Structural and biomolecular changes in aorta and pulmonary trunk of patients with aortic aneurysm and valve disease: implications for the Ross procedure

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Abstract

Objectives: A higher incidence of pulmonary autograft dilatation is assumed in patients with ascending aortic dilatation and bicuspid aortic valve disease. To examine whether structural abnormalities are present in the ascending aorta as well as in the pulmonary trunk (PT) we specifically addressed molecular mechanisms and signalling pathways for aneurysm formation in ascending aortic aneurysms and PT of patients with different aortic valve pathology undergoing an extended Ross procedure.

Methods: Wall segments resected from aortic aneurysms (20 patients, 7 bicuspid aortic valves BAV, and 13 tricuspid aortic valves TAV) and from PTs were submitted to analysis of leukocyte infiltration (immunohistochemistry), smooth muscle cell (SMC) apoptosis (in situ end-labelling of DNA-fragments TUNEL), and expression of death-promoting proteins perforin, granzyme B, Fas/FasL (immunoblotting).

Results: Degenerative changes including rarefication and apoptosis of SMCs were significantly more severe in BAV than TAV disease (apoptotic index 9.2 ± 3.2 vs. 11.9 ± 6.2, \( P = 0.02 \)). Immunohistochemistry confirmed presence and activation of death-promoting mediators in aneurysmal tissue whereas pulmonary tissue displayed only few apoptotic cells, occasional Fas + cells, rarely colocalized with FasL. By Western blot analysis extracts from BAV and TAV but not pulmonary artery wall contained appreciable amounts of perforin, granzyme B, and Fas/FasL. Conclusion: Aneurysm formation is associated with SMC apoptosis and local signal expression of activated cells in patients with bicuspid as well as TAV. The PT itself is not pathologically involved with only minor degenerative changes. Although the disease process in the aorta appeared to be more severe in patients with BAV, there was similarity of histological and molecular changes of the pulmonary artery wall in all patients. Dilation of the pulmonary autograft seems not to be the result of histopathological and biomolecular mechanisms in the PT.

Keywords: Bicuspid aortic valve; Apoptosis; Aneurysm; Ross procedure

1. Introduction

The superior hemodynamic performance of the pulmonary autograft in the aortic position is increasingly recognised. At present the technique of complete aortic root replacement has become the preferred method of performing the Ross procedure. A number of studies have demonstrated that the pulmonary root arranges with the pressure and flow demands of the aortic root. A significant proportion of patients undergoing pulmonary autograft aortic valve replacement presents with congenital bicuspid aortic valve (BAV). A higher incidence of pulmonary autograft dilatation is assumed in patients with ascending aortic dilatation and BAV disease [1]. Although histological abnormalities of the ascending aorta and pulmonary trunk (PT) in patients with BAV disease have been described [2],


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available data on the pathogenetic basis and the causal relationship of dilation of the pulmonary autograft after the Ross procedure are controversial [3].

In this study we investigated whether the BAV is associated with an underlying pathological process in the aorta and in the pulmonary artery, which may have implications for the Ross procedure. Our experiments were designed to specifically address molecular mechanisms and signalling pathways for aneurysm formation in ascending aortic aneurysms and in the PT of patients with different aortic valve pathology.

2. Material and methods

2.1. Aneurysmal aortic and nonaneurysmal pulmonary trunk tissue

From 1996 to 2003, 94 patients with a mean age of 42.7 ± 9.8 years, range 18–65 years, underwent the Ross procedure at the University Hospital Regensburg. During the same period 20 (21%) of the cases included repair of an ascending aortic dilatation (diameter 3.5–4.9 cm) or ascending aortic aneurysm (diameter 5.0–7.4 cm). Aortic dilatation was treated by a vertical aortoplasty (n = 14). Patients who presented with an aneurysm had an interposition Dacron graft replacement of their ascending aorta (n = 6). Table 1 depicts the clinical profile of these patients. For comparison, normal ascending aortic and main pulmonary artery wall segments were obtained during multiorgan harvesting from five organ donors, who had no evidence for aneurysmal or atherosclerotic disease (average age 47.8 ± 8.8 years, range 31–57 years).

