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Extracellular matrix in deoxycholic acid decellularized aortic heart valves

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Only limited information is available regarding the influence of decellularization on the extracellular matrix in heart valves. Within the extracellular matrix proteoglycans (PG) play a central role in the structural organization and physical functioning of valves and in their capability of settling with endothelial and interstitial cells partially myofibroblasts. We have therefore estimated the effects of decellularization using deoxycholic acid on the structure of the extracellular matrix and PG's in porcine aortic valves.

Material/Methods: Cupromeronic blue was used, alone or in combination with OsO_4/thio-carbo-hydrazide/OsO_4 for electron microscopic visualization. For PG and glycosaminoglycan (GAG) investigation a papain digestion was employed in combination with photometric determination using dimethylmethylene blue.

Results: The results indicate that deoxycholic acid affects the compartmentation of the PG-associated interstitial network not significantly. Compared to controls the PG-rich network was preserved even after deoxycholic acid treatment for 48 h. In parallel to electron microscopy immune assays (ELISA) showed smooth muscle cell α-actin to be reduced to 0.96%±0.71 and total soluble protein to 6.68%±2.0 (n=3) of untreated controls. Protein loss corresponded well with the observations in electron micrographs of rupture and efflux of cell content. Further signs of lysis were irregular cell contours and loss of the basement membrane.

Conclusions: Efficient cell-lysis without disintegration or loss of integrity of the interstitial PG network can be achieved by treatment of aortic valves with deoxycholic acid for 48h. This protocol might also be suitable for clinical use to optimize conditions for growth and autologous remodelling of valves.

key words: **tissue engineering • xenograft • aortic valve • replacement • *in vitro* studies • bioengineering**

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Background

Decellularization of heart valves is a promising approach to overcome the limitations associated with conventional manufacturing techniques of bioprostheses. During the last years xenografts prepared with methods of decellularization have been used clinically [1–5]. However, there were also drawbacks with some of these products, mostly related to incomplete cell removal [6]. Nevertheless promising results have been achieved with deoxycholic acid decellularized pulmonary heart valves. These xenografts showed growth and remodelling of the valves in the juvenile sheep [1,7]. Furthermore these valves show excellent clinical performance for now up to 10 years [8–10] and also lack immunological response by the patient [11]. Encouraged by these results, the focus has now been set on the influence of the deoxycholic acid treatment on the preservation of the interstitial fine structure of aortic valves, in which proteoglycans (PG) represent a major component.

PGs are involved in the organization, hydration, electrolyte regulation, cell surface interaction, antithrombogenicity, prevention of calcification, and in the binding of growth factors [12–14]. Therefore, they might also play a significant role in mechanisms related to remodelling and growth of decellularized valves in patients. Due to their molecular composition PGs are ideal candidates for ultracytochemical investigations by electron microscopy. They contain polysulfated glycosaminoglycan (GAG) side chains which are covalently attached to a filamentous core protein. These GAGs interact with tetracationic dyes such as cuprolinic blue or cupromeronic blue (CMB) [15,16]. Both dyes offer a number of sophisticated methods for electron microscopic detection and characterization of PGs *in situ*. In each case, electron contrast must be enhanced by Na_2WO_4. PG molecules then appear as long-shaped precipitates and can be discriminated by criteria like size, location, critical electrolyte concentration and their susceptibility towards GAG-degrading enzymes as shown previously in arterial and valve tissues [15,17–20].

In the present electron microscopic study we have focussed our interest on the integrity of the interstitial compartments, in which decorin as a collagen-associated PG and versican in the soluble matrix are dominating [21,22]. Decorin is a small PG with one dermatan-sulphate (DS) GAG side chain. DS controls the fibrillogenesis and is responsible for the interspaces between collagenfibrills. Versican is one of the larger PGs and contains multiple GAG side chains, most of them being chrondroitin-sulphate (CS). CS possesses the ability to bind large amounts of water to build a gel like layer. This layer plays an important role in the tissue and supports to resist compressive deformations and to absorb loads. Combined DS and CS represent 90% of the total GAG content in heart valves [17]. Decellularization experiments with the aim to produce low antigenetic bioscaffolds suitable as xenografts must take care of this three-dimensional extracellular network in which these different types of PG are present and specifically located. When applied alone, the CMB method excludes collagen and elastic fibers to be stained intensively. Therefore, an overall staining of PG-related structures was applied also by extending CMB staining in some specimens and by using OsO_4/thiocarbohydrazide/OsO_4 (OTO) before dehydration and embedding of the tissues in epoxy resin [20].

