E-Selectin Ligands Mediate Tumor Necrosis Factor–Induced Neutrophil Sequestration and Pulmonary Edema in Guinea Pig Lungs

Siou K. Lo, Michael B. Bevilacqua, Arsal B. Malik

Abstract We have previously shown in perfused guinea pig lungs that tumor necrosis factor-α (TNF-α) pretreatment of lungs enhanced neutrophil sequestration as reflected by a 2.4-fold increase in lung myeloperoxidase (MPO) activity. Subsequent perfusion of phorbol 12-myristate 13-acetate (PMA) to activate the sequestered neutrophils produced an approximately threefold increase in the pulmonary capillary hydrostatic pressure and fulminant pulmonary edema. Using this ex vivo model of lung injury, we studied the role of three putative E-selectin ligands, sialyl–Lewis X, Lewis X, and dimeric sialyl–Lewis X, in mediating neutrophil sequestration and pulmonary edema. We pretreated neutrophils with monoclonal antibodies (mAbs) directed against these E-selectin ligands. Pretreatment of neutrophils with mAbs to sialyl–Lewis X and Lewis X reduced the neutrophil sequestration, as evidenced by 45% and 27% reductions in MPO activity from control levels, respectively. This occurred in parallel with inhibition of neutrophil adhesion to the TNF-α-activated endothelial cells in vitro. The mAbs to dimeric sialyl–Lewis X and an isotype-matched control mAb against lactosamines present on neutrophils had no effect on lung MPO activity and neutrophil adhesion. All mAbs to sialyl–Lewis X, Lewis X, and dimeric sialyl–Lewis X reduced the increases in the pulmonary capillary hydrostatic pressure after challenge of the sequestered neutrophils with PMA and also reduced lung weight gain by 71%, 45%, and 38%, respectively. The control mAb to the lactosamines had no effect on the pulmonary capillary hydrostatic pressure and lung weight gain. These data indicate that E-selectin ligands contribute to the TNF-α–induced neutrophil sequestration in lungs and that adhesive interaction between E-selectin and sialyl–Lewis X and its related carbohydrates is critical in the neutrophil-dependent increases in pulmonary vascular pressures and edema. (Circ Res. 1994;75:955-960.)

Key Words • E-selectin ligands • sialyl–Lewis X • vascular injury • adhesion • neutrophils

The vascular endothelium expresses E-selectin that interacts with specific carbohydrate structures to recruit neutrophils, monocytes, and a subset of T lymphocytes into sites of inflammation.1,2 The lectin domain on E-selectin is believed to recognize and bind to an array of specific carbohydrate moieties expressed on the neutrophil via a calcium-dependent mechanism.3 It is generally accepted that sialyl–Lewis X is the major recognition ligand for E-selectin.4 Monoclonal antibodies (mAbs) against sialyl–Lewis X or mucins containing this carbohydrate structure were shown to inhibit E-selectin–mediated neutrophil adhesion.5,4 Desialylation or removal of the fucose residues reduced leukocyte binding to E-selectin,5 indicating that both sialic acid and fucose are essential for E-selectin recognition. However, the data also point to the possibility that E-selectin can recognize and bind to an array of sialyl–Lewis X–related structures.5,6 In this regard, Lewis X (CD15) and dimeric sialyl–Lewis X (CD65) are both abundant on leukocyte plasma membrane,7 having been implicated in the binding to E-selectin, albeit at a lower affinity than sialyl–Lewis X.

We have previously shown that infusion of the cytokine (tumor necrosis factor-α [TNF-α]) in neutrophil-perfused guinea pig lungs markedly enhanced the neutrophil sequestration in the pulmonary vascular bed.8 The neutrophil uptake was dependent on the adhesive interaction between CD11/CD18 integrins on neutrophils and intercellular adhesion molecule-1 (ICAM-1) present on pulmonary vascular endothelial cells.8 Activation of the sequestrated neutrophils with phorbol ester resulted in the development of fulminant pulmonary edema,8 which was in part dependent on the ICAM-1/CD18 interactions because mAbs against CD11/CD18 or vascular ICAM-1 prevented the pulmonary edema. Since the adhesion of neutrophils is believed to involve a sequential adhesive event initiated by the E-selectin–induced upregulation of CD11/CD18 activity,9–11 we speculated that preventing adhesion of E-selectin to its carbohydrate ligands may prevent the lung neutrophil sequestration and lung injury. In the present study, we tested the functions of three putative E-selectin ligands, sialyl–Lewis X, Lewis X, and dimeric sialyl–Lewis X, in mediating TNF-α–induced lung neutrophil sequestration and edema formation.

