BASIC RESEARCH

Expression of bone-regulatory proteins in human valve allografts

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Objective: To test the hypothesis that valve allograft (VA) calcification results from an ossification process in which bone-regulatory proteins are expressed.
Methods: 15 VA that were explanted at the time of surgery for dysfunction were studied. VA were analysed and compared with normal aortic valves (n = 20).
Results: All the VA (5 aortic, 10 pulmonary) exhibited heavy calcification and important fibrosis. Immunohistochemistry studies showed that the bone-specific transcription factor Cbfa-1 was expressed by stromal cells. Bone alkaline phosphatase was expressed in calcified regions. Immunostaining for α smooth muscle (α-SM) actin was increased in VA compared with normal valves and in 6 of the 15 valves formed cellular clusters close to the calcified nodules. In VA osteopontin and osteonectin were expressed by stromal cells, whereas osteocalcin was closely associated with the calcified regions. Furthermore, analysis of the bone-regulatory proteins that control bone resorption showed that receptor activator of nuclear factor κB ligand (RANKL), receptor activator of nuclear factor κB (RANK) and osteoprotegerin (OPG) were differentially expressed in calcified VA and normal valves. Normal valve leaflets expressed OPG, whereas OPG expression was absent or faint in calcified VA. RANKL and RANK were not detected in normal valves, whereas calcified VA expressed RANKL and RANK.
Conclusion: These data suggest that calcification of VA results from an ossification process, which relies on tight control of bone-regulatory protein expression. The expression pattern of the RANKL/RANK/OPG system suggests that it may have a regulatory role not only in osteoclastogenesis but also in the calcification of human VA.

The clinical performance of valve allografts (VA) is good but the longevity of these valve substitutes is impeded by the calcification process, especially in young patients.[2] Calcification of VA is associated with structural valve failure that eventually leads to reoperation.[2] In the past, heterotopic calcification was considered to be a passive mechanism through which calcium is deposited in degenerative tissues. More than a decade ago the works of Demer's and Giachelli's groups established that calcification of vascular structures was not the result of a mere passive deposition of calcium but was rather related to an active cellular process.[3 4] Recently, calcification of valve tissue was also linked to an active transformation of resident interstitial cells to bone-forming cells.[5] Calcification of VA has been related to immune mechanisms and inflammation within the grafts.[6] Regulation of the ossification process has, however, not been thoroughly described in VA.

In bone, ossification is an exquisitely regulated process that is under the control of numerous molecules including phosphatase, growth factors, extracellular matrix proteins and matricellular proteins. Osteoblast differentiation is regulated through the expression of the bone-specific transcription factor Cbfa-1, which also regulates the rate of bone matrix deposition by differentiated osteoblasts.[7] Proper calcification of bone tissue is dependent on the expression and activity of alkaline phosphatase (ALP).[8] Furthermore, the biomineralisation process is regulated through the production of non-collagenous bone proteins including the matricellular proteins, which are secreted biomolecules that interact with cell surface receptors, extracellular matrix and growth factors but do not themselves have a direct structural role. Osteonectin and osteopontin are matricelullar proteins that regulate the communication between cells and the extracellular matrix and participate in the control of the ossification process.[9 10] Osteocalcin is the most abundant non-collagenous bone protein but its role in the regulation of the biomineralisation process is not completely elucidated.[11] Recently, a group of molecules from the tumour necrosis factor-related family has been shown to have a key role in skeletal and vascular mineralisation. The receptor activator of nuclear factor κB ligand (RANKL) is expressed by T cells and osteoblasts especially in regions of active bone remodelling. RANKL binds to the receptor activator of nuclear factor κB (RANK) on the surface of monocyte/macrophages lineage cells, including osteoclasts, and promotes cell survival and activation.[12] Osteoprotegerin (OPG) is a secreted protein that regulates bone mass and osteoclast activation by binding to RANKL. Mice deficient in OPG develop osteoporosis and severe vascular calcification.[13] OPG is secreted by the vascular wall and may participate in the protection of blood vessels against heterotopic calcification. In this study we hypothesised that during calcification of human VA leaflets, bone-regulatory proteins including the RANKL/RANK/OPG system are produced.

