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Ultrasound-based molecular imaging and specific gene delivery to mesenteric vasculature by endothelial adhesion molecule targeted microbubbles in a mouse model of Crohn's disease

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ABSTRACT

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract (GI) for which treatments with immunosuppressive drugs have significant side-effects. Consequently, there is a clinical need for site-specific and non-toxic delivery of therapeutic genes or drugs for CD and related disorders such as inflammatory bowel disease. The aim of this study was to validate a gene delivery platform based on ultrasound-activated lipid-shelled microbubbles (MBs) targeted to inflamed mesenteric endothelium in the CD-like TNFΔARE mouse model. MBs bearing luciferase plasmid were functionalized with antibodies to MAdCAM-1 (MB-M) or VCAM-1 (MB-V), biomarkers of gut endothelial cell inflammation and evaluated in an in vitro flow chamber assay with appropriate ligands to confirm targeting specificity. Following MB retro-orbital injection in TNFΔARE mice, the mean contrast intensity in the ileocecal region from accumulated MB-M and MB-V was 8.5-fold and 3.6-fold greater, respectively, compared to MB-C. Delivery of luciferase plasmid to the GI tract in TNFΔARE mice was achieved by insonating the endothelial cell-bound agents using a commercial sonoporator. Luciferase expression in the midgut was detected 48 h later by bioluminescence imaging and further confirmed by immunohistochemical staining. The liver, spleen, heart, and kidney had no detectable bioluminescence following insonation. Transfection of the microcirculation guided by a targeted, acoustically-activated platform such as an ultrasound contrast agent microbubble has the potential to be a minimally-invasive treatment strategy to ameliorate CD and other inflammatory conditions.

1. Introduction

Crohn's disease (CD) is an autoimmune disorder that leads to a chronic and debilitating form of inflammatory bowel disease (IBD). The pathogenesis of CD is unknown and likely results from a confluence of genetic and environmental factors [1,2]. Conventional treatment of CD with steroids and other immunosuppressive drugs results in amelioration of acute symptoms, but therapy must be carefully monitored to minimized negative side-effects that are attendant with long-term usage [3,4]. Alternative therapies using biological agents targeted at regulatory elements of the immune system have shown positive clinical outcomes in many cases, and allow respite from some of the negative side effects of long-term steroid use [5–7]. The most widely-used biologics to date have been humanized monoclonal antibodies that block the activity of the cytokine, tumor necrosis factor alpha (TNF-α) [8,9]. In addition, antibodies to cytokines such as IL-23, IL-17, IL-6, and alpha 4 integrin are in clinical trials or have been approved as treatments for CD [10,11]. While promising as therapies, all biologics developed to date have significant drawbacks, including limited efficacy in some patients, risks of adverse immune responses with repeated administration, and high cost

Abbreviations: AU, arbitrary units; CD, Crohn's disease; EC, endothelial cell; GALT, gut-associated lymphoid tissue; GI, gastrointestinal; ICAM-1, intercellular adhesion molecule-1; IV, intravenous; IHC, immunohistochemical; IBD, inflammatory bowel disease; MAdCAM-1, mucosal addressin cellular adhesion molecule-1; MBs, microbubbles; MB-M, plasmid-bearing targeted microbubbles to MAdCAM-1; MB-V, plasmid-bearing targeted microbubbles to VCAM-1; MB-C, plasmid-bearing control microbubbles; rmMAdCAM-1, recombinant murine MAdCAM-1; ROI, region of interest; TNF-α, tumor necrosis factor alpha; IP, intraperitoneal; UCA, ultrasound contrast agents; US, ultrasound; VCAM-1, vascular cell adhesion molecule-1; rmVCAM-1, recombinant murine VCAM-1.

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[5,11–13]. Gene therapy strategies aimed at addressing the underlying disease components may therefore represent an alternative approach for long-term treatment of CD and IBD.