Material and Methods

Preparation of heart valves

Porcine hearts were obtained from a local slaughterhouse. The aortic valves were dissected and stored at 4°C in antibiotic solution (200 U/ml Penicillin, 200 mg/ml Streptomycin and 10 µg/ml Amphotericin B). Incubation with 1% deoxycholic acid (sodium salt, Fluka, Deisenhofen) was performed for 24 h and for 48 h at 37°C in physiological saline (PBS). Deoxycholic acid and products of lysis were removed afterwards by washing the specimens several times with PBS alone at room temperature, as described earlier [1]. As reference tissues, aortic valves were prepared and stored at 4°C in antibiotic solution without deoxycholic acid treatment. For ultracytochemical processing, small tissue samples were chemically fixed with 2.5% glutaraldehyde.

Protein quantification

In parallel to our ultrastructural investigations immune assays were used to quantify the loss of cellular proteins by deoxycholic acid treatment compared to native tissue expressed as percent of residual smooth muscle cell-(SMC)-α-actin and of soluble total protein. SMC-α-actin is present in most valvular cells and is therefore a adequate marker for cells in heart valves. Treated and untreated aortic tissues were homogenized in PBS (0.5 g/ml, pH 7.4) with an Ultra-Turrax (Jahnke & Kunkel, Germany). The homogenates were centrifuged at 2000 × g for 10 min. The supernatant was collected and kept at –80°C until use. Protein determinations were performed according to Bradford [23].

Sandwich ELISA was initiated by binding of anti-SMC-α-actin (clone E184, Epitomics, CA, USA, 1:500) for 120min to anti-mouse IgG coated microtiter plates (Pierce, IL, USA). Then 250 µl heart valve samples were added and incubated for 120 min. After washings with PBS 4 µg/ml of second, different primary anti-SMC-α-actin antibody (clone AC-40, Sigma, Deisenhofen, Germany, 1:500) was added and incubation continued for 120 min. Then primary antibodies were labelled with a biotinylated anti-mouse IgG secondary antibody (Rockland, PA, USA, 100 µl, 1:10.000) followed by streptavidin-horseradish-peroxidase (HRP, Rockland, 100 µl, 1:10.000). A colour reaction was obtained with peroxidase reagent after 25 min (100 µl, TMB: 3.5',5.5'-tetramethylbenzidine, Pierce). The reaction was terminated with TMB stop reagent (100 µl, Sigma). Optical density was measured at 450 nm with 620 nm as reference using an Anthos Labtec HATII plate reader (Anthos, Austria). Incubation steps with antibodies, samples or enzymes were followed by washings with PBS (3×250 µl each). All samples were determined in triplicate. Standard curves were estimated with supernatant of untreated aortic valves covering two fold dilutions of 15 steps. α-actin content of treated valves were estimated from the standard curves as percentage of the α-actin content of untreated valves.

Glycosaminoglycan determination

For the determination of the GAG content in the tissues the method of Farndale was used [24]. Briefly, the tissues were homogenated first and interfering proteins were digested for 16 h at 60°C with papain. Following digestion iodoacetic

acid was added to a final concentration of 10 mM and the sample was filled up to a final volume of 5 ml with 50 mM TRIS/HCL (pH 8.0). For further purification of the GAGs the samples were treated twice with ethanol to precipitate the GAGs. Following solubilization the samples were divided each into three tubes. The first was digested with chondroitinase ABC, the second with chondroitinase AC and to the third solely buffer was added. Following 30 minutes of incubation at 37°C the samples were mixed with the colour reagent and the resulting optical density was determined at 540 nm. The chondroitinase ABC digests chrondroitin- and dermatan-sulphate whereas chondroitinase AC solely digests chrondroitin-sulphate. For the chondroitin-sulphate the reduction achieved by the chondroitinase AC digestion compared to buffer alone was calculated. For the determination of the dermatan-sulphate the reduction achieved by the chondroitinase ABC digestion was calculated in relation to the chondroitinase AC digestion. With previously maintained standard curves the concentrations of the samples were estimated.

Ultrahistochemical processing

Treated and untreated tissue samples of aortic heart valves were fixed with 2.5% glutaraldehyde. Cupromeronic blue (Seikagaku, Japan) staining was done in acetate buffer at pH 5.6 and counterstaining was performed with Na_2WO_4 according to Rothenburger et al. [18]. In parallel to CMB staining alone, CMB staining was extended with a sequence of OsO_4/thio-carbo-hydrazide/OsO_4 (OTO) as described [19]. After washing, specimens were dehydrated in a graded series of ethanol and embedded in epoxy resin for ultra thin sectioning. They were digitally photographed in a transmission electron microscope (Philips EM 201, FEI, Germany) using imaging plates (DITABIS, Aschaffenburg, Germany).

