

**Bleomycin increases neutrophil adhesion to human vascular endothelial cells**

**independently of upregulation of ICAM-1 and E-selectin**

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Abbreviations: AEC, alveolar epithelial cell; EC, endothelial cell; ET-1, endothelin-1;  
HUVEC, human umbilical vein endothelial cell; IPF, idiopathic pulmonary fibrosis; PMVEC,  
pulmonary microvascular endothelial cell

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## Abstract

**Aim of the Study:** Bleomycin-induced lung disease is a serious complication of therapy characterised by alveolar injury, cytokine release, inflammatory cell recruitment, and eventually pulmonary fibrosis. The mechanisms underlying bleomycin-induced pulmonary fibrosis may be relevant to other progressive scarring diseases of the lungs. Pulmonary vascular endothelial cells are critically involved in immune cell extravasation at sites of injury through adhesion molecule expression and cytokine release. We sought to determine the effects of bleomycin on adhesion molecule expression and cytokine release by pulmonary vascular endothelial cells, and their functional relevance to inflammatory cell recruitment.

**Methods:** The effects of pharmacologically-relevant concentrations of bleomycin on adhesion molecule expression and cytokine release by human vascular endothelial cells *in vitro* were studied by flow cytometry, qPCR, and ELISA. A flow chamber model was used to assess the functional consequences on adhesion of flowing human neutrophils to endothelial cell monolayers.

**Results:** Bleomycin increased ICAM-1 (CD54), VCAM-1 (CD106), and E-selectin (CD62E) expression, and increased MCP-1 and IL-8 release by endothelial cells. Increases in protein expression were accompanied by increased mRNA transcription. In contrast, there was no direct effect of bleomycin on the pro-fibrotic cytokines TGF- $\beta$ , PDGF-BB, or endothelin-1. Under flowing conditions, endothelial cells exposed to bleomycin supported increased neutrophil adhesion which was independent of ICAM-1 or E-selectin.

**Conclusion:** Our findings demonstrate that bleomycin promotes endothelial-mediated inflammation and neutrophil adhesion. These mechanisms may contribute to the development of pulmonary fibrosis by supporting immune cell recruitment in the lungs.

## Introduction

Lung fibrosis is a serious side effect of therapy with bleomycin [1, 2], a potent cytotoxic drug used for treating head and neck cancers, lymphomas, and testicular cancer. Whilst toxicity can be minimised by careful attention to dosing, bleomycin-induced pulmonary fibrosis causes significant morbidity and mortality once it is established. A better understanding of its pathogenesis could lead to more effective preventative strategies. Furthermore, the potential mechanisms involved may also be relevant in idiopathic pulmonary fibrosis (IPF) and connective tissue disease-associated pulmonary fibrosis .

Direct instillation of bleomycin into the lungs of rodents has been used to model IPF, but has proved to be a poor pre-clinical predictor of beneficial interventions in humans [3]. Systemic delivery of bleomycin to animals results in pulmonary fibrosis similar to that seen in human patients, with evidence of injury to the lung endothelium, sub-pleural fibrosis, alveolar epithelial cell (AEC)-I loss, AEC-II hyperplasia, and immune cell accumulation [4-7]. The pulmonary vascular endothelium is the first lung tissue to encounter systemically administered bleomycin. Expression of adhesion molecules and cytokines by endothelial cells (ECs) is critical for the adhesion and transmigration of inflammatory leukocytes, and ECs may also directly promote fibrosis by releasing pro-fibrotic cytokines.

We aimed to characterize role of endothelium in the inflammatory response preceding bleomycin-induced pulmonary fibrosis. We hypothesized that pharmacologically-relevant concentrations of bleomycin would induce expression of adhesion molecules and release of cytokines by human ECs *in vitro*, leading to increased adhesion of flowing neutrophils to bleomycin-treated EC monolayers.

## **Materials and methods**

### **Bleomycin**

Bleomycin sulphate was from Carbosynth, UK. Concentrations were chosen based on peak plasma concentrations reported following intravenous dosing in human subjects (0.1-6.5µg/ml depending on dose and infusion method) [8, 9].

