Decellularization does not Eliminate Thrombogenicity and Inflammatory Stimulation in Tissue-Engineered Porcine Heart Valves

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Background and aim of the study: **In tissue engineering of heart valves using decellularized xenogenic valves, it has been suggested that cell elimination would result in a biologically inert matrix. The aim of this in-vitro investigation was to evaluate different decellularization methods in regard to the completeness of cell removal, inflammatory response, and thrombocyte activation.**

Methods: **Decellularized porcine Synergraft® valves were compared with porcine pulmonary conduits decellularized with Triton X-100, sodium deoxycholate, Igepal CA-630® and ribonuclease. Completeness of decellularization was evaluated with staining for nuclei and α-Gal epitope. Decellularized heart valves with and without seeding with endothelial cells (ECs) were incubated with human platelet-rich plasma and stained for CD41 and PAC-1 to evaluate thrombocyte activation. Samples were processed for laser scanning microscopy (LSM) and scanning electron microscopy (SEM). Migration of human monocytic cells towards extracted valve proteins was tested.**

Results: **In contrast to the Synergraft, complete cell removal and elimination of the α-gal epitope was achieved with the new decellularization method. Numerous adherent and activated platelets were found on the decellularized matrix. This was inhibited by seeding with ECs. Even in completely cell-free valve tissue extracellular matrix proteins attracted human monocytic cells as in early inflammation, depending on whether porcine or human tissue was used.**

Conclusion: **Important differences were found in the decellularization efficacy of treatment methods. However, even complete elimination of cells and their remnants did not result in a biologically inert matrix. The decellularized porcine heart valve matrix has the potential to attract inflammatory cells and to induce platelet activation. These findings suggest that it will be important to control the different inflammation-stimulating factors if porcine tissues are to be used successfully in tissue engineering.**

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End-stage valvular heart disease is a significant cause of morbidity and mortality, and therefore requires surgical treatment. Currently, heart valve replacement performed with either mechanical or biological prostheses has major disadvantages. In particular, replacement with mechanical prostheses may lead to thromboembolic events and bleeding due to a need for lifelong anticoagulation therapy (1-4). The durability of biological prostheses is age-dependent, with degeneration and calcification leading to early tissue failure occurring especially in patients aged less than 35 years (5-8). Different factors which contribute to this degeneration have been identified, but no theory explains satisfactorily the triggers for calcification of glutaraldehyde cross-linked heart valve prostheses. One of the major problems in heart valve replacement is the lack of living materials with the potential for regeneration and growth. These limitations lead, especially in pediatric patients, to the need for reoperation and also cause increases in both morbidity and mortality.

Tissue engineering represents a promising concept to overcome these disadvantages, the goal being to create a physiological, biologically inert tissue. This requires a biodegradable scaffold which can then be either seeded with recipient autologous cells in vitro using

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bioreactors, or repopulated in vivo after implantation (9-14). Various scaffold materials such as synthetic polymer matrices, fibrin gel matrices or decellularized xenogene matrices have been studied (15-19). The choice of an appropriate matrix appears crucial (20), the most important point being that the ideal matrix should not elicit a specific immunological or unspecific inflammatory response. The decellularized xenogenic heart valve is attractive because of its natural anatomical geometry. It has been shown previously that decellularization protocols vary importantly in the completeness of cell removal, and subsequently a protocol was developed which results in a completely cell-free and cell debris-free, well-preserved heart valve matrix (21,22). However, the effectiveness of decellularization to create a biologically inert heart valve scaffold is, as yet, undetermined. The focus of this in-vitro investigation was to document the importance of complete cell removal, the potential for thrombocyte activation, and the induction of inflammation by the decellularized porcine pulmonary heart valve matrix.

Materials and methods

Valve conduits

Porcine pulmonary valve conduits were obtained from a local slaughterhouse and transported in RPMI 1640 medium containing multiple antibiotics. Sterilization was performed according to the standard procedure for homografts. The valve conduits were incubated with ciprofloxacin (3 mg/l), vancomycin (12 mg/l), amikacin (12 mg/l) and metronidazole (12 mg/l).

