Transpl Int (2000) 13 [Suppl 1]: S528–S531

F.B.S. Oei
M.J.P. Welters
L.M.B. Vaessen
R.L. Marquet
P.E. Zondervan
W. Weimar
A.J.JC. Bogers

Heart valve dysfunction resulting from cellular rejection in a novel heterotopic transplantation rat model

F.B.S. Oei (✉) · A.J.JC. Bogers
Departments of Thoracic Surgery,
Room Bd. 158,
University Hospital Rotterdam-Dijkzigt,
P.O. Box 2040, 3000 CA Rotterdam,
The Netherlands
e-mail: oei@inw1.azr.nl,
Tel.: + 31-10-4635420,
Fax: + 31-10-4635430

M.J.P. Welters · L.M.B. Vaessen · W. Weimar
Department of Internal Medicine,
University Hospital Rotterdam-Dijkzigt,
3000 CA Rotterdam, The Netherlands

R.L. Marquet
Department of Experimental Surgery,
University Hospital Rotterdam-Dijkzigt,
3000 CA Rotterdam, The Netherlands

P.E. Zondervan
Department of Pathology,
University Hospital Rotterdam-Dijkzigt,
3000 CA Rotterdam, The Netherlands

Abstract Structural failure of heart valve allografts may be related to technical factors or immunological reactions. To circumvent nonimmunological factors a new rat implantation model was developed to study whether alloreactivity results in histopathological changes and valve dysfunction. Syngeneic (WAG-WAG, DA-DA) and allogeneic (WAG-BN, WAG-DA) transplantation was carried out using this new technique, and the function of explanted valves was assessed 21 days later by retrograde comptence testing. Additionally, grafts were examined using standard histological and immunohistochemical techniques. There was no leakage during retrograde injection in nine of tem syngeneic and two of ten allogeneic grafts. Microscopically, syngeneic valves appeared normal without fibrosis or intimal thickening, although $CD8^+$ lymphocytes and macrophages were found in necrotic myocardial rim and adventitia. In contrast, allogeneic valves were deformed and noncellular, with extensive infiltration of $CD4^+$, $CD8^+$ and $CD68^+$ cells in adventitia and media. Absence of fibrosis and intimal thickening in syngeneic transplanted valves indicated circumvention of nonimmunological factors. Allogeneic valve transplantation induces cellular infiltration in the graft with subsequent graft failure.

Key words Implantation model · Aortic valves · Valve dysfunction · Rejection · Rat

Introduction

Human aortic valve allografts (AVA), as used in valve replacement surgery, were successfully introduced in the 1960s [11]. To improve the clinical performance and durability of these AVAs different methods of decontamination and storage have been evaluated [1]. The rationale is to preserve viable fibroblasts in these valves. Fibroblasts are needed for maintenance of the extracellular matrix in the valve, thereby preventing structural valve deterioration. O'Brien et al. introduced cryopreservation as a method for viable storage of heart valves and experienced good clinical results [9]. On the other hand, implantation of human heart valves with viable cells could evoke a specific immune response, which could be the reason for early graft failure, which is often observed in children [2].

Several experimental and clinical studies have demonstrated the immunogeneicity of AVAs, even after cryopreservation [4, 13]. Yet, it is still unclear whether, or to what extent, activation of the immune system by AVA implantation is responsible for valve allograft failure. To investigate the relationship between the immune response and the degeneration of AVAs, an adequate in vivo model is required. Current in vivo transplantation models in the rat include heterotopic interposition of the AVA in the abdominal aorta [3, 10, 12]. To prevent local thrombosis one valvular cusp has to be rendered

incompetent, so that washout of the sinuses of Valsalva is ensured. The adjustment of the valvular anatomy and the mismatched anastomosis, owing to the circumferential difference between the aortic annulus and the abdominal aorta, may create both surgical trauma and flow turbulence. As a result, accelerated valvular fibrosis and intimal proliferation may occur [6]. Therefore, the current AVA transplantation models are not suitable for evaluating the effect of the alloreactivity on the morphology and function of the AVA. The aim of the present study is to evaluate a new heterotopic aortic valve implantation technique as a suitable model to study transplantation of AVAs without the bias of size mismatch or surgical trauma. This new implantation technique allows the valve to maintain the normal mobility of the three valve cusps and therefore includes the possibility of analysing the valve competence as a measure of valve function. With this model we were able to investigate the histological and functional consequences of the immune response to the MHC-mismatched donor AVA, without technical bias.

