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Biomaterials differentially regulate Src kinases and phosphoinositide 3-kinase-γ in polymorphonuclear leukocyte primary and tertiary granule release

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ABSTRACT

In the foreign body response, infiltrating PMNs exocytose granule subsets to influence subsequent downstream inflammatory and wound healing events. In previous studies, we found that PMNs cultured on poly(ethylene glycol) (PEG)-containing hydrogels (i.e., PEG and gelatin + PEG hydrogels) had enhanced primary granule release, yet similar tertiary granule release compared with PMNs cultured on polydimethylsiloxane or tissue culture polystyrene. PMN primary granules contain microbicidal proteins and proteases, which can potentially injure bystander cells, degrade the extracellular matrix, and promote inflammation. Here, we sought to understand the mechanism of the enhanced primary granule release from PMNs on PEG hydrogels. We found that primary granule release from PMNs on PEG hydrogels was adhesion mediated and involved Src family kinases and PI3K-γ. The addition of gelatin to PEG hydrogels did not further enhance PMN primary granule release. Using stable-isotope dimethyl labeling-based shotgun proteomics, we identified many serum proteins – including Ig gamma constant chain region proteins and alpha-1-acid glycoprotein 1 – that were absorbed/adsorbed in higher quantities on PEG hydrogels than on TCPS, and may be involved in mediating PMN primary granule release. Ultimately, this mechanistic knowledge can be used to direct inflammation and wound healing following biomaterial implantation to promote a more favorable healing response.

1. Introduction

Infiltrating PMNs play a critical role in the inflammatory response to biomaterials. PMNs contain nearly 300 granule proteins, which are compartmentalized into four granule subsets (i.e., primary granules, secondary granules, tertiary granules, and secretory vesicles) [1–3]. Of the four granule subsets, secretory vesicles are released first and most readily, upon PMN adhesion to the endothelium. PMN tertiary granules are released next, during PMN diapedesis, and contain extracellular matrix (ECM)-degrading enzymes such as matrix metalloproteinase-9 (MMP-9) [4]. PMN secondary and primary granules are released after PMN extravasation to the site of inflammation, and contain antimicrobial reagents and ECM-degrading enzymes such as myeloperoxidase (MPO), cathelicidin LL-37, human neutrophil peptides 1–3 (HNP1–3), cathepsin G, and elastase [4]. PMN primary and secondary granule proteins also facilitate monocyte (MC) chemotaxis and mediate MC/macrophage activation and function [4–15]. Selected PMN granule proteins and their functions are shown in Supplementary Table 1, demonstrating their importance in inflammatory and wound healing events.

Previous studies from our group showed that PMNs cultured on poly(ethylene glycol) (PEG)-containing hydrogels (i.e., PEG hydrogels and gelatin + PEG [GP] hydrogels) had significantly greater primary granule release than PMNs cultured on

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polydimethylsiloxane (PDMS) or tissue culture polystyrene (TCPS) [16,17]. To understand the underlying mechanisms of this enhanced primary granule release, we investigated the following:

(1) The kinetics of PEG hydrogel-mediated PMN degranulation to confirm that primary granule release is a cell adhesion-mediated event.

(2) The role of gelatin in promoting PMN degranulation. Other groups have demonstrated that ECM components such as collagen and RGD tripeptide can attenuate the pro-inflammatory foreign body response to synthetic materials [18–23].

(3) The role of Src family kinase and phosphoinositide 3-kinase-γ (PI3K-γ) signaling in PMN degranulation in the presence or absence of bacterial peptide formyl-Met-Leu-Phe (fMLP). We previously found that Src family kinases were involved in primary granule release from PMNs cultured on PEG hydrogels, TCPS, and GP hydrogels and in tertiary granule release from PMNs cultured on PEG hydrogels, PDMS, TCPS, and GP hydrogels [17]. PI3K-γ is a signaling molecule that has been shown to be involved in early, adhesion-independent, fMLP-induced PMN activation [24–26].

(4) The role of adsorbed proteins on PEG hydrogels and TCPS in PMN degranulation.