### **Cells**

Pulmonary microvascular endothelial cells (PMVECs) were from Promocell GmbH (Heidelberg, Germany). Human umbilical vein endothelial cells (HUVECs) were purchased from TCS Cellworks (Buckingham, UK) or Promocell. Cells were cultured to passage 3-5 in either Promocell endothelial cell growth medium (HUVECs) or endothelial cell growth medium MV (PMVECs) containing the supplied supplements, penicillin (100µg/ml)-streptomycin (100U/ml) (PAA, Pasching, Austria), and L-glutamine (2mM) (Lonza, Basel, Switzerland). Cells were incubated in an atmosphere containing 5% CO<sub>2</sub> at 37°C. Cytotoxicity of bleomycin to HUVECs over 6 and 24 hours was tested by MTS and SRB assays. Only bleomycin concentrations greater than 10µg/ml caused greater than 50% cell death, whereas lower concentration caused minimal toxicity.

### **Flow Cytometry**

Confluent cells were treated with bleomycin or TNF-α (Promocell) for 6 and 24 hours, or left untreated in fresh medium. Cells were dissociated using TrypLE™ Express (Gibco, Paisley, United Kingdom), pelleted by centrifugation, and re-suspended in medium. Cell suspensions were pipetted to wells of a 96-well multi-plate (Costar) and centrifuged. Cells were incubated with 20 µg/ml purified mouse anti-human (IgG1) antibodies against CD31 (clone WM59) CD50 (MEM-17), CD51/61 (23C6), CD54 (HA58), CD62E (HAE-1f), CD62P (AK4),

CD106 (STA), and CD162 (KPL-1) (Biolegend) for 30 minutes, washed, and incubated with 20 $\mu$ g/ml rabbit F(ab')<sub>2</sub> anti-mouse IgG:FITC (Serotec). Cells were washed, suspended in 50 $\mu$ l PBS/0.1% BSA, and 5-10,000 events per sample were analysed using a FACSCalibur flow cytometer (Becton Dickinson).

### **Enzyme linked immunosorbent assays**

ECs were re-seeded at a concentration of  $2 \times 10^5$ /ml and treated with bleomycin or left untreated. Cells were treated with TNF- $\alpha$  (10ng/ml) or human plasma thrombin (Calbiochem, MerckMillipore, Feltham, UK) as positive controls. Supernatants were aspirated and stored at -80°C. ELISA kits were purchased from Biolegend (IL-8, MCP-1, latent TGF- $\beta$ ), R&D Systems (Endothelin-1), and Sigma Aldrich (PDGF-BB). Assays were performed according to supplied instructions. Plates were read on a Thermo Multiskan FC plate-reader (Thermo Scientific).

### **Quantitative PCR**

RNA was extracted using the ReliaPrep™ RNA Cell Miniprep System (Promega, Madison, Wisconsin, USA). Total RNA in each sample was determined using a Qubit quantification kit. Reverse transcription was conducted using RevertAid Premium Reverse Transcriptase (Thermo Scientific) using 1 $\mu$ g total RNA and Oligo(DT) primer (Thermo Scientific) to generate samples containing 50 ng/ $\mu$ l RNA. “No RT” samples were also generated. Reverse transcription was done in a Techne TC-3000 thermal cylinder (Bibby Scientific, Stone, Staffordshire, UK). Purity of cDNA samples was confirmed by PCR using a GAPDH primer (MWG Eurofin, Luxembourg). Primer specificity was confirmed by PCR using primers for human ICAM-1, E-selectin, VCAM-1, TGF- $\beta$ , PDGF-BB, Endothelin-1, MCP-1, IL-8, and reference gene UBC (Primer Design, Southampton, UK). The presence of single bands of

appropriate size indicated that reverse transcription had been successful, samples were not genomic DNA-contaminated, and primers were specific.