Decellularization procedure

The porcine heart valve conduits were decellularized according to a protocol which was developed in the authors' laboratory (21,22). In brief, the conduits were treated with 0.05% Triton X-100 (BioRad, Hercules, CA, USA), 0.05% sodium deoxycholate (Merck, Darmstadt, Germany) and 0.05% Igepal CA-630® (ICN Biomedicals Inc., Ohio, USA) under permanent shaking for 48 h at 4°C. Subsequently, the conduits were incubated with ribonuclease, 100 µg/ml (RNase; Roche Diagnostics GmbH, Mannheim, Germany) and deoxyribonuclease, 150 IU/ml (DNase; Sigma, St. Louis, Missouri, USA) with 50 mmol/l $MgCl_2$ in Dulbecco's phosphate-buffered saline (D-PBS) (Invitrogen, Paisley, UK) under permanent rotation for 24 h at 37°C. The samples were washed thoroughly with M-199 medium (Gibco, Paisley, UK) and 0.2% EDTA (Merck) under permanent shaking for 5 days at 4°C and again washed with M-199 medium alone under permanent shaking for 5 days at 4°C.

Histology

Histological examination was performed for the detection of tissue integrity and cell removal. Slices of each conduit were embedded in paraffin. For general morphology, three sections (10 µm) of each slice were stained with hematoxylin and eosin (H&E) and examined by two independent assistants using high-power field light microscopy.

Alpha (α)-gal-epitope

Specimens of decellularized pulmonary porcine valve conduits (n = 5) were compared with samples of the commercially available Synergraft model 700 (Cryolife Inc., USA) (n = 5) in regard to cell removal and elimination of the α-gal-epitope.

Thrombogenicity

Platelet separation

After providing their informed consent, blood samples were drawn by venipuncture from healthy adult volunteers who had not taken antiplatelet drugs during the previous 10 days. Blood was collected into a 4.0 ml test tube (Greiner, Kremsmuenster, Austria) containing 3.8% sodium citrate as anticoagulant, without the use of a tourniquet. Samples were centrifuged at 300×*g* for 5 min at room temperature to obtain platelet-rich plasma (PRP), or at 1900×*g* for 10 min to obtain platelet-poor plasma (PPP). PRP was then diluted with PPP and adjusted to 200,000 platelets per µl.

Endothelial cell seeding and platelet/matrix adhesion experiments

Human umbilical vein endothelial cells (HUVEC) were prepared using a procedure as described previously (23). Cryostat sections (10 µm thickness) of decellularized heart valve conduit wall tissue were either left untreated (group 1, n = 5) or seeded with HUVEC (group 2, n = 5) on 5% bovine serum albumin (BSA)-coated adhesive microscope slides with $2\times10^4/cm^2$ cells until confluent (at least 24 h) under static conditions in a humidified incubator (37°C, 5% CO_2). Samples of both groups were incubated with PRP for 20 min in a humidified incubation chamber at room temperature and intensively washed with D-PBS (3×5 min).

Laser scanning microscopy (LSM) studies

Three LSM studies were initiated. The first was to demonstrate the morphologic integrity of the conduit matrix after decellularization, samples were stained for porcine collagen types I and III with 0.5 µg/ml lyophilized IgG antiserum (Monosan®; Uden, The Netherlands) and elastin with monoclonal anti-elastin, clone BA-4 mouse IgG_1 isotype, 1:2000 (Sigma).

The second study was to detect cells and the α-gal-epitope in samples of the leaflets and conduit walls.

Cryostat sections of all samples were stained for residual nuclei with 1 µmol/ml of the DNA-specific dye TO-PRO 3® (Molecular Probes, Leiden, The Netherlands). The α-gal-epitope was stained in all samples using 40 µg/ml fluorescein isothiocyanate (FITC)-conjugated *Bandeiraea simplicifolia* (BS) isolectin B4 (Sigma). The incubation time for all stainings was 1 h.

The third study was to document the adhesion and activation of platelets on seeded or non-seeded matrix specimens, as well as their activation. Cryostat sections were incubated with an R-phycoerythrin (R-PE)-conjugated mouse anti human CD41 antibody (clone P2; Immunotech, Marseille, France) and a FITC-conjugated mouse PAC-1 antibody for 20 min in the dark. PAC-1 is a pentameric IgM κ-immunoglobulin that recognizes an epitope on the gpIIb/IIIa ($\alpha_{IIb}\beta_3$) complex of activated platelets at or near the platelet fibrinogen receptor (24). After staining and washing, specimens were fixed for 1 h with 1% paraformaldehyde (pH 7.4) at room temperature in the dark. Nuclei of HUVEC seeded onto the matrix samples were stained with the DNA-specific dye TO-PRO 3 for 10 min at room temperature and washed repeatedly.

Scanning electron microscopy (SEM) studies

Specimens of the decellularized heart valve conduits were seeded with $2\times10^4/cm^2$ HUVEC (n = 5) until confluent for at least 24 h in a humidified incubator (37°C, 5% CO_2), or incubated with HUVEC culture medium alone (n = 5). Samples of both groups were incubated with PRP (200,000 platelets/µl layered onto the luminal side) for 20 min in the wells of a 24-well cell culture plate at room temperature. After intense washing (3×5 min) with D-PBS, specimens were fixed in 2% glutaraldehyde, dehydrated in graded ethanol solutions, critical-point-dried over CO_2, and finally coated with gold-palladium for examination by SEM.