Data analysis

Data given are mean and standard deviation (SD) of the number of independent experiments indicated (n). GraphPad Prism (GraphPad Inc., CA, USA) was used to analyse ELISA experiments by curve fitting. The evaluation and standard curves for the biochemical investigations of chondroitin- and dermatan-sulphate were performed with GraphPad Prism as well.

Results

For the preparation of bioscaffolds from porcine aortic heart valves a protocol employing deoxycholic acid has been used to remove all cells. The effects of deoxycholic acid lysis were estimated after 24 h and 48 h of incubation with regard to protein and GAG content and, by electron microscopy, with regard to the preservation of the topography of the PGs in the interstitial space.

As shown by ELISA the residual content of smooth muscle cell (SMC) α-actin in decellularized valves was 0.96%±0.71 (n=3). The low amount of remaining α-actin indicated a high efficiency of cellular lysis. After a 24 h deoxycholic acid treatment the total soluble protein content representing intra and extracellular protein (6.68%±2.0, n=3) did not decline further even when the incubation time of the treatment was extended to 48 h.

Similar results were obtained with regard to the CS and DS content in the tissues investigated. Here we achieved a considerable higher GAG concentration in decellularized compared to untreated tissues. The measured CS concentration was app. 1.2±0.5 mg CS/g fresh weight (fw) in decellularized tissues and 0.6±0.2 mg CS/g fw in native tissues. The DS concentration in the treated tissues was 0.5±0.3 mg DS/g fw and in native tissues 0.3±0.1 mg DS/g fw.

These findings were confirmed by electron microscopic observations. CMB-OTO overall staining showed disruption of the nuclear envelope and of the cytoplasmic membrane as well as the release of nuclear filaments through the cytoplasm into the proximity of the cells (Figure 1). Cytosceletal filaments were not visible any more, the shape of the cells appeared rough, and their basement membrane was largely removed.

The influence of cell lysis and protein extraction by deoxycholic acid on the integrity of the PG-rich network in the interstitial space was investigated in valve tissues exclusively stained with CMB (n=3). These specimens revealed faintly stained elastic layers and collagen bundles but intensively and more clearly defined PG-CMB precipitates (Figure 2). We observed small PG-CMB precipitates aligned along collagen-fibrils formerly characterized as dermatansulfate/chondroitin-4-sulfate PGs (decorin), or at elastic fibers as well as on the cell surface, as heparansulfate-rich ones [15,17]. Large PG-CMB precipitates about 200 nm in length were located outside the collagen-fibrils in an area called soluble matrix. These PGs were described as chondroitin-6-sulfate-rich ones (versican). When compared with controls high magnification electron micrographs revealed that the molecular network of these different types of PG was not disrupted by the deoxycholic acid treatments, neither after 24 h nor after 48 h of incubation (Figure 2).

Discussion

In the present study a similar method as recently described for porcine pulmonary heart valves has been used for decellularization of porcine aortic heart valves applying deoxycholic acid [1]. The focus of this study was set on biochemical and electron microscopic investigations of aortic valve tissue following deoxycholic acid treatment, with special emphasis to PGs and their GAG side chains. As a major component of the interstitial space PGs are essential for maintaining physical and physiological properties of arterial tissue and, therefore, must resist deoxycholic acid induced destruction of their extracellular network to be clinically applicable. The biochemical investigation of the tissues revealed an even higher concentration of relevant GAGs following decellularization. This increase can be most likely be explained by the reduction of the specific weight of the tissue by decellularization due to the considerable reduction of cellular components like proteins, i.e. the protein content of treated valves was reduced by 93% compared to native valves. Alternatively it is also possible that the assay is being affected by cellular components in native tissues following papain digestion. Thus, some GAGs, which are associated with cells, might be lost during purification. Nevertheless these results underline that there is no overall decrease in GAG concentration following decellularization of the tissue. The present data are consistent with previous investigations

