Effects of preservation techniques on in vivo expression of adhesion molecules by aortic valve allografts

Methods of sterilization and preservation of aortic valve allografts influence graft longevity. The effect of storage techniques on valve durability may be mediated by alterations in the immunologic properties of the allograft, which are reflected by expression of leukocyte adhesion molecules. Rat aortic valve grafts were transplanted in the fresh state, after cryopreservation (-196°C), or after storage at 4°C for 1 to 21 days. Syngeneic and strongly allogeneic valves were transplanted for 4 hours to 21 days and were retrieved for immunohistochemical staining for expression of leukocyte adhesion molecules. Unimplanted valves and transplanted syngeneic valves, regardless of storage methods, exhibited little or no expression of leukocyte adhesion molecules. Fresh allogeneic valves expressed all molecules, indicating up-regulation, at all time intervals studied. Cryopreserved allogeneic valves demonstrated no leukocyte adhesion molecules at 4 hours or 2 days and weak reactivity at 10 and 21 days. Allogeneic valves stored at 4°C , regardless of the duration of storage, demonstrated weak expression of all molecules at 10 days and strong expression at 21 days. Expression of leukocyte adhesion molecules requires an allogeneic environment and may precede immune-mediated injury. Reduced expression of leukocyte adhesion molecules resulting from storage may predict a diminished immunologic response. Cryopreservation (-196°C) causes the greatest delay and diminution of expression of leukocyte adhesion molecules. (J THORAC CARDIOVASC SURG 1994;107:717-23)

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Human valve allografts have been successfully used to treat a number of cardiac diseases. Improved methods of valve treatment and storage have improved both the availability and the longevity of these tissues. In some centers, allograft valves are stored at 4°C , and satisfactory clinical results have been reliably achieved.¹ More recently, cryopreservation of allograft valves has succeeded in improving the availability of these scarce resources. Cryopreservation may result in diminished endothelial

cellularity and in a reduction in the expression of class I surface antigens. These phenomena may be related, because endothelial cells constitute the major source of antigen presentation.² However, endothelial cells play an important role in preventing thrombus formation, inhibiting calcification, and providing nutrition to the fibroblasts of the allograft.³ Thus whether the loss of endothelial cells has a positive, negative, or neutral effect on long-term allograft structure and function remains unknown. In this regard, O'Brien and colleagues⁴ have observed superior long-term performance with cryopreserved allograft valves, surpassing even those results obtained in their prior experience using fresh allografts.

Expression of the leukocyte adhesion molecules by vascular beds predicts inflammatory response. Endothelium-leukocyte adhesion molecule-1 (ELAM-1), for example, is required in neutrophil-mediated lung injury in rats.⁵ Intercellular adhesion molecule-1 (ICAM-1) is expressed on canine cardiac monocytes⁶ and is a mediator of cardiac allograft rejection in cynomolgus monkeys.⁷

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In both acute cardiac allograft rejection and chronic kidney allograft rejection in rats there is up-regulation of ICAM-1 and increased ICAM-1 specific binding of lymphocytes.^{8,9} Expression of vascular cell adhesion molecule-1 (VCAM-1) is detected in murine cardiac allografts beginning 3 days after transplantation or 3 to 4 days before rejection is complete.^{10,11}

Previous studies investigating these three important molecules examined fresh tissues. Whether tissues stored for prolonged periods at 4° C or those subjected to cryopreservation express these molecules is unknown. The present studies were designed to examine the effects of preservation methods and immunologic differences on adhesion molecule expression by *in vivo* aortic valve grafts. Because adhesion molecule expression effects lymphocyte migration and accumulation,⁸ the modulation of adhesion molecule expression according to the selected method of graft preservation may affect the immune response. This may in turn affect long-term survival and function of valve allografts.

