Role of E-selectin in bleomycin induced lung fibrosis in mice

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Received 19 April 1999
Returned to authors 28 June 1999
Revised manuscript received 18 October 1999
Accepted for publication 20 October 1999

Abstract

Background—Bleomycin (BLM), a well known anti-cancer drug, often causes acute lung injury and fibrosis by mechanisms that are not well understood. It is suspected that some proteases and active oxygen species generated from inflammatory cells cause the lung injury and subsequent lung fibrosis. It was therefore hypothesised that inhibition of adhesion of inflammatory cells to the endothelium might prevent these developments.

Methods—BLM (100 mg/kg) was injected into the tail veins of ICR mice to evaluate the induction of E-selectin, an adhesion molecule known to induce neutrophil attachment on endothelial cells. E-selectin mRNA induction was detected by reverse transcriptase polymerase chain reaction (RT-PCR). The myeloperoxidase (MPO) activities in the lung tissues of BLM treated and control mice were compared to evaluate neutrophil infiltration. Pathological changes in the lungs of soluble E-selectin transgenic mice (TG) and their TG negative (non-TG) littermates after BLM treatment were also compared. Serum samples of TG mice and non-TG mice were tested for their ability to block the binding of sialyl Lewis^x to recombinant E-selectin in vitro.

Results—E-selectin mRNA was maximally induced at six hours after BLM treatment in the ICR mice. The soluble form of E-selectin which can competitively inhibit the binding of sialylated antigens on inflammatory cells to E- and P-selectins on the endothelium was detected in the serum of TG mice. BLM induced lung fibrosis occurred in non-TG mice but not in TG mice. This result confirms the finding that the serum of TG mice inhibits the binding of sialyl Lewis^x to E-selectin in vitro.

Conclusion—E-selectin plays an essential role in BLM induced lung fibrosis through the induction of neutrophil and other inflammatory cell accumulation, and soluble E-selectin may be of use in the prophylactic treatment of lung fibrosis.

(*Thorax* 2000;55:147–152)

Keywords: E-selectin; sialyl Lewis^x; bleomycin; lung fibrosis

Bleomycin (BLM) lung injury and subsequent lung fibrosis in animals is a widely used experimental model of acute lung injury and fibrosis in humans.[1–3] The mechanisms of this lung injury and fibrosis, however, are not yet understood in detail. The progression of lung fibrosis is predominant after intravenous administration of BLM, indicating that the subpleural area of the lung is more susceptible to BLM induced fibrosis than the hilar area. Some proteases and active oxygen species generated from leucocytes have recently been reported as causing lung injury and fibrosis.[4–7] Leucocytes migrate into inflamed tissues in response to several stimuli. The first phase of the cascade of leucocyte adhesion to activated endothelium is initiated by weak binding mediated by selectins which cause leucocytes to roll along the inflamed endothelium.[8 9] Selectins induced on endothelium bind sialyl Lewis^x oligosaccharides, which are associated with E-selectin ligand-1 (ESL1),[10] and P-selectin glycoprotein ligand-1 (PSGL-1), which are expressed on leucocytes.[11] During the next phase leucocyte adhesion depends upon firm integrins binding to their ligands, including immunoglobulin superfamily members, thus promoting inflammatory cell migration to the inflamed sites.[12] We therefore hypothesised that inhibition of the first step of leucocyte adhesion to endothelium might prevent neutrophil and other inflammatory cell infiltration and subsequent cell mediated lung injury and lung fibrosis. We have therefore examined the role of E-selectin in BLM induced lung fibrosis in mice.

