Regenerative medicine in cardiovascular disease: from tissue engineering to tissue regeneration
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CHAPTER 4: DECELLULARIZATION OF RAT AORTIC VALVE ALLOGRAFTS REDUCES THE CELLULAR IMMUNE RESPONSE RESULTING IN A STRUCTURALLY INTACT COLLAGEN NETWORK IRRESPECTIVE OF COLLAGEN DENATURATION

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ABSTRACT

Background  There is increasing evidence that the immunogenicity of aortic valve allografts (AVA) is associated with valvular degeneration. Chemically induced cell extraction is expected to reduce an immune response. However, whether this will translate into reduced structural damage is unclear. The present study focussed at characterizing the immune response to cellular and acellular AVA and correlation of results to structural damage.

Material and Methods  Rat aortic valve conduits were decellularized by a recently developed 2-step detergent-enzymatic extraction method. Cellular allogeneic (n = 8), acellular, allogeneic (n = 8), and syngeneic (n = 8) aortic valve conduits were grafted into the descending aorta for either 7 (n = 4) or 21 (n = 4) days. Immuno-histochemistry to detect lymphocyte and macrophage infiltration (CD8 and CD68) and apoptosis, as well as histological and chemical analysis of collagen damage were performed.

Results  A severe influx of CD8+ and CD68+ cells accompanied by interstitial cell apoptosis was observed 1 week after AVA transplantation, followed by extensive fragmentation of the collagen matrix at 3 weeks. Decellularisation resulted in an absent cellular infiltration with preservation of the collagen matrix. Chemical analysis revealed an increase in denatured collagen after decellularization (7.8 ± 0.7 % vs. 12.0 ± 1.2 %, p < 0.05), which did not change 3 weeks after transplantation (12.0 ± 1.8 %, p = ns), in contrast to the increase in cellular AVAs (12.3 ± 1.5 %, p < 0.05).

Conclusion  Transplantation of acellular AVA results in an absent early immune response, with a histological preservation of the valvular collagenous structure at 3 weeks. The amount of denatured collagen increases after decellularization, but does not increase further after transplantation, in contrast to cellular AVAs.
INTRODUCTION

There is mounting evidence that the immunogenicity of aortic valve allografts (AVA) is associated with valvular degeneration. Recently it was shown that human leukocyte antigen (HLA) mismatch correlated strongly with echocardiographic valve failure [1]. Others showed that, especially in recipients younger than 50 years of age, a strong immunization of donor-specific HLA antibodies was associated with progressive aortic valve incompetence [2]. These results are consistent with studies in rat aortic valve transplantation models that showed extensive infiltration of (CD8+) T-lymphocytes and macrophages 7 days after allogeneic transplantation with loss of the extracellular matrix structure on the long term [3]. Furthermore, there is increasing evidence that the cellular elements of the AVA are the main source of alloreactivity [4,5]. Modification of the AVA to reduce immunogenicity is therefore considered to be a promising approach in overcoming allograft valve failure.

Chemically induced cell extraction depletes antigen-presenting cells and is expected to reduce the immune response. Decellularised allografts could then be used as scaffolds for regenerative medicine purposes. Recently we have developed a two-step detergent-enzymatic extraction method to produce completely decellularized rat aortic valve conduits with minimal damage to the ECM [6]. In a rat aortic valve transplantation model [7] transplantation of decellularised AVA between MHC-mismatched rats, valves were still morphologically intact after 3 weeks, whereas transplanted cellular allografts became deformed and swollen with major morphological changes to the ECM, especially collagen [6].

An important question is whether an absent immune response in the decellularised valves will translate into improved long-time durability. A recent study showed early failure of non-fixed, decellularised porcine heart valves after implantation in children [8]. Furthermore, studies in our own laboratory showed that different cell extraction methods resulted in moderate to severe changes in ECM constitution [9], which could lead to problems in valve functionality. Moreover, few studies regarding valve decellularization have linked the immune response to structural changes in the ECM. Although the ECM of decellularised valve scaffolds is remodelled in vivo, the exact molecular composition and post translational modifications of the ECM components is rarely considered. Therefore, new methodologies for investigating the in vivo degradation of decellularised valve scaffolds are needed.

