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Alternative strategies to manipulate fibrocyte involvement in the fibrotic tissue response: Pharmacokinetic inhibition and the feasibility of directed-adipogenic differentiation

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ABSTRACT

Fibrocytes have previously been identified as important mediators in several inflammatory and fibrotic diseases. However, there is no effective treatment thus far to reduce fibrotic tissue responses without affecting wound healing reactions. Here we investigate two strategies to alleviate fibrocyte interactions at the biomaterial interface, reducing collagen production and scar tissue formation. First, in an indirect approach, TGF-β inhibitor-SB431542 and IL-1β/TNF-α inhibitor SB203580 were locally released from scaffold implants to block their respective signaling pathways. We show that the inhibition of IL-1β/TNF-α has no influence on overall fibrotic tissue reactions to the implants. However, the reduction of localized TGF-β significantly decreases the fibrocyte accumulation and myofibroblast activation while reducing the fibrotic tissue formation. Since fibrocytes can be differentiated into non-fibrotic cell types, such as adipocytes, we further sought a more direct approach to reduce fibrocyte responses by directing fibrocyte differentiation into adipocytes. Interestingly, by initiating fibrocyte-to-adipocyte differentiation through sustained differentiation cocktail release, we find that adipogenic differentiation forces incoming fibrocytes away from the traditional myofibroblast lineage, leading to a substantial reduction in the collagen formation and fibrotic response. Our results support a novel and effective strategy to improve implant safety by reducing implant-associated fibrotic tissue reactions via directing non-fibrotic differentiation of fibrocytes.

1. Introduction

The localized fibrotic response to biomaterials continues to be an ever-daunting challenge in the design of artificial implants. It is recognized that the degree of inflammation and the persistence of inflammatory cells and products at the interface may drive the resulting long-term fibrotic response to the implant. Unfortunately the interactions between inflammation and fibrotic responses are poorly understood. It is well established that the interactions of immune cells (neutrophils, macrophages and mast cells) with the biomaterial prompt the release of a variety of pro-inflammatory and pro-fibrotic cytokines [1–5]. Some of these mediators, including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), platelet activating factor and platelet-derived growth factor (PDGF) have been shown to prompt the recruitment and activation of fibroblasts, which may lead to localized fibrotic tissue formation and collagen production [6–9]. Fibroblasts, however, often have organ-specific functions in promoting tissue homeostasis such as extracellular matrix (ECM) and cytokine production [10]. In addition, fibroblasts are often quiescent in tissue and must activate to myofibroblasts before participating in wound healing or tissue remodeling [11]. Recent evidence suggests that circulating fibroblast-like cells termed fibrocytes may be responsible for the extent of fibrotic reactions, presenting an alternative model of repair. These cells are highly migratory and have been shown to be multi-potent, differentiating to myofibroblasts as well as adipocytes [12,13]. More importantly, these cells have been shown to be responsive to immune/inflammatory cells, migrating with the inflammatory cascade, and reactive to the initiating response of mast cells [14]. In addition, fibrocyte recruitment corresponds directly with the amount of collagen production in wound healing and pulmonary fibrosis [14,15]. By manipulating these versatile fibroblast-like cells, either through upstream immune/inflammatory cell interactions, or through altered differentiation aspects, it

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may be possible to reduce localized collagen formation and alleviate the fibrotic response.

TGF-β is a well-known inflammatory and fibrotic mediator shown to promote pro-fibrotic and wound healing responses [16–18]. In addition, the cytokine has previously been linked to fibrocyte migration and proliferation in a lung fibrosis model [18–20]. Furthermore, TGF-β can also influence myofibroblast differentiation of resident tissue fibroblasts, which may be perpetuated by circulating fibrocytes, leading to extensive collagen production [12,21,22]. To alter fibrocyte-mediated responses and their innate differentiation to myofibroblasts, porous tissue scaffolds were made to release TGF-β inhibitor SB431542 using a fabrication technique established previously in our laboratory [23]. SB431542 is a potent ALK-5 inhibitor which has been shown to protect the cardiac conduction system in Chagas' disease, inhibit scar formation after glaucoma surgery and inhibit ECM formation of fibronectin and collagen in vitro [24–26]. We therefore explore both the acute and long-term responses of fibrocytes and their involvement in the degree of biomaterial-mediated fibrotic reactions during localized inhibition of TGF-β. Similarly, SB203580 is a p38 MAPK inhibitor shown to be effective at inhibiting inflammatory agents such as IL-1β and TNF-α [27,28]. SB203580 has been shown to suppress the development of endometriosis, improve renal disease, alleviate arthritis and reduce bone resorption in rodent models by down-regulating pro-inflammatory cytokines [27,29,30]. In the inflammatory/fibrotic cascade there is a known up-regulation of IL-1β and TNF-α after adhesion of monocytes to material surfaces [5]. IL-1β may further be a potent mitogen for fibrocytes [31], and function to maintain fibrocytes in a pro-inflammatory state, driving further recruitment of inflammatory cells [32]. Therefore we also assess the influence of localized SB203580 release from scaffolds.