Materials and Methods
Monoclonal Antibodies

Hybridomas of mAbs 1B2 (IgM, against type II lactosamine chains) and CSLEX1 (IgM, against sialyl–Lewis X, or sialyl–CD15) (Table) were obtained from American Type Culture
Carbohydrate Structures Recognized by Monoclonal Antibodies Used to Block Polymorphonuclear Leukocyte Sequestration in Tumor Necrosis Factor-α-Challenged Guinea Pig Lungs

<table>
<thead>
<tr>
<th>mAbs</th>
<th>Nomenclature</th>
<th>Structure</th>
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<tbody>
<tr>
<td>1B2</td>
<td>Type II chains</td>
<td>Gal(\beta)(_1)GlcNAc</td>
</tr>
<tr>
<td>PM81</td>
<td>Lewis X</td>
<td>Gal(\beta)(_1)GlcNAc</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fuc(_\alpha)(_1)-3</td>
</tr>
<tr>
<td>CSLEX1</td>
<td>Sialyl-Lewis X</td>
<td>NeuNAC(_\alpha)(_2)GlcNAc</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fuc(_\alpha)(_1)-3</td>
</tr>
<tr>
<td>VIM2</td>
<td>Dimeric sialyl-Lewis X</td>
<td>NeuNAC(_\alpha)(<em>2)GlcNAc,Glcp(</em>\alpha)(<em>1)GlcNAc,Glcp(</em>\alpha)(_1)Galp</td>
</tr>
</tbody>
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mAbs indicates monoclonal antibodies.

Collection. Ascites of these mAbs were prepared in mice by use of the standard protocols. Purification of murine IgM from the ascites fluid followed the manufacturer's (Windsor Park Laboratory Inc.) procedure. Purified mAb PM81 (IgM, against Lewis X, or CD15) was obtained from Medarex. mAb VIM2 (purified IgM, against dimeric sialyl–Lewis X, or CD65) was a generous gift from Dr. Knapp, AN.Der.Grub, Kaumberg, Austria. The carbohydrate structures recognized by these mAbs are detailed in the Table. All of these mAbs bind to human neutrophils as determined by flow cytometry within the concentration range of 1 to 10 μg/mL; we observed saturation binding at 10 μg/mL. Binding did not increase at higher concentrations of 20 to 40 μg/mL. We used the mAb-saturating concentration of 10 μg/mL in studies in vitro and 20 μg/mL in studies ex vivo. All mAbs (1B2, CSLEX1, PM81, and VIM2) were shown to bind abundantly to human neutrophils by flow cytometry (data not shown).

Materials

Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma Chemical Co. Recombinant human TNF-α was obtained from Cetus; the endotoxin concentration of the TNF-α preparation was <20 pg/mL by Limulus amebocyte lysate assay. The specific bioactivity of TNF-α was 2.4 × 10⁹ U/mg with 1 U TNF-α defined as the amount producing 50% cytoxicity of L929 cells in 18 hours at 37°C.

Adhesion Assay Neutrophils to Endothelium

E-Selectin–mediated adhesion of human neutrophils to the activated endothelium was carried out on Terasaki plates, detailed elsewhere. Human umbilical vein endothelial cells were isolated and grown on fibronectin precoated Terasaki plates for 2 to 3 days. On the day of the experiment, the cells were treated with TNF-α (200 U/mL for 4 hours), and excess TNF-α was removed by washing. Human neutrophils were isolated according to established methods, and the purity of neutrophils was >97% by morphological criteria. The adhesion assay involved adding freshly isolated neutrophils (1 × 10⁶ cells per milliliter, 10 μL) onto the endothelial cell monolayers with the following modification: the assay was performed at 4°C for 15 minutes to eliminate the CD11/CD18-dependent adhesion. Flow cytometry studies revealed the endothelial cells expressed E-selectin after TNF-α treatment (data not shown). Unbound neutrophils were removed by washing (three times), and adhesion was quantified by counting the bound neutrophils.

Guinea Pig Lung Preparation

The isolated/perfused guinea pig lung preparation has been described elsewhere. The surgical procedures were in accordance with institutional guidelines. In brief, the animals were anesthetized with pentobarbital sodium (50 mg/kg IP, Abbott Laboratories), and then the trachea was cannulated. The heart and lung were removed and suspended on a counterbalanced beam balance. Catheters were positioned in the pulmonary artery and left atrium and connected to pressure transducers. The lungs were perfused with a defined perfusate containing phosphate-buffered saline plus 0.5 g/L albumin. The perfusion rate was adjusted to 28 mL/min with a reservoir volume of 300 mL. The pulmonary capillary hydrostatic pressure was estimated by the double-occlusion method. Airway pressure of 1 cm H₂O was applied throughout the experiment to maintain lung inflation. The temperature of the perfusate was regulated at 37°C. This ex vivo lung preparation was stable for 4 to 5 hours, which allowed simultaneous continuous measurement of pulmonary artery pressure, capillary hydrostatic pressure, and the lung weight gain (a measure of continuous edema formation).