The present study focussed at characterizing the immune response to cellular and acellular AVA and aimed to correlate these results to structural damage using standard histology and biochemical analysis of collagen damage. This will provide further insights in the use of acellular scaffolds in regenerative medicine.
MATERIAL AND METHODS

Animals

Female and male inbred WAG-Rij (RT-1\(^h\) / RijHSD) and female Brown Norway (RT-1\(n\) / RijHSD) rats 9-12 weeks old were obtained from Harlan CPB, Horst, the Netherlands. Female WAG and Brown Norway rats were used as syngeneic and allogeneic donors respectively. Male WAG rats were used as recipients. All experiments were approved by the committee on animal welfare of the Leiden University Medical Centre (LUMC), The Netherlands. Rats were housed under standard conditions and were given standard diet and water *ad libitum*, and have received humane care in compliance with the Guide for the Care and Use of Laboratory Animals.

Experimental Design

In this study we included 3 different groups of animals, consisting of cellular allogeneic, acellular allogeneic and acellular syngeneic aortic valve conduit transplantations. Each group consisted out of 8 animals, sacrificed either at day 7 (\(n=4\)) or at day 21 (\(n=4\)). The explanted U-shaped aortic valve conduit was cut just above the sinuses and the remaining piece was split longitudinally into 3 valve cusp-containing symmetric rectangular pieces for immunohistochemistry and chemical collagen quantification. Non-implanted cellular (\(n=5\)) and acellular (\(n=5\)) valve conduits were used as controls.

Decellularization of Aortic Valve Conduits

The aortic valve conduits were decellularized by a recently described 2-step detergent-enzymatic extraction method [6]. In short, the valve conduits were treated for 24 hours at 4 °C with 0.1% SDS in distilled water after which they were placed in a solution containing RNase A (20 \(\mu\)g/ml) and DNase (0.2 \(\mu\)g/ml) for 1 hour in HBSS at room temperature. Subsequently, conduits were washed with HBSS at 4 °C for 48 hours to remove residual substances.

Surgical Technique

All operations were performed using a modification of a technique [6] first described by Oei et al. [7]
**Histology**

From each animal a valve cusp-containing symmetric rectangular piece was immersion-fixed in 4% paraformaldehyde at 4 °C for 48 hours, dehydrated in graded ethanol and xylene and subsequently embedded in paraffin. Serial sagittal sections of 5 μm were cut and mounted onto egg white/glycerin-coated glass slides for histological and immunohistochemical analysis. Parallel sections were routinely stained with Sirius red to detect collagen.

**Immunohistochemistry**

Apoptosis was detected by a monoclonal rabbit anti cleaved caspase-3 antibody (Cell signaling Technology Inc.) Macrophages were visualized with a monoclonal mouse IgG1 recognizing the CD68 receptor (clone ED-1; Camon, Wiesbaden, Germany) and cytotoxic T-lymphocytes (CTL) were detected with the a monoclonal mouse antibody (Clone OX8; Caltag, Burlingame, CA, USA) recognizing the CD8 receptor present on CTLs. Sections were deparaffinated and dehydrated in xylene and graded alcohol. Endogenous peroxidase was quenched for 20 minutes in 0.3% H₂O₂ in phosphate-buffered saline solution (PBS). Sections were incubated overnight with the primary antibodies caspase-3 (1:300), ED-1 (1:25) and OX-8 (1:200).

