Soluble P-Selectin Glycoprotein Ligand 1 Inhibits Ocular Inflammation in a Murine Model of Allergy

Erich C. Strauss,1,4 Kirsten A. Larson,1 Ina Brenneise,1 C. Stephen Foster,2 Glenn R. Larsen,3 Nancy A. Lee,1 and James J. Lee1

PURPOSE. To assess the anti-inflammatory modality of a soluble extracellular form of P-selectin glycoprotein ligand 1 (sPSGL-1) in a mouse model of ocular allergic response.

METHODS. Potential anti-inflammatory effects of sPSGL-1 were investigated in SWR/J mice sensitized by topical application of short ragweed pollen to the nasal mucosa followed by a challenge of the ocular mucosa with the same allergen. Five experimental groups were included in these studies: A, mice neither sensitized nor challenged with pollen (control group 1); B, animals sensitized but not challenged (control group 2); C, animals not sensitized but challenged (control group 3); D, animals sensitized and challenged; and E, sensitized animals treated with sPSGL-1 before pollen challenge. All experimental groups were evaluated for gross morphologic ocular changes, and histologic assessments were made to determine the onset/progression of inflammatory reactions and to look for evidence of eosinophil infiltration.

RESULTS. Mice sensitized and challenged with pollen developed clinical signs consistent with human allergic conjunctivitis. These signs correlate with histologic changes in the conjunctival epithelium and stroma (e.g., edema and extensive eosinophil infiltration). Moreover, the ocular changes also correlated with evidence of eosinophil degranulation. However, sensitized and challenged mice concurrently treated with sPSGL-1 displayed no inflammatory ocular changes associated with a ragweed-induced type-1 hypersensitivity reaction. The lack of ocular changes included the absence of histologic late-phase inflammatory changes of the conjunctiva and a 97% reduction in the presence of eosinophils, a major inflammatory cell in the IgE-mediated late-phase reaction. Current medical therapy for ocular allergic disorders is primarily symptomatic. We have used a murine model that closely resembles human allergic conjunctivitis to assess the efficacy of new treatment modalities and to study the pathophysiology of allergic inflammation.3 SWR/J mice develop clinical (conjunctival congestion and erythema) and histologic (eosinophil infiltration and edema) signs of allergic conjunctivitis after exposure to clinically relevant allergens such as short ragweed pollen. Because eosinophils are a hallmark of the late-phase response, the mechanisms that mediate their recruitment are integral to an understanding of ocular allergic inflammation.

Leukocyte recruitment involves the distinct phases of rolling, firm adhesion, and diapedesis. The selectin family consists of three transmembrane adhesion receptors that mediate the initial rolling interaction between leukocytes and the vascular endothelium before extravasation.4,5 P- and E-selectin are expressed differentially on activated endothelial cells, whereas L-selectin is constitutively expressed on leukocytes.6 The structural organization of the selectins is similar and includes an amino-terminal lectin domain that binds a variety of sialylated and fucosylated oligosaccharides.8 However, physiologically relevant ligands for the selectins appear to be restricted to a discrete group of cell surface glycoproteins.9 P-selectin glyco-
protein ligand 1 (PSGL-1) is a 220-kDa mucin-like transmembrane glycoprotein expressed on the cell surface of most leukocytes and a counter receptor for P-selectin. PSGL-1 has also been shown to bind E-10, 13-15 and L-selectin. 16-18 A soluble extracellular form of human PSGL-1 (sPSGL-1) has been engineered and expressed in COS cells, and in vivo studies in rodents have shown that it is capable of binding P- and E-selectin. Because rolling is an early event in leukocyte recruitment, the inhibition of selectin function is likely to disrupt the subsequent steps of firm adhesion and diapedesis of inflammatory cells. For example, gene-targeted mice lacking selectin expression display deficiencies in leukocyte rolling and leukocyte-dependent inflammatory responses. 20-25 In addition, recent evidence suggests that oligosaccharide competitors of specific selectin ligands can provide a protective effect against inflammation in animal models. 24 25 These findings offer persuasive support for the development of antiselectin therapeutic strategies to inhibit acute inflammatory responses. In this study, we assessed the efficacy of human sPSGL-1 to antagonize eosinophil recruitment and ocular allergic inflammation in the SWR/J mouse model.