Materials and methods

Valve harvest, sterilization, and preservation. Male rats, 125 to 175 gm body weight, of the Lewis and Brown-Norway strains (strains that are strongly histoincompatible at both the RT1 and the non-RT1 loci) were subjected to general pentobarbital anesthesia. A median sternotomy was performed. The aortic valve with a short portion of ascending aorta was excised and rinsed in chilled, heparinized saline. Some valves were transplanted immediately. Other valves were placed in chilled saline, placed on ice, and transported to CryoLife, Inc. (Marietta, Ga.), for cryopreservation. At the time of implantation, the valves were thawed by immersion in 37° C saline and rinsed thoroughly before use. The remaining valves were placed in 4° C RPMI 1640 tissue medium containing 10% fetal calf serum and the following antibiotics: gentamicin (160 mg/L), piperacillin (1000 mg/L), oxacillin (50 mg/L), metronidazole (200 mg/L), and amphotericin B (100 mg/L). This combinations of antibiotics was selected on the basis of two previous studies, one from this laboratory, which showed that this solution resulted in endothelial viability without bacterial growth after as much as 3 weeks of storage at 4° C,¹² and one from Strickett, Barratt-Boyes, and MacCulloch,¹³ which characterized the tissue toxicity of other antibiotics previously used for allograft sterilization.¹³ After 24 hours, the tissues were transferred to RPMI 1640 culture medium with 10% fetal calf serum without antibiotics at 4° C. The tissue was maintained in this solution for 1, 3, 7, 10, 14, or 21 days. At the time of implantation, the valves were rinsed in 0.9% saline solution before use.

Operative preparation. Heterotopic transplantation of aortic valve allografts was performed as described originally by Yankah and colleagues.¹⁴ Male Brown-Norway rats, 175-250 gm body weight, underwent general anesthesia with sodium pentobarbital, 60 mg/kg body weight, administered by intraperitoneal injection. Rats underwent laparotomy and dissection of the abdominal aorta under a 14X operating microscope. The recipient aorta was occluded proximally and distally

and divided. An end-to-end interposition of the allograft was performed with 8-0 polypropylene suture. The anterior leaflet of the aortic valve was incorporated into the suture line to prevent valve competence, which may predispose to thrombosis. The abdomen was closed and the animal allowed to recover. Animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985).

Valve retrieval and preparation. The valves remained in place for 4 hours, 2 days, 10 days, or 21 days. After the designated interval the animals were again subjected to general anesthesia, the grafts were retrieved, and the animals were killed. The retrieved grafts were rinsed in saline and washed free of salts with distilled water. They were then embedded in Tissue-Tek O.C.T. (Miles Inc., Elkhart, Ind.) and frozen in liquid nitrogen. Sections were cut 8 µm thick and mounted on poly-L-lysine-coated slides for immunoperoxidase staining. Two or three valves of each strain, with each method of preservation, for each duration of implantation, were studied. Fifty sections of each specimen were stained and graded for evaluation in a blinded fashion.

Monoclonal antibodies. The antibodies used for immunoperoxidase staining were all monoclonal immunoglobulin G, and were directed against ELAM-1 (CL-3), ICAM-1 (1A29) or VCAM-1 (CL-348). CL-3 (a gift from C. W. Smith, Baylor College of Medicine, Houston, Tex.) was prepared by immunization of BALB/C mice with interleukin-1-stimulated endothelial cells from the human umbilical vein. After hybridoma formation and subcloning the antibody was found to be cross-reactive with interleukin-1-stimulated rat endothelial cells but not leukocyte populations.⁵ 1A29 (a gift from M. Miyasaka, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) was raised specifically against the rat ICAM-1 homolog as previously described.^{15,16} CL-348 (a gift from R. Lobb, Bogen Corporation, Cambridge, Mass.) was raised against the human antigen and was found to be cross-reactive in the rat.¹⁷⁻¹⁹

Frozen sections were mounted on poly-L-lysine-coated glass slides, fixed with methanol containing 0.3% hydrogen peroxide at room temperature for 30 minutes, and washed with phosphate-buffered saline. They were then incubated with CL-3, CL-348, and 1A29 for 45 minutes. For control studies, other sections were similarly incubated with a nonspecific immunoglobulin G (MOPC 21, mouse myeloma). The slides were washed with phosphate-buffered saline and stained for bound monoclonal antibodies with the Vectastain biotin/avidin-peroxidase system for mouse immunoglobulin G (Vector Laboratories Inc., Burlingame, Calif.). After hematoxylin counterstaining, sections were coated with Aquamount (Lerner Laboratories, Pittsburgh, Pa.) and examined by light microscopy for the presence of reaction products of peroxidase.

Results

The results of the immunohistochemical staining studies are summarized in Tables I to III. Unimplanted valves, regardless of storage methods, were not reactive for ICAM-1, VCAM-1, or ELAM-1 (Fig. 1). Syngeneic valves that were transplanted in the fresh state did not stain for the adhesion molecules at the 4-hour or 2-day

Fig. 1. Allogeneic valve implanted after cryopreservation and retrieved after 2 days, stained for ELAM-1, and counterstained with hematoxylin. Absence of reddish-brown coloration indicates absence of ELAM-1. ($\times 220$.)