Prior to incubation with the caspase-3 and ED-1 antibodies, the sections were submitted to antigen retrieval by heating in a microwave oven (97 °C) in 0.01M citric buffer of pH 6.0. Specific labeling was detected with biotinylated horse anti-mouse (1:200; Vector) with normal horse serum (1:66; Vector) or biotinylated goat anti-rabbit (1:200; Vector) with normal goat serum (1:66; Vector). Finally peroxidase-conjugated streptavidin (1:400; Dako) was added and detected by diaminobenzidine tetrahydrochloride for 8 minutes at room temperature. Sections were counterstained with hematoxylin for 10 seconds, dehydrated in graded ethanol, and mounted in Entellan (Merck, Darmstadt, Germany).

**Semi-quantitative Scoring**

The coded sections were examined in a blinded fashion independently by two of the authors (RG and MR). The density of infiltration was graded on a scale of 0 to 4, as compared to a non-transplanted valve (0 = comparable to a non-transplanted valve, 1 = incidentally a positive cell, 2 = multiple positive cells, 3 = severe infiltration of positive cells). A similar scale was used to assess leaflet structural changes, based on collagen staining (0 = normal, 1 = mild, 2 = moderate, 3 = moderate to severe, 4 = severe).
**Quantification of Denatured Collagen**

A selective digestion technique was used to determine the amount of denatured collagen molecules (as a percentage of the total amount of collagen) as previously described [10]. In short, valve cusps were carefully cut out of the sinuses and digested at 37 °C in α-chymotrypsin solution that selectively dissolves degraded collagen. The degraded and intact collagen was then separated by removing the supernatant (degraded collagen) from the remaining insoluble matrix (intact collagen). Thereafter, the amounts of degraded and intact collagen were determined by measuring the collagen specific amino acid hydroxyproline in both fractions. Finally, the percentage of denatured collagen was calculated by dividing the hydroxyproline concentration in the supernatant by the sum of hydroxyproline concentration in the supernatant and remaining matrix.

**Statistical Analysis**

Collagen damage was expressed as percentage of denatured collagen. Means were obtained from 4 animals in the 1 week and 3 weeks groups, and 5 animals in the control groups. Data are reported as mean and standard error of the mean. Analysis of variance testing was used to compare multiple groups, with Bonferroni’s Multiple Comparison Test used to compare individual groups. Significance was assumed at P < 0.05.
RESULTS

Immuno-histochemistry of Infiltrating Cells

Characterisation of the immune infiltrating cells was carried out by immuno-histochemistry against CD8+ T-lymphocytes and CD68 positive macrophages. After 1 week a moderate to severe infiltration of CD8+ (Figure 1A) and CD68+ (Figure 2A) cells was observed in the cellular aortic valve allograft leaflets (Table 1).

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Table 1. Immuno-histochemistry and leaflet damage. (a) Semiquantitative scoring of infiltration of CD68 and CD8 positive cells: 0 = comparable to a non-transplanted valve, 1 = incidentally a positive cell, 2 = multiple positive cells, 3 = severe infiltration of positive cells. (b) Leaflet damage, based on collagen staining: 0 = normal, 1 = mild, 2 = moderate, 3 = moderately severe, 4 = severe.
In contrast to cellular grafts, decellularised aortic valve allografts elicited virtually no CD8+ (Figure 1B) and CD68+ (Figure 2B) cellular infiltration after 7 days (Table 1). These findings were also not different from the acellular syngeneic leaflets (Figures 1C and 2C).

Figure 1. Representative immunohistochemistry for cytotoxic T-lymphocytes (CTL) (CD8) one week (left panel) and three weeks (right panel) after heterotopic transplantation of cellular allogeneic (A and D), acellular allogeneic (B and E) and acellular syngeneic (C and F) valve transplantation. Arrows indicate the CD8+ CTLs 1 week after cellular allogeneic valve transplantation. (Bars indicate 50 μm)

Figure 2. Representative immunohistochemistry for macrophages (CD68) one week (left panel) and three weeks (right panel) after heterotopic transplantation of cellular allogeneic (A and D), acellular allogeneic (B and E) and acellular syngeneic (C and F) valve transplantation. Arrows indicate the CD68+ macrophages 1 week after cellular allogeneic valve transplantation. (Bars indicate 50 μm)
After 21 days (Table 1) all aortic valve allograft leaflets had become completely acellular with only sporadically CD8+ (Figure 1D) and sometimes CD68+ positive cells (Figure 2D). At 21 days, acellular allogeneic and syngeneic leaflets showed still no infiltration of CD8+ cells (Figure 1E-F), and only none to mild infiltration of CD68+ cells (Figure 2E-F).

