Distribution of selected elements in calcific human aortic valves studied by microscopy combined with SR-μXRF: Influence of lipids on progression of calcification

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A B S T R A C T
Calcified heart valves display a significant imbalance in tissue content of trace and essential elements. The valvular calcification is an age-related process and there are data suggesting involvement of lipids. We studied elemental composition and lipid distribution in three distinct regions of calcified human aortic valves, representing successive stages of the calcific degeneration: normal, thickened (early lesion) and calcified (late lesion), using SR-μXRF (Synchrotron Radiation Micro X-ray Fluorescence) for elemental composition and Oil Red O (ORO) staining for demonstration of lipids. Two-dimensional SR-μXRF maps and precise point spectra were compared with histological stainings on consecutive valve sections to prove topographical localization and colocalization of the examined elements and lipids. In calcified valve areas, accumulation of calcium and phosphorus was accompanied by enhanced concentrations of strontium and zinc. Calcifications preferentially developed in lipid-rich areas of the valves. Calcium concentration ratio between lipid-rich and lipid-free areas was not age-dependent in early lesions, but showed a significant increase with age in late lesions, indicating age-dependent intensification of lipid involvement in calcification process. The results suggest that mechanisms of calcification change with progression of valve degeneration and with age.

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1. Introduction

Calcific degeneration of aortic valve is the most common type of valvular heart disease in the Western world, causing significant morbidity and mortality (Jung et al., 2003; Otto et al., 1999). It shares many features with vascular atherosclerosis. Ectopic mineralization, a hallmark of the process, was previously regarded as passive deposition of calcium salts in pathologically altered tissue areas. Recent years brought strong evidence supporting the concept of active mechanisms participating in the pathophysiology of the process, which is preceded by endothelial damage, inflammatory infiltration, lipid deposition and oxidation of lipoproteins (O’Brien et al., 1996; Otto et al., 1994; Kaden et al., 2005; Aikawa et al., 2007a). It is not clear, however, whether these processes driven by a variety of cells and cell mediators can explain all aspects of calcific valve degeneration. Some new data suggests that cardiovascular calcification, previously regarded as a simple unidirectional pathway, is in fact a multi-faceted process, leading to divergent consequences, including inhibition or even reversal of valve destruction (Shanahan, 2007; Nicoll and Henein, 2013). The calcification largely depends on the local tissue milieu. In atherosclerosis and valvular calcific degeneration, involvement of lipids and lipoproteins both locally accumulated and circulating is well established (O’Brien et al., 1996; Otto et al., 1994). Recent publications, however, emphasize that their mode of action seems to be much more complex than suggested earlier and is still far from clarification (Pflanzagl, 2006; Kontush et al., 2003; Colles et al., 2001; Demer and Tintut, 2011).
Physico-biological properties and appropriate function of aortic valves depend on their structural and biochemical integrity including elemental composition. Calcified heart valves display a significant imbalance in tissue content of calcium, phosphorus, iron, zinc and copper (Nystrom-Rosander et al., 2002; Tohno et al., 2002; Sarnowski et al., 2001). There is very few data, however, on their distribution in particular valve areas and changes in concentration accompanying the progression of valve degeneration. Up to now, studies investigating jointly concentration and localization of elements in different regions of the cardiovascular system have been performed on human atherosclerotic arteries (Rojiers et al., 2011; Cichocki et al., 1985), while aortic valves have been examined only in animal models (Gajda et al., 2008). Degenerating aortic valves are highly heterogeneous, showing areas with different levels of pathology as well as areas with normal histomorphology. As recently proposed by Nagy et al. (2013), these morphologically distinct areas may represent the successive stages of aortic valve degeneration.

The biological samples, such as aortic valves, are usually studied by histological, histochemical and immunofluorescence methods. However, the contemporary physics offers new tools, suitable also for the biological studies. SR-μXRF (Synchrotron Radiation Micro X-Ray Fluorescence) is a multimodal and highly sensitive analytical technique based on the excitation of an extremely small sample area (a few micrometers) by the synchrotron radiation source and the resulting, element-specific X-ray fluorescence emitted from different areas of the sample allows to examine the concentration and lateral distribution of the elements. The use of high-intensity focused beam obtained from synchrotron source makes possible analysis of elements that occur in biological material even at extremely low concentrations. Since synchrotron delivers a beam of high flux and brightness, no preconcentration procedure for samples is required and the concentration of different elements can be determined with high accuracy (Wang et al., 2010; Twining et al., 2003). This technique was also successfully implemented to the studies on distribution of selected elements in atherosclerotic plaques in a murine model (Gajda et al., 2008, 2010).

