Regenerative medicine in cardiovascular disease: from tissue enginering to tissue regeneration

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PART I

Tissue Engineering of Aortic Valve Leaflets

CHAPTER 2

HISTOLOGICAL EVALUATION OF DECELLULARIZED PORCINE AORTIC VALVES: MATRIX CHANGES DUE TO DIFFERENT DECELLULARIZATION METHODS

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ABSTRACT

Background Several decellularisation techniques have been developed to produce acellular matrix scaffolds for the purpose of tissue engineering, mostly comprising (non-)ionic detergents or enzymatic extraction methods. However, the effect of chemically induced decellularisation on the major structural and adhesion molecules as well as glycosaminoglycans, and the possible replenishment of lost compounds has escaped attention.

Methods Porcine aortic valves were treated with two different methods: detergent Triton X-100 and enzymatic Trypsine cell extraction. (Immuno-) histochemistry was used to address changes in extracellular matrix constitution (elastin, collagen, glycosaminoglycans, chondroitin sulfate, fibronectin and laminin) and the production of extracellular matrix components by seeded endothelial cells.

Results The Trypsine treated group showed a fragmentation and distortion of elastic fibers. Changes in collagen distribution were observed in both groups. An almost complete washout of glycosaminoglycans and chondroitin sulfate was observed in the Triton and Trypsin treated group, but the latter with a smaller glycosaminoglycans reduction. Both treatments resulted in a considerable washout of the adhesion molecules laminin and fibronectin. Furthermore, seeded endothelial cells were capable of synthesizing laminin, fibronectin and chondroitin sulfate.

Conclusions Chemically induced decellularisation by Triton or Trypsine resulted in changes in the extracellular matrix constitution, which could lead to problems in valve functionality and cell growth and migration. Seeded endothelial cells were capable of synthesizing extracellular matrix components lost by cell extraction. Further studies on tissue engineering should focus more on the effect of chemically induced cell extraction on the extracellular matrix of the remaining scaffold and the in vitro or in vivo replenishment of lost compounds.

INTRODUCTION

Currently used biological heart valves are shown to have poor long–time durability. Fresh and cryopreserved heart valve homografts containing viable cells are capable of inducing an immune response, resulting in valvular degeneration [1, 2, 3]. Gluteraldehyde preserved porcine xenograft valves on the other hand are considered to have a limited durability due to the lack of viable cells inside the matrix [4]. Furthermore homograft and xenograft valve conduits have no ability to grow, which is of particular relevance to the pediatric population.

To overcome these problems replacement of the immunogenic donor cells by non-immunogenic autologous cells is considered to be a promising approach. Decellularised allogeneic [5, 6] or xenogeneic [7, 8, 9] heart valve scaffolds can be reseeded with autologous cells of the recipient prior to implantation, or be repopulated by recipient cells *in vivo*. These so-called tissue engineered heart valves are believed to be non-immunogenic and to have growth and regenerative potentials.

Several groups have described methods to obtain decellularized heart valve leaflets comprising ionic [3, 9, 10] and non-ionic [7] detergents, as well as enzymatic extraction methods [5, 8]. These methods showed sufficient decellularisation capacity with promising results from *in vivo* animal models [8, 10]. Furthermore the first human implantation clinical trials have already been undertaken [6, 11].

A tendency exists to focus on cell extraction, while the effect of the decellularisation procedure on structural ECM molecules is limited to collagen and elastin fibers. However, the effect of chemically induced cell removal on glycosaminoglycans and adhesion proteins such as laminin and fibronectin has escaped attention.

The aim of the present study was to address histological changes in porcine ECM constitution induced by two different cell extraction methods; a non-ionic detergent Triton X-100 and an enzymatic Trypsin decellularisation method. Furthermore, the potential of seeded arterial endothelial cells to produce extracellular matrix components was assessed.