The prevalence of interstitial cells undergoing apoptotic cell death was evaluated by anti-cleaved caspase 3 antibodies. One week after transplantation a considerable amount of apoptotic nuclei was observed in the cellular allogenic valve leaflets (Figure 3).

**Histological Assessment of Collagenous Structure**

Decellularisation of aortic valves did not result in any detectable ECM changes, as determined by collagen staining, compared to cellular valves (Figure, 4A, B). One week after cellular aortic valve allograft transplantation leaflet morphology was mild to moderately changed which was accompanied by thickening of the leaflets, compared to non-transplanted cellular valves (Figure 4C and Table 1). Transplanted acellular allogeneic and syngeneic leaflets did not show any major changes in the collagenous structure after 7 days and remained thin (Figure 4D, E). Furthermore, 3 weeks after transplantation of the cellular aortic valve allograft leaflets morphology was moderately to severely changed with pronounced valvular thickening and a disruption in the collagenous structure (Figure 4F). This is in contrast to the decellularised allogeneic and syngeneic valves, where the collagenous structure remained unchanged at 3 weeks (Figure 4G and H).

**Chemical Quantification of Collagen Damage**

The amount of denatured collagen was determined at baseline, and after 7 and 21 days for all three groups (Figure 4I). At baseline, cellular non-treated valve cusps contained 7.8 ± 0.7%
Figure 4. Representative Sirius red staining of cellular and acellular aortic valve leaflets at baseline (left panel), and 1 week (middle panel) and 3 weeks (right panel) after transplantation. Transplantation of cellular allogeneic valves (A), leads to time dependent morphological changes in collagen structure (B and C). Transplantation of decellularised allogeneic and syngeneic aortic valves (D) resulted in a preservation of the leaflet structure for both the allogeneic (E and F) and syngeneic (G and H) valve leaflets. (Bars indicate 50 μm). Quantification of the percentage of denatured collagen in the valve leaflets at baseline and 1 and 3 weeks after transplantation (I). * = p < 0.05 as compared to cellular valve leaflets at baseline.
denatured collagen, which was significantly increased after the decellularisation procedure to 12.0 ± 1.2 % (p < 0.05).

Seven days after transplantation the amount of denatured collagen did not change in both the acellular allogeneic and acellular syngeneic valve cusps (11.0 ± 0.7 % and 10.8 ± 1.1 % respectively). Also after 21 days this remained unchanged (12.0 ± 1.8 % and 12.3 ± 0.9 % respectively). Furthermore, for all time-points no differences between the allogeneic and syngeneic valve cusps were observed.

Transplantation of the cellular allogeneic valves resulted in a non significant trend towards an increase in denatured collagen after 7 days (7.8 ± 0.7 vs. 10.0 ± 0.8 %; p = 0.07). However, after 21 days the percentage of denatured collagen was significantly increased compared to baseline (7.8 ± 0.7 vs. 12.25 ± 1.5; p = 0.02). Furthermore, after 7 and 21 days the amount of denatured collagen in the transplanted cellular allogeneic leaflets did not differ from the acellular transplanted valves.
DISCUSSION

In the present study we showed that cellular AVA elicit an early immune-response 1 week after transplantation, which resulted in a disorganisation of the collagen structure of the leaflets and subsequent thickening of the leaflets after 3 weeks. Decellularization of aortic valve allografts AVA attenuated this cellular immune response, resulting in preservation of the collagen organisation. However, despite the major morphological differences in collagen structure, both the cellular and acellular AVA showed increased amounts of denatured collagen in the valve leaflets.

