A Chemokine Self-Presentation Mechanism Involving Formation of Endothelial Surface Microstructures

Catherine Whittall, Oksana Kehoe, Sophie King, Antal Rot, Angela Patterson and Jim Middleton

J Immunol 2013; 190:1725-1736; Prepublished online 16 January 2013; doi: 10.4049/jimmunol.1200867
http://www.jimmunol.org/content/190/4/1725

Supplementary Material
http://www.jimmunol.org/content/suppl/2013/01/16/jimmunol.1200867.DC1

Why The JI?

- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Speedy Publication! 4 weeks from acceptance to publication

*average

References
This article cites 38 articles, 16 of which you can access for free at:
http://www.jimmunol.org/content/190/4/1725.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
A Chemokine Self-Presentation Mechanism Involving Formation of Endothelial Surface Microstructures

Catherine Whittall,* Oksana Kehoe,* Sophie King,† Antal Rot,‡ Angela Patterson,§ and Jim Middleton*†

Endothelial surface microstructures have been described previously under inflammatory conditions; however, they remain ill-characterized. In this study, CXCL8, an inflammatory chemokine, was shown to induce the formation of filopodia-like protrusions on endothelial cells; the same effects were observed with CXCL10 and CCL5. Chemokines stimulated filopodia formation by both microvascular (from bone marrow and skin) and macrovascular (from human umbilical vein) endothelial cells. Use of blocking Abs and degradative enzymes demonstrated that CXCL8-stimulated filopodia formation was mediated by CXCR1 and CXCR2, Duffy Ag/receptor for chemokines, heparan sulfate (HS), and syndecans. HS was present on filopodial protrusions appearing as a meshwork on the cell surface, which colocalized with CXCL8, and this glycosaminoglycan was 2,6-O- and 3-O-sulfated. Transmission electron microscopy revealed that CXCL8-stimulated filopodial and microvilli-like protrusions that interacted with leukocytes before transendothelial migration and removal of HS reduced this migration. iTRAQ mass spectrometry showed that changes in the levels of cytoskeletal, signaling, and extracellular matrix proteins were associated with CXCL8-stimulated filopodia/microvilli formation; these included tropomyosin, fascin, and Rab7. This study suggests that chemokines stimulate endothelial filopodia and microvilli formation, leading to their presentation to leukocytes and leukocyte transendothelial migration. The Journal of Immunology, 2013, 190: 1725–1736.

Endothelial cells (ECs) are major cells involved in the immune response and inflammation. They play a role in the regulation of leukocyte extravasation, angiogenesis, cytokine production, protease and extracellular matrix synthesis, vasodilation and blood vessel permeability, and Ag presentation (1). In inflammatory conditions, such as psoriasis and rheumatoid arthritis, ECs have a predominant role undergoing activation, expressing adhesion molecules, and presenting chemokines, which leads to leukocytes migrating from the blood into the tissue.

Although the method of leukocyte extravasation has been widely studied, little is known about the function of EC surface microstructures in leukocyte migration. There have been several reports on the formation of filopodial and microvillous structures by ECs under inflammatory conditions. EC filopodia form during angiogenesis and in response to the inflammatory mediators TNF-α and bradykinin (2–4). Endothelial projections have been shown to be prominent in the inflamed synovium of arthritic patients (5), and microvillous structures are associated with ECs in atherosclerotic plaques (6) and in experimental allergic encephalomyelitis (7), suggesting their relevance in inflammatory disease. When human skin was injected with the chemokine CXCL8 after 30 min, numerous protrusions of the EC luminal membrane were visible and leukocyte extravasation occurred (8). Similarly, when CXCL8 was injected into human and rabbit skin, immunoelectron microscopy showed that the chemokine localized on the luminal EC surface where it was particularly concentrated on projections and microvillous processes (9). The concentration of chemokine on these structures suggested that the chemokine binding sites, such as glycosaminoglycans (GAGs) and potentially Duffy Ag/receptor for chemokines (DARC), may also be concentrated on these microstructures (9, 10). Such data imply that chemokines are presented to blood leukocytes on EC microstructures leading to leukocyte transendothelial migration. In addition, Feng et al. (11) injected the chemotactrant N-formyl-methionyl-leucyl-phenylalanine intradermally into guinea pigs and found that the vascular endothelium developed increased surface wrinkling and neutrophil recruitment occurred. Therefore, EC protrusive microstructures appear to be involved in leukocyte extravasation, but little is known concerning their composition, the receptors involved in their formation, and the associated intracellular changes.

