Aortic valve grafts in the rat: evidence for rejection.

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Objective: The histopathologic changes of fresh rat aortic valve allografts over time and the effect of cryopreservation were examined. Methods: Fifty-six syngeneic and allogeneic rat aortic valves were transplanted, either fresh or after cryopreservation, and then at different time points they were explanted and histologically examined in a blinded fashion. Results: Histopathologic changes in the first week are similar in syngeneic and allogeneic grafts. Fresh syngeneic grafts and leaflets retained normal structure up to 56 days. Allogeneic grafts showed retrovalvular thrombus formation with leaflet ghosts and neointimal proliferation. Cryopreservation did not alter this process. Conclusions: Cardiac allograft valves in the rat model undergo changes that are characteristic of cell-mediated rejection and lead to valve failure. (J Thorac Cardiovasc Surg 1997;114:891-902)

A number of studies have demonstrated the superiority of cryopreserved aortic valve allografts compared with other bioprosthetic valves over long-term follow-up in regard to durability and low incidence of valve-related complications.1-4 Despite their advantages, however, aortic valve allografts usually fail in the aortic position, the effective life of the average aortic homograft in an adult being about 15 years.5 In children valve failure occurs faster for reasons that remain unknown.5,6 The cause of homograft failure in children and adults is most likely multifactorial and includes mechanical, immunologic, and other factors. In particular, the role of immune-mediated rejection in the failure of allograft heart valves in human beings is uncertain.7-9 Although there is clear evidence of a donor-specific systemic immune response to allograft aortic valve implants in human beings and rats, the effects of such a response are unclear.9,10 Short retrieval to cryopreservation times that would be expected to maximize viability and therefore antigenicity have been associated with increased failure of allograft valves in children.11 The use of heart valve transplants between syngeneic and allogeneic strains of rats permits investigations into the role of immune-mediated rejection of these grafts. Evidence suggesting an immunologic basis has been presented by numerous authors.12-16 This study describes the pathologic changes in rats, over time, after transplantation of syngeneic versus allogeneic and fresh versus cryopreserved valves and identifies changes indicative of rejection. These changes could be in the form of either an acute or chronic rejection process. Acute rejection is microscopically characterized by a lymphocytic infiltrate. Chronic rejection has been described in human and animal solid organ transplants and in the rat aortic allograft model and includes persistent perivascular inflammation, generalized concentric intimal thickening, and medial necrosis.17,18 An in-depth examination of the pathologic changes and mechanisms underlying valve failure may help to define the relative role, time sequence, and magnitude of the main components of an immunologic reaction. This would have important implications for the choice and timing of therapeutic interventions aimed at modifying this response.

Materials and methods

Animals. Lewis and Brown Norway rats (Harlan Sprague Dawley, Inc., Indianapolis, Ind.) weighing 225 to 300 gm were used. Different strain combinations were used with all recipient rats being Lewis and donor rats
Fig. 1. Longitudinal section through the leaflet and myocardial cuff of a fresh syngeneic rat aortic valve graft at 2 days. Beginning myocellular necrosis and mild infiltration with polymorphonuclear neutrophils (arrow) is noted. (Original magnification ×100.)

Table I. Number of rats in each experimental group at each time point

<table>
<thead>
<tr>
<th>Group</th>
<th>Days after transplant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>Fresh syngeneic</td>
<td>1</td>
</tr>
<tr>
<td>Fresh allogeneic</td>
<td>3</td>
</tr>
<tr>
<td>Cryopreserved syngeneic</td>
<td>—</td>
</tr>
<tr>
<td>Cryopreserved allogeneic</td>
<td>—</td>
</tr>
</tbody>
</table>

being either Lewis (syngeneic) or Brown Norway (allogeneic). Grafts were transplanted either fresh or cryopreserved, so that there were four experimental groups: fresh syngeneic, fresh allogeneic, cryopreserved syngeneic, and cryopreserved allogeneic. All animals were housed and fed ad libitum at the Dalhousie University Animal Care Center in accordance with the guidelines of the Canadian Council of Animal Care. 19

Surgical technique. Aortic valve transplants were performed as described by Yankah, Wottge, and Muller-Ruchholtz. 13 An intraperitoneal injection of sodium pentobarbital, 65 mg/kg, was used to anesthetize the rats. None of the animals received antibiotics or heparin at any time.