PATIENTS AND METHODS

Patients

We examined 15 cryopreserved VA that had been implanted for periods ranging from 2–16 years (median 8.7 years). Five

Abbreviations: ALP, alkaline phosphatase; α-SM, α smooth muscle; OPG, osteoprotegerin; RANK, receptor activator of nuclear factor κB; RANKL, receptor activator of nuclear factor κB ligand; VA, valve allografts

aortic and 10 pulmonary VA had been implanted in the pulmonary position for reconstruction of the right ventricular outflow tract. The mean age of patients was 17 (SD 6) (range 2–44) years; nine were men and six were women. The mean age of VA donors was 21.4 years (range 12–33). Reasons for explantation were stenosis in nine patients and a predominant regurgitation in six patients. For controls, we selected 20 heart transplant recipients who had no valve disease, from whom the aortic valve was obtained at the time of transplantation. Samples were taken at the time of surgery. Cusps including the conduit were immediately transported to the laboratory in cold HEPES solution. Tissues were decalcified in Cal-Ex (Fisher, Nepean, Ontario, Canada) for 24 h. Then one cusp was fixed in formaldehyde 10% for histological processing and one cusp was embedded in optimum cutting temperature compound (TissueTek, Miles Laboratories, Elkhart, Indiana USA) and frozen in liquid nitrogen for immunohistological analysis.

Histological analysis

Decalcified and fixed tissues were processed for routine paraffin embedding. Valve samples were excised vertically to the base at the midpoint. Sections of 5 μm were obtained and stained with haematoxylin and eosin and trichrome Masson. Calcification of VA was determined by alizarin red coloration (Sigma, Oakville, Ontario, Canada).

Immunohistological analysis

Cryostat sections were analysed immunohistologically with the following mouse monoclonal antibodies: anti-collagen type I (Sigma, St Louis, Missouri, USA), anti-bone ALP (R&D systems, Minneapolis, Minnesota, USA), anti-α smooth muscle (α-SM) actin (Sigma, Oakville), antiosteonectin (US Biological, Swampscott, Massachusetts, USA), anti-osteocalcin (US Biological), anti-OPG (Chemicon International, Temecula, California, USA), anti-RANKL (Imgenex, San Diego, California, USA) and anti-RANK (R&D Systems). The following polyclonal antibodies were used: rabbit anti-Cbfa-1 (US Biological) and goat anti-osteopontin (R&D Systems). Slides were then incubated with a biotin-conjugated anti-mouse immunoglobulin antibody (Jackson ImmunoResearch, West Grove, Pennsylvania, USA), an anti-goat immunoglobulin antibody (BioCan Scientific, Mississauga, Ontario, Canada) or an anti-rabbit