Monocyte migration experiments

Preparation of tissue extracts

Frozen porcine or human pulmonary wall (100 mg), as well as leaflet tissue, were homogenized in 2 ml ice-cold Dulbecco's Modified Eagle Medium (DMEM; Bio Whittaker, USA) using a mortar and pestle. The homogenization was carried out mechanically at 700 r.p.m. for 10 min on ice. The homogenates were aspirated, transferred into centrifuge tubes, and the debris of each homogenate was sedimented by centrifugation at 10,000×*g*, at 18°C, for 30 min. An aliquot (1.6 ml) of each supernatant was carefully withdrawn, and 100 µl of each sample was removed for measurement of protein content using a modified Bradford assay (BioRad, Heidelberg, Germany). The remaining 1.5 ml was used for migration experiments. DMEM without protein extracts (1.5 ml) was used as a negative control.

U937 mononuclear phagocyte cell culture

U937 monocytic cells (25) were purchased from ATCC and cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; Promocell, Germany) and antibiotics at 37°C and 5% CO_2. Fresh medium was added every four days, and cell number was determined using a Microdiff hematology analyzer (Coulter, USA). For differentiation to the monocyte/macrophage phenotype, the cells were treated with 10 nmol/l 1,25-$(OH)_2$-calcitriol (26) for 72 h. Before plating into assay chambers, the cells were washed with DMEM and subsequently resuspended in DMEM without any supplements, to a final concentration of 1.0×10^6 cells/ml.

Monocyte migration assay

The in-vitro cell migration assays were performed using a six-well culture plate with a PET- membrane (Falcon™ cell culture inserts; Beckton Dickinson Labware, France) placed in each well. The pore size of the membrane used in all experimental settings was 3 µm. The cell inserts divided the wells into a lower (LCH) and an upper chamber (UCH). The bottom of each well was covered with 1.5 ml of the protein extracts of heart valve specimens or negative control

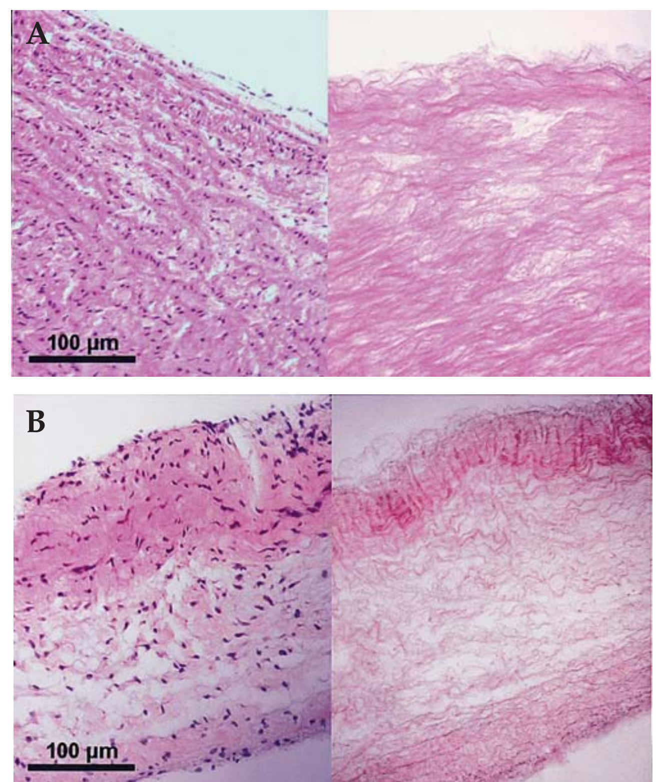


Figure 1: Hematoxylin and eosin staining of native (left) and decellularized (right) porcine pulmonary heart valves. Light microscopy, original magnification, ×400. Upper: Conduit wall. Lower: Leaflet.

medium. Onto the upper side of the filter were placed 1.5×10^6 U937 cells. The chambers were incubated in a humidified 5% CO_2 atmosphere for 24 h at 37°C. After the incubation period, the cell inserts were removed and the bottom adhesive cells loosened. The transmigrated U937 cells were centrifuged at 300×*g* for 10 min and the supernatant was discarded. Cells were stained by adding 200 µl of crystal violet solution and counted by an independent assistant, using a hemocytometer.

