Endothelialized biological heart valve prostheses in the non-human primate model

G. Lehner a, T. Fischlein a,*, G. Baretton b, J.G. Murphy c, B. Reichart a

a Department of Cardiac Surgery, University of Munich, Medical Center Großhadern, Marchionistrasse 15, D-81377 Munich, Germany
b Department of Pathology, University of Munich, Medical Center Großhadern, Marchionistrasse 15, D-81377 Munich, Germany
*Max Planck Institute for Biochemistry, Department of Cell Biology, D-82152 Martinsried, Germany

Received 14 March 1996; revised 29 May 1996; accepted 1 July 1996

Abstract

Objective: The main disadvantage of implanted cardiac valve bioprostheses remains primarily tissue failure due to calcification. Coating of bioprostheses with viable autologous endothelial cells may delay or even eliminate tissue calcification and subsequent cardiac dysfunction. Methods: Glutaraldehyde-preserved Hancock bioprostheses (n = 5), pretreated with glutamic acid (8%) and cryopreserved allografts (n = 5) were lined, using endothelial cells harvested from the external jugular vein. Coated specimens were cultivated for 9 days in Medium 199 supplemented with 20% fetal calf serum and basic fibroblast growth factor. Endothelialized grafts were anastomosed into the descending thoracic aorta of adult Chacma baboons. Untreated valve bioprostheses (n = 4) served as controls. Forty days after implantation the prostheses were examined morphologically and immunohistochemically. Results: After implantation endothelialized prostheses showed a positive Factor VIII related antigen reaction by immunohistochemistry on all valve surfaces. Scanning electron microscopy showed confluently lined leaflets with transplanted endothelial cells and displayed cobblestone morphology on all coated allografts. In contrast, the surface of pretreated xenograft valves revealed uncoated areas with platelet and leucocyte aggregates. No endothelium was observed on the leaflets of untreated controls 40 days after implantation. Conclusion: In vitro endothelialization of cardiac valve bioprostheses with autologous endothelial cells is possible. The newly created endothelium is shear stress resistant and the antithrombotic as well as the antiaggregatory capacity of the transplanted cells were retained. Lining with autologous endothelial cells could improve the durability and clinical outcome of biological valve prostheses. © 1997 Elsevier Science B.V.

Keywords: Antithrombogenicity; Biological cardiac valves; Endothelialization; Shear stress resistance; Valve leaflet morphology

1. Introduction

For approximately 30 years biological cardiac valves have been in clinical use [4]. Their hemodynamic functions are superior to mechanical valves and anticoagulation therapy is not required [2,18]. Moreover, undesirable side effects caused by mechanical prostheses (e.g. thromboembolism, infection) are considerably reduced or do not occur at all [14]. However, despite these advantages a strict indication is required due to their limited durability [15,16,18].

A reason for the latter may be the absence of viable cells on the valve surface due to the standardized glutaraldehyde fixation of commercially available valve bioprostheses. Thus, a protecting barrier which is physiologically provided by an intact endothelium is missing [11]. This may result in platelet and fibrin deposits on the valvular surface, tissue calcification, bacterial infection, immunological reactions and finally graft degeneration [11,12,17]. Such a protection barrier does not exist on antibiotic treated and cryopreserved homografts either because most of the cellular components of the valve structure are destroyed due to the conservation process.
Transplantation of autologous, endothelial cells (ECs) onto the graft surface may delay early degeneration or even prevent it. In vitro endothelialization of commercially available bioprostheses has already been achieved successfully in our laboratory [9]. However, several questions remain for a promising clinical application: will transplanted ECs withstand the shear stress of the blood stream and retain their antiaggregatory and antithrombogenic function after implantation? Does the new created endothelium behave as a similar efficient barrier as endocardial ECs do in vivo? Thus, we performed this non-human primate study to verify the technique of bioprosthetic valve endothelialization.

2. Materials and methods

2.1. Endothelial cell cultures

Endothelial cells were enzymatically (0.1% collagenase II, Worthington, USA) harvested from segments of the external jugular vein, 2–3 cm in length, as described previously [10]. Once isolated, ECs from each animal were grown in six well culture plates (Costar, MA) which had been precoated with human fibronectin (28 μg/ml, Seralab, Sussex, UK). ECs were cultivated in Medium 199 (Flow, Irvine, UK) containing gentamycin (50 mg/ml), 20% fetal calf serum (Flow, Irvine, UK) and endothelial cell growth supplement (ECGS 75 μg/ml, Seralab, Sussex).

