

Flow-Dependent Re-Endothelialization of Tissue-Engineered Heart Valves

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Background and aim of the study: The generation of a functional, non-immunogenic, non-thrombogenic construct based on autologous cells seeded onto an acellular extracellular matrix is the major goal in heart valve tissue engineering. The study aim was to identify culturing conditions required to achieve a stable endothelial cell (EC) layer under physiological flow conditions, a prerequisite for the requested characteristics.

Methods: Eleven detergent-decellularized ovine pulmonary valves (PVs) were statically reseeded in special bioreactors with ovine venous ECs (1.2×10^7 cells per valve). The dynamic culture was started with 0.1 l/min in eight bioreactors. In four bioreactors the initial flow rate was slow, and increased by 0.1 l/min twice each day until maximal flow was 0.5 l/min and pulsation rate (PR) was 20 beats/min; in four other bioreactors the flow was increased by 0.7 l/min/day and reached 2.0 l/min with a PR of 50 beats/min. The mean system pressure was maintained at 25 ± 5 mmHg during the whole dynamic cultivation in both groups. Three statically reseeded valves served as baseline. After achieving maximal appointed flow, the valves were investigated morphologically (hematoxylin and eosin staining, electron microscopy, von

Willebrand factor, endothelial nitric oxide synthase immunostaining) and for metabolic activity (MTS assay).

Results: After reseeded, the endothelium appeared on the luminal surface of the PV as a non-confluent monolayer. Moderate pulsatile circulation induced complete confluence of EC monolayers on both cusp sides and the pulmonary wall. A high flow rate led to a partial loss of cells on the wall surface with large defects, and to complete cell wash-off from cusps. Cusp and wall metabolic activity was significantly higher after culture under moderate flow ($p < 0.001$) than in other groups, and was absent from cusps in high-flow bioreactors.

Conclusion: Moderate pulsatile flow with small increments stimulates EC proliferation on the ovine decellularized valve scaffold. A rapid increase in bioreactor flow to physiological levels leads to significant damage of the reseeded endothelium and complete loss of cusp cellularity. This effect may be responsible for the in-vivo failure of static reseeded tissue-engineered valves exposed to physiological hemodynamic forces.

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Improvements in current reseeded methods for the endothelialization of decellularized biological or polymer matrices in dynamic culture represent important issues in heart valve tissue engineering. Recognized crucial functional properties of the endothelium include: (i) the modulation and regulation of platelet

and leukocyte adhesion; (ii) the regulation of exchanges between the bloodstream and surrounding tissues; and (iii) the organization of growth and the development of connective tissue cells, which emphasizes the importance of the re-endothelialization of artificial heart valve constructs (1).

As compared to static conditions, several research groups have recently demonstrated the advantages of a dynamic culture with application of mechanical shear stress with regard to endothelial cell (EC) characteristics, including cell-cell and cell-matrix connections, EC growth, EC orientation and migration, as well as the expression of both specific and unspecific EC proteins (2-6).

The achievement of stable cell-matrix connections of

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reseeded ECs and their mechanical resistance to shear-stress in the physiological circulation represents one of the main difficulties in the technological process of matrix re-endothelialization (7-9). An efficient dynamic valve repopulation with building of a complete, stable EC-monolayer on the matrix surface, and especially of the leaflets, may play an important role in the long-term durability and functionality of tissue-engineered heart valve prostheses.

In the present study, investigations were made into the influence of pulsatile flow conditions on the stability of reseeded ECs on a decellularized biological matrix in a dynamic valve bioreactor.

Materials and methods

Ovine pulmonary valves

All animal experiments and surgical procedures were conducted in compliance with the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication No. 85-23, revised 1996) and approved by local animal care committees.

Valve conduits (pulmonary valves, PV) including short subvalvular myocardial cuff, valve annulus, valve leaflets and pulmonary artery (PA) wall (3 cm length) were harvested from juvenile sheep (body-weight 15-20 kg) under sterile conditions. Following the removal of any adherent fat, the PVs were stored in phosphate-buffered saline (PBS) at 4°C.