Statistical analysis

All values were reported as mean ± SEM. The Wilcoxon rank sum test was used to assess the differences between the groups. A p-value <0.05 was considered to be statistically significant.

Results

Decellularization and α-gal epitope

Specimens of the porcine matrix obtained after treatment with Triton X-100, sodium deoxycholate, Igepal CA-630 and ribonucleases according to the study protocol were completely cell-free in the leaflets and the conduit wall (Fig. 1a and b). Staining for collagen types I and III and elastin showed good preservation of the matrix architecture. In accordance with the complete cell removal shown after staining with the DNA-specific dye TO PRO 3, the BS isolectin B4 staining demonstrated complete elimination of the α-gal-epitope in the wall and leaflets of the treated porcine tissues.

In contrast, in the porcine graft decellularized with Synergraft technology, multiple residual nuclei within the matrix of the conduit wall were seen after staining with TO PRO 3 (Fig. 2a). In accordance with this finding, the α-gal-epitope was detected in the Synergraft.

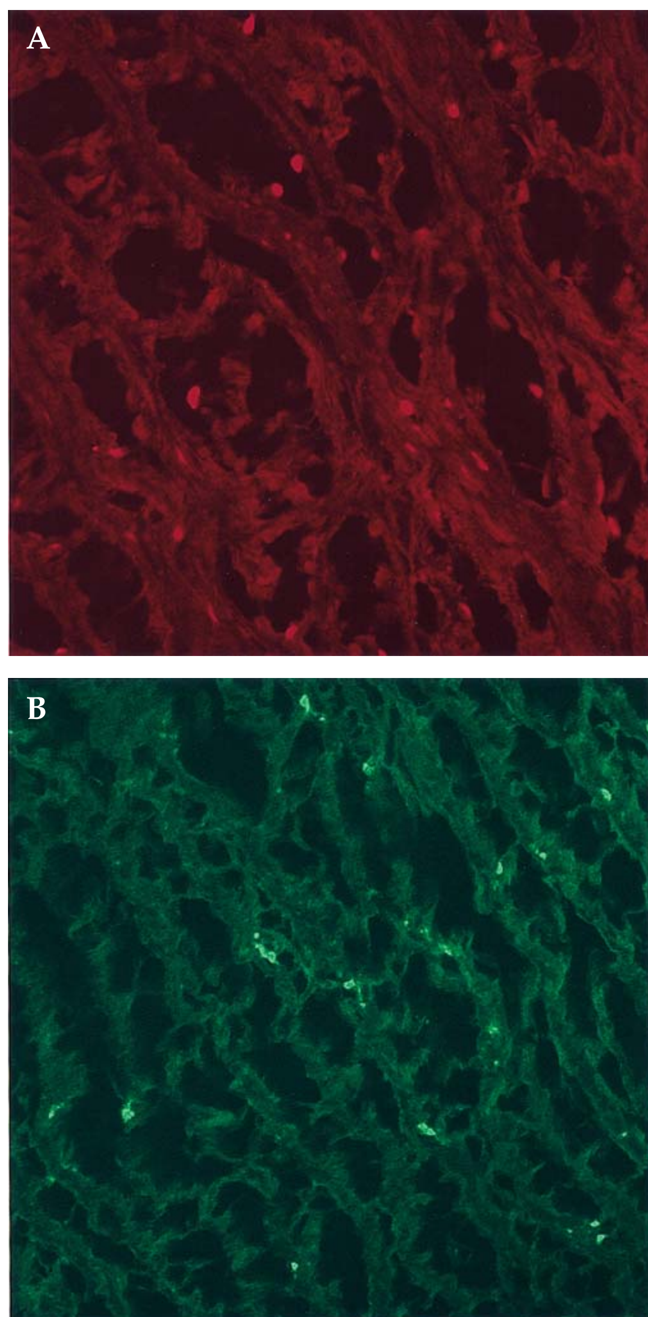


Figure 2: Upper: Synergraft: Multiple residual nuclei within the matrix of the conduit wall (staining for DNA with TO PRO 3: red). Lower: Synergraft: α-gal-epitope within the matrix of the conduit leaflet (staining for α-gal with BS isolectin B4: green). (Laser scanning microscopy, original magnification, ×400.)