2. Materials and methods

2.1. Biomaterial substrate preparation

PEG diacrylate (molecular weight 3350) and cysteine/PEG-modified gelatin (gel-PEG-cys) were synthesized as previously described [27] and characterized by proton NMR (Varian Unity-Inova 400 MHz; Varian Inc., Palo Alto, CA). To prepare PEG hydrogels, we dissolved 10 weight percent PEG diacrylate (molecular weight 3350) and 0.1 weight percent 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone (Sigma–Aldrich, St. Louis, MO) in water with heat (37 °C). GP hydrogels were prepared by dissolving 10 weight percent PEG diacrylate (molecular weight 3350), 0.1 weight percent 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone and 10 weight percent gel-PEG-cys in water with heat (37 °C). The PEG and GP hydrogel solutions were injected into Teflon molds held between two pre-cleaned glass slides and polymerized under a UV light (UV CF1000 LED, λ_{max} = 365 nm; Clearstone Technologies, Minneapolis, MN) for 3 min per side. The PEG and GP hydrogel films were incubated in 70% ethanol (Decon Labs, Inc., King of Prussia, PA) for 45 min and equilibrated overnight in Dulbecco's phosphate-buffered saline (DPBS; Cellgro, Manassas, VA). We used biopsy punches (Miltex, Inc., York, PA) to cut the PEG and GP hydrogel films into 8-mm diameter disks. The hydrogel disks were placed into 48-well plates (Corning Inc., Corning, NY) and incubated in 70% ethanol for 45 min. Hydrogels containing varying compositions of PEG and gelatin were prepared by mixing PEG diacrylate and gelatin (type B, bloom 75, from bovine skin) at the indicated weight percentages in water containing 0.1 weight percent 2-hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone. These hydrogel solutions were injected into Teflon molds held between two glass slides, polymerized under a UV light, and cut into 8-mm diameter disks as described above.

Gelatin hydrogels were prepared by dissolving 15 or 20 weight percent gelatin (type B, bloom 75, from bovine skin; Sigma–Aldrich, St. Louis, MO) in water with heat (37 °C). The gelatin solution was added to wells of a 48-well plate (200 μl/well). The well plate was incubated at −20 °C for 5 min to allow the hydrogels to gel. The hydrogels were then crosslinked with 4% paraformaldehyde in PBS (Affymetrix, Inc., Cleveland, OH) for 20 min at room temperature. The 4% paraformaldehyde solution was removed and the gelatin hydrogels were washed three times with DPBS. The hydrogels were washed three more times with DPBS per day for another two days prior to seeding with PMNs.

PDMS sheets (0.762 mm thickness, non-reinforced vulcanized gloss/gloss; Specialty Manufacturing Inc., Saginaw, MI) were cut into 8-mm diameter disks with biopsy punches and sonicated in a 0.5 weight percent solution of sodium dodecyl sulfate (Amresco, Solon, OH) for 10 min. The PDMS disks were rinsed with deionized water, placed into 48-well plates, and incubated in 70% ethanol for 45 min. PEG hydrogels, GP hydrogels, hydrogels containing varying compositions of PEG and gelatin, and PDMS disks were washed three times with DPBS and equilibrated in RPMI-1640 basal medium (Cellgro, Manassas, VA) prior to seeding with cells. TCPS wells (Corning Inc., Corning, NY) were also washed three times with DPBS and equilibrated in RPMI-1640 medium prior to cell seeding.

2.2. Culture and analysis of human blood-derived PMNs

Peripheral whole blood was obtained from healthy adult donors in accordance with protocols approved by the University of Wisconsin–Madison Institutional Review Board. PMNs were isolated as previously described using a density gradient method [28]. Unless otherwise indicated, PMNs were adjusted to 1×10^6 cells/ml in RPMI-1640 medium supplemented with 10% autologous human serum (AHS) and were statically seeded onto the biomaterial substrates in 48-well plates (0.5 ml/well for a density of approximately 5.3×10^3 PMNs/mm^2). The cells were incubated in a humidified atmosphere at 37 °C with 5% CO_2.

Metabolic capacity as an indicator of cell viability was measured using CellTiter-Blue® Reagent (Promega Corporation, Madison, WI). At pre-determined time points, the culture media was aspirated from PMN cultures and replaced with a solution containing 10% CellTiter-Blue® Reagent in RPMI-1640 medium supplemented with 10% AHS. PMN cultures containing the CellTiter-Blue® Reagent were incubated for 4 h in a humidified atmosphere at 37 °C with 5% CO_2. The culture media was then transferred to wells in a 96-well plate (100 μl/well; Corning Inc., Corning, NY) and the fluorescent signal (584-nm excitation, 620-nm emission) was measured using a plate reader (FLUOstar OPTIMA; BMG Labtech, Cary, NC). Fluorescent intensities (FI) of PMN samples were normalized to biomaterial control samples that did not contain cells.

