The localisation of inflammatory cells and expression of associated proteoglycans in response to implanted chitosan

Brooke L. Farrugia [a], John M. Whitelock [a], MoonSun Jung [a,1], Barbara McGrath [b], Robert L. O'Grady [a], Simon J. McCarthy [b], Megan S. Lord [a,*]

[a] Graduate School of Biomedical Engineering, The University of New South Wales, Sydney, NSW 2052, Australia
[b] HemCon Medical Technologies, Inc., Portland, OR, USA

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ABSTRACT

Implantation of a foreign material almost certainly results in the formation of a fibrous capsule around the implant however, mechanistic events leading to its formation are largely unexplored. Mast cells are an inflammatory cell type known to play a role in the response to material implants, through the release of pro-inflammatory proteases and cytokines from their α-granules following activation. This study examined the in vivo and in vitro response of mast cells to chitosan, through detection of markers known to be produced by mast cells or involved with the inflammatory response. Mast cells, identified as Leder stained positive cells, were shown to be present in response to material implants. Additionally, the mast cell receptor, c-kit, along with collagen, serglycin, perlecan and chondroitin sulphate were detected within the fibrous capsules, where distribution varied between material implants. In conjunction, rat mast cells (RBL-2H3) were shown to be activated following exposure to chitosan as indicated by the release of β-hexosaminidase. Proteoglycan and glycosaminoglycans produced by the cells showed similar expression and localisation when in contact with chitosan to when chemically activated. These data support the role that mast cells play in the inflammatory host response to chitosan implants, where mediators released from their α-granules impact on the formation of a fibrous capsule by supporting the production and organisation of collagen fibres.

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1. Introduction

One of the major problems associated with the implantation of foreign materials is their propensity to induce inflammation and fibrosis, which may result in a loss of implant function. Implantation of materials into the body results in a cascade of events starting with the host's response to injury, inflammation. The most likely long-term host response is fibrosis and formation of a fibrous capsule around the implant [1] and although widely recognised, research into the mechanistic events that occur are still relatively unknown. Strategies to reduce fibrosis generally revolve around alteration of material surfaces to make them anti-fouling or protein resistant, but the role of inflammatory cells in mediating fibrosis capsule formation during the body's inflammatory response to an implanted material remains largely unexplored. Upon implantation

of a foreign material, injury to the vasculature occurs, resulting in haemorrhage, infiltration of neutrophils and activation of the complement cascade [2]. A number of products of complement activation function as mediators of inflammation, by recruiting various inflammatory cells. Many different cell types are known to be associated with inflammation and are quite often studied when assessing implant-induced inflammation, including neutrophils, macrophages and foreign-body giant cells, yet the role that mast cells play in response to implanted materials is only now starting to be investigated.

Mast cells, a population of cells associated with inflammation, are derived from hematopoietic stem cells. They are a heterogeneous cell population, and are widely distributed in the connective tissues where they are normally located in close proximity to blood vessels. They are concentrated in tissue at sites directly exposed to the environment including skin, airways and gastrointestinal tract [3]. Mast cells are important for the induction of inflammatory reactions to implanted materials through their release, upon activation, of pro-inflammatory proteases and mediators including cytokines that attract cells to the site of implantation. Mast cells have also been linked with chronic inflammation resulting in

fibrosis [4], suggesting that these mediators have a positive effect on the expression of collagen. Mast cell activation can occur through a number of different pathways, and results in the release of the contents of their α-granules, which are bound to the proteoglycan serglycin [3]. Serglycin is an intracellular proteoglycan (PG) produced by hematopoietic and endothelial cells, consisting of a protein core to which chondroitin sulphate (CS), heparan sulphate (HS) and/or heparin chains are attached depending on the origin and species of the producing cell [5]. Mast cells, along with other inflammatory cells, have previously been shown to be present following implantation of foreign materials [6]. Degranulation of mast cells has been correlated with the inflammatory reaction within the surrounding tissue [7]. Mast cells are known to play a role in the acute inflammatory response [8], where they have been shown to regulate the infiltration of neutrophils [9], and macrophages [10,11]. In addition to their role in the acute inflammatory response, the long term presence of mast cells may be related to the degree of fibrotic encapsulation [12,13].

