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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-1β gene expression. Fibroblasts impacted macrophage phenotype leading to increased iNOS, IL-1β 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.

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 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

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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 CO_2 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 cm^2 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 ^{1}H NMR.

Monoacrylated PEG tyrosine-arginine-glycine-aspartic acid-serine (Acryl-PEG-YRGDS) was synthesized as described previously [11]. Briefly, 1.1 M YRGDS (GenScript, Piscataway, NJ) was reacted with 1.0 M monoacrylated-PEG_{3400}-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 ^{1}H 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) photoinitiator 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/cm^2 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 10^7 cells/mL of pre-hydrogel solution, ii) acellular hydrogels were seeded with 200 μL of RAW 264.7 macrophages at a density of 1.3×10^3 cells/mm^2 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 μg/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, eBiosciences, San Diego, CA, and IL-1β, Pierce, Thermo Scientific, Rockford, IL) were carried out according to the manufacturers' instructions.