Donor operation. The donor aortic valve was removed with a cuff of ventricular muscle and the ascending aorta. The coronary arteries were tied with a 10-0 nylon suture (Ethilon, Ethicon Inc., Somerville, N.J.). One cusp of the valve was sutured to the aortic wall to render the valve incompetent and prevent thrombosis. Any excess myocardial muscle was removed from the valve. The donor graft was then placed in cold isotonic saline solution before transplantation for the fresh experiments or cryopreserved as described. Both the donor and recipient operations were performed with a Weck OM-1206 operating microscope (Pilling Weck, Research Triangle Park, N.C.) under 7× to 10× magnifications.

Recipient operation. The abdominal aorta was mobilized from the inferior vena cava from the level of the left renal artery almost down to the aortic bifurcation and divided between two vessel clamps. The aortic valve allograft was then interposed between the stumps of the recipient aorta with a continuous 10-0 nylon suture.

Cryopreservation. Valves were used immediately after harvesting (fresh) or after 2 to 8 weeks of cryopreservation. Cryopreservation was performed with the use of standard clinical protocols. The explanted aortic valve allografts were incubated in Hanks balanced salt solution containing gentamicin (80 mg/L) and cefazolin (1 gm/L) and stored at 4°C for 24 hours. The valves were then removed from this solution, cryopreserved in Hanks balanced salt solution with 10% dimethylsulfoxide, and pack-

Table II. Preserved leaflets seen at different time points

<table>
<thead>
<tr>
<th>Group</th>
<th>28 days</th>
<th>56 days</th>
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<tbody>
<tr>
<td>Fresh syngeneic</td>
<td>7/7</td>
<td>3/5</td>
</tr>
<tr>
<td>Fresh allogeneic</td>
<td>8/8</td>
<td>0/6*</td>
</tr>
<tr>
<td>Cryopreserved syngeneic</td>
<td>2/5</td>
<td></td>
</tr>
<tr>
<td>Cryopreserved allogeneic</td>
<td>0/6†</td>
<td></td>
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</tbody>
</table>

Data shown are grafts with preserved leaflets/total successful grafts.
*Leaflet ghosts were seen in five of six grafts.
†Leaflet ghosts were seen in all six grafts.
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Fig. 2. Fresh syngeneic graft at 7 days showing massive infiltration of polymorphonuclear neutrophils in media (arrow) and adventitia. (Original magnification ×100.)

Table III. Schematic representation of the histologic appearance of the aortic valve allograft at different time points

<table>
<thead>
<tr>
<th>Grafts</th>
<th>Intimal thickening</th>
<th>Media necrosis</th>
<th>Perivascular inflammation</th>
<th>Preserved leaflets</th>
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<tbody>
<tr>
<td>FS 2 days</td>
<td>0</td>
<td>0</td>
<td>0→+</td>
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</tr>
<tr>
<td>FA 2 days</td>
<td>0</td>
<td>0</td>
<td>0→+</td>
<td>Yes</td>
</tr>
<tr>
<td>FS 7 days</td>
<td>0</td>
<td>+→++</td>
<td>+</td>
<td>Yes</td>
</tr>
<tr>
<td>FA 7 days</td>
<td>0</td>
<td>+→++</td>
<td>+</td>
<td>Yes</td>
</tr>
<tr>
<td>FS 28 days</td>
<td>0→+, focal</td>
<td>0</td>
<td>0</td>
<td>Yes</td>
</tr>
<tr>
<td>FA 28 days</td>
<td>+→++</td>
<td>+→++</td>
<td>+</td>
<td>Yes</td>
</tr>
<tr>
<td>FS 56 days</td>
<td>+→++</td>
<td>0→++, focal</td>
<td>0</td>
<td>Yes</td>
</tr>
<tr>
<td>FA 56 days</td>
<td>+→++</td>
<td>++</td>
<td>+</td>
<td>No, leaflet ghosts</td>
</tr>
<tr>
<td>CS 56 days</td>
<td>++</td>
<td>+++</td>
<td>0</td>
<td>Yes</td>
</tr>
<tr>
<td>CA 56 days</td>
<td>+→++</td>
<td>+</td>
<td>0</td>
<td>No, leaflet ghosts</td>
</tr>
</tbody>
</table>