<table>
<thead>
<tr>
<th>Sex</th>
<th>TAV (n = 13)</th>
<th>BAV (n = 7)</th>
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</thead>
<tbody>
<tr>
<td>Male</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>Female</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Age (years; range)</td>
<td>49.8 ± 9.4 (42–65)</td>
<td>36.1 ± 6.3 (18–56)</td>
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</table>

<table>
<thead>
<tr>
<th>New York Heart Association</th>
<th>TAV (n = 13)</th>
<th>BAV (n = 7)</th>
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<tr>
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<tr>
<td>Functional class II</td>
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<tr>
<td>Functional class IV</td>
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<td>–</td>
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<tr>
<td>Left ventricular ejection fraction (%; range)</td>
<td>51.4 ± 10.7</td>
<td>46.4 ± 6.5</td>
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<td>Aortic valve pathology</td>
<td></td>
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<tr>
<td>Stenosis</td>
<td>9</td>
<td>5</td>
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<tr>
<td>Insufficiency</td>
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<td>2</td>
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<tr>
<td>Mixed lesion</td>
<td>3</td>
<td>–</td>
</tr>
<tr>
<td>Ascending aorta diameter (CT-scan) (mm; range)</td>
<td>58.4 ± 11.2 (35–54)</td>
<td>61.3 ± 13.0 (37–74)</td>
</tr>
</tbody>
</table>

Full thickness aortic and PT wall specimens were obtained during the surgical procedure from seven patients presenting with bicuspid and from 13 patients presenting with tricuspid aortic valves (TAV). Tissue samples were excised just distal to the sinotubular junction from the anterior wall of the ascending aorta and PT, resp. The average age of patients with a BAV was 36.1 ± 6.3 years (range 18–56 years), and of patients with a TAV was 49.8 ± 9.4 years (range 42–65 years). Fresh tissue was divided and either immediately snap frozen in liquid nitrogen or paraffin embedded for immunohistochemistry, detection of apoptotic cells, and immunoblot isolation. Institutional review board approval and written informed consent was obtained before sample collection.

2.2. In situ detection of apoptotic cells by the TUNEL assay

Apoptosis is characterized by cell shrinkage, chromatin margination, membrane blebbing and nuclear condensation. DNA fragmentation in apoptotic cells is followed by cell death. In order to quantitatively examine the occurrence of nuclear DNA fragmentation, we used the TUNEL (in situ end-labelling of DNA fragments) assay with an ApopTag detection kit (Intergen).

Principles of the procedure comprised formalin-fixed, paraffin-embedded tissue sections, rehydration and proteinase K pre-treatment (20 μg/ml). Incubation with 3.0% hydrogen peroxide provided quenching of endogenous peroxidase. TdT (terminal deoxynucleotidyl transferase) enzyme was added to label DNA strands. After repeated washing anti-digoxigenin peroxidase conjugate was applied to the specimen and incubated. Nuclei staining were performed with 0.5% methyl green, and counter-staining with peroxidase substrate diaminobenzidine resulted in brown coloured condensed nuclei in apoptotic cells. Finally specimens were mounted and viewed under light microscopy. For each specimen, at least four sections aiming at 100 cells were analysed, and the counts were averaged. The cells with nuclear labelling were defined as TUNEL-positive cells. Apoptotic index was calculated as percentage of TUNEL-positive cells using the formula:

apoptotic index = 100 × (number of TUNEL+ cell nuclei/ number of total cell nuclei)

2.3. Immunohistochemical techniques

Immunohistochemical stains of aortic and pulmonary wall tissue were performed using mouse primary monoclonal antibodies directed against T cells (CD3), B cells (CD20), macrophages (CD68), smooth muscle cells (SMC) (HHF35), obtained from Dako, and for perforin, Fas/FasL, and granzyme B, obtained from Transduction Laboratories. Paraffin-embedded sections (6 μm) were prepared and slide mounted. After rehydration
and pre-treatment with target retrieval high pH solution (Dako) and 0.3% H₂O₂ to block endogenous peroxydase, sections were incubated for 30 min in a blocking solution of 10% normal horse serum and then stained with a panel of monoclonal antibodies. After washing in tris-buffered saline incubation was performed with biotin-conjugated anti-mouse IgG antibody (Camon) for 60 min at room temperature. Bound antibodies were then recognized through avidin–biotin-complex formation by an avidin–alkaline phosphatase-fast reagent (Vectastain ABC kit, Vector). Normal mouse IgG (Sigma Chemical) served as the control for the immunostains. In each section, 100 cells were examined by two independent investigators.