The aim of the present study was to compare the elemental distribution in morphologically defined areas of calcifying aortic valves and to establish the relation between the distribution of selected elements, calcification and accumulation of lipids as hallmarks of aortic valve degeneration. Two-dimensional maps and precise point spectra of SR-μXRF recordings, acquired from microscopically selected areas, were compared with histological/histochemical stainings on adjacent sections to prove topographical co-localization of the examined elements and lipids. Since the age is a significant factor involved in both calcification and lipid accumulation (Stewart et al., 1997; Sell and Scully, 1965), we also checked whether the relation between calcium and lipids is age-dependent.

2. Materials and methods

2.1. Material harvesting and preparation

The study material comprised 15 human aortic valves. Eleven valves were excised during routine surgery (six men and five women, mean age 68.58 ± 8.17 yrs) due to severe stenosis without rheumatic etiology. Four normal (non-stenotic, macroscopically unchanged) valves (three men and one woman, mean age 49.5 ± 5.07 yrs) were collected at autopsies. The study protocol was approved by the Bioethical Committee of the Jagiellonian University Medical College and the patients informed consent was obtained.

2.2. Macroscopic and microscopic analysis of valve cusps

The valve cusps were first examined under Stemi 2000C stereomicroscope (Zeiss, Germany) coupled to Coolpix 990 digital camera (Nikon, Japan).

In each valve, three distinct areas representing the successive stages of calcific valve degeneration were analyzed: (1) normal – thin, semitranslucent, pliable and showing unchanged microscopical structure, (2) thickened – opaque with prominent thickening characteristic for early lesions and (3) calcified – containing focal calcifications significantly distorting valve surface, typical for late lesions (Fig. 1).

Serial 10 μm thick unfixed frozen sections of the abovementioned valve cusp areas were thaw-mounted on 3 μm-thick Mylar foil, immediately air-dried, stored in tightly sealed dishes at room temperature and subjected to SR-μXRF measurements. Adjacent sections were used for histology and histochemistry.

2.3. Histology and histochemistry

Sections were fixed in 4% buffered formaldehyde for 5 min and stained with hematoxylin and eosin for general morphology, with Oil Red O (ORO) for lipids and with von Kossa method for calcifications. They were examined under bright field/fluorescence Olympus BX50 microscope (Olympus, Japan). Images were recorded using DP-71 digital CCD camera (Olympus, Japan) coupled to IBM PC-class computer equipped with AnalySIS-FIVE® (Soft Imaging System GmbH, Münster, Germany) image analysis system. Histochemically stained sections served to determine the locations of lipid-rich and calcified areas in the adjacent sections used for SR-μXRF measurements.

2.4. SR-μXRF

Precise point spectra and two-dimensional maps of phosphorus, sulphur, calcium, iron, strontium, copper and zinc were performed using SR-μXRF at the bending magnet beamline L of the synchrotron radiation source DORIS III (DESY, Hamburg, Germany). The experiment was conducted in the air, at room temperature. The samples were mounted on a remotely controlled stage equipped with high-precision stepping motors allowing micrometric movement in xyz space. Samples were fixed at an angle of 45° to the incident beam. The primary photon energy was set to 17.5 keV by a multilayer double monochromator, that enables the analysis of elements with atomic number between 14 (Si) and 38 (Sr). The Vortex SDD semiconductor detector was used for spectral acquisition. 2D maps were acquired from the studied areas of the valves (normal, early lesions and late lesions), with the resolution of 15 μm, for 3 s/pixel. They were used for qualitative assessment of element distribution in the valves. Point spectra from these areas (10 points per area in each valve) were recorded with the resolution of 15 μm, for 300 s/point and used for quantitative analysis of the elemental content. The obtained spectral data were analyzed by AXIL software and peak areas in XRF spectra were calculated, including background subtraction and integration of the area under the peak of the Kα line of the selected elements. For each element, the peak areas were normalized to the incident beam flux and to the peak area under the Compton peak to compensate for the differences in thickness and density of valve sections and the inhomogeneity of sample matrix composition that would influence the beam penetration depth. The concentrations of studied elements were expressed in arbitrary units, proportional to the number of counts.
2.5. Statistical analysis

The variables were expressed as median [lower–upper quartile values] or mean ± SD depending on their type and distribution. The Kolmogorov–Smirnov test was used to assess conformity with the normal distribution. The statistical analysis included Student’s t-test for variables with normal distribution, Mann–Whitney U-test if departure from normality was found and Wilcoxon matched pairs test for comparison of the elemental content in pairs of microscopically selected areas in the valves. The Kruskal–Wallis test followed by Dunn’s post hoc test was used for multiple group comparison. Spearman rank correlation was employed to assess the association between continuous variables. Statistical analysis related to elemental content was based on SR-μXRF precise point spectra measurements. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software Inc., USA). In all tests, two-tailed p values < 0.05 were considered statistically significant.