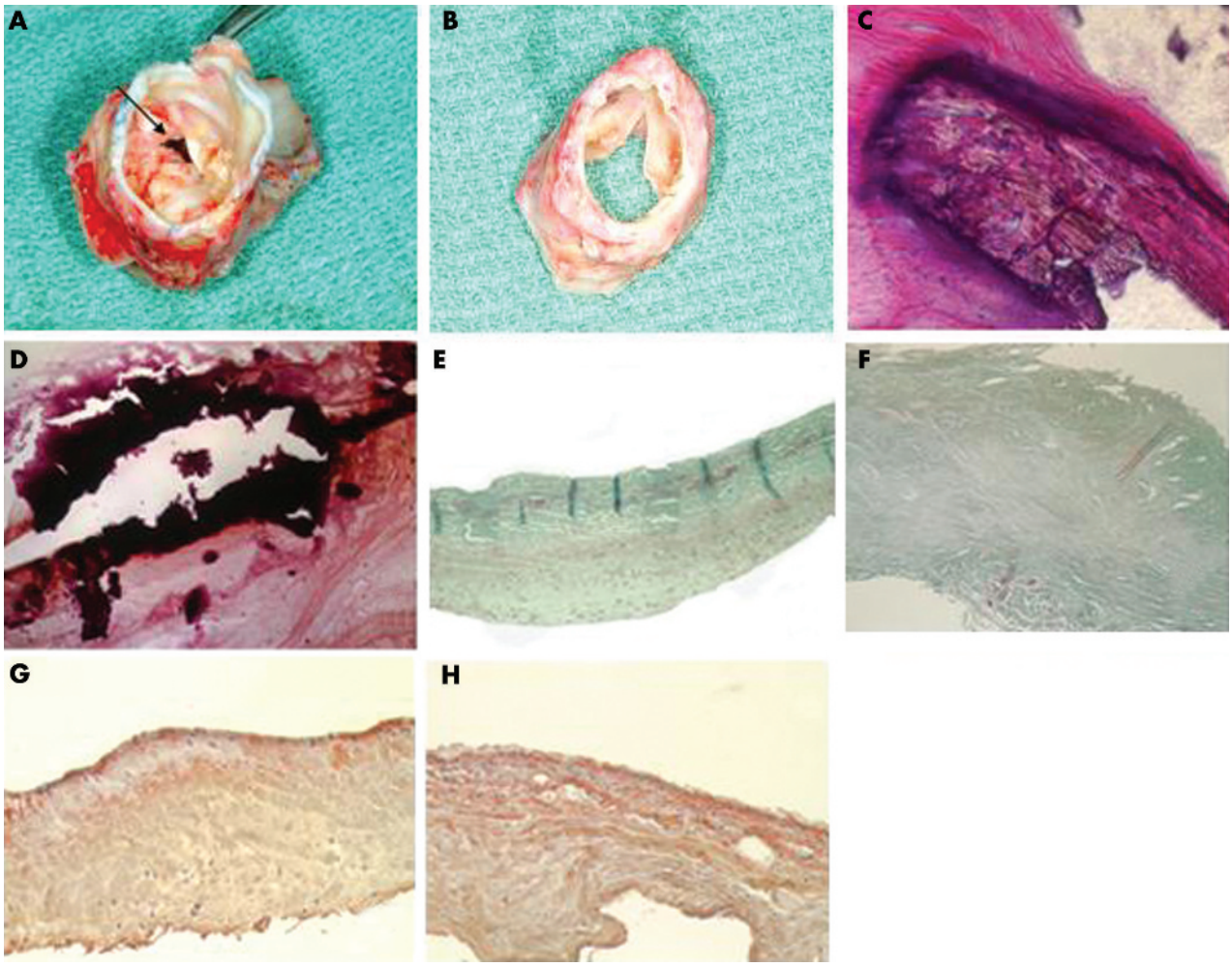

Figure 1 Macroscopic inspection of explanted valve allografts shows that all the conduits were mineralised. (A) Leaflets were heavily calcified in patients with a predominant stenosis leaving only a small aperture for blood passage (arrow). (B) In patients with a regurgitant allograft leaflets appear retracted. (C) Small nodules of calcification were located at cusp insertion as seen on haematoxylin and eosin examination (original magnification 400×). (D) Alizarin red colouration showed extension of calcified foci into the leaflet matrix (original magnification 400×). (E) Trichrome Masson staining in normal valves showed deposition of collagen (green) in the fibrosal layer (original magnification 100×). (F) Valve allografts with dense deposition of collagen had thickening of leaflets (original magnification 100×). Immunostaining for collagen type I showed (G) the presence of collagen in normal valves (original magnification 100×) and (H) increased collagen in leaflets of valve allografts (original magnification 100×).