F, Fresh; C, cryopreserved; S, syngeneic; A, allogeneic; 0, none; +, mild; ++, moderate; ++++, severe.

aged separately in pouches (Kenpak Convertors Incorporated, City of Commerce, Calif., and Kapak Corporation, St. Louis Park, Minn.). The packaged aortic valves were placed in the precooled freezer chamber of a programmable controlled-rate freezer (Planer KRYO 10, Diamed Laboratory Supplies, Mississauga, Ontario, Canada) and cooled at a controlled rate of −1°C/min to −5°C/min to a temperature of −80°C, and then labeled and stored at below −135°C (Revco Ultralow Freezer, Rheem Manufacturing Company, Asheville, N.C.). For transplantation the packaged cryopreserved valve was thawed in a water bath at 37°C for 10 minutes, removed from the package, and implanted.

Experimental groups. The animals were put to death for explantation and histologic examination of the grafts at different time points after transplantation according to Table I. These times were chosen on the basis of previous experience in mouse and rat aortic allografts in which we20 have shown that chronic rejection is evident by 28 days after transplantation and well established by 56 days. Surgical failures include death of the animal before the time of planned death, obliteration of the graft at explantation, and aneurysms at the anastomotic site of the graft. These were excluded from analysis.

Histology. The grafts were explanted with a short segment of adjacent recipient aorta and immediately fixed in 10% buffered formalin solution for at least 24 hours. With the use of a syringe and a No. 25 plastic cannula, 10% buffered formalin was gently injected into both ends of the explanted aortic graft to remove any air and ensure proper fixation. The leaflet area was marked with India ink on the outside of the graft. The grafts were then embedded in paraffin and completely sectioned through with 4 μm thick sections and stained with hematoxylin-eosin. We used
Fig. 3. A, Horizontal cross section of a fresh syngeneic graft at 28 days showing preserved normal leaflets (arrow). (Original magnification ×20.) B, Fresh syngeneic graft at 28 days showing normal structure of preserved leaflet, normal media, and adventitia with mild, focal intimal thickening (arrow). (Original magnification ×100.)

longitudinal sections for the 2- and 7-day grafts and horizontal sections for the 28- and 56-day grafts.

Analysis. Histopathologic examination was performed by two pathologists blinded to the original protocol and study design. All layers of the aortic valve transplants were systematically examined.

Results

Surgical results. Of 56 animals subjected to transplantation, 54 survived the planned time period until they were put to death. When the animals were put to death, 45 grafts were patent and acceptable for histopathologic assessment, six grafts were obliterated, and three had aneurysms. No differences were seen in complications by comparison of cryopreserved grafts with fresh grafts.

Histologic results. Tables II and III summarize the histopathologic findings of the rat aortic valve allografts at the examined time points.

