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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-v}, and their congenic controls. Indeed, mast cell deficient mice prompted substantially less fibrocyte and myofibroblast responses in comparison to C-57 wild type mice controls. Most interestingly, subcutaneous mast cell reconstitution of WBB6F1/J-*Kit*^W/*Kit*^{W-v}/J mice almost completely restored the fibrocyte response in comparison to the C-57 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

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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-1 β (IL-1 β), 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-1 β 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 both granulocyte and phagocyte responses, and associated inflammatory products, are essential to fibrotic reactions (14, 26-31).

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-1 α /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 (α -SMA⁺) 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 diapedesis 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, 113kDa, 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 ~ 1mm. 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 5mm 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 15mm 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-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 75cm² flasks in DMEM with 10% low IgG serum (Invitrogen, Carlsbad, CA) supplemented with 10ng/mL SCF (Prospec) and 10ng/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 cytopun onto slides and stained with Toluidine Blue. Mast cells were then injected in deficient mice subdermal 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 7µm. 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 $\pm$ 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 to 14. Interestingly by day 4 there is an observed decrease in granulocytic cells with a crossover to round and spindle morphology throughout the matrix. The influx of CD45⁺/Col1⁺ fibrocytes (Figure 1A), becomes significant at 4 days corresponding with an observed decrease in leukocytes (neutrophils, monocytes/macrophages) (CD11b⁺) cells after their peak at 1013 ± 100 cells/mm² on day 2. Following the recruitment of leukocytes, a significant increase in fibrocyte numbers is observed (121% increase from day 4 to day 10) reaching a maximum 10 days after implantation at 419 ± 86 cells/mm² (Figure 1B). This increase in fibrocyte numbers is mirrored by the collagen content within the capsule. Total collagen production, observed through Masson Trichrome staining, demonstrates a shift in the inflammatory and fibrotic response between day 7 and 10 with a doubling ($21\% \pm 1.8\%$ to $41\% \pm 3.9\%$) in the amount of collagen present in the biomaterial interface (Figure 1C). Interestingly, we have also observed a decrease in the capsule thickness (160 ± 19 μ m at day 10, to 113 ± 16 μ m at day 14) and CD11b leukocytes (727 ± 152 cells/mm² at day 10, to 287 ± 77 cells/mm² at day 14). Finally a correlation between the percent total collagen in the interface with the number of fibrocytes present demonstrates a linear relationship ($R^2=0.902$), indicating a strong link between fibrocyte recruitment and the shifting change from inflammation to fibrosis within the biomaterial interface (Figure 1D). This shifting interaction and correlation determination demonstrate

the importance of recruited fibrocytes however the question remains how fibrocytes are recruited to the biomaterial implantation sites.

3.2. Mast cell responses dictate biomaterial-mediated fibrocyte responses

Some previous work has established the importance of mast cell responses in controlling phagocyte chemotaxis to the site of biomaterial implantation (40). To investigate whether variable mast cell responses could initiate a change in the fibrocyte and tissue reactions to biomaterial implants, PLGA films incorporating either mast cell stabilizing or destabilizing chemicals were developed. The downstream effects of mast cell modulation on the fibrotic response were analyzed at 2 weeks. Surprisingly histological staining of the implants revealed some significant differences related to both the organization and thickness of the infiltrated cell layer at the interface (Figure 2, H&E). Specifically, localized activation of mast cells by compound 48/80 treatment resulted in a significant increase in cell number (41% increase over the control unmodified film) (Figure 2A & 2B), and capsule thickness (Figure 2A & 2C). On the other hand, by comparison with controls, the localized release of cromolyn substantially diminished the extent of cell recruitment (Figure 2A & 2B) and fibrotic capsule thickness (Figure 2A & 2C).