Decellularization method

Decellularization was performed by detergent treatment of the PVs (n = 16) in a solution of 0.5% sodium deoxycholate (Sigma) and 0.5% sodium dodecylsulfate (SDS; Carl Roth) for 24 h, followed by six wash cycles (12 h each) with PBS supplemented with 100 IU/ml penicillin-streptomycin (PenStrep; Biochrom) in order to remove any residual detergents and cell debris.

Cell source and culture

For cell isolation, jugular veins were harvested from juvenile lambs under sterile conditions. The ECs were digested from the vessel wall with 2% collagenase A (Roche Diagnostics) in M199 (Gibco) and resuspended in culture medium (CM) consisting of Endothelial Cell Basal Medium-2 (Clonetics), supplemented with SingleQuot Kit (Clonetics), 10% fetal calf serum (FCS; Biochrom), PenStrep (100 µg/ml), and finally seeded into culture flasks.

Dynamic bioreactor system

Cell seeding was performed using a specially developed bioreactor system for dynamic cultivation. The system was filled with 500 ml CM and kept in a conventional incubator at 37°C (MCO-20AIC, Sanyo); this

allowed pulsatile circulation. The system was able to mimic the physiological conditions of the pulmonary circulation. Flow rate and pulsation were measured continuously (Flowmeter, Medi-Stim) and adjusted accordingly. Pressure and temperature were constantly maintained and monitored (Model-64S, Hewlett-Packard). Gas exchange occurred by constant medium surface aeration inside the oxygenation/compliance chamber. Fresh gas (average 94% air, 6% CO₂) was transported into the reservoir by a roller pump (Ismatec). The pH in the circulating CM was adjusted by the level of CO₂ supply. During dynamic culture, lactate, glucose, pO₂, pCO₂ and pH levels were monitored repeatedly.

EC repopulation and dynamic culture

Reseeding

Decellularized PVs (n = 11) were inserted into the bioreactors after preincubation in CM for 24 h. In three rounds, a total of 1.2×10⁷ ECs (second or third passage) was injected precisely into the valve lumen through specially designed cell seeding inlets. Each seeding step was followed by a 12-h period of slow rotation of the bioreactor (0.1 rotation/min), thereby exposing the entire valve surface in order to achieve optimal attachment conditions. When reseeded had been completed, three PVs were analyzed not only morphologically but also for the metabolic activity of adherent cells (group A).

Dynamic culture

Following reseeded, eight bioreactors were attached to a pulsatile pump, with the pulsatile circulation initially 0.1 l/min. In four bioreactors the initial flow rate was increased by 0.1 l/min twice each day until a maximal flow of 0.5 l/min and a pulsation rate of 20 beats/min were reached (moderate flow, group B). The mean system pressure was maintained at 25 ± 4 mmHg during the whole dynamic cultivation.

In the other four bioreactors the initial flow rate was rapidly increased by 0.35 l/min twice each day until a maximal flow of 2.0 l/min and a pulsation rate of 50 beats/min was attained; this simulated physiological conditions (high-flow, group C). In this group the mean system pressure was also maintained at 25 ± 6 mmHg during culture.

When maximally appointed bioreactor flows were achieved in both groups, the valves were removed from the bioreactors and analyzed for the morphology of reseeded valves and their metabolic activity.

Histology and immunohistochemistry

Extracellular matrix (ECM)

The structural and morphological analysis of the ECM of five decellularized PVs was performed on formalin-fixed, paraffin-embedded valvular tissue sec-

tions using standard hematoxylin and eosin (H&E) and Movat pentachrome staining.

The integrity of collagen IV and laminin, as the main proteins of the basement membrane, was evaluated immunohistochemically (avidin-biotin-peroxidase technique) using a mouse monoclonal anti-collagen IV antibody (Dako) and a rabbit polyclonal anti-laminin antibody (Dako). Native ovine pulmonary valve tissue samples served as positive controls.

Reseeded valves

Histological evaluation of the reseeded valves was performed with standard H&E staining. Repopulated ECs were tested for endothelial phenotype by fluorescence staining using a rabbit polyclonal anti-endothelial nitric oxide synthase (eNOS) antibody (Dianova) and a von Willebrand factor (vWF) mouse monoclonal antibody (Dako). The presence of primary vWF antibody was detected using the avidin-peroxidase method as described previously (10,11). The eNOS-stains were counterstained with DAPI, and vWF-stains with Meyer's hematoxylin solution. Native ovine pulmonary valve tissue samples served as positive controls.