Experimental Protocols

The Ringer's albumin–perfused lungs were challenged with TNF-α (1000 U/mL) injected into the pulmonary arterial catheter. At the end of 90 minutes, freshly isolated human neutrophils (2 × 10⁶ cells) were infused slowly via the same catheter for 5 minutes. In mAb blockade studies, the neutrophils were pretreated with mAbs (concentration, 20 μg/mL) for 20 minutes on ice followed by 30 minutes at 37°C. In some experiments with mAbs, we determined the lung myeloperoxidase (MPO) activity as a measure of neutrophil sequestration (see below). In other experiments, the sequestered neutrophils were challenged with PMA (10⁻⁴ mol/L) to determine the effects of activation of the sequestered neutrophils on pulmonary hemodynamics and pulmonary edema formation.

Measurement of MPO Activity

Neutrophil sequestration in lungs was quantified by lung tissue MPO activity. Lungs were collected at the end of the experiment and homogenized on ice in hexadecyltrimethylammonium bromide. The samples were freeze-thawed three times. The tissue was centrifuged at 40000g for 10 minutes at 4°C, and the MPO activity of the supernatant was assayed with O-dianisidine dihydrochloride and hydrogen peroxide. The MPO activity was expressed as the change in absorbance per minute per gram lung tissue.

Statistical Analysis

Data are expressed as mean±SEM. Statistical analysis was performed by one-way repeated-measures ANOVA followed by multiple comparisons using Tukey's test. Statistical significance was accepted at P < .05.

Results

Reduction of E-Selectin–Mediated Adhesion by mAbs to Sialyl–Lewis X and Related Carbohydrate Structures In Vitro

mAbs to sialyl–Lewis X (sialyl–CD15), Lewis X (CD15), dimeric sialyl–Lewis X (CD65), and type II
Fig 1. Bar graph showing effects of monoclonal antibodies (mAbs) against putative E-selectin ligands on neutrophil (PMN) adhesion to tumor necrosis factor-α (TNF)-activated endothelial cells (ECs). Ctrl indicates control; SLe\(^a\), sialyl-Lewis X; and Le\(^a\), Lewis X. Cultured human umbilical vein ECs were pretreated with 200 U/mL TNF for 4 hours at 37°C before the addition of freshly prepared human PMNs. Adhesion assays were performed as detailed in "Materials and Methods." PMNs were incubated with various mAbs at a concentration of 10 \(\mu\)g/mL before the adhesion assay. Values are shown as mean±SEM from quadruplicates of four separate independent experiments. *P<.05 vs the no mAb group.

Fig 2. Bar graph showing the effects of monoclonal antibodies (mAbs) against putative E-selectin ligands on lung uptake of neutrophils (PMNs) after tumor necrosis factor-α (TNF) treatment as determined by alterations in lung tissue myeloperoxidase activity. Ctrl indicates control; SLe\(^a\), sialyl-Lewis X; and Le\(^a\), Lewis X. Guinea pig lungs were perfused with Ringer's albumin solution. In the TNF-challenged group, lungs received TNF (1000 U/mL) infusion for 90 minutes before the perfusion of human PMNs (2×10\(^7\) cells). In mAb-treated groups, PMNs were preincubated with various mAbs at 20 \(\mu\)g/mL for 20 minutes on ice, followed by 30 minutes at 37°C. Values are shown as mean±SEM from five independent experiments in each group. *P<.05 vs the no mAb group.

**E-selectin Ligands Mediate TNF-Induced Neutrophil Sequestration and Edema**

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MPO activity to 4.8±1.3 A·min\(^{-1}\)·g\(^{-1}\) (45% reduction from control), and Lewis X (mAb PM81) reduced the value to 6.3±0.7 A·min\(^{-1}\)·g\(^{-1}\) (27% reduction from control) (Fig 2). mAbs VIM2 (against dimeric sialyl-Lewis X) and 1B2 (against lactosamines) had no inhibitory effect after TNF-α challenge (10.9±2.0 and 8.8±0.3 A·min\(^{-1}\)·g\(^{-1}\), respectively) (Fig 2).