Chitosan, is a naturally occurring polysaccharide polymer, consisting of glucosamine and N-acetyl glucosamine linked by β(1–4) glycosidic bonds and is commercially produced by the deacetylation of chitin, which is most frequently derived from crustaceans. Typically, deacetylation of chitosan ranges from around 65–95%, though lower and higher degrees are possible depending on the method of processing. Due to the broad degree of deacetylation and molecular weight range (5–500 kDa) available, the material properties of chitosan have a wide range of variability. The applications of chitosan span numerous fields, including agriculture, water treatment, cosmetics and the biomedical sciences [14]. It has been investigated for a variety of biomedical applications [15] including wound healing [16], bone regeneration as well as being developed as a drug and growth factor delivery vehicle [17]. All of these applications make use of the antibacterial, haemostatic and non-toxic properties of this naturally derived polymer.

The aim of this study was to investigate mast cell interactions, including activation, with chitosan *in vivo* and *in vitro*. This study investigated early infiltration of mast cells *in vivo* following implantation of chitosan, with or without acid pre-treatment, subcutaneously into rats and as well as Surgicel, a commercially available haemostatic cellulose dressing. Histological assessment was carried out following 7 days implantation to investigate mast cell infiltration in response to the implanted material, along with expression of markers known to be produced by mast cells or involved in the inflammatory response. *In vitro* experiments on the rat mast cell line, RBL-2H3, were also carried out to investigate activation and the expression of PGs and glycosaminoglycans (GAGs) produced by these cells upon contact with chitosan.

2. Materials and methods

2.1. Materials

Chemicals were purchased from Sigma–Aldrich (Castle Hill, Australia) unless stated otherwise. Materials used for implantation included Surgicel (Johnson and Johnson, USA) and chitosan (Ultrapure FMC-NovaMatrix, Norway, Batch no# 1148013, 460 kDa, 91% degree of deacetylation) were used as received and prepared for implantation as described below.

2.2. Rat implantation model

Preparation of chitosan for implantation involved either an acid or non-acid process. Chitosan gels were prepared, 2% (w/v) chitosan in 2% (v/v) acetic acid, and frozen at −40 °C for 2 h to achieve a micro-dispersed lamella phase structure within a continuous ice phase, followed by lyophilisation to remove the water resulting in a chitosan sponge. The sponges were compressed at 80 °C to a thickness of 3 mm (density approximately 0.12 g/cm^3) and placed in a convection oven (80 °C) reducing the acetic acid fraction to approximately 16% w/w. The non-acid processed sponges were then soaked in a neutralising solution, 2 M NaOH/EtOH for 1 h, followed by thorough washing in deionised water and lyophilisation. Samples for implantation were cut and sterilised by gamma irradiation (11–19 kGy, Steris, USA).

The *in vivo* implantation study was performed in an accredited facility by the Association for Assessment and Accreditation of Laboratory Animal Care under Institutional Animal Care and Use Committee approval. The study was divided into three blinded arms with eight rats in each arm, two of the arms contained exposure to chitosan and the third exposed to Surgicel. Samples were subcutaneously implanted into the lumbar region of female Sprague-Dawley rats weighting between 80 and 100 g. Animals were anesthetised and clipped dorsally over the flank region, with samples (1 × 0.5 cm) implanted through 1.5 cm transverse skin incisions into both the left and right lumbar regions. Animals were sacrificed at 7 days and the implant and 0.5 cm of surrounding tissue dissected out and placed in formalin.

2.3. Histological analysis

Grafts were fixed in 10% neutral buffered formalin and paraffin embedded as previously described [18]. Paraffin embedded samples were sectioned (4–5 μm) with up to 20 serial sections collected per sample and stained for analysis as follows. Sections were washed twice, 5 min each, with xylene to remove paraffin and the slides were immersed in a series of ethanol solutions for 3 min each (twice in 100% (v/v), once in 95% (v/v), once in 70% (v/v)) followed by several exchanges of water. Histological characterisation was undertaken through the use of hematoxylin and eosin, picrosirius red, Leder stain, in addition to immunolocalisation of PGs and GAGs of interest. Following rehydration, slides were stained with Harris haematoxylin (Fronine, Australia) for 10 min, followed by eosin (Fronine, Australia) for 5 min, before dehydrating and mounting. Picrosirius red sections were stained following rehydration with Weigert's haematoxylin for 8 min, followed by picrosirius red solution (0.1% Sirius red in saturated picric acid) for 1 h, washed with acidic water, dehydrated and mounted. Leder stain detects the presence of chloroacetate esterase (Naphthol AS-D chloroacetate (NADC), Sigma 91C kit) and was carried out as per the manufacturer's protocol. Briefly, following rehydration, sections were fixed in the citrate-acetone-formaldehyde solution (30 s), washed in deionized water, and immediately placed into the NADC solution and incubated for 15 min at 37 °C. Slides were then rinsed in pre-warmed deionized water for 2 min followed by counterstaining with hematoxylin (Gill #3) for 2 min further and then rinsed with deionized water. A semi-quantitative analysis of Leder positive cell was performed by taking images (6) at random positions along the fibrous capsule. The distances from the material/tissue interface to the positively stain cells was measured to determine infiltration of the positively stained cells in response to the implanted materials.