Table I. Expression of adhesion molecules by untransplanted aortic valve grafts

	Storage method						
	Fresh	Cryopreserved	4° C×1 day	4° C×3 days	4° C×7 days	4° C×14 days	4° C×21 days
ELAM-1	—	—	—	—	—	—	—
ICAM-1	—	—	—	—	—	—	—
VCAM-1	—	—	—	—	—	—	—

+, Positive staining for indicated adhesion molecule; —, absence of staining for molecule; $\pm$, weak or minimal staining for molecule.

Table II. Expression of adhesion molecules by transplanted syngeneic aortic valve grafts

Duration implanted	Storage method						
	Fresh	Cryopreserved	4° C $\times$ 1 day	4° C $\times$ 3 days	4° C $\times$ 7 days	4° C $\times$ 14 days	4° C $\times$ 21 days
4 Hours							
ELAM-1	—	—	—	—	—	—	—
ICAM-1	—	—	—	—	—	—	—
VCAM-1	—	—	—	—	—	—	—
2 Days							
ELAM-1	—	—	—	—	—	—	—
ICAM-1	—	—	—	—	—	—	—
VCAM-1	—	—	—	—	—	—	—
10 Days							
ELAM-1	—	—	—	—	—	—	—
ICAM-1	$\pm$	—	—	—	—	—	—
VCAM-1	—	—	—	—	—	—	—
21 Days							
ELAM-1	$\pm$	—	—	—	—	—	—
ICAM-1	$\pm$	—	—	—	—	—	—
VCAM-1	$\pm$	—	—	—	—	—	—

+, Positive staining for indicated adhesion molecule; —, absence of staining for molecule; $\pm$, weak or minimal staining for molecule.

intervals. At 10 days there was only minimal reactivity for ICAM-1 and no reactivity for ELAM-1 or VCAM-1. By 21 days the fresh syngeneic valves demonstrated minimal reactivity for all three adhesion molecules. Cryopreserved

syngeneic valves and syngeneic valves stored at 4° C for any duration showed no reactivity for ICAM-1, ELAM-1, or VCAM-1 at any of the time points examined.

Allogeneic valves that were transplanted in a fresh state

Table III. Expression of adhesion molecules by transplanted allogeneic aortic valve grafts

Duration implanted	Storage method						
	Fresh	Cryopreserved	4° C×1 day	4° C×3 days	4° C×7 days	4° C×14 days	4° C×21 days
4 Hours							
ELAM-1	+	—	—	—	—	—	—
ICAM-1	+	—	—	—	—	—	—
VCAM-1	+	—	—	—	—	—	—
2 Days							
ELAM-1	+	—	—	—	—	—	—
ICAM-1	+	—	—	—	—	—	—
VCAM-1	+	—	—	—	—	—	—
10 Days							
ELAM-1	+	±	±	±	±	±	±
ICAM-1	+	+	±	±	±	±	±
VCAM-1	+	—	±	±	±	±	±
21 Days							
ELAM-1	+	±	+	+	+	+	+
ICAM-1	+	+	+	+	+	+	+
VCAM-1	+	±	+	+	+	+	+

+, Positive staining for indicated adhesion molecule; —, absence of staining for molecule; ±, weak or minimal staining for molecule.

showed strong reactivity at all time points examined for ELAM-1, ICAM-1, and VCAM-1 (Fig. 2). When allogeneic donor valves underwent cryopreservation before implantation, there was delayed and diminished expression of all three adhesion molecules. In contrast to the freshly transplanted allografts, cryopreserved allografts showed no reactivity at 4 hours or 2 days for any of the adhesion molecules. Staining for ELAM-1 did not become positive until 10 days after transplantation and even then it was relatively weak. By 21 days, the staining for ELAM-1 had decreased considerably. Moderate staining for ICAM-1 was evident at 10 days and persisted at 21 days. Staining for VCAM-1 was not evidenced until 21 days after transplantation and even then was minimal. The allogeneic valves stored at 4° C showed a uniform response for all durations of storage. No adhesion molecules were expressed at 4 hours or 2 days, all were expressed weakly at 10 days, and all were expressed strongly at 21 days. Inflammatory infiltrates were observed in grafts in proportion to the intensity of adhesion molecule expression (Fig. 3).