Scanning electron microscopy (SEM)

Evaluation of the morphological integrity of decellularized scaffold and semi-quantitative estimation of PV reseeding were performed using SEM. Samples (one cusp of each PV and wall) were fixed in 2.5% glutaraldehyde (Polyscience) in 0.1 M sodium cacodylate buffer (Merck) at 4°C, for a minimum of 4 h, followed by dehydration in ascending concentrations of ethanol. Samples were critical point-dried (Balzers CPD 030) and sputtered with an ultrathin gold layer (Polaron SEM Coating System). Intraluminal surface and sharp cut cross-sectional areas of the specimens were examined using SEM (Phillips SEM-505).

The presence of cells with a cobblestone-like morphology (typical of ECs) was the inclusion criteria for the semi-quantitative analysis. The area of successful reseeded PV surface was determined by three independent investigators in semi-quantitative manner by estimating the degree of coverage of cusps and PA wall, and classified as: (i) complete monolayer with surface covering >95%; (ii) incomplete monolayer with partial surface covering from 50 to 95%; (iii) incomplete monolayer with partial surface covering from 5 to 49%; or (iv) an absence of ECs (surface covering <5%).

Metabolic activity test (MTS assay)

The CellTiter 96® AQ_{ueous} One Solution Cell Proliferation (MTS) assay (Promega) was used to assess the metabolic activity of re-endothelialized valve tissue according to the manufacturer's protocol.

Briefly, this test is based on a MTS tetrazolium compound (Owen's reagent), which is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion is accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells (12).

Samples of PV wall and cusp (surface ~25 mm²) were incubated in wells containing 700 µl CM and 140 µl MTS at 37°C. After 2 h incubation, formazan production in a sample of 100 µl was measured photometrically at 490 nm in a 96-well plate ELISA Reader MRX (Dynatec), as described previously (13). Three replicates were prepared for each sample. The photometric parameter of the assay medium was used as a baseline value.

Statistical analysis

All data were recorded as mean ± SD. An unpaired Student's *t*-test was used for analyses. Statistical sig-

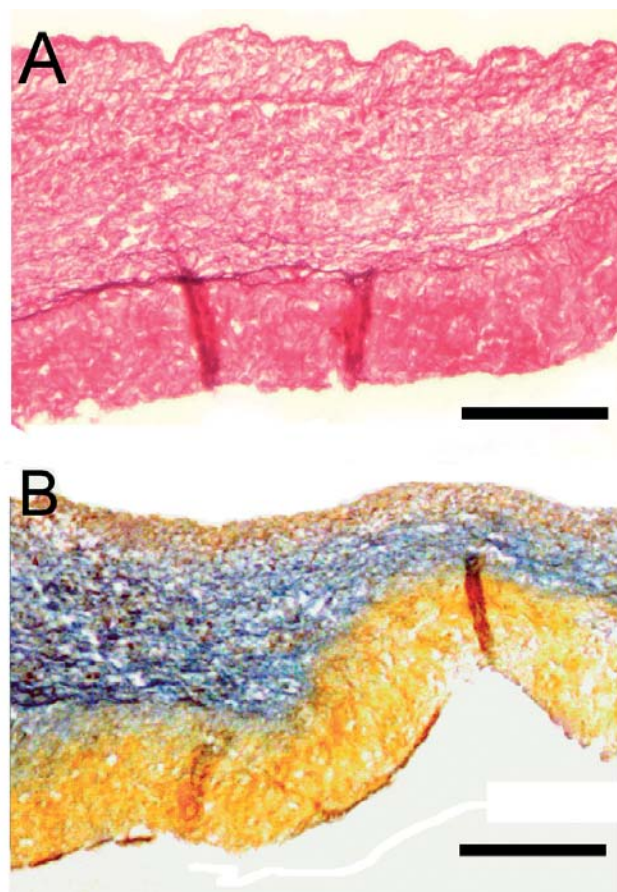


Figure 1: Histology of decellularized pulmonary valve cusp showing complete removal of cells and efficient preservation of the extracellular matrix. A) H&E staining: nuclei show blue; connective tissue shows red. B) Movat pentachrome staining: collagen shows yellow; elastic fibers show red; proteoglycans show blue/green; nuclei show dark purple. Scale bars = 100 µm.