Identification of a reference gene was performed using primers for GAPDH, B2M, UCB, ATP5BN, YWHAZ, and RPL13A. cDNA from treated and untreated cells were diluted in RNase/DNase free water to a concentration of 0.5ng/ $\mu$ l. Precision MasterMix with SYBR Green (Primerdesign) (10 $\mu$ l), RNase/DNase free water (4 $\mu$ l), and diluted primer (1 $\mu$ l) were combined to generate a Master mix. This Master mix (15 $\mu$ l) was pipetted into each well of a 48-well qPCR plate (Primerdesign) and 5 $\mu$ l of diluted cDNA was added. The plate was placed in the Illumina ECO qPCR machine (Illumina, Little Chesterton, Essex, UK) and subject to 10 minutes at 95<sup>0</sup>C for polymerase activation; 40 cycles of PCR comprising 15 seconds at 95<sup>0</sup>C and 60 seconds at 60<sup>0</sup>C, and a melt curve period for which the plate was heated to 95<sup>0</sup>C for 15 seconds, 55<sup>0</sup>C for 15 seconds, and 95<sup>0</sup>C for 15 seconds. Results from each geNorm experiment were compiled and analysed using the qBase+ program (Vandesompele, et al., 2002). The most stable reference gene overall was UBC (ubiquitin C gene).

To determine primer efficiency, standard curves were generated in duplicate. cDNA (1ng/ $\mu$ l) was generated by combining 1 $\mu$ l cDNA with 49 $\mu$ l DNase/RNase free water; tenfold dilutions were then prepared. Five  $\mu$ l of each dilution was then added to 15 $\mu$ l of master mix (described above) containing each specific primer in a 48-well plate and qPCR (described above) was conducted. Standard curves were generated using the Eco software (version, 5.0.16.0, Illumina). The results from duplicate standard curve experiments were plotted on linear regression graphs using GraphPad (version 5.04) and the efficiency of each primer was determined.

All samples were run in triplicate for target genes, reference genes, and no cDNA samples.

No RT samples were run for each primer-sample combination. As all primers were shown to have comparable efficiency, fold-change was calculated using the Livak equation.

### **Neutrophil adhesion experiments**

Uncoated  $\mu$ -Slide I 0.4 Luer flow chambers (ibiTreat, tissue culture treated, sterile), sterile silicone tubing, and Luer elbow connectors were purchased from Thistle Scientific (Glasgow, United Kingdom). A flow system was created using a 50ml glass syringe (Hawksley Lancing, Sussex, United Kingdom), BD Connectas, and 10ml plastic syringes. HUVECs were cultured to 90% confluence and used between passages 3 and 5. Cells were re-suspended to a concentration of  $2.5 \times 10^6$ /ml in medium and 100 $\mu$ l was pipetted into the chambers. Chambers were incubated for 15 minutes, after which time 60 $\mu$ l of fresh media was added to each media reservoir. The chambers were then incubated in an atmosphere containing 5% CO<sub>2</sub> at 37°C. After 4 hours, medium was aspirated and replaced with fresh medium (220 $\mu$ l) prior to incubations for a further 20 hours. Media was aspirated and replaced with endothelial cell basal medium (220 $\mu$ l) containing bleomycin, TNF- $\alpha$ , or no treatment (control). For E-selectin blocking experiments, endothelial cells in flow chambers were incubated in culture medium containing 20 $\mu$ g/ml anti-CD62E antibody (clone P2H3) (Novus Biologicals, UK) or Mouse IgG1 $\kappa$  isotype control concurrently with bleomycin or TNF- $\alpha$  treatment and incubated for 6 hours to ensure monolayer stability.

Ethical approval was obtained from Hull York Medical School for blood neutrophil isolation from consenting healthy volunteers. Neutrophils were isolated from 15ml acid citrate dextrose-anticoagulated whole blood diluted 1:1 in PBS using a single-step density gradient (15ml histopaque 1077) centrifuged at 400 x g for 30 minutes. Erythrocytes in the pellet were lysed by adding 40ml deionised water (pH 6.8) and agitating gently. 10xPBS was added to

restore isotonicity. Neutrophils were suspended in PBS with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , 0.15% (w/v)

BSA, and 5mM D-glucose (supplemented PBS), and purity assessed by morphology on cytospin preps stained with Rapi-Diff (Atom Scientific, Cheshire, UK). For CD18 blocking experiments, neutrophils ( $4 \times 10^6$  cells/ml) were incubated for 30 minutes with 20 $\mu\text{g/ml}$  anti-CD18 antibody (clone TS1/18) (low endotoxin, azide-free, Biolegend) or IgG1 $\kappa$  isotype control (low endotoxin, azide-free, Biolegend) and washed.