Numerous protocols have been described to obtain decellularised heart valve leaflets comprising ionic [6] and non-ionic [9,11], as well as enzymatic extraction methods [12,9]. Furthermore, several studies assessed the effect of decellularisation on the biomechanical properties of a valve [13]. However, detailed studies that assess the immunologic response to cellular and acellular AVA and its effect on the valves structural integrity are lacking. Therefore, in the present study we used a recently developed two-step detergent-enzymatic extraction method [6] and a rat aortic transplantation model [6,7] to investigate the relationship between the cellular immune response and structural damage.

One week after transplantation, cellular aortic valve allografts displayed a significant infiltration of CTL and macrophages. After 3 weeks, however, almost no immune infiltrating cells could be observed. This is consistent with previous studies on rat aortic valve allografts [3]. Decellularisation resulted in a virtual absent CTL and macrophage infiltration in both the allogeneic and syngeneic valve grafts 1 week after transplantation. Furthermore, after 3 weeks still no CTL immune infiltration was observed with only a mild infiltration of CD68+ positive cells. These findings are in line with previous work by Meyer et al. who observed significant reductions in CD8+ lymphocytes 1, 2, and 4 weeks after transplantation of acellular allogeneic and syngeneic valve grafts [14].

The difference with our results, however, is that we observed almost no CD8+ T-lymphocytes after 1 and 3 weeks. This difference could possible be explained by the use of a non-ionic detergent (Triton) [14] instead of the ionic detergent SDS used in our study, which has been shown to have better cell extraction capacities [15].

In the present study we used a classical histochemical Sirius Red staining to assess morphological changes in the collagen distribution. Decellularization of allogeneic and syngeneic aortic valve leaflets did not result in major structural changes in the collagen structure. However,
transplantation of cellular AVA resulted in valvular thickening and a morphological disruption of the collagenous structure. In contrast, acellular syngeneic and allogeneic aortic valve leaflets remained thin over the three week period. Although histochemical staining against collagen is a useful tool in general matrix diagnosis, it is not sufficient to determine the biochemical state (e.g. amount of denaturation and cross linking) of the tissue.

Several authors have used biomechanical tests to determine the impact of decellularization on the structural strength of the collagen network [13]. However, because of the relatively small size of rat aortic valves, this technique is not suitable for this model.

Hydroxyproline assays have been used to estimate collagen as a proportional percentage of tissue dry weight, in porcine [13] and rat [16] aortic valves. However, hydroxyproline assays only provide a proportional percentage of the total ECM composition, but not the structural integrity of the collagenous structure. For that reason, in the present study we used a previously described selective digestive technique that distinguishes between denatured and intact collagen molecules [10]. Normal collagen molecules have a typical triple helical structure, whereas the denatured collagen is characterised by unfolding and cleavage of the triple helixes [10]. However, due to the presence of cross-links between collagen molecules, denatured collagen molecules remain incorporated in the collagen network [17].

In the present study we used a 2-step detergent-enzymatic extraction method involving SDS in a hypotonic solution in combination with RNase and DNase [6] for the decellularisation of the AVAs. SDS is an ionic detergent that is effective in solubilising both cytoplasmatic and nuclear cellular membranes, but also has a tendency to denature proteins by disrupting protein-protein interactions [18]. Other protocols comprising SDS to decellularize anterior cruciate ligaments showed a significant reduction in GAG and increased tissue susceptibility to the enzyme trypsin, indicating denaturation of the collagen network [19]. However, SDS does not appear to remove collagen from the tissue [15]. Indeed, our biochemical investigations determined a distinct increase in denatured collagen after the decellularisation procedure. However, transplantation of decellularised allogeneic and syngeneic aortic valve conduits did not change the percentage of denatured collagen. In contrast, cellular allogeneic valve cusps showed a time-dependant increase in denatured collagen, accompanied by marked histological differences in the collagen matrix.