**METHODS**

**Animals**

Eight-week-old female SWR/J mice purchased from Jackson Laboratories (Bar Harbor, ME) were maintained in microisolator cages housed in the specific pathogen-free animal facility at the Mayo Clinic Scottsdale. Mice were cared for in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. 26

**sPSGL-1**

sPSGL-1 (Genetics Institute, Cambridge, MA) is a recombinant soluble form of PSGL-1. sPSGL-1 represents the mature extracellular domain of PSGL-1 truncated at the isoleucine corresponding to position 316. This soluble ligand construct was coexpressed with fucosyltransferase in COS cells to mediate the necessary posttranslational modifications (i.e., glycosylation) necessary for selectin binding. The details of COS cell expression display deficiencies in leukocyte rolling and leukocyte-dependent inflammatory responses. A single lot of purified sPSGL-1 was used during this study.

**Experimental Design**

Mice were sensitized and challenged with short ragweed pollen, Ambrosia artemisiifolia, in powder form (International Biologicals, Piedmont, OK). Animals were sensitized with 2.5 mg of allergen by topical administration to the nasal mucosa for 6 consecutive days (days 0-6). The conjunctival inferior fornix of the right eye was subsequently challenged with 1.25 mg of allergen 3 days later (day 9). Pollen was delivered to the conjunctival sac with a micropipette under a stereodissecting microscope. Before challenge, mice were anesthetized with 50 μl of a 4:1 mixture of ketamine (100 mg/ml; Fort Dodge Laboratories, Fort Dodge, IA) to xylazine (20 mg/ml; Miles, Shawnee Mission, KS). Control groups for these experiments included animals neither sensitized nor challenged (control group 1), animals sensitized but not challenged (control group 2), and animals not sensitized but pollen challenged (control group 3). Mice of all groups received either an intraperitoneal (IP) injection of 300 μl of sterile phosphate-buffered saline (PBS) or were treated with 200 μg of sPSGL-1 in PBS 30 minutes before the ocular ragweed pollen challenge. The dosing schedule for sPSGL-1 was based on preliminary studies that have assessed inhibition of neutrophil recruitment in a mouse model of thioglycolate-induced peritonitis. During the 12-hour postchallenge period, groups of animals continued to receive IP injections of PBS (300 μL) or sPSGL-1 (200 μg in PBS) every 4 hours. Clinical signs of ocular inflammation were evaluated in room light under a stereodissecting microscope after 12 hours. The postchallenge time course corresponds to the characteristic peak interval for the allergic late-phase response. 27

**Histology and Immunofluorescence**

Mice were killed by carbon dioxide asphyxiation, orbits were exenterated, and ocular tissues were fixed in 10% buffered formalin for 24 to 48 hours at room temperature. Specimens were embedded in paraffin and 2-μm to 4-μm sections processed for light microscopy and immunofluorescence. Sections were stained with hematoxylin–eosin for light microscopy. Serial sections were placed on sialylated slides for immunofluorescence studies. Sections for immunofluorescence were deparaffinized using a xylene/ethanol/water gradient, treated for 30 minutes in 0.1% trypsin/0.1% calcium chloride (pH 7.5), rinsed well with water, and blocked overnight at 4°C in a mixture of PBS, 10% goat serum, and 0.1% sodium azide. The blocked tissue sections were rinsed in PBS, a rabbit antimurine eosinophil granule major basic protein polyclonal serum, 28 diluted (1:25) or prebleed serum (diluted 1:25) was applied, and the sections were incubated for 45 minutes. The slides were washed with PBS (3 × 5 minutes) and blocked with 0.1% Chromotrope 2R (J. T. Baker, Phillipsberg, NJ)/PBS solution for 30 minutes at room temperature. The sections were rinsed well in PBS and stained with fluorescein isothiocyanate-conjugated anti-rabbit IgG (diluted 1:40; Sigma, St. Louis, MO) for 30 minutes at 37°C. The slides were washed in PBS, and coverslips were mounted using a mixture containing 90% glycerol, 10% PBS, and 0.1% phenylendiamine (Sigma). Conjunctival tissues were visualized and photographed using a compound fluorescent microscope (Axiphot; Zeiss, Oberkochen, Germany).

**Blocking Studies Using Anti–P-Selectin and Anti–E-Selectin Monoclonal Antibodies**

Specific blocking of P- and/or E-selectin function was achieved in vivo through the IP administration of monoclonal antibodies. Anti–E-selectin antibodies (Rat IgG2b; 106G) and anti–P-selectin antibodies (Rat IgG1; 5H1) were gifts from Barry Wolitzky (Hoffmann-La Roche, Nutley, NJ). Each antibody was administered IP at a dose of 0.5 mg on day 9 of the sensitization/challenge protocol 2 hours before the ocular pollen challenge. Clinical signs of ocular inflammation were evaluated 12 hours after challenge.