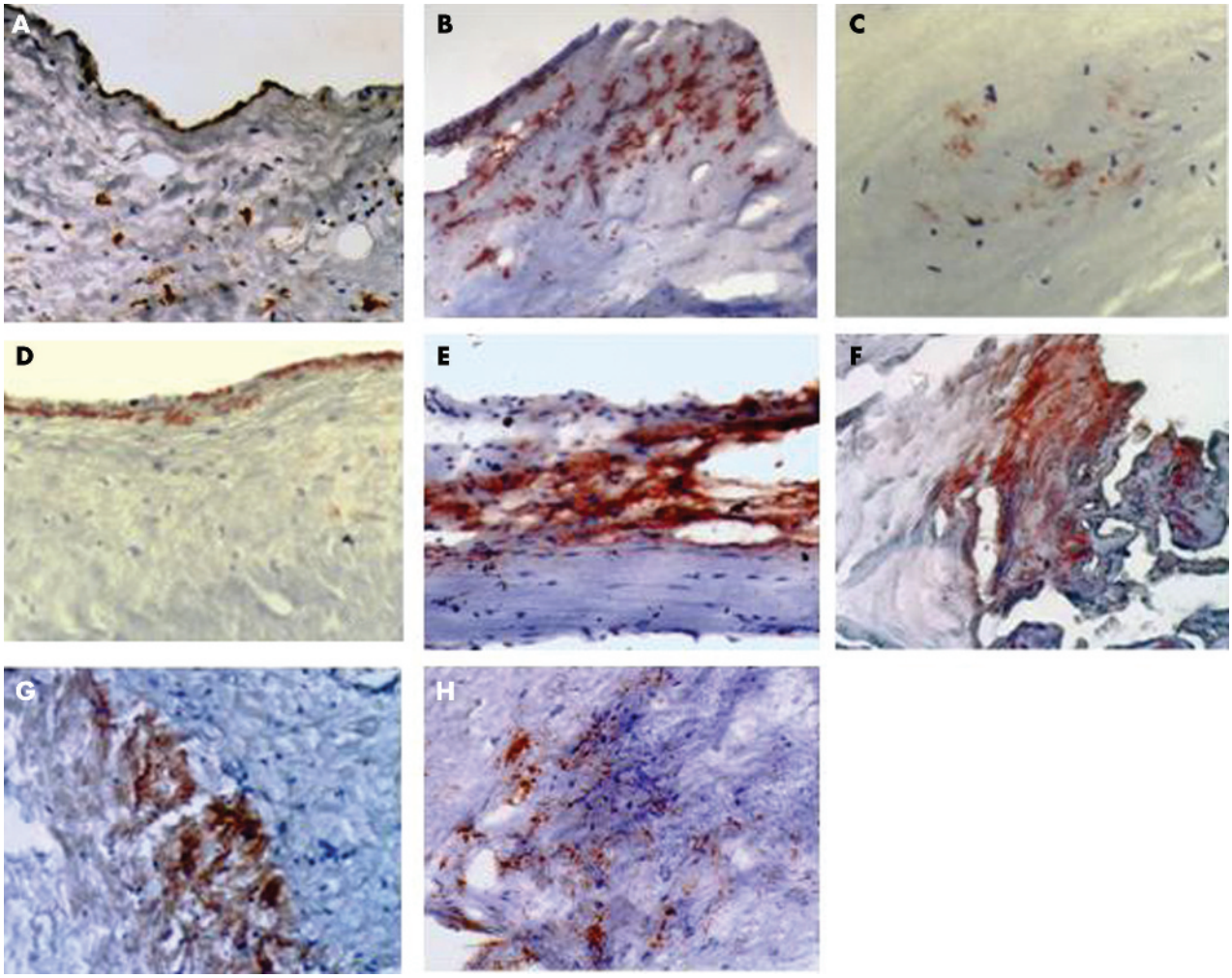

Figure 2 (A) Immunostaining for α smooth muscle (α-SM) actin in normal valves showed dispersed positive cells within the matrix. (B) Valve allografts had numerous α-SM actin-positive cells forming cellular clusters (original magnification 200×). (C) In valve allografts stromal cells were positive for bone-specific transcription factor (Cbfa-1) (original magnification 400×). (D) Normal valves expressed bone alkaline phosphatase (ALP) located in the fibrosal layer. (E) In valve allografts ALP immunostaining was increased and located in calcified areas (original magnification 200×). (F) Osteocalcin was immunodetected only in allografts and located in calcified areas. (G) Osteopontin and (H) osteonectin were located in the stromal cells (original magnification 200×).

immunoglobulin antibody (BioCan Scientific), followed by horse radish peroxidase-conjugated streptavidin and ABC substrate (Dako, Mississauga, Ontario, Canada). Tissue sections were counterstained with haematoxylin. Secondary antibodies without the primary antibody were used as controls in all the experiments; in this study isotype-matched irrelevant antibodies were not used.