MATERIALS AND METHODS

Dissection of Porcine Aortic Valves

Aortic valve conduits of 21-week-old pigs were obtained from a local abattoir. Immediately after the arterial heart valves were grossly excised from the heart, they were stored in Hanks balanced salt solution (HBSS) at 4 °C to shorten warm ischemia time. At the laboratory the aortic valves were dissected from the pulmonary valves and freed from fat and most of the myocardium, leaving only a small rim of subvalvular myocardium.

Decellularization Procedures

For decellularisation of the aortic heart valves two different methods were applied: the non-ionic detergent Triton X-100 and a Trypsin enzymatic cell extraction technique.

Triton method The aortic valve conduits were placed in phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} containing 1 % tert-octylphenyl-polyoxyethylen (Triton X-100®) in 0.02 % ethylene-diamine-tetra-acetic acid (EDTA), 0.02 mg/ml Gentamicin, 0.2 mg/ml DNase and 20 mg/ml RNAse-A for 24 hour at 37 °C under continuous shaking as previously described [7]. They were then washed for 2 x 24 hours at 4 °C under continuous shaking to remove residual substances.

Trypsin cell extraction The aortic valve conduits were placed in a solution of 0.5 % trypsin, 0.05 % EDTA, 0.02 % Gentamicin, 0.2 mg/ml DNase and 20 mg/ml RNase A in Milli-Q for 1 to 17 hrs under continuous shaking at 37 °C. The valves were then washed 2 x 24 hrs with HBSS at 4 °C.

Endothelial Cell (Ec) Culture and Seeding

Porcine ECs were harvested from the descending thoracic aorta using 0.2 % collagenase A (Boehringer Mannheim) in phosphate-buffered saline (pH 7.4) for 15 min at 37 °C as previously described [7]. Primary ECs were cultured in Iscoves modified DMEM containing l-glutamine (Gibco BRL), 10 % fetal calf serum (Gibco BRL), 5 ng/ml ECGF (Roche Molecular Biochemicals), 100 U/ml penicillin, 100 mg/ml streptomycin and 5000 U/ml preservative-free heparin. At confluence the Po cells were trypsinized (0.05 % in EDTA), pelleted at 300g and subsequently seeded onto the lamina fibrosa (LF) of Triton decellularized valvular leaflets. After 10 days culture the leaflets were fixed and proceeded for immunohistochemistry.

(Immuno-)histochemistry

The specimens were fixed in 2 % acetic acid / 98 % ethanol for 48 hrs at 4 °C. To wash out acetic acid the fixed tissues were further dehydrated in 100 % ethanol (2x 2 hrs) and xylene (2x 2 hrs) and subsequently embedded in paraffin. Sections of 10 mm were cut and mounted serially onto protein-glycerin coated glass slides. Immunohistochemical staining was performed by overnight incubation at room temperature as described previously [13]. The primary antibodies used were the polyclonal rabbit anti-human fibronectin (1:400, Dako, Denmark), laminin (1:15, Biogenex, USA), rabbit anti-human von Willebrand Factor (1:250, Dako, Denmark) and a monoclonal mouse anti-human chondroitin sulfate (1:2000, Bio-Yeda, Israel). To enhance immunoreactivity of laminin the sections were pre-treated with pronase E (0.1 nU/ml) for 5 min at 37 °C. Bounded primary antibodies were visualized with horseradisch peroxidase-conjugated swine anti-rabbit (1:250, Dako, Denmark) or rabbit anti-mouse (1:250, Dako, Denmark) antibody. Sections were shortly counterstained with haematoxilin, while adjacent sections were stained with standard Azan (collagen), Resorcin/Fuchsin (elastin) and Alcian blue (glycosaminoglycans).