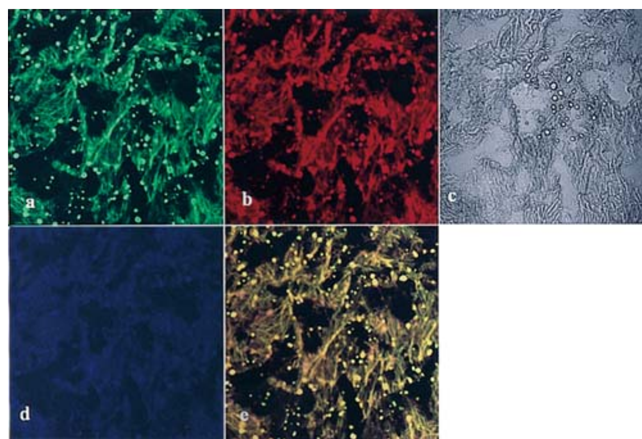


Figure 3: Decellularized porcine matrix after incubation with platelet-rich plasma (PRP) for 20 min (laser scanning microscopy, original magnification, ×400). a) Fluorescent staining with PAC 1 (green) showing multiple activated platelets; b) fluorescent staining for CD41 (red) showing the presence of multiple platelets; c) image taken using differential interference contrast (Nomarski); d) staining with TO PRO 3 (blue); e) overlay of (a), (b), (c) (co-localized CD 41 and PAC 1 signals: yellow).

Samples of the leaflets were completely free of cells, indicating better tissue penetration of the thin leaflets as compared to the much thicker wall with this particular decellularization protocol. Surprisingly, after staining with BS isolectin B4, the α-gal epitope was still present in the leaflets (Fig. 2b).

Platelet adhesion and activation

Examination by LSM revealed numerous activated platelets on the non-seeded matrix after incubation with PRP. The overlaid signals of co-localized CD-41 and PAC-1 resulted in orange to yellow staining on the decellularized tissue sections (Fig. 3). In contrast, after seeding the decellularized matrix with HUVEC, only very isolated platelets were detected. Overlay of the CD-41 and PAC-1 signal resulted in red staining corresponding to the 543 nm laser line exciting CD-41 (Fig. 4). That the cells seen were in fact platelets was proven by positive staining for CD-41 (red). Activation of platelets was demonstrated by positive staining with PAC-1 (green). The seeded matrix did not lead to platelet activation.

Examination by SEM showed an accumulation of aggregated platelets adhering to the surface of the extracellular matrix (Fig. 5a). Seeding with HUVEC achieved a confluent monolayer on the surface of the decellularized matrix. After incubation with PRP, only few isolated platelets were detected on the surface (Fig. 5b). In samples with a partially denuded endothelial monolayer, platelets adhered to areas of exposed matrix and extended their pseudopodia along the fibers (not shown).

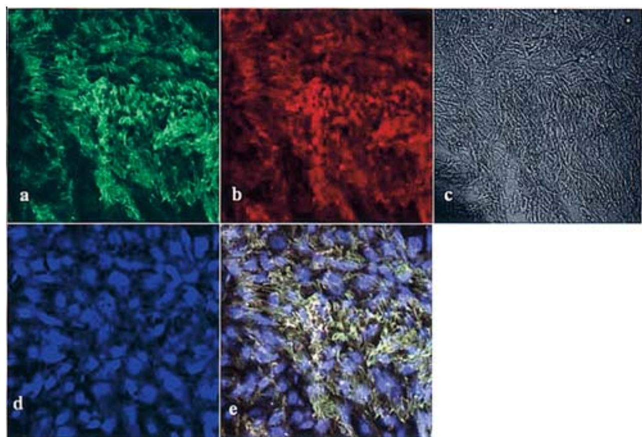
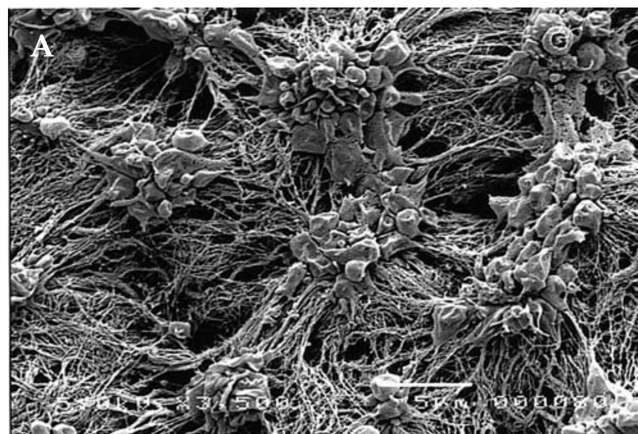


Figure 4: Decellularized porcine matrix repopulated with human umbilical vein endothelial cells (HUVEC) after incubation with platelet-rich plasma (PRP) for 20 min (laser scanning microscopy, original magnification, ×400). a) Fluorescent staining with PAC 1 (green) showing very isolated activated platelets; b) fluorescent staining for CD 41 (red) showing the presence of platelets; c) image taken using differential interference contrast (Nomarski); d) nuclei of HUVECs stained with TO PRO 3 (blue); e) overlay of (a-d) (co-localized CD 41 and PAC 1 signals: yellow; nuclei of HUVEC: blue).

