Apoptosis in rheumatic and degenerative aortic valve stenosis. A progress toward understanding

By

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Key words: Apoptosis – rheumatic - degenerative – aortic stenosis

Abstract:

Background: Although cardiologists and cardiac surgeons encounter aortic valve stenosis on a frequent basis, the molecular biology of cuspal calcification is poorly understood. Now, there is compelling histopathologic data suggesting that apoptosis is involved in calcification of degenerative stenotic aortic valves. Little is known about its contribution to calcification of rheumatic valves.

The aim of this study was to investigate the possible role of apoptosis in cuspal calcification in both rheumatic and degenerative aortic valve stenosis**.**

Material and Methods: The study population included 20 patients undergoing aortic valve replacement for aortic valve stenosis. Ten cases were rheumatic (age 28 ± 8.6 years) and ten cases were degenerative (age 65.3 ±7.4 years). The severity of aortic valve disease was determined preoperatively by echocardiography. We performed histological, histochemical and immunohistochemical studies on formalin-fixed, paraffin-embedded stenotic aortic valve leaflets removed during aortic valve replacement. Masson trichrome stain was performed to highlight fibrotic changes. Immunohistochemical studies were performed according to avidin-biotin-peroxidase complex (ABC) method using polyclonal rabbit antihuman Bax antibody. An immunoreactive score (IRS) was calculated by multiplying the grade of percentage of positive cells by the grade of intensity of Bax immunostaining.

Results: All the studied valves showed positive Bax immunostaining that was predominantly detected in the cytoplasm of interstitial fibroblasts "especially in areas adjacent to calcification" as well as the endothelial cells of the new-capillary sprouts present in the valvular interstitium. A differentiating feature was the positive Bax immunostaining, detected only in the cytoplasm of the valvular surface endothelial cells of rheumatic cases. Also, areas of neovascularization were more abundant in degenerative aortic valves and in the vicinity of calcification of these valves. The IRS was higher in degenerative aortic valve stenosis compared to rheumatic valves.

Conclusions: Our data attest that apoptosis contributes to calcification of stenotic aortic valve cusps. However, the interplay of endothelial cells and fibroblasts apoptosis in the pathogenesis of rheumatic and degenerative aortic stenosis seems to be different. Understanding the role of apoptosis and angiogenesis in the pathogenesis of both rheumatic and degenerative aortic stenosis offers the potential to develop targeted therapeutic regimens.

Introduction:

Rheumatic heart disease is the most common cause of valvular heart disease in developing countries including Egypt. Despite the high prevalence, increased morbidity, and well-described histopathological findings of this disease, little is known about the cellular mechanisms responsible for calcification in these valves. Until recently, this has been thought to be due to a passive accumulation of calcium along the surface of the valve leaflet. [1]

On the other hand, calcific aortic valve disease is a slowly progressive disorder more common in the elderly and is the most common acquired valvular disorder found in developed countries. [2] It represents a disease spectrum that spans *aortic sclerosis* (mild valve thickening without obstruction of blood flow) to *aortic stenosis* (severe calcification with impaired leaflet motion and obstruction to left ventricular outflow). [3]

There has been a long-held notion that this disease was "degenerative", because of time-dependent wear-and-tear of the leaflets with passive calcium deposition and therefore unmodifiable, condition. [4]

Now, there is compelling histopathologic data suggesting that calcification in either rheumatic or non-rheumatic aortic valve stenosis is an active regulated disease process. [1, 5] In degenerative aortic stenosis, it includes the apparent early involvement of apoptotic vesicles [6] and apatite nucleation of these sites with calcification of devitalized cells.[5] In rheumatic aortic stenosis, the data on involvement of apoptosis in calcification of rheumatic valves are scarce.

Apoptosis means programmed cell death which describes the orchestrated collapse of a cell. [7] Apoptotic cell death plays an important role in maintenance of the normal physiological state and in the pathogenesis of different diseases in the body. [8]

Proteins of the Bcl-2 family are key regulators of apoptosis. Certain members promote cell survival (e.g. Bcl-2, Bcl-xL, Bcl-w and A1/Bfl-1) while others promote cell death (e.g. Bax, Bak, Bad and Bim), and their relative abundance in any cell may determine its fate. [9]

The aim of this study was to investigate the possible role of apoptosis in cuspal calcification in both rheumatic and degenerative aortic valve stenosis**.**

Material and Methods:

Tissue: Twenty stenotic aortic valves represent the material of this study. The etiology of aortic stenosis was rheumatic in 10 cases and degenerative

in 10 cases. The ten patients with degenerative aortic stenosis had no past history of rheumatic fever or infective endocarditis showing only isolated aortic valvular stenosis. The stenotic aortic valves were collected from patients undergoing aortic valve replacement and they were fixed immediately in the operating room in 10% formalin.

