample (Fig. 1D–F) with different sized spherical and cubic formed particles, with the majority being spherical in form with equal variation between the healthy and diseased donors.

3.3. Chemical composition of calcified particles

The majority of the particles, both spherical and cubic, revealed calcium (Ca), phosphorus (P), and smaller amounts of magnesium (Mg) (Fig. 1G and H). However, in a small amount of particles, a small amount of carbon (C) and sodium (Na) was present (not shown). There were no differences in the elemental composition of the particles for healthy and diseased donors (Fig. 1H).

3.4. Calcified particles increase von Willebrand factor (vWF) secretion in VECs

Treatment with calcified particles for 7 days showed no difference in staining of vWF, CD31, α-SMA, and SM22 [17] (Fig. 2A), and no osteoblast markers were observed in VECs before and after 7-day treatment with the calcified particles (not shown). Only a very small percentage of the cells from one isolate in the positive control expressed α-SMA and did not express α-SMA in the control samples. There was no difference between the healthy and diseased calcified particle treated groups.

After 21 days, a higher number of cells demonstrated increased intensity of vWF and decreased intensity of CD31 staining in the calcified particle treated groups compared to the control (Fig. 2B). Expression of α-SMA was seen in the positive control for one isolate, while no cells were positive in the control. No expression of SM22 was seen for each group. Weakly positive cells for osteopontin and no osteocalcin were found in treated groups, including the positive control.

There was no nodule formation after 3 weeks but some increase in alizarin red and ALP staining; however, osteogenic media failed to induce any significant increase in these markers in VECs. No von Kossa positive staining was detected in any treated group after 21 days (Fig. 3A). There was no difference between the healthy and diseased calcified particle treated groups.

3.5. Calcified particles induce a change in phenotype of VICS

A 7-day treatment was not performed as this was deemed insufficient for osteogenic changes. Vimentin expression decreased in the positive control and treated groups after 21 days. No α-SMA was detected in the control group and positive control. In contrast, 8.4%±1.7% of VECs showed α-SMA-positive cells after treatment with calcified particles (Fig. 4).

Control cells expressed no osteopontin, osteocalcin, or collagen I with baseline expression of collagen III. The positive control showed a very slight increase in expression of collagen I and III. The calcified
particle treated groups showed very weak expression of osteopontin and osteocalcin and moderate expression of collagen I and III (Fig. 4). Alizarin red and ALP staining of VICs (Fig. 3) showed positive staining in over 30%±2.4% of VICs and von Kossa staining showed positive staining in over 10%±1.3% of VICs when treated with the calcified particles compared to being negative for the control and positive control. There was no difference between the healthy and diseased calcified particle treated groups.

3.6. Calcified particles induce myofibroblastic and limited osteoblastic changes

For the calcified particle treated VECs, there was a significant decrease in VE-CAD (P=.04) and in CD31 (P=.009), an increase in vWF (P=.02), no change in α-SMA (P=.82), and no change in osteopontin (P=.35) (Fig. 5). The collagen antibodies did not work on Westerns; CBFA-1 and osteocalcin were not detected in VECs before or after treatment.

For the VICS treated with calcified particles, there were a significant increase in α-SMA (P=.03) and osteopontin (P=.01) and no change in CBFA-1 (P=.93) (Fig. 5). There was no difference between the healthy and diseased calcified particle treated groups for VECs and VICS.

3.7. Cell viability is reduced for high concentrations of particles

Overall, VEC viability remained high after treatment for 7 days (>80%) using the lower volumes of 10 and 20 μl of [0.25] and 10 μl of [1.0]. However, for the highest dose of particles (absorbance reading of 1.0, 20, and 50 μl per 500 μl of media), VECs were significantly less viable compared to the control (93.83%±4.7% vs. 65.38%±24.68%, P=.03) (Fig. 6A and C). There was no difference between the healthy and diseased calcified particle treated groups.

VICS were significantly less viable compared to the control (93.43%±9.0%) when treated with the lower dose of calcified particles (absorbance reading of 0.25 and 10 μl per 500 μl of media (84.57%±11.5%) and 20 μl per 500 μl (79.4%±16.4%), P=.02). For the highest doses of calcified particles (absorbance reading of 1.0 and 20 μl per 500 μl and 50 μl per 500 μl of media), VICS were significantly less viable compared to the control (93.43%±9.0% vs. 79.9%±18.3% and 93.43%±9.0% vs. 54.6%±22.3%, P=.04) (Fig. 6B and D). There was no difference between the healthy and diseased calcified particle treated groups.