Materials and methods

Animals

Male inbred Brown Norway (RT1^n/RyHSD), DA (RT1^a/RyHSD) and WAG (RT1^u/RyHSD) rats (Harlan CPB, Horst, The Netherlands) weighing 200–250 g, were used. All animals received food and water ad libitum. The experimental protocols were approved by the Committee on Animal Research of the Erasmus University of Rotterdam, The Netherlands and complied with the *"Principles of Laboratory Animal Care"* (1985).

Donor procedure

The heart of each anaesthetised donor rat (WAG or DA) was removed, followed by dissection of the U-shaped aortic valve conduit. The graft, consisting of the myocardial rim, ascending aorta, aortic arch and descending aorta, was flushed with heparinised saline (50 U/ml). After ligation of the coronary and jugular arteries with 8–0 nylon sutures (Ethicon, Sommerville, N.J.), the grafts were stored in cold heparinised saline until transplantation.

Recipient procedure

After ether anaesthesia, midline laparotomy was performed. The infra-renal part of the abdominal aorta was dissected and cross-clamped with a modified mosquito clamp. Two incisions were made for the end-to-side anastomoses using continuous 9–0 monofilament nylon sutures (Ethicon) (Fig. 1). The native abdominal aorta segment was ligated with 6–0 silk sutures (Ethicon) to complete the bypassing blood flow through the graft.

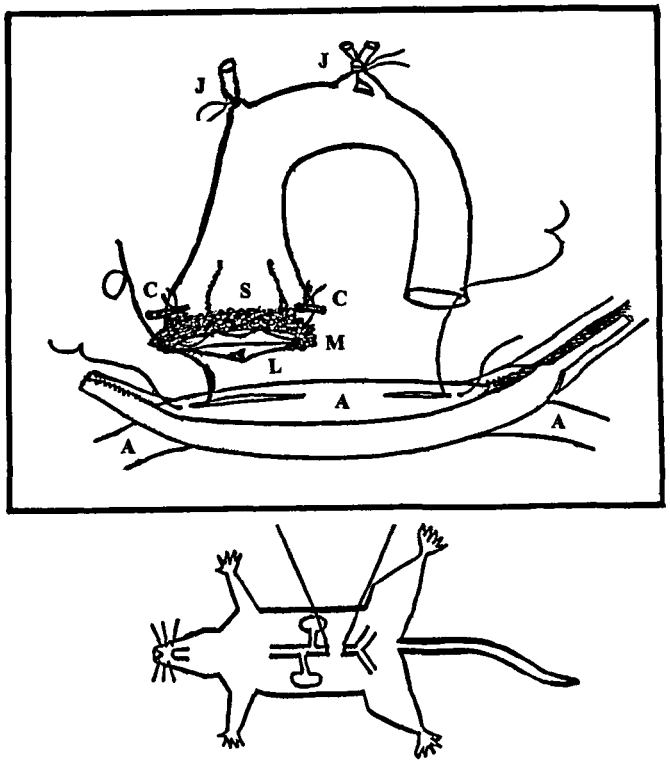

Fig. 1 In the new "Rotterdam" model the normal anatomy of the aortic arch is used to create a U-shaped graft. The jugular arteries (*J*) and coronary arteries (*C*) are ligated before implantation. After completion of the proximal and distal end-to-side anastomosis, the recipient abdominal aorta (*A*) will be ligated to ensure the bypass (*M* myocardial rim, *L* anterior mitral valve leaflet, *S* sinuses of Valsalva)