The mean age of our patients in the rheumatic group was (28 ± 8.6 years) with male to female ratio of 3:2. On the other hand, the mean age of the patients in the degenerative group was (65.3 ±7.4 years) with male to female ratio of 2.33:1 .

Echocardiography:

Pre-operatively all patients were subjected to complete echocardiographic study using commercially available machines. The studies were recorded on videotapes for revision off-line.

M-mode and 2D were done in standard parasternal long axis, short axis and apical four and five chamber views. The aortic valve morphology was assessed with characterization of the severity and extent of the pathological process in patients with aortic valve disease. The anatomic abnormalities of the stenotic aortic valve were determined as thickening, calcification, fusion of the commissures and restriction of the leaflet motion. The left ventricular internal dimensions, wall thickness, FS and EF were measured according to the recommendations of the American Society of echocardiography. [10]

Color Doppler examination was done to assess the degree of aortic stenosis :[11]

Indicator	Mild	Moderate	Severe
Jet Velocity m/s	Less than 3	3 -4	>4
Mean Gradient (mm Hg)	<25	25-40	>40

Aortic regurgitation was assessed by color Doppler echocardiography and graded to mild, moderate and severe according to the following parameters:

Jet area/LVOT area [12]

<20%→mild, 20-40%→moderate, 40-60%→moderate to severe, >60%→severe.

Tissue processing and staining:

All explanted valve specimens were fixed in 10% formalin, subsequently; the valves were embedded in paraffin. Paraffin-embedded sections were subjected to:

1- **Haematoxylin and Eosin (H&E) staining** for routine microscopy.
2- **Masson's trichrome staining** to highlight fibrotic changes. The procedure of Masson's trichrome staining was performed according to Bancroft and Gamble (2002). [13]

3-Immunohistochemistry:

Immunohistochemistry was performed on paraffin-embedded 3-5µ sections according to avidin-biotin-peroxidase complex (ABC) method. [14] Briefly, sections were deparaffinized with xylene and rehydrated with graded alcohol series. Antigen retrieval was done by immersing the sections in 10 m mol /L citrate buffer (pH 6.0) for 10 minutes at 100˚ C in microwave. Endogenous peroxidase activity was blocked with H_2O_2 (0.6% in methanol). After thorough washing of the sections with phosphate buffered saline, incubation was done for 30 minutes with non-specific blocking reagent "normal goat serum" to prevent non-specific binding. Subsequently, an overnight incubation of the sections with polyclonal rabbit antihuman Bax antibody (Bax-A3533 polyclonal rabbit antihuman-Dako) was done at a dilution 1:1000 at room temperature in a humidity chamber. The sections were then washed with PBS and the following steps were performed:

1. Incubation with biotinylated secondary antibody for 30 minutes.
2. Incubation with avidin-biotin-peroxidase complex solution for 30 minutes.
3. The reaction products were visualized using 3-3`-diamino-benzidine-tetra-hydrochloride (chromogen).
4. Sections were then counterstained with Mayer's haematoxylin, dehydrated in alcohol and mounted in DPX.

The germinal centers of a reactive lymph node were used as positive controls. Negative controls were prepared by omission of the primary antibody.

Immunohistochemical analysis of Bax immunostaining:

Bax positivity was indicated by cellular brownish cytoplasmic staining. A semiquantitative immunostaining analysis was done in which the percentage of cells positive for Bax was determined and graded as follows: **0:** 0%–5%, **1:** 6%–25%, **2:** 26%–50%, **3:** 51%–75%, and **4:** 76%–100%, and the intensity of Bax staining was graded as follows: **0:** None, **1:** Weak, **2:** Moderate, and **3:** Intense staining. An **immunoreactive score (IRS)** [15] was calculated by multiplying the grade of percentage of positive cells by the grade of intensity of staining. In cases of heterogeneous staining intensities within a sample, each component was scored independently and the results

were summed. The cases were categorized according to their IRS into the following groups:
Group I: IRS 1 - <3, **group II:** IRS 3 - <6, **Group III:** IRS 6 - <9, and **group VI:** IRS 9-12. The mean and the standard error of the mean of the scores of each of the degenerative group and the rheumatic group were calculated and the data were expressed as mean± standard error of the mean.