2.4. Western blotting

Expression of candidate mediators of cell death (Fas, perforin, granzyme B) was analysed by Western blotting. Vascular wall tissue samples were snap-frozen in liquid nitrogen, crushed and mixed with 0.5 ml of SDS protein extraction buffer (10% SDS, 20 mmol/l NaCl, 100 mmol/l Tris–HCl, pH 7.6). Centrifugation at 13 000 rpm was performed for 20 min at 4 °C. After collection of the supernatant, protein concentration was determined using BSA as a standard. For determination of activated Fas aggregate a 7.5% SDS-PAGE gel, otherwise a 12.5% SDS-PAGE minigel was used. The samples (30 µg protein/lane) were separated by electrophoresis for 1.25 h at 100 V. After transfer of the proteins to a membrane, the membranes were blocked with 5% low fat dried milk dissolved in TBS (5 g/100 ml) and incubated for 12 h at room temperature. After proper washing of the membranes with TBS subsequent incubation in HRP (horseradish peroxidase)-labelled secondary antibody (anti-mouse IgG) was performed for 1 h. The washed membranes were evaluated with a light emitting nonradioactive enhanced chemiluminescence Western blotting kit as described by the manufacturer’s instructions (ECL™, Amersham, Montreal, Canada). Densitometric quantification of the immunoblots was performed using an automated image scanner (Sharp JX-330) supported by relevant software (Imagemaster 1D Elite 2.0, Amersham Biosciences).

2.5. Statistical analysis

All of the results are representative of at least two experiments performed repeatedly. Continuous variables are presented as mean ± one SD. Statistical differences between groups were evaluated by use of the unpaired t-test or the Mann–Whitney test when indicated. Categorical variables were analysed by the Fisher exact test. P values less than 0.05 were considered significant.

3. Results

3.1. Ascending aorta and pulmonary trunk histological features

Follow-up is complete for all patients, and ranges from 3 to 91 months. Dilatation of the reconstructed ascending aorta or aortic root has not been demonstrated by postoperative echocardiographic evaluation performed annually. In agreement with our earlier results degenerative as well as inflammatory changes were present in aneurysmal aortic tissue of patients with bicuspid and TAVs [4]. In contrast, the media of PT wall segments was normally dominated by immunoreactive α-actin positive vascular SMCs. SMCs were well arranged within orderly-organised layers of elastic laminae. Although hypocellular areas depicting cell fragmentation and disruption were present both in BAV and TAV aneurysmal aortic tissue the severity of histological changes was most pronounced in patients with BAV. In order to quantify a reduction in the absolute number of SMCs in the aneurysmal wall, the number of nuclei per cross-sectional area was determined and expressed as percent of values for healthy aorta as described previously [4]. This calculation demonstrated a 23% decrease in the nuclei per unit area in TAV aneurysms, and a 36% decrease in BAV aortic tissue, respectively, when compared to normal aorta (P = 0.03). The PT was not dilated in any of the patients studied. There was no difference in nuclear density in PT wall between the TAV and BAV group. In addition, values in pulmonary arterial samples from patients with diseased aorta (BAV and TAV) were not statistically different from samples from organ donors with nondiseased aorta.

3.2. Immunohistochemical stains

The relative numbers of mononuclear cells present in the intima and media of the vascular walls that were positive for T cells (CD3), B cells (CD20), macrophages (CD68), SMCs (HHF35), perforin, Fas/FasL, and granzyme B were graded from 0 to 3 + (none to greatest) as has been described by Fox et al. [5]. As seen in Table 2, CD3- and CD20-positive mononuclear cells were identified by antibody staining in the media of aortic aneurysms but not in the pulmonary vessel walls. Mononuclear cells were localised in proximity to endothelial cells as well as within the media of aortic wall irrespective of aortic valve morphology. Fas/FasL- and perforin-positive cells were identified in the media of nine of thirteen of the aneurysm specimens derived from TAV-patients, and in all seven specimens derived from segments of BAV-aneurysms. Pulmonary tissue displayed very few Fas + cells, which were only occasionally co-localized with FasL.
3.3. Smooth muscle cell apoptosis

