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Tunable physiologic interactions of adhesion molecules for inflamed cell-selective drug delivery

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ABSTRACT

Dysregulated inflammation contributes to the pathogenesis of various diseases. Therapeutic efficacy of anti-inflammatory agents, however, falls short against resilient inflammatory responses, whereas long-term and high-dose systemic administration can cause adverse side effects. Site-directed drug delivery systems would thus render more effective and safer treatments by increasing local dosage and minimizing toxicity. Nonetheless, achieving clinically effective targeted delivery to inflammatory sites has been difficult due to diverse cellular players involved in immunity and endogenous targets being expressed at basal levels. Here we exploit a physiological molecular interaction between intercellular adhesion molecule (ICAM)-1 and lymphocyte function associated antigen (LFA)-1 to deliver a potent anti-inflammatory drug, celastrol, specifically and comprehensively to inflamed cells. We found that affinity and avidity adjusted inserted (I) domain, the major binding site of LFA-1, on liposome surface enhanced the specificity toward lipopolysaccharides (LPS)-treated or inflamed endothelial cells (HMEC-1) and monocytes (THP-1) via ICAM-1 overexpression, reflecting inherent affinity and avidity modulation of these molecules in physiology. Targeted delivery of celastrol protected cells from recurring LPS challenges, suppressing pro-inflammatory responses and inflammation-induced cell proliferation. Targeted delivery also blocked THP-1 adhesion to inflamed HMEC-1, forming barriers to immune cell accumulation and to aggravating inflammatory signals. Our results demonstrate affinity and avidity of targeting moieties on nanoparticles as important design parameters to ensure specificity and avoid toxicities. We anticipate that such tunable physiologic interactions could be used for designing effective drug carriers for *in vivo* applications and contribute to treating a range of immune and inflammatory diseases.

1. Introduction

Imbalance between pro- and anti-inflammatory responses of host immune system contributes to the pathogenesis of various human diseases of modern society [1]. In particular, prolonged and excessive inflammation, which comprises persistently reinitiating acute and chronic inflammatory responses between non-immune (epithelium, endothelium, etc) and immune cells, has been implicated in cardiovascular diseases [2], obesity [3], neurodegenerative diseases [4], and cancer [5]. Subsequently, anti-inflammatory agents such as corticosteroids, nonsteroidal anti-inflammatory drugs, cyclo-oxygenase-2-selective inhibitors, and statins have been used clinically to treat acute and chronic inflammatory diseases [6–9]. Long-term and high-dose enteral or parenteral administration of these drugs, however, have been limited due to adverse systemic side effects that included gastrointestinal disturbances, renal, ocular and liver toxicities, skeletal and muscle damages, and increased risk of cardiovascular diseases [10–13].

Current advances in site-directed drug delivery systems [14] would thus contribute much benefit towards safer and more effective clinical use of anti-inflammatory agents. Numerous studies have targeted drug carriers to the endogenous molecules of endothelium such as ICAM-1 [15,16], vascular cell adhesion molecule (VCAM)-1 [17,18], platelet endothelial cell adhesion molecule (PECAM)-1 [19], E- and P-selectins [20,21], and $\alpha_v\beta_3$ integrin [22,23], which belong to a family of cell adhesion molecules. Some of the cell adhesion molecules such as E-selectins and $\alpha_v\beta_3$ integrin are more exclusively expressed in vascular endothelium, while others may be present in both non-immune and immune cells. The difference in their response to inflammation is also observed in terms of the levels of

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induction as well as their basal expression prior to inflammation. ICAM-1 has been of particular interest for its superior inducible and localized expression upon inflammatory stimuli both in immune and non-immune cells such as endothelial, smooth muscle, epithelial cells, fibroblasts, lymphocytes, and myeloid cells [24,25]. Specific delivery of drug carriers or nanoparticles to inflamed cells has traditionally been achieved by molecular interactions with cell surface molecules created by attaching antibodies or short peptides to the surface of nanoparticles [26,27]. However, most prior approaches have failed to address affinity and avidity modulation as important design criteria for efficient nanoparticle binding to target cells, which will be drastically different from those for free molecule binding due to much larger detachment force on nanoparticles caused by hydrodynamic stress *in vitro* and *in vivo*. Alternative to non-native interactions, the use of physiological ligands or their engineered variants [28–30] conjugated to nanoparticles at an optimal density for tunable affinity and avidity may prove advantageous in regard to ensuring specificity against target receptors and a lower risk of unwanted immune response.

