EFFECTS OF IN VIVO SOLUBLE SELECTIN GENE INTRODUCTION ON LPS-INDUCED LEUKOCYTE ACCUMULATION IN THE MURINE LUNG

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Abstract—The selectin family adhesion molecules exert a crucial role in accumulation of leukocytes at the site of inflammation. To test the biological effects of soluble selectin on lung inflammation, we introduced an expression plasmid vector of soluble selectin gene via HVJ-liposome into a murine model of LPS-induced lung injury. The myeloperoxidase activity in LPS-injected mice was suppressed by the in vivo injection of soluble P-selectin gene relative to control mice. On the contrary, soluble E- or L-selectin genes did not exert suppressive effects. Our observations suggest that P-selectin plays a crucial role in the initial steps of lung inflammation, and exogenous introduction of soluble P-selectin by in vivo gene transfer method may be a useful strategy for regulating inflammation of the lung.

INTRODUCTION

Inflammatory reactions play a critical role in host defense mechanisms, but excessive and sustained inflammation leads to various illnesses. In the lung, endotoxemia, aspiration pneumonia or exposure to an elevated concentration of oxygen induces serious pathologic inflammatory reactions, such as acute lung injury (ALI) or adult respiratory distress syndrome (ARDS). The pathogenesis of ALI or ARDS has not been fully clarified, but it is suggested that excessive accumulation of activated neutrophils and release of oxygen radicals, proteinases and arachidonic acid derivatives from the cells are crucial to the pathophysiology of the diseases (1). To ameliorate the lung injury, trials to regulate activity of oxygen radicals and arachidonic acid derivatives have been successfully conducted (1, 2). The outcomes of ALI and ARDS have improved, but the mortality rate is still high (3). Hence, it is necessary to evaluate the therapeutic effects of...
new strategies. One potential strategy may be anti-adhesion therapy. Leukocyte accumulation takes place in the initial step of inflammation, and regulation of this step may potentially regulate the cascade leading to lung injury (4–6). In inflammatory responses, adhesion molecules and humoral factors are involved in accumulation of leukocytes (7, 8). The initial steps in leukocyte emigration in response to inflammation involve 1) rolling, 2) adhesion, and 3) transmigration. In rolling, leukocytes intermittently adhere to vascular endothelial cells by interaction between members of the selectin family of cell surface adhesion molecule and their ligands. The selectin family includes L-, E-, and P-selectins. L-selectin is expressed constitutively on the majority of leukocytes, and the counter-receptors of L-selectin on endothelial cells are sialomucin glycoproteins, GlyCAM-1, CD34 and MAdCAM-1. E-selectin is expressed on the cell surface of the activated endothelial cells, and P-selectin is found on activated endothelial cells and platelets. The counter-molecules of E- and P-selectin have structural features of sialylated Lewis X (sLeX), and they are designated E-selectin ligand-1 (ESL-1) and P-selectin glycoprotein ligand-1 (PSGL-1), respectively (9, 10). Introduction of anti-selectin antibodies or selectin-IgG chimera molecules inhibited the various types of experimental lung injuries (11–14). These observations suggest that inhibition of binding of selectins to their counter-molecules may prevent lung injury. Hence, introduction of soluble selectin molecules may regulate leukocyte accumulation, thus inhibiting it. In this study, we introduced the expression vector of soluble selectin genes into the murine model of lipopolysaccharide (LPS)-induced lung injury (14) by hemagglutinating virus of Japan (HVJ)-liposome (15), which is an effective in vivo gene transfer method. To evaluate in vivo the anti-inflammatory effects of this strategy, we tested various parameters of lung inflammation.