3. Results

3.1. Aortic valve morphology

Microscopic examination of stenotic valve leaflets confirmed the occurrence of the three areas differing in histomorphology. The pathological changes: lipid-rich areas and focal calcifications were mostly located in the superficial fibrosa layer. Prominent lipid-rich areas were observed in early and late lesions. The early lesions showed the presence of fine granular calcifications, while the late lesions contained massive calcified deposits. In normal regions of stenotic valves (as well as in nonstenotic valves) lipid accumulations and calcifications were absent. In early lesions, lipid-rich areas were located superficially, along generally unaltered fibrosa. In late lesions, lipids were located within focal calcifications and in their vicinity (Fig. 1).

3.2. Elemental content of the examined areas of stenotic valves

Analysis of stenotic valves revealed significant differences in the elemental content of normal areas, early lesions and late lesions (Fig. 2). Even though microscopic examination of normal valves and macroscopically normal parts of stenotic valves did not reveal significant differences, elemental analysis showed higher calcium and lower potassium content in normal areas of stenotic valves as compared to normal valves (64.51 [54.26–74.93] vs 34.80 [33.44–50.12]; p = 0.001 and 1.07 [0.32–1.97] vs 3.64 [1.34–6.06]; p = 0.006). There were no significant differences in the content of other elements between these groups.

Generally, the examined elements showed a tendency to accumulate in early and late lesions, as compared to normal areas of the valves. Significantly higher concentrations of calcium, copper and sulphur were observed in early lesions, and of all studied elements except iron in late lesions. In case of calcium, phosphorus, strontium and zinc, late lesions showed significantly higher content of these elements than early lesions (Fig. 3).
Since the normal valves were collected from significantly younger individuals than stenotic valves (49.5 ± 5.07 vs 68.58 ± 8.17; \( p = 0.001 \)), we checked if lower calcium levels in normal valves as compared to morphologically unaltered regions of stenotic valves can be related to age differences. Indeed, calcium content was positively correlated with age in both normal areas of stenotic valves and in early lesions but not in late lesions. A strong calcium/age correlation was also found when normal areas of stenotic valves and nonstenotic valves were analyzed jointly (Fig. 4a).

### 3.3. Lipids and elemental content in early lesions

The representative distribution of the studied elements in early lesion is shown in Fig. 5. The ORO-positive lipid-rich areas contained significantly more calcium, phosphorus, potassium,
copper and strontium as compared to ORO-negative, lipid-free areas (Table 1). There was a strong correlation in calcium content between these areas \((r = 0.84, p = 0.001)\). The accumulation of lipids did not influence positive calcium/age correlation which was observed in both, lipid-rich and lipid-free areas, although the progression of calcification was more substantial in the former ones (Fig. 4b).

The other elements did not show significant correlation with age except from strontium which was positively correlated with age in lipid-free areas \((r = 0.68, p = 0.02)\).

In both areas the calcium content was positively correlated with phosphorus and strontium (lipid-rich: \(r = 0.74, p = 0.01\) for both elements; lipid-free: \(r = 0.73, p = 0.01\) and \(r = 0.85, p = 0.0008\), respectively). Correlation of calcium with zinc and copper in lipid-rich areas was close to significance \((r = 0.6, p = 0.05\) and \(r = 0.58, p = 0.06\) respectively).

3.4. Lipids and elemental content in late lesions

The representative distribution of elements in late lesion is shown in Fig. 5. In such lesions, lipid-rich areas contained more calcium, phosphorus, potassium and strontium (Table 1), but in contrast to early lesions, calcium content in both, lipid-rich and lipid-free areas did not correlate significantly with each other \((r = 0.49, p = 0.13)\). Likewise, positive calcium/age correlations seen in both areas of early lesions were not found in late lesions (Fig. 4c).

