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Understanding the host response to cell-laden poly(ethylene glycol)-based hydrogels

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Abstract

Poly(ethylene glycol) (PEG)-based hydrogels are promising in situ cell carriers for tissue engineering. However, their success in vivo will in part depend upon the foreign body reaction (FBR). This study tests the hypothesis that the FBR affects cells encapsulated within PEG hydrogels, and in turn influences the severity of the FBR. Fibroblasts were encapsulated within PEG hydrogels containing RGD to support cell attachment. Macrophages were seeded on top of cell-laden hydrogels to mimic in vivo macrophage interrogation and treated with lipopolysaccharide to induce an inflammatory phenotype. The presence of activated macrophages reduced fibroblast gene expression for extracellular matrix molecules and remodeling, but stimulated VEGF and IL-1 β gene expression. Fibroblasts impacted macrophage phenotype leading to increased iNOS, IL-1 β and TNF- α expressions. Syngeneic cell-laden and acellular hydrogels were also implanted subcutaneously into C57bl/6 mice for 2, 7 and 28 days. Encapsulated fibroblasts secreted collagen type I during the first week, but tissue deposition and cellularity decreased by 28 days. The presence of encapsulated fibroblasts led to greater acute inflammation, but did not influence the fibrotic response. In summary, this work emphasizes the importance of the host response in tissue engineering, and the potentially deleterious impact it may have on cell-laden synthetic scaffolds.

Keywords

Poly(ethylene glycol); Hydrogel; Co-culture; Monocyte/macrophage; Fibroblast; Tissue engineering

1. Introduction

Hydrogels formed from non-cytotoxic macromolecular precursors and under benign reaction conditions offer a promising platform for cell encapsulation and in vivo cell delivery.

Poly(ethylene glycol) (PEG) hydrogels have become one of the most widely investigated

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2012.10.037>.

synthetic hydrogel platforms for cell encapsulations because of their ease of use and tunable properties. They enable the introduction of a range of biological moieties creating instructive environments for cells such as cell adhesion ligands [1] and proteolytically degradable linkers, which together facilitate cell spreading, proliferation, and migration [2] and the entrapment or tethering of biologically active molecules to support cell survival and/or function [3]. Due to their high degree of tunability, PEG-based hydrogel platforms have been successfully used to encapsulate a wide range of cell types.

Injectable cell-laden hydrogels are promising from a clinical perspective because cells are delivered minimally invasively [4], the act of curing the hydrogel in situ improves adhesion to the adjacent tissue [5], and the host environment provides a number of growth factors and other signaling molecules specific to the niche. However, when synthetic materials are implanted into the body, invariably an inflammatory response is invoked. The foreign body reaction (FBR) is characterized by the presence of activated macrophages and foreign body giant cells, which secrete inflammatory cytokines, enzymes, and reactive oxygen and nitrogen species in an effort to degrade the implant. If the implant persists, stabilization of the FBR eventually occurs leading to the walling off the implant with a largely avascular collagenous fibrous capsule [6]. From a tissue engineering perspective, the early stages involving inflammation and late stages involving a fibrous capsule may have adverse consequences on the performance and integration of a tissue engineered construct [7]. Many of the inflammatory molecules released by macrophages and foreign body giant cells have known detrimental effects on tissue producing cells leading to apoptosis [8], inhibition of tissue synthesis [9], and/or stimulation of matrix degrading enzymes [6]. To realize the full potential of cell-laden synthetic hydrogels in vivo, it is important to understand the effects that the FBR has on cells residing within synthetic hydrogels.

Recent work from our laboratory has shown that when PEG hydrogels, prepared from poly(ethylene glycol) diacrylate (PEG-dA) precursors, are implanted subcutaneously into immunocompetent mice, they elicit an inflammatory response characterized by a prolonged presence of macrophages at the hydrogel–tissue interface [10–12]. The severity of the FBR was reduced, although not abrogated, by the incorporation of biological moieties [13] and reducing the stiffness of the hydrogel [12]. However, macrophages were still present at the implant surface and a dense fibrous capsule had formed. In vitro studies have confirmed that bone marrow derived murine macrophages cultured in direct contact with PEG hydrogels leads to a classically activated phenotype which is further elevated in the presence of lipopolysaccharide [11], a molecule commonly used to simulate the in vivo inflammatory environment in vitro [14].

Our previous findings have led us to hypothesize that this adverse host response may have adverse effects on cells encapsulated within PEG hydrogels formed from PEG-dA precursors, which in turn may influence the severity of the FBR. Specifically, in the present study we asked the following questions: i) Do macrophages when activated by direct contact with the hydrogel or in combination with lipopolysaccharide, affect cells that are encapsulated in PEG hydrogels? ii) Do cells encapsulated in the hydrogel affect macrophage phenotype and hence their activation state? iii) Do cells encapsulated in the hydrogel affect or are affected by the in vivo host response? To address these questions in the context of tissue engineering, we chose a skin tissue engineering model with fibroblasts encapsulated in PEG hydrogels. This model was chosen such that the fibroblasts residing in the hydrogel would be implanted into their native niche where our in vivo model for studying the FBR is subcutaneous implantation in a mouse model.

