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Diminished adhesion and activation of platelets and neutrophils with CD47 functionalized blood contacting surfaces

Matthew J. Finley [a,b], Lubica Rauova [b,c], Ivan S. Alferiev [a,b], John W. Weisel [b,d], Robert J. Levy [a,b], Stanley J. Stachelek [a,b,*]

[a] Division of Cardiology, Department of Pediatrics, The Children's Hospital of Philadelphia, PA 19104-4318, USA
[b] The University of Pennsylvania, Perelman School of Medicine, PA, USA
[c] Division of Hematology, Department of Pediatrics, The Children's Hospital of Philadelphia, PA, USA
[d] Department of Cell and Developmental Biology, The University of Pennsylvania, Perelman School of Medicine, PA, USA

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ABSTRACT

CD47 is a ubiquitously expressed transmembrane protein that, through signaling mechanisms mediated by signal regulatory protein alpha (SIRPα1), functions as a biological marker of 'self-recognition'. We showed previously that inflammatory cell attachment to polymeric surfaces is inhibited by the attachment of biotinylated recombinant CD47 (CD47B). We test herein the hypothesis that CD47 modified blood conduits can reduce platelet and neutrophil activation under clinically relevant conditions. We appended a poly-lysine tag to the C-terminus of recombinant CD47 (CD47L) allowing for covalent linkage to the polymer. SIRPα1 expression was confirmed in isolated platelets. We then compared biocompatibility between CD47B and CD47L functionalized polyvinyl chloride (PVC) surfaces and unmodified control PVC surfaces. Quantitative and Qualitative analysis of blood cell attachment to CD47B and CD47L surfaces, via scanning electron microscopy, showed strikingly fewer platelets attached to CD47 modified surfaces compared to control. Flow cytometry analysis showed that activation markers for neutrophils (CD62L) and platelets (CD62P) exposed to CD47 modified PVC were equivalent to freshly acquired control blood, while significantly elevated in the unmodified PVC tubing. In addition, ethylene oxide gas sterilization did not inhibit the efficacy of the CD47 modification. In conclusion, CD47 modified PVC inhibits both the adhesion and activation of platelets and neutrophils.

1. Introduction

Aberrant biocompatibility between blood and the polymeric biomaterials that comprise the blood conduits used in such clinical procedures as cardiopulmonary bypass and renal dialysis are associated with post-procedural complications [1,2]. Investigators have attempted to address this issue by modifying the blood contacting surfaces with various moieties such as heparin, thrombotic inhibitors, and self assembled monolayers of alkylthiols [3–5]. As these technologies are in various states of development, a therapeutic strategy to address the untoward effects observed when large volumes of blood are exposed to synthetic surfaces remains an unmet need in biomaterials research.

We previously described and characterized a surface modification in which recombinant human CD47, a ubiquitously expressed transmembrane protein that functions as a molecular marker of self, was biotinylated (CD47B) and appended to avidin modified polyvinyl chloride (PVC) and polyurethane (PU) surfaces [6]. We further demonstrated, using protein specific antibodies, that the anti-inflammatory properties of CD47 functionalized polymeric surfaces were mediated by surface immobilized CD47 and Signal Regulatory Protein alpha 1 (SIRPα1) [6], a transmembrane protein expressed in cells of myeloid origin that is the cognate receptor of CD47 [7]. Work by others has largely defined the anti-inflammatory properties of CD47 mediated SIRPα1 signaling, via src homology region 2 containing phosphatase 1 (SHP-1) and SHP-2 secondary messengers interacting with the immune receptor tyrosine inhibitory motif (ITIM) of SIRPα1, within the context of its cytoskeletal effects [8,9]. Thus, in our previous studies, we largely defined biocompatibility as a reduced affinity of inflammatory cells for the CD47 modified surfaces [6]. However, we did observe that the expression of CD18, a surface marker of neutrophil activation was significantly reduced when whole blood was perfused over the luminal surface of CD47 modified blood conduits [6]. Based upon these observations, we test herein the hypothesis that the

* Corresponding author. Division of Cardiology, Department of Pediatrics, The Children's Hospital of Philadelphia, Abramson Research Building, 3615 Civic Center Blvd., Suite 702, PA 19104-4318, USA. Tel.: +1 215 590 0157; fax: +1 215 590 5454.
E-mail address: stachelek@email.chop.edu (S.J. Stachelek).