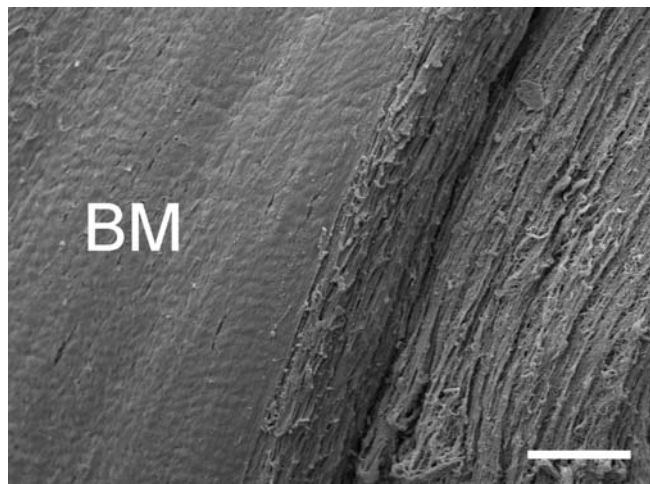


Figure 2: Scanning electron micrograph showing transversal section of decellularized ovine pulmonary valve cusp and presence of the basal membrane (BM) on the luminal surface. Scale bar = 100 μm .

nificance was defined as a p-value <0.05 . A statistical software package for Windows (SPSS, v. 11.0) was used to carry out the statistical analysis.

Results

Morphological characterization of decellularized PV

Histological analysis revealed that treatment with detergents for 24 h resulted in the elimination of cells in the graft below to a detectable threshold (Fig. 1A and B).

In decellularized scaffolds, the microscopic structures remained intact, as shown histologically (Fig. 1A and B). In this respect, SEM revealed an efficiently preserved three-dimensional network of ECM fibers with complete maintenance of basement membrane all along the inner surface of the PA wall and on both sides of the leaflet (Fig. 2). The presence of collagen IV and laminin confirmed the structural integrity of the basement membrane of resultant scaffolds after decellularization (Fig. 3A and B), which indirectly implied maintenance of the adhesion functionality of the scaffold.

PV after reseeding (group A)

After static seeding in the bioreactor for two days (group A), ECs were detected on the luminal surface of the PVs as an incomplete monolayer with covering $\geq 50\%$ (Fig. 4). In this group, no significant differences regarding the degree of EC covering were found between the cusps and the PA wall.

Moderate flow group (group B)

Under dynamic, pulsatile flow at 0.5 l/min, the ECs

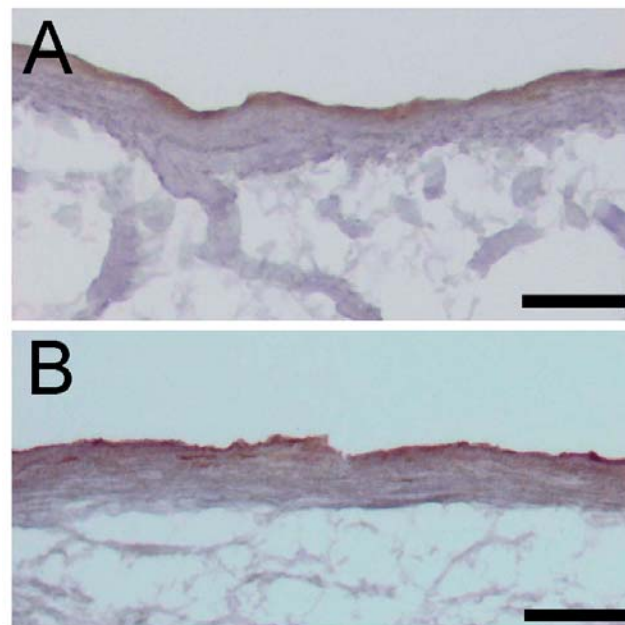


Figure 3: Immunohistochemistry of decellularized pulmonary valve showing the maintenance of collagen IV and laminin as main proteins of the basal membrane after decellularization. A) collagen IV (brown); B) laminin (brown). Scale bars = 50 μm

reached cell confluence and formed a complete monolayer with surface covering $>95\%$ (Fig. 5). The degree of EC covering of the ventricular and arterial sides of valve cusps, as well as of the luminal PA wall surface, was comparatively equal.