2. Materials and methods

2.1. Cells

NIH/3T3 fibroblasts and RAW 264.7 macrophages used for the in vitro studies were purchased from American Type Culture Collection (Manassas, VA). Fibroblasts were expanded in high glucose Dulbecco's modified eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Thermo Scientific, Logan, UT) and penicillin/streptomycin/Fungizone® (PSF) (Invitrogen). Macrophages were expanded in DMEM (ATCC) supplemented with 10% FBS (HyClone) and PSF.

Primary dermal fibroblasts were used for the implantation study. In brief cells, were isolated from six- to eight-week old C57bl/6 male mice (Charles River Laboratories, Kalamazoo, MI) as described previously by Rogers et al. [15]. Briefly, mice were sacrificed via CO₂ asphyxiation and cervical dislocation. The skin was removed of hair and disinfected with ethanol. Sections of skin (one by 5 cm) were dissected and the dermis was separated from the epidermis. Dermal skin sections were washed with sterile PBS, cut into smaller pieces, treated thrice with 0.25% trypsin (Invitrogen) at 37 °C for 15 min, followed by treatment with 200 U/mL of collagenase type I (Worthington Biochemical, Lakewood, NJ) for 20 min at 37 °C. Freshly isolated cells were recovered by centrifugation. Primary dermal fibroblasts were cultured in DMEM (ATCC) supplemented with 15% FBS (Atlanta Bio, Lawrenceville, GA), 4 mM L-glutamine (Invitrogen), and PSF on 225 cm² tissue culture treated polystyrene flasks (Greiner Bio One, VWR, Denver, CO). Primary dermal fibroblasts were used at passage two.

2.2. Hydrogel synthesis, formation and cell encapsulation

Poly(ethylene glycol) diacrylate (PEG-dA) was synthesized as described previously [11]. Briefly, PEG-dA was synthesized by reacting 0.4 M acryloyl chloride (Sigma-Aldrich, St. Louis, MO) with 0.1 M PEG (3000 Da, Fluka, St. Louis, MO) in the presence of 0.44 M triethylamine (Sigma-Aldrich) in dry toluene at room temperature overnight with constant stirring and protected from light. PEG-dA was filtered over alumina, purified by precipitation in cold diethyl ether, dried, and stored at 4 °C. Percent acrylation based on two acrylates per PEG molecule was determined to be 95 via ¹H NMR.

Monoacrylated PEG tyrosine-arginine-glycine-aspartic acid-serine (Acryl-PEG-YRGDS) was synthesized as described previously [11]. Briefly, 1.1 M YRGDS (Gen-Script, Piscataway, NJ) was reacted with 1.0 M monoacrylated-PEG₃₄₀₀-Succinimidyl Carboxymethyl (Laysan Bio, Arab, AL) in 50 mM sodium bicarbonate buffer (pH 8.4) for 2 h. The monoacrylated PEG-peptide was purified by dialysis, lyophilized and stored under argon at 4 °C. The extent of conjugation of the peptide to monoacrylated PEG was determined to be 90% via ¹H NMR.

Pre-hydrogel solutions contained 10% (w/w) PEG-dA, 2.5 mM acryl-PEG-YRGDS, 0.05% (w/w) I2959 (Irgacure 2959, Ciba Specialty Chemical, Tarrytown, NY) photo-initiator in PBS. Hydrogel disks (five mm diameter and one mm thickness) were formed by polymerizing the pre-hydrogel solution under 365 nm light at an intensity of 5–10 mW/cm² for 10 min. For in vitro experiments, hydrogel disks were secured at the bottom of 96 well culture plates with a small amount of sterile vacuum grease. Three conditions were prepared: i) NIH/3T3 fibroblasts were encapsulated in PEG-RGD hydrogels at 10⁷ cells/mL of pre-hydrogel solution, ii) acellular hydrogels were seeded with 200 µL of RAW 264.7 macrophages at a density of 1.3 × 10³ cells/mm² in culture medium into wells of 96 well plates, and iii) RAW 264.7 macrophages were seeded as described above onto fibroblast-laden hydrogels. Each condition was cultured in growth medium described above. Cultures

were not disturbed for 24 h to allow for macrophage attachment. Medium was exchanged at 24 h (referred to as time 0 h) with fresh growth medium supplemented with or without 1 $\mu\text{g}/\text{mL}$ lipopolysaccharide (LPS) from *S. enterica* (Sigma–Aldrich). The experimental design is depicted in Fig. 1a.

2.3. Cell visualization

Hydrogels were fixed in 4% paraformaldehyde for 4 h and stored in 15% sucrose at 4 °C. Hydrogels were washed three times, 5 min each with 0.025% TritonX-100® (Sigma Aldrich) in tris buffered saline (TBS) to permeabilize the cell membrane, and blocked in 10% normal goat serum (Invitrogen) with 1% bovine serum albumin (BSA, Invitrogen) in TBS with agitation for 2 h at room temperature. Immunocytochemistry was performed overnight with agitation at 4 °C using rat anti murine F4/80, a membrane macrophage marker (Caltag Laboratories Inc., Burlingame, CA, 1:300 dilution) and rabbit anti murine β -actin (Abcam, Cambridge, MA, 1:1000 dilution), a ubiquitous cytoskeletal protein found in both cell types. Appropriate secondary antibodies were chosen for co-localization (goat anti-rat AlexaFluor® 546 for F4/80 (1:400) and donkey anti-rabbit AlexaFluor® 488 for β -actin (1:400), Molecular Probes, Invitrogen). Samples were incubated in the dark for 1 min with a nucleic acid stain, DAPI (Molecular Probes, 300 nM), to visualize the nuclei. Samples were imaged via confocal microscopy (Zeiss LSM5 Pascal).