Neutrophils ( $5 \times 10^5$  cells/ml in supplemented PBS) and wash buffer (PBS with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) were loaded into separate plastic syringes. The tubing was flushed at 10ml/min for 30 seconds. Wash buffer was run through the flow chamber at 5 dyn/cm<sup>2</sup> for 30 seconds to remove any non-adherent endothelial cells and all culture medium. The neutrophil suspension was run through at 5 dyn/cm<sup>2</sup> for 30 seconds to introduce the cells to the system. This flow rate precluded unwanted granulocyte adhesion to the endothelial monolayer. The shear stress [10] was decreased to 0.5 dyn/cm<sup>2</sup> for 160 seconds, allowing approximately  $5 \times 10^5$  neutrophils to pass through the chamber. Photographs (20 per field) were taken of 5 fields along the midline using a QCapture Pro version 6 software using a light microscope (Nikon Eclipse TS100, Nikon, Tokyo, Japan) with a QImaging Retiga 2000R Fast1394 camera (QImaging, Surrey, Canada).

Total numbers of adherent, slow-rolling, and rolling cells were recorded from each series of stills. The results of each series of experiments were expressed as the mean number of adhesion, rolling, and slow rolling events per field (1mm<sup>2</sup>) under each treatment condition (five fields per slide). Adherent neutrophils were identified as small, round, static phase-bright cells. Spreading neutrophils were partially bright-phase, larger, but not clearly round. Rolling neutrophils were adherent but moved slowly along the endothelium and were often tethered. Speed was determined by measuring the distance of travel between still photographs

*This is an Accepted Manuscript of an article published by Taylor & Francis in Experimental lung research on 31 October 2016, available online: <http://www.tandfonline.com/doi/full/10.1080/01902148.2016.1243742> (one second apart). Events in which rolling or slow-rolling neutrophils became firmly*

adherent were regarded as new adhesion events.

### **Statistical analyses**

Flow cytometry, neutrophil adhesion, and ELISA data were analysed by Mann-Whitney-U tests, and qPCR and adhesion data by ANOVA. Analyses were carried out using SPSS v.19 or Graphpad InStat v.3 software. Quantitative values are presented as mean +/- standard error. A *P* value  $\leq 0.05$  was regarded as a statistically significant difference.

## **Results**

### **Bleomycin increases expression of adhesion molecules and release of pro-inflammatory cytokines by endothelial cells**

PMVECs were treated with bleomycin at concentrations in a similar range to levels reported in plasma following intravenous dosing in human subjects [8, 9]. Bleomycin significantly increased expression of ICAM-1 (CD54) (10 $\mu$ g/ml bleomycin for 6 hours and 1 $\mu$ g/ml or 10 $\mu$ g/ml bleomycin for 24 hours) and VCAM-1 (CD106) (1 $\mu$ g/ml or 10 $\mu$ g/ml bleomycin for 6 hours and 24 hours) compared with untreated cells (figure 1). E-selectin (CD62E) expression was low, but increased in response to 10 $\mu$ g/ml bleomycin at 6 hours. Release of IL-8 and MCP-1 increased in concentration- and time-dependent manners in bleomycin-treated PMVECs (figure 1).

HUVECs are widely available and have been more commonly studied than PMVECs, so we assessed the value of HUVECs as surrogates for pulmonary vascular ECs by measuring expression of adhesion molecules ICAM-1, E-Selectin, and VCAM-1 following treatment with bleomycin for 6 and 24 hours in similar patterns to PMVECs (figure 2). ICAM-1 expression increased significantly when HUVECs were treated with 10 $\mu$ g/ml bleomycin for 24 hours, VCAM-1 and E-selectin expression when HUVECs were treated with 10 $\mu$ g/ml bleomycin for 6 hours, and E-selectin when treated with all concentrations of bleomycin for 24 hours. When mRNA transcript expression was assessed using qPCR, there were significant increases in ICAM-1, E-selectin, and VCAM-1 transcript levels in samples treated with bleomycin (figure 2) which broadly reflected surface expression demonstrated by flow cytometry.

Bleomycin treatment increased IL-8 and MCP-1 release by HUVECs in a dose-dependent manner, which was associated with increased mRNA transcription demonstrated by qPCR (figure 3).