Methods

DETECTION OF E-SELECTIN MESSENGER RNA

The time dependent induction of murine E-selectin messenger RNA (mRNA) was assessed by reverse transcriptase polymerase chain reaction (RT-PCR).[13] The E-selectin and P-selectin primers used have been described elsewhere.[14 15]

ICR MICE AND TRANSGENIC (TG) MICE

In seven week old ICR mice purchased from Charles River Co (Boston, USA) soluble E-selectin (sE-selectin) transgenic (TG) mice were established as previously described.[16] The established mice, back crossed to MRL+/+ mice five times, had serum sE-selectin levels ranging from 5 to 50 μg/ml. In their transgenic negative (non-TG) littermates used as controls the serum level of sE-selectin was below the limit of detection.[16] Leucopenia was not seen in either group. Soluble E-selectin in this model was produced only in liver tissue under the control of the α_1-antitrypsin promoter containing truncated sequences encoding a portion of the extracellular domain (nucleotides 83–

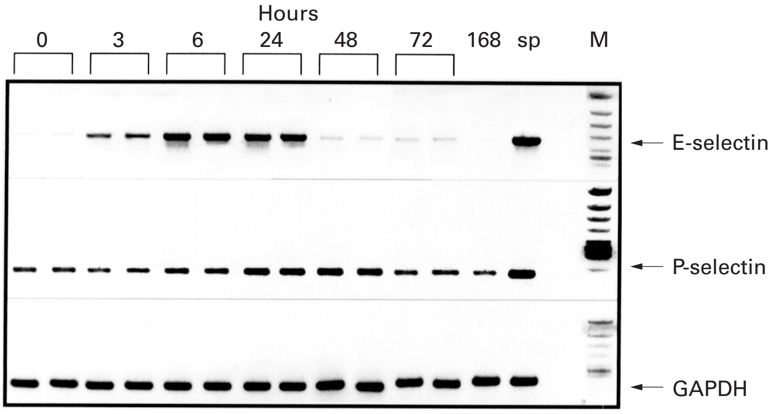

Figure 1 Time courses of E-selectin and P-selectin mRNA induction in lung tissues of ICR mice treated with BLM as detected by RT-PCR. E-selectin mRNA expression (271 bp) was strongest six hours after BLM treatment. In contrast, expression of P-selectin mRNA (181 bp) was constitutive and reached a maximum at 24 hours. sp = spleen cells from MRL-1pr mice; M = molecular marker.

1506). There was no significant difference in activation state of peripheral neutrophils between TG (originally described as TgnEsol) and non-TG mice. TG mice were healthy and did not develop characteristic pathological lesions (unpublished observation).[16]

ASSAY OF INHIBITION OF SIALYL LEWIS^x BINDING TO E-SELECTIN

Ninety six-well microtitre plates (Sumilon MS-8596F) were coated with either 50 µl/well E-selectin IgG 5 µg/ml (provided by Dr S Watson, Genentech Inc, San Francisco, California, USA) or human IgG (Sigma) in PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ [PBS(+)] at 4°C overnight. Two hundred µl of 3% BSA-PBS(+) were added and, after washing with PBS(+), the plates were incubated at 4°C for eight hours. Following this, 40 µl of 50 µg/ml anti-E-selectin monoclonal antibody BBIG-E4 (mouse IgG_1), 10 mM EDTA, or 40 µl of washing buffer alone were added and the plates were kept at room temperature for 20 minutes. Ten µl/well of 10 µg/ml biotinylated sialyl Lewis^x polymeric probe (sLe^x BP probe; Seikagaku Kogyo, Tokyo, Japan) was added with or without 50 µg/ml 2H5 monoclonal antibody, normal mouse IgM, or test serum samples (diluted tenfold) and the plates were incubated for 40 minutes at room temperature. After washing, 50 µl/well of peroxidase conjugated streptavidin (diluted 500 fold) in washing buffer was added and the plates were incubated for one hour at room temperature; 50 µl/well *o*-phenylenediamine in 0.05 M citrate and 0.1 M phosphate buffer (pH 5.2) were then added and the plates were incubated for five minutes. Fifty µl of 8N H_2SO_4 was added to each well and the optical densities (OD) at 490 nm were measured.[17]

TREATMENT WITH BLM

BLM purchased from Nippon Kayaku Co (Tokyo, Japan) was dissolved in normal saline (0.3 ml per mouse) and administered intravenously to ICR mice (n = 10) in a dosage of 100 mg/kg, nearly one third the dose at which 50% of the animals would have died (LD_{50}). Ten control mice received 0.3 ml saline. Four weeks after administration of BLM fibrotic foci were observed, predominantly in the subpleural area (data not shown). BLM was also administered intravenously to both TG and non-TG mice in a dosage of 100 mg/kg.