2.4. Real-time RT-PCR

Hydrogels were removed from culture at 0, 4, 24, and 48 h after medium exchange, and immediately dipped into TRK lysis buffer (Omega, Norcross, GA) followed by snap freezing. To isolate RNA from the two cell populations, the following technique was developed. Co-culture hydrogels containing encapsulated fibroblasts and macrophages were dipped into TRK lysis buffer for 20 s to collect RNA predominately from macrophages. The hydrogel was subsequently rinsed in PBS, and dipped in a second lysis buffer to extract RNA predominantly from fibroblasts. RNA was isolated using E.Z.N.A microelute columns (Omega) per manufacturer's instructions. The isolated RNA was transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Samples were stored at -80 °C until use. Quantitative RT-PCR was performed on a 7500 Fast system (Applied Biosystems) using Fast SYBR Green Master Mix (Applied Biosystems). Custom primers were designed and validated. Primer sequence, efficiencies, and accession numbers can be found in Supplemental Table 1. Data are presented as expression relative to the housekeeping gene L32 as follows:

$$\text{Relative Expression} = \frac{E_{\text{HKG}}^{C_{t(\text{HKG})}}}{E_{\text{GOI}}^{C_{t(\text{GOI})}}}$$

where C_t is the cycle number where fluorescence crosses the threshold, E is the efficiency of the primer, HKG is the housekeeping gene, and GOI is the gene of interest. Data for co-cultures where macrophages are seeded over a fibroblast-laden hydrogel (CC+) or for LPS treated constructs (LPS+) are also presented as fold change by comparing to monoculture (CC-) or the absence of LPS (LPS-), respectively, for each cell type as follows:

$$\text{Fold change} = \frac{\text{RE}_{\text{CC+}}}{\text{RE}_{\text{CC-}}} \&= \frac{\text{RE}_{\text{LPS+}}}{\text{RE}_{\text{LPS-}}}$$

2.5. Secreted protein quantification

Supernatant was collected from samples at 0, 4, 24, and 48 h, snap frozen in liquid nitrogen, and stored at -80°C until further processing. ELISAs (IL-10, eBio-sciences, San Diego, CA, and IL-1 β , Pierce, Thermo Scientific, Rockford, IL) were carried out according to the manufacturers' instructions.

2.6. Endotoxin testing

Hydrogels with and without encapsulated fibroblasts used for in vitro and in vivo experiments were confirmed to be free of endotoxins. Testing was performed using an E-TOXATE Kit (Sigma-Aldrich) per manufacturer's instructions.

2.7. Implantation study

Hydrogel disks (five mm diameter and one mm thick) were prepared in PBS with and without encapsulated primary dermal fibroblasts as described above. Immediately after photopolymerization, hydrogels were stored in warm PBS until implantation, up to 3 h. Hydrogels were implanted subcutaneously into dorsal pockets in nine- to ten-week-old male C57bl/6 mice. Four pockets, one over each hip and shoulder, were formed, and a single construct was placed into each pocket. Incisions were closed with surgical staples and the animals were monitored daily for abnormalities at the wound site. Animals were sacrificed 2, 7, and 28 days post implantation by CO₂ asphyxiation and cervical dislocation.

2.8. (Immuno) histological analysis

Implants and surrounding tissue were excised at prescribed time points ($n = 6$), and fixed in 4% paraformaldehyde at room temperature for 24 h. The explants were stored in 15% sucrose at 4°C until further processing. Standard protocols were followed for dehydration and paraffin embedding.

Ten-micron sections were stained with Masson's Trichrome or Hematoxylin and Eosin via standard protocols. Immunohistochemistry staining was performed for the detection of Col I. Briefly for Col I staining, tissue sections were deparaffinized, blocked, and incubated with rabbit polyclonal primary antibody to Col I (Abcam, Cambridge, MA) at a 1:500 dilution followed by an HRP conjugated goat anti-rabbit secondary antibody (Abcam) at a 1:1000 dilution. The samples were subsequently developed with 3,3'-Diaminobenzidine (DAB, BD Biosciences) and counterstained with methyl green (Vector Labs, Burlingame, CA).

All tissue sections were imaged by light microscopy (Zeiss, Axioskop 40) and a digital camera (Diagnostic Instruments, MN 14.2 Color Mosaic) using SPOT Software v. 4.6. Inflammatory cells and fibrous capsule formation were evaluated semi-quantitatively using NIH ImageJ software.

2.9. Statistical analysis

Data are presented as mean with standard deviation for error bars of 4 replicates unless otherwise specified per condition. Statistical significance for PCR was determined via three-way analysis of variance (ANOVA) using the general linear model within Minitab 16. Additionally, significance was determined via ANOVA and a Tukey's post hoc analysis in KaleidaGraph with $\alpha = 0.05$ or a student's t -test. Factors were culture time and LPS or culture condition (mono- versus co-culture). The assumptions underlying ANOVA, including normal distribution were verified by examining a normality plot of the residuals using Minitab 16. P values less than 0.05 were considered statistically significant.