**Statistical Analysis**

Differences between groups were analyzed by the two-sample t-test and the Wilcoxon rank sum test. A value of P < 0.01 was considered statistically significant.
RESULTS

Ocular Allergic Model to Assess the Anti-Inflammatory Efficacy of sPSGL-1

The effects of sPSGL-1 on eosinophil recruitment were determined using a modified version of a previously described experimental model of allergic conjunctivitis in SWR/J mice. The modification used limited the sensitization of the animals to ragweed pollen to topical treatment of the nasal mucosa (i.e., as opposed to both transnasal and ocular exposures). This modification thus separated the sensitization and challenge periods and restricted the recruitment of eosinophils to the conjunctiva to the post-ocular challenge period. The study divides mice into five experimental groups (n = 8 per group) designated A, B, C, D, and E, including three control cohorts. Group A consisted of SWR/J mice that were neither sensitized nor challenged with allergen (control group 1). Group B mice were sensitized but not challenged with allergen (control group 2). Group C consisted of mice not sensitized but challenged with allergen (control group 3). Group D mice were sensitized and challenged with ragweed pollen. Group E mice were also sensitized and challenged with ragweed pollen; however, these mice were treated with sPSGL-1 before being challenged with allergen. The data presented in this study were derived from a single cohort of 40 animals (i.e., 5 groups of n = 8) and a single lot of sPSGL-1; however, we have successfully repeated these studies using additional groups of animals (i.e., these experiments have been repeated with more than 5 additional groups of n = 6 animals) and 3 different lots of sPSGL-1 ligand.

sPSGL-1 Effects on the Clinical Signs of Ocular Allergic Inflammation

Figure 1 illustrates the appearance of the right eye of representative mice from each group. Animals that were neither sensitized nor challenged (Fig. 1A), sensitized but not challenged (Fig. 1B), and not sensitized but challenged (Fig. 1C) displayed a normal appearance, with the eye and periorcular regions failing to show any signs of irritation or inflammation. These data indicate that the modified sensitization protocol alone did not affect the clinical appearance of the ocular or periorcular structures. In striking contrast, group D mice that were sensitized and challenged displayed prominent gross changes to the conjunctiva and periorcular area (Fig. 1D). The changes include conjunctival hyperemia, lid redness and edema, and tearing, all classic signs of allergic conjunctivitis. However, as shown in Figure 1E, when mice were sensitized, treated with sPSGL-1, and challenged with ragweed pollen, the physical appearance of the right eye and its surrounding external structures was indistinguishable from the control groups.
FIGURE 2. Histologic evidence that sPSGL-1 inhibits allergic inflammation including eosinophil recruitment and degranulation. Histologic and immunofluorescence analyses of experimental groups A, B, C, D, and E described in Figure 1 are shown. Panels are representative 4-μm serial sections of the right (challenged) eye examined by hematoxylin–eosin staining (top panels), immunofluorescence with antimurine eosinophil MBP polyclonal antibody (center panels), and negative control prebleed serum (bottom panels). Pertinent ocular structures are displayed: conjunctival epithelium (c) and stroma (s), ciliary body (cb), and iris (i). Arrows in the hematoxylin–eosin section of (D) and (E) identify intact infiltrating eosinophils. Scale bars, 25 μm.

(Figs. 1A, 1B, 1C). The sPSGL-1-treated group showed no visible signs of irritation or inflammation. These gross observations suggested that sPSGL-1 treatment prevented allergic conjunctivitis and also implied that sPSGL-1 could represent a novel treatment for selectin-mediated allergic inflammation.

Histologic Effects of sPSGL-1 on Eosinophil Recruitment and Late-Phase Inflammation