Results of metabolic activity assays performed on cells on the reseeded valves (PA walls and valve cusps) in all groups are detailed in Table I. The ECs on both wall and valve cusps showed a significantly higher

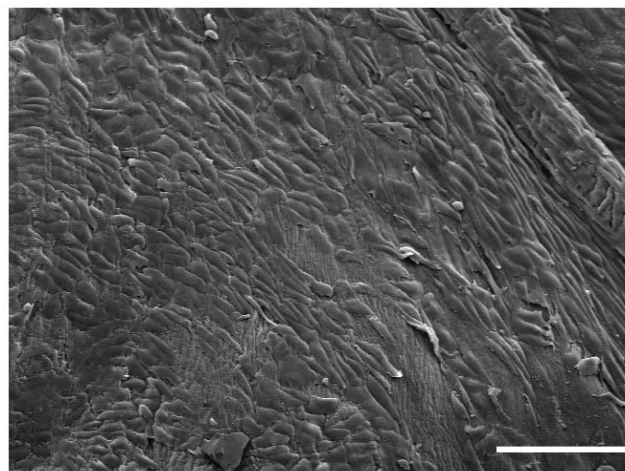


Figure 4: Scanning electron micrograph showing incomplete endothelial cell monolayer on a statically reseeded pulmonary valve cusp with a surface coverage $\geq 50\%$. Scale bar = 100 μm .

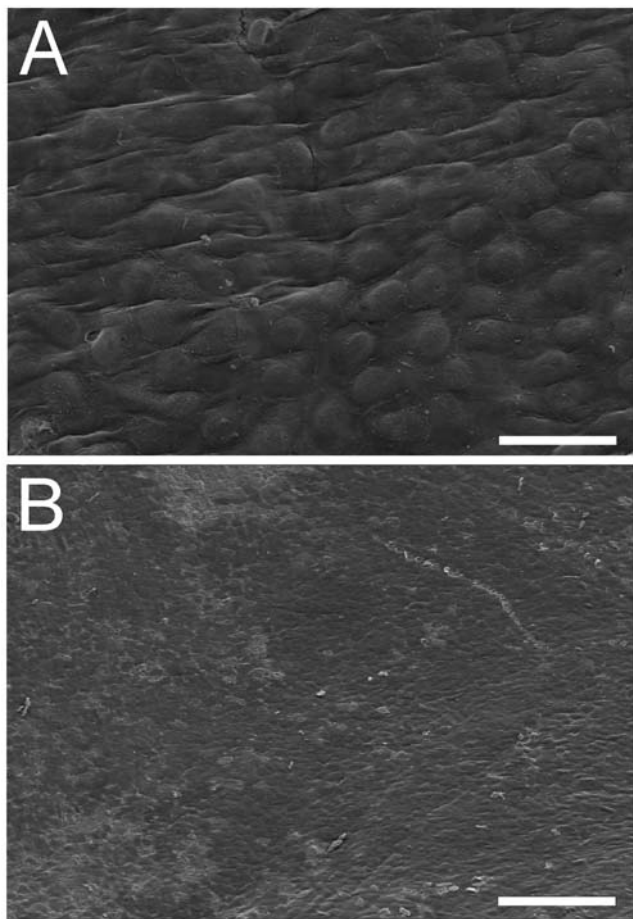


Figure 5: Perfect re-endothelialization of the pulmonary valve (PV) surface under moderate bioreactor flow (0.5 l/min). Scanning electron micrograph of the PV cusp. A) High-power magnification; scale bar = 20 μm . B) Low-power magnification; scale bar = 200 μm .