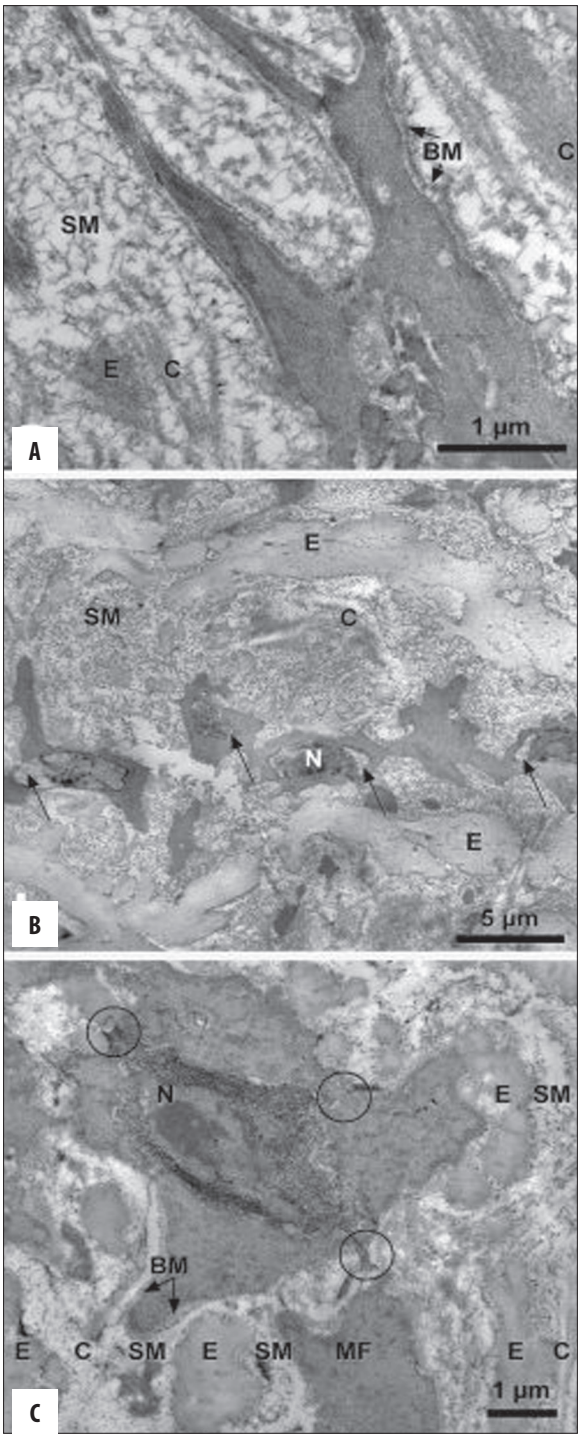

Figure 1. Electron micrographs of porcine aortic heart valve tissues double-stained with CMB/OTO. (**A**) The outside of a native myofibroblast is covered with a basement membrane layer (BM) and an interstitial network around with areas of soluble matrix (SM), elastic fibers (E) and collagen fibrils (C). PGs are seen as fuzzy long-shaped precipitates cross-linking the extracellular matrix (**B**). The overview electron micrograph of valve tissue treated with deoxycholic acid for 24 h shows adjacent myofibroblasts (MF) with ruptured nuclei (N, arrows). The integrity of the extracellular matrix is not disturbed. (**C**) 48 h of deoxycholic acid incubation shows bursts of cells releasing their nuclear and cytoplasmic content (circles) into the pericellular space.