2.10. IACUC approval

NIH guidelines for the care and use of laboratory animals (NIH Publication #85-23 Rev. 1985) have been observed. The University of Colorado at Boulder Institutional Animal Care and Use Committee approved all animal protocols.

3. Results

3.1. Characterization of macrophage and fibroblast hydrogel cultures

The presence of macrophages at the outer surface of the hydrogel and fibroblasts embedded within the hydrogel were confirmed in the co-cultures containing both cell types by immunocytochemistry and by comparing to monocultures after 72 h. Macrophage monocultures stained strongly for both F4/80 and β -actin (Fig. 1b). Antibodies were able to penetrate into the hydrogel and fibroblasts stained positive for β -actin, but not F4/80 (Fig. 1c). In the co-culture system, the majority of cells on the surface of the hydrogel (Fig. 1d) stained positive for both F4/80 and β -actin, the former being indicative of macrophages, while those 40 μ m into the hydrogel were only positive for β -actin, indicative of fibroblasts (Fig. 1e). In addition, cell spreading, which is characteristic of fibroblast morphology in 2D culture, was not evident for cells at the hydrogel surface indicating that any cells that did not stain positively for F4/80 may be encapsulated fibroblasts near the hydrogel surface.

A two-step process was developed that enabled RNA extraction from each cell population in the co-culture system (Supplementary Fig. 1). RNA that was predominantly from macrophages was collected from the first 20 s dip in lysis buffer. The hydrogel was subsequently rinsed in phosphate buffered saline solution, and dipped in a second lysis buffer to extract RNA that was predominantly from fibroblasts. Using fibroblast monocultures, the amount of contaminating RNA extracted from entrapped fibroblasts in the first dip in the co-culture was estimated to be ~10%. Using macrophage monocultures, there was no detectable RNA extracted in the second lysis buffer dip, indicating that the RNA extracted in the co-culture system from the fibroblasts contained negligible amounts of contaminating macrophage RNA.

3.2. Gene expression of fibroblasts in mono- and co-cultures

NIH/3T3 fibroblasts encapsulated in hydrogels were cultured in monocultures and in co-cultures with macrophages seeded on top of the hydrogel with and without LPS. Fibroblast gene expression was assessed for genes related to tissue production: collagen type I (Col I), collagen type III (Col III); tissue remodeling: matrix metalloproteinase 2 (MMP-2); tissue breakdown: interleukin 1 beta (IL-1 β); anti-inflammation: interleukin 10 (IL-10); and angiogenesis: vascular endothelial growth factor-A (VEGF-A). The relative expression as a function of culture time is shown in Fig. 2a–f and the accompanying statistical analysis is given in Table 1. Culture time was significant for Col I ($p < 0.001$), Col III ($p < 0.001$) and MMP-2 ($p < 0.01$). The presence of LPS was significant for Col III, IL-1 β , and IL-10 ($p < 0.05$) and VEGF expressions ($p < 0.01$). The presence of macrophages was a significant factor for Col I, Col III, IL-10, MMP-2, and VEGF expression ($p < 0.001$) and was a factor, although not statistically significant for IL-1 β expression ($p = 0.056$).

To isolate the effects that macrophages have on fibroblasts in the absence and presence of LPS, fibroblast relative expression for each gene in co-culture was normalized to the relative expression of that gene in fibroblast monocultures. Over the course of 48 h, expressions of Col I (Fig. 3a), Col III (Fig. 3b), MMP-2 (Fig. 3e), and IL-1 β (Fig. 3c) were either not affected or significantly lower in the presence of macrophages with no LPS. In the co-culture, VEGF-A (Fig. 3f) expression was significantly higher by 24 and 48 h while IL-10 (Fig. 3d) mean expression was also higher, but not statistically significant. With LPS, Col I,

Col III and MMP-2 expressions were largely down-regulated in the presence of macrophages (all significant by 48 h) while IL-10 and IL-1 β expression increased 317-fold ($p < 0.05$) and 3-fold ($p < 0.01$) at 4 h, respectively. However, high IL-1 β expression was not sustained and was down-regulated at 48 h ($p < 0.05$) (Fig. 3c).

To isolate the effects of LPS, fibroblast relative expression for each gene in the LPS treated hydrogels was normalized to the relative expression of that gene from the no LPS treated hydrogels (Fig. 4a–f). In monocultures, LPS treatment affected the fibroblasts leading to early up-regulations in Col I, Col III, and VEGF at 4 h, but which subsided by 24 h. LPS treatment did not affect IL-10, IL-1 β , or MMP-2 with the exception of MMP-2 at 48 h, which was down-regulated by 1.25-fold ($p < 0.05$). In co-cultures, LPS treatment led to significant up-regulations in expressions of IL-1 β up to 11-fold at 4 h ($p < 0.01$), IL-10 up to 45-fold at 4 h ($p < 0.05$), and VEGF up to 4-fold at 24 h ($p < 0.05$), but had no effect on Col I, III, or MMP-2.