Leukocyte diapedesis is a critical component for immune system function and inflammatory responses. This occurs by the migration of leukocytes either directly through individual ECs (transcellular) or between them (paracellular) (12). The endothelial cytoskeleton plays an essential role in transcellular migration; ICAM-1– and VCAM-1–enriched endothelial projections termed “transmigratory cups” or “docking structures” have been shown to partially embrace transmigrating leukocytes both in vivo and in vitro. These structures may serve as guidance structures to facilitate the initiation of both trans- and paracellular diapedesis (12–14).

*Leopold Muller Arthritis Research Centre, Institute for Science and Technology in Medicine, Medical School, Keele University, Robert Jones and Agnes Hunt Orthopaedic Hospital, Oswestry, Shropshire SY10 7AG, United Kingdom; †Department of Oral and Dental Science, Faculty of Medicine and Dentistry, University of Bristol, Bristol BS1 2LY, United Kingdom; ‡School of Immunity and Infection, Immune Regulation, University of Birmingham, Birmingham B15 2TT, United Kingdom; and §Rowett Institute of Nutrition and Health, University of Aberdeen, Aberdeen AB21 9SB, United Kingdom.

Received for publication March 20, 2012. Accepted for publication December 6, 2012.

This work was supported by Keele University (U.K.), the Medical Research Council (U.K.), and the Institute of Orthopaedics, Robert Jones and Agnes Hunt Orthopaedic Hospital (Oswestry, U.K.).

Address correspondence and reprint requests to Prof. Jim Middleton, Faculty of Medicine and Dentistry, Department of Oral and Dental Science, Lower Maudlin Street, University of Bristol, Bristol BS1 2LY, U.K. E-mail address: jim.middleton@bristol.ac.uk

The online version of this article contains supplemental material.

Abbreviations used in this article: DARC, Duffy Ag/receptor for chemokines; EC, endothelial cell; GAG, glycosaminoglycan; HBMEC, human bone marrow EC; HMVEC, human dermal microvascular EC; HS, heparan sulfate; HSPG, HS proteoglycan.

Copyright © 2013 by The American Association of Immunologists, Inc. 0022-1767/13/S16.00
This study examined the formation of EC surface protrusions by microvascular and macrovascular ECs in response to various chemokines. The composition of their chemokine binding molecules, the receptors involved in their formation, and their association with transmigrating leukocytes have been investigated.

Furthermore, mass spectrometry and Western blot analysis were performed on chemokine-stimulated ECs to determine the intracellular changes that occurred related to EC microstructure formation. The results suggest that by chemokines stimulating formation of filopodial and microvillous protrusions, they may enhance their own presentation.

Materials and Methods

**Cell culture**

For comparisons of endothelial microstructure formation between cells, various ECs were used: immortalized human bone marrow ECs (HBMECs), donated by Prof. B. B. Weksler (15), maintained in DMEM-F12 (Lonza, Wokingham, U.K.); HUVECs extracted from umbilical cords following informed consent, maintained in 0.1% gelatin-coated tissue culture dishes in EGM-2 (Lonza); and human dermal microvascular ECs (HMVECs; Lonza) maintained in EGM-2–MV (Lonza), all containing 10% FBS (Invitrogen, Paisley, U.K.) and 50 μM penicillin/streptomycin (Lonza). All ECs were incubated at 37°C in a humidified incubator with the addition of 5% CO2 and grown to around 70% confluence before being used in the following experiments.

**EC activation**

ECs were activated by incubating monolayers with recombinant human CXCL8 (IL-8), CXCL10 (IP-10), or CCL5 (RANTES; PeproTech EC, London, U.K.) at concentrations ranging from 0 to 500 ng/ml in serum-free medium for 30 min at 37°C. They were also activated with cytokines recombinant human TNF-α and IFN-γ (both at 100 ng/ml; PeproTech) for 16 h at 37°C. Negative controls contained no chemokines or cytokines.

The effect of chemokine on filopodial protrusion formation was determined by counting the total number of cells per random field of view (n = 10 fields of view) at original magnification ×200 and the number of those cells that had formed the microstructures. The percentages of cells with filopodial protrusions were then calculated and means ± SE determined.