Migration assay

Compared to the negative control ($10.3 \pm 2.1\times10^3$ cells in LCH; n = 12), a strongly increased migration of U937 cells across the PET-membrane was seen within 24 h when protein extracts of native porcine pulmonary wall ($364.5 \pm 59.6\times10^3$; n = 10, $p < 0.01$) or the homograft conduit wall ($452.9 \pm 58.9\times10^3$; n = 10, $p < 0.01$) were added into the LCH of the transmigration chamber (Fig. 6a). These data indicate that a gradient of extracted soluble valve tissue proteins leads to the migration of monocytic cells.

Compared to conventional homograft arterial wall tissue, the proteins of the decellularized porcine pulmonary artery caused a significantly decreased (75.4 ±



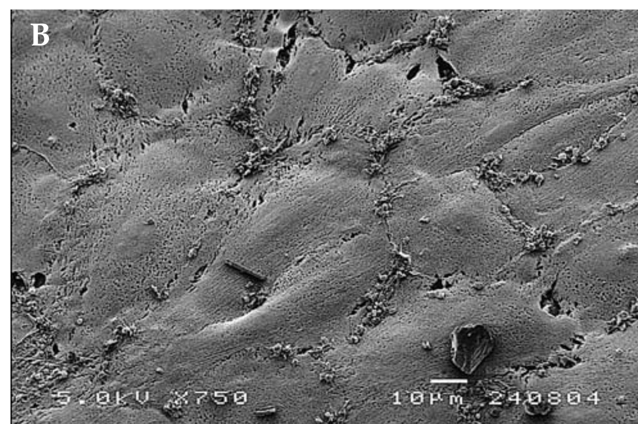


Figure 5: a) Decellularized porcine matrix after incubation with platelet-rich plasma (PRP) for 20 min. Accumulation of aggregated platelets adhering to the surface of the decellularized porcine heart valve. b) Decellularized porcine matrix repopulated with human umbilical vein endothelial cells after incubation with PRP for 20 min. Confluent endothelial cell monolayer with only isolated adherent platelets (both images prepared using scanning electron microscopy).

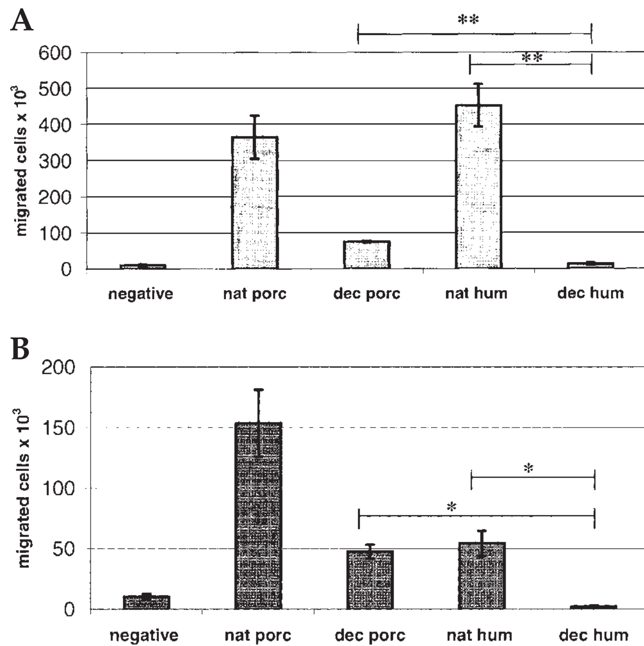


*Figure 6: Migration of U-937 cells towards extracts of pulmonary conduit wall (a) and cusps (b). nat porc: Native porcine; dec porc: Decellularized porcine; nat hum: Cryopreserved pulmonary homograft; dec hum: Decellularized pulmonary homograft. Wilcoxon rank sum test: *p <0.05; **p <0.01.*

2.1×10^3, n = 10, p <0.01) migration of monocytic cells. However, migration towards the decellularized human pulmonary arterial wall tissue ($13.1 \pm 2.8 \times 10^3$; n = 10) was significantly lower (p <0.01) than that of the porcine equivalent, and comparable to that of the negative control (Fig. 6a).