3.3. Gene expression in macrophages in mono- and co-cultures

RAW 264.7 macrophages seeded onto hydrogels were cultured in monocultures and co-cultures with fibroblasts with and without LPS treatment. Macrophage gene expression was assessed for genes related to inflammation: inducible nitric oxide (iNOS), IL-1 β , tumor necrosis factor alpha (TNF- α); wound healing: arginase type I; and immunoregulation: IL-10. The relative expression as a function of culture time is shown in Fig. 2g–k and the accompanying statistical analysis is given in Table 1. Culture time was a significant factor in macrophage gene expression for arginase, IL-1 β , IL-10, and iNOS ($p < 0.001$), and for TNF- α ($p < 0.05$). LPS was a significant factor for IL-1 β , IL-10, iNOS, and TNF- α ($p < 0.05$). Overall, the presence of fibroblasts was not a significant factor on macrophage gene expression, however there were several differences at specific time points, which are worth noting.

To isolate specific effects of fibroblasts on macrophages, macrophage relative expression for each gene in the co-culture system was normalized to relative expression of that gene from macrophage monocultures. The presence of fibroblasts without LPS led to small, but significant up-regulations of arginase and IL-1 β expressions ($p < 0.05$) at early culture times, but had no effect on iNOS, TNF- α , or IL-10 expressions throughout the culture. With LPS, the presence of fibroblasts led to higher expressions of IL-1 β , TNF- α , IL-10, but lower expressions of arginase at various culture times.

To isolate the effects of LPS, macrophage relative expression for each gene in LPS treated hydrogels was normalized to the relative expression of that gene from the untreated hydrogels (Fig. 4g–k). In monoculture, LPS treatment affected macrophages leading to significantly higher expressions of IL-1 β by 220-fold ($p < 0.05$), iNOS by 58-fold ($p < 0.05$), TNF- α by 2-fold ($p < 0.05$), and IL-10 by 10-fold ($p < 0.001$), but only at early culture times and which subsided by 24 h. Contrarily arginase was significantly up-regulated by 5-fold ($p < 0.05$), but only at late culture times. In co-culture, LPS treatment led to significant up-regulations in expressions of iNOS up to 106-fold at 4 h ($p < 0.001$), IL-1 β up to 285-fold at 4 h ($p < 0.001$), TNF- α up to 38-fold at 48 h ($p < 0.01$), IL-10 up to 25-fold at 4 h ($p < 0.001$), but had no effect on arginase expression.

3.4. Cytokine secretion

Two cytokines were selected; one that represented an inflammatory response, IL-1 β (Fig. 5a), and one that represented an anti-inflammatory response, IL-10 (Fig. 5b). Secretion was assessed in fibroblast monocultures, macrophage monocultures, and co-cultures with and without LPS treatment. No IL-1 β was detected in the culture medium in the absence of LPS.

In the presence of LPS, the co-culture system had the highest mean amounts of IL-1 β in the culture medium and was ~3-fold higher than in the macrophage monoculture ($p = 0.18$). No IL-1 β was detected in the LPS treated fibroblast monocultures. Without LPS, there was no IL-10 detected in the fibroblast monoculture and co-cultures and minimal levels detected in the macrophage monoculture. Secretion of IL-10 into the culture medium was most pronounced in the co-culture system treated with LPS. IL-10 was detected in the culture medium in the fibroblast and macrophage monocultures, but the levels were 24 ($p = 0.09$) and 5 ($p = 0.28$) fold less than the co-culture system, respectively, although not statistically significant.

3.5. In vivo response

The in vivo reaction to acellular and cell-laden hydrogels when implanted subcutaneously in C57bl/6 mice was evaluated. Representative (immuno) histological micrographs are shown in Fig. 6 for Masson's Trichrome (a–f), which assesses collagen deposition and fibrous capsule formation, for Hematoxylin and Eosin (H&E) (g–l), to examine the overall response at the material–tissue interface, and Col I (m–o) for encapsulated fibroblast matrix secretion. A layer of inflammatory cells was present at the hydrogel surface for both acellular and cell-laden hydrogels over the course of four weeks. By the end of the study, a dense collagenous fibrous network surrounded all implants. Ventral side inflammatory cells surrounding fibroblast-laden hydrogels were greater by 1.3-fold than acellular hydrogels ($p = 0.051$) at day 2, although not statistically significant. By day 28, the fibrotic response was similar for both acellular and fibroblast-laden hydrogels (Fig. 7). The encapsulated fibroblasts appeared to be affected long-term showing a reduction in Col I deposition from day 7 to day 28, which is accompanied by a loss of cells.

4. Discussion

The present study investigated the hypothesis that the harsh environment associated with the FBR affects cells residing in the hydrogel, and in turn influences the severity of the FBR. Through a combination of in vitro and in vivo experiments, findings from this study support our hypothesis. In vitro data demonstrated that activated macrophages affect encapsulated cells and that crosstalk between encapsulated cells and macrophages appears to elevate the severity of the response. In vivo data demonstrated an overall trend of reduced fibroblast-associated tissue deposition with implantation time concomitant with an elevated acute inflammatory reaction with the presence of encapsulated cells. However, there were no obvious effects on the late stage fibrotic response to cell-laden hydrogels.