To further deduce a possible mast cell influence the structure of deposited collagen was examined using Picrosirius Red staining (Figure 2). Unmodified films had random, discontinuous collagen I fiber deposition, pronounced near the implant interface becoming segmented away from the implant. Compound 48/80 embedded implants had well formed collagen I bands, extending from the implant surface throughout the interface tissue and running parallel to the biomaterial interface. Cromolyn embedded implants had a mixture of discontinuous collagen I with some collagen bands evident near the implant interface, though noticeable less deposition throughout the biomaterial interface. As expected compound 48/80 embedded implants have significantly higher percentage of collagen I deposition ($53 \pm 3.5\%$), in comparison to both PLGA unmodified ($37 \pm 2.5\%$) and cromolyn embedded films ($23 \pm 2\%$) (Figure 2D). In addition, cromolyn embedded implants had significantly reduced collagen I deposition compared to PLGA unmodified films. The total collagen content was also investigated prior to picrosirius red staining using Masson trichrome staining. These results, presented in supplementary figure S1, concur with the significant differences observed for cell number and capsule thickness however are less significant ($P < 0.05$) than that of the collagen I specific response ($P < 0.01$).

Further studies were carried out to assess the effect of Compound 48/80 and Cromolyn on the pathogenesis of biomaterial-mediated fibrotic tissue formation. As expected, we observe a significant increase ($P < 0.05$) in the leukocyte (CD45⁺) density for compound 48/80 treatment to either the unmodified PLGA implant or the cromolyn treated film (Figure 3B). Similarly there is also a reduction of the leukocyte response in cromolyn treated films from the unmodified PLGA films. In addition, compound 48/80 release led to an elevated percentage of fibrocytes (CD45⁺/CI⁺) at the interface (471 ± 59 cells/mm²) in comparison to PLGA controls (373 ± 62 cells/mm²) or cromolyn (173 ± 32 cells/mm²) (Figure 3A & C). Interestingly fibrocytes are more localized to the biomaterial side for both PLGA and cromolyn films where in the compound 48/80 samples they seem to be more dispersed throughout the matrix (figure 3A). With the observed alterations in fibrocyte percentages, and the significant differences in collagen deposition at the interface, we expected to observe differences in the numbers of CD45⁺ α -SMA⁺ myofibroblasts. Indeed, similar to the reduction in leukocytes and fibrocytes, cromolyn treated films resulted in a significantly lower cell density of myofibroblasts (296 ± 90 cells/mm²) at the interface compared with compound 48/80 treated films (713 ± 192 cells/mm²) and unmodified PLGA films (471 ± 94 cells/mm²) (figure 3D). Our results thus far suggest that the treatment of mast cell activator and stabilizer affect fibrocyte/ myofibroblast responses and subsequent fibrotic reactions.

However, it is not clear whether mast cell responses are solely responsible for biomaterial-mediated fibrotic tissue reactions.

3.3. Effects of mast cell deficiency on biomaterial-mediated fibrotic responses

As a more direct test of the possible role of mast cells in fibrotic tissue reactions, we employed mast cell deficient mice WBB6F1/J-*Kit*^W/*Kit*^{W-v}/J, mast cell reconstituted WBB6F1/J-*Kit*^W/*Kit*^{W-v}/J mice and their wild type controls. After implantation with unmodified PLGA films for 2 weeks, both groups of mice were analyzed for their fibrotic tissue responses (Figure 4A). Very interestingly we observe more significant changes in the tissue response to mast cell deficiency than was observed with chemical modification. The overall cellular density response is observed to decrease by 60% with a mast cell deficiency in comparison to the control C57 wild type response. Surprisingly after a six week subcutaneous mast cell reconstitution the biomaterial-mediated cellular response was almost completely restored and exerts inflammatory cell recruitment similar to controls (Figure 4B & C). The capsule thickness correlates with the cellular density at ($102 \pm 9 \mu\text{m}$) for the C57 wild type, ($87 \pm 9 \mu\text{m}$) with the reconstituted sample and only a thickness of ($64 \pm 3 \mu\text{m}$) with a mast cell deficiency. The cells as well as the collagen stained with Aniline Blue (supplemental figure S2) appear to have a higher degree of organization forming parallel layers with the wild type and reconstituted response. Collagen type I fibers however are observed to be discontinuous and segmented. Quantification of the collagen content shows a 40% reduction in total collagen and 17% reduction in collagen type I content with the mast cell deficiency. The collagen content is similarly almost entirely restored with mast cell reconstitution and in fact the collagen I content is slightly higher, though not significant, at $45.8 \pm 4.5\%$ for reconstitution and $43.4 \pm 3.2\%$ for the C57 wild type response (Figure 4D). With a mast cell deficiency there is significantly less cell density and collagen content at the interface. More importantly mast cell reconstitution almost completely restores the wild type response. We thus hypothesized that mast cell deficiency hinders the recruitment of fibrocytes while reconstitution restores recruited fibrocyte and myofibroblast populations.