RESULTS

Macroscopic findings in VA valves showed that all the conduits were heavily calcified, whereas valve cusps had two different patterns of calcification. The VA cusps from patients with VA stenosis were severely calcified, whereas the VA cusps from patients with a regurgitant VA were less calcified but had leaflet retraction (fig 1A, B). Histological analysis showed that all the VA cusps were calcified at the cusp insertion (fig 1C). In 11 patients calcified nodules not only were present at the cusp insertion but also extended through the leaflet matrix (fig 1D). Trichrome Masson staining and immunostaining for collagen type I showed that VA were densely infiltrated with collagen compared with normal valves (fig 1E–H).

In VA we found that α-SM actin immunostaining was increased when compared with normal valves. Moreover, in six VA, α-SM actin-positive cells formed clusters close to the calcified nodules (fig 2A ,B). The bone transcription factor Cbfa-1 was not found in normal valves, whereas it was expressed by stromal cells within VA (fig 2C). In normal valves the ossification marker ALP was immunodetected in the fibrosal layer whereas it was increased and located in calcified areas in VA (fig 2D, E). We found no osteocalcin, osteopontin or osteonectin in normal valves, whereas we did detect them in VA. Immunohistochemical studies showed that osteocalcin was closely associated with calcified areas, whereas osteopontin and osteonectin were immunodetected in stromal cells within the leaflet matrix (fig 2F–H).

Analysis of normal valves showed that their cusps had a strong immunoreactivity for OPG (fig 3A), whereas RANKL and RANK were not detected. As opposed to normal valves, VA had no or faint expression of OPG (fig 3B), whereas RANKL and RANK were immunodetected (fig 3D, F). RANKL was detected as an extracellular protein and it was not detected in stromal cells (fig 3E). RANK was detected in VA and immunolocalised to stromal cells that on occasion formed cellular aggregates (fig 3F).