Results:

Pathological findings:

Macroscopic findings:

Inspection of the aortic valves during surgical excision indicated that all the stenotic valves were obviously thickened and irregular. Rheumatic valves were tricuspid with variable degree of commissural fusion and calcification. Heavy calcification was noticed in 3 cases (30%). On the other hand, 9 cases (90%) of the degenerative valves were tricuspid with no commissural fusion while only one patient (10%) had fusion of one commissure. Heavy calcification was observed in 8 cases (80%).

Histopathological findings:

Histopathological examination of the studied cases revealed that both rheumatic and degenerative stenotic aortic valves displayed the same histopathological picture in the form of subendothelial thickening, abundant interstitial fibrosis (Fig. 1), infiltration by inflammatory cells mainly lymphocytes and macrophages (Fig. 2), and foci of dystrophic calcification ranging from minute foci to extensively calcified nodules (Fig. 2,3&4).
The only differentiating point on the histopathological level was the presence of numerous newly formed capillary-like sprouts (neovascularization) with irregularly-sized lumens within the cusps of the degenerative stenotic aortic valves which were more evident close to the areas of calcification (Fig. 4). These new capillary sprouts were observed in 8 cases (80% of cases) of degenerative aortic stenosis while they were not frequently observed in the studied rheumatic stenotic aortic valves as they were observed only in 2 cases (20% of cases).
Fibrosis within the studied valves was highlighted by the Masson's trichrome stain, the collagen fibers showed green color (Fig. 5).

Immunohistochemical analysis of Bax immunostaining in cases of degenerative aortic valve stenosis:

Bax immunohistochemistry studies of specimens of degenerative aortic valve stenosis revealed that all the studied valves showed positive Bax immunostaining, which was predominantly detected in the cytoplasm of the interstitial fibroblasts "especially in areas adjacent to calcification" (Fig. 6&7) as well as the endothelial cells of the new capillary sprouts present in the valvular interstitium and the perivascular cells (Fig. 8&9).

Eight cases (80% of cases) showed Bax positivity in both sites (interstitial fibroblasts and endothelial cells), while two cases (20% of cases) showed Bax positivity only in the interstitial fibroblasts. No difference in the staining intensity was observed between fibroblasts and new-capillary vascular endothelial cells. None of the studied cases showed Bax positivity in the valvular surface endothelium (Fig. 10).

Bax-positive cases of degenerative aortic valve stenosis were categorized according to the Bax immunoreactive score (IRS) as follows (Table 1):

-Three cases (30% of cases) were group I (IRS 1 - <3).
- Four cases (40% of cases) were group II (IRS 3 - <6).
- Two cases (20% of cases) were group III (IRS 6 - <9).
- One case (10% of cases) was group IV (IRS 9 - <12).

Immunohistochemical analysis of Bax immunostaining in cases of rheumatic aortic valve stenosis:

Bax immunohistochemistry studies of specimens of rheumatic aortic valve stenosis revealed that all the studied valves showed positive Bax immunostaining, which was predominantly detected in the cytoplasm of the valvular surface endothelial cells "8 cases, 80%" (Fig. 11&12), interstitial fibroblasts "7 cases, 70%" (Fig. 11,12&13), and the new-capillary vascular endothelial cells "2 cases, 20%" (Fig. 14).

The intensity of staining was higher in the valvular surface endothelial cells lining the valve leaflets than both the interstitial fibroblasts (Fig. 11&12) and the new-capillary endothelial cells.

Bax-positive cytoplasmic remnants of fragmented fibroblasts were seen adjacent to the calcified areas (Fig. 15).

Bax-positive cases of rheumatic aortic valve stenosis were categorized according to the Bax immunoreactive score (IRS) as follows (Table 1):

-Six cases (60% of cases) were group I (IRS 1 - <3).
-Four cases (40% of cases) were group II (IRS 3 - <6).

Table 1: Bax immunoreactive score (IRS) in both degenerative and rheumatic aortic valve stenosis.

Bax immunoreactive score (IRS)	Degenerative valves n=10		Rheumatic valves n=10	
	n	%	n	%
Group I (IRS 1 - <3)	3	30	6	60
Group II (IRS 3 - <6)	4	40	4	40
Group III (IRS 6 - <9)	2	20	-	-
Group IV (IRS 9 - <12)	1	10	-	-
Mean±SE	5.3±0.94		3.2±0.49	

Clinical and echocardiographic findings:

Diagnosis:

The diagnosis of the 20 patients who underwent aortic valve replacement is illustrated in Table 2.