MATERIAL AND METHODS

Construction and Plasmid Vector. Extracellular domain of human P-, gift of Roger P. McEver, University of Oklahoma, (16), E-, gift of Tucker Collins, Harvard Medical School, (17) and L-, gift of Thomas F. Tedder, Duke University Medical Center, (18) selectin cDNAs were synthesized by polymerase chain reaction (PCR) with primers depicted as follows:

- P-selectin sense primer; 5'-GAGATGGCCAACCTGCCAAATAGCC-3',
- P-selectin antisense primer; 5'-TCAGGCTCCTGGATAGTCAATGG-3';
- E-selectin sense primer; 5'-CTTGAAGTCATGATTGCTTCACAG-3',
- E-selectin antisense primer; 5'-TCAGGGAATGTTGGACTCAGT-3';
- L-selectin sense primer; 5'-TCAGGGAATGTTGGACTCAGT-3',
- L-selectin antisense primer; 5'-TCAGGGAATGTTGGACTCAGT-3'.

The sense primers were designed to include upstream sequences of initiation codons, and antisense primers were designed to include upstream sequences of the transmembrane portion of
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Fig. 1. Design of PCR primers to amplify the extracellular region of selectin cDNA. Thin line represents selectin cDNA. Translated region of cDNA is shown by thick line and transmembrane region is shown by open box. Right and left arrows denote sense and antisense primers, respectively.

selectin genes (Figure 1). Reaction mixture contained 1× PCR buffer (Takara, Kyoto, Japan), 0.2 mM of each deoxynucleotide triphosphate, 400 mM sense and antisense primers and 1.25 U Taq Polymerase (Takara, Kyoto, Japan) and cDNA in 50 μl. Amplification was performed in Thermal Cycler MP (Takara, Kyoto, Japan) for appropriate cycles (denaturation at 94°C for 90 s, annealing at 60°C for 30 sec, extension at 72°C for 1 min).

The PCR product was cloned into pCAGGS expression vectors (19) which contain the chicken beta-actin promoter, and soluble P-, E-, and L-selectins were designated pCA-sP-sel, pCA-sE-sel, and pCA-sL-sel, respectively (Figure 2). The plasmids were grown in Escherichia coli JM109, prepared using Qiagen Endofree Plasmid Mega Kit (Chatsworth, California). To confirm the presence of human soluble selectin expression, the vectors were transfected into A549, a human lung adenocarcinoma cell line, using FuGENETM6-Transfection Reagent (Boehringer Mannheim, Germany). Concentration of each selectin in the culture supernatant was measured by enzyme-linked immunosorbent assay (ELISA) (Bender MedSystems, Vienna, Austria). The concentrations of soluble P-, E-, and L-selectins in the culture supernatant were 7.3, 19.7, and 5.6 ng/ml, respectively. In the culture supernatant of untransfected A549 cells, the concentration of each selectin was below the sensitivity of ELISA.

Fig. 2. Structure of soluble selectin expression vector. (pCA-sP-sel, pCA-sE-sel, pCA-sL-sel).
Hemagglutinating Virus of Japan (HVJ)-Liposome Preparation. Hemagglutinating Virus of Japan (HVJ)-liposome was prepared as previously described (15). Briefly, lipids, phosphatidylcholine, phosphatidylserine and cholesterol were mixed at a ratio of 24:5:10 (w/w/w). The lipid mixture (10 mg) in tetrahydrofuran was placed in a rotary evaporator. Plasmids and high mobility group-I (HMG-I) proteins were incorporated into liposomes by shaking and sonication. The liposomes and HVJ, which were inactivated by ultraviolet irradiation (198 mJ/cm²), were incubated at 4°C for 10 min and then at 27°C for 60 min with gentle shaking. This solution was centrifuged on a sucrose gradient. The top layer was collected and diluted with balanced salt solution (BSS).