A co-culture system was developed to mimic in vivo macrophage interrogation of a material. It was critical that macrophages interact directly with the hydrogel as it is well known that macrophage interaction with a biomaterial influences their activation and is dependent on a number of biomaterial properties including chemistry, porosity, roughness, and stiffness [12,16–18]. Simulating the in vivo inflammatory environment in vitro was also critical, as studies have suggested a synergism between biomaterial-induced activation and inflammation [19]. Thus, in this configuration, macrophages were activated by the material itself and in combination with an inflammatory environment (i.e. LPS). Because the hydrogel crosslinks create a mesh-like network, with an average distance between crosslinks ~150 Å [20], a physical barrier exists between macrophages and cells residing within the hydrogel. As such, the primary interaction between the two cell types will be paracrine signaling. In this co-culture system, paracrine signaling will be dynamic enabling real time crosstalk between encapsulated cells and interrogating macrophages, which can influence cell responses [21].

Macrophages are phenotypically diverse cells involved in inflammation, immunoregulation and wound healing [22]. They interact with a number of different cell types in the body primarily through paracrine signaling by the secretion of a panoply of growth factors and cytokines. In this study, we demonstrate that in vitro, macrophages provide signals to encapsulated fibroblasts, which lead to down-regulation of Col I and to a lesser extent Col III concomitant with a reduction in MMP-2 gene expression, a matrix remodeling collagenase important to wound healing [23]. Interestingly, this down-regulation was independent of macrophage activation by LPS suggesting that molecules secreted by macrophages or biomaterial-activated macrophages are sufficient to elicit this response in fibroblasts. In the absence of LPS, secretion of IL-1 β protein was not detected suggesting that the down-regulation of these genes was not mediated by IL-1 β or a classically activated macrophage phenotype. Contrarily, IL-10 was detected in the culture medium of macrophages in the absence of LPS. IL-10 has been shown to inhibit TGF- β induced Col I expression and synthesis in fibroblasts [24], inhibit Col I, Col III and MMP-2 expression in fibrotic animal models [25], and has been linked to altered wound healing [26]. In addition, macrophages also led to up-regulations in VEGF-A gene expression and down-regulations in IL-1 β gene expression in fibroblasts. While not measured here TGF- β , which is known to be secreted by macrophages at different stages of activation [27], has been shown to stimulate VEGF-A expression [28] and inhibit IL-1 β expression in fibroblasts [29]. However, other macrophage-secreted factors are also likely playing a role [30]. In the absence of LPS, macrophages appear to display an immunoregulatory or wound healing phenotype, corroborated by the macrophage gene expression data. This phenotype appears to have an impact on fibroblasts confirming that interrogating macrophages can affect cells residing within a hydrogel.

The initial stages of the FBR are characterized by an acute inflammatory reaction with inflammatory cells, namely macrophages displaying a classically activated phenotype [6]. LPS has been used in vitro as a means to better capture the in vivo macrophage inflammatory phenotype [13,14]. Our own work has also shown good correlation between the in vitro response of LPS-activated macrophages and the severity of the FBR in vivo [11-13]. In this study, LPS-activated macrophages had a pronounced effect on fibroblast expression of cytokines, leading to higher IL-1 β and IL-10 mRNA and protein levels in the co-culture. Because LPS appeared to have little effect on encapsulated fibroblasts, at least with respect to the genes analyzed, this finding is largely attributed to paracrine signaling from the activated macrophages. Others have reported that fibroblasts secrete IL-10 and IL-1 β in response to inflammatory cues [31,32]. IL-1 β has been shown to have a deleterious effect on tissue synthesis and to increase tissue degradation through elevated MMP activity [33]. While we did not observe any additional effects on fibroblast gene expression related to tissue synthesis and remodeling by LPS-activated macrophages, IL-1 β could lead to long term effects. In addition, activated macrophages led to higher up-regulations of VEGF-A expression in fibroblasts, which is not surprising as it is well known that a number of pro-inflammatory molecules secreted by activated macrophages lead to angiogenesis [34].

Overall macrophages appeared to be less affected by the presence of fibroblasts, which is not surprising as macrophages have been recognized as one of the major sources of cytokines in wound repair [35]. However there were several differences worth noting. Most notably, LPS-activation of macrophages appeared to be heightened by the presence of fibroblasts leading to elevated levels of iNOS, TNF- α , and IL-10 at various time points, while maintaining high IL-1 β expression levels. Our findings agree largely with findings from Holt et al. [21], which showed elevated cytokine production in fibroblast-macrophage co-cultures when compared to monocultures. They attributed their finding to the real time crosstalk between fibroblasts and macrophages and implicated monocyte chemotactic protein-1 (MCP-1) as a primary mediator. MCP-1 is known to be secreted by fibroblasts and

macrophages [36], has been shown to elevate pro-inflammatory cytokines in macrophages, is thought to be a key signaling molecule in the FBR and is closely associated with several pathological diseases [37]. However, MCP-1 is also important in immune modulation [38]. Therefore, additional studies will be required to elucidate the exact factors secreted by fibroblast, which contribute to the elevated macrophage activation observed in this study.