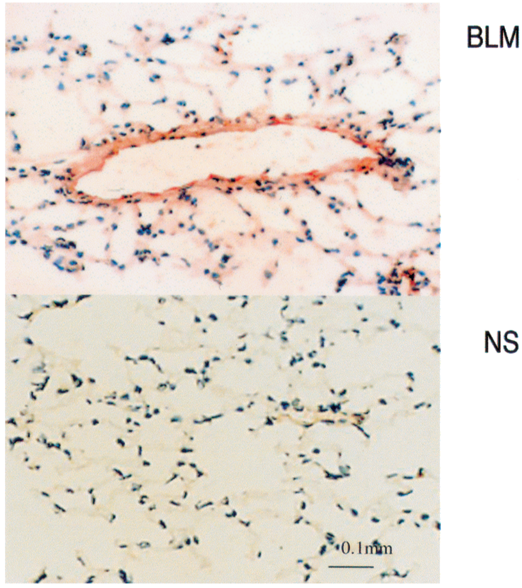

Figure 2 E-selectin expression on the vascular endothelium of lungs of mice treated with bleomycin (BLM). E-selectin was expressed on the endothelium of middle sized vessels and capillaries of BLM treated mice (upper figure) but not in the tissues of control mice treated with normal saline (NS, lower figure).

MYELOPEROXIDASE (MPO) ASSAY

Myeloperoxidase activity in the lung tissues of ICR mice was measured by spectrophotometric assay. In brief, the lung tissue was minced and sonicated on ice, then centrifuged at 40 000*g* for 15 minutes. The supernatant was assayed for MPO activity as previously described.[18] The MPO activity in BLM treated ICR mice (n = 10) was compared with that in normal saline (NS) control mice at 0, 3, 6, 12, 24, 72, and 168 hours after BLM or saline treatment. In TG mice (n = 10) the MPO activity was measured at 0, 24, 72, and 168 hours. Statistical significance was tested using repeat measures ANOVA.

PATHOLOGICAL ANALYSIS

The pathological features of lung fibrosis and leucocyte counts in the sE-selectin TG mice (n = 10) were evaluated by three independent observers with Elastica Masson Goldner (EMG) stain and Giemsa stain, respectively, under light microscopy. Three longitudinal sections from each mouse lung were used for analysis. The degree of BLM induced lung fibrosis, including collagen and elastic fibres in the lung interstitium, was assessed according to Ekimoto's score[19] as follows: grade = total score of specimens in group/number of specimens in group; incidence = number of mice with fibrosis in group/number of mice tested in group. Pathology was rated as follows:—(0) = absence of fibrosis; +/− (1) = presence of area with suspected fibrosis in alveolar septa; + (2) = some fibrotic foci, often in subpleural area; ++ (4) = scattered fibrotic foci; +++ (6) = diffuse fibrosis.

Table 1 Time course of myeloperoxidase (MPO) activities in U/ml in lung tissues of bleomycin (BLM) treated and control ICR mice. Maximum activity was seen at 12 hours after BLM treatment and was significantly higher than in normal saline (NS) control mice. MPO activities of NS group remained low and stable.