**Blocking and degrading CXCL8 binding sites**

Before stimulation with CXCL8, the cells were treated with heparanases and Abs using titrations; concentrations ranged from 5 to 20 U/ml for heparinase I (Sigma-Aldrich, Dorset, U.K.); 0.5–2 U/ml for heparinase III (Sigma-Aldrich); 0.5–2 μg/ml for anti-CXCR1 (R&D Systems, Oxfordshire, U.K.); 2.5–10 μg/ml for anti-CXCR2 (R&D Systems); 50–400 ng/ml for anti-DARC (donated by Prof. D. Blanchard, University of Nantes, Nantes, France); 2.5–10 μg/ml for both anti–syndecan-3 and –syndecan-4 (donated by Prof. G. David, University of Leuven, Leuven, Belgium). The cells were then treated with the optimum concentrations of heparinase I (10 U/ml) or heparinase III (2 U/ml) for 1.5 h at 37°C to remove the heparan sulfate (HS). To block CXCL8 binding sites, we treated ECs with mouse monoclonal blocking Abs (IgG2A) to human CXCR1 (optimum concentration, 2 μg/ml) or human CXCR2 (2.5 μg/ml) for 30 min at 37°C; mouse IgG2A (Dako, Cambridge, U.K.) was used as an isotype control; a mouse mAb (IgG1) to human DARC Fab6 (2C3, 50 ng/ml) for 30 min at 37°C; and mouse IgG1 (Dako) was used as an isotype control. To examine syndecan-3 and –4 internalization, we treated the cell monolayers with cell culture medium at 4°C or 37°C in the presence of mouse mAbs to syndecan-3 and –4 (IC7 and 8G3; both at 5 μg/ml) or mouse IgG1 (isotype control) and maintained them at this temperature for 30 min (in an atmosphere of 5% CO2). In addition, control cells were not treated with blocking Abs or enzymes and were without chemokine stimulation; in addition, cells were left untreated, yet with CXCL8 (100 ng/ml) stimulation for 30 min. After the treatments, the cells were fixed with ice-cold 1:1 acetone-methanol in the absence of permeabilization before being analyzed with phase-contrast microscopy. The percentage of ECs with filopodial protrusions was determined as described previously.

**Immunofluorescence**

A total of 1 × 10^5 HBMECs in 500 μl serum-free DMEM-F12 was seeded into 8-well chamber slides (Cote-Parmer Instrument Company, London, U.K.) and incubated at 37°C for 48–72 h until the levels of confluence achieved as described earlier. Immunofluorescence of HS was performed using phage display-derived single-chain Abs with a VSV-G tag (donated by Prof. T. Kuppevelt, University of Nijmegen, Nijmegen, The Netherlands). CXCL8-stimulated (100 ng/ml for 30 min) or unstimulated ECs were incubated with 0.5 U/ml A40B8, which recognizes the GlcNS6S-IdoUA2S-GlcNS6S epitope (16), or HS4C3, which recognizes the 3-O-sulphation epitope (17) (both at 0.5 μg/ml in 2% BSA/PBS), at room temperature for 1.5 h. Cells were washed three times for 5 min in PBS. Primary Abs were detected using a mouse mAb (IgG1) to the VSV-G tag (clone PD54; 1:100 in 2% BSA/PBS; Sigma-Aldrich) for 1 h followed by incubation with Alexa Fluor 488 goat anti-mouse IgG1 (1:400 in 2% BSA/PBS; Invitrogen) and washed before being counterstained with DAPI for 3 min. The cells were mounted with Hydromount (Fisher Scientific) or Prolong Gold anti-fade reagent (Invitrogen) and visualized with a light microscope (Olympus IX51) and analyzed with Cell^F software, or a confocal microscope (Leica TCS SP5) analyzed with Leica Application Suite. PBS was used as a negative control of the phage display Abs as a negative control. Mouse IgG1 (0.5 μg/ml) was used as an isotype control.

For double labeling, the experiment was repeated except that goat anti-human CXCL8 (5 μg/ml in 2% BSA/PBS; R&D Systems, Abingdon, U.K.) was added with anti-HS, and Alexa Fluor 594 donkey anti-IgG1 (1:200 in 2% BSA/PBS; Invitrogen) added with Alexa Fluor 488 goat anti-mouse IgG1 (as described earlier). Goat IgG1 (5 μg/ml in 2% BSA/PBS; Dako) was used instead of primary anti-CXCL8 Ab as control.

