The allograft valve in heart transplantation and valve replacement

Genetic assessment of the origin of the cells by means of deoxyribonucleic acid profiles

Assessment of the cellular origin of allograft valves is essential in comprehending their biologic behavior and in improving preparation methods. In this study we retrospectively analyzed 10 allografts obtained from patients who underwent valve replacement or heart transplantation. Histologic evaluation and deoxyribonucleic acid amplification by polymerase chain reaction technology with fluorescence labeled primers was performed on different parts of the valve leaflets. Automated analyses of the obtained amplimers showed in the heart transplantation group the presence of receptor cells interspersed with native donor cells in three cases. Preliminary results for the valve replacement group are inconclusive as yet. (J THORAC CARDIOVASC SURG 1995;109:218-23)

João Q. Melo, MD, PhD^a (by invitation), Carolino Monteiro, MSc, PhD^b (by invitation), José Neves, MD (by invitation), Ricardo Santos, MSc^{a*} (by invitation), Ana Martins, MD^a (by invitation), Sância Ramos, MD^a (by invitation), Carol Calta, MSc^a (by invitation), António Matoso-Ferreira, PharmD^a (by invitation), João Viana, MD^a (by invitation), José Rueff, MD, PhD^b (by invitation), and Manuel Machado-Macedo, MD, PhD, *Carnaxide and Lisbon, Portugal*

Allograft valve replacement is considered in two different clinical situations: valve replacement and heart transplantation. In the valve replacement group, the valves are usually cryopreserved and the recipients are not immunosuppressed. The late results of these substitutes are good, with acceptable valve gradients and a low incidence of thromboembolism and endocarditis. However, the durability of

cryopreserved allografts is limited and the failure rate increases with time.^{1,2} In the heart transplantation group the valves are fresh and viable, and their recipients are immunosuppressed. During a period of up to 6 years these valves functioned normally and there were no cases of endocarditis or thromboembolism. Histologic studies were made to determine the structure of the explanted leaflets. Pathologic specimens were assessed genetically to evaluate the origin of the cells from fragments of the valve leaflets. Polymerase chain reaction (PCR) technology³ was used with fluorochrome-labeled primers to amplify (CA)_n hypervariable deoxyribonucleic acid (DNA) target regions^{4,5} from very small amounts of DNA extracted from various portions of explanted aortic valve leaflets.

Material and methods

Ten valves were retrospectively analyzed from five patients who had undergone allograft valve replacement and from five who had had heart transplantation. All valves from the valve replacement group were removed during reoperations and valves from the heart transplantation group were collected during postmortem examination. These valves were obtained with approval from the

From the Instituto do Coração and Hospital de Santa Cruz,^a P-2795 Carnaxide, Portugal, and Departamento de Genética, Faculdade de Ciências Médicas UNL,^b R Junqueira 96, P-1300 Lisbon, Portugal.

Supported in part by the Programa CIENCIA/JNICT, Fundação Calouste Gulbenkian, The Portuguese Health Ministry, and CMDT/UNL.

Read at the Seventy-fourth Annual Meeting of The American Association for Thoracic Surgery, New York, N.Y., April 24-27, 1994.

Address for reprints: João Q. Melo, MD, Instituto do Coração, Av. Prof. Reynaldo dos Santos, 27 Carnaxide P-2795, Portugal.

*Recipient of a BIC scholarship from Programa CIENCIA/JNICT.

Copyright © 1995 by Mosby-Year Book, Inc.