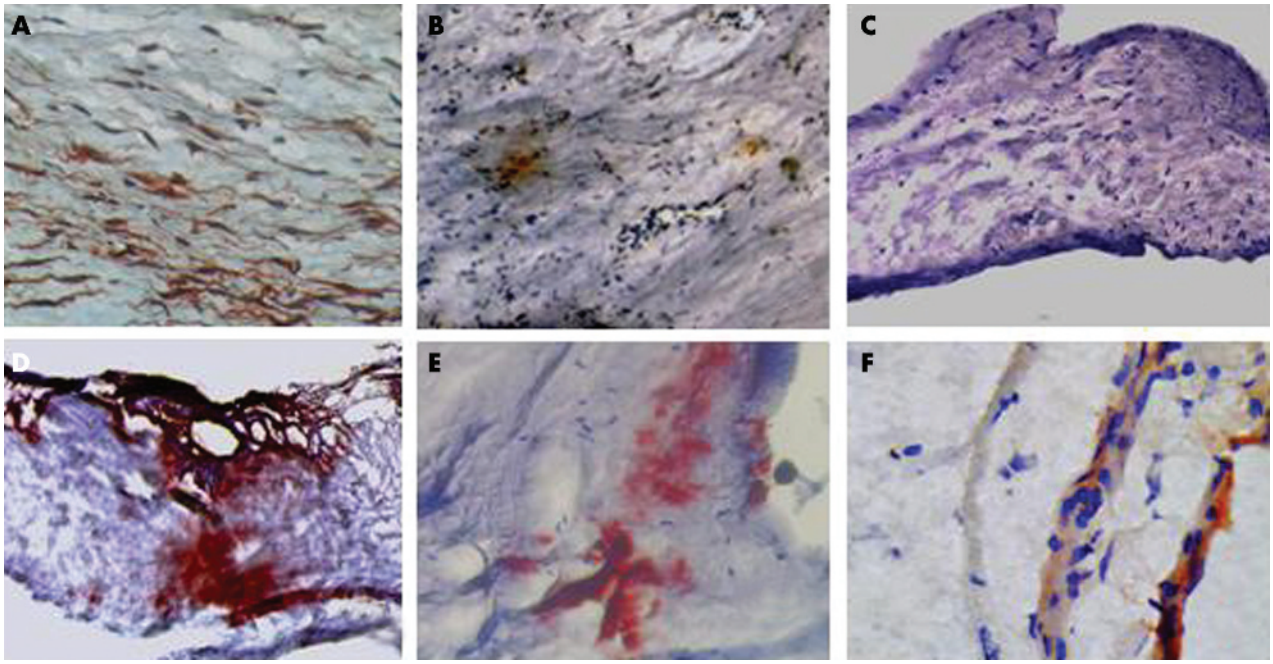

Figure 3 Osteoprotegerin (OPG) was immunodetected in (A) normal leaflets but (B) not or weakly detected in valve allografts (original magnification 200×). (C) Receptor activator of nuclear factor κB ligand (RANKL) was absent in normal leaflets but (D) was immunodetected in valve allografts (original magnification 100×) (E) as a soluble protein (original magnification 400×). (F) Receptor activator of nuclear factor κB (RANK) was not detected in normal valves (not shown) but it was immunodetected in stromal cells of valve allografts (original magnification 400×).

DISCUSSION

VA structural failure is closely associated with graft calcification. Calcification of extraosseous tissue, once considered a passive process, is now viewed as an active cellular mechanism through which bone-specific proteins are produced. Leaflets are composed of valve interstitial cells represented by fibroblasts and myofibroblasts. Myofibroblasts expressing α-SM actin are active synthetic cells that take part in active tissue remodelling.[14] We have observed that normal valves were composed of mainly fibroblasts with the presence of occasional myofibroblasts expressing α-SM actin, which were dispersed within the leaflet matrix. On the other hand we have found that VA valves were composed of myofibroblasts, which in some grafts formed cellular clusters close to calcified regions. In VA, the presence of α-SM actin-positive cells near calcified regions may reflect the active synthesis of extracellular matrix in areas where calcified matrix is deposited. In vitro isolated interstitial cells calcify when cultured in appropriate conditions, indicating the plasticity of this cellular population and their potential implication in the extraosseous ossification process. Isolated valve interstitial cells lose the α-SM actin marker and acquire a bone phenotype during mineralisation.[5] It has been shown in an experimental model that calcification of aortic allografts is associated with the transformation of α-SM actin-positive cells into chondrocytes followed by endochondral ossification, indicating that α-SM actin-positive cells are precursors of bone-forming cells within allografts.[15]

VA leaflets were associated with dense deposition of collagen type I along with calcification that started at the cusp insertion and extended into the leaflet matrix. Ossification is closely associated with the synthesis of collagen type I, it is the most abundant protein in osseous tissue, and is necessary for proper mineralisation of bone.[16]

Fibrosis and retraction of leaflets were predominant features of regurgitant VA, whereas stenotic VA were characterised by extensive leaflet calcification. Factors that drive a profibrotic and a retraction process of VA leaflets compared with a predominant calcifying response are unknown but potential mechanisms related to a differential production of growth factors may be involved.

Maturation of osteoblasts depends on the coordinated expression of many genes and transcription factors. Differentiation of precursor cells into osteoblasts is marked by the expression of bone-specific proteins and transcription factors such as Cbfa-1.[17] We have observed in VA a population of cells expressing the bone transcription factor Cbfa-1, indicating the presence of cells with a bone-like phenotype within VA. In extraosseous mineralised tissues such as calcified atherosclerotic aorta, bone matrix proteins along with their specific bone transcription factors such as Cbfa-1 have been detected.[18] The transcription targets of Cbfa-1 include bone sialoprotein, collagen type I and osteopontin.[19]

ALP, long recognised as an osteoblastic marker, is a membrane-associated enzyme that converts organic phosphate into orthophosphate to form hydroxyapatite crystals.[20] A high level of bone ALP activity is necessary to ensure adequate mineralisation of bone, as a genetic deficiency in the activity of this enzyme is associated with impaired skeletal development. In vitro, blockage of ALP inhibits the formation of calcified nodules in isolated leaflet interstitial cells.[5] In the present study, normal valves expressed ALP within the fibrosal layer, whereas VA had an increased expression that was associated with calcified regions. Although the role of ALP in normal valves is still unknown, it is believed that increased enzymatic activity in calcified tissue is necessary to achieve mineralisation.[21]