We therefore examined the biomaterial interface for differences in the leukocyte, fibrocyte, and myofibroblast cell populations due to mast cell deficiency. Very interestingly we observe, as might be expected from the previous results, significantly altered the leukocyte, fibrocyte, and myofibroblast response within the implant interface. In each of the target cell groups we see an approximate (50%) reduction in cell density with mast cell deficiency. More striking however is that by simple subcutaneous mast cell reconstitution we see a full recovery in the fibrocyte ($287 \pm 34 \text{ cells/mm}^2$ for C57 and $273 \pm 80 \text{ cells/mm}^2$ for reconstituted) and the fibrocyte-derived myofibroblast ($522 \pm 166 \text{ cells/mm}^2$ for C57 and $485 \pm 30 \text{ cells/mm}^2$ for reconstituted) cellular densities (Figure 5).

4. Discussion

After implantation for varying periods of time, almost all biomaterial implants are surrounded with layer(s) of fibrotic tissue (1, 2). However, little is known about the cell types and associated mechanisms governing such common foreign body reactions. Given their prominence in other fibrotic diseases and conditions the study of fibrocytes has become increasingly important (36, 38, 48-49). Our investigation first verified participation of fibrocytes in biomaterial-mediated tissue responses. This observation is supported by an early finding that polymer-based wound chamber implants can be used to induce fibrocyte infiltration (50). In addition, we have very recently shown that fibrocytes, as myofibroblast pre-cursors, are present around a biomaterial implant at 2 weeks and that their presence is influenced by the surface topography of the implant (51). The immigrated fibrocytes and associated cellular responses are likely to be responsible for tissue reactions. This assumption is supported by an earlier observation that there is a good relationship between

the extent of fibrocyte recruitment and collagen production in a lung fibrosis animal model (38). In our investigation we have similarly observed that the fibrocyte response to a PLGA biomaterial implant correlates with the collagen production at the biomaterial interface and the progressing fibrotic reaction out to 14 days. The potential role of fibrocyte responses on fibrotic reactions has been investigated and documented in many recent works (37, 52-53). These studies suggest that fibrocytes participate in fibrotic reactions through differentiation to myofibroblasts (α -SMA⁺) and secretion of collagen I, vimentin, and other proteins which influence the developing fibrotic matrix (33-37). In support of this it has been shown that as the fibrocyte shifts toward myofibroblast differentiation the expression of identifying leukocyte CD34 and CD45 markers diminish as expression of α -SMA develops (32, 38, 54). It has further been suggested that certain stimuli, such as IL-1 β , may function to maintain fibrocytes in a pro-inflammatory state leading to an increase in the inflammatory cell population during wound healing (55). In addition, the stimulation of cultured fibrocytes with fibrogenic cytokines such as TGF- β has shown transition to myofibroblast phenotype in vitro (36). In correlation with these previous studies, we observed that fibrocytes reach a maximal accumulation at 10 days after implantation and then begin to decrease in number potentially as the shift to myofibroblast differentiation occurs.

The governing mechanisms of fibrocyte recruitment to the biomaterial are not totally understood. It has previously been shown in a pulmonary fibrosis model that fibrocytes traffic to the inflamed areas and migrate with inflammatory cells (38). It has been generally assumed that inflammatory products may provide the essential signals for fibrocytes immigration. Indeed, literature supports the potential role of the SDF-1 α /CXCR4 axis in fibrocyte recruitment to a site of injury or inflammation, and the subsequent role of the fibrocyte in facilitating fibrotic responses (32, 38). There are however conflicting observations on the primary chemotactic regulator of fibrocyte responses with several factors implicated including CCL2, CCL21, and CCL12 (54, 56-58).