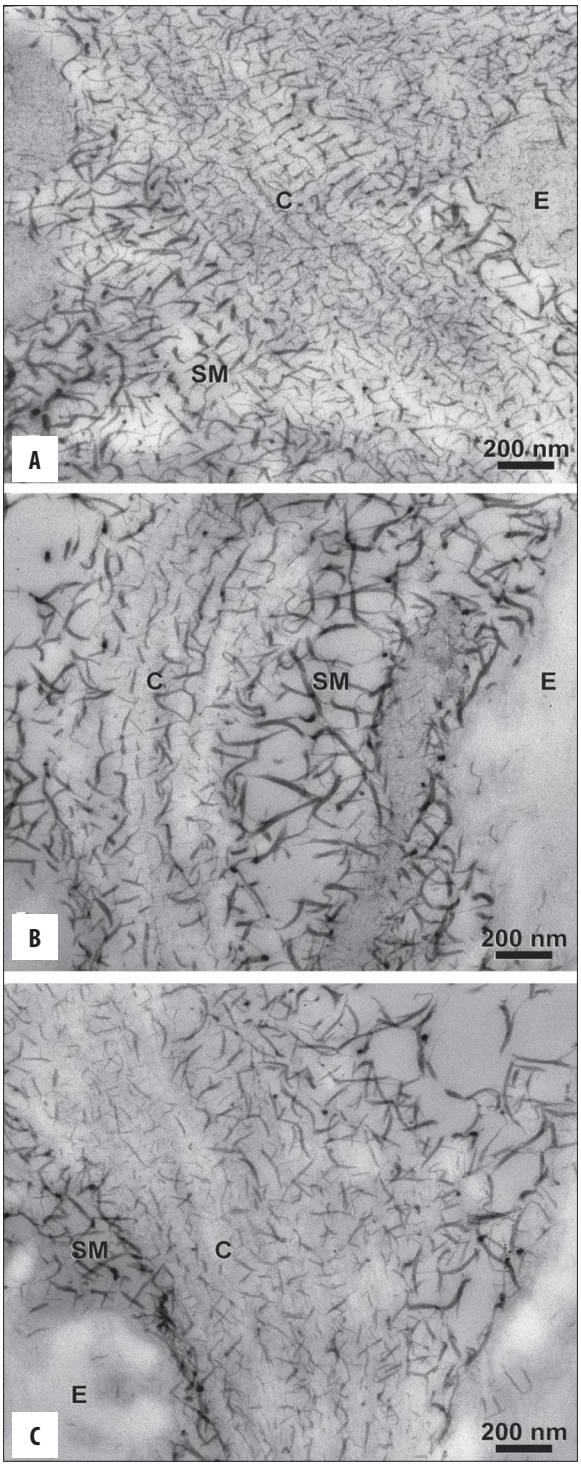

Figure 2. Electron micrographs of porcine aortic heart valve tissues stained with CMB. PGs are stained strongly and show a characteristic pattern of compartmentation. Large CMB precipitates are located inside the soluble matrix (SM), which represents amorphous areas in the space between collagen bundles (C), elastic fibers (E) and the cell surface. Small precipitates are preferentially located along collagen fibrils (C), the surface of elastic fibers (E) and the cell surface. The electron micrographs in (**A**) control, (**B**) 24 h deoxycholic acid treatment, (**C**) 48 h deoxycholic acid treatment show that there is no significant change in the patterns of distribution and compartmentation of the PGs.

of Grand-Allen et al who estimated a total GAG content of 0.69 mg/g in aortic valves [25], which is close to the GAG content achieved in this study. The preservation of GAGs following decellularisation could be confirmed by electron micrographs showing PGs and their associated extracellular structure *in situ* to be intact after 48 h deoxycholic acid treatment. Prior to the ultra-histological examinations the effectiveness of lysis and the loss of cellular components was checked by estimating residual SMC-α-actin and total protein content. As the total protein content remained stable and did not decline further in the prolonged deoxycholic acid treatment of 48 h there is evidence that the soluble cellular proteins were sufficiently and quantitatively extracted. SMC-α-actin is presumably a suitable indicator for effectiveness of decellularisation of vascular segments of the valves, since it is a contractile protein which needs solubilization as prerequisite for its complete removal from the tissue. Estimating residual proteins after decellularization has advantages over methods of measuring residual RNA and DNA content of the tissues. Complete removal of the filamentous nucleotides is achieved hardly without the use of DNAase or RNAase [1]. In principle, the use of enzymes during decellularization procedures for the production of bioscaffolds might lead to adverse immunological reactions in patients if they are not removed completely including the residuals and therefore limits their value in clinical applications. As seen in electron micrographs cellular and filamentous nuclear material was released from bursting cells by the deoxycholic acid treatment even in deep regions of the valve tissue. This aim in the preparation of bioscaffolds was accompanied now by the electron microscopic finding that the fine structure of the PG-associated interstitial network remained well preserved in the aortic heart valves during one or even two days of deoxycholic acid treatment. This was best seen in specimens selectively stained with CMB alone.

Preservation of the extracellular matrix with respect to the suitability of different decellularization techniques is also in the focus of other investigators [26–30]. The reason for this is that the mechanical properties of the scaffolds might not be sufficient if structures of the extracellular matrix are altered as it has been shown for trypsin-EDTA decellularized valves [28]. But on the other side incomplete decellularization leads to adverse immunological reactions [6]. Therefore it is now more and more accepted that detergent based decellularization techniques are preferable to other methods [31].

Previous work indicated that the extracellular matrix and its PGs play an important role in the development of cardiac valves [12] and therefore might be important for remodelling and growth of decellularized tissues [1]. Until now there is only limited information available whether and how decellularization techniques affect the distribution and structure of PGs and GAGs within the extracellular matrix of heart valves [28]. This study demonstrates that deoxycholic acid treatment of porcine aortic valves for up to 48 h does not affect the extracellular matrix architecture and its network of PGs inside the valves. Whether this is also true in porcine pulmonary valves and whether this is responsible for the observed remodelling and growth of the valves *in vivo* [7] needs further investigation. There is evidence that an intact extracellular matrix plays an important role in remodelling but less is known which co-factors are additionally required for the recellularization of bioscaffolds *in vivo*.

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