Study design

To exclude rat strain variability, WAG to BN or WAG to DA allogeneic and WAG to WAG or DA to DA syngeneic transplantations were performed. There were five animals in each group. After implantation, the patency of the graft was checked twice weekly by palpation of the abdomen. All rats were sacrificed on postoperative day 21 and the AVA removed. Qualitative functional examination of the graft was performed by retrograde injection of saline. Subsequently, the grafts were dissected longitudinally in three symmetric pieces, each containing one valve cusp, and prepared for histological and immunohistochemical evaluation.

Histology

After fixation in 10% buffered formalin solution for at least 24 h and embedding in paraffin, longitudinal sections of 4 μm were cut, followed by staining with standard haematoxylin-eosin.

Immunohistochemistry

After embedding in Tissue-Tek (Miles Diagnostic Division, Elkhart, Ind.) and frozen in liquid nitrogen, 5-μm cryosections were stained by the three-layer immunoperoxidase technique as de-

Table 1 Surgical procedures, morphology and competence of the aortic valve graft after 21 days of transplantation (*Sy* syngeneic, *Al* allogeneic, *DP* donor procedure time, *IT* heart valve implantation time, *VC* valve competence, *PI* perivascular inflammation, *Throm* retrovascular thrombus, *CD4*$^+$ cells stained positively with mouse anti-rat antibody W3/25, *CD8*$^+$ cells stained positively with mouse anti-rat antibody Ox8, *CD68*$^+$ cells stained positively with mouse anti-rat antibody ED1, – none, + mild, + + severe, *nd* not determined, *A* adventitia, *I* intima, *M*, media, *PVL* preserved valve leaflets)

Grafts	Combination	DP	IT	VC	PVL	PI	Throm	CD4$^+$	CD8$^+$	CD68$^+$
Sy/Al 1	WAG-WAG/WAG-BN	15/15 min	50/45 min	Yes/No	Yes/Def	–/++	–/–	–/I + A	–/I + M + A	–/I + M + A
Sy/Al 2	WAG-WAG/WAG-BN	20/20 min	40/40 min	Yes/No	Yes/Throm	–/++	–/+	nd/A	nd/M + A	nd/M + A
Sy/Al 3	WAG-WAG/WAG-BN	15/10 min	40/45 min	Yes/Yes	Yes/Def	+/++	–/–	–/A	A/A	A/M + A
Sy/Al 4	WAG-WAG/WAG-BN	10/15 min	35/50 min	Yes/No	Yes/Throm	+/++	–/+	–/nd	A/nd	A/nd
Sy/Al 5	WAG-WAG/WAG-BN	15/20 min	40/50 min	Yes/No	Yes/Def	–/++	–/–	nd/A	nd/A	nd/M/A
Sy/Al 6	DA-DA/WAG-DA	20/15 min	50/45 min	Yes/No	Yes/Def	–/++	–/–	nd/nd	nd/nd	nd/nd
Sy/Al 7	DA-DA/WAG-DA	20/15 min	45/50 min	Yes/Yes	Yes/Def	+/++	–/–	nd/nd	nd/nd	nd/nd
Sy/Al 8	DA-DA/WAG-DA	15/10 min	40/60 min	No/No	Throm/Throm	+/++	+/+	nd/nd	nd/nd	nd/nd
Sy/Al 9	DA-DA/WAG-DA	15/10 min	40/50 min	Yes/No	Yes/Def	–/++	–/+	nd/nd	nd/nd	nd/nd
Sy/Al 10	DA-DA/WAG-DA	15/15 min	50/45 min	Yes/No	Yes/Def	–/++	–/+	nd/nd	nd/nd	nd/nd
Mean	5/5/5/5	20 min	47.5 min	Yes: 9/2	Yes: 9/0	None: 6/0	None: 9/5			

scribed previously [5]. The primairy antibodies (Serotec, Oxford, UK) used were: W3/25 (CD4; dilution 1:300), ED1 (CD68; dilution 1:200), Ox8 (CD8; dilution 1: 300), and an irrelevant IgG as a negative control.