Animals. Seven-week-old male C3H/HeN mice were purchased from SLC Japan, Inc. (Hamamatsu, Japan) and were kept under specific pathogen-free conditions. HVJ-liposome containing pCA-sP-sel, pCA-sE-sel, or pCA-sL-sel was intraperitoneally injected in a total volume of 0.3 ml, which contained about 5 to 10 µg of plasmid DNA. In control mice, the same volume of HVJ-liposome suspension containing BSS was injected. Three days after the HVJ-liposome injection, the mice were challenged with 2 mg/ml of lipopolysaccharide (LPS), and they were sacrificed 0, 1, 3, and 7 h after the LPS challenge by inhalation of sevoflurane (Maruishi Pharmaceutical Co., Ltd., Osaka, Japan). The lungs were excised after perfusing with 8 ml of 0.1 M of pH 7.0 of K₂HPO₄ buffer. The right lung was used for assessing myeloperoxidase (MPO) activity, and the left lung was fixed with 10% buffered formaldehyde, and 2 µmeter thick sections were stained with hematoxylin/eosine and analyzed pathohistologically.

Determination of Myeloperoxidase (MPO) Activity. MPO activity was determined as previously described (20). Briefly, the right lung was homogenized with 1.5 ml 0.1 M (pH 7.0) K₂HPO₄ buffer by Polytron (Kenematica AG, Luzern, Switzerland), and the homogenate was centrifuged at 40,000 G for 30 min. The ppt was resuspended in 1 ml (pH 7.0) K₂HPO₄ buffer, and sonicated by Sonifier B-12 (Branson Sonic Power Company, Danbury, Connecticut) at level 7 for 90 s. The suspension was then centrifuged at 12,000 G for 10 min, and 0.1 ml of the supernatant was added to 0.3 ml 0.25% BSA containing HBSS, 0.25 ml 0.1 M K₂HPO₄ buffer pH 7.0, 0.05 ml 1.25 mg/ml o-dianisidine, and 0.05 ml 0.05% H₂O₂. The mixture was incubated at 25°C for 15 min, and then the reaction was stopped by adding 0.05 ml 1% NaN₃. The absorbance was measured at 460 nm.

Flowcytometric Analysis. Three days after intraperitoneal injection of pCA-sP-sel or BSS by HVJ-liposome, heparinized blood was drawn from the mice. The whole blood cells were incubated with FITC-labeled anti-human P-selectin antibody (Southern Biotechnology Association, Inc., Birmingham, Alabama) at room temperature for 30 min. The red blood cells were removed by hemolysis, and fluorescence was determined by FACSort (Becton Dickinson, New Jersey).

Titration of Serum TNF-α Activity. Before, and 2 to 4 h after LPS challenge, serum was obtained from mice transfected with pCA-sP-sel or BSS by HVJ-liposome. TNF-α activity of the serum was titrated with mouse TNF-α ELISA kit (Genzyme, Cambridge, Massachussetts).

Statistical Analysis. All data were expressed as mean ± SD. The significance for the difference between the groups was assessed using the Student t test. Values of P < 0.05 were considered significant.

RESULTS

We tested the effects of soluble P-selectin gene expression on MPO activity in LPS-injected mice. The expression plasmid vector pCA-sP-sel or BSS was intraperitoneally introduced by the HVJ-liposome method. Three days after injection, the mice were challenged with 2 mg/kg LPS or BSS, and MPO activity was measured 3 h after challenge (Figure 3). The increase in MPO activity after
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Fig. 3. Myeloperoxidase levels in perfused lungs of mice that received control HVJ-liposome + LPS, sP-sel HVJ-liposome + LPS, control HVJ-liposome alone, or sP-sel HVJ-liposome alone. On day –3, mice were ip injected with BSS encapsulated in HVJ-liposome (BSS) or with soluble P-selectin expression vector plasmid encapsulated with HVJ-liposome (sP-sel). On day 0, mice were ip injected with 2 mg/kg LPS (LPS +) or buffer (LPS –). Myeloperoxidase activity was measured 3 h after LPS challenge. Bars represent mean absorption + SD. *: P < 0.05.