Discussion

The immunologic stimulus caused by an allogeneic mismatch appears capable of inducing adhesion molecule expression by heterotopically transplanted aortic valves. The present studies further indicate that subjecting donor valves to a period of cryopreservation delays and suppresses the regulation of the endothelial adhesion molecule. This could result from loss of endothelial cells from the donor grafts after preservation. Previous investigations from this laboratory have shown that cryopreserved

human arteries and valves retain identifiable endothelial cells in only 16% of specimens examined.²⁰ Loss of donor endothelium may increase the possibility that host endothelium ultimately resurfaces the donor valve. Other models have shown host endothelial repopulation of allograft vasculature.^{21, 22} Because endothelial antigens present the primary immunologic stimulus, such an effect of cryopreservation may delay adhesion molecule expression (while endothelial surfaces are regenerated). Furthermore, reendothelialization by host endothelial cells could render the valves less immunogenic, and adhesion molecule expressions would presumably be diminished. This proposed sequence of events would agree precisely with the results seen in our studies after cryopreservation.

Regardless of the mechanism, the attenuation of adhesion molecule expression by the application of preservation techniques may diminish immune-mediated destruction of transplanted valves. Endothelial cells express adhesion molecules when stimulated by cytokines, thereby stimulating leukocyte adherence (Fig. 4). This observation is supported by recent findings wherein monoclonal antibodies to various adhesion molecules have been used to protect against acute inflammatory injury. ELAM-1 is required for the development of lung injury and the accumulation by neutrophils in lungs after the deposition of immune complexes.⁵ VLA-4 is the ligand for VCAM-1, and infusion of an anti-VLA-4 inhibits adherence of rat T-cells to VCAM-1 on stimulated endothelial cells. Anti-VLA-4 also selectively inhibits migration of lymphocytes enriched in T memory cells into sites of cutaneous and joint inflammation.²³ CD18 is the common β -subunit of the B₂ integrin adhesion mol-

Fig. 2. Allogeneic valve implanted fresh and retrieved after 2 days, stained for ELAM-1, and counterstained with hematoxylin. Reddish-brown coloration indicates presence of ELAM-1. ($\times 220$.)

Fig. 3. Fresh allogeneic valve implanted for 10 days. An acute inflammatory infiltrate is demonstrated. ($\times 190$.)

ecules. Two of the B_2 integrins, LFA-1 and MAC-1, are recognized by ICAM-1. Murine monoclonal antibodies to CD18 and ICAM-1 in various animal models have also demonstrated that the CD18 integrins and ICAM-1 are

required for leukocyte accumulations at inflammatory sites.^{24, 25} Anti-ICAM-1 has been used to prolong renal and cardiac allograft survival in monkeys.⁷

The importance of immunologic differences between

Fig. 4. Vascular endothelial cells when stimulated by tumor necrosis factor, interleukin-1, or other cytokines express the adhesion molecules ELAM-1, VCAM-1, and ICAM-1. The ligands for these molecules include LAM-1, MAC-1, and LFA-1, which are found on neutrophils, and VLA-4, which is found on monocytes.

donor and recipient on the structure and function of aortic valve allografts remains uncertain. Current clinical practices regarding allograft use place little emphasis on tissue typing of donors and recipients, immunosuppression of allograft recipients, or avoidance of allografts in previous allograft recipients. Studies in patients with ABO blood group-compatible valves compared with those with incompatible valves have demonstrated no differences in allograft longevity. Nevertheless, it is the opinion of many practitioners that ABO matching is preferred if circumstances permit.²⁶

Evidence that allograft immunogenicity may affect graft function has included isolated reports of accelerated calcification in recipients of multiple allografts. This evidence is supported by experimental studies from this and other laboratories that observed a positive correlation between immunologic differences and allograft calcification.^{27,28} If immunologic differences are important in determining the pathologic fate of allograft valves, it is possible that methods of harvest, storage, and preservation should be tailored to minimize such differences. Cryopreservation or storage at 4° C may both be acceptable methods for doing so. The outstanding results that have been reported with cryopreserved valve allografts may in part be a reflection of more profound immunologic alterations.

In summary, ELAM-1 has been demonstrated for the first time to be up-regulated in transplanted tissues. VCAM-1 and ICAM-1, which have previously been observed in whole organ transplants, are observed in

allograft valves as well. The expression of each of these adhesion molecules by allograft valves is attenuated by clinically used methods of graft storage. This attenuation of inflammatory mediators may in turn blunt the host response to the valves, perhaps improving graft longevity.

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