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anti-inflammatory capacity of CD47 functionalized surfaces extends beyond inhibiting inflammatory cell attachment to synthetic surfaces and that CD47-SIRPα1 interactions can alter the inflammatory activation state of blood cells.

Understanding the signaling relationship between CD47 functionalized surfaces and platelets is essential to applying this strategy to effectively inhibit the inflammatory response observed when large volumes of blood are exposed to synthetic surfaces. Platelets are important mediators of the acute inflammatory response observed in clinically used blood conduits [10–12]. To date, the effects of CD47-SIRPα1 signaling in platelets were never investigated. In these current investigations we begin to profile the platelet response to CD47 functionalized surfaces. Specifically, we ascertain the possibility of SIRPα1 mediated signaling mechanisms functioning in platelets and characterize the surface expression of proinflammatory markers of platelet activation when whole blood is exposed to CD47 modified blood conduits.

In addition to the biological response of CD47 modified surface, this current work begins to address the pragmatic challenges associated with the potential clinical application of CD47 modified blood conduits. Our previous attachment strategy for appending CD47 to polymeric surfaces was based on covalently binding avidin to the polymer surface via photoactivation chemistry and then appending biotinylated CD47B to the avidin functionalized surface [6] via Avidin-Biotin affinity binding. Although this attachment proved effective in reducing the affinity of inflammatory cells for CD47 functionalized surfaces under *in vitro, ex vivo,* and long term *in vivo* studies, there exists a concern that the avidin-biotin system may not be appropriate for all applications, due to potential avidin immunogenicity [13]. Thus, in the present studies, we also tested the hypothesis that molecular modifications can be made to the extracellular domain of recombinant CD47 that allows it to be covalently immobilized onto polymeric surfaces, via thiol chemistry, whereupon it can effectively enhance biocompatibility under dynamic conditions. This approach would eliminate the need for initially immobilizing avidin to the surface, and would hypothetically increase the resiliency of the CD47 protein by covalently attaching the recombinant protein to the biomaterial surface. In these studies, we compared, with respect to anti-inflammatory and durability endpoints, our original biotinylated CD47B with our recently developed lysine tagged (CD47L) attachment strategy.

Our goals of these studies were: 1. Investigate a chemical attachment strategy to directly link recombinant CD47 to polymeric surfaces using a lysine tag methodology, 2. Study the biologic response of platelets and neutrophils to CD47 functionalized PVC surfaces versus unmodified surfaces, 3. Assess the retention and effectiveness of the CD47 modification upon functionalized PVC surfaces following biophysical challenges as well as ethylene oxide gas sterilization.

2. Materials and methods

2.1. Materials

Clinical grade polyvinyl chloride tubing conduits were acquired from Terumo Cardiovascular Systems (Ann Arbor, MI). A FITC-conjugated mouse monoclonal antibody directed against CD47 (Clone: B6H12) was purchased from BD Biosciences (Franklin Lakes, NJ). Ultrapure avidin was purchased from Life Technologies (Carlsbad, CA). N-Succinimidyl 3[2-pyridyldithio]-propionate (SPDP), tris(2-carboxyethyl) phosphine hydrochloride (TCEP), and all other chemicals and solvents, unless otherwise specified, were purchased from Sigma Aldrich (St. Louis, MO).

2.2. Recombinant CD47 protein production and purification

We used a modification of the previously published methodology to produce recombinant human CD47 from human whole blood [6]. Briefly, the extracellular domain of human CD47 (GenBank accession number NM_174708) was amplified from human cDNA using gene specific primers. Forward Primer:5′ – ATAAGCTTATGTGGCCCCTGG – 3′. Reverse Primer: 5′ –GCGGATCCTCTGGTTCAACCCT – 3′. The PCR conditions were as follows: 95 °C for 30 s denaturation, 59 °C for 45 s annealing, and 72 °C for 1 min extension for a total of 35 cycles.