RESULTS

Morphology of a Normal Valve

Normal porcine aortic valve leaflets display a typically three layered structure as seen in human aortic valves, a lamina ventricularis (LV), spongiosa (LS), and fibrosa (LF) can be recognized (fig 1). These layers differ both in architecture and extracellular matrix components. Radially aligned elastin fibers are found predominantly at the ventricular side in the LV (Fig 1a). The most abundant component of the porcine aortic valve is collagen with densely packed fibers in the LV and LF, and only loosely arranged fibers in the LS (Fig 1d). Glycosaminoglycans (GAG) are found predominantly in the LS with also some staining at the arterial side of the LF (Fig 1g). Chondroitin sulfate staining was evident throughout all layers of the leaflet but with a higher expression in the LS, LV and arterial side of the LF (Fig 2a). Laminin expression was found throughout the entire leaflet but with higher expression pattern in the LF, predominantly in the basal lamina and some spots in the LS (Fig 2d). Fibronectin expression was distributed throughout the entire leaflet with stronger expression patterns in the two basal laminae of the LF and LV (Fig 2g).

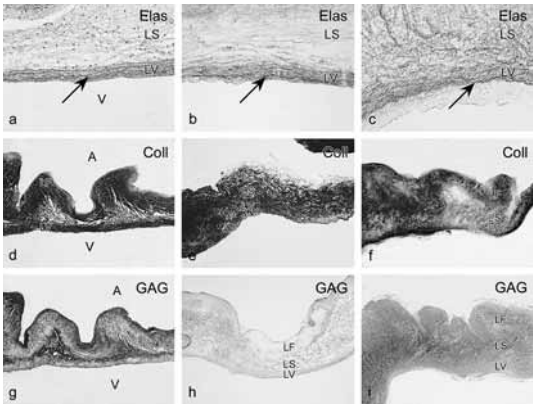

Figure 1. (a,d, g) Histology of a normal porcine aortic valve (left panel). Elastin fibers are found at the ventricular side of the lamina ventricularis (LV) (1a, arrow)). Collagen is mainly found in the LV and lamina fibrosa (LF) (1d). Glycosaminoglycans (GAGs) are especially found in the lamina spongiosa (LS) and the arterial side of the LF (1g). (b, e, h) Histology of a Triton X-100 treated porcine aortic valve (middle panel). No distortion or fragmentation of elastic fibers was observed (1b, arrow). A widening of the interfibrillar spaces was observed especially in the LF (1e). There was also an almost complete washout of GAGs from all layers (1h). (c, f, i) Histology of a Trypsin treated porcine aortic valve (right panel). There was a distortion and fragmentation of elastic fibers in the LV (1c, arrow), with a less compact appearance of the collagen formations (1f). There was a smaller reduction of GAG as compared to Triton treatment (1i). Arterial (A) and ventricular (V) side of the leaflet. Magnification (a-c, 40x, d-i 20x)

Acellularity

Triton X-100 treatment resulted in a complete loss of cellular structures from the entire valve leaflet. Cellular remnants were only found in the myocardium and in the aortic wall. The layering within the leaflets and aortic wall was preserved, while the valve leaflets were still competent.

Using trypsin to decellularize the valves the layering of the leaflet was unimpaired. It contained shrunken cells with picnotic nuclei, which had lost contact with the extracellular matrix. Substraction of the cells from the leaflet, myocardium and vessel wall was impossible, even with extended washings. Prolonged treatment with trypsin for up to 17 hours reduced the cell number, but affected the normal valve configuration and resulted in a substantial damage.