Fresh syngeneic grafts. At 2 days in the fresh syngeneic aortic valve grafts (Fig. 1) the myocardial cuff showed evidence of beginning myocellular necrosis. There was a mild infiltration of the myocardial cuff and adventitia with polymorphonuclear neutrophils (PMNs). The media looked normal. No
intima was present. The leaflet was preserved but lost its endothelium. At 7 days (Fig. 2) there was an extensive necrosis of the myocardial cuff. Concomitantly, a massive infiltration of PMNs and macrophages throughout the myocardial cuff, adventitia, and media was present. The intima was lost completely and PMNs were adherent to the lumen. At 28 days in the fresh syngeneic grafts (Fig. 3, A and B) sections at the valvular level and at the more distal aortic level show the adventitia, media, and leaflet to be normal. Focal intimal thickening was present. The endothelium was generally present on the intima and leaflet. No retrovalvular thrombus was seen. At 56 days the fresh syngeneic grafts (Fig. 4, A and B) continued to show no changes in the adventitia. There were focal areas of medial cell loss of varying degrees, but the elastin layers were continuous. There was diffuse, but concentric intimal thickening, which was more prominent than at 4 weeks. The leaflets were thin and appeared viable and without significant changes. Both the leaflet and the neointimal proliferation were still covered with endothelium.
Fig. 5. Longitudinal section through the leaflet and myocardial cuff of a fresh allogeneic rat aortic valve graft at 2 days. Histopathology is similar to syngeneic counterpart. Again, beginning myocellular necrosis and mild infiltration with polymorphonuclear neutrophils (arrow) is noted. (Original magnification ×100.)

Fig. 6. Fresh allogeneic graft at 7 days showing preserved leaflet. Inflammatory cell infiltration of media and adventitia (arrow) is seen. (Original magnification ×100.)

Fresh allogeneic grafts. At 2 and 7 days in the fresh allogeneic aortic valve grafts the histopathologic characteristics do not differ significantly from those of the syngeneic graft (Figs. 5 and 6). At 28 days in the fresh allograft sections at the valvular level (Fig. 7, A and B) showed the adventitia to be densely infiltrated with mainly lymphocytes and macrophages. Areas of prominent vascularity in the adventitia were identified. Medial necrosis was variable but in many places full thickness. There was limited presence of inflammatory cells within the wall itself. The arrangement of spindle cells and elastic fibers appeared normal in the unaffected media. The changes in the media were far less prominent in the aortic conduit than in the aortic root. The aortic lumen showed retrovalvular thrombus development, with early organization of the thrombus in many cases. Luminal narrowing had
occurred. Focal areas of mild to moderate intimal thickening were present, consisting mainly of non-inflammatory cells and covering the leaflets on the luminal side. As yet there were limited changes to the leaflet itself other than edema, considerable, often complete loss of vitality of valvular stromal cells, and loss of the lining endothelium. At 56 days in the fresh allogeneic transplants (Fig. 8, A and B) the dense lymphocytic cell infiltrate in the adventitia remained. There was almost complete and diffuse medial necrosis. Free valve leaflets were not present. The aortic lumen showed complete retrovalvular thrombosis. The thrombus was now organized, and hemosiderin deposits and leaflet ghosts were seen within. Significant neointimal proliferation with luminal narrowing was seen between the leaflet ghosts and the lumen. Distally in the aortic conduit the intima was still moderately to markedly thickened, consisting of mainly spindle cells and a few inflammatory cells.

Cryopreserved syngeneic grafts. At 56 days cryopreserved syngeneic grafts (Fig. 9, A and B) showed an unremarkable adventitia. The media showed more extensive areas of medial necrosis than in the fresh

Fig. 7. A, Fresh allogeneic graft at 28 days showing preserved, but dysplastic leaflet (arrow). (Original magnification ×20.) B, Section through the dysplastic leaflet and sinus of Valsalva of a fresh allogeneic graft at 28 days showing retrovalvular thrombus development (arrow). (Original magnification ×100.)
syngeneic grafts at 56 days, and only focal areas of normal media were present. There was moderate and diffuse neointimal proliferation. Preserved leaflets were present. Both the leaflet and the neointimal proliferation were covered with endothelium. No retrovalvular thrombus was seen. In the aortic conduit neointimal proliferation was rather eccentric.