Ossification and control of the mineralisation process is complex and partially understood. Many reports have documented the presence of three non-collagenous matrix proteins, osteonectin, osteopontin, and osteocalcin, in bone and in mineralised tissues outside the skeleton.[22] Osteonectin is a calcium-binding protein that has an affinity for collagen,

suggesting that it might support the formation of hydroxyapatite. In bone osteonectin promotes osteoblast formation and survival supporting the concept that it is a positive regulator of bone formation.[23] Osteopontin is an acidic phosphoprotein with a hydroxyapatite-binding site suggesting that it regulates the calcification process. It accumulates in injured blood vessels and in calcified areas. Osteopontin has been shown to have a potent inhibitory action on the calcification process.[24] Osteocalcin is a vitamin K-dependent protein that strongly inhibits hydroxyapatite nucleation. Osteocalcin expression has been documented in mineralised areas of atherosclerotic plaques and aortic stenosis.[25] We have found in VA that osteonectin and osteopontin were immunodetected in stromal cells whereas osteocalcin was detected in calcified areas. The presence of non-collagenous bone proteins within VA suggests that the mineralisation process of allografts is similar to bone formation.

The recently discovered RANKL/RANK/OPG system appears to mediate important interactions between the immune system, bone tissue and blood vessels. Animals deficient in OPG develop osteoporosis and severe vascular calcification, supporting the hypothesis that OPG has a role—although undefined at the moment—in the protection against vascular calcification.[13] Furthermore, systemic administration of OPG in rodents prevented warfarin and vitamin D-induced extraosseous calcification.[26] Tissue expression of OPG is greatly reduced in calcific aortic stenosis, reinforcing the hypothesis that modulation of OPG in peripheral tissue may take part in the regulation and control of extraosseous calcification.[27] We have immunodetected OPG in normal aortic leaflets, where it was abundant, whereas in VA its expression was faint or absent, suggesting that OPG may have a protective role against calcification in allografts. RANKL and RANK were immunodetected only in VA, with diffuse extracellular immunomarking of RANKL and cellular expression of RANK. Kaden *et al*[27] have shown in isolated valve interstitial cells that RANKL induced matrix calcification and increased expression of Cbfa-1, osteocalcin and ALP. RANKL is initially synthesised as a membrane-anchored molecule that can be cleaved as a soluble molecule by the metalloprotease-disintegrin tumour necrosis factor-α convertase.[28] In VA the immunomarking for RANKL suggested that it was secreted, but its cellular origin and the mechanisms for its production are unknown The membrane-bound protein RANK is expressed by osteoclasts, chondrocytes and dendritic cells. Osteoclast-like cells that express RANK have been described in calcified atherosclerotic plaque, suggesting that mineral resorption may be a defensive mechanism of the arterial wall.[29] Differential expression of the RANKL/RANK/OPG system in VA therefore suggests that, along with its implication in the control of bone resorption, this pathway may contribute to the calcification of allografts.

In this study, expression of bone proteins was documented by immunohistochemistry studies as the sole technique, which can introduce some limitations to the interpretation of the results. Results obtained from other studies that used either immunohistochemistry studies or other techniques such as mRNA amplification in calcified vascular tissue or aortic stenosis have, however, yielded comparable results.[22 25] Calcification of VA, long recognised as an important mechanism for structural failure, is now perceived as an active cellular process involving numerous and complex interactions between growth factors, bone-regulatory proteins and the extracellular matrix. To our knowledge this is the first study to document the differential expression of the RANKL/RANK/OPG system in the calcification process of human VA. Although not fully understood, the expression pattern of bone-regulatory proteins including the RANKL/RANK/OPG system in VA suggests that an active cellular mechanism related to bone ossification takes part in the mineralisation process of allografts. Understanding the mechanisms that contribute to the mineralisation process of VA may help to design new treatments aimed at preventing VA structural failure.

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IMAGES IN CARDIOLOGY

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64-slice cardiac computed tomography: appearance of a complex coronary to pulmonary arterial fistula with conus artery aneurysm

A 63-old-patient with drug refractory paroxysmal atrial fibrillation underwent 64-detector row cardiac computed tomography (CT) in order to facilitate a pulmonary vein isolation procedure. Previous clinical and echocardiographic information suggested that the heart was structurally normal.

A complex, unsuspected coronary artery to pulmonary artery fistula was diagnosed and clearly delineated using three-dimensional volume rendering software (GE Healthcare Technologies, Waukesha, Wisconsin, USA). There are a number of components to this anomaly (see panels).