LPS challenge was significantly reduced by pCA-sP-sel introduction compared to the BSS treated controls. In both control and pCA-sP-sel introduced mice, MPO activity significantly increased after LPS challenge compared to the activity before. To test the effects of in vivo gene introduction, MPO activity without LPS injection was also tested. The MPO activity in the pCA-sP-sel introduced murine lung was not significantly different from the activity of the control mice (data not shown).

The activity of other classes of selectin family molecules was also tested. Soluble E- and L-selectins slightly reduced MPO activity, but no significant differences were found between the activity in tested versus control animals. To test whether E- and P-selectin exert any synergistic effect, we introduced 300 µl each of P-sel and sE-sel genes into mice (Figure 4). The activity of co-introduced sP-sel and sE-sel genes was not different from the activity of sP-sel alone, and no synergistic activity was observed.

The chronological change in MPO activity after soluble P-selectin gene introduction was tested (Figure 5). One hour after LPS challenge, both groups of
Fig. 4. Effects of LPS-stimulation on the lung pretreated with soluble selectin gene introduction. *: \( P < 0.05 \). Control; BSS-HVJ-liposome, sP-sel; soluble P-selectin, sE-sel; soluble E-selectin, sL-sel; soluble L-selectin. Three hundred micro liters sL-sel HVJ-liposome, sE-sel HVJ-liposome, or both were ip injected into the mice. Bars represented mean absorption + SD.

Fig. 5. Change in myeloperoxidase levels of perfused lungs of mice that received control HVJ-liposome or sP-sel HVJ-liposome followed by LPS injections. *: \( P < 0.05 \) v.s. control group. Open circles show soluble P-selectin HVJ-liposome treated mice. Closed circles show control HVJ-liposome treated mice.
Animals showed an increase in MPO activity. Three hours after LPS challenge, the MPO activity of the sP-sel treated group was significantly reduced compared to the control group. However, the reduction was transient, and 7 h after LPS challenge, the MPO activity increased in the pCA-sP-sel treated group. There was no significant difference compared to the control group.

To confirm the results of MPO activity, pathohistologic changes were tested (Figure 6). In the murine lung three hours after LPS challenge, marked cellular infiltration, most of which was polymorphonuclear cells, was observed (Figure 6-a). On the contrary, cellular accumulation induced by LPS challenge was markedly suppressed in the lung of the sP-sel introduced group (Figure 6-b). Peroxidase staining of the lung section showed that most polymorphonuclear cells in the lung were neutrophils. Introduction of pCA-sP-sel without LPS challenge did not induce any notable pathohistologic change (data not shown).

Soluble human P-selectin expression in vivo was confirmed by flowcytometric analysis. Three days after intraperitoneal sP-sel introduction, peripheral blood cells were stained with FITC-labeled anti-human P-selectin antibody, and
In vivo soluble human P-selectin expression. Three days after intraperitoneal sP-sel introduction, peripheral blood cells were stained with FITC-labeled anti-human P-selectin antibody, and the signals on the cell surface of the leukocyte were analyzed by FACSort.

On the cell surface of the leukocytes from the sP-sel introduced mice, human P-selectin molecule was detected (Figure 7).

To assess the effect of soluble selectin gene introduction, serum TNF-α concentration, which is one of the major inducers of selectins, was tested. The level of serum TNF-α was transiently elevated after LPS challenge, as shown in the closed symbols in Figure 8. The serum TNF-α level of pCA-sP-sel introduced mice did not differ from the serum level of control mice.

**DISCUSSION**

Our investigation has shown evidence that P-selectin is crucial for rolling in inflamed lung tissue, and expression of soluble P-selectin molecule by in vivo gene transfer method effectively delivered soluble P-selectin molecules into the lung for attenuation of leukocyte accumulation in the lung. As previously reported, selectin family molecules have a principal role in leukocyte accumulation at the site of inflammation. The evidence suggests that P-selectin is the most essential of the selectin family molecules for lung injury induced by LPS. In our experimental system, dramatic reduction in leukocyte accumulation was observed 3 h after LPS injection, and it did not persist more than 7 h. These results suggest that P-selectin exerts its activity in the early stages of inflammation. Mayadas et al. reported that P-selectin-deficient mice generated by
gene targeting in embryonic stem cells exhibited virtually no leukocyte rolling in mesenteric venules, and delayed recruitment of neutrophils to the peritoneal cavity upon experimentally induced inflammation (21). Our present investigation supports these findings on the role and biological characteristics of P-selectin.