Tissue sections of the right eye from groups A, B, C, D, and E were prepared for light and immunofluorescence microscopy to correlate macroscopic observations of the eye with histologic changes (Fig. 2). Serial sections were examined by hematoxylin–eosin staining (top panels), immunofluorescence staining with a rabbit polyclonal serum generated against the murine eosinophil major basic protein (mMBP) (center panels), and prebleed negative control serum (bottom panels). mMBP is an abundant protein found within the eosinophil secondary granule. The anti-mMBP polyclonal antibody permits the identification of infiltrating tissue eosinophils and provides evidence of eosinophil degranulation, leading to the extracellular deposition of MBP.26 Group A (unsensitized, unchallenged) mice illustrated the normal conjunctiva and adjacent ciliary body and iris. In the hematoxylin–eosin section (Fig. 2A, top), the conjunctival epithelium is a discrete 1 to 2 cell layer, and the conjunctival stroma appears as a compact structure. A few endogenous cells lacking characteristic features of eosinophils were evident in the subepithelial region of the stroma. Anti-mMBP staining (Fig. 2A, center) was indistinguishable from a serial section stained with the negative prebleed control (Fig. 2A, bottom), demonstrating that resident eosinophil populations were essentially absent from the normal conjunctiva. Group B (sensitized, unchallenged) and group C (unsensitized, challenged) animals showed that the normal structural characteristics of the conjunctival epithelium and stroma have been maintained (top panels in Fig. 2B and Fig. 2C, respectively), and there was no evidence of eosinophil infiltrate (center and bottom panels in Fig. 2B and Fig. 2C, respectively). We concluded that allergen sensitization by topical exposure of the nasal mucosa without ocular challenge or ocular challenge without prior sensitization did not alter the architecture of the conjunctiva. Neither treatment led to eosinophil influx of the eye or the development of allergic inflammation. However, group D mice (sensitized and challenged with ragweed pollen) exhibited dramatic and reproducible changes in histology (Fig. 2D, top). One of the most striking features was the overall expansion of the tissue dimensions as a consequence of edema associated with late-phase inflammation. The conjunctiva from group D occupies the entire frame and essentially excludes the ciliary body and iris from the visual field. In addition, a substantial eosinophil infiltrate was evident (Fig. 2D, top). The specific presence of eosinophils was confirmed by anti-mMBP immunofluorescence staining (Fig. 2D, center and bottom panels). Moreover, anti-mMBP immunofluorescence showed evidence of degranulation associated with the prominent eosinophil influx. Thus, eosinophil activation and deposition of secondary granule proteins occurred concomitantly with the late-phase inflammatory changes in the conjunctiva. In group E animals, sPSGL-1 administration abrogated the pronounced inflammatory changes associated with ragweed pollen sensitization and ocular chal-
FIGURE 3. Quantitative assessment of eosinophil cellularity in the conjunctiva of experimental groups A, B, C, D, and E. For each group, eight hematoxylin–eosin-stained right eye sections were counted in a blinded fashion by two investigators at a magnification of ×630. The number of eosinophils in each entire section were counted. *P < 0.01.

challenged. The conjunctival epithelium and stroma were normal in appearance, although a few eosinophils were apparent by hematoxylin–eosin (arrows in the top panel of Fig. 2E) and immunofluorescence staining (Fig. 2E, center and bottom panels). Interestingly, the anti-mMBP immunofluorescence showed only the intense focal labeling pattern of intact eosinophils without extensive degranulation, a finding consistent with the absence of late-phase inflammatory changes to the conjunctiva and periocular structures.

Quantitative Assessment of sPSGL-1 Administration on Eosinophil Recruitment

The eosinophil infiltrate associated with hematoxylin–eosin tissue sections was quantified for each experimental group to provide a more precise determination of the effects that sPSGL-1 had on eosinophil recruitment (Fig. 3). Mice that were neither sensitized nor challenged (group A) were shown to have only very low numbers of eosinophils in the periocular region. A comparison of group A with groups B and C (control groups 1, 2, and 3, respectively) revealed no statistical difference in the number of eosinophils in the conjunctiva. Thus, sensitization or challenge alone did not contribute to eosinophil influx into the conjunctiva. In contrast, mice sensitized and challenged (group D) showed an almost 500-fold increase in eosinophil numbers. However, treatment of sensitized and challenged mice with sPSGL-1 (group E) resulted in a 97% inhibition of eosinophil recruitment to the conjunctiva.

It is likely that inhibition of eosinophil recruitment in this model results from the blockade of E- and P-selectins. Figure 4 presents representative photomicrographs of hematoxylin–eosin–stained sections of sensitized and challenged mice that were also administered with either neutralizing anti-E-selectin antibodies (Fig. 4B), anti-P-selectin antibodies (Fig. 4C), or both anti-E- and anti-P-selectin antibodies (Fig. 4D). These data demonstrate that administration of either one of these antibodies alone had virtually no effect (relative to control animals, Fig. 4A) on conjunctival edema or the recruitment of tissue-infiltrating eosinophils. However, the coadministration of anti-E-selectin and anti-P-selectin monoclonal antibodies does provide significant inhibitory effects on ragweed-induced ocular inflammation, including the inhibition of eosinophil movement at levels similar to the effect achieved by the administration of sPSGL-1 (Fig. 4D versus Fig. 2E). These results provide strong evidence that sPSGL-1 is a potent antagonist of eosinophil recruitment and the late-phase response through the blockade of E- and P-selectins and suggest a potential therapeutic role for this agent in ocular allergic inflammation.