Analysis

The slides were microscopically examined (blinded) by two independent investigators. Each slide was scored for the presence of preserved valve leaflets, perivascular infiltration, retrovalvular thrombosis and inflammatory cells. For comparison, normal nontransplanted valves were evaluated.

Results

Transplantation procedure

The time needed to obtain and prepare the donor valve was comparable for all rats and ranged between 10 and 20 min, while the implantation procedure time varied from 35 to 60 min (Table 1). Routine physical examination showed continuous pulsations of the AVA in all the animals until the end of the study period (postoperative day 21), but functional testing of the explanted valves revealed one of the ten syngeneic and eight of the ten allogeneic valve leaflets to be incompetent (Table 1; Student t-test $P < 0.05$).

Explanted grafts

Macroscopically, nine of the ten syngeneic valve leaflets showed a normal morphology, whereas all allogeneic leaflets were shrivelled and dysmorphic (Table 1; preserved valve leaflets). Microscopic analysis showed major structural difference between the two groups (Table 1). The valve leaflets in syngeneic grafts appeared normal, and retrovalvular thrombosis occurred in only one rat. No early fibrosis or intimal proliferation was observed. Marginal cellular infiltrates were found in the myocardial rim and the adventitium of these syngeneic grafts (Table 1; perivascular inflammation). Immunohistochemical evaluation of three syngeneic AVAs showed (Table 1) that the cellular infiltration consisted mainly of macrophages (CD68$^+$) and some cytotoxic T cells (CD8$^+$). The allogeneic valve leaflets were deformed and noncellular as well as having lost extracellular matrix structure. Furthermore, in six of the ten cases, initial formation of retrovalvular thrombi was seen in the Valsalva sinuses. No thickening or infiltration of the tunica intima was seen, except in one explanted graft. In the allogeneic grafts studied immunohistochemically, the adventitia was extensively infiltrated with CD4$^+$, CD8$^+$, and CD68$^+$ cells (Table 1). Additionally, in these grafts the amount of elastin fibres in the media was reduced and CD68$^+$ cells were found between these fibres.

Discussion

The effect of the immune response on the morphological and functional changes to AVAs is still not clear and can only be studied in a suitable in vivo model. Previous heterotopic transplantation models in rats are not appropriate, because several nonimmunological factors mask the immune-specific morphological changes. In the present study, a new model for heterotopic implantation of AVAs in rats was used to determine structural and functional consequences of the antidonor immune response on the AVA transplanted across a MHC barrier.

A technical adaptation of the anastomotic mismatch (end-to-side anastomosis) made it possible to reduce the implantation time and the surgical trauma. Consequently, no peri- and postoperative complications were

observed. In addition, by adapting the size mismatch, the nonimmunological degeneration, characterized by early fibrosis and intimal proliferation in the syngeneic transplantation [6], was minimized. Syngeneic transplanted AVAs, using the "Rotterdam" model, showed no histological evidence of structural changes, while allogeneic transplanted AVAs were severely damaged. Therefore, this novel model can be used to study the morphological and functional consequences of an allogeneic immune response.

Our data indicate that the vascular wall of fresh AVAs implanted across a MHC barrier were infiltrated by mononuclear cells (MNC), while the valve leaflets were deformed and noncellular. These infiltrating T-lymphocytes ($CD4^+$ and $CD8^+$) and macrophages ($CD68^+$) are regarded as effector cells in solid organ and tissue rejection. Their appearance is localized mainly in the tunica adventitia and the outer layer of the tunica media of the aortic wall and not in the intimal layer, except for one explanted graft. This phenomenon could be explained by the abundancy of endothelial cells in the complex vascular network of the adventitia, while the tunica intima bears only a monolayer of endothelial cells. These endothelial cells are regarded as the main target cells in the immune response directed against AVA [7]. The noncellularity and deformation of the valve leaflets at 3 weeks after transplantation is probably the result of early cellular destruction of the leaflet components.