Cell cultures were stored in an incubator at 37°C and 5% CO₂ (Heraeus, Hanau, Germany) until confluency was reached. Primary cultures were passaged into a 75 cm² culture flask (Costar, MA) using 0.25% trypsin–EDTA (Inotech, Zurich, Switzerland). Additionally, a fraction of those ECs were transferred into one well of a six well plate as a back up in case of microbiological contaminations.

2.2. Bioprostheses and tissue pretreatment

Five Hancock xenograft heart valves sewed in a dacron tube (Medtronic, Irvine, USA) and five antibiotic-treated, cryopreserved allografts which had been obtained from non human primates (Chacma baboons) during autopsy were used for autologous endothelialization in this study. Unlined xenografts (n = 2) and allografts (n = 2) were implanted as controls.

Prior to EC-seeding, the commercially available xenografts had to be pretreated in order to neutralize free aldehydes [7]. The specimens were first rinsed with phosphate buffered saline (PBS) and subsequently stored in a 200 mM glutamic solution (Sigma, MO), adjusted to pH 3.5, for 48 h at room temperature. After two days grafts were rinsed again with PBS.

Allografts were thawed in Medium 199 and rinsed with PBS three times in order to remove dimethylsulphoxide (DMSO), which was used for cryopreservation.

2.3. Endothelialization

Graft seeding was performed when EC-confluence was reached in the 75 cm² culture flasks. Pretreated bioprostheses were precoated with 28 μg/ml human fibronectin, placed in six well culture plates and seeded with ECs (300 000 EC/cm²) on both sides for 30 min. Subsequently, grafts were placed in a glass container, filled with their EC-suspension and rotated for 1 h (12 rotations) in order to facilitate an even cell distribution. Following the seeding procedure, each valve prosthesis was cultivated in 300 ml of Medium 199 containing 20% fetal calf serum and 7 ng/ml basic fibroblast growth factor for nine days.

2.4. Surgical technique

Adult Chacma baboons, weighing 18–20 kg, were dewormed and prepared for four weeks in quarantine before graft implantation was performed. All animals received human care in compliance with “Guide for the care and use of laboratory animals” published by the National Institute of Health (NIH publication No. 85-23, revised 1985). Valve bioprostheses were implanted as a conduit in an end-to-end manner into the descending thoracic aorta via a left posterolateral thoracotomy (fourth intercostal space). Baboons were anaesthetized with 5 mg/kg Tiletamia/Zolazepan i.v. (Zoletil 100, Laboratories Reading, Carros) and halothane (0.5%) after intubation was performed. For graft implantation the descending thoracic aorta was prepared, body temperature was reduced (32°C) and the aorta was clamped distal to the origin of the left subclavian artery. Preceding graft implantation one leaflet of each valve was partially removed in order to verify sufficient endothelialization by means of scanning electron microscope (SEM) and light microscope investigations. Postoperatively patency of prostheses was guaranteed by Doppler ultrasound examination. After five weeks animals were euthanized with 200 mg sodium pentabarbiton (Eutanaze, Centaurlabs, Isando 1600, S.A.) heparinized and valve prostheses were explanted.

2.5. Evaluation of explanted valve prostheses

Immediately after explantation, valves were flushed with PBS, carved longitudinally and inspected for thrombotic deposits. Thereafter one part was fixed in buffered formalin for light microscopical analysis and the other part was placed in 2% glutaraldehyde–0.05 M
Fig. 1. Scanning electron micrograph of an endothelialized allograft leaflet before implantation. The surface is confluently lined with venous ECs exhibiting the characteristic cobblestone morphology (magnification 500 ×).

cacodylate buffer (pH 7.2) for scanning electron microscopy (SEM).

For light microscope analysis the tissues were embedded in paraffin, cut into slices of 3–4 μm and stained according to the hematoxylin eosin (HE) method and by immunohistochemistry for Factor VIII related antigen (moab M616 Dako, Copenhagen, Denmark, Avidin–Biotin complex method).

For SEM the specimens were dehydrated with acetone to the critical point of carbon dioxide (PCD 030 Balzers, Lichtenstein), coated with gold palladium (SCD 050 Balzers) and examined with an electron microscope JEOL-JSM35C (Jeol, Tokyo, Japan).

3. Results

Before implantation endothelialized grafts displayed a confluent endothelium with cobblestone morphology demonstrated by SEM analysis (Fig. 1).

