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IgG deposition and activation of the classical complement pathway involvement in the activation of human granulocytes by decellularized porcine heart valve tissue

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Abstract

Decellularization treatment of heart valves has been thought to eliminate tissue immunogenicity. Early failure of tissue-engineered xenogeneic heart valves was seen in children and has been a major drawback in this promising field of research. This study was designed to characterize the effects of acellular porcine heart valve tissue on immune activation in vitro. Incubation of decellularized porcine tissue with human plasma led to adsorption of IgG, activation of the classical complement pathway and adhesion of activated polymorphonuclear leukocytes (PMN). This inflammatory response was strongly inhibited by proteins extracted from native porcine tissue which might indicate that inhibitors of PMN activation present in the extracellular matrix (ECM) are lost during the decellularization process.
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1. Introduction

Heart valve tissue engineering is a field of intense research and various approaches for providing supporting structures exist. Decellularized allograft or xenograft scaffolds may serve as templates for cell attachment and retain many of the mechanical and structural properties of native heart valves. Moreover, decellularization treatment has been thought to eliminate immunogenicity and thereby to increase implant durability [1–3]. In a recent publication we have demonstrated that decellularization of vascular tissue effectively reduces monocyte and lymphocyte recruitment, whereas the migration of polymorphonuclear leukocyte (PMN), known to be involved in the early immune response, is hardly affected [4]. This residual immuno-stimulatory activity of decellularized porcine vascular tissue toward PMN observed in vitro, correlates with in vivo findings with decellularized human and porcine heart valves [5–7].

Neutrophilic granulocytes are the most abundant leukocytes in the human body and release a complex assortment of agents that can destroy normal cells and dissolve connective tissues. They have little intrinsic ability to differentiate between foreign and host antigens and rely on other arms of the immune system (e.g. complement) to select their targets. In contrast to neutrophils, the complement system has the unique ability to discriminate between ‘‘self’’ and ‘‘non-self’’ structures. Through the interaction of CD11b/CD18 (CR3) with surface-bound iC3b activated neutrophils can be attached to foreign surfaces [8].

Biomaterials intended for use in contact with whole blood (e.g. dialysis tubing, cardiopulmonary bypass devices) should

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not activate the complement and coagulation systems, however, their activity has been noted and studied for many years [9,10]. Acellular tissues, designated for use in tissue-engineered constructs, have been less well studied and may be over-estimated with respect to their biological inertness. It is known that when a foreign matrix comes in contact with blood plasma, the plasma proteins immediately bind and cover the surface [11], but this fact has not yet been addressed for acellular scaffolds. Clinical findings with decellularized heart valves have suggested that extraction or at least structural changes of protective matrix constituents in the course of the decellularization procedure occurs. These changes may be associated with strong immune activation that has been observed in vivo [5–7]. The goal of the present study was therefore to investigate (i) whether the contact of human blood with a decellularized xenogeneic heart valve matrix leads to immunoglobulin deposition and complement activation in vitro and (ii) whether complement deposits trigger the binding and activation of PMN, as described for some polymeric biomaterials [12].

2. Material and methods

2.1. Decellularization procedure

Porcine pulmonary heart valve conduits ($n = 10$) were obtained from a local slaughterhouse. After cold sterilization in sterile water containing multiple antibiotics [13], each trunk was divided into three longitudinal sections. Two parts were left untreated (native), the third part was decellularized as described previously [13]. Briefly, the conduits were placed into 100 ml sterile water containing 0.05% *tert*-octylphenyl-polyoxyethylene (Triton X-100, Biorad, Hercules, CA), 0.05% sodium-deoxycholate (Merck), and 0.05% octylphenyl-polyethylene glycol (IGEPAL-CA630, ICN) for 48 h at 4 °C. Conduits were subsequently treated with ribonuclease (100 μg/ml, Roche Diagnostics GmbH, Mannheim, Germany) and deoxyribonuclease (150 IU/ml, Sigma, Steinheim, Germany) with 50 mmol/l $MgCl_2$ in Dulbecco's phosphate buffered saline (PBS, Gibco, Paisley, UK) for 24 h at 37 °C. To remove residual detergents, the heart valve conduits were subsequently washed for 12 days in PBS that was changed every second day, with an additional antibiotic sterilization carried out for 3 days at the end of the washing procedure. All steps were conducted with continuous shaking. Two longitudinal slices (5 mm) of each conduit were excised for histology ($n = 40$). Tissue specimens of the remaining native and decellularized porcine pulmonary wall tissue were snap-frozen in liquid nitrogen and stored at −80 °C until used in the experiments. For detection of tissue integrity and cell removal, histological examination was performed as in Ref. [13].