Proteins extracted from native porcine leaflet specimens caused $153.7 \pm 27.7 \times 10^3$ cells to transmigrate across the membrane, and the decellularization procedure reduced the number of U-937 cells in the LCH to $47.7 \pm 5.7 \times 10^3$ (Fig. 6b). The extracts of the human native pulmonary cusps elicited a smaller number of monocytic cells ($54.4 \pm 11.4 \times 10^3$) to transmigrate towards the heart valve proteins. The decellularized human pulmonary valve leaflets again induced only $2.1 \pm 0.9 \times 10^3$ cells to migrate through the membrane, which was within the range of random migration (range: 1 to 19×10^3 cells in LCH).

Discussion

Tissue engineering of heart valves represents a promising approach to overcome the current limitations of common heart valve replacement. The decellularized porcine heart valve, when used as a scaffold for heart valve tissue engineering, represents an attractive possibility because of its anatomic similarity to human valves. Various decellularization procedures have been studied to obtain a cell-free, antigen-reduced valve matrix (27-29). The considerable differences in the efficiency of different protocols have been documented previously, and a method has been developed which enables a completely cell-free scaffold to be achieved (21,22). It has been suggested that cell elimination would result in a biologically inert matrix - that is, decellularization eliminates antigenicity - and therefore a concept of implantation and in-vivo repopulation with endothelial and interstitial cells is proposed (12-14). However, the first commercially available decellularized porcine heart valve, Synergraft, failed due to severe inflammatory response in humans (30). Therefore, the present authors' specific interest was focused on the responsible mechanisms of inflammatory response to the xenogenic matrix.

In this in-vitro study, the efficiency of the decellularization protocol developed for the complete removal of all cells in pulmonary valves was confirmed. It could be shown that samples of the Synergraft conduit were incompletely decellularized. Corresponding with the presence of cells, the α-gal-epitope was identified within the wall and the leaflet matrix, but was not detectable in tissues treated with the 'Vienna-protocol'. The α-gal-epitope is known to be responsible for hyperacute rejection in xenotransplantation. The interactions of preformed anti-pig Ig (xenoreactive natural antibodies) with the terminal disaccharide structure galactose-1,3-galactose (α-gal-epitope), which is present on the surface of porcine vascular endothelium, initiates activation of the classical complement pathway with concomitant endothelial cell activation, resulting in hyperacute rejection and ultimately graft failure (31-33). In a recent study, the α-gal-epitope was detected in commonly used porcine bioprostheses, and it has been documented that some patients, following implantation of these porcine bioprostheses, developed a significant increase in xenoreactive natural antibodies towards α-gal (34). Thus, it was suggested that the remaining α-gal within the matrix of porcine bioprostheses and tissue-engineered porcine heart valves might induce a xenograft-specific immune response. Indeed, this must be considered if porcine valves are decellularized in order to obtain a valve scaffold for tissue engineering, as even very low level expression of the α-gal-epitope may have the potential for initiating an inflammatory response, leading to graft failure.

It was also documented in this in-vitro experiment that the decellularized porcine heart valve matrix is a highly thrombogenic surface. Platelets adhere readily to the fibrous matrix and are activated. Seeding the matrix with endothelial cells effectively abolishes platelet adhesion and activation. Platelet activation is

even seen in samples with an incomplete endothelial cell layer in areas in which the decellularized matrix is not covered, suggesting that an intact endothelial lining is crucial. It is well known that the exposition of collagen in vascular injury leads to an immediate activation of platelets and, through activation of the coagulation pathway, to sealing of the wound (35). Therefore, it is not surprising that the uncovered collagen fibers of the decellularized matrix would exhibit a strong prothrombotic stimulus. In the case of the Synergraft, however, thrombosis was not a dominant finding and the present results underline the importance of an intact endothelium in cardiovascular tissue engineering. All currently used biological prostheses, as well as homografts which lack a functional endothelial coverage, have limited durability (36,37). Various mechanisms have been identified as being responsible for degeneration, but no theory provides any satisfactory explanation of the triggers for calcification of glutaraldehyde cross-linked heart valve prostheses (5,6). It is hypothesized that platelet adhesion - which is known to be involved in proinflammatory processes - may also be a contributing factor. Platelet adhesion supports leukocyte adhesion and transmigration through the interaction of platelet P-selectin with leukocyte P-selectin glycoprotein ligand-1 (38). Furthermore, proinflammatory cytokines and chemokines leading to the activation of monocyte integrins and increased monocyte recruitment are expressed by activated platelets (39,40). Therefore, the influence of platelet-leukocyte interactions seems to be crucial in the context of tissue engineering, and the elimination of thrombocyte activation is important not only from the point of view of graft thrombosis but also to mitigate the inflammatory response of the recipient towards the implant.