### **Bleomycin does not directly affect synthesis or release of pro-fibrotic cytokines by endothelial cells**

Endothelial cells treated with bleomycin did not show any significant change in TGF- $\beta$  release (figure 4). This was mirrored by qPCR results which showed stable transcript levels when cells were treated with bleomycin for 24 hours (figure 4). The expression of PDGF-BB was also unchanged in response to bleomycin treatment over 6 or 24 hours, and was mirrored by stable mRNA transcript levels in qPCR experiments. Bleomycin treatment had no consistent effects on ET-1 release or transcript levels (figure 4).

### **Increased neutrophil adhesion to bleomycin-treated endothelial monolayers in flow chambers**

Significantly increased adhesion of flowing human neutrophils occurred when endothelial cell monolayers were treated with bleomycin (1 $\mu$ g/ml and 10 $\mu$ g/ml) for 6 or 24 hours (figure 5). As expected, increased neutrophil adhesion to monolayers was also induced by treatment with TNF- $\alpha$  (10ng/ml). The number of new adhesion events, whereby neutrophils which had not previously adhered to the endothelium in the initial image were captured, slowed, and rolled along the endothelium, was greatest in the TNF-treated monolayers. Very few spreading events were recorded in any of the experiments (data not shown).

Pre-treatment of neutrophils with  $\beta$ 2 integrin (CD18)-blocking antibody reduced adhesion of neutrophils to TNF-treated monolayers by around 30%, but had no effect on neutrophil adhesion to bleomycin-treated monolayers. When endothelial monolayers were pre-treated with function-blocking E-selectin antibody and neutrophils flowed over the monolayer, a decrease in adhesion of neutrophils to TNF-treated ECs of approximately 60% was observed.

However, there was no effect of E-selectin blockade on neutrophil adhesion to bleomycin-treated endothelial monolayers (figure 5).

## Discussion

We characterized the effects of bleomycin on adhesion molecules, chemokines, and pro-fibrotic cytokines at protein and mRNA levels in human endothelial cells *in vitro*. Bleomycin increased expression of ICAM-1, VCAM-1, and E-selectin by human PMVECs and HUVECs, with evidence that increased protein expression was the result of increased mRNA transcription. Bleomycin treatment of endothelial monolayers increased adhesion of flowing neutrophils, which in contrast to TNF-mediated adhesion, was not inhibited by blocking  $\beta$ 2 integrins (the neutrophil ligands for ICAM-1) or E-selectin.

We were careful to use concentrations of bleomycin compatible with those seen in the plasma following systemic dosing in humans. Previous studies have used much higher drug concentrations, for example Fichtner et al [11] reported increased ICAM-1 protein and mRNA expression in PMVECs treated with 100 U/ml (equivalent to  $>50\mu\text{g/ml}$ ) bleomycin for 24 hours. Effects of lower doses of bleomycin on E-selectin expression by HUVECs have been previously reported [12] [13].

In the present study a dose-dependent increase in release of IL-8 and MCP-1 by ECs treated with bleomycin was observed. Miyamoto, et al [12] reported significant increases in IL-8 release at 12 hours, but also reported high levels of cell death at higher bleomycin concentrations, a phenomenon that we were careful to exclude. Both IL-8 and MCP-1 are synthesised and stored in granules and the Golgi body, and both may be released in response to inflammatory stimuli or secretagogues [14-16] [17, 18]. As both *de novo* synthesis of IL-8 mRNA and increased release were noted at both time points tested it appeared that bleomycin was actively inducing IL-8 transcription, and the same is likely to be true for MCP-1.

Although we did not directly assess exocytosis of granules, our results showed concordance between inflammatory cytokine release and mRNA transcription suggesting that bleomycin is not acting as a secretagogue. The role of the increased synthesis and release of MCP-1 and

IL-8 may be of importance in the recruitment of immune cells to the endothelium, and their subsequent activation, prior to diapedesis and the movement of these cells into the lung.