3.1. Morphology of implanted endothelialized prostheses

Macroscopically no thrombus formations were detectable on the valvular surfaces immediately after explantation (Fig. 2). All valve prostheses were patent and showed intact and flexible leaflets. One endothelialized Hancock bioprosthesis showed a haemorrhage in one leaflet.

SEM evaluations revealed that all endothelialized allografts were confluently lined with ECs with no signs of structural damage after implantation. The surface of the leaflets and of the graft aortic walls presented the typical cobblestone morphology (Fig. 3A). The newly created host endothelium remained intact under physiological shear stress of the blood stream. Neither fibrin deposits nor platelet aggregates were detectable on the surfaces. Additionally, no leucocyte adhesions were present.

In contrast two of five endothelialized Hancock valves showed several uncoated areas with fibrin deposits and platelet aggregates. Interestingly, adhesions of leucocytes have been observed all over the endothelialized leaflet surfaces (Fig. 3B). The dacron tube of the endothelialized prostheses was almost confluently lined without platelets or leucocytes adhesions. In contrast the dacron wall of non-endothelialized prostheses was coated with a fibrin layer and some platelets.

Fig. 2. Macroscopical evaluation of an endothelialized xenograft after an implantation period of 40 days. No thrombus formations can be seen on the graft surface.
The proof of Factor VIII related antigen demonstrated entirely endothelialized valve surfaces on all coated allografts by immunohistochemistry (Fig. 4A). On the other hand Hancock valves again showed partially endothelialization.

Additionally, endothelialized Hancock bioprostheses showed an inflammatory reaction observed under light microscope. The infiltrate consisted of some lymphocytes and plasma cells, fibroblasts and histiocytes. Granulation tissue appeared to have grown from the outside into the wall of the dacron prostheses.

Interestingly, the allograft stroma turned out to be cell poor. The stroma of the cusps was edematous with broken and denaturated elastic fibers. Only a few of the plasma cells and lymphocytes were interspersed subendocardially. Penetration by these cells into the depth of the leaflet was not noted.

3.2. Morphology of non-endothelialized implanted prostheses

By macroscopical examination of the xeno- and allografts no thrombus formation on the cusps and on the surface of the dacron prostheses could be observed.
Fig. 4. Light microscopy (10×) shows a positive immunoreactivity of the new created endothelium for Factor VIII related antigen. A, explanted leaflet of an allograft, confluent ly lined with ECs (arrow). B, no positive immunoreactivity for Factor VIII related antigen on the leaflet of an implanted non-endothelialized allograft.

No ECs were detectable on the leaflet surface of the non-endothelialized control grafts by SEM investigations. Only directly around the anastomoses a few ECs of the host aorta were present. Furthermore, fibrin deposits, platelet aggregates, leukocytes and red blood cells were observed on the graft surfaces.

Factor VIII assays indicated that no ECs were present on the valve surface of the non-endothelialized xeno- and allografts (Fig. 4b). Only in the region of the anastomoses of the dacron prostheses ECs from the host aorta were detectable again. Nonendothelialized allografts showed an acellular stroma under light microscope. Only a few plasma cells and lymphocytes were detectable subendocardially.

4. Discussion

With this experimental baboon study shear stress resistance as well as antithrombotic and antiaggregatory capacity of the new created valve endothelium was demonstrated after implantation of autologous coated bioprostheses. Like human cardiac valves, which are covered by an intact EC-layer on both sides, autologous endothelialized biological heart valves may possess the same physiological qualities. Commercially available Hancock bioprostheses have no vital ECs on their surface due to the standard glutaraldehyde fixation technique. Even after a long implantation period no spontaneous endothelialization of these prostheses occur due to continuous release of cytotoxic aldehydes.
The absence of vital ECs on glutaraldehyde treated valves is known to be one major reason for tissue degeneration and calcification after implantation [3,11]. We have previously shown that endothelialization of commercially available Hancock bioprostheses with autologous ECs is feasible after appropriate detoxification [9]. With this animal study we demonstrated the feasibility of valve endothelialization for the first time in vivo. Moreover, the anticoagulative properties were preserved after implantation. SEM investigations and Factor VIII assay both revealed that two of five endothelialized and implanted xenografts showed uncovered areas postoperatively. An explanation could be a release of cytotoxic aldehydes despite special pretreatment [1]. The reoccurring release of aldehydes after implantation would explain the adhesion of leucocytes on the Hancock valve surfaces due to EC activation [18]. Interestingly, all endothelialized xenograft prostheses exhibited an intact EC-layer on the dacron wall surface postoperatively. In these areas no leucocyte adhesions were present. One reason for these findings may be that in contrast to glutaraldehyde-fixed valve leaflets no residual aldehydes were released from the dacron tube after implantation. Thus, improvements are necessary regarding the detoxification pretreatment in order to allow EC growth on xenograft prostheses or even better an alternative non-toxic graft preservation procedure should be applied [1,6,7,18]. Additionally, proteins cross-linked by glutaraldehyde fixation also could have caused an immunological reaction [5]. SEM showed that the non-endothelialized implanted xenografts were covered with fibrin and thrombotic deposits on their surfaces. The dacron wall, especially, was densely covered with fibrin deposits and platelet aggregates. The endothelialized specimens, however, were free of such deposits. This suggests that ECs do not lose their antithrombotic and antiaggregatory properties following transplantation and implantation. We have already demonstrated in the laboratory that venous ECs still release prostacyclin after transplantation onto pretreated Hancock valves [9]. Histological examinations of the valve stroma showed no substantial differences between endothelialized and non-endothelialized xenografts. However, we did not expect any fundamental changes within an implantation period of 40 days.