	0 h	*3 h*	*6 h*	*12 h*	*24 h*	*72 h*	*168 h*	*Overall mean difference (95% CI)*
BLM	1.9 (0.12)	7.8 (0.08)	9.3 (0.46)	9.3 (0.66)	7.5 (0.30)	4.2 (0.30)	2.9 (0.16)	3.4 (2.6 to 4.2)
NS	2.4 (0.17)	2.4 (0.13)	3.9 (0.25)	3.3 (0.22)	3.6 (0.10)	1.6 (0.20)	1.7 (0.07)	(p<0.001)

Values are mean (SE).

E-selectin immunostaining was performed using a Vectastain ABC enhancement kit. Sixteen hours after BLM treatment frozen sections of lung were incubated with anti-mouse E-selectin antibody (10E9.6, rat IgG_{2a}, PharMingen, San Diego, California, USA) for one hour at room temperature, then with biotinylated anti-rat immunoglobulin (Vector Laboratories, Burlingame, California, USA) as a secondary antibody, then the substrate system 3-amino-9-ethylcarbazole (AEC) (Dako Corp, Carpinteria, California, USA) for staining. Leucocyte and endothelial cell numbers in three Giemsa stained lung sections from TG and non-TG mice were counted using light microscopy.

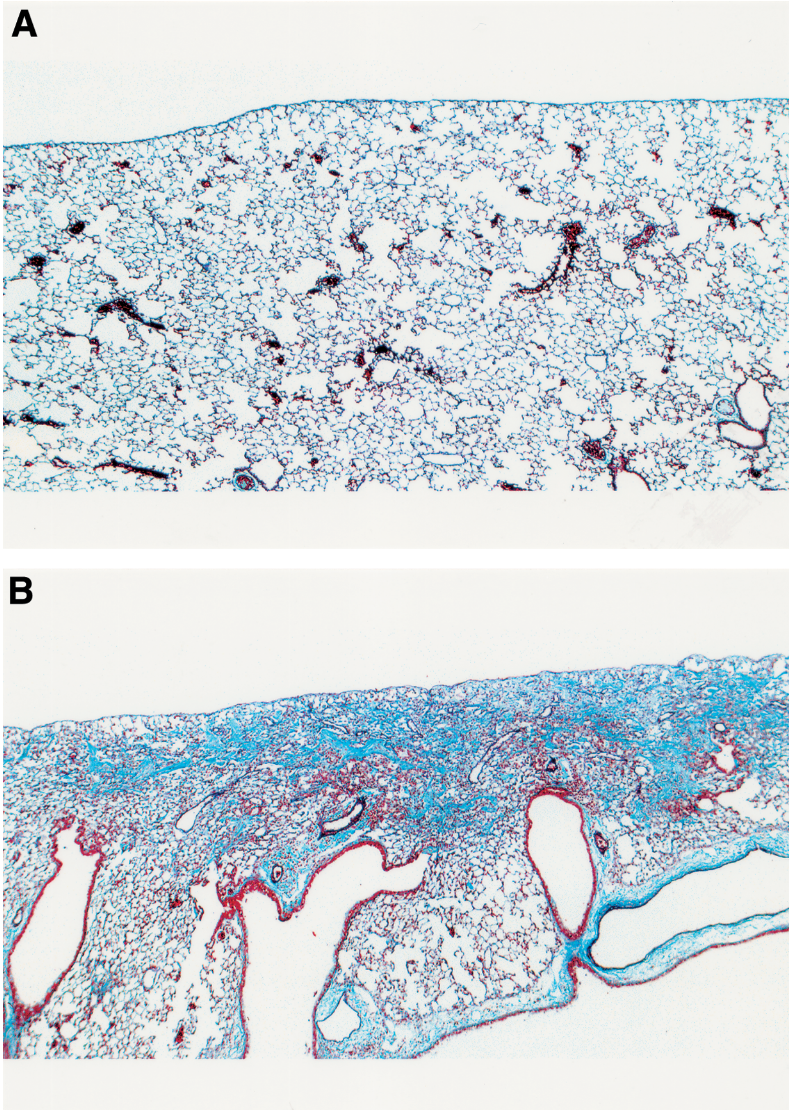

Figure 3 Pathological features of lung fibrosis in soluble E-selectin gene transgenic (TG) mice four weeks after intravenous administration of BLM (Elastica Masson Goldner (EMG) staining; × 40). Lung fibrosis was absent in TG mice (A), in contrast to the marked progression in their non-TG littermates (B). Fibrotic foci in non-TG mice showed deposition of collagen and elastic fibres in the parenchyma and alveolar lumen.