Table 2: Echocardiographic diagnosis of the study population

Diagnosis/ Echocardiography	Degenerative valves n=10	Rheumatic valves n=10
Severe AS	10	6
Severe AS+ mild AR	-	4
Mean transvalvular gradient	91.3±6.7 (mmHg)	80.7±6.4 (mmHg)

Relation between echocardiographic parameters and pathological findings:

It was observed that echocardiographic examination underestimated the degree of fibrosis and calcification in the excised aortic valves. However, there was a general trend of matching between the two different methods i.e. patients with higher jet velocity and mean transvalvular gradient showed higher degree of fibrosis, calcification and vascularization.

Discussion:

Many studies to date have concentrated on elucidating the similarities between rheumatic and non-rheumatic aortic valve stenosis, while explanatory studies explaining the observed discrepancies are lacking. We

believe that continuous study of the disease process in aortic stenosis will provide important information on the treatment of valvular heart disease.

Calcification:

In addition to fibrosis, calcification is a defining feature of aortic valve lesions. Aortic valve stenosis characteristically progresses due to cuspal calcification, often necessitating valve replacement surgery. For nearly a century, the mechanical failure of calcified heart valves was attributed to a passive process. But now, it has been shown unequivocally to be an active, rather than a passive, process. Valvular calcium deposits contain both calcium and phosphate as hydroxyapatite, [16] the form of calcium-phosphate mineral present in both calcified arterial tissue [17] and bone.

In stenotic aortic valves, we have shown a positive immunoreactivity to a pro- apoptotic marker (Bax) in both endothelial cells and valve fibroblasts. Calcific deposits were frequently observed in association with the apoptotic fibroblasts. These findings reiterate the results of previous investigators who demonstrated that, initiation of apoptosis of valvular interstitial cells was a mechanistic event in cuspal calcification. They showed that TGF-B1 was involved in this process. [18]

However, a major difference between rheumatic and degenerative valves was evident. Positive Bax immunoreactivity was demonstrated only in surface endothelial cells of the rheumatic valves while fibroblasts in both rheumatic and non-rheumatic valves were positive. This paradox suggests that the underlying disease processes determine which type of cells predominantly undergoes apoptotic changes.

Based on the above finding, our results provide circumstantial evidence that apoptosis in rheumatic valves may play an important role in the alterations of endothelial integrity. It is possible that this will lead to increased filtration of calcium into the deeper layers of the valve tissues. Then, the cellular degradation products and organelles extruded from the apoptotic interstitial cells provide the substrates for calcium binding with progressive development of calcification in the valve tissue.

Interestingly, this observation may explain a common macroscopic and echocardiographic finding, i.e. early commissural fusion in rheumatic valves and sparing the commissures in degenerative stenotic aortic valves. The

involvement of surface endothelium and stronger staining intensity in these cells compared to the interstitial fibroblasts indicate a more active disease process along the rheumatic valve surface. This may elicit an intense inflammatory response and initiate the development of calcific deposits in a coaptation pattern (along the line of cusp coaptation) leading to early commissural fusion in rheumatic valves.

Angiogenesis:

Another finding in this study was the presence of areas of neovascularization in 80% of cases of degenerative aortic valve stenosis compared to 20% of cases in rheumatic valves. Such areas have previously been described in both rheumatic and degenerative heart valves. [1, 4]

The process of angiogenesis is thought to involve a stereotypical cascade of events, based mainly on study of development, pathologies and on extensive literature studying in vitro systems. [19] It is generally believed that new vessels are derived from the invasion of tissues by new capillary beds made of the proliferation of adjacent capillary and venular endothelium. [20] It is assumed, but has not been established, that this cascade holds true for physiological angiogenesis. In addition, there is evidence that different patterns of angiogenesis can occur in vivo when the mechanical environment, both inside and outside vessels, is changing. [21] It has also been suggested that the recruitment of interstitial fibroblasts may also participate in angiogenesis. [22]

The role of angiogenesis in the pathogenesis of aortic stenosis remains under investigation. It is possible that these areas of neovascularization are a response of tissue to injury as they are known to be associated with wound healing in general [23] or they result from autoimmune process. Rheumatoid arthritis, an autoimmune disease, is known to have such areas [24] and rheumatic heart disease is considered to have an autoimmune etiology. [25] In addition, Olsson et al 1994, [26] postulated that immune response plays an important role in the progression of degenerative aortic stenosis.

Previous investigators suggested that mineralization of rheumatic cardiac valve tissue is similar to skeletal bone formation that is associated with neoangiogenesis. [1] Mohler et al 2001 [27] demonstrated the association of angiogenesis to ossification occurring in degenerative aortic valves.