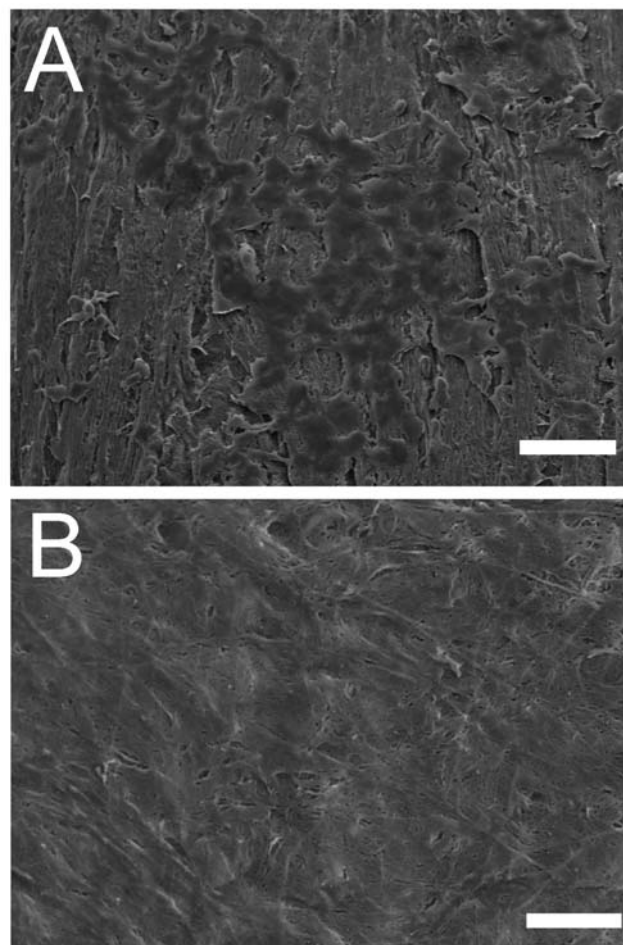


Figure 6: Scanning electron micrograph showing incomplete endothelial cell (EC) monolayer on the pulmonary artery wall <50% (A) and an absence of ECs on the intact basement membrane surface of the pulmonary valve cusp (B) following culture under high-flow conditions (2 l/min). Scale bar = 50 μm .

Table I: Metabolic characteristics of endothelial cell-reseeded pulmonary valves.

| Group/Location | Metabolic activity [†] |
|-------------------------|---------------------------------|
| Group A (static) | |
| Wall | 0.176 \pm 0.029 |
| Cusp | 0.194 \pm 0.023 |
| Group B (moderate flow) | |
| Wall | 0.265 \pm 0.025* |
| Cusp | 0.301 \pm 0.031* |
| Group C (high flow) | |
| Wall | 0.132 \pm 0.015** |
| Cusp | 0.026 \pm 0.021** |

[†]MTS assay; absorbance at 490 nm.

Values are mean \pm SD.

*p < 0.01; **p < 0.001 versus group A.

degree of activity compared to those of group A (p < 0.01).

High-flow group (group C)

A rapid increase in flow rate to 2.0 l/min (group C) resulted in an incomplete EC monolayer with large defects attached to the luminal surface of the PA wall and surface covering <50% (Fig. 6A). In addition, no ECs were detectable on both sides of the valve cusps and valve sinus (Fig. 6B). This absence of cells on the valve cusps was confirmed, using the MTS assay, by a lack of metabolic activity (Table I). The metabolic activity of the reseeded wall was also significantly reduced compared to that of group A (p < 0.001).

Phenotypic analysis of reseeded cells

By using immunohistochemistry, the cells on the surface of reseeded valves in all groups were seen to