Table 2 Comparison of fibrotic changes between soluble E-selectin TG and non-TG mice

Transgenic mice	*Incidence*	*Grade*	*HOP (µmol/g)*
(+)	0/5	0/25 = 0 (0)	23.8 (1.6)
(−)	5/5	46/25 = 1.84 (0.08)*	44.6 (2.3)*
Mean difference (95% CI)		1.84 (1.6 to 2.0)	20.9 (14 to 23)

Degree of fibrosis (grade, mean (SE)) was evaluated according to Ekimoto's score.[19] Mean (SE) levels of hydroxyproline (HOP, µmol/g) in the right lung were measured by modification of the method of Woessner.[20]
Mean differences (95% CI) of grade and hydroxyproline levels between TG(+) and TG(−) mice are shown.
*p<0.001.

HYDROXYPROLINE MEASUREMENT

The total collagen content of the right lung was determined by hydroxyproline assay.[20] After acid hydrolysis of the right lung with 12N HCl at 100°C for 20 hours in a sealed glass tube (Iwaki, Tokyo, Japan), the hydroxyproline content was determined using high performance liquid chromatography (HPLC).

STATISTICAL ANALYSIS

The differences between the two groups (grade of fibrosis, hydroxyproline, leucocyte counts in sE-selectin TG and non-TG mice, values of OD 490 nm in photometric assay) were tested for significance using the Mann-Whitney U test. Differences in the mean in vitro binding inhibition assay were tested using the Student's *t* test. p values of less than 0.001 were considered to be statistically significant. The repeat measures ANOVA was used to evaluate differences in MPO activities and leucocyte counts of the two groups with time. Data are presented as mean (SE) values. Mean differences and 95% confidence intervals (CI) between the two groups are given.

Results

E-selectin mRNA was maximally induced six hours after BLM administration and disappeared by 72 hours whereas the constitutive expression of P-selectin mRNA reached a maximum at 24 hours (fig 1). Upregulation of E-selectin protein in the lung vessels 16 hours after administration of BLM was confirmed histologically (fig 2). Changes in P-selectin protein expression by immunohistochemical analysis were not significantly different (data not shown), but the visible changes in mRNA levelled out. It should be noted that our method does not distinguish between surface P-selectin and intracellular P-selectin (see discussion). Lung fibrosis was predominantly in the subpleural area of these mice 4–5 weeks after administration of BLM (data not shown).

MPO activity was increased in the lung tissues of ICR mice treated with BLM, peaking 12 hours after administration (table 1) with an

