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The pivotal role of fibrocytes and mast cells in mediating fibrotic reactions to biomaterials

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ABSTRACT

Almost all biomaterial implants are surrounded by a fibrotic capsule, although the mechanism of biomaterial-mediated fibrotic reactions is mostly unclear. To search for the types of cells responsible for triggering the tissue responses, we used poly-L glycolic acid polymers capable of releasing various reagents. We first identified that $CD45^+$/Collagen 1^+ fibrocytes are recruited and resided within the fibrotic capsule at the implant interface. Interestingly, we found that the recruitment of fibrocytes and the extent of fibrotic tissue formation (collagen type I production) were substantially enhanced and reduced by the localized release of compound 48/80 and cromolyn, respectively. Since it is well established that compound 48/80 and cromolyn alter mast cell reactions, we hypothesized that mast cells are responsible for triggering fibrocyte recruitment and subsequent fibrotic capsule formation surrounding biomaterial implants. To directly test this hypothesis, similar studies were carried out using mast cell deficient mice, WBB6F1/J-Kit^W/$Kit^{W\text{-}v}$/, and their congenic controls. Indeed, mast cell deficient mice prompted substantially less fibrocyte and myofibroblast responses in comparison to C57 wild type mice controls. Most interestingly, subcutaneous mast cell reconstitution of WBB6F1/J-Kit^W/$Kit^{W\text{-}v}$/J mice almost completely restored the fibrocyte response in comparison to the C57 wild type response. These results indicate that the initial biomaterial interaction resulting in the stimulation of mast cells and degranulation byproducts not only stimulates the inflammatory cascade but significantly alters the downstream fibrocyte response and degree of fibrosis.

1. Introduction

Despite substantial improvements in biomaterial design, almost all biomaterial implants initiate fibrotic responses [1,2]. It is well documented that biomaterial-mediated fibrotic responses are at least partially responsible for the failure of many medical implants, including a variety of biosensors, spine/joint implants, breast implants, encapsulated tissues/cells, drug delivery systems, neural electrodes and eye implants [3–13]. Unfortunately, the mechanisms governing biomaterial-mediated fibrotic responses are poorly understood. Since biomaterial implants are often surrounded with large numbers of inflammatory cells prior to fibrotic tissue formation, it is generally believed that implant-induced inflammatory reactions are responsible for launching subsequent host fibrotic responses. This is supported by both acute and chronic inflammatory reactions. First, in the acute phase of inflammation, mast cells have been shown to regulate neutrophil accumulation [14] and subsequently have shown a correlation with fibrosis around silicone implants [15]. Second, in continued inflammation fibroblast proliferation and collagen production are strongly influenced by many phagocyte-derived proteins, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), platelet activating factor, and platelet-derived growth factor [16–19]. We and many others have shown that adherent phagocytes produce and release significant amounts of pro-inflammatory and pro-fibrotic cytokines, such as IL-1β and TNF-α [1,18–21]. Third, by suppressing the activation - but not the presence - of phagocytes (specifically macrophages) with corticosteroids, *in vivo* biomaterial-mediated fibrotic tissue formation can be dramatically reduced [5,22–25] (although we should temper this by pointing out that steroids may have phagocyte-independent effects on fibroblast proliferation and wound healing). Finally, intensive research of wound healing reactions has found that that both granulocyte and phagocyte responses, and associated inflammatory products, are essential to fibrotic reactions [14,26–31].

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Little is known about the source and function of fibroblast-like cells responsible for fibrotic tissue formation surrounding biomaterial implants. Recent work on the mechanisms of fibrosis has led to the discovery of the fibrocyte, a $CD34^+$ $CD45^+$ blood leukocyte which arrives at injury sites within approximately 4 days after injury, possibly through the same SDF-1α/CXCR4 axis which has been linked with peripheral stem cell mobilization [32]. After arrival at a wound site, fibrocytes ($CD45^+$/$CD34^+$/collagen I^+/vimentin$^+$) participate in fibrotic reactions through differentiation to myofibroblasts (αSMA^+) and secretion of collagen I, vimentin, and other proteins which influence the developing fibrotic matrix [33–37]. Most importantly it has been demonstrated in pulmonary fibrosis that fibrocyte recruitment corresponds directly with collagen production [38]. However, it is not clear whether fibrocytes and fibrocyte-associated cellular responses are essential to the fibrotic tissue reactions of biomaterials and medical implants.

In an attempt to determine the factor(s) governing biomaterial-mediated fibrotic reactions, we have previously found that mast cell activation is accountable for the recruitment of inflammatory cells to the implantation site [39,40]. Mast cell degranulation releases several mediators such as histamine and heparin as well as interleukin 1-beta, monocyte chemoattractant protein (MCP-1), and several growth factors such as TGF-β [41]. These mediators, especially histamine, have been linked with subsequent inflammatory cell diapedisis and adherence to biomaterial implants [40]. In fact, the long term presence of mast cells at the implantation site may be related to the degree of fibrotic encapsulation [42]. In support of this work a recent study has indicated that suppression of the mast cells response may lead to a reduction in tissue reactions surrounding synthetic mesh implants [43]. However, the potential effect of mast cells and degranulation factors on peripheral tissue fibrotic responses to biomaterial implants has yet to be demonstrated in detail.

Based on the above observations, we hypothesized that mast cell activation and subsequent fibrocyte responses are directly associated with the fibrotic pathogenesis of biomaterial implants. To model implant-mediated fibrotic tissue formation, we employed films made of poly-L glycolic acid (PLGA), materials commonly used to fabricate tissue engineering scaffolds. Some of these implants were made to release a variety of histamine blockers, mast cell stabilizer or activator. Using these films, we investigated the potential role and interactions of fibrocytes and mast cells on the pathogenesis of biomaterial-mediated fibrotic reactions.