Cryopreserved allogeneic grafts. At 56 days cryopreserved allogeneic grafts (Fig. 10, A and B) showed a moderate to severe mononuclear cell infiltrate in the adventitia, consisting mainly of lymphocytes and macrophages. There was complete and diffuse medial necrosis. Free valve leaflets were not present but leaflet ghosts were identified in all grafts. The aortic lumen showed complete retrovalvular thrombosis. As in the 56-day fresh allogeneic grafts, the thrombus was organized with hemosiderin deposits. Between the leaflet ghosts and the lumen there was significant neointimal proliferation with luminal narrowing. In the aortic conduit there

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**Fig. 8.** A, In the fresh allogeneic graft at 56 days no preserved leaflets are seen. Marked concentric intimal thickening is present (arrow) with luminal narrowing. (Original magnification ×20.) B, Fresh allogeneic graft at 56 days showing leaflet ghost (small arrow) with neointimal proliferation toward the lumen (open arrow) and complete medial necrosis (large arrow). Lymphocytic infiltration of the adventitia persists. (Original magnification ×100.)
still was neointimal proliferation but less prominent and eccentric. Endothelium was seen occasionally.

**Discussion**

Homograft valves are commonly used in cardiac surgery, often in children and young adults. Unfortunately, they are prone to fail and require replacement, particularly in children. The precise cause of this failure is unknown, but one main area of research has been into the possible immunogenicity of these homografts. It has been shown that recipients of homovital, noncryopreserved homografts produce donor HLA-specific antibodies of the immunoglobulin G class and that recipients of cryopreserved homografts form immunoglobulin G- and T cell-mediated reactions to donor HLA antigens. However, it is unknown whether this alloreactivity ultimately results in the observed histopathologic changes that lead to valve allograft failure. This study demonstrates that aortic valve allografts in the rat model fail through retrovalvular thrombosis and neointimal proliferation. In addition, widespread medial necrosis and a lymphocytic adventitial infiltration are present in the allografts...
but not in the syngeneic grafts. These findings are characteristic of acute and chronic rejection and have been described in the rat aortic allograft model, which does not include the aortic valve. Retrovalvular thrombosis in the allograft could therefore represent one additional expression of alloreactivity. Potential effector mechanisms include secretion of cytokines, growth factors, and other factors by lymphocytes, endothelial cells, and smooth muscle cells, resulting in the up-regulation of cell adhesion molecules and leading to thrombosis. Already in the early time points there is a progressive, massive infiltration of the adventitia with PMNs in both the syngeneic and allogeneic grafts. This nonspecific inflammatory reaction is at least partly caused by the concomitantly transplanted myocardial cuff, which undergoes ischemic necrosis. We postulate that this PMN invasion then spills over to the media, resulting in medial cell loss again in both the syngeneic and allogeneic grafts,
which could be the source of release of cytokines, growth factors, and other factors. This results in intimal thickening in the syngeneic transplants, which was observed beginning at 28 days and progressing at 56 days. At each time point allogeneic grafts demonstrate more extensive neointimal proliferation than their syngeneic controls, as intimal thickening in the allogeneic transplants is a result of both nonspecific inflammatory response to the necrotic muscle cuff and ongoing allostimulus of the graft itself. The importance of the transplanted myocardial cuff has been referred to earlier. By 28 days the fresh syngeneic graft has regenerated its media, and one would expect that eventually intimal thickening in the syngeneic grafts should regress once the media is restored. However, we did not look at this beyond 56 days. In the allogeneic grafts the allostimulus persists and therefore the media does not regenerate but continues to mount an immune-mediated response, resulting in further medial necrosis and thus further intimal thickening. Syngeneic grafts are expected to not generate an immune response and survive indefinitely. The fact that we observed intimal thickening in our fresh and cryopreserved syngeneic grafts at 56 days leads us to believe that the ultimate histopathologic structure of this group of grafts has not yet been reached. Intimal thickening in syngeneic grafts has been briefly mentioned in the literature without an attempt at explanation.

Beyond the first 7 days, which were characterized by a nonspecific inflammatory response, and throughout all further time periods a chronic lymphocytic infiltrate persists in the adventitia of the allogeneic grafts. This has been described by others and could be another source of ongoing production of cytokines and other growth factors responsible for smooth muscle cell destruction, migration, or transformation, resulting in medial necrosis and neointimal proliferation.