**Anti-E-Selectin Ligand mAbs Reduce TNF-α-Mediated Pulmonary Hypertension**

Challenge with PMA (10\(^{-9}\) mol/L) in lungs in which neutrophils were sequestered increased pulmonary arterial pressure. At 30 minutes after PMA, the pulmonary capillary pressure and the mean pulmonary arterial pressure increased approximately threefold, from 5.0±0.2 to 18.2±0.7 mm Hg and from 7.0±0.2 to 26.8±2.4 mm Hg, respectively. Pretreatment of neutrophils with mAbs to sialyl-Lewis X, Lewis X, and dimeric sialyl-Lewis X blunted the increases in the mean pulmonary capillary pressure (Fig 3). Neither pressure was reduced from control values (ie, no mAb group) in the presence of neutrophils treated with mAb to the lactosamines.

**Anti-E-Selectin Ligand mAbs Reduce Lung Edema Formation**

Fulminant lung edema developed in the guinea pig lungs after challenge of the sequestered neutrophils with PMA. The wet lung weight increased 5.3±0.3 g at 30 minutes after the phorbol infusion (Fig 4). Pretreatment of neutrophils with mAbs to sialyl-Lewis X, Lewis X, and dimeric sialyl-Lewis X significantly decreased the wet lung weight gain by 71% (to 1.6±0.6 g), 44% (to 2.9±0.6 g), and 37% (to 3.4±0.5 g), respectively. In contrast, the control mAb to the type II chains had a minimum effect.
Fig 3. Bar graphs showing temporal changes in mean pulmonary arterial pressure (P_{pa}) and pulmonary capillary hydrostatic pressure (P_{cp}). Guinea pig lungs were challenged with tumor necrosis factor-α (TNF-α, 1000 U/mL) for 90 minutes, followed by perfusion with control human neutrophils (no mAb). After 5 minutes, the sequestered neutrophils were challenged with phorbol 12-myristate 13-acetate (10^{-6} mol/L) added to the pulmonary artery catheter. TNF-α-challenged lungs were perfused with neutrophils pretreated with monoclonal antibody (mAb) to sialyl-Lewis X (antiSLex, n=5), neutrophils pretreated with mAb to Lewis X (anti-Le^x, n=5), neutrophils pretreated with mAb to dimeric sialyl-Lewis X (anti-dimeric SLe^x, n=5), and neutrophils pretreated with mAb to type II chains (anti-type II chain, n=5). Values are shown as mean±SEM. *P<.05 vs time-match control (no mAb) group.

Discussion

In a previous study, we have shown that TNF-α challenge of guinea pig lungs for 90 minutes caused marked neutrophil sequestration in the pulmonary vascular bed as reflected by twofold to fourfold increases in the lung tissue MPO activity. The lung uptake of neutrophils was shown to be dependent in part on the adhesive interaction between ICAM-1 expressed on the endothelium and the CD11/CD18 integrins present on neutrophils. In the present study, we show that the E-selectin ligands (ie, sialyl-Lewis X and Lewis X) participate in mediating neutrophil sequestration in the pulmonary vascular bed. The conclusion is based on the use of mAbs against the putative E-selectin ligands. Monoclonal antibodies against both sialyl-Lewis X and Lewis X attenuated the increase in lung neutrophil uptake as measured by the tissue MPO activity. The ability of these mAbs to reduce the neutrophil sequestration ex vivo was correlated with their ability to inhibit E-selectin–dependent adhesion of neutrophils to the vascular endothelial cells in vitro. Although sialyl-Lewis X and Lewis X antibodies reduced neutrophil adhesion in the TNF-α–treated vascular endothelial cells as well as the increase in MPO activity, the effect of the Lewis X antibody was less than that of the sialyl-Lewis X antibody. This finding is consistent with the hypothesis that sialyl-Lewis X is the major carbohydrate on neutrophils recognizing E-selectin. Although we have not measured the binding affinities, the binding of sialyl-Lewis X to E-selectin is greater than the binding of Lewis X. Therefore, the greater inhibitory effect of anti-sialyl-Lewis X mAb on neutrophil adhesion and sequestration is in accord with the high affinity of sialyl-Lewis X to E-selectin.

The anti–dimeric sialyl-Lewis X mAb did not affect neutrophil sequestration, although it did reduce to some extent neutrophil adhesion. Evidence from liposome studies suggests that dimeric sialyl-Lewis X may be an alternate ligand for E-selectin. Although dimeric sialyl-Lewis X may bind to E-selectin with a relatively low affinity under static conditions, the present results suggest that it may not play a critical role in neutrophil interaction with E-selectin under shear stress conditions encountered in the intact pulmonary microcirculation.