2. Materials and methods

2.1. PLGA film fabrication and chemical/drug loading

All chemicals were from Sigma Aldrich (St. Louis, MO) unless otherwise specified. PLGA films (75:25, 113 kDa, Medisorb Inc., Birmingham, AL) were fabricated as previously described [44], with slight modifications to incorporate chemicals for mast cell stabilization and destabilization experiments. Films were incorporated with either the mast cell stabilizing agent cromolyn supplemented at 640 μg/kg body wt/day, mast cell destabilizing agent compound 48/80 at 1 mg/kg body wt/day, or no treatment unmodified PLGA films [40,45,46]. For cromolyn embedded films, cromolyn salt was mixed with the polymer solution and cast into molds and evaporated as per control films. For compound 48/80 embedded films, compound 48/80 was dissolved in dimethylsulfoxide. The solution was then blended with the polymer solution and cast into molds. For all film conditions, the resulting film had a thickness of ~ 1 mm. By incubating the drug-loaded films with PBS for various periods of time, we determined that the average release rates of compound 48/80 and cromolyn to be approximately 6.25 and 4.0 μg/day, respectively. Films were cut into 5 mm disks and stored at −20 °C until implantation.

2.2. PLGA film implantation

C57 mice (Jackson Labs) were selected for equal age and sex prior to housing by implantation condition. For film implantation, mice were anesthetized and a dorsal midline incision was created as previously described [47]. Briefly, each mouse was implanted with two films of equal treatment condition, placed laterally on either side of the incision tucked into the subcutaneous space approximately 15 mm away from the incision. The incision was then closed with surgical clips. The mice were subsequently returned to housing and monitored daily for irritation around the implantation for 1 week or 2 weeks until explanation.

2.3. Mast cell deficient model

The 2 week fibrotic response to PLGA films was assessed in three treatment groups at $n = 6$. Groups consisted of control C57 mice, mast cell deficient mice (WBB6F1/J-Kit^W/$Kit^{W\text{-}v}$/J, Jackson Labs, and dermally reconstituted mast cell deficient mice. Peripheral mast cell reconstitution was achieved as previously described [40]. Briefly, bone marrow flushes were taken from C57 mice ($n = 8$). Cells were placed in 75 cm^2 flasks in DMEM with 10% low IgG serum (Invitrogen, Carlsbad, CA) supplemented with 10 ng/mL SCF (Prospec) and 10 ng/mL IL-3 (Prospec). Cells were transferred every 3 days into new flasks and supplemented with fresh mast cell differentiation media for 4 weeks. To verify mast cell phenotype, cells were cytospun onto slides and stained with Toluidine Blue. Mast cells were then injected in deficient mice sub-dermal at 3×10^6 cells per mouse in PBS. Six weeks later, compound 48/80 was injected subcutaneously to verify peripheral mast cell engraftment. The response to films was assessed at 2 weeks.

2.4. Histological evaluation

Films and surrounding tissue were removed and embedded into OCT for frozen sectioning. Cross sections were cut at 7 μm. H&E staining was used to visualize the extent development of the infiltrated cell layer. Quantifications of interface response were performed as previously reported using Image J [47]. Briefly, data presented as average of multiple counts taken from H&E stains, with images captured on the skin side of the biomaterial interface. Measurements were taken from the biomaterial perpendicular toward where the capsule met native healthy tissue. Fibrosis was assessed using both Masson Trichrome and Picrosirius Red staining. Thickness of the collagen layer was quantified. Picrosirius Red staining was visualized using polarized light microscopy. The degree of collagen I in the fibrotic layer was quantified using Image J to measure the percentage of red/yellow birefringence per total interface area (between the implant and hypodermis).

All antibodies used in this study were purchased from Santa Cruz Biotech (Santa Cruz, CA) unless otherwise specified. The density of inflammatory cells and fibrocyte/myofibroblasts around the implant was assessed using immunohistochemistry. Cells were analyzed using the following conventions: inflammatory cells/leukocytes (CD11b or CD45), fibrocyte-derived myofibroblasts (CD45 co-expressed with α-SMA), fibrocytes (CD45 co-expressed with collagen I). The appropriate fluorescent secondary antibodies isotype conjugated to either FITC or Texas Red was used for each primary antibody (ProSci, Poway, CA). For all stains, nuclei were visualized using DAPI (Invitrogen, Carlsbad, CA). Interface density of expressing cell was quantified per interface area as previously described [47]. Cell densities were calculated as the number of positive cells per area, approximately similar areas were used in each case calculated from Image J. Stained sections were visualized using a Leica microscope and imaged with a CCD camera (Retiga EXi, Qimaging, Surrey BC, Canada).

2.5. Statistical analysis

GraphPad (La Jolla, CA) was used for all statistical operations. Results are reported as the means ± standard deviations. We have assumed Gaussian distributions and performed parametric tests. Statistics were calculated with ANOVA using the Bonferroni post test and considered significant when $P < 0.05$.

3. Results

3.1. Deployment of inflammatory cells and fibrocytes to the implantation site

To determine the cells responsible for biomaterial-mediated fibrotic tissue reactions, we began by analyzing the types of cells recruited to the interface of the subcutaneously implanted PLGA films. After implantation for different periods of time, the influx of cells to the subcutaneous implants was analyzed histologically. Indeed, results reveal accumulation of inflammatory cells, between the biomaterial and the native skin tissue, reaching a maximum 2 days after implantation. This dynamic interface is characterized by a mixture of cells mostly having a granulocyte morphology up to 4 days after implantation, with a gradual increase in morphologically spindle-shaped cells prominent in the layer immediately next to the implant by day 7–14. Interestingly by day 4 there is an observed