In contrast to the effects on inflammatory cytokine release, we found that bleomycin treatment did not impact on mRNA transcript or secretion of the pro-fibrotic cytokines TGF- $\beta$ , PDGF-BB, or ET-1. There are few published works assessing pro-fibrotic cytokine expression by bleomycin-treated ECs. Miyamoto, et al.[12] found no significant difference in TGF- $\beta$ 1 release by bleomycin-treated ECs, but it is not clear whether active or latent TGF- $\beta$  was measured or what the baseline expression levels were. Increased TGF- $\beta$  release by endothelial cells in bleomycin-treated rodents could be due to a direct effect on endothelial cells, or an indirect effect via other cells and mediators [19]. There have been no previous reports of the expression of ET-1 and PDGF-BB by ECs exposed to bleomycin.

The results of the present study are consistent with a pro-inflammatory effect of bleomycin on ECs, with no evidence of a direct pro-fibrotic effect.

E-Selectin expression may be regulated by activation of its promoter which contains three binding sites for NF- $\kappa$ B[20, 21] , and blockade of NF- $\kappa$ B activation and binding reduced E-Selectin expression [13] [22-24]. NF- $\kappa$ B activation is also required for the transcription of ICAM-1, VCAM-1, IL-8, and MCP-1, and therefore this may be the mechanism by which adhesion molecule and inflammatory cytokine upregulation occurs in response to bleomycin. Expression of adhesion molecules and release of inflammatory cytokines in response to bleomycin were similar in HUVECs and PMVECs. We concluded that the more readily available HUVECs could be used as surrogate cells for PMVECs when assessing responses to bleomycin. Recent studies has suggested that the phenotype of ECs is dependent on their environment *in vivo* [25, 26]. When cultured *ex-vivo*, ECs from different vascular sites may display similar phenotypes.

In the systemic circulation, the first event in migration of neutrophils into injured tissue is rolling on vascular endothelium mediated by interactions between neutrophil ligands and endothelial selectins, including E-selectin. This slowing and tethering of neutrophils permits stronger adhesion via interactions between neutrophil integrins such as LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) and endothelial ICAM-1. The firm binding of neutrophil integrins to ICAM-1 requires integrin activation, mediated by cytokines such as IL-8 [27]. Spreading and transmigration then leads to the movement of neutrophils across the endothelium into the site of injury. The processes of adhesion and transmigration are not the same in all tissues. The main site of neutrophil sequestration in the lungs is the pulmonary capillary bed, an area devoid of vascular selectins. Here, initial binding of neutrophil integrins to ICAM-1 promotes leukocyte adhesion to the capillary endothelium [28]. Adhesion of flowing neutrophils to endothelial monolayers increased when the ECs were exposed to bleomycin for 6 or 24 hours. We hypothesised that increased adhesion may be mediated by E-selectin – upregulated quickly and reaching a peak at 4-6 hours, or ICAM-1 – upregulated slowly and reaching a peak at 24 hours, or a combination of the two. However, the present study demonstrated that neither molecule was involved in increased neutrophil adhesion to bleomycin-treated EC monolayers. Since the role of interactions between neutrophils and endothelial VCAM-1 have not been well defined, VCAM-1 was not assessed in this study.

The number of adherent neutrophils was similar in monolayers treated with bleomycin, shown to induce a modest increase in adhesion molecule expression, and those treated with TNF- $\alpha$ , which induced larger increases in adhesion molecule expression. It is possible that neutrophil adhesion to TNF-treated monolayers was the maximum achievable using this number of neutrophils, or that the functional effect of bleomycin is comparable to that of TNF- $\alpha$ , at least in this system. Published works assessing neutrophil adhesion have used more

neutrophils, but typically in systems whereby neutrophil boluses are perfused and

measurement takes place once all cells have entered the chamber. We believe that the method used in the present study is physiologically relevant, although differences in methodology mean the results of our work may not be directly comparable to those of other groups.