2.2. Blood sampling, preparation of plasma, and isolation of PMN

Venous blood was collected from healthy adult volunteers ($n = 12$, f/m: 6/6, mean age 32 ± 10.2) who were not using any medications and who gave informed consent (approved by the ethical committee, Medical University of Vienna). Whole blood was anticoagulated with 5 IU/ml heparin and centrifuged at $2000\times g$ for 15 min to obtain plasma. Human PMN were retrieved from EDTA-anticoagulated venous blood by lysing 1 ml blood with 5 ml 0.9% (w/v) ammonium chloride for 15 min at 4 °C, followed by centrifugation at $160\times g$ and 4 °C for 10 min [14]. The supernatant was discarded and the cell pellet washed three times with PBS. Cells were subsequently resuspended in RPMI-1640 medium (BioWhittaker™, Verbiers, Belgium) to a final concentration of 5000/μl and immediately used for the experiments. The cell suspensions comprised $82.2 \pm 2.9\%$ PMN, $13.5 \pm 2.7\%$ lymphocytes, and $3.8 \pm 1.9\%$ monocytes. The platelet contamination was less than 0.1 platelet per PMN.

2.3. Adsorption of plasma proteins and complement to tissue specimens

Porcine native ($n = 4$) and decellularized ($n = 4$) tissue specimens, prepared as described above, were incubated with increasing concentrations (1, 10, 20, 50, 100%) of heparinized plasma (diluted in PBS containing 0.15 mmol/l Ca^{2+} and 0.5 mmol/l Mg^{2+}) on a rocking platform for 5 h each at 37 °C. Human sera with decreasing levels of IgG ($n = 3$) were used to pre-treat the tissue specimens described above in order to test the effect of immunoglobulin fixation on complement activation on decellularized porcine heart valves in vitro. In another set of experiments, decellularized specimens ($n = 4$) were exposed to either factor B-depleted serum (50% [v/v] in PBS) (Quidel, San Diego, CA), factor C2-depleted serum (50% [v/v] in PBS), or a mixture of both on a rocking platform for 5 h each at 37 °C. In some experiments ($n = 3$), the C1-esterase inhibitor Berinert® (kindly provided by CSL Behring, Vienna, Austria) was added to human plasma samples at a final concentration of 5 mg/ml to inhibit the activation of the classical activation pathway prior to incubation of the tissue specimens. To totally inhibit complement activation plasma was supplemented with 10 mmol/l EDTA and subsequently used for pre-treatment of tissue specimens ($n = 3$). In another set of control experiments ($n = 4$), tissue samples were incubated with human serum albumin (20% (v/v) in PBS). At the end of the incubations tissue specimens were removed, thoroughly rinsed with PBS, embedded in Tissue-Tec OCT compound, and frozen in liquid nitrogen. Twenty-micrometers thick cryostat sections were used for histologic evaluation and for performing the PMN/matrix experiments.

2.4. Staining for IgG, IgM, and iC3b

Cryostat sections were fixed with acetone for 20 min at room temperature. Samples were washed with PBS (3×5 min) and blocked with 10% goat serum (in PBS) for 1 h. Specimens were then incubated with mouse anti-human iC3b (monoclonal mouse IgG, 1:100, Quidel) followed by Alexa 546-conjugated goat anti-mouse IgG (1:1000, Molecular Probes, Leiden, The Netherlands). Fixation of plasma IgG and IgM on tissue samples was determined using Alexa 488-conjugated goat anti-human IgG (1:1000, Molecular Probes) or Alexa 488-conjugated goat anti-human IgM (1:1000, Molecular Probes). Negative control sections were incubated in parallel, with omission of the first antibody. Samples were mounted with Pro-Long Antifade Plus mounting medium (Molecular Probes) and sealed with glass cover slips.

2.5. Preparation of tissue extracts

Frozen samples of native porcine pulmonary wall tissue were washed with cold PBS and homogenized in 750 μl ice-cold RPMI-1640 medium (Bio Whittaker), using a mortar and pestle. The homogenization was carried out mechanically at 700 rpm for 10 min on ice. Sterile technique was used throughout the experiments. The homogenates were aspirated and transferred into centrifuge tubes, and the debris of each homogenate was sedimented by subsequent centrifugation at $10{,}000\times g$ and 18 °C for 30 min. Then, 500 μl of each supernatant was carefully withdrawn. Next, 100 μl of each sample was drawn, and the protein content was determined using the Quick Start™ Bradford protein assay (Biorad). The remaining 400 μl was used for the granulocyte/matrix experiments. RPMI-1640 medium without protein extracts was used as the negative control.

2.6. Granulocyte/matrix adhesion experiments

Plasma used for pre-treating the tissue samples and granulocytes was obtained from the same donor during each experiment. Porcine native ($n = 12$) and decellularized ($n = 12$) tissue specimens (0.5×0.5 cm) were either incubated with 20% of heparinized autologous plasma (diluted in PBS containing 0.15 mmol/l Ca^{2+} and 0.5 mmol/l Mg^{2+}) on a rocking platform for 5 h each at 37 °C, or left untreated. Cryostat sections ($n = 144$) of the four groups were incubated with 5000 PMN/μl for 15 min in a humidified incubation chamber at room temperature (RT). This incubation time was chosen since it proved

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