Antigen epitope retrieval was undertaken by immersing the slides, following rehydration, in 0.01 M sodium citrate (pH 6), followed by heat treatment in a decloaking chamber (Applied Medical CA, USA) at 120 °C for 4 min. The slides were then rinsed with deionised water followed by blocking with 3% (v/v) H$_2$O$_2$ for 10 min. Some slides were also treated with chondroitinase ABC (C'ase ABC) (0.05 U/mL) in Dulbecco's phosphate buffered saline (DPBS) pH 7.2 for 3 h at 37 °C. The slides were washed with 50 mM Tris–HCl, 0.15 M NaCl, 0.05% (w/v) Tween-20, pH 7.6 (TBST) and then blocked with 1% (w/v) bovine serum albumin (BSA) in TBST for 1 h at room temperature (RT). The slides were incubated with primary antibodies diluted in 1% w/v BSA in TBST at 4 °C for 16 h. Primary antibodies used included polyclonal rabbit anti-serglycin (1:500, gift from Dr. Achilleas Theocharis, University of Patras, Greece), polyclonal anti-c-kit (CD117, 1:100, AbCam, Cambridge, USA) or polyclonal rabbit anti-perlecan (ab906, 1:500), and mouse monoclonal antibodies reactive to the 4-sulphated linkage region of chondroitin sulphate (CS) chains following C'ase ABC digestion (2B6, conditioned media 1:400) gift from Prof. Bruce Caterson, Cardiff University, UK). Slides were then washed twice with TBST before incubating with the appropriate biotinylated secondary antibodies (GE Healthcare, Sydney, Australia, 1:500) for 1 h at RT. The slides were washed twice with TBST then incubated for 30 min with streptavidin-HRP (1:250), rinsed four times with TBST before colour development with NovaRED™ chromogen stain (Vector Laboratories, Burlingame CA, USA). The slides were then counterstained with hematoxylin (Gill's #3, Vector Laboratories, Burlingame CA, USA) for 3 s and rinsed with deionised water.

2.4. Mast cell adhesion

Tissue culture polystyrene (TCPS) 24-well plates (Greiner) were coated with chitosan (1% (w/v) in 2% (v/v) acetic acid) for 24 h at 60 °C and the wells were washed with sterile PBS prior to the addition of rat basophilic leukaemia cells (RBL-2H3) (1 × 10^5 cells/well) in RPMI-1640 medium containing 10% (w/w) foetal bovine serum (FBS) and 100 U/mL penicillin and 100 μg/mL of streptomycin and maintained in a humidified incubator (5% CO$_2$/95% air atmosphere at 37 °C). Cell adhesion was analysed using the Trypan blue exclusion assay at 24 h after cell seeding.

Additionally, adhesion was assessed by staining the actin cytoskeleton adhered cells. RBL-2H3 cells were seeded at a density of 1 × 10^5 cells/well in 24-well plates in RPMI-1640 medium as described above and maintained in a humidified incubator (5% CO$_2$/95% air atmosphere at 37 °C) for 24 h. Wells were rinsed with PBS followed by fixation with 4% (w/w) paraformaldehyde and 1% (w/w) sucrose in PBS for 15 min at 37 °C. Cells were then washed with PBS, permeabilised with 0.5% (w/v) Triton X-100 at 4 °C for 5 min and stained with rhodamine–phalloidin (1:100 in 1% (w/v) BSA in PBS, Life Technologies, Sydney, Australia) for 1 h at 37 °C. Cells were counterstained with Hoechst 33342 (1:1000 in 1% (w/v) BSA in PBS) for 10 min at 37 °C. Cells