The PCR product was ligated into a thymidine/adenine vector containing the rat CD4 domains 3 and 4 (rCD4D3+4) coding sequence. Either a biotin coding sequence (B), required for the subsequent biotinylation of the final recombinant protein, or poly-lysine coding sequence (L) was cloned into the vector at the three prime end of the rat CD4 sequence to aid in protein purification and appendage to the polymeric surfaces. The expression cassette (5′ – hCD47-rCD4d3+4-Biotin – 3′ or 5′ – hCD47-rCD4d3+4-Poly-Lysine – 3′) was then subcloned into pcDNA5-FRT (Invitrogen, Carlsbad, CA) by digesting vector and insert with Hind III and BamHI and then ligating using T4 DNA ligase. All vectors were sequenced by the Children's Hospital of Philadelphia Research Institute Nucleic Acid Facility and confirmed to be free of coding errors and in-frame for accurate protein production. The pcDNA5-FRT-hCD47-rCD4d3+4-Biotin (hCD47B) and pcDNA5-FRT-hCD47-rCD4d3+4-Poly-Lysine (hCD47L) were co-transfected with pOG44, used to transiently provide the recombinase enzyme required for integration of the hCD47-rCD4d3+4 into the genomic DNA of the host cell, into CHO Flp-In cells to allow for genomic integration of the expression cassette. Recombinant protein was isolated from the cell culture medium, concentrated, desalted, and purified using an avidin protein purification column or anti-CD47 amino-link protein purification column both obtained from Thermo Fisher Scientific (Wilmington, DE). Protein concentration and purity were analyzed by the Bradford assay and SDS-PAGE Western blot analysis.

2.3. Casting PVC films

PVC was dissolved in dimethylacetamide, and then solvent cast as films with thickness ranging from 159 to 220 μm as used in prior studies [6]. Films were cast onto flow streamer slides specifically for the Flex-Flow parallel plate shear delivery system by Flex-Cell International (Hillsborough, NC) and subsequently used in shear studies as previously described [14].

2.4. Appending of poly-lysine tagged recombinant proteins to synthetic polymeric surfaces

PVC surfaces were reacted with 2-pyridyldithio-, benzophenone- and carboxy-modified polyallylamine (PDT-BzPh) and reduced with TCEP to obtain a thiol reactive surface, as previously described [6]. The poly-lysine tail of the recombinant CD47L protein was reacted with SPDP for 1 h to form thiol-reactive groups. SPDP was removed via a desalting protein cut off column and the poly-lysine CD47 protein was incubated with the synthetic surface overnight at 4 °C. Fig. 1 is provided as a graphic illustration of the chemistry described in this section. Confirmation of recombinant protein attachment was determined by conjugation with a FITC-tagged antibody specific for human CD47 (BD Biosciences, Franklin Lakes, NJ). Where indicated, blood conduits were sterilized by ethylene oxide (EtO) gas sterilization by the Children's Hospital of Philadelphia Instrument Sterilization Facility.

2.5. Analysis of recombinant CD47 poly-lysine appendage with synthetic polymeric surfaces

PVC films, modified with either CD47B or CD47L (6 μg/cm^2), were exposed to shear flow of 1× phosphate buffered saline for 2 h at physiologically relevant levels of shear stress (20 dyn/cm^2), using the computer controlled Flex-Cell Streamer instrument [14]. Control films were not exposed to shear conditions. Following the protocol, films were stained with FITC-conjugated antibody specific for CD47. Briefly, the polymeric films were blocked using 5% bovine serum albumin (BSA) in 1× tris buffered saline with tween-20 (TBST) for 1 h at room temperature. Following blocking, the films were stained with 100 μg FITC-conjugated CD47 antibody in 5% BSA 1× TBST for 1 h at room temperature. Films were washed three times in 1× TBST and imaged by fluorescence microscopy. In order to estimate the concentration of recombinant hCD47 protein, the antibody was titrated to form a standard curve, which was then used to determine the concentration of recombinant hCD47 protein on the polymeric surfaces. To quantify results, the fluorescence intensity was collected for each film and correlated to a standard curve in order to determine the approximate protein concentration in micrograms.

2.6. Chandler Loop analysis of CD47 modified polymeric blood conduits

PVC tubing (1/4″) was modified with either CD47B or CD47L. CD47B was attached to the polymeric surface using thiol reactive avidin, as previously described [6]. Whole blood isolated aseptically from healthy volunteers, per an IRB protocol approved by the University of Pennsylvania IRB after informed consent, was either kept as control or loaded into PVC, CD47B modified PVC, or CD47L modified PVC tubing. Blood was perfused over the polymeric surfaces for 3 h to simulate the effect of whole blood interaction with the conduit polymeric surface and blood samples were withdrawn for flow cytometry analysis. As detailed below, at the termination of the protocol, whole blood was collected for flow cytometry analysis and scanning electron microscopy (SEM) was performed on the tubing.