0022-5223/95 \$3.00 + 0 12/6/61139

Table I. Donor and receiver information for allograft valve replacement and heart transplantation groups

Case	Age		Sex		Blood group		Cold IT (hr)	Cryo storage (days)	Follow-up (mo)
	D	R	D	R	D	R			
A1	23	72	M	M	A-	A+	36	54	2
A2	21	62	M	F	A+	A+	24	19	6
A3	40	64	M	M	A+	A+	24	33	2
A4	30	45	M	F	NA	A+	48	14	12
A5	42	23	M	M	A+	O+	168	0	72
T1	29	53	M	M	O-	O+	2.0		1
T2	28	53	M	M	A+	A+	2.2		2
T3	19	42	F	M	A-	A+	2.8		5
T4	25	60	F	M	A+	A+	1.5		4
T5	41	61	M	M	A+	A-	2.0		38

A, Valve replacement group; T, heart transplantation group; D, donor; R, receiver; IT, Ischemic time; NA, not available.

national institutional review board. In the valve replacement group the donor valves were retrieved from brain dead donors (three cases) and from cadavers (two cases). In the heart transplantation group all the hearts were obtained from brain dead donors. After heart transplantation all patients were given cyclosporine, azathioprine, and prednisone for immunosuppression. Four patients incurred one to three treated rejection episodes. All patients in the heart transplantation group died with no evidence of rejection, except for patient T1, who died 1 month after the operation with signs of mild rejection. The mean age, sex distribution, blood type, and valve information for the valve replacement and heart transplantation groups are outlined in Table I.

One leaflet of each valve was used for genetic evaluation. The leaflet was divided into one to four fragments for the valve replacement group and into four to 21 fragments in the heart transplantation group depending on the size and quality of the sample. Blood samples or small portions of native aortic tissue were collected as a source of constitutional DNA for comparative studies. From solid tissue, DNA was obtained by the standard proteinase K method followed by phenol-chloroform extraction, whereas the guanidine-hydrogen chloride method was used for blood.⁶ DNA was amplified by PCR technology by means of primers in which one primer is fluorescently labeled. Oligonucleotide primers flanking the hypervariable (CA)_n repeat regions⁷⁻¹⁰ were synthesized by a 381 DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.). A fluorescent dye (5-carboxyfluorescein [FAM] or 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein [JOE]) was attached to the 5' end of the pair of PCR primers using Aminolink 2 (Applied Biosystems, Inc.) and were purified by high-performance liquid chromatography, as described by Gibbs and associates.¹¹ The size of the (CA)_n-containing amplicons is determined by referencing them to the internal lane size standards by means of the GENESCAN 672 software (Applied Biosystems, Inc.).^{12, 13}

Results

Histologic studies indicated that the valve leaflets from the valve replacement group lost their normal

structure. There was evidence of hyalinization and predominant ground substance, sometimes with focal calcification. Small quantities of fibroblasts were found only in cases A1 and A2. Endothelial cells were not found in any of the cases and in three cases there was evidence of focal polymorphonuclear infiltration. In the heart transplantation group, histologic evaluation was almost normal with a slight decrease in the number of cells at the free edge of the leaflets. The endothelium was focally maintained and none of the leaflets showed inflammatory infiltrations.

The valve replacement group was genetically assessed with marker D5S82, which yielded a heterozygous profile in two samples (A1 and A4) and homozygous profiles for the remaining three samples. An electrophoretogram with more than two peaks was not encountered for any of the valve replacement cases, so that the results were inconclusive. Sample A4 exhibited two different sized amplicons in the interior region of the leaflet and one amplicon with a very small area in the exterior region of the leaflet. DNA controls from the receiver were obtained in cases A1 and A2, in which the same genotypes as those present in the valves were found. For the heart transplantation group, three different sized amplicons in three cases (T1, T3, and T5) were obtained. In two cases (T1 and T5) we found a very high difference in the area of one amplicon as compared with the other, demonstrating the presence of one population with a homozygous profile and another with a heterozygous profile (Fig. 1). We were unable to find any of these types of electrophoretograms in case T2. Analysis of T4 indicates a homozygous profile in the valve and a heterozygous profile for the control DNA (recipient). Results for case T5, showing three different

Fig. 1. Electrophoretograms of PCR products with primers for D5S299, case T5 (*top*, fragment A; *bottom*, control) corresponding to data in Table II. The *abscissa* indicates size in base pairs and the *ordinate* indicates fluorescence intensity.

sized amplimers using marker D5S299 and two different sized amplimers with areas differing by a factor of 2 using marker D5S346, are presented in Table II.