The TUNEL method detects apoptotic cells containing fragments of genomic DNA in their nuclei. We employed this method to analyze apoptosis in arterial tissues derived from ascending aortic aneurysms and PT. In aneurysmal tissue, the majority of TUNEL $^{+}$ cells appeared in the media, and cells demonstrating TUNEL positive nuclei were predominantly SMCs. Determination of the apoptotic index displayed significantly increased numbers of dead SMCs in aneurysmal sections from BAV patients when compared to TAV patients (BAV: AI = 11.9 ± 6.2; TAV: AI = 9.2 ± 3.2; P = 0.02). In contrast to the aorta samples, TUNEL stained few cells in the tunica media of pulmonary vascular segments. The overall level of TUNEL $^{+}$ cells was < 3% (AI = 2.34 ± 1.4). These data indicate that apoptosis occurred at a limited rate in the pulmonary artery wall of our patients (Fig. 1).

3.4. Expression of death-promoting molecules

Levels of Fas/FasL, perforin, and granzyme B proteins in extracts of ascending aorta and pulmonary artery wall were assessed by means of immunoblotting. PT tissue exhibited only insignificant levels of these candidate mediators of cell death. Consistent with the results of immunohistochemistry there was definite activation of Fas/FasL, granzyme B, and perforin in the tissue of diseased aorta of BAV and TAV patients. Extracts from BAV and TAV aorta contained appreciable amounts of perforin, granzyme B, and Fas/FasL by Western blot analysis (Fig. 2). Further studies evaluated the intensity of the protein bands on Western blotting membranes by densitometry. When the results of densitometric analysis for Fas were plotted against granzyme B for all tissue samples we were able to discriminate two distinct groups of patients. One group depicted only low intensity for Fas and granzyme B whereas the other group demonstrated elevated levels for both proteins. The group with limited expression of Fas and granzyme B represented...
pulmonary artery tissue samples. (Fig. 3). Finally there was a well defined subgroup of specimens, which was characterized by the highest levels of Fas as well as of granzyme B activation. These tissue samples originated from ascending aorta of patients with BAV disease.

4. Discussion

For patients with aortic valve disease requiring surgical valve replacement, there is the option of mechanical, biological, and autologous valve substitutes. Among the array of implants for aortic valve replacement the pulmonary autograft has proven benefits such as viability, avoidance of anti-coagulation, ability to grow, and prospect of definite repair [6]. A significant number of patients undergoing autograft aortic valve replacement presents with a congenital BAV. Pathology studies have clearly demonstrated an association between BAV and aortic medial abnormalities suggesting a common underlying developmental disease or genetic predisposition [7,8]. With respect to a common embryonic origin of the aortic and pulmonary roots, the conotruncus [9], the pulmonary root, which is completely transferred when an aortic root replacement technique is performed, may potentially be also involved in wall abnormalities. Moreover, a BAV is frequently associated with dilatation of the ascending aorta, irrespective of the functional status of the valve. Dilatation of the pulmonary autograft is assumed to be the most common cause of failure of the Ross procedure [1,2].

In our study we examined histological features of aneurysmal ascending aorta and main pulmonary artery of patients undergoing an extended Ross procedure. In a previous study we have described that degenerative and inflammatory changes in the ascending aorta were more severe in patients with BAV disease than in patients with tricuspid valve disease [4]. The most striking finding in the present study was that the histopathologic features seen in the ascending aorta were not present in pulmonary artery tissue layers derived from patients with BAV or TAV disease. We were able to characterize the level of α-actin, a highly conserved protein and major component of both the cytoskeletal and contractile apparatus, as a marker of structural deterioration. The decrease in cellularity in aortic tissue may originate either from reduced presence of SMCs expressing a contractile phenotype or from mediators that induce apoptosis in association with infiltration of T lymphocytes and macrophages [10]. Consistent with those findings was a significant leukocyte infiltration in sections derived from aneurysmal aorta whereas pulmonary artery segments demonstrated normal major structural and cellular elements. Another important finding of our study was the demonstration of evident SMC apoptosis and of expression of cell death-promoting mediators only in aortic tissue. The low level of inflammatory cells in pulmonary artery medial layers may have resulted in a lower expression of Fas/FasL, perforin, and granzyme B but cannot explain the almost complete absence of these proteins in segments from main pulmonary artery walls.