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**Fig. 4.** Progression of aortic valve calcification with age and the involvement of lipids. (a) Comparison of calcium/age relation in normal areas, early lesions and late lesions. Is means lesions; (b and c) comparison of calcium/age relation in lipid-rich (ORO+) and lipid-free (ORO−) areas of early (b) and late (c) lesions; (d and e) changes in proportion of calcium content in lipid-rich (ORO+) areas to calcium content in lipid-free (ORO−) areas of early (d) and late (e) lesions in relation to age. Points represent median values. For each graph, Spearman’s rank correlation coefficient \((r)\) and \(p\) values are specified.

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**Fig. 5.** Corresponding areas of degenerating aortic valve showing localization of Oil Red O (ORO) stained lipids and relative concentrations of elements (SR-µXRF maps) in early lesion (upper panel) and late lesion (lower panel). *Nodular calcification in lower lesion.
In both areas of late lesions, calcium content was positively correlated with phosphorus and strontium in lipid-rich areas as compared to lipid-free areas (Table 1), but also a significant increase in calcium content in lipid-rich regions of late lesions as compared to lipid-rich regions of early lesions (524.4 [469.1–743.7] vs 123.7 [79.9–174.0], p = 0.004). Such increase was not observed in lipid-free areas (173.6 [122.7–363.9] vs 93.5 [66.1–121.8], p = 0.2).

3.5. Calcification develops preferentially in lipid-rich areas

Wilcoxon matched pairs test employed for comparison of calcium concentration in lipid-rich and lipid-free areas of early and late lesions showed not only higher concentration of calcium in lipid-rich areas as compared to lipid-free ones (Table 1), but also a significant increase in calcium content in lipid-rich regions of late lesions as compared to lipid-rich regions of early lesions (524.4 [469.1–743.7] vs 123.7 [79.9–174.0], p = 0.004). Such increase was not observed in lipid-free areas (173.6 [122.7–363.9] vs 93.5 [66.1–121.8], p = 0.2).

3.6. Involvement of lipids in calcification related to age is different in early and late lesions

We analyzed the influence of age on the proportion of calcium located in lipid-rich and lipid-free areas (Fig. 4d and e). In early lesions, calcium concentration ratio was not significantly related to age, while in late lesions it significantly increased with patients' age (r = 0.38, p = 0.25) and (r = 0.69, p = 0.02, respectively).

4. Discussion

Our study has demonstrated that changes in valve histomorphology reflecting the successive stages of the calcific degeneration are accompanied by substantial changes in the content of tissue-bound elements in the altered valves. In early lesions, characterized by valve thickening without macroscopically detectable calcification, SR-μXRF revealed not only substantial accumulation of copper and sulphur but also significant increase in calcium, reflecting the initial phase of diffuse calcification observed microscopically. In late lesions showing the presence of large focal mineral deposits, a strong increase in the concentration of calcium, phosphorus, strontium as well as zinc was found.

Increased content of sulphur in early lesions can reflect matrix remodeling leading to accumulation of highly sulphated proteoglycans. Increased proteoglycan and decreased collagen content in early valve lesions were found in mouse model of aortic valve disease (Krishnamurthy et al., 2012).

The increase in copper concentration in early lesions suggests significance of this trace element in early valve matrix remodeling. Formation of late lesions, however, was not associated with further accumulation of copper, suggesting a limited (if any) role of this element in the progression of valve degeneration. Decreased copper content in heavily calcified human aortic valves as well as atherosclerotic lesions in cholesterol-fed rabbits was found by others (Nystrom-Rosander et al., 2002; Rajendran et al., 2007).

Since various forms of calcium phosphates including hydroxyapatites typical for bone are the predominant components of cardiovascular calcifications, the observed increase in calcium and phosphorus in late lesions can be expected. In both early and late lesions, calcium and phosphorus colocalized with strontium and zinc. Such colocalization was also observed in calcified atherosclerotic plaques of apoE/LDLr-double knockout mice (Gajda et al., 2008).

The role of zinc seems to be more complex. This essential trace element is required for matrix mineralization in various tissues (Kwun et al., 2010; Yusa et al., 2011). Zinc was demonstrated to colocalize with calcifications in atherosclerotic arteries and its involvement in early deposition of calcium salts was suggested (Rooijers et al., 2011). There are, however, also contradictory results, showing decreased concentration of zinc in atherosclerotic lesions (Minqin et al., 2003). We found that zinc correlates with calcification in lipid-rich areas. Zinc can be involved in prevention of lipid peroxidation, since it is not only a structural component of superoxide dismutase, but also competes with redox-active iron and copper, indirectly limiting their peroxidative activity (Bettger, 1993). On the other hand zinc is present in matrix metalloproteinases (MMPs), endopeptidases able to degrade all organic components of extracellular matrix and proved to participate in stenotic valves remodeling (Kaden et al., 2005; Soini et al., 2001). It is thus possible that zinc in lipid-free areas is involved in different processes, not related directly to calcification.