An alternative to glutaraldehyde treated xenograft prostheses for biological valve replacement are allografts. Because these grafts are clinically used more frequently during recent years considerations of factors which improve long-term survival became necessary. Cell viability was suggested as one important point with respect to long term valve durability [19,20]. Another concern is an immunologically mediated destruction of the graft [13]. Due to possible immunologic reactions induced by donor valve cells endothelialization with autologous cells could be more advantageous and may improve the clinical outcome. Furthermore, clinical results of pulmonary autografts, which are provided with an intact autologous endothelium and vital stroma cells, are excellent when used for aortic valve replacement. In this study SEM investigations showed in all cases of endothelialized allografts a confluent endothelium, with no fibrin and platelet deposits on the valvular surfaces. There was no adherence of leucocytes either, which were present on the surfaces of the detoxified and endothelialized xenografts.

In conclusion, endothelialization of biological heart valves with autologous ECs is feasible. The transplanted ECs withstand the shear forces of the blood stream in vivo and the new created endothelium retains the anticoagulative properties after implantation. The newly created autologous endothelium can act as a physiologic barrier between blood and tissue as ECs normally do in vivo. Thus, successful endothelialization of biological heart valve prostheses can lead to an improvement of durability and clinical outcome.

Acknowledgements

We thank Prof. Pat Kelly, Department of Clinical Veterinary Studies, University of Zimbabwe and Dr. Jan Still, Faculty of Veterinary Science, University of Southern Africa, for their assistance and helpful suggestions during the animal study.

References

Appendix A. Conference Discussion

Dr Armand Piwnica (Paris, France): Do you have any idea of other physiologic functions of your endothelial cells?

Dr Lehner: Human heart valves are physiologically covered by a monolayer of ECs. Some of the most essential properties of an intact endothelium includes barrier functions between the graft tissue and the blood, the exchange of biological materials across their luminal and abluminal surfaces, their anti-inflammatory and immunological role, generation of growth factors and interaction with different cell types.

Dr Gregor Zund (Zürich, Switzerland): Congratulations for this beautiful paper. Let me ask you a question. You were isolating endothelial cells by collagenase. How do you know that you obtained a pure endothelial cell culture rather than a mixed cell culture?

Dr Lehner: None of our cultures showed areas of overgrowing or typically spindle shaped smooth muscle cells. Beside the typical morphological appearance of pure EC cultures, the immuno-histochemically tested ECs were positive for factor VIII related antigen.

Dr Zund: My experience is that when we isolate cells with collagenase, usually we get mixed cell populations with endothelial cells, smooth muscle cells and fibroblasts.

Dr Lehner: We didn’t get mixed cell populations. The digestion time of the collagenase is 15 min and after this time period we only isolate endothelial cells from the vessel.

Dr Gino Gerosa (Padova, Italy): You stated that endothelialization of valve prostheses should improve durability of currently used bioprostheses. Do you have any further data supporting this statement? Did you test endothelialized bioprostheses in an animal model like the growing sheep model?

Dr Lehner: No. The hypothesis is that an intact endothelium could improve the durability and clinical outcome of biological valves. We only applied them in our baboons for 40 days to test the shear stress resistance of the transplanted venous ECs on the valve leaflet.