While in vitro studies allow for a more controlled assessment of macrophage response to a material, the FBR is much more complex and involves other cell types such as neutrophils and leukocytes [39]. Implantation of syngeneic dermal fibroblast-laden hydrogels demonstrated that the in vivo acute inflammatory response appeared to be enhanced by the presence of encapsulated cells leading to a greater amount of inflammatory cells interrogating the hydrogel; an observation supported by our in vitro data. The higher number of inflammatory cells on the ventral side is attributed to increased vascularity associated with the adjacent fatty tissue as compared to the adjacent skin tissue on the dorsal side. During the first week post-implantation encapsulated fibroblasts appear to be viable based on collagen type I deposition. However, long-term the cellularity and deposition of COL is reduced. Our working hypothesis is that there exists a dynamic crosstalk between encapsulated fibroblasts and inflammatory cells, where by fibroblasts secrete molecules that recruit a higher number of inflammatory cells at the implant–host interface, which in turn leads to secretion of pro-inflammatory mediators [40,41] that stimulate catabolism and reduced cellularity in the encapsulated cells. However, other factors may have contributed to the observed response. If the encapsulated cells are actively dying, they could elicit a response that recruits inflammatory cells. More long term, the reduced cellularity could result from a lack of vasculature and oxygen delivery to the encapsulated cells. Therefore, additional studies are needed to confirm our hypothesis. Interestingly, the resolution phase of the FBR with the formation of a fibrous capsule was not affected by the presence of encapsulated cells. This finding suggests that either the presence of encapsulated cells had little effect on the pathways involved in orchestrating fibrous capsule formation and FBR stabilization or the majority of the encapsulated cells were no longer viable, and thus had little paracrine signaling contributions to the latter stages of the FBR.

Fibroblasts were chosen as the model cell type to better understand the effects of the FBR in tissue engineering when cell-laden scaffolds are implanted into their native niche. However, we recognize that macrophages and fibroblasts have a unique interaction in normal and abnormal wound healing. As such, each cell type can secrete instructive signaling molecules designed to mediate the function of the other cell type [40]. Interestingly, it has been shown that the type of 3D matrix and its mechanical properties alter fibroblast sensitivity to cytokines and growth factors [42], suggesting that when fibroblasts are encapsulated in a hydrogel they may respond differently to macrophages and the FBR. Nonetheless, the FBR represents abnormal wound healing and leads to a classically activated macrophage phenotype and the secretion of pro-inflammatory molecules [43]. From a tissue engineering perspective, a sustained inflammatory environment is known to have detrimental effects on tissue producing cells and in fact may lead to the recruitment of additional macrophages via the secretion of MCP-1, which is not only produced by fibroblasts but also other tissue producing cells such as chondrocytes [44] and osteoblasts [45].

We recognize several limitations of this study. The in vitro study explored the interaction between macrophages and fibroblasts using immortalized cell lines. It has been shown that secretion of pro-inflammatory cytokines is lower in cell lines compared to primary cells [21]. Therefore, it is possible that the differences observed in our in vitro studies, may have been underestimated. A second limitation is the use of non-degrading PEG-based hydrogels, at least on the time scales of this experiment. Tissue engineering requires the use of degradable scaffolds, which is important for tissue elaboration and as well, will likely

change the FBR. However, recent studies have shown that degradable thermo-reversible synthetic hydrogels led to the infiltration of a large number of neutrophils and macrophages [46], suggesting that the acute inflammatory response may still have an impact on the tissue engineering outcome even for degradable materials.

While our studies indicate that the FBR may affect cells residing within a scaffold, and in particular PEG-dA-based hydrogels, it is important to recognize that there have been in vivo successes reported with similar cell-laden hydrogels containing islets [47], chondrocytes [48], and stem cells [49]. However, many of these studies were performed in immunocompromised mice, which do not capture the full complexity of the FBR [50]. PEG-based hydrogels have also been used commercially as tissue sealants (e.g., FocalSeal™ from Genzyme and CoSeal™ from Baxter) in several tissues such as intestine [51]. While successful, studies have reported a chronic inflammatory response to FocalSeal™ [52] supporting findings from our laboratory. Incorporating biological moieties into PEG-based hydrogels may reduce the severity of the FBR to PEG-based hydrogels [11], where for example PEG-fibrinogen acellular hydrogels have been used in vivo and supported bone regeneration in rats [53].

5. Conclusions

Our in vitro co-culture system, which was designed to mimic the in vivo macrophage interrogation of a cell-laden hydrogel, demonstrated that macrophages impacted cells residing within the hydrogel. Our data strongly suggest that there exists a dynamic crosstalk between the two cell types. In an inflammatory environment, the encapsulated cells appear to enhance the classically activated phenotype in macrophages, as suggested by high iNOS and TNF- α expressions and sustained IL- β expression. Our in vivo findings corroborate our in vitro findings demonstrating an overall trend that the acute inflammatory reaction is enhanced by the presence of encapsulated cells, and in turn may have led to a reduction in viability and tissue deposition by encapsulated fibroblasts longer term; although additional studies are needed to determine the exact mechanisms involved. Our findings suggest that targeting the secretion of pro-inflammatory molecules (e.g., interleukin 1 β) from activated macrophages on biomaterials may be one strategy to improve the performance of the encapsulated cells. Our work further highlights that macrophages and the FBR may play an undesirable role in tissue engineering when synthetic hydrogels are employed. Our findings emphasize the need for intelligently designed scaffolds that are capable of attenuating this potentially deleterious host response and capturing the normal wound healing response such as that observed when natural materials are employed [54].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