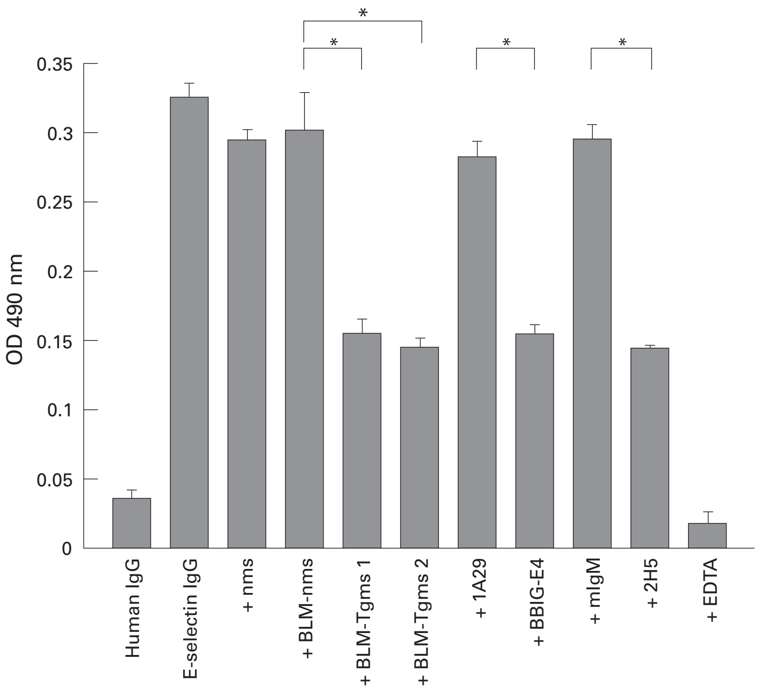

*Figure 4 Blocking assay for sialyl-Lewis^x binding to E-selectin. Serum of TG mice treated with bleomycin (BLM) blocked the binding of sialyl Lewis^x to E-selectin, as did anti-E-selectin antibody (BBIG-E4; 50 μg/ml) and anti-sialyl Lewis^x antibody (2H5; 50 pg/ml), but serum of non-TG mice treated or not treated with BLM did not inhibit this binding. nms = normal mouse serum; Tgms = serum of transgenic mouse; 1A29 = irrelevant isotype matched control mouse monoclonal antibody; mIgM = murine IgM. Median differences (95% CI) between +BLM-nms and BLM-Tgms1, +BLM-nms and BLM-Tgms2, + 1A29 and +BBIG-E4, and +mIgM and +2H5 were 0.151 (0.12 to 0.18), 0.161 (0.13 to 0.19), 0.130 (0.12 to 0.14), and 0.157 (0.15 to 0.16) OD 490 nm, respectively. *p<0.001.*

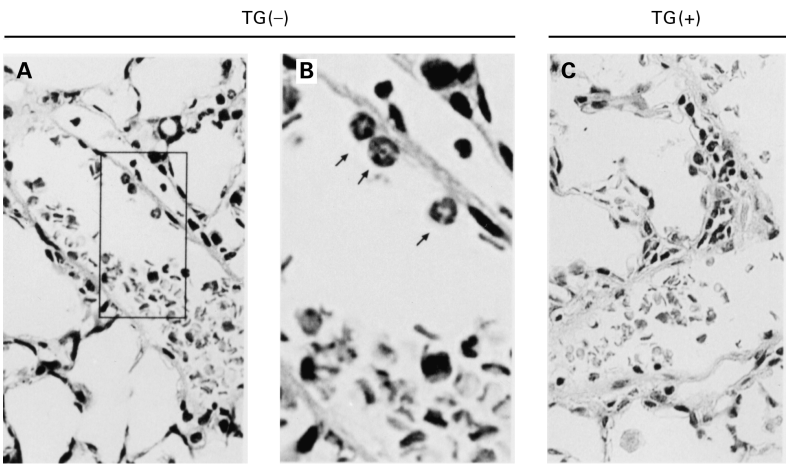

Figure 5 Typical view of neutrophil attachment to vascular endothelium in non-TG mice (TG(–)) 12 hours after BLM inoculation (A = low magnitude; B = high magnitude of rectangle in A). Neutrophils were rarely detected on the vascular endothelium of sE-selectin TG(+) mice (C).