Discussion

The aim of this study is to determine allograft leaflet cellular origin with and without the influence

of immunosuppression by means of a new method of fluorescent primers in PCR and electrophoresis. This type of automated DNA analysis using primers in a PCR reaction in which one of them is fluorescently labeled enables us to accurately genotype and quantify the obtained amplimers. Cell origin (donor or recipient) was not always visually apparent, especially when the control and valve DNA samples were

Table II. Genetic profiles for case T5 of the heart transplantation group for different sections of the leaflet indicating the obtained size in base pairs (bp) for each amplimer and their respective areas

Fragment	D5S299						D5S346			
	Size	Area	Size	Area	Size	Area	Size	Area	Size	Area
A	158	498	178	516	184	535	114	5761	116	3101
B	158	570	178	1917	184	1585	114	7207	116	2571
C	*	*	*	*	*	*	*	*	*	*
D	158	2455	178	703	184	347	114	20192	116	9875
Control	158	38555					114	35618	116	33092

The receiver DNA is represented as the control. Fragment segment locations are labeled A through D, with A as the outermost segment and D as the innermost segment.

*No DNA amplification.

amplified to the same size in the PCR reaction. This verifies that quantified areas evaluated by the GENESCAN 672 software are crucial for assessing quantification. With respect to scoring accuracy, the electrophoretograms obtained show that homozygous genotypes can be readily distinguished from heterozygotes, which differ by only two nucleotides in the size of their alleles.

The most striking factor for the valves in the valve replacement group was the marked decrease in cellularity. The presence of infection in a few of these valves made the DNA assessment of the native cells controversial because the obtained DNA amplification may be due to infection-related leukocytes. So that this problem could be avoided, five slices of 5 μ m each from the same region as that used for genetic assessment were histologically examined and found to contain no white blood cells. Results in the valve replacement group indicated a heterozygous profile in A1 and a homozygous profile in A2. The respective DNA controls had similar profiles indicating coincidental genotypes for recipient and donor in both cases or total inhabitation of the cells of the valves by host cells. For the heart transplantation group a pattern of three DNA profiles was found in three cases, and within these a pattern of two DNA profiles was found by the use of different markers in which the area of one allele is notably different from the other. This enables us to deduce the genotype of the donor as homozygous for the more concentrated band. These findings were confirmed by the use of three different markers, proving a double origin of these cells. In one case we encountered recipient cells within all fragments of the leaflet and a patchy distribution of endothelial cells. Morphologic studies showed that the number of endothelial cells was negligible as compared with the number of fibroblasts. There-

fore, we conclude that the cells yielding DNA for amplification are the most abundant cells, the fibroblasts. For case T5 with the marker D5S299, the amount of DNA-amplified product showed that the cell population at the free edge of the cusps was predominately from the recipient and the cells at the base were from mixed origins. These differences in location of cell population along the leaflet require further research before any conclusion can be reached.

The overall method used in this study represents a promising alternative to conventional genetic screening. The combination of PCR with the sensitivity of the fluorescent PCR alternative and the use of specific software to screen and to quantify the obtained amplimers results in a time-saving method with enhanced sensitivity and accuracy of detection. Further use of new genetic assessment methods will definitely improve our knowledge of the cellular content of the allograft valve in heart transplants and valve replacement.

We thank Dr. Eric Mayrand and Dr. Margaret Galvin from Applied Biosystems, Inc., Foster City, California, for their assistance in automated DNA analysis.

