Discovery of a potent nanoparticle P-selectin antagonist with anti-inflammatory effects in allergic airway disease[1]

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SPECIFIC AIMS

In the present study we aimed to identify novel P-selectin antagonists that would inhibit leukocyte attachment and rolling in vitro and have potent anti-inflammatory effects in vivo in a murine model of allergic airway disease.

PRINCIPAL FINDINGS

1. Potent P-selectin antagonists were designed based on a polymerized lipid nanoparticle (PLNP) that displays ligand mimetics simulating PSGL-1. The inhibitors specifically blocked P-selectin-dependent attachment and rolling of leukocytes in vitro

The novel inhibitors display only the key elements of PSGL-1 responsible for adhesion to P-selectin; therefore, all but the key carbohydrate unit fucose were removed from the sialyl Lewis X (sLex) carbohydrate. This carbohydrate was displayed along with polyvalent sulfate ester groups to emulate the sLe^x/sulfated tyrosine super ligand motif shown to be critical for P-selectin binding. Nanoparticle formulations were tested in which the ratio of fucose, sulfate, and polyethylene glycol were varied. The optimal ratio of the four lipids was found to be fucose:sulfate:PEG:matrix 5:25:1:69. The efficacy of formulation changes were evaluated in an in vitro recirculating loop assay (Proteo-Flow®) in which interactions between PSGL-1-expressing human leukocytes (U-937) and P-selectin-coated glass capillary surfaces at physiological shear rates were monitored. In the absence of PLNP, the number of U-937 cells interacting with the coated capillary tubes gradually increased with time. Administration of PLNP after establishing the leukocyte/selectin rolling interaction reversed the existing rolling, and completely inhibited new cell attachment (**Fig. 1*a***). By increasing the PEG lipid level from 1% to 15%, selectin inhibitory activity of the PLNP was abolished (Fig. 1*a*), providing a nanoparticle that could be used as a negative control PLNP in other experiments. The leukocyte/P-selectin inhibition activity of the PLNP showed a dose-dependent decrease (Fig. 1*b*), with no effect on leukocyte/E-selectin interactions (Fig. 1*c*).

2. After intravenous administration of selectin PLNP antagonists in a murine endotoxin model of systemic activation, PLNP binding was almost exclusively restricted to endothelial cells within the lung. The absence of PLNP binding in E/P-selectin −/− mice confirmed that this pattern of distribution was consistent with selectin-specific attachment of the inhibitor to the endothelium

After identifying potent PLNP inhibitors in vitro, their ability to bind preferentially to selectins on activated endothelial cells was confirmed in vivo. Wild-type C57Bl6 (WT) mice and mice deficient in E- and P-selectin ($E/P^{-/-}$) expression were injected with i.v. LPS, then PLNP 2 h later. Tissue samples were collected for histological analysis 3 h after PLNP injection and the distribution of autofluorescent PLNP was examined in the lung. In the absence of LPS, few PLNPs were found in contact with the endothelium in WT mice. After LPS treatment, extensive PLNP binding was visible mainly on the endothelial cells within the lung vasculature. In $E/P^{-/-}$ mice stimulated with LPS, there was little or no direct binding of the PLNP to the endothelial cells although some PLNPs were associated with a small number of leukocytes.

3. A single administration of selectin PLNP in a murine model of allergic airway disease led to prolonged attachment of PLNP to the surface of endothelial cells after allergen challenge, resulting in a reduction in the level of allergen-induced peribronchial inflammation and airway hyper-responsiveness in the lungs

The in vivo efficacy of the selectin PLNP was assessed in a selectin-dependent murine model of cockroach aller-