Previous studies indicate that mAb against CD11/CD18 prevented the neutrophil sequestration and that a cross-reacting anti–ICAM-1 mAb caused ~50% reduction in neutrophil uptake. In the present study, mAbs against E-selectin ligands also provided a partial inhibition in neutrophil sequestration. These data are compatible with the hypothesis that neutrophil sequestration in the lungs requires the adhesive interaction of E-selectin and CD11/CD18 occurring in a sequential manner. We have shown that E-selectin–mediated binding of neutroph-
Pulmonary hypertension and edema were evident after PMA challenge in lungs in which the neutrophils were sequestered in the pulmonary microcirculation as a result of TNF-α pretreatment. The increase in lung weight may be the consequence of both increased pulmonary capillary hydrostatic pressure and vascular endothelial permeability. In the present study, we observed that the increases in pulmonary capillary hydrostatic pressure and lung water content were reduced after PMA challenge when the neutrophils had been pretreated with the anti–sialyl–Lewis X antibody, anti–Lewis X antibody, or anti–dimeric sialyl–Lewis X antibody. Pulmonary capillary hydrostatic pressure and lung water content did not decrease significantly from the control group after PMA challenge in lungs perfused with neutrophils pretreated with control mAb to the type II chains. In the case of the mAbs to sialyl–Lewis X and Lewis X, the degree of protection was related to the ability of these antibodies to reduce the neutrophil sequestration. The adhesion may provide a close proximity of the neutrophils and the endothelial cells and thereby enhance the degree of neutrophil activation or allow the accumulation of mediators (eg, oxygen radicals and protease) implicated in the pathogenesis of vascular injury and edema formation. However, since the mAb to dimeric sialyl–Lewis X did not reduce neutrophil uptake but significantly reduced the pulmonary capillary hypertension and lung weight gain from the control group, the protective effect of this mAb cannot be solely attributed to reduction in neutrophil sequestration. This mAb may have other effects such as inhibition of thromboxane production by neutrophils, which has been implicated in the development of pulmonary hypertension and edema.

Mulligan et al have recently shown that IgG immune complex deposition in rat lungs was dependent on E-selectin expression and was inhibited by an anti–E-selectin antibody. Gundel et al have also indicated a role for E-selectin in the pathogenesis of acute airway inflammation and late-phase airway obstruction in the primate model of asthma. These studies provide evidence for the involvement of E-selectin in inflammatory diseases. The present study indicates that E-selectin ligands are also involved in the pathogenesis of lung neutrophil uptake and vascular injury. These data point to the essential contribution of sialyl–Lewis X and Lewis X in the mediation of neutrophil sequestration and edema formation possibly through the interaction of these E-selectin ligands with the E-selectin on TNF-α–activated pulmonary vascular endothelial cells. In light of the evidence that E-selectin causes neutrophil rolling on the endothelium, we would predict that both anti–sialyl–Lewis X and Lewis X mAbs would exert their protective effects by inhibiting neutrophil rolling as in the case of anti–E-selectin mAbs.

The lack of cross-reacting anti–E-selectin mAbs in the guinea pig did not allow us to assess the role of E-selectin in this model. However, E-selectin may have been expressed in guinea pig lung microvessels after TNF-α challenge, because data in cultured human endothelial cells and in intact microvessels of rats and primates indicate that upregulation of E-selectin can occur within a 1- to 2-hour period after cytokine challenge and decreases to control levels after 4 hours. A 5-minute period of TNF-α challenge of the guinea pig

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**Fig. 4.** Bar graphs showing changes in lung weight gain in guinea pig lungs that received 90 minutes of tumor necrosis factor-α challenge, followed by perfusion of lungs with control neutrophils (no monoclonal antibody [no mAb], n=5), neutrophils pretreated with mAb to sialyl–Lewis X (anti-SLeα, n=5), neutrophils pretreated with mAb to Lewis X (anti-Leα, n=5), neutrophils pretreated with mAb to dimeric sialyl–Lewis X (anti-dimeric SLε, n=5), and neutrophils pretreated with mAb to type II chains (anti–type II chains, n=5). Values are shown as mean±SEM. *P<.05 vs control (no mAb) group.
lungs did not result in sialyl-Lewis X–dependent neutrophil uptake in these lungs (data not shown), further consistent with the finding that E-selectin expression is a time-dependent process requiring de novo protein synthesis.1

In summary, the present data indicate that the sialyl-Lewis X and Lewis X antigens contribute to the neutrophil sequestration in TNF-α–challenged lungs. Both antigens are also important in the development of pulmonary hypertension and edema, probably by the binding of these carbohydrates to E-selectin expressed on TNF-α–activated pulmonary vascular endothelium.

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References


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