In an alternative strategy, we investigate the influence of localized fibrocyte-to-adipocyte differentiation on fibrotic tissue reactions surrounding the implant. Fibrocytes have recently been shown to possess differential plasticity with the ability to differentiate not only to myofibroblasts but also to adipocytes [12,33], osteoblasts [34] and chondrocytes [34]. The differentiation of fibrocytes to these various lineages, however, has so far only been investigated in vitro. It is unclear whether fibrocyte differentiation to myofibroblasts is essential to implant-associated fibrotic tissue reactions. To test this hypothesis, fibrocyte myofibroblast differentiation was drastically reduced by inducing adipogenic differentiation with sustained release of a specific mitogenic cocktail. The feasibility of this approach is supported by several lines of evidence. First, human fibroblasts have been characterized to have myofibroblastic or lipofibroblastic phenotypes with Thy-1+ and Thy-1− subsets [35]. Second, it has been shown in vitro that differentiation of fibrocytes to adipocytes is driven by the peroxisome proliferator-activated receptor PPAR-γ and that TGF-β drives fibrocyte-to-myofibroblast differentiation [12]. Interestingly, the two pathways were found to reciprocally inhibit each other [12,36]. This paradigm was investigated both in vitro and in vivo.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma–Aldrich (St Louis, MO) unless otherwise specified. Poly(D,L-lactic-co-glycolic acid) (75:25, 113 kDa) was purchased from Medisorb Inc. (Birmingham, AL). The near-infrared fluorophore Xsight 761 was obtained from Carestream Health (New Haven, CT). Mini-osmotic pumps (Alzet Model 1002) were purchased from Alza Corporation (Palo Alto, CA) and the corresponding polyvinyl chloride catheters were also obtained from Alzet (Durect Corporation, Cupertino, CA). For differentiation studies, StemPro Adipogenesis Differentiation media (Kit A10070-01) was purchased from Invitrogen (Grand Island, NY). Both antagonistic compounds SB431542 and SB203580 were obtained from Selleck Chemicals (Houston, TX). All primary antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA) and all secondary antibodies were obtained from ProSci (Poway, CA).

2.2. Fibrocyte culture and differentiation

Isolation and culture of fibrocytes was performed by an established procedure in which cells are harvested from the spleens of Balb/c mice [37]. Briefly, the spleen is finely diced and then digested with collagenase (Invitrogen, Grand Island, NY) and hyaluronidase for 30 min at 37 °C. RPMI media is then used to dilute the sample for cell straining and centrifugation. The sample is then resuspended in 1 ml of red blood cell lysis buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM ethylenediaminetetraacetic acid) for 3 min at room temperature. Cell lysis is neutralized by the addition of phosphate buffered saline (PBS) before being further cultured. The cells are cultured in Dulbecco's modified Eagle medium supplemented with M-CSF and IL-13 as previously described for 7 days [37]. Fibrocytes were then positively identified by surface markers CD45 and Collagen 1 (Santa Cruz Biotechnology) through immunohistochemistry. For in vivo imaging, some cells were incubated with 5 μM of near-infrared fluorophore (Xsight 761) for 3 h. Following labeling, 2×10^6 cells in 200 μl PBS were administered by intravenous (IV) injection, as described in the previous work [38,39].

For adipogenesis differentiation studies, fibrocytes were subcultured after the initial 7 days in culture and re-plated on glass cover slips in a 24 well plate. Cells were plated at 10,000 cells per well and allowed to adhere overnight. StemPro Adipogenesis Differentiation media was then used, according to the manufacturer's instructions, to stimulate adipogenic differentiation. The medium was replaced every 3 days by removing half the old medium and supplementing with an equal volume of new medium. Differentiation of fibrocytes to adipocytes was carried out for 14 days. Control cells were similarly seeded and supplemented with a half change of medium every 3 days, maintained in the original fibrocyte media containing M-CSF and IL-13. Adipocyte differentiation was confirmed through Oil Red O staining for lipid droplet accumulation. To assess the degree of differentiation, some samples were stained with Oil Red O for lipids while other samples were stained with Sirius Red to identify collagen. The two stains were then extracted from the cells and the degree of staining was assessed by colorimetric absorbance micro-assay, as previously described [40,41]. Briefly, Oil Red O was extracted by the addition of isopropyl alcohol to the cells. The absorbance of the dye was then read at a wavelength of 510 nm on a microplate reader (Infinite® M200; Tecan Group Ltd, Mannedorf, Switzerland). For the Sirius Red assay, the dye was extracted by the addition of a 0.1 N sodium hydroxide solution and read at a wavelength of 550 nm.

2.3. Scaffold synthesis and characterization

Protein microbubble scaffolds were used as model implants capable of releasing anti-inflammatory agents in a controlled fashion. Microbubble scaffold formation was based on our previous method for bovine serum albumin microbubble scaffolds [23]. Briefly, poly(D,L-lactic-co-glycolic acid) (75:25, 113 kDa), was dissolved in 1,4-dioxane at a 7.5% w/v ratio. Microbubbles of bovine gelatin were produced by ultrasonication at 20 kHz for 10 s of a 10% w/v gelatin solution under nitrogen gas. The resulting gelatin microbubbles were immediately added to the polymeric solution at a 1:1 v/v ratio. The quasi-stable mixture is agitated gently and