Interestingly, the infiltration of CD8+ T-lymphocytes and macrophages 1 week after transplantation of the cellular allografts was accompanied by apoptosis of the leaflet interstitial cells, as indicated by the caspase-3 staining. This is in line with the results of Legare et al., where a CD8+ CTL influx in allogenic valve leaflets was accompanied by cells undergoing apoptotic cell death [20]. These results therefore suggest that the observed cell loss three weeks after the AVA
transplantation is mediated by the alloreactive CTLs that eliminate the leaflet interstitial cells by apoptotic mechanisms.

Interstitial valvular cells and endothelial cells are able to secrete proteolytic enzymes such as matrix metalloproteinases (MMPs) that mediate ECM degradation [21]. Therefore, the increased degradation and fragmentation of collagen observed after 3 weeks could be the result of the release of MMPs by apoptotic valvular interstitial and endothelial cells. Moreover, MMP activity can also be influenced by the local concentration of inflammatory cytokines [22]. Furthermore, several MMPs are secreted by inflammatory cells, including macrophages [23]. This is consistent with the high amount of macrophages and CD8+ CTL in the valve interstitium 1 week after AVA transplantation. MMP release as a result of the cellular immune response could therefore have influenced collagen cross-linking, impairing the elastic restraining force of the collagen network in these regions [24]. The presence of remaining glycosaminoglycans (GAGs) leads to water imbibition, subsequently resulting in the observed histological changes as swelling of the valve leaflets.

Therefore, in our study the present results indicate a link between the early inflammatory response and late ECM remodelling, which was not observed in the decellularised valves. This finding could be associated with an improved durability of the decellularised valve grafts. However, the effects of the increase in denatured collagen after decellularization remains unclear at present. A recent study demonstrated that cleavage and unwinding of triple helices of collagen molecules significantly changed the mechanical integrity of the collagen network in bone [25]. For that reason, the exact effects of decellularization on collagen denaturation and aortic valve mechanical integrity needs to be defined in future studies.

We would like address the following limitations for this study. Firstly, we used a 2-step detergent-enzymatic extraction method involving an ionic detergent in a hypotonic solution in combination with RNase and Dnase. It is not clear whether the present results can be extrapolated to other decellularization protocols comprising non-ionic and enzymatic extraction methods that display different effects on the ECM. Secondly, in the present study we only performed biochemical quantification of the collagen molecules. Detailed assessment of other components of the ECM (e.g. elastin and GAGs) should also be performed in future studies. Thirdly, it is known that rat heart valve leaflets differ in their structure from larger animals, and respond differently to decellularization protocols [6]. Finally, the present data only reflects a 3 week follow-up. Additional longer term studies are needed to to assess the possible beneficial or detrimental effect of acellular aortic valve allograft transplantation.
In summary, the present results demonstrate that our 2-step detergent-enzymatic extraction method in rat aortic valve allografts results in an attenuation of the cellular immune response and a histological preservation of the collagen matrix structure. However, transplantation of both the cellular and acellular AVA resulted in an increase in denatured collagen in the valve leaflets. Our observations are of relevance with respect to the development of acellular matrices for the purpose of regenerative medicine. They also have implications for the current practice of heart valve banks that aim to preserve (vital) cellularity as much as possible in the process of cryopreservation while no attention is given to ABO or HLA differences between donor and recipient.
REFERENCES


CHAPTER 4: DECELLULARIZATION OF RAT AORTIC VALVE ALLOGRAFTS REDUCES THE CELLULAR IMMUNE RESPONSE RESULTING IN A STRUCTURALLY INTACT COLLAGEN NETWORK IRRESPECTIVE OF COLLAGEN DENATURATION