MNC were also observed at 21 days after transplantation in the syngeneic aortic vascular wall. This infiltration is totally different from the allogeneic transplanted valve, in cell type, intensity and localization. Only $CD68^+$ cells (macrophages) were present predominantly in the necrotic myocardial rim, and a minority of these cells was found in the adjacent adventitium of the aortic wall. The necrotic tissue probably induces this nonspecific immune response and subsequently the spread of cellular infiltration into the adventitia [8].

The histological signs of cellular infiltration and graft injury corresponded to the results of functional analysis of the AVAs; that is to say extensively infiltrated valves showed more leakage.

This study demonstrates that AVAs induce invasive and destructive cellular immune responses when implanted in a recipient with MHC disparity. In addition, cellular infiltration and destruction do indeed result in valve dysfunction. Still further studies using the Rotterdam implantation model are needed to evaluate the kinetics of the cellular immune response and valve dysfunction. Further relevant issues include the role of cryopreservation and immunosuppression in modifying valve infiltration and injury are currently under investigation.

Acknowledgements The authors would like to thank Mrs C. Peekstok for her preparation of the histological sections. This study was partly financed by The Netherland Heart Foundation (grant no. 96.177).

References

1. Barratt-Boyes BG (1965) A method for preparing and inserting a homograft aortic valve. Br J Surg 52: 847
2. Baskett RJ, Ross DB, Nanton MA, Murphy DA (1996) Factors in the early failure of cryopreserved homograft pulmonary valves in children:preserved immunogenicity? J Thorac Cardiovasc Surg 112: 1170–1179
3. Green MK, Zhao XM, Senewiratne S, McGiffin DC (1992) A micro-surgical rat model for aortic valve allografts. Transplant Proc 24: 2286
4. Hoekstra FM, Witvliet M, Knoop CJ, Bogers AJ, Weimar W (1997) Donor-specific anti–human leukocyte antigen class I antibodies after implantation of cardiac valve allogarfts. J Heart Lung Transplantation 16: 570–572
5. Kouwenhoven EA, de Bruin RWF, Heemann UW, Marquet RL, IJzermans JNM (1999) Late graft dysfunction after prolonged cold ischemia of the donor kidney. Inhibition by cyclosporin. Transplantation (in press)
6. Kouwenhoven EA, Marquet RL, Bonthuis F, IJzermans JN, de Bruin RW (1997) The role of alloantigen independent factors in transplant arteriosclerosis. Transplant Proc 29: 1721–1722
7. Lupinetti FM, Christy JP, King DM, El Khatib H, Thompson SA (1991) Immunogenicity, antigeneicity and endothelial viability of aortic valves preserved at degrees C in a nutrient medium. J Cardiac Surg 6: 454–461
8. Moustapha A, Ross DB, Bittira B, Lannon CL, Lee TD (1997) Aortic valve grafts in the rat:evidence for rejection. J Thorac Cardiovasc Surg 114: 891–902
9. O'Brien MF, McGiffin DC, Stafford EG (1991) Allograft aortic valve replacement: long-term comparative clinical analysis of tyhe viable cryopreserved and antibiotic 4 degree C stored valves. J Cardiac Surg 6: 534–535
10. Pacifico AD, Kirklin JW (1977) Homografts for replacement of the aortic valve. Circulation 55: 353–361
11. Ross DN (1962) Homograft replacement of the aortic valve. Lancet II:487–490
12. Yankah AC, Wottge HU, Muller-Ruchholtz W (1988) Prognostic importance of viability and a study of a second set allogarft valve: an experimental study. J Cardiac Surg 3: 263–264
13. Zhao XM, Green M, Frazer IF, Hogan P, O'Brien MF (1994) Donor–specific immune response after aortic valve allografting in the rat. Ann Thorac Surg 57: 1158–1163