The intestinal inflammation characteristic of CD is associated with recruitment of naïve lymphocytes into the gut-associated lymphoid tissue [14]. Animal studies suggest that CD in time takes on characteristics of chronic inflammatory disease, such as continuously dysregulated production of pro-inflammatory cytokines, overexpression of adhesion molecules such as mucosal addressin cell adhesion molecule-1 (MAdCAM-1) [13,15], intercellular adhesion molecule-1 (ICAM-1) [12,16,17], and in some models, vascular adhesion molecule-1 (VCAM-1) [18,19]. Notably, increased expression of MAdCAM-1 has been reported in the intestinal microvascular endothelium in several CD-like mouse models [20,21], as has VCAM-1 [22].

One approach to molecularly image the vascular inflammation characteristic of CD and IBD is to target ultrasound microbubble contrast agents to receptors on inflamed endothelium. In this approach, microbubbles (MBs), ranging from 2 to 4 μm, are functionalized with antibodies or peptides to endothelial markers such as MAdCAM-1 that then support adhesion to the blood vessel wall in the tissue of interest [21,23,24]. Consequently, contrast (generated by the MB echogenicity) maps to the expression of the respective antigens on the vascular endothelium. One advantage of contrast enhanced ultrasound imaging is that the microbubble's size constrains it to the lumen of the blood vessel and results in rapid clearance by the lung and spleen. Repeated applications should therefore be possible to probe the expression patterns of different antigens [25].

Ultrasound contrast MBs have also been used to sonoporate cells, a process in which acoustic energy induces bubble collapse, generating microjets that create transient pores in the cell membrane. The combination of membrane pore creation and microjet formation facilitates transport of large molecules and nanoparticles into the cell cytoplasm [26–34]. The net result is that an intravascularly administered MB can deliver DNA plasmids, siRNAs, proteins, or conventional drugs to the cells of the blood vessel wall if appropriately activated by ultrasound.

Previously, we have used a targeted MB formulation as an imaging modality to detect and evaluate MAdCAM-1 expression noninvasively in ileitis in a mouse model of chronic intestinal inflammation [21]. Plasmid-bearing untargeted MBs have also been used to deliver genes to the rat hindlimb skeletal muscle [35], achieving luciferase gene transfer by localized application of ultrasound. In this study, MBs were formulated to adhere to the vascular endothelium via the receptors MAdCAM-1 and VCAM-1, known to be upregulated in the mesentery of TNFΔARE mice, an animal model of IBD-like disorders. Additionally, the targeted MBs were modified to carry DNA plasmids coding for luciferase, a reporter gene. Using this platform, we demonstrated its ability to detect MAdCAM-1 and VCAM-1 expression in chronically inflamed mesenteric vasculature of TNFΔARE mice and with ultrasound we initiated targeted transfection of the luciferase plasmid to the diseased intestinal tissue.

2. Materials and methods

2.1. Luciferase plasmid

The plasmid pCMV-GL3 was derived from pGL-3 (Promega, Madison, WI). The pCMV-GL3 plasmid drives the expression of the firefly luciferase gene from the CMV promoter, with a molecular weight of 38 kDa. The plasmid was amplified in Epicurian Coli XL10 gold ultracompetent cells (Stratagene, La Jolla, CA) and then isolated and purified using QIAGEN plasmid giga kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Plasmid concentration was assessed by photometric absorption at 260 nm.