Of note, our results showed the presence of neovessels in the vicinity of calcified areas in degenerative stenotic aortic valves that are similar to those distributed in relation to an atherosclerotic plaque. However, studies supporting similarities between calcific aortic valve disease and atherosclerosis have produced, at best, circumstantial evidence without providing clear evidence of a direct causative pathway. Barger et al 1984 [28] supposed that neovessels are essential for growth of atherosclerotic lesion and may be contributing to the morbidity and complications of the disease process. The hypothesis of Barger was buttressed in a paper published by the group of Folkman. They showed that inhibition of angiogenesis by endostatin caused a reduction of atherosclerotic plaque growth, suggesting a direct role of angiogenesis in the progression of atherosclerotic plaque. [29] Similar implications could be made for the role of angiogenesis in the pathogenesis of degenerative aortic stenosis.

Our study showed that the newly-formed vessels in aortic valves are abnormal with irregularly-sized lumens and multi-layered in some sections especially in degenerative aortic valves. Such abnormalities have been previously described in tumors and these vessels were highly permeable. [30] We postulate that an increased permeability of those abnormal vessels could result in exposing a calcific deposit or valvular tissue to many cytokines and growth factors that normally are confined to the plasma, and through this indirect mechanism stimulate fibrosis and calcification or increase the size of calcific lesions.

Survival of vascular endothelium:

Once new vessels have assembled, the endothelial cells become remarkably resistant to exogenous factors, and are quiescent, with survival measured in years. Diminished endothelial survival-or endothelial apoptosis is characterized by vascular regression. [31] The list of factors identified that regulate endothelial apoptosis is extensive, [32] and these vary considerably according to the development time point, the specific site, function and type of vessel, in addition to surrounding physiological and/or pathological stimuli.

Molecular mechanisms implicated in mediating cell cycle arrest and survival of vascular endothelial cells include several factors involved in regulation of cell cycle and apoptosis such as p53,p21 and Bax. [33]

In this study, positive pro-apoptotic Bax immunoreactivity was detected in the cytoplasm of endothelial cells of the new-capillary sprouts present in the valvular interstitium and the perivascular cells.

Although the endothelium has received the most attention in angiogenesis research, the surrounding peri-endothelial cell layers are critical for ongoing structural and functional support of the vascular network. Vascular smooth muscle cells stabilize nascent vessels by inhibiting endothelial migration and proliferation. Indeed vessels regress more easily when not covered by smooth muscle cells in case angiogenic stimuli become limiting. [34] A similar phenomenon of neovascularization and subsequent regression of newly formed vessels has been observed in tumor vessels. [35]

We think that apoptosis of endothelial cells of single layered vessels and/or apoptosis of perivascular cells will interfere with integrity and increase the permeability of these new vessels and ultimately lead to vessel regression. This will lead to inclusion of a new area of the valve in the pathologic process (fibrosis and/or calcification). This seems to be predominant in degenerative aortic stenosis rather in rheumatic aortic stenosis.

Clinical implications:

As results from studies on the pathogenesis and progression of aortic valve stenosis emerge, targeted pharmacotherapeutic regimens to interfere with the disease pathways to either slow or halt the disease process are being proposed. Clinical implementation of pharmacological regimens will require rigorous validation in experimental models and prospective intervention trials, as well as from retrospective databases.

Retrospective studies have demonstrated strong associations between statin use and decreased risk of progression of aortic valve calcification.[36] In addition, Thompson 1995 [37] suggested that members of the Bcl-2 family involved in apoptosis, could provide ideal targets for therapeutic intervention. Promising results were obtained using several agents in a variety of cardiovascular apoptotic models. [38] Also, the therapeutic goal, that is to mitigate against angiogenesis during pathological processes, has become realizable at the clinical level. [39]

Our data provide new insights into the mechanisms of rheumatic valvular disorders and open new perspectives for prevention of progression and treatment of rheumatic aortic stenosis.

Until benefits of potential pharmacological therapies are well established, conventional treatment of aortic valve stenosis should be guided by conventional recommendations. These include diligent clinical follow-up to monitor for symptoms onset, with surgical valve replacement as the preferred option of treatment.

Conclusions:

Our results confirm that apoptosis contributes to calcification of stenotic aortic valve cusps. However, the interplay of endothelial cells and fibroblasts apoptosis in the pathogenesis of rheumatic and degenerative aortic stenosis seems to be different. Further studies are mandatory in order to clarify the mechanism for the initiation of apoptotic process in the endothelial cells and fibroblasts.

Understanding the role of apoptosis and angiogenesis in the pathogenesis of both rheumatic and degenerative aortic stenosis offers the potential to develop targeted therapeutic regimens.

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