In any attempt to deliver drugs via ICAM-1 targeting, one needs to ensure specific delivery of drug carriers to inflamed cells but not to normal cells, given the observation that ICAM-1 is broadly expressed in the body albeit at much lower levels than post exposure to inflammatory stimuli [31]. In order to design drug carriers against ICAM-1 to be robust and amenable to fine-control in terms of affinity and avidity, we have chosen a targeting moiety based on a native molecule called the inserted or I domain, derived from a physiological receptor to ICAM-1 called lymphocyte function associated antigen (LFA)-1 integrin [25,28]. In contrast to commonly used monoclonal antibodies (~150 kDa) against ICAM-1 for targeting, LFA-1 I domain is small (~20 kDa) and suitable for a low-cost, large-scale production in bacteria, and can be modified for optimal affinity to ICAM-1 and facile conjugation to drug carriers. Among various nanoparticles suitable for drug delivery, liposomes have been used in this study, which have been approved for clinical use to take advantage of diverse functionalized phospholipids and large compartments for encapsulation of both hydrophilic and hydrophobic molecules. To the surface of liposomes, high affinity LFA-1 I domain (Id-HA) was attached via non-covalent, His-tag binding to nickel ions chelated onto phospholipid molecules. As a model anti-inflammation drug, we incorporated celastrol, a quinone methide triterpene, into the liposomes. Celastrol possesses potent anti-inflammatory, anti-oxidative, and anti-proliferative activities via the inhibition of NF-κB signaling and proteasome activity [32–34].

In this study, we emphasized the delivery of celastrol selectively to inflamed cells without causing cytotoxicity while maintaining potent therapeutic effect. To implement this, the density of Id-HA on the surface of liposomes was adjusted for optimal dose and specificity. We then examined whether such targeted delivery conditions could elicit the anti-inflammatory effect of celastrol in inflamed endothelium and myeloid cells in a manner dependent on ICAM-1 and LFA-1 interaction. As for the measure of selective delivery of celastrol, we investigated its ability to inhibit gene and protein expression of pro-inflammatory mediators and to block the migration of monocytes, which together may form a key barrier to immune cell accumulation and to aggravating inflammatory responses in physiology.

2. Materials and methods

2.1. Cell culture

Human dermal microvascular endothelial cells (HMEC-1) were obtained from the Center for Disease Control. HMEC-1 were cultured in MCDB 131 medium (Invitrogen) supplemented with 10% FBS (Atlanta Biologicals), 10 mM L-glutamine, Pen-strep (100 units/ml penicillin and 100 μg/ml streptomycin), 1 μg/ml hydrocortisone (MP Biomedicals), and 10 ng/ml recombinant human epidermal growth factor (Invitrogen). HMEC-1 were trypsinized when confluent, and gently centrifuged and washed to remove residual trypsin before plating. Human monocytic leukemia THP-1 cells (ATCC) were grown in RPMI 1640 medium (Invitrogen) with 10% FBS and Pen-strep. HeLa cells were propagated in Advanced DMEM medium (Invitrogen) containing 10% FBS and 2 mM L-glutamine, and Pen-strep. Mouse brain microvascular endothelial cells (bEnd.3, ATCC) were maintained in Advanced DMEM medium supplemented with 4 mM L-glutamine, Pen-strep, and 10% FBS. For induction of inflammation, HMEC-1, THP-1, or bEnd.3 cells were treated with 1 μg/ml of LPS (*Escherichia coli*. 026:B6, Sigma). All mammalian cells were maintained at 37 °C in a 5% CO_2 humidified incubator.