Since fibrocyte migration is mediated by inflammatory signals (32, 38, 54, 56) and mast cell activation is essential to biomaterial-mediated inflammatory responses (40), we thus hypothesized that mast cell activation is responsible for facilitating biomaterial-mediated fibrotic responses through fibrocyte interactions. In support of our hypothesis, we have observed that mast cell deficient mice failed to prompt fibrotic tissue reactions to subcutaneous implants. Furthermore, the localized replenishment of mast cells restores fibrotic tissue formation surrounding the implants. These observations are supported by the results from several earlier investigations of liver and lung fibrosis suggesting mast cells play a role in the fibrotic response (59-61). A few studies have also attributed persistent mast cell activation to fibrotic responses in inflamed tissue (42, 62). A previous study using this strain of mast cell deficient mice did note deficiencies in inflammatory cell responses in the dermis of these mice attributed to mast cell deficiency. In addition, these mice did have impairments in fibrotic responses during the wound healing phase (63). Although the deficiency in c-kit has also been associated with deficiencies in other processes such as angiogenesis (64) which may account for the previously observed impairments. Despite this possible limitation the restoration of tissue responses by mast cell transplantation demonstrates the important role of mast cell responses in fibrotic tissue reactions to biomaterial implants.

The potential role of neutrophils in fibrotic tissue responses has attracted research interests. These studies however have shown some contradicting results. Mast cell deficiency has been shown to significantly alter the acute inflammatory response with the reduction of neutrophil, but not macrophage, recruitment using a dermal wound healing model (14). However, in a subcutaneous biomaterial implantation model, we have shown that mast cell deficiency reduced the recruitment of both macrophages and neutrophils to the implant (40).

It has also been suggested that early neutrophil recruitment may have an influence on the degree of fibrous capsule formation around implants (15). On the other hand, the numbers of recruited neutrophils decreased significantly one week after material implantation (65). In addition, at two weeks after implantation no myeloperoxidase-positive neutrophils were found around subcutaneous implants suggesting that most of the residual neutrophils are no longer activated or functional (66).

This study has uncovered that mast cell and fibrocyte responses are essential to biomaterial-mediated fibrotic tissue reactions. However, the processes and factors governing these cellular responses are mostly undetermined. It is likely that biomaterial implantation prompted the activation of mast cells. Activated mast cells secrete a number of mediating cytokines, chemokines, growth factors and granules, such as SDF-1 α , TGF- β , IL-1B, SLC, and MCP's, which may trigger further mast cell activation, and the onset of phagocyte and leukocyte trafficking (41, 67). Some of these mast cell products, including histamine, TGF- β , TNF- α , MCP-1, and SDF-1 α may be potent in triggering inflammatory cells and fibrocyte immigration in vivo (38, 40, 67-69). As stated earlier several homing axis's for fibrocyte recruitment have been identified and therefore it may be that mast cells exert a dominant cofactor effect on one or more of these axis's such as the CXCL12/CXCR4 pathway (70-72).

In addition to identifying the processes governing fibrotic tissue responses to biomaterial implants, we also explored the idea of incorporating various reagents into biomaterials to alter localized tissue responses. Although we do realize that the differences of cellular responses between treated and control groups are not as great as systemic administrations as used in many previous works (40, 43, 45-46), the results do indicate a significant response from the localized release. This demonstrates the high potential of mast cell governing agents to be incorporated into biomaterials for future development of better medical implants.

To the best of our knowledge, these results constitute the first direct evidence that biomaterial-mediated fibrotic reactions are both mast cell- and fibrocyte-dependent. At this juncture, we still do not know the potential mechanism(s) governing mast cell activations and subsequent fibrocyte responses. However, we believe that improved understanding of such responses would help to enhance the longevity of the implants and the safety of the patients.

5. Conclusion

Due to their prominence in lung, renal, and hepatic fibrosis as well as other inflammatory diseases the study of fibrocytes is becoming increasingly important. Little attention however has been paid to the potential influence of fibrocytes on the biomaterial-mediated fibrotic response to an implant. Here we show that the mast cell may represent a critical link in the subsequent recruitment of fibrocytes to the biomaterial interface. Through paracrine interactions with inflammatory cells these fibrocytes are stimulated to produce collagen and differentiate into myofibroblasts which then contract the fibrotic capsule deposited around the biomaterial. Upstream modulation of mast cell responses can reduce the percentage of fibrocytes at the biomaterial interface and reduce fibrotic responses, implicating mast cells and fibrocytes as critical regulators of biomaterial-mediated fibrotic responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix

Supplemental material is available with the online version. Figure S1 shows Masson trichrome staining and quantification for collagen content revealing significant ($P<0.05$) alteration in the total collagen response to mast cell stabilizing and destabilizing chemicals. Similarly Figure S2 shows Masson trichrome staining and quantification for total collagen percentage in control, mast cell deficient, and reconstituted animals indicating a significant ($P<0.01$) decrease with mast cell deficiency from both the control and reconstituted samples. More importantly subdermal mast cell reconstitution of WBB6F1/J-*Kit^W/Kit^{W-v}*/J mice was able to completely restore the collagen content within the biomaterial interface.