The calcified particle treated groups showed a significantly increased number of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive VECs compared to control (P=.005) (Fig. 6E),
Fig. 2. Immunostaining of VECs after 7 days (A) and 21 days (B) of treatment. Control is untreated VECs, and positive control is treated with TGF-β1. VECs are double stained for CD31 (red) and vWF, and α-SMA (red) and SM22. Positive control is VECs cultured with osteogenic media. VECs are double stained for CD31 (red) and vWF, α-SMA (red) and SM22, and osteopontin (red) and osteocalcin (red). Calc Part = calcified particles. Scale bars represent 50 μm, n=3.
Fig. 3. Alizarin red, ALP, and von Kossa staining of VECs (A) and VICs (B) after 21 days of culturing with two different donors of particles (calc part). Positive control is with osteogenic media, and the control for VECs and VICs is untreated. Scale bars represent 100 μm, n=3.
Fig. 4. Immunostaining of VICs after 21 days of culturing with calcified particles. Control is untreated, and positive control is VICs with osteogenic media. Calc Part = calcified particles. Scale bars represent 50 μm; inserts for osteopontin show higher magnification; n=3.
and VICS showed no difference in TUNEL positivity compared to control (Fig. 6F). However, it was noted that there were fewer cells present for the treated groups compared with both controls.

3.8. Proliferative capacity is unchanged by addition of calcified particles

Proliferative capacity for VECs was not changed for the groups with particles compared with the control after 7 and 14 days (Fig. 6G and H, respectively). Proliferative capacity for VICS was unchanged after 7 and 14 days (Fig. 6I and D, respectively); however, the diseased [0.25] group had a higher proliferative capacity compared with the control after 14 days (Supplementary Fig. 1) (P=0.05, n=3). For VECs, there was no difference between the healthy and diseased donor groups.

3.9. Calciﬁed particles are taken up by VECs

As VECs are the first to encounter the calcified particles, we assessed their interaction with the calcified particles. SEM showed some embedding of the calcified particles within cell membranes (Supplementary Fig. 2). However, using focused ion beam and SEM, a range of different-sized calcified particles were detected within VECs (Supplementary Fig. 3). The calcified particles persisted when cells were treated with them and did not degrade. We did not see a change in the size distribution of the calcified particles during the time course of these experiments. This was consistent between VECs and VICS.

4. Discussion

Effects of crystalline calcified particles on human valvular cells have not been previously reported, and we have demonstrated that calcified particles isolated from healthy and diseased aortic donors have the capacity of inducing phenotypic and pathological remodeling of human VECs and VICS as well as decreasing viability and inducing apoptosis in VECs.

Turbidity was able to gauge a measure of the particles, and this was reproducible and consistent over different days and has been used with other calcified particles [14,18]. The heterogeneity in the calcified particles has been linked to the physicochemical parameters of their native growth niche and to the local condition of the extracellular matrix [13].

The isolated calcified particles demonstrated identical size, shape, distribution, and spectra to the in vivo detected calcified particles [10]. Electron diffraction patterns of the in vivo calcified particles were typical of highly crystalline hydroxyapatite, whereas bone consists of a poorly crystalline apatite [10,19,20]. This characterization of the isolated particles validated the isolation process, ensuring the identical calcified particles detected in vivo were isolated. The origin of these calcified particles is the subject of ongoing research, and they could arise due to a number of different cellular mechanisms and from a number of different cells. A variety of bodies are released from cells such as apoptotic bodies, exosomes, matrix vesicles, extracellular vesicles, and microparticles, and there is significant overlap in their size and structure [21,22]. The size range of these calcified particles is considerably larger, ranging from 100 nm up to 2.5 μm in diameter (data from these current samples and from [10]), compared to that of extracellular vesicles released from smooth muscle cells in normal and calcifying media which was shown to be between 30 and 300 nm [23]. This lower range also holds true for exosomes, matrix vesicles, and microparticles [24] which are different entities to the crystalline calcified particles used here. The larger size of these calcified particles may arise due to extracellular nucleation of hydroxyapatite through the deposition of Ca^{2+} and P_{i} in the hole zone regions of collagen fibrils within the matrix [25]. Elevated levels of extracellular calcium have been shown to induce mineralization of vascular-smooth-muscle-cell-derived matrix vesicles [26], and both smooth muscle cells [27] and matrix vesicles [28] have been demonstrated in calcified valves.

Calcified particles significantly increased the expression of vWF in VECs and decreased CD31 and VE-cadherin after 21 days of incubation. In general, vWF plays a key role in hemostasis by recruiting platelets to
Cell viability of VECs (A) and VICs (B) with different amounts of particles 10, 20, and 50 μl per 500 μl media of the ODs 0.25 and 1.0 after 7 days. Green represents live, red represents dead cells, and control is untreated. Percentage of living VECs (C) and VICs (D) as determined from the images with MATLAB for healthy and diseased donors. Data are depicted as mean and S.D. TUNEL staining of VECs (E) and VICs (F) after 7 days of treatment with calcified particles. Green represents apoptotic cells. Scale bars represent 100 μm. Controls are untreated VECs and VICs. Proliferation of VECs (G, H) and VICs (I, J) after 7 days (G, I) and 14 days (H, J) of treatment with different amounts of particles. Red is the positive control, yellow is the control (0.4% fetal calf serum in media), blue represents an OD of 0.25, and green indicates that an OD of 1.0 used for the particles. Data are depicted as mean and S.D. * P<.05, n=3.
sites of vascular damage [29]. A higher expression of vWF might be an early sign of endothelial activation [30] and damage [29,31], an initiating factor in calcific aortic valve disease. Calcium phosphate particles were shown to stimulate IL-8 expression and NF-κB activity in human gingival epithelial cells, which are both involved in the process of inflammation [32]. VECs treated with calcified particles showed no evidence of endothelial to mesenchymal (EMT) or osteogenic transformation over 21 days, suggesting that human VEC isolates, compared to VICs, are more resistant to a phenotypic change when exposed to these calcified particles. One VEC isolate was able to undergo EMT when treated with TGFβ1, and TGFβ1 is a key initiator for EMT [33]. VEC isolates in osteogenic medium did not undergo osteogenic differentiation, which contrasts to clonal populations of ovine VECs [34]; this may be related to species, age, gender [35], and definitely isolate type.