In the present studies it was also possible to show, in a migration assay, that the decellularization of porcine heart valve tissue reduces monocyte migration towards extractable proteins, but does not completely eliminate transmigration. In contrast, in decellularized human heart valves transmigration was effectively abolished. Non-cellular integral components such as glycosaminoglycans are known to have the capacity for interaction with cytokines and chemokines (41), and their elimination might play an essential role in the avoidance of an immune response. While various cell types are involved in the initial inflammatory response, monocytes represent the first line of action.

In conclusion, the results of the present study indicated that the decellularization of xenogenic heart valve tissue did not result in a biologically 'silent', inert matrix. Decellularization protocols were seen to vary in their efficiency of cell removal, and incomplete decellularization was associated with the presence of the α-gal-epitope which may be an early inflammatory stimulus. It was further shown that the decellularized matrix induced platelet adhesion and activation. Seeding with endothelial cells effectively abolished platelet adhesion and activation. It was also shown that the migration of monocytic cells towards decellularized porcine heart valve extractable proteins was reduced, but not eliminated, in contrast to human decellularized tissue. The present findings suggest that a number of inflammatory stimuli are active within decellularized xenogenic tissue, and this will need to be addressed in tissue engineering efforts using a xenogenic matrix if graft failure is to be avoided.

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Meeting discussion

SIR MAGDI YACOUB (London, UK): You detected α-gal by immunocytochemistry, but have you performed any antibody testing to see if the α-gal would fix naturally occurring antibodies in humans, and whether it was complement-fixing, or not?
DR. MARIE-THERES KASIMIR (Vienna, Austria): We did not try that, but a recent report indicated that after the implantation of porcine biological bioprostheses patients developed increased levels of antibodies against α-gal.
MR. YACOUB: But you did not see it fixed to the valve, or to be complement-fixing?
DR. LLOYD WOLFINBARGER (Virginia Beach, Virginia, USA): Many groups use a combination of DNase and RNase in their decellularization protocols. As you only used RNase did you have any complications leaving the nucleic acids in your matrix? When we decellularize, we can often see residual nucleic acid staining. Did you encounter any problems by not using DNase?
MR. YACOUB: So you did not stain for nucleic acids afterwards to see whether there was complete clearance?
DR. KASIMIR: We stained specifically for DNA, and there were no residual cells.
DR. PETER ZILLA (Cape Town, South Africa): It would be interesting to know how the thrombogenicity of the non-endothelialized group compares with the standard thrombogenicity of bioprostheses. Did you examine the same tissue after glutaraldehyde fixation, because it is thought that glutaraldehyde cross-linking might reduce thrombogenicity?
DR. KASIMIR: No, we have not yet tried that.
DR. SVEN BEHOLZ (Berlin, Germany): Most patients having a Ross operation will be prescribed aspirin for two to three months. Would you also see this type of thrombogenicity in patients given aspirin?
DR. KASIMIR: I don't know - it depends on the response to aspirin. The non-seeded matrix is a highly thrombogenic surface. I can't believe that taking aspirin would be enough to prevent thrombogenicity.
DR. BEHOLZ: Would this be of any consequence for your postoperative anticoagulation management?
DR. KASIMIR: Perhaps.
MR. YACOUB: A consequence of what?
DR. BEHOLZ: Perhaps the patient should be given warfarin?
DR. FRANCISCO DA COSTA (Curitiba, Brazil): Your results reinforce our studies, where we used decellularized allografts. We performed a study in humans that showed there is no immune response, no antibody production against human matrices. Do you think that if you were to use proper decellularization, you could use porcine or other heterograft matrices instead of the allograft?
DR. KASIMIR: We showed that the matrix components had induced an inflammatory response which seen in human decellularized tissue. So, we must identify the triggers in the porcine matrix.
MR. YACOUB: Looking at the cell proliferation and the chemotactic points, you used a cell line for your response?
DR. KASIMIR: Yes.
MR. YACOUB: Was this a monocyte cell line?
DR. KASIMIR: It was monocytic cell line, U-9036.
MR. YACOUB: Did you consider using T cells of either CD4 or CD8?
DR. KASIMIR: It was a tumor cell line of monocytic cells, which we did not stain at all.
MR. YACOUB: And you chose that in preference to other cells because you thought it was important for the xenograft reaction or for an immune response or a foreign body reaction? You used the word 'inflammation' rather than immune response.
DR. KASIMIR: For testing the chemoattractants of the decellularized matrix.
DR. SIMON P. HOERSTRUP (Zurich, Switzerland): These types of study are very important because they clearly show that decellularization is not everything. We have to look beyond that, to matrix immunogenicity. Importantly, we must also be careful with everything that is xenogenic.
MR. YACOUB: Preclinical testing is also very important.