DISCUSSION

The presence of eosinophils in tissues is frequently associated with the pathogenesis of allergic inflammation. There is considerable evidence implicating eosinophils in the pathophysiology of allergic asthma, atopic dermatitis, eosinophilic gastroenteritis, allergic rhinitis, and the ocular allergic disorders, which include seasonal conjunctivitis, giant papillary conjunctivitis, vernal keratoconjunctivitis, and atopic keratoconjunctivitis. Consequently, preventing the accumulation of eosinophils may provide a therapeutic benefit in allergic states.
Leukocyte recruitment to sites of allergic inflammation involves a multistep sequence of receptor-ligand interactions and signaling events leading to the adherence of circulating leukocytes to the vascular endothelium. The selectin family of adhesion molecules mediates the initial rolling attachment of leukocytes to venular endothelial cells at inflammatory sites and, thus, the inhibition of these interactions prevents the subsequent steps of firm adhesion, diapedesis, and migration. Recent discussions of the physiological significance of PSGL-1 as a ligand for the selectins suggest that these receptor–glycoprotein ligand interactions are critical for leukocyte adhesion. The use of soluble cell-surface glycoproteins as therapeutic agents against viral infections (CD4 immunoadhesions for AIDS), a soluble form of intracellular adhesion molecule 1 against rhinovirus) and modulators of immune function (a soluble form of interleukin-1 receptor) have been investigated. Previous studies also suggest that competitors targeted against specific selectins may inhibit inflammatory responses. In a rat model of P-selectin–dependent lung injury, the intravenous infusion of sialyl-Lewis X containing oligosaccharides provided a protective effect against neutrophil-dependent inflammation. Furthermore, in mouse models of thioglycolate-induced peritonitis, the administration of a L-selectin immunoglobulin chimera and heparin oligosaccharides that bind L- and P-selectin reduced neutrophil recruitment. Because selectins have been implicated in pathologic leukocyte recruitment and inflammatory disorders, and because PSGL-1 can mediate adhesion interactions with the selectins, soluble forms of PSGL-1 may provide a therapeutic anti-inflammatory effect. In a recent study with sPSGL-1, a potential inhibitory role for the ligand was suggested in a rat model of renal ischemia/reperfusion injury.

Our data provide persuasive evidence that sPSGL-1 is an efficacious antagonist of eosinophil recruitment and ocular allergic inflammation in a mouse model system. The striking inhibitory effect of sPSGL-1 was presumed to be mediated by specific blockade of both P- and E-selectin because only the coadministration of anti-P-selectin and anti-E-selectin monoclonal antibodies mimics the inhibitory effect of sPSGL-1 in this model. Recently, however, L-selectin has also been shown to bind sPSGL-1, although at reduced affinity relative to P- and E-selectins (Larsen G, Sypek J, unpublished data). This observation suggests that appropriate sPSGL-1 dosage regimens may provide effective blockade of all three selectins. Preliminary pharmacokinetic studies with sPSGL-1 suggest a postadministration half-life of approximately 2 hours for the form of the ligand used in this study. The short term sPSGL-1 dosage protocol used in our investigation prevented the development of allergic late-phase inflammatory changes. Moreover, no adverse side effects were associated with the sPSGL-1 treatment regimen in our model of ocular allergic inflammation. Our study has demonstrated the efficacy of IP administration of sPSGL-1, although alternative routes of delivery to target tissues are of considerable interest. For example, although the intravenous infusion of sPSGL-1 has reduced neutrophil influx in a mouse model of thioglycolate-induced peritonitis, the subcutaneous administration of sPSGL-1 has achieved only minimal success in this inflammatory model (Larsen G, Sypek J, unpublished observations). In an ideal clinical setting, elimination of ocular inflammation through the competitive inhibition of selectins with sPSGL-1 would be achieved by topical administration of the eye. Investigations are currently in progress to address alternative drug delivery options. In addition, other constructs and chimeric forms of sPSGL-1 are being developed to enhance the duration of anti-inflammatory activity associated with this antagonist. With the rational design of alternative sPSGL-1 structures that are stable in vivo and permit specific delivery to appropriate target tissues, this strong inhibitor may have extensive applications beyond ocular allergic inflammation (e.g., acute allergic asthma therapy to inhibit eosinophil recruitment and pulmonary inflammation).

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