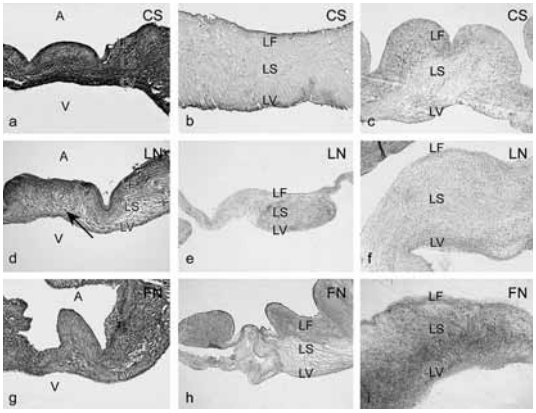

Figure 2. (a,d, g) Immuno-histochemistry of a normal porcine aortic valve (left panel). Chondroitin sulfate (CS) expression was evident throughout all layers but with a higher expression in the lamina spongiosa (LS), lamina ventricularis (LV) and arterial side of the lamina fibrosa (LF) (2a).Laminin (LN) expression was present in all layers of the leaflet with higher expression in the basal membrane of the LF and some spots in the LS (2d). Fibronectin (FN) expression was also present in all layers with a higher expression in the two basal membranes (2g). (b, e, h) Immuno-histochemistry of a Triton X-100 treated porcine aortic valve (middle panel). There was a strong reduction in CS expression from all layers of the leaflet with only some expression at the arterial and ventricular side (2b). There was also a strong decrease of LN (2e) and FN (2h) expression with loss of their specific distribution paterns. (c, f, i) Immuno-histochemistry of a Trypsin treated porcine aortic valve (right panel). There was no detectable CS staining (2c), with also a considerable washout of LN (2f) and FN (2i) with loss of their specific distribution patterns. Arterial (A) and ventricular (V) side of the leaflet. Magnification (a-i, 20x)

Morphology of Decellularised Valves (Table 1)

Triton treatment No distortion or fragmentation of elastic fibers was observed compared to a fresh leaflet (Fig 1b). There was a loss of collagen density in the LF and LV with widening of the interfibrillar spaces, especially in the LF (Fig 1e). There was an almost complete washout of GAGs in the LS and arterial side of the LF (Fig 1h), and also a strong reduction in chondroitin sulfate expression from all layers of the leaflet with only some minor expression at the arterial and ventricular side (Fig 2b).
A considerable washout of both laminin and fibronectin in all layers of the leaflet was observed, while the specific distribution patterns where lost (Fig 2e, h).

Trypsin treatment A distortion and fragmentation of elastic fibers in the LV was observed (Fig 1c). A reduction in collagen staining was observed, especially in the LF and LV where the collagen formations were less compact (Fig 1f). There was a smaller reduction of GAGs as compared to the Triton treated leaflets (Fig 1i). However, there was no detectable staining of chondroitin sulfate in all layers of the valve leaflet (Fig 2c). A considerable washout of both laminin and fibronectin with loss of the specific distribution patterns was observed (Fig 2f, i).

EXTRACELLULAR MATRIX COMPOSITION

		extracellular matrix composition					
		Elastin	Collagen	GAG	Cs	Ln	Fn
Triton X-100	LF	-	↓↓	↓↓↓↓	↓↓	↓↓	↓↓
	LS	-	=	↓↓↓↓	↓↓↓↓	↓↓	↓↓
	LV	=	↓	↓	↓↓↓	↓↓	↓↓
Trysin	LF	-	↓↓	↓↓	↓↓↓↓	↓↓↓↓	↓↓↓
	LS	-	=	↓	↓↓↓↓	↓↓↓↓	↓↓↓
	LV	↓↓↓	↓↓	↓↓	↓↓↓↓	↓↓↓↓	↓↓↓

Table 1. Changes in extracellular matrix composition due to Triton X-100 and Trypsin treatment. LF, lamina fibrosa; LS, lamina spongiosa; LV, lamina ventricularis. GAG, glycosaminoglycans; Cs, chondroitin sulfate; Ln, laminin; Fn, fibronectin. Compared to a normal leaflet, no changes (=), minor changes (↓), moderate changes (↓↓), strong changes (↓↓↓), severe changes (↓↓↓↓).

Endothelial Cell Seeding

After ten days approximately 80 % of the surface of the leaflet was covered with a monolayer of vWF-positive endothelial cells (Fig 3a,b). The cells did not migrate into the leaflet. Sometimes small clusters of cells consisting of two to four cell layers were observed. The endothelial cell monolayer was also strongly positive for fibronectin (Fig 3c), laminin (Fig 3d) and chondroitin sulfate.