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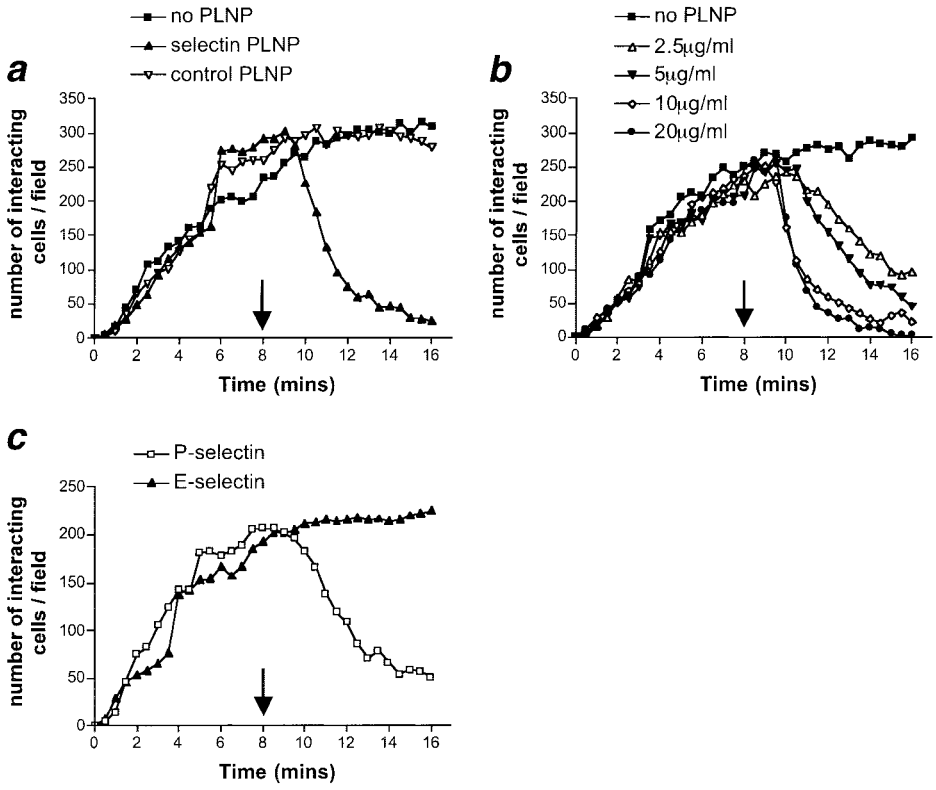

Figure 1. Inhibition of P-selectin- but not E-selectin-mediated leukocyte cell tethering/rolling by PLNP under shear in vitro. Selectin-blocking PLNPs (1% PEG) or negative control PLNPs (15% PEG) were administered after U-937 cell rolling was established on P-selectin-coated capillary tubes and the number of cells interacting with the wall of the capillary tube was determined (*a*). Rolling of U937 cells was established on P-selectin chimera-coated capillary tubes and the dose-dependent effect of P-selectin blocking PLNP was determined (*b*). Comparison of the inhibitory effect of selectin-blocking PLNP on U-937 interactions with P- or E-selectin chimera-coated capillary tubes (*c*). All data are representative of 3–4 independent experiments showing similar results.

gen (CRA)-induced allergic airway inflammation. Selectin PLNPs administered 2 h after allergen challenge were still visible on the endothelial cell surface almost 24 h later. These PLNPs were also seen in association with the alveolar walls but were not present in large airways. Negative control PLNPs were not detected in association with leukocytes or endothelial cells within the lung vasculature. Treatment with selectin PLNP altered the responses in CRA-induced lung inflammation compared with mice treated with control PLNP or allergen alone. Methacholine-induced airway hyperresponsiveness and peribronchial inflammation (**Fig. 2**) were both significantly reduced in mice treated with P-selectin-specific PLNP compared with those treated with CRA alone or control PLNP.

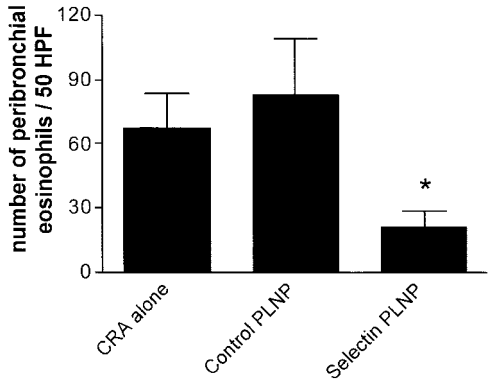

Figure 2. Inhibition of peribronchial eosinophil recruitment by selectin PLNP. The number of peribronchial eosinophils was determined in 50 high-power fields from H&E-stained lung sections (×1000). Data are representative of 3 independent experiments and expressed as mean ± SE (n=5–6 mice per group).

CONCLUSIONS AND SIGNIFICANCE

Some studies have identified selectins as potential targets for the design of novel anti-inflammatory agents, and research has focused on identifying the structural features required for high selectin binding affinity. Therapeutically viable P-selectin inhibitors have proved difficult to realize due to the weak binding of the monovalent ligands and high cost of producing physiological ligand-based inhibitors such as sLe^x or PSGL-1. Identification of critical interactions that occur between P-selectin and the physiologic ligand PSGL-1 served as a starting point for the design of our inhibitor. Although as an unmodified monosaccharide fucose has no reported P-selectin blocking activity, some sulfated carbohydrates and sulfated fuco-oligosaccharides do show inhibition. If fucose and sulfate could be presented to P-selectin in the proper orientation on a synthetic carrier, a relatively simple inhibitor could be designed. We and others have demonstrated that polyvalent display of fucose or fucosylated oligosaccharides in proximity to multiple sulfate ester groups creates a potent inhibitor for P-selectin-mediated adhesion. Our PLNPs display fucose and sulfate groups on a macromolecular structure created by mixing lipids with these molecules to direct self-assembly into liposome bilayers.