The three classes of selectins have similar domain structures, those being an amino-terminal domain that is homologous to calcium-dependent animal lectins, followed by an EGF-type domain, repeats of a complementary regulatory protein domain, and a short cytoplasmic tail site. Hence, their biological role in inflammation is common to the selectin family, but each selectin has its own mode of expression (9, 17, 22). L-selectin is constitutively expressed on the surface of leukocytes, and it is shed from the cell surface by the activation of protein kinase C (22). Constitutive expression of E-selectin is minimal, although by stimulation with endotoxin or cytokine, its expression is induced on the surface of endothelial cells. The E-selectin mRNA expression is NF-kB dependent, and the level peaks 4 to 6 after stimulation (17). P-selectin contrasts with these two classes of selectins. The transcription level of P-selectin is constitutive and the gene product is stored in Weibel-Palade bodies of the endothelial cells, or a-granules of platelets. By degranulation, P-selectin is expressed on the cell surface 2 to 5 min after stimulation by histamine or thrombin. The duration of expression is short and it rapidly disappears from the cell surface (9). Evidence from earlier studies suggested that P-selectin regulates binding of leukocytes to vascular endothelial cells at the initial steps of inflammation. It may be pertinent that as the process of inflammation advances, de novo synthesized E-selectin is expressed, and the key molecule in inflammation may switch from P-selectin to E-selectin.
The findings of our present investigation showed that the expression of soluble P-selectin features was rapid and transient. The results were compatible with the mode of expression of P-selectin, which were reported in earlier investigations.

In this investigation, the inhibitory effect of E- or L-selectin was weak or minimal on neutrophil accumulation in the lung induced by LPS. The difference in the intensity of inhibitory effects may be explained in part by the amount of ligand. The principal ligand of P-selectin is PSGL-1, which is highly specific for P-selectin, and P-selectin weakly binds sLeX in the absence of PSGL-1. On the other hand, the specificity of E-, or L-selectin for the ligand is not as limited as P-selectin. E-selectin can bind to sLeX on various glycoproteins or glycolipids, and it can also bind to sLeX alone (9), and L-selectin can bind to glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1), CD34 or MAdCAM-1 (9). This evidence suggests that excess amount of ligand for E- or L-selectin may be present at the site of inflammation. The amount of ligand expressed by our gene transfer system may not have been enough to inhibit the activities of E- or L-selectin. It has also been reported that soluble forms of E-selectin directly promote neovascularization at the site of inflammation of rheumatoid arthritis, malignant tumor or healing site of a wound by binding sugar chain (23). This observation suggests that soluble selectin does not act as antagonist for E-selectin. Further studies are needed to clarify whether the soluble E-selectin can act as antagonist.

TNF-α is one of the most important cytokines, and it induces expression of P- and E-selectin (24, 25). In vivo P-selectin gene introduction did not affect the serum TNF-α expression, and our present investigation did not show the presence of a negative-feed back circuit. Moreover, by flowcytometric analysis, human P-selectin was detected on the cell surface of murine leukocytes. These findings suggest that human soluble selectin binds to ligand on the surface of murine leukocytes. Thus the major mechanism of suppression of leukocyte accumulation in the lungs by soluble P-selectin may be competitive inhibition of the P-selectin ligand. However, the contribution of molecules other than P-selectin has not been ruled out by our study, and further investigation is needed. Investigation of the selectin family, and the strategy of in vivo soluble selectin gene introduction may lead to development of a new field of antiinflammatory therapy in the lung.

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