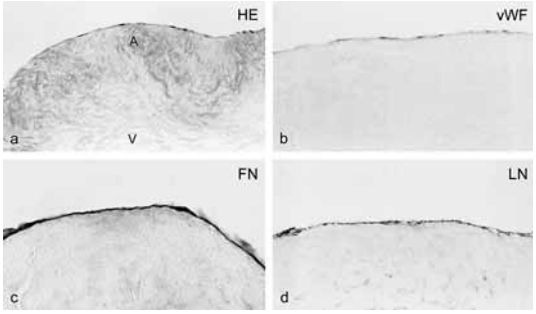

Figure 3. Endothelial cell seeding of Triton X-100 decellularised valves. Seeded endothelial cells stained positive for vWF (a), seeded cells were capable of producing laminin (b), fibronectin (c) and chondroitin sulfate (d). Arterial side valve leaflet (A). Magnification (40x)

DISCUSSION

In this study we compared the effect of chemically induced decellularisation on different extracellular matrix molecules between two methods; a non-ionic detergent Triton X-100 and trypsin enzymatic cell extraction. Valves treated with Triton X-100 showed a completely cell-free structure across the complete thickness of the valve leaflet, which is consistent with earlier results by Bader et al. [7]. However, Kim and coworkers failed to obtain effective decellularised valves using Triton X-100. They presumed that this was due to a technical difficulty with the additional RNase and DNase for the exclusion of potential cellular remnants [14].
Trypsin treatment has previously been reported to be a successful method for decellularization of ovine [8] and human [5] heart valves. However, in this study treatment of porcine valves with trypsin for a period up to 17 hours did not result in a sufficient removal of leaflet cells. The cells, however, did loose their contact with the ECM. An explanation could be that we applied a shorter treatment interval than other studies, where treatment durations up to 48 hours were applied [5, 8]. However, in a recent study trypsin treatment for up to 96 hours also failed to produce completely acellular valves with multiple residual nuclei within the matrix [25].
After decellularization changes in the ECM constitution were examined by (immuno-)histochemistry for both decellularisation methods. After Triton X-100 cell extraction no changes in elastin distribution were observed, however, trypsin treatment resulted in a distortion and fragmentation of elastic fibers in the LV. Elastin present in the normal aortic valve leaflet is coupled to collagen fibers and is predominantly present in the LV as a large continuous sheet of amorphous or compact mesh elastin that covers the entire layer [15].
The elastin in the LV is considered to maintain a specific collagen fiber configuration, and restores collagen fiber structures back to their radially compressed state between consecutive loading cycles [16]. Damage to elastin would therefore alter mechanical behavior of the valve leaflet, resulting in a reduced extensibility and increased stiffness [17].
Changes in elastin configuration due to chemically induced cell extraction may therefore contribute to early valve degeneration and reduced long-time durability.

In both methods minor changes in collagen distribution were detected, Triton X-100 cell extraction resulted in a decrease of collagen density with widening of the interfibrillar spaces, which is consistent with earlier findings by Bader et al. [7]. Furthermore, cell extraction by trypsin resulted in a less compact appearance of collagen formations in the LF and LV. Collagen is mainly found in the LV where it provides strength and stiffness to maintain coaptation during diastole [12]. Therefore, although there were only minor changes, a reduction in the quantity of the collagen fiber network could result in loss of a valve's biomechanical function [18]. This could possibly lead to valvular insufficiency after implantation.

Triton X-100 treatment resulted in an almost complete washout of GAGs from the LS and also, but to a lesser extent, from the trypsin treated valve leaflets. Furthermore, both methods resulted in a complete loss of the GAG chondroitin sulfate expression.
The LS from the aortic valve leaflet acts as a cushioning layer between the other structural layers because of its high content of hydrophilic GAGs that readily absorb water to form a gel, which resists deformations during valve function [12]. Changes in GAG distribution of the LS could therefore lead to altered internal shear properties and may increase internal stresses during opening and closing, contributing to early valve failure [19].
In both the valves treated with Triton X-100 and trypsin the loss of fibronectin was comparable to that seen for laminin. A considerable washout of these adhesion molecules from the leaflets with loss of their specific distribution patterns was observed. Fibronectin is a dimeric glycoprotein found in the extracellular matrix of most tissues and serves as a bridge between cells and the interstitial collagen meshwork. Furthermore, it plays a roll in cell growth, proliferation and migration [20]. Laminin promotes the attachment of epithelial cells to the basal lamina and is also involved in the migration and growth of these cells [21, 22]. Therefore loss of these adhesion molecules by chemically induced cell extraction may lead to a disturbance in migration and growth of cells after *in vivo* or *in vitro* repopulation.
Although the use of acellular xenografts and homografts as biological scaffolds for the purpose of tissue engineering seems a promising approach, the effect of currently used chemicals for cell extraction on the remaining ECM has to be further elucidated.