Design of new selectin inhibitors has been hampered by the lack of high-throughput physiologic assays that rigorously measure the potency of potential inhibitors

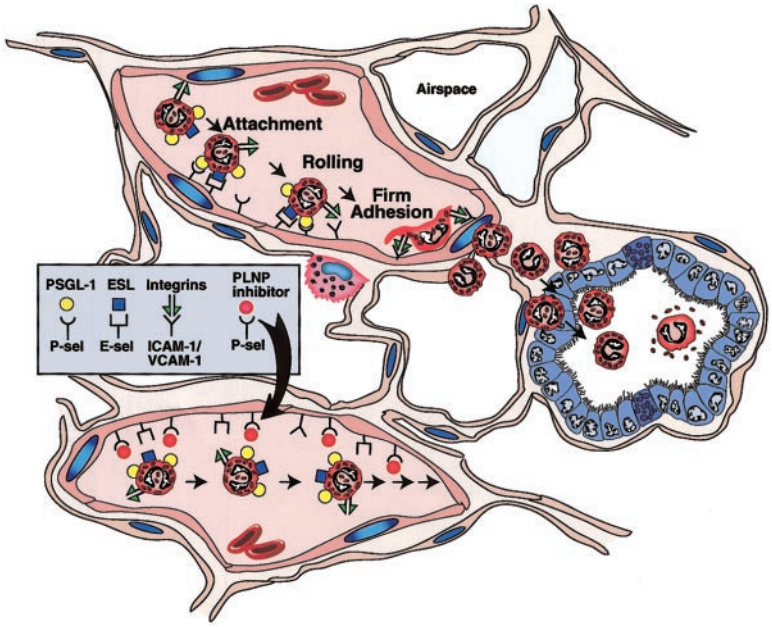

Figure 3. Inhibition of eosinophil recruitment by selectin-targeted nanoparticles. Eosinophil recruitment to peribronchial areas of the lung plays an important role in mediating airway hyper-responsiveness in allergic airway disease. Extravasation of eosinophils from the vasculature to the airways is preceded by P-selectin-mediated attachment and rolling of leukocytes on the endothelial cell surface. Inhibition of these processes by the binding of PLNP to P-selectin prevents the subsequent integrin-mediated firm adhesion of eosinophils to the endothelium, a prerequisite step for migration through the blood vessel wall and toward the airways.

under conditions that truly mimic blood flow. Many reported selectin inhibitors have typically been assayed under static conditions. While this may provide a starting point from which to design inhibitors, the inhibition measured under static conditions with this type of adhesion protein can be irrelevant when subjected to shear forces.

To simulate the microenvironment a drug and leukocyte would experience in vivo, we used an in vitro system where the luminal surfaces of capillary tubes were coated with P-selectin proteins. The "vessels" are integrated into a loop system in which fluid can be recirculated via a peristaltic pump. PSGL-1-expressing U-937 cells were injected into the system and their interaction with the protein monolayer was monitored by video microscopy. Potential P-selectin inhibitors are infused into the assay and their effect on leukocyte/protein interactions is readily measured. We have demonstrated that the PLNPs are targeted primarily to P-selectin on the endothelium and preferentially inhibit established P-selectin- but not E-selectin-dependent leukocyte rolling/attachment in vitro.

Our studies indicated that binding of nanoparticles to the endothelium was selectin-specific in both LPS- and CRA-induced inflammation and that the PLNPs remain in contact with the endothelium, acting as inhibitors of selectin-mediated leukocyte attachment and rolling over a prolonged period. The severity of allergic airway disease is primarily dependent on the extent of the inflammatory response, and attenuation of leukocyte infiltration in the lung is frequently correlated with clinical improvement. Studies of selectin involvement in the pathogenesis of allergic airway disease have identified a key role for E- and P-selectin; therefore, the efficacy of our selectin antagonists was tested in a murine model of this disease. P-selectin-specific PLNP significantly attenuated methacholine-induced airway hyper-responsiveness and peribronchial eosinophilia 24 h after allergen challenge. At 24 h, PLNP could still be identified in association with the endothelial cell surface in the lung vasculature, and it is conceivable that a single administration of PLNP may inhibit leukocyte recruitment for much longer.

In summary, these studies demonstrate the discovery of a P-selectin inhibitor with potent in vivo activity by using a physiologically relevant in vitro shear assay system. The use of novel glycomimetics to specifically deliver sterically inhibitory P-selectin binding nanoparticles to the endothelium may have therapeutic potential for allergy/asthma as well as other inflammatory diseases. FJ