2.2. Cell culture preparation and flow cytometry analysis

Three cell lines were used in this study. SVEC4-10 (murine endothelial cell line derived by SV40 transformation) was purchased from ATCC (Manassas, VA), and was used to investigate MB adhesion to VCAM-1. SV-LEC, a mouse lymphatic endothelial cell line from the mesenteric adventitial tissue, was generated as described previously [36]. SV-LEC cells express MAdCAM-1 following TNF-α stimulation. SV-LEC cells were maintained in Dulbecco modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen) and 1% (v/v) penicillin/streptomycin (P/S; Invitrogen) in a 95% air–5% CO_2 humidified atmosphere at 37 °C. The SVEC4-10 cells were similarly maintained, except for the addition of 10% heat-inactivated FBS. The endothelioma line bEND.3 was a gift from Dr. Eugene Butcher and was maintained under identical conditions as SV-LEC cells. For the functional binding flow chamber assay, 35-mm culture dishes (Corning, Corning, NY) were pre-coated with 2 μg/ml fibronectin (Millipore, Temecula, CA) for 3 h at room temperature (RT) and both cell lines were seeded at a density of 1.5×10^5 cells/mL and grown to confluence. SV-LEC cells were cultured in the presence or absence of 20 ng/mL recombinant murine TNF-α (PeproTech, Rocky Hill, NJ) for 24 h prior to the parallel flow chamber experiment. The presence of VCAM-1 in SVEC4-10 cells and MAdCAM-1 in SV-LEC cells was verified by FACS analysis (data not shown) using Alexa Flour 647-conjugated anti-VCAM-1 (clone: 429, eBioscience, San Diego, CA) or FITC-conjugated anti-MAdCAM-1 (clone: MECA-367, eBioscience, San Diego, CA), respectively. bEND.3 cells cultured with 20 ng/mL murine TNF-α were positive for VCAM-1 and MAdCAM-1. All flow cytometry data were analyzed with FLOWjo Software (www.treestar.com/flowjo).

2.3. Targeted plasmid-bearing microbubble preparation

Biotinylated monoclonal murine antibodies against MAdCAM-1 (clone: MECA-367), VCAM-1 (clone: 429), Alexa Fluor 647 (AF647) conjugated mouse anti-Rat IgG and IgG2a isotype control (clone: EBR2a) were purchased from eBioscience (San Diego, CA). We utilized an experimental MB manufactured by Targeson, Inc. (San Diego, CA), known as TS-02-008 [26]. MBs were composed of a gaseous decafluorobutane core encapsulated by distearoylphophatidylcholine (DSPC), a lipid shell, and polyethylene glycol (PEG) stearate (Supplemental Fig. A). The bubble shell contains a small amount of lipid 1,2-distearoyl-3-trimethylammoniumpropane (DTAP; Avanti, Alabaster, AL) which provides a net positive surface charge. The MB also contains 1% of lipid–PEG–biotin, which enables conjugation of targeting ligands using biotin–streptavidin conjugation chemistry. Conjugation of biotinylated antibodies to the MB was performed as previously described [21]. Briefly, anti-MAdCAM-1, anti-VCAM-1 or isotype control antibodies were added at 5 μg per 1×10^7 MBs, followed by addition of pCMV-GL3 plasmid (20 μg per 1×10^8 MBs) to MBs being used for gene delivery. MBs were resuspended to a final concentration of 2×10^7 MB/mL. For the targeted MB construct characterization by FACS analysis, luciferase plasmid-bearing MBs were labeled with the nucleotide-avid fluorophore YOYO-1 (Molecular Probes, Eugene, OR), 1 μg per 1×10^8 MBs and second staining was conducted with AF647 conjugated anti-rat IgG antibody (5 μg per 1×10^7 MBs) at a final concentration of 5×10^6 MB/mL for FACS analysis. To compare the polydisperse MB population with known bead sizes by FACS, some MB samples were incubated with polystyrene microbeads (2.022 ± 0.046 μm and 5.919 ± 0.354 μm from Polyscience, Inc.; Warrington, PA). MB concentration and size distribution were assessed by electrozone sensing using a Coulter Multisizer 4 (Beckman–Coulter, Miami, FL).

2.4. Laminar flow adhesion assay

35-mm nontreated polystyrene Petri dishes (BD Falcon, Franklin Lakes, NJ) were used to absorb recombinant murine MAdCAM-1