Our findings are in contradiction to observations by the Toronto group [1,3,11] who believe that dilatation of the pulmonary autograft is related to the histopathologic nature and degenerative changes in the PT especially in patients with a BAV. We were not able to document any association between morphological abnormalities in enlarged aorta and histological features in the pulmonary artery in either group of patients. Our findings are in line with the results of a clinico-pathological study performed Luciani et al. [3]. In their study they were not able to identify any correlation between autograft dilatation and pulmonary wall histology.

The potential biological processes involved in arterial wall remodelling are many, including SMC proliferation and rarefaction, net production of extracellular matrix, and migration of mononuclear cells [10,12–16]. SMC apoptosis is seen as the major process governing SMC numbers, and the reduction in SMCs in remodelling is mainly due to differences in apoptotic rates [17]. The biochemical hallmark of apoptosis is the fragmentation of genomic DNA; an irreversible event that commits the cell to die and occurs before changes in plasma membrane permeability, the so-called pre-lytic phase. We used in situ labelling of fragmented DNA (TUNEL stain) to analyze numbers of cells with DNA damage. TUNEL technique is a well-established and reproducible tool to detect apoptosis. Apoptosis is regulated by a complex interplay between cell surface signals and the expression of specific intracellular gene products. Thus, different pathways may be responsible for inducing apoptosis in SMCs, which finally activate a cascade of cysteine proteases (caspases). A powerful caspase-activating system is mediated by cytokine receptors of the tumor necrosis factor family, notably fas/ apo-1/CD95. These receptors, on receiving a death stimulus from binding their ligand, initiate a series of protein-protein interactions which eventually convert caspase 8 from its inactive to the active form [18]. Caspases appear to be present in most cells in inactive form awaiting activation by cleavage. Masson and colleagues [19] found that in the process of cell death channels are formed in target cell membranes by the pore-forming protein perforin that is secreted by T lymphocytes. Co-secreted granzyme B enters the target cell cytoplasm via these pores, triggers these latent proenzymes, and induces programmed disintegration of the target cell.

Mononuclear leukocyte infiltration of the aneurysmal aortic wall was the central histological feature of aneurysm formation in our study. There was evidence of more severe inflammatory processes in wall segments derived from patients with BAV- than with TAV-disease. We also observed a concomitant up-regulation in Fas- and granzyme B expression in specimens from aneurysmal aorta. These findings are in line with observations performed by Pascoe and associates [20] that complete agreement with clinical
substrates is only obtainable when raised levels of perforin, granzyme B, and Fas-ligand are encountered simultaneously.

Our observations of SMC rarefaction, inflammatory infiltration of leukocytes and expression of death-promoting mediators in BAV and TAV aneurysmal sections but not in pulmonary artery tissue provide biochemical evidence for restriction of the remodelling alterations to the aortic side. To our knowledge, there are with respect to pulmonary autograft aortic valve replacement only histological but not biomolecular observations on tissue derived from aorta and PT.

The present data support the view that the pathogenesis of aneurysm formation may depend on expression of cell death-promoting proteins by infiltrating immune cells. Lack of Fas/FasL-, perforin, and granzyme B expression in pulmonary artery tissue verify the hypothesis that structural and biomolecular changes of the ascending aorta in patients with TAV, and specifically with BAV disease, are not present in the pulmonary artery trunk at the time of the Ross procedure. Based on the finding that the PT is not pathologically affected by this process of vascular remodelling [21,22], autograft dilatation following autograft aortic valve replacement seems not to be the result of SMC apoptosis and expression of cell death-initiating proteins. Further studies should be performed to offer more insights into the pathogenesis and inherent molecular defects of the native PT in patients with a BAV that regulate the disease process at the molecular level.

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References