Recent decellularisation studies comprising ionic detergents such as sodium-dodecyl-sulfate (SDS) [3, 9] or combination of ionic- and nonionic detergents [25] showed excellent cell removal capacity with preservation of the major structural ECM molecules. However, also disintegration of collagen fibers after SDS treatment has been reported, even in concentrations of 0.01% [25]. Another study showed fragmentation and swelling of the collagen after SDS treatment [26].
In a recent study from our laboratory we showed that SDS treatment of rat aortic valves resulted in a preservation of the collagen structure, but in a loss of chondroitin sulfate and fibronectin [3], comparable to what we observed in the present study. These contradictory results regarding the ECM damage described in literature could be caused by the kind of detergent used but detergent concentration, duration of treatment, presence of protease inhibitors and species differences, could be of influence too. Detergents are water-soluble molecules that are divided in an ionic and non-ionic group according to their hydrophilic/hydrophobic character and ionic groups. These differences determine the pattern of protein-detergent interactions and possibly their ultimate effect on the ECM constitution. Others have hypothesized that the discrepancies observed between various decellularisation techniques are due to activation of proteases, which can lead to autolysis of ECM proteins [27]. In the present study EDTA an inhibitor of MMPs,

was added to both used protocols, to reduce the effect of protease activation. Therefore it is not very likely that the observed differences in ECM damage is caused by the protease activation.

Recently Leyh and coworkers showed in a sheep implantation model that the source of decellularized valve matrix conduits (allogeneic or xenogeneic) influences in vivo repopulation and early calcification [23]. They hypothesized that this is due to different ECM microenvironments of different biological matrices or to a species-specific ECM component damaging effect of the decellularization procedure. Furthermore, early failure of non-fixated, decellularized porcine heart valves after implantation in pediatric patients has already been reported with calcific deposits and no cell repopulation of the matrix [24]. These results indicate that even minor changes in the ECM scaffold microenvironment could have significant effects on their use as a scaffold in tissue engineering.

Besides the effect of cell extraction on the ECM, the possibility of production of ECM compounds by *in vitro* reseeded cells was also investigated. Cultured and seeded von Willebrand factor positive endothelial cells were capable of synthesizing laminin, fibronectin and chondroitin sulfate. All components that were lost during the decellularisation treatment.

Steinhoff and coworkers showed that endothelial cells and myofibroblasts seeded on ovine acellular matrix scaffolds were capable of procollagen I synthesis *in vivo* [8]. Furthermore, studies in our own laboratory on rat aortic valves decellularized with a 2-step detergent-enzymatic extraction method showed that α-smooth muscle positive cells infiltrating the valve leaflet were capable of replenishment of lost fibronectin and chondroitin sulfate [3]. The possibility of restoration of lost compounds by in vitro or in vivo reseeded cells should therefore be further investigated.

In conclusion, we studied the effect of detergent and enzymatic cell extraction on the remaining ECM of aortic valves for the purpose of scaffolds in tissue engineering. Furthermore, synthesis of valvular ECM components by seeded endothelial cells was investigated. Changes in the ECM constitution were found as compared to fresh valves, which could lead to problems in valve functionality and cell growth and migration. Seeded endothelial cells were capable of producing ECM components lost by cell extraction.

Further studies on tissue engineering should focus more on the effect of chemically induced cell extraction on the ECM of the remaining scaffold and the *in vitro* or *in vivo* restoration of lost compounds.

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