Table 3 Comparison of neutrophil counts and myeloperoxidase (MPO) activity between soluble E-selectin TG mice (n = 10) and non-TG mice (n = 10)

	TG	*0 h*	*24 h*	*72 h*	*168 h*	*Overall mean difference (95% CI)*
MPO activity	(+)	3.1 (0.3)	2.8 (0.2)	3.2 (0.4)	2.3 (0.4)	4.2 (3.0 to 5.4)*
	(–)	3.8 (0.3)	12.8 (0.5)	7.2 (0.2)	4.5 (0.3)	
Neutrophil count	(+)	28 (2.0)	32 (1.9)	32 (1.4)	46 (1.0)	136.5 (109 to 164)*
	(–)	34 (2.3)	248 (3.1)	230 (3.2)	168 (4.1)	

Mean neutrophil counts (/cm^2) of five sections of lung tissue stained with Giemsa; myeloperoxidase activity (U/ml) was measured at several time points after injection of BLM. Both parameters differed significantly between TG and non-TG mice (*p<0.001).

overall mean (95% CI) difference of 3.4 (2.6 to 4.2) U/ml (p<0.001). Changes in MPO activity parallelled those in E-selectin mRNA expression, as described above.

These data suggest that E-selectin might have an important role in the development of lung fibrosis by BLM. To examine this hypothesis we used sE-selectin TG mice in which serum sE-selectin levels ranged from 5 to 50 μg/ml and had activity to alter the direction of E-selectin dependent tumour metastasis.

In contrast to the marked lung fibrosis seen in non-TG mice, lung fibrosis was nearly absent in the TG mice. In non-TG mice (with no detectable sE-selectin) the lung fibrosis was predominantly peripheral and subpleural, with interstitial and intraluminal fibrotic foci. There were numerous inflammatory cells including macrophages (fig 3). Lung fibrosis scores and hydroxyproline content differed significantly between the TG and non-TG mice (table 2). The inhibition test of E-selectin binding to sialyl Lewis^x was performed to characterise further the sE-selectin molecule in the serum of TG mice and the results showed that the serum of TG mice significantly inhibited the binding of biotinylated sialyl Lewis^x to human E-selectin coated plates (p<0.001), as did 2H5 (monoclonal antibody against sialyl Lewis^x) and anti-E-selectin (BBIG-E4) monoclonal antibody (fig 4). Mean (95% CI) differences between +BLM-nms and BLM-Tgms1, +BLM-nms and BLM-Tgms2, +1A29 and +BBIG-E4, and +mIgM and +2H5 were 0.151 (0.12 to 0.18), 0.161 (0.13 to 0. 19), 0.130 (0.12 to 0.14), and 0. 157 (0.15 to 0.16) OD 490 nm, respectively. These results show clearly that sE-selectin in the serum of TG mice strongly inhibited E-selectin mediated binding.

Finally, the acute phase of BLM induced lung injury in this system was analysed. TG mice injected with BLM showed no neutrophil adhesion to vascular endothelial cells, whereas significant neutrophil adhesion was seen in non-TG mice (fig 5). MPO activity and neutrophil numbers in the lungs of non-TG mice 24 hours after administration of BLM were significantly increased while baseline levels were maintained in TG mice (table 3).

Discussion

These results demonstrate the essential role of E-selectin in the progression of BLM induced lung fibrosis. Although the mechanism of pulmonary fibrosis in BLM treated mice remains unclear, many investigators believe that neutrophil mediated lung injury is necessary for initiation and/or propagation of the fibrogenic process.[21–23] Neutrophil adhesion to vascular endothelial cells is an important process in cell mediated lung injury with the release of proteases and free radical formation. Since selectins have been implicated in neutrophil mediated acute lung injury,[24] we investigated whether selectins are critically involved in cell mediated tissue injury in BLM inoculated mice.

The maximal expression of E-selectin mRNA at six hours after BLM administration (fig 1) and the subsequent expression of E-selectin on the vascular endothelium (fig 2) indicate that E-selectin is, in fact, involved in the interaction between inflammatory cells and endothelium. The parallel changes in MPO activity in lung tissues and the level of E-selectin mRNA add support to the idea that neutrophils have an important role in inducing lung fibrosis (fig 1, table 1). Furthermore, macrophages—which were frequently seen in the fibrotic foci induced by BLM—have a significant role in lung fibrosis and their adhesion to the endothelium may be inhibited by sE-selectin in this model. The effect of sE-selectin will apply to both types of inflammatory cells.