Treated VECs showed an increase in myofibroblastic differentiation and some osteoblastic changes. Although there was no change in CBFA-1, alizarin red and ALP staining was increased and osteopontin was significantly upregulated as an adaptive mechanism. Many studies rely on the expression of CBFA-1 and levels of ALP as indicators of osteogenic differentiation [36]; however, calcification can proceed in the absence of ALP [37]. Uptake of nanocrystals by vascular smooth muscle cells selectively induced limited osteogenic gene expression that did not involve CBFA-1, only BMP2 and osteopontin [37]. [3-Tricalcium phosphate crystallized micron particles have been shown to enhance calcification of human mesenchymal stem cells in vitro and decrease cell viability [38]. Porcine VCs have been compared with osteoblastic cell types at different stages of differentiation, and it has been suggested that VCs may not need to progress through an activated myofibroblast stage before reaching an osteoblast-like phenotype [39]. However, there is a relationship between myofibroblastic porcine VEC activity and initial calcific nodule formation [40], and differentiation of VCs to myofibroblasts was a key mechanistic step in the process of early mineralization [41]. Crucially, it must be noted that VCs from humans that are fibroblastic in nature [15] and of an older age were used here, which may have slowed their rate of differentiation, while other studies used younger and animal VCs which normally are activated in vitro and may have a higher differentiation potential.

VEC and VIC viability was significantly decreased for the highest dose of healthy particles. In the treated groups, only a few apoptotic cells can be seen, while 50% of dead cells were seen in the cell viability staining. This may suggest that both donors mainly cause necrosis instead of apoptosis at high doses. However, a lower percentage of cells were present in the treated groups compared with the control, implying that dead cells were washed away or already had undergone apoptosis. Apoptosis has been documented in human calcified valves [42,43] and in vascular smooth muscle cells by calcium phosphate crystals [44]. Apoptosis has been thought to lead to growth of calcium phosphate crystal structures, exacerbating the calcification process [45]. Although the proliferative capacity for VCs was not affected by the particles after 7 days, a significant increase for the low dose of particles from the diseased donor (absorbance reading of 0.25 and 10 μl per 500 μl) was seen after 14 days. This could mean that these VCs become more activated and thus proliferate more. However, proliferation was not seen for the high concentration (absorbance reading of 1.0), suggesting that this might be causing cell death.

Differences between the effect of the calcified particles on cell viability and proliferation for VECs and VICs may be due to the differences in size or native of the two cell types. VECs are much smaller than VICs, meaning that a smaller surface area is available to interact with the particles compared with the larger VICs. This could have led to a higher VEC viability and could explain the lack of changes in proliferation and osteogenic differentiation. On the contrary, smaller particles would be easier to engulf and have intracellular effects, and small calcium phosphate crystals were shown to be more potent in inducing tumor necrosis factor-α in monocytes/macrophages, with larger pieces of crystals having no effect on vascular smooth muscle cell viability [44]. A range of different-sized calcified particles were observed in our isolated fractions and in vivo [10]. This is also true for extracellular vesicles that manifest as spotty microlocalizations and large calcifications [46]. This can be explained by the growth and nucleation of microlocalizations through the sequential process of accumulation, aggregation, fusion of membranes, and mineralization for the extracellular vesicles [46]. We did not observe a change in distribution of size of the calcified particles over the time course of our experiments.

There was no difference in the effects of the calcified particles from the healthy and diseased donors at the same concentration. Healthy cardiovascular tissue harbors low amounts of these calcified particles, and to counteract these, there are natural inhibitors present in the serum and the cells such as matrix Glα-protein [47,48] and fetuin-A [49] that actively suppress calcification.

This work warrants further investigations into the paracrine role of these calcified particles and a comparison to the effects by adherent calcified particles. Identification of downstream signaling pathways would potentially provide a means of inhibiting their effects.

In conclusion, this study demonstrates that crystalline calcified particles isolated from human aortae, used at these doses, have pathologic significance on human valvular cells, which has not been previously reported. Calcified particles are able to activate a high percentage of human VECs and a subset of VCs to the myofibroblastic phenotype with some osteoblastic changes. The particles also have a significant effect on the viability of both VECs and VICs, signifying their clinical importance. Further work is warranted in limiting the number and size of these calcified particles and in understanding their paracrine roles.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jcarpath.2017.02.006.

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