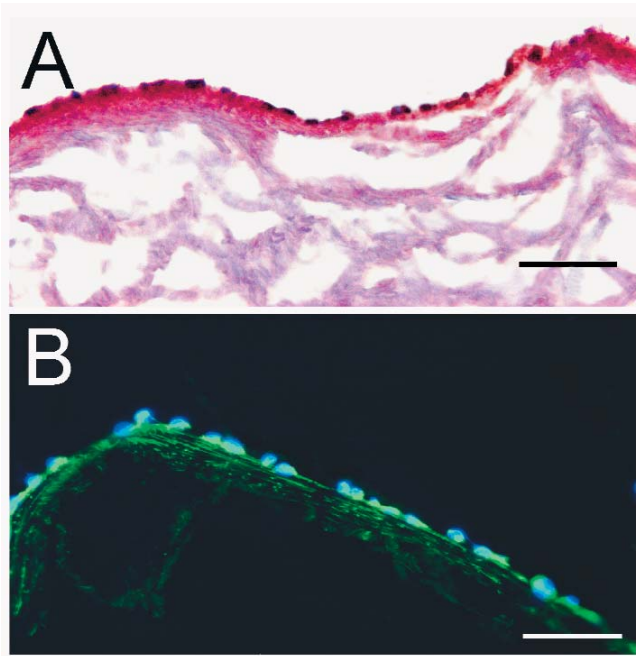


Figure 7: Representative immunostainings showing the expression of (A) von Willebrand factor (vWF; brown) and (B) eNOS (green) and DAPI (blue) as co-immunofluorescence of reseeded endothelial cells on the pulmonary valve, implicating the maintenance of endothelial cell function. Scale bars = 50 μ m.

express vWF and eNOS, thereby demonstrating an endothelial origin and implying the maintenance of functional activity of reseeded ECs (Fig. 7A and B).

Discussion

The effect of two different flow regimens on the culturing success of ECs, which were seeded on detergent-decellularized biological valve matrices in a specially designed bioreactor, was investigated. Recently, several investigations demonstrated the importance of endothelial covering of tissue-engineered valves with regard to improving their anti-thrombogenicity in vivo (2,14). The absence of an endothelial layer on decellularized valves predisposes the unprotected matrix surface to thrombosis and intimal hyperplasia, with subsequent graft failure.

It is known that a dynamic culture under fluid shear stress improves the functional characteristics of the ECs. The endothelium in vivo acts as a signal transduction interface for hemodynamic forces, and the application of physiological shear stress determines the structure, shape, orientation and function of the ECs (3-6).

The building of an EC layer on polymer or biological valve scaffolds under static conditions is relatively straightforward, and has been reported elsewhere

(10,12,15). In contrast, maintenance of the structure and function of reseeded endothelium in a physiological circulation poses an immense challenge for tissue engineering research. The application of high shear-stress on the valve surface, especially in the area of the cusps, during turbulent opening and closing valve action in vivo demands for a high resistance of the ECs to applied hemodynamic forces. Disruption of newly reseeded ECs under high flow in vitro on the polyurethane vascular prosthesis and glutaraldehyde-preserved valve matrices has been described by Gulbins et al. (7). These authors showed that a rapid transfer of the static EC-repopulated constructs in the high-flow circulation led to an interruption of cell-matrix connections and to a cell wash-off effect. However, a stable adhesion between the ECs and studied grafts could be achieved only by slow cell adaptation to flow.

Correspondingly, in the present study was found that a rapid increase in pulsatile bioreactor flow similar to physiological conditions and following the static reseeding of ECs also led to significant interruption of the endothelium from a detergent-decellularized ovine scaffold. Consequently, the damage to ECs was especially distinct in the high-turbulence valve cusp area. Thus, it was hypothesized that after an implantation of tissue-engineered valves reseeding under static conditions, the in-vivo shear forces may similarly damage the EC layer; indeed, this may be the reason for increased valve thrombogenicity in vivo, leading potentially to graft failure.

In contrast, the moderate pulsatile circulation with a stepwise slow increase in flow stimulated the proliferation of seeded ECs on the ovine detergent-decellularized pulmonary scaffold. It is believed that small flow increments may cause adaptation of the seeded ECs, resulting in efficient resistance against any wash-off phenomenon. This would be a crucial step in avoiding EC layer injury under physiological conditions in tissue-engineered valves.