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Figure 1. Characterization of the biomaterial interface

Immunohistochemistry staining for fibrocytes (CD45/Cl) and inflammatory cells (CD11b) (A) showing spindle and round morphology respectively. Masson trichrome staining (A) shows a varying degree of collagen present throughout the interface. All images are shown at the day 10 time point corresponding to the maximal fibrocyte numbers. Analysis reveals early arrival of CD11b inflammatory cells (leukocytes) followed by an influx of fibrocytes beginning after day 2 and the maximal leukocyte recruitment (B). Corresponding analysis of the collagen percent shows maximal deposition occurring at day 10 as recruited fibrocytes similarly reach peak concentrations (C). Further correlation links the presence of fibrocytes to the amount of collagen in the interface (D). Results are calculated from $n=4$ per group and reported as the mean $\pm$ standard deviation. For collagen/ fibrocyte correlation the mean values were compared at each of the seven time points.

Figure 2. Histological staining and quantification of response to mast cell stabilizing and destabilizing chemicals

(A) H&E staining (first column) indicates cellular influx and matrix deposition within the biomaterial interface. Picrosirius red staining (second column) shows varying degrees of collagen I deposition and organization. Quantification of the capsule thickness (B) and cellular density (C) are presented as the mean $\pm$ standard deviation for $n=6$ per group in 2 separate experiments. Collagen type I percentage (D) similarly shows significant differences for treatment groups. Significant differences are calculated with ANOVA by Bonferroni comparison and taken as significant at $*P<0.05$ and $**P<0.01$

Figure 3. Cellular response to Mast Cell stabilizing and destabilizing chemicals

(A) Immunohistochemical staining of fibrocytes (dual positive CD45/ collagen I cells are shown in yellow) demonstrates morphology and cell dispersion through the implant interface. Quantification of the cellular density presents leukocytes (CD45⁺)(B), fibrocytes (CD45⁺ CI⁺)(C) and myofibroblasts (CD45⁺ αSMA⁺)(D) within the biomaterial interface. Results are calculated from n=6 per group in 2 separate experiments and show the mean ± standard deviation. Significant differences are calculated with ANOVA by Bonferroni comparison and taken as significant at *P<0.05 and **P<0.01 or not significant (NS).

Figure 4. Histological staining and quantification for response to Mast Cell deficiency
 (A) H&E staining (first column) and Picrosirius Red (second column) show the altered tissue response to mast cell deficiency. Quantification of the capsule thickness (B) and cellular density (C) show significant decreases for mast cell deficiency from both the control and reconstituted samples. Collagen I content similarly shows significant differences for mast cell deficiency. In each case the difference between control and reconstituted samples was not significant. Results are calculated from n=6 per group in 2 separate experiments and show the mean $\pm$ standard deviation. Significant differences calculated with ANOVA by Bonferroni comparison and taken as significant at * $P < 0.05$ and ** $P < 0.01$.

Figure 5. Cellular response to Mast Cell deficiency and reconstitution

(A) Immunohistochemical staining of fibrocytes (shown in yellow) demonstrates morphology and alignment of cells. Quantification of the cellular density presents leukocytes (CD45⁺) (B), fibrocytes (CD45⁺ CI⁺) (C), and myofibroblasts (CD45⁺ αSMA⁺) (D), within the biomaterial interface. For each cellular density the mast cell deficient samples were significantly less than the control or reconstituted samples. In addition the control and reconstituted samples were not significant from each other. Results are calculated from n=6 per group in 2 separate experiments and show the mean ± standard deviation. Significant differences are calculated with ANOVA by Bonferroni comparison and taken as significant at *P<0.05 and **P<0.01.