2.2. Production of I domains and GFP-Id-HA fusion protein

The wild-type (WT), D137A, and F265S/F292G mutants of LFA-1 I domains (Id-WT, Id-D137A, and Id-HA) were produced as previously described [28,30]. Briefly, the I domains (Asn129 to Tyr307) followed by a stop codon were subcloned into pET28a vector (Novagen) between NheI and XhoI for expression with a His-tag at the N-terminal. The I domains were expressed in *E. coli* BL21 (DE3) cells (Novagen) as inclusion bodies. Cells were initially grown to OD600 of 0.4–0.5 and induced with 1 mM IPTG (isopropyl-β-D-thiogalactoside) for 6 h at 37 °C. To isolate the inclusion bodies, cells were resuspended in washing buffer (50 mM Tris-Cl (pH 8.0), 23% (w/v) sucrose, 1 mM EDTA, 0.5% (v/v) Triton X-100) and sonicated. Inclusion bodies were washed by repeating cycles of centrifugation, removal of supernatant, and sonication until pure pellets were obtained. Inclusion bodies were then solubilized in denaturing buffer (50 mM Tris-Cl (pH 8.0), 6 M guanidine HCl) and diluted in refolding buffer (50 mM Tris-Cl (pH 8.0), 15% glycerol, 1 mM $MgCl_2$) to a volume such that the final concentration of guanidine HCl was less than 25 mM. Refolded proteins were concentrated and subjected to gel filtration chromatography using Superdex S200 column in HBS (20 mM HEPES, 140 mM NaCl, pH 7.4) connected to AKTA Purifier (GE Healthcare). Some of the I domains were conjugated to Alexa Fluor 488 (AF488, Invitrogen) according to the instruction of the vendor. For GFP-Id-HA fusion protein, eGFP (Val2 to Lys239) [35] was first inserted into pET28a vector between NheI and BamHI and then Id-HA (Asn129 to Tyr307) followed by a stop codon was placed between BamHI and XhoI. GFP-Id-HA was produced from soluble fractions in BL21 cells. Cells were grown to OD600 of 0.4–0.5, induced with 1 mM IPTG at 25 °C for 6 h and recovered by centrifugation. Cells were then resuspended and sonicated in NTA (nitrilotriacetic acid) binding buffer (50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 10 mM imidazole). Soluble proteins were separated from cell debris by centrifugation, purified by flowing the supernatant through a Ni-NTA column (Novagen) and gel filtration chromatography using S200 column.

2.3. Immunofluorescence flow cytometry and microscopy

Antibodies used for this study include anti-ICAM-1 monoclonal antibody (mAb) LB-2 (Santa Cruz) and anti-VCAM-1 mAb P3C4 (Developmental Studies Hybridoma Bank). If necessary, phycoerythrin (PE)-labeled goat anti-mouse IgG (Santa Cruz) was used for the detection of primary mAbs. For flow cytometric analysis (Beckman Coulter EPICS XL-MC), cells were trypsinized, washed with ice-chilled labeling buffer (PBS (pH 7.4), 0.5% (w/v) BSA, 10 mM $MgCl_2$), and incubated on ice for 20 min. Antibodies and proteins were used at 10 μg/ml in 200 μl of the labeling buffer and were incubated with cells on ice for 30 min. Cells were washed twice in 500 μl of the labeling buffer between each step of labeling when secondary antibodies were needed. After final washing, cells were resuspended in 300 μl of the labeling buffer and subjected to flow cytometer. For immunofluorescence microscopy, HMEC-1 were fixed with 3.7% paraformaldehyde for 30 min, washed and labeled with mAbs LB-2 or P3C4 in labeling buffer (PBS, 1% BSA, 0.02% (v/v) Tween 20) on a rocker at room temperature for 2 h. DAPI (4′,6-diamidino-2-phenylindole, Invitrogen) was used at 300 nM in PBS for 10 min for nucleus staining. To estimate fluorescence and nuclei density from DAPI staining, microscopic images of 4 random spots were acquired and processed by Image-Pro Plus (Media Cybernetics) for automated intensity measurement and object counting. Similarly, confluent bEnd.3 cells were incubated with AF488 conjugated Id-HA. Stained cells were washed and imaged with a fluorescence microscope (Zeiss Axio Observer Z1). For confocal microscopy (Leica TCS SP2), HeLa cells were labeled with 10 μg/ml of mAb LB-2 or GFP-Id-HA in 200 μl of labeling buffer on ice for 30 min.

2.4. LFA-1 or Mac-1 I domain-displaying yeast binding to HMEC-1

Mammalian cell surface binding of yeast cells displaying various I domains was performed as previously described [35]. In short, yeast cells expressing WT or activated mutants of LFA-1 and Mac-1 I domains were washed and resuspended in washing buffer (PBS (pH 7.4), 0.5% BSA, 10 mM $MgCl_2$). 300 μl of yeast cell suspension containing approximately 5×10^7 cells was applied to each well of confluent HMEC-1 in a 24-well plate. Yeast cells were allowed to bind to HMEC-1 for 1 h at room temperature. Cells were then washed with washing buffer by gentle shaking on an orbital shaker for 15 min.