REFERENCES

1. Angell W, Oury JH, Lamberti JJ, Koziol J. Durability of the viable aortic allograft. *J THORAC CARDIOVASC SURG* 1989;98:48-56.
2. Barratt-Boyes BG. Cardiothoracic surgery in the antipodes. *J THORAC CARDIOVASC SURG* 1979;78:804-22.
3. Saiki RK, Gelford DH, Stoffel S, et al. Primer—direct enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 1988;239:487-91.
4. Weber JL, May PE. Abundant class of human DNA polymorphism which can be typed using the polymerase chain reaction. *Am J Hum Genet* 1989;44:388-96.

5. Litty M, Luty FA. A hypervariable microsatellite by in vitro amplification of a dinucleotide repeat within the cardiac muscle actin gene. *Am J Hum Genet* 1989;44:397-401.
6. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning—a laboratory manual, 2nd ed. New York: Cold Spring Harbor Laboratory Press, 1989.
7. Breukel C, Tops C, van Leeuwen C, et al. CA repeat polymorphism at the D5S82 locus, proximal to adenomatous polyposis coli (APC). *Nucleic Acids Res* 1991;19:5804.
8. van Leeuwen C, Tops C, Breukel C, van der Klift H, Fodde R, Khan PM. CA repeat polymorphism at the D5S299 locus linked to adenomatous polyposis coli (APC). *Nucleic Acids Res* 1991;19:5805.
9. van Leeuwen C, Tops C, Breukel C, et al. CA repeat polymorphism within the MCC (mutated in colorectal cancer) gene. *Nucleic Acids Res* 1991;19:5805.
10. Spirio S, Joslyn G, Nelson L, Leppert M, White R. A CA repeat 30-70 KB downstream from the adenomatous polyposis coli (APC) gene. *Nucleic Acids Res* 1991;19:6348.
11. Gibbs RA, Nguyen P-N, McBride LJ, Koepf SM, Caskey CT. Identification of mutations leading to the Lesch-Nyhan syndrome by automated direct DNA sequencing of in vitro amplified cDNA. *Proc Natl Acad Sci U S A* 1989;86:1919-23.
12. Mayrand PE, Corcoran KP, Ziegler JS, Robertson JM, Hoff LB, Kronick MN. The use of fluorescence detection and internal lane standards to size PCR products automatically. *Applied and Theoretical Electrophoresis* 1992;3:1-11.
13. Ziegler JS, Su Y, Corcoran KP, et al. Application of automated DNA sizing technology for genotyping microsatellite loci. *Genomics* 1992;14:1026-1031.

Discussion

Dr. A. C. Yankah (Berlin, Germany). Dr. Melo, I agree with you that allografts are antigenic. The viable allografts and even the allografts are subjected to immunologic attack and trauma. Consequently, the endothelial cells as well as the fibroblasts and the collagen structures would be destroyed after implantation after an immune response by the host. The immunologic complications are our major concern, especially in children. Unless the immune response is delayed or arrested by means of a nontoxic dose of immunosuppression such as cyclosporine to inhibit T-lymphocyte activation and prevent structural deterioration, the durability of the viable allograft valve would be limited. It has been demonstrated in our animal experimental laboratory that in rats a short course of cyclosporine therapy with a dose of 10 mg/kg for 14 days beginning from the day of operation can achieve unresponsiveness of the host to the allograft. Subsequently, the endothelial cells and the fibroblasts could be preserved. The cells could survive to replicate, the structures and valve integrity could be maintained, and therefore durability of the allograft could be prolonged. In certain rat strains (weakly

allogeneic) with major histocompatibility-compatible, non-major histocompatibility-incompatible encoded endothelial cells, prolonged if not indefinite valve durability could be achieved after a short-course nontoxic dose of cyclosporine.

If these findings are applicable in the clinical situation, then this implies that patients who are at risk for forming antibodies against allograft valves could benefit from a tissue typing especially for HLA-DR compatibility and an additional short-course nontoxic dose of immunosuppressive therapy for about 3 months. Observations made in heart transplant patients in whom aortic valve function was always normal during acute or chronic rejection support and justify the use of some type of immunosuppression in high-risk patients who are electively scheduled to receive an allograft.