Treatment of isolated neutrophils with anti-CD18 blocking antibody reduced the binding of neutrophils to TNF-treated endothelial monolayers by approximately 30%, in keeping with previous reports[29-31]. However, neutrophil adhesion to bleomycin-treated monolayers was not reduced by CD18 blockade, implying that ICAM-1 is not involved in neutrophil adhesion to bleomycin-treated endothelial cells. This was despite the fact that ICAM-1 was the adhesion molecule most upregulated by bleomycin, and was clearly involved in neutrophil adhesion to TNF-treated monolayers. Previous reports using mouse models identified associations between pulmonary venule and capillary ICAM-1 expression and the accumulation of leukocytes at the vessel wall and in the perivascular space in response to bleomycin[32, 33]. However, the results of the present work suggest that, in human cells, one or more alternative adhesion molecules must be involved in neutrophil adhesion to endothelial cells in response to bleomycin treatment. Blockade of E-selectin was assessed to determine whether this molecule was responsible for the increased adhesion observed in response to bleomycin. There was a 60% decrease in adhesion of neutrophils to TNF- $\alpha$  treated monolayers, but blockade of E-selectin did not affect neutrophil adhesion to bleomycin-treated monolayers. It appears that other adhesion molecules other than ICAM-1 and E-selectin were responsible for the increased adhesion of neutrophils mediated by bleomycin. It would be of interest to assess the expression of other adhesion molecules in response to bleomycin, including ICAM-2, implicated in leukocyte crawling along the endothelium[34, 35], or GlyCAM-1, which like PSGL-1, binds neutrophil L-selectin, but

appears more involved in lymphocyte than neutrophil tethering[36], though the potential role of this molecule in extravasation is poorly understood.

**Bleomycin-induced neutrophil adhesion to human endothelial cells may contribute to the inflammatory cell influx seen in the lungs during bleomycin-induced lung injury and subsequent pulmonary fibrosis. Future work should seek to further define the adhesion molecule(s) responsible.**

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### **Declaration of Interests**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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JDW designed the study and performed the experiments; LRS supervised the experiments; MGC designed the study; JG designed the study and supervised the experiments; SPH designed the study and supervised the experiments. All authors contributed to writing the manuscript.

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## **Figure legends**

### **Figure 1**

Adhesion molecule expression and cytokine release by PMVECs. (A) representative flow cytometry histograms showing ICAM-1 (CD54) expression by bleomycin-treated PMVECs. Dotted line, control Ab; black line, untreated; dark grey line, 0.1  $\mu\text{g/ml}$  bleomycin; grey line, 1  $\mu\text{g/ml}$ ; light grey line, 10  $\mu\text{g/ml}$ . (B-D) Surface expression of ICAM-1, E-selectin (CD62E), and VCAM-1 (CD106) by bleomycin-treated PMVECs. (E-F) Release of IL-8 and MCP-1 release from PMVECs in response to bleomycin.  $n=3$ ; \*  $P \leq 0.05$  compared to untreated cells; for MCP-1 (24 hours) the dose-dependent increase was not statistically significant ( $P=0.275$ , untreated vs 10  $\mu\text{g/ml}$  bleomycin).

### **Figure 2**

Adhesion molecule expression is accompanied by increased mRNA transcription. (A-C) ICAM-1, VCAM-1, and E-selectin expression by HUVECs following exposure to bleomycin. (D-F) Bleomycin increases ICAM-1, VCAM-1, and E-selectin mRNA transcript levels.  $n=3$ ; \*  $P < 0.05$  compared to untreated cells; for ICAM-1,  $P=0.0585$  (6 hours).

### **Figure 3**

Pro-inflammatory cytokine release is accompanied by increased mRNA transcription. (A) IL-8 and (B) MCP-1 release by HUVECs following exposure to bleomycin. (C) IL-8 and (D) MCP-1 mRNA transcript levels following exposure to bleomycin.  $n=3$ ; \*  $P < 0.05$  compared to untreated cells; for IL-8,  $P=0.0962$  (6 hours),  $P=0.0501$  (12 hours).

### **Figure 4**

release from HUVECs following treatment with bleomycin (n=3). (B) TGF- $\beta$ , (D) PDGF-

BB, and (F) endothelin-1 mRNA transcript levels in bleomycin-treated endothelial cells.

n=3.

## **Figure 5**

Neutrophil adhesion to bleomycin treated endothelial monolayers. (A) Representative HUVEC monolayer after exposure to flowing human neutrophils. Adherent neutrophils were identified by their characteristic size and shape (yellow circles). (B) Neutrophil adhesion to HUVEC monolayers following treatment with bleomycin or TNF for 6 hours and (C) 24 hours. (D) Effects of anti-CD18 and (E) anti-E-selectin blocking antibodies on neutrophil adhesion to bleomycin-treated HUVECs. n=3; \* P<0.05 compared to untreated endothelial cells.