Efficient preservation of the ECM - and especially of the basement membrane - during decellularization might be a prerequisite for successful repopulation with ECs. In the present study, the decellularization method used allowed good preservation of the basement membrane, and of major cell-binding proteins such as collagen IV and laminin. Clearly, the molecular mechanisms of cell-matrix connections for biological decellularized matrix and autologous cells are not well understood, and further investigations are required in this respect. Here, several factors play decisive roles. Cell adhesion is mediated by many different types of interaction between the cell-surface receptors and ligands in the ECM or on the surface of other cells. Recognition of these interactions should help to

achieve better stabilization of reseeded EC layers, improving the outcome of valve tissue engineering. Consequently, several alternative approaches, including polymer binding or protein matrix coating, have shown promise in terms of their preliminary results (16,17).

In conclusion, evidence was provided that a high pulsatile turbulent flow negatively influences the formation of an intact EC monolayer created by reseeded ECs, especially on the cusp surfaces of decellularized biological valve matrices. By using small flow increments and moderate pulsatile flow in a bioreactor, complete re-endothelialization of the pulmonary valves could be achieved. Resistance of the reseeded endothelium to shear-stress might be the consequence of an adaptation process based on the development of a more stable cell-matrix connection as a result of gradually increasing the flow rate in the bioreactor. However, further investigations are needed to provide a deeper insight into the process of dynamic matrix-cell binding at the molecular level.

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Meeting discussion

MR. ADRIAN CHESTER (London, UK): Did you investigate the alignment of the endothelial cells after they had been exposed to flow? In heart valves the endothelial cells align perpendicularly to the flow - it is intriguing whether any endothelial cell type would do that, or is it a specialized function of valve endothelium?

DR. ARTUR LICHTENBERG (Hannover, Germany):

We did not check specifically the alignment of the cells, but on the ventricular side of cusps their distribution was in the direction of flow.

DR. CRAIG SIMMONS (Toronto, Canada): Did you investigate the expression of eNOS and von Willebrand factor on opposite sides of the leaflet? We have shown that in native valves there are differences depending on whether there is a high shear flow or a more circulatory flow on the reverse side of the valve. You took cells from a venous system which does not experience these flows and put them into a system where they experience very different flows. Did you show any differences?

DR. LICHTENBERG: We did not see any differences in eNOS expression between the ventricular and arterial sides of the leaflets or of the wall, but this was not a specific point of the investigation - it was only a subjective view of the investigator.

DR. SIMMONS: So you saw no differences qualitatively at this point?

DR. LICHTENBERG: No.

DR. IVAN VESELY (Los Angeles, California, USA): You said that turbulence may be responsible for these cells being washed off, but can you estimate the degree of turbulence relative to the physiologic situation? I noticed that the shape of the outflow tract is not smooth - it doesn't have sinuses. I wonder whether you had inadvertently subjected these cells to far more turbulence than they would normally experience in either the left or rightsided circulation?

DR. LICHTENBERG: That's why we said similar to physiological condition. But comparison to the normal condition in the pulmonary flow through the pulmonary valve is not easy - the only parameters are

flow, pulsation rate and pressure. I don't know if the leaflet motion under these conditions is the same as in the normal pulmonary valve.

DR. VESELY: I was commenting on the shape of the space behind the leaflets - you may inadvertently obtain much more turbulence than you would normally.

DR. LICHTENBERG: The pulsation curve is stalling the systolic motion of the leaflet. This high-flow condition in vitro may be more traumatic than in vivo.

DR. BRANDON TRAVIS (Arhus, Denmark): I have a response to that in terms of turbulence. I don't think the cells would be removed from the surface just because there is a boundary layer between the surface and the turbulent flow - there is essentially no turbulence in the boundary layer. It would be difficult for the turbulence alone to remove cells from the surface. What may happen in this halfloose state is that there would be a greater viscous shear stress on that area, and that could cause the cells to leave the surface.

DR. VESELY: I always thought that turbulence causes shear stress, but perhaps I am wrong.

DR. LICHTENBERG: The valve is a complex three-dimensional structure, so it's difficult to investigate shear stress at the leaflet or the wall.

MR. CHESTER: In one of your groups there was a similar loss of endothelial cells on both sides of the cusp, so there is more laminar flow as the valve opens, but turbulent flow as it shuts. Were the endothelial cells lost to the same extent on both sides?

DR. LICHTENBERG: Yes, the same covering was detected on both sides. A flow rate of 500 ml per min is good for the proliferation of endothelial cells, but less traumatic for achieving a washoff effect.