a) Experimental design for in vitro studies. b–e) Immunocytochemistry analysis of RAW 264.7 macrophage monocultures (macrophages seeded onto acellular gels) (b), NIH/3T3 fibroblast monocultures (encapsulated fibroblasts) (c), and co-cultures (RAW 264.7 macrophages seeded on top of a fibroblast-laden hydrogel) at the surface (d) and 40 μ m deep into the hydrogel (e). Cells were stained for F4/80 (red), a macrophage marker and β -actin (green), which stains both cell types. Nuclei were stained with DAPI (blue). Images were taken after 72 h of culture. Scale bar is 20 μ m and is the same for all images. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Relative gene expression for NIH/3T3 fibroblast (a–f) and RAW 264.7 macrophage (g–k) mono- and co-cultures in the presence and absence of LPS: a) collagen type I, b) collagen type III, c) interleukin 1 β , d) interleukin 10, e) matrix metalloproteinase 2 (MMP-2), f) vascular endothelial growth factor-A (VEGF-A), g) arginase type I, h) interleukin 1 β , i) interleukin 10, j) inducible nitric oxide synthase (iNOS), k) tumor necrosis factor (TNF)- α . Data ($n = 4$) are presented as mean with standard deviation as error bars.

Fig. 3.

Gene expression represented as a fold change of relative expression in co-culture over relative expression in monocultures for fibroblasts (a–f) and macrophages (g–k). Monocultures and co-cultures were cultured in the absence and presence of lipopolysaccharide (LPS). Genes analyzed were a) collagen type I, b) collagen type III, c) interleukin 1 β , d) interleukin 10, e) matrix metalloproteinase 2 (MMP-2), f) vascular endothelial growth factor-A (VEGF-A), g) arginase type I, h) interleukin 1 β , i) interleukin 10, j) inducible nitric oxide synthase (iNOS), k) tumor necrosis factor (TNF)- α . Data ($n = 4$) are presented as mean with standard deviation as error bars. * indicates significance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 4.

Gene expression represented as a fold change of relative expression in cultures with LPS over relative expression in cultures without LPS for fibroblasts (a–f) and macrophages (g–k). Effects of co-culture and culture time on gene expression of a) collagen type I, b) collagen type III, c) interleukin 1 β , d) interleukin 10, e) matrix metalloproteinase 2 (MMP-2), f) vascular endothelial growth factor-A (VEGF-A), g) arginase type I, h) interleukin 1 β , i) interleukin 10, j) inducible nitric oxide synthase (iNOS), k) tumor necrosis factor (TNF)- α . * indicates significance. Data ($n = 4$) are presented as mean with standard deviation as error bars. * indicates significance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 5.

Cytokine secretion from mono- and co-cultures: a) Interleukin 1 β after 48 h of culture, b) Interleukin 10 after 24 and 48 h. F is NIH/3T3 fibroblast monocultures, CC is co-culture of RAW 264.7 macrophages seeded atop NIH/3T3 fibroblast-laden hydrogels, M is RAW 264.7 macrophage monocultures, and LPS is lipopolysaccharide. Cytokines in the culture medium were quantified by ELISAs.

Fig. 6.

Representative (immune) histological micrographs of acellular and syngeneic cellular PEG-based hydrogels implanted subcutaneously into C57BL/6 male mice for 2 (a, d, g, j, m, p), 7 (b, e, h, k, n, q), and 28 (c, f, i, l, o, r) days. Acellular tissue sections were stained with Masson's Trichrome (a, b, c). Tissue sections were stained with Masson's Trichrome (d, e, f) and Hematoxylin and Eosin (g, h, i). Histological images of the center of cell-laden hydrogels were stained with Hematoxylin and Eosin (j, k, l). Tissue sections were stained with anti-collagen type I antibody and counterstained with methyl green. Tissue sections with nuclei stained with DAPI (p, q, r). Scale bar is 100 μ m.

Fig. 7.

Semi-quantitative analysis of acellular and cellular hydrogels implanted subcutaneously for up to 28 days. Inflammatory cell thickness was measured for the dorsal (a) and ventral (b) sides of the implanted hydrogels after 2, 7 and 28 days. Fibrous capsule thickness was measured at day 28 on both the dorsal and ventral sides of the implant (c). Data ($n = 4$) are presented as mean with standard deviation as error bars.

Table 1

Three-way ANOVA results for relative expression.

	ANOVA factors		
	Culture time	Lipopolysaccharide	Co-culture
	<i>p</i>	<i>p</i>	<i>p</i>
NIH-3T3			
<i>Collagen type I</i>	<0.001	=0.349	<0.001
<i>Collagen type III</i>	<0.001	0.031	<0.001
<i>Interleukin 1 Beta</i>	0.261	0.010	0.056
<i>Interleukin 10</i>	0.165	0.023	<0.001
<i>Matrix metalloproteinase 2</i>	0.004	0.640	<0.001
<i>Vascular endothelial growth factor-A</i>	0.544	0.003	<0.001
RAW 264.7			
<i>Arginase I</i>	0.001	0.080	0.699
<i>Interleukin 1 Beta</i>	<0.001	<0.001	0.372
<i>Interleukin 10</i>	<0.001	<0.001	0.086
<i>Inducible nitric oxide synthase</i>	<0.001	<0.001	0.817
<i>Tumor necrosis factor alpha</i>	0.015	0.002	0.744