Supernatants from PMN cultures on the biomaterials (seeded at a density of approximately 5.3×10^3 PMNs/mm^2) were collected and centrifuged at 2500 × g for 10 min at 4 °C to remove cells and cellular debris. MPO and MMP-9 in supernatants from PMN cultures were measured using a multi-analyte Luminex® screening assay kit (R&D Systems, Inc., Minneapolis, MN) as biomarkers for the release of primary and tertiary granule subsets, respectively. The assay was read using a Bio-Plex 100 Suspension Array System (Bio-Rad Laboratories, Hercules, CA). MPO and MMP-9 in the 10% AHS culture medium ranged from 3.8–10.3 ng/ml and 7.1–15.2 ng/ml, respectively, depending on donor.

2.3. PMN pre-treatment with Src family kinase inhibitor PP1 and/or PI3K-γ inhibitor AS604850

After isolation, PMNs were suspended at a concentration of 2×10^6 cells/ml in RPMI-1640 medium supplemented with 10% AHS and 10 μM Src family kinase inhibitor PP1 (4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo-d-3,4-pyrimidine; EMD Millipore, Billerica, MA), 1 μM PI3K-γ inhibitor AS604850 (Echelon Biosciences Inc., Salt Lake City, UT), or an equivalent volume of dimethyl sulfoxide (DMSO; Sigma–Aldrich, St. Louis, MO). These doses were selected based on previous reports [25,29,30]. The PMNs were statically seeded onto PEG hydrogel, PDMS, TCPS, GP hydrogel, and 15 weight percent gelatin hydrogel surfaces in 48-well plates (0.25 ml/well for a density of approximately 5.3×10^3 PMNs/mm^2). The plates were incubated in a humidified atmosphere at 37 °C with 5% CO_2 for 10 min. After 10 min, an additional 250 μl of RPMI-1640 medium supplemented with 10% AHS or 10% AHS containing 200 nM fMLP was added to the wells, resulting in a final fMLP concentration of 100 nM for half of the wells. This concentration of fMLP is used to stimulate PMN degranulation [31]. The well plates were incubated in a humidified atmosphere at 37 °C with 5% CO_2. After 2 h, cell viability, MPO, and MMP-9 were measured as described above.

2.4. Serum protein adsorption on PEG hydrogels and TCPS

Peripheral blood was drawn from fasted human donors into a container without anticoagulant. The blood was incubated in a 37 °C water bath for 2 h to allow for clotting. The clotted blood was centrifuged for 10 min at 1600 × g at room temperature. Serum was collected, filtered through a 0.22 μm filter, and stored at −20 °C. Serum was fractionated using PEG 4000 to reduce albumin [32,33]. This method has been validated previously in our group: removal of albumin had no effect on the relative concentration of total proteins adsorbed on PEG hydrogels or TCPS, as determined by Fourier transform infrared spectroscopy and bicinchoninic acid test [32]. Briefly, PEG (MW 4000; Sigma–Aldrich, St. Louis, MO) was added to the serum at a concentration of 1 g PEG per 10 ml serum (i.e., 3.5 g PEG to 35 ml serum). The solution was stirred in a beaker on ice. After 60 min, the solution was centrifuged at 800 × g for 30 min at 4 °C. The pellet was set aside as Fraction 1. The supernatant was added into a new beaker and the same amount of PEG as used previously (i.e., 3.5 g) was added to the beaker. The solution was stirred on ice for 30 min. The solution was centrifuged at 800 × g for 30 min at 4 °C. The pellet was set aside as Fraction 2 and the supernatant was removed and set aside as Fraction 3. Fractions 1 and 2 were combined and resuspended to the original volume of the serum (i.e., 35 ml) with DPBS. PEG hydrogel disks 74 mm in diameter were prepared as described above and placed into tissue culture dishes with an 80-mm internal diameter and 60.1 cm^2 growth area (TPP, Trasadingen, Switzerland). RPMI-1640 supplemented with 10% of serum fractions 1 and 2 was added to the dishes containing PEG hydrogels and to empty TCPS dishes (12 ml per dish) and the dishes were placed in a humidified atmosphere at 37 °C with 5% CO_2 for 2 h. Because PEG hydrogels are porous, both adsorption and absorption of proteins could occur, so we will use the term adsorption to refer to both adsorption and absorption.

2.5. On-surface enzymatic digestion, dimethyl labeling, and nanoLC-MS^2 analysis

All reagents were purchased from Fisher Chemical (Fair Lawn, NJ) unless otherwise indicated. After 2 h, the dishes containing PEG hydrogels and TCPS were removed from a humidified atmosphere at 37 °C with 5% CO_2 and washed 2 times with 12 ml DPBS for 10 min at 37 °C with gentle rocking. Proteins adsorbed on PEG hydrogels and TCPS were denatured and disulfide bonds were reduced by adding 5 ml per dish of a solution containing 5 mM dithiothreitol (DTT), 8 M urea, and 50 mM