Leaflet viability may be important for long-term allograft valve function. In this study the leaflets of fresh syngeneic grafts retained normal structure and cellularity for up to 56 days. Fresh allogeneic leaflets were dysplastic at 28 days and demonstrated an obliterative thrombosis in the sinus of Valsalva at 56 days. Cryopreservation did not alter the leaflet structure of syngeneic grafts nor did it prevent retrovalvular leaflet thrombosis in allogeneic grafts. However, cryopreserved syngeneic grafts showed more extensive medial necrosis than their fresh counterparts, which could be partly due to some cytotoxic effect of cryopreservation.

A criticism of this study is that it is a nonworking valve model, which may explain the thrombosis of the valves that was seen in the rats and is not seen in human beings. Nevertheless, the preservation of valve leaflets exclusively in the syngeneic rats is strong evidence of immune-mediated damage occurring only in the allogeneic grafts. We postulate that this thrombosis is a marker for such damage. In human allografts this injury may lead to loss of viability, which will ultimately cause the valve to fail.

This study demonstrates that aortic valve allografts in the rat model undergo histopathologic changes that are characteristic of both acute and chronic rejection. This rejection process ultimately leads to allograft valve failure via a progressive obliterative retrovalvular thrombosis that is destructive to the valve leaflets. Further studies are needed to determine whether similar events might be occurring in homografts and which mediators are involved. On the basis of these and other results, investigations into therapies to modify this response appear justified. This could be achieved either by tissue typing and matching donor and recipient or by subjecting the recipient to immune suppression for an unknown time period.

We gratefully acknowledge the financial support of the Izak Walton Killam Hospital for Children to this study. We thank Mr. Lewis Page, Mr. André Le Prairie, and members of the Regional Tissue Bank, Queen Elizabeth II Health Sciences Centre, Halifax, Canada, for their assistance with the cryopreservation. In addition we would like to thank Mrs. Mary Wile for her expertise with the histologic preparation.

REFERENCES

Discussion

Dr. Charles A. Yankah (Berlin, Germany). I would like you to differentiate between cellular and humoral rejection. The homograft conduit with myocardium around the root, depending on the cellular viability, might undergo cell-mediated rejection, as you said, or you will observe a humoral rejection with production of anti-endothelial cell antibodies. If you implant an acellular or nonviable allograft without any myocardial tissue, you might have a humoral type of rejection, which might be an ongoing process or a chronic process. Could you differentiate this type of rejection process in your study?

Dr. Moustapha. We tried to trim the myocardium as much as possible. With aortic grafts, that is very difficult because of the insertion of the leaflet. We were not able to transplant grafts without myocardium, so we were not able to differentiate that. We always had a minimal myocardial rim there.

Mr. Magdi Yacoub (London, England). My question is twofold. First, where did you put the grafts exactly? Were these anastomosed in the descending aorta? Do you think that site has something to do with it? If you had them in the subcoronary position, would that be different?

Second, which cells in the graft play a role in this process of rejection, particularly with regard to antigen presentation? Do you think that dendritic cells and/or endothelial cells are important, and what is the role of myofibroblasts? We have some preliminary data. We have early data to suggest important interaction between these cells. I would like to know your views.

Dr. Moustapha. With respect to your first question, these were heterotopic transplants into the abdominal aorta right underneath the renal vein and above the bifurcation.

With respect to your second question, which cell is important? What we saw were basically clinical signs of both acute and chronic rejection. I think that in the acute rejection phase, the lymphocytic infiltrate is the main thing. The lymphocytes, I think, are the dominating factor here.

With respect to the element that we saw with the chronic rejection, I think that it is mostly a combination of all of the elements that you mentioned. We saw that in the endothelium and the syngeneic grafts. In the allogeneic grafts, the endothelium was gone, so I think that plays a lesser role. But the myofibroblasts and the lymphocytes I speculate are the ones that are causing the chronic damage.