The main fistula is formed from the conus artery, arising as a separate origin from the right aortic sinus where it is aneurysmal (white arrow), and the pulmonary artery. This branches to form two proximal limbs that take a tortuous course superiorly between the aorta (Ao) and pulmonary trunk (PT), which then unite and pass anterior to the PT just above the level of the pulmonary valve (white arrowheads). This vessel becomes progressively more dilated before fistulating into the left anterolateral aspect of the PT. There are also two aberrant branches of the proximal left anterior descending artery (LAD); the more proximal of these can be seen to drain directly into the pulmonary outflow tract with the more distal branch communicating directly with this conus arterial fistula.

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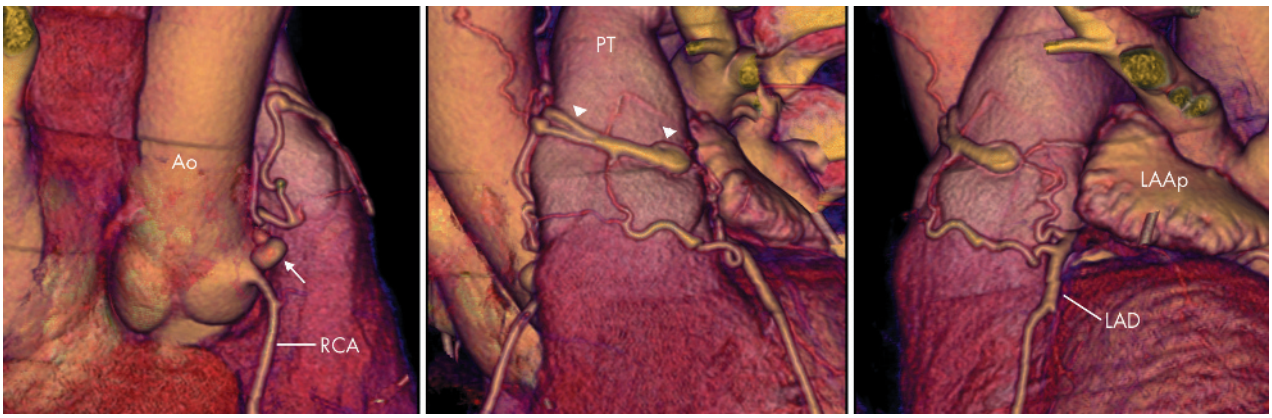

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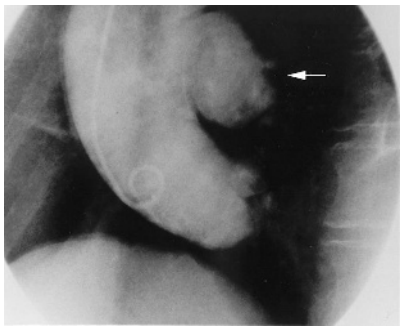
A footprint of brucella infection: enormous saccular aneurysm of the ascending aorta



A 78-year-old woman, with a longstanding history of hypertension, was referred to our institution for a scheduled cardiac catheterisation because of an atypical chest pain and an electrocardiographically positive stress test. The coronary angiogram revealed atherosclerotic coronary vessels without any significant stenosis. For clarification of her clinical syndrome aortography was performed, portraying an enormous saccular aneurysm of the posterior wall of the ascending aorta (see panel). The chest *x* ray was unremarkable while concomitant aortic valve disease was not present. The nature of this very large aneurysm, however, remained to be determined. Her past history excluded any injury. It was questionable if hypertension alone could explain this localised finding, as there was no diffuse dilatation of the aorta and no aortic valve disease that hypertension primarily enhances. Detailed questioning of the patient's history brought to light a successfully treated *Brucella mellitensis* infection a couple of years previously. Taking into account the localised character of the saccular aneurysm, the brucella infection, although rare, could be the major aetiologic component of this lesion. Since the risk of rupture and dissection was high, aneurysmectomy was scheduled without delay. Histopathological examination of the excised tissue verified the mycotic nature of the aneurysm and tissue PCR revealed the presence of *Brucella mellitensis* DNA in the lesion. At six months follow-up, the patient was doing well and had no symptoms.

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Heart

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Notes