Macroscopically undetectable (diffuse) calcifications have been demonstrated by various modalities in early lesions of aortic valves (Aikawa et al., 2007a; Lis et al., 2003). This form of calcification has the tendency to increase with age, even in healthy aortic valves and aorta, as detected by compositional analyses based on a dry-weight basis (Tohno et al., 2002; Ohnishi et al., 2003). Indeed, we have demonstrated that in normal areas and early lesions of stenotic valves calcium concentration increases with age and that fine calcifications as well as lipids are present in early lesions, what supports the notion that both calcifications and lipids are involved in valve degeneration in the initial phase of the process (O'Brien et al., 1996; Otto et al., 1994). Lipids present on the surfaces of cells and matrix components can act as calcium nucleators during calcification of aortic valves (Ortolani et al., 2010). Since in this study higher amounts of calcium were found to colocalize with lipids in both early and late lesions, our results suggest a pathophysiological link between lipid accumulation and calcification in human aortic valves. This supports the lipid hypothesis of cardiovascular calcification proposed by Demer (1997).

Since potassium is the main intracellular cation, its higher concentration in lipid-rich areas indicates that calcification progresses in a milieu rich in cell debris (e.g. apoptotic vesicles) and/or matrix vesicles, both influencing calcification (Jian et al., 2003; Kim, 1976; Galeone et al., 2013). Highly oxidized lipids are cytotoxic for cells (Colles et al., 2001; Gajda et al., 2008), although their impact critically depends on local pH and lipid concentration (Pfanzagl, 2006; Brodeur et al., 2008).
We showed that in early lesions calcification increased with age in both lipid-rich and lipid-free areas and that proportion of calcium deposited in these areas was not significantly modified by age. This may suggest that although in early phase of the disease lipids facilitate calcification, the process is regulated mainly by other factors, such as inflammatory cells and their mediators (Otto et al., 1994; Aikawa et al., 2007b). This thesis can be supported by reports on the use of statins in the treatment of aortic stenosis. Their anti-inflammatory activity seems to be independent of lipid-lowering effect and indeed, promising results are expected rather in early but not in advanced phase of the disease (Dimitrów and Jawień, 2010; Antonini-Canterin et al., 2008).

In late lesions, total calcium concentration as well as calcium content in lipid-rich and lipid-free areas was not significantly related to age, but proportion of calcium content in lipid-rich and lipid-free areas significantly increased with age, pointing to a lesser involvement of lipids in the calcification process in younger individuals. This is a seemingly confusing result, since age-dependent increase in calcium proportion should be accompanied by a similar increase in calcium concentration, at least in lipid-rich areas. It cannot be excluded that this discrepancy results from much higher dispersion of calcium concentration values in advanced lesions, requiring larger number of valve samples to achieve a reliable statistical evidence of the tendency for its increase with age, observed in lipid-rich areas. Moreover, calcium in lipid-free areas did not show such tendency at all (Fig. 4c).

These differences between early and late lesions suggest that in advanced lesions either new players are involved or the tissue response to factors acting in early lesions is substantially different. The latter possibility can be supported by results of Aikawa et al. (2007b) who demonstrated that in mouse model of atherosclerosis, calcification was positively correlated with inflammatory macrophages in early lesions, but negatively in more advanced ones. The role of lipids can also be different in early and late lesions. Lipid oxidation products enhance formation of osteoclasts (Tintut et al., 2004) which occur in stenotic valves (Nagy et al., 2013; Lis et al., 2014) and are able to decalcify bone tissue. Hence, it cannot be excluded that the role of lipids in late lesions is bimodal: on one hand they promote calcification, being important components of the permissive matrix, on the other hand they may facilitate mobilization of mechanisms inhibiting calcification.

In conclusion, we have demonstrated that aortic valve degeneration leading to formation of focal calcifications is associated with significant changes in the elemental content and that calcifications are preferentially located in lipid-rich areas. In early lesions, lipids seem to be less intimately associated with the progression of calcification than in the late ones, where (especially in older patients) lipid-rich areas accumulate much higher proportion of calcium. This suggests that calcification mechanisms are modulated with the progression of calcific aortic valve stenosis and with age.

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References