The two slides confirm the results of the animal experiments we performed at the Institute of Immunology at the University of Kiel, Germany. Allografts in rats not treated with cyclosporine A lost their endothelial cells after a humoral rejection, leaving bare collagen structures in the blood circulation. Subsequently the fibroblasts died away and the valve became acellular. The lost endothelium, however, was replaced by a thin layer of fibrous neointima. Very interestingly, no thrombotic formations were observed on the valve surfaces.

In the cyclosporine-treated rats the endothelial cells were maintained until postoperative day 250, when the experiment was terminated. The endothelial cells could be identified by factor VII and *Ulex europaeus* lectin identification test, and endothelial cell origin was shown by means of donor and recipient specific monoclonal antibodies as well as monoclonal antibodies (OX6) directed at major histocompatibility class II rat endothelial antigens.

In the control syngeneic grafts or the isografts (herein referred to as autografts), no immune response was detected by direct immunofluorescence study. The isografts were viable and durable at the completion of the experiment on day 250.

I would like to address one question to Dr. Melo. Can you apply your method to identify the donor or recipient endothelial cells?

Dr. Melo. This method is very useful. Of course, the key issue regarding this study concerns which cells we are looking at. Are they endothelial cells or fibroblasts?

Evaluating the allograft group is easy because we could not show, in any of these specimens that were kept in pathology, any endothelial cells on the surface of these allografts. We were able to get PCR amplification, but because we had just one band, it could be either from the donor or from the recipient. However, this DNA definitely did not come from the endothelial cells of the donor.

The second issue concerns the transplantation group. The endothelial cells assessed by cytology are scarce, the main reason being that all these examinations have at least a 24-hour delay after death. At histologic examination all the controls showed the endothelial cells to be present, but in a patchy distribution. In two of the three samples both donor and recipient cells were present in all the pieces studied, which would be impossible if the DNA were from the endothelial cells. This indicates that we are

looking at fibroblasts. Besides, this method is quantitative, which gives you an idea of the number of cells present. This is a second reason to believe that most DNA is from the fibroblasts.

Mr. Magdi H. Yacoub (*Harefield, England*). Dr. Melo, I have two questions for you. What were the genetic markers used for the PCR? What was the exact sequence and what genes were you targeting and why?

Second, what is the interval between insertion and examination in both types?

Dr. Melo. Regarding your first question, I will have my genetics colleagues provide you all the information. I think they chose their markers because of the specificity of the Portuguese population. One of the characteristics of a

small country like ours is that it is much more homozygous than a population in which there is a greater crossing of races.

Regarding the second question, in patients having aortic valve replacement there is a very large delay between implantation and removal, which varied from 2 months to 6 years. In the transplantation group the average time was 7 months.

Dr. Yacoub. Did you evaluate the effect of time?

Dr. Melo. Yes, and we could show exactly on these three allografts where we have seen donor and recipient cells; one allograft had 1 month, another 6 months, and the other 4 years after implantation. They were in the shortest, medium, and longest time after transplantation.

Availability of JOURNAL back issues

As a service to our subscribers, copies of back issues of THE JOURNAL OF THORACIC AND CARDIOVASCULAR SURGERY for the preceding 5 years are maintained and are available for purchase from the publisher, Mosby-Year Book, Inc., at a cost of \$12.00 per issue. The following quantity discounts are available: 25% off on quantities of 12 to 23, and one third off on quantities of 24 or more. Please write to Mosby-Year Book, Inc., Subscription Services, 11830 Westline Industrial Drive, St. Louis MO 63146-3318, or call (800)453-4351 or (314)453-4351 for information on availability of particular issues. If unavailable from the publisher, photocopies of complete issues are available from University Microfilms International, 300 N. Zeeb Rd., Ann Arbor, MI 48106, (313)761-4700.