The contribution of P-selectin to the adhesion of inflammatory cells to the endothelium was not evaluated because of its constitutive expression in immunohistochemical analysis (data not shown), although a slower increase in the level of P-selectin mRNA was seen in the lungs of BLM challenged mice (fig 1). It is well known that expression of P-selectin protein on the cell surface is mainly regulated by transport step, not by protein synthesis, and P-selectin is expressed on the surface minutes after activation of endothelium.[25] However, MPO activity in the lung tissue of BLM treated ICR mice did not increase rapidly. These findings suggest that P-selectin is not a significant molecule in BLM induced lung fibrosis, although we cannot rule out the contribution of P-selectin to neutrophil recruitment by BLM since our immunohistochemical method does not distinguish between surface and intracellular P-selectin.

To investigate further the role of E-selectin in the progression of BLM induced lung fibrosis we used TG mice in which sE-selectin is secreted in the serum.[16] Importantly, the TG mice developed no apparent lung fibrosis following BLM challenge, in marked contrast to their non-TG littermates (fig 3, table 2). It is possible that sE-selectin blocked one or more ligands for E-selectin on neutrophils.[26 27] This theory was supported by our finding that serum from TG mice inhibited the binding of sialyl Lewis^x to recombinant E-selectin (fig 4). E-selectin binds to several sialylated ligands,[26] including ESL-1[28] and PSGL-1,[29] and to some gangliosides.[26] sE-selectin from TG mice probably binds to several E-selectin ligands, resulting in inhibition of E-selectin mediated extravasation of neutrophils to the lung. In line with our findings, neutrophil dependent acute lung injury was significantly inhibited by administration of soluble selectins.[30] We confirmed that sE-selectin in this model was produced only in liver tissue under the control of the α_1-antitrypsin promoter containing sequences encoding a portion of the extracellular domain of E-selectin (nucleotides 83–1506). There was no significant difference in the activation state of peripheral neutrophils between TG and non-TG mice. TG mice were healthy and did not develop characteristic pathological lesions (unpublished data).

Studies have been published which show the lack of a role for neutrophils in BLM induced pulmonary fibrosis.[31 32] Several studies have also shown that even neutropenic patients can develop adult respiratory distress syndrome.[33 34] Other cells, such as monocytes or lymphocytes, may contribute to the progression of pulmonary fibrosis in this model; however, our study strongly suggests that neutrophils play an important part in pulmonary fibrosis, even in the absence of neutropenia.

The induction of E-selectin expression is clearly not the sole factor contributing to the generation of fibrosis since E-selectin expression is ubiquitous in the lung vasculature whereas fibrosis is not homogeneous but predominates in the subpleural area. The relationship between selectin induction and the action of BLM remains unclear. Several recent reports have suggested that tumour necrosis factor α (TNFα) is a strong inducer of pulmonary fibrosis in a BLM model.[35 36] TNFα is a potent inducer of selectins on vasculatures.[37 38] One possible mechanism for BLM induced lung fibrosis is via TNFα enhancement of selectin expression. Further studies will be required to resolve this issue. The results of the present study strongly suggest that sE-selectin plays an essential role in the inhibition of BLM induced lung injury and fibrosis by blocking the adhesion of inflammatory cells to pulmonary endothelial cells. sE-selectin may therefore be useful as a prophylactic agent against lung fibrosis. Inhibition of the initial phase of the inflammatory cell adhesion cascade, particularly neutrophil adhesion to inflamed capillary endothelium, may be a key strategy in preventing BLM induced lung injury and fibrosis.

The authors thank Dr S Watson for her kind gift of human recombinant selectins.

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