CXCL8-stimulated HBMECs were pretreated with heparinase I or II (as described previously) before staining as described earlier. The expression of the A40B8 and HS4C3 epitopes (± heparinase treatment) was quantified by counting the total number of cells per field of view (from 10 fields of view) and the number of those cells that had positively stained filopodia. An EC was scored positive when the HS-positive filopodia/meshwork was associated with it. The percentage of cells expressing the epitopes on the filopodia was calculated, and means ± SE determined.

**Transendothelial migration**

**Leukocyte isolation.** A total of 4 ml blood was obtained in EDTA-containing tubes, after informed consent, from healthy volunteers. Blood was added to 16 ml ice-cold ammonium chloride and incubated on ice for 15 min before adding heparin (20 U/ml) to remove erythrocytes. The leukocytes were washed three times with HBSS; at 1000 rpm for 10 min at 4°C, the supernatant was removed, and the solution was resuspended in 15 ml sterile HBSS. After further centrifugation, the supernatant was removed and the leukocytes resuspended in HBSS to a final concentration of 1 × 10^6 cells/ml.

**Neutrophil migration through an endothelial layer.** A total of 2 × 10^5 ECs in 500 μl medium was cultured on 3-μm pore transwell filters (Millipore UK, Watford, U.K.) in 24-well flat-bottom microplates (SLS, Nottingham, U.K.) at 800 μl medium/well, until a monolayer was formed (24–48 h at 37°C). The solution in the apical chamber was replaced with 500 μl fresh serum-free DMEM-F12, and the solution in the basal chamber was replaced with a solution of serum-free medium containing 100 ng/ml CXCL8; for controls, serum-free medium containing no CXCL8 was used. The samples were incubated at 37°C for 30 min for filopodia/microvilli formation to occur. A total of 5 × 10^6 leukocytes were then added to the apical chambers, followed by the basal chambers until a monolayer was formed (30 min in continued presence of CXCL8). Initial experiments compared 30- and 60-min migration times, and 30 min was found to be optimal and sufficient for 30% of neutrophils to migrate across microvascular HBMECs. These time points are in general agreement with those reported for CXCL8-stimulated neutrophil migration across HUVECs (18). Neutrophil migration was quantified using flow cytometry, as described later.

**Inhibition of neutrophil migration.** Endothelial monolayers were pretreated with 500 μl serum-free medium in the apical chambers, containing either 10 U/ml heparinase I or 2 U/ml heparinase III for 1.5 h at 37°C; the enzymes were removed before CXCL8 stimulation (as described earlier). As a control, neutrophils were added to a CXCL8-stimulated endothelial monolayer that had not been treated with any enzymes. Neutrophil migration was quantified by flow cytometry.

**Flow cytometry.** After leukocyte transendothelial migration, the solutions from the basal chambers were analyzed at 1400 rpm for 6 min. The supernatant was removed and the pellets resuspended in 150 μl HBSS; the solutions were analyzed on a flow cytometer (FACScan; Becton Dickinson, Oxfordshire, U.K.). A solution of the original leukocyte preparation was analyzed primarily so gates could be applied on the neutrophil population; after this the migrated samples were analyzed and the numbers of neutrophils in each well were determined. The percentage of neutrophils that had migrated was calculated by counting the neutrophils that may have adhered to the plastic surfaces of the basal chambers, thereby...
artificially reducing the numbers collected for flow cytometry. Microscopy revealed no such adherence to the plastic surfaces of the lower chamber.

Transmission electron microscopy

Electron microscopy analysis was performed on HBMEC monolayers grown on 3-μm pore transwell filters, in the presence and absence of leukocytes, as described earlier. HBMECs were stimulated with 100 ng/ml CXCL8, or left unstimulated, before the addition of the leukocytes. The leukocytes were left to migrate for 15 or 30 min at 37°C before the filters were washed with PBS and fixed for 2 h at room temperature in 0.1 M sodium cacodylate (Sigma-Aldrich) and 2 mM CaCl2 (buffer A; Sigma-Aldrich) containing 2.5% glutaraldehyde (Sigma-Aldrich); the filters were removed from the inserts and stored in buffer A containing 0.1% glutaraldehyde. The samples were washed three times for 5 min in buffer A, before postfixation using 0.1% osmium tetroxide in buffer A for 1 h. The samples were then washed again and stored overnight in 70% ethanol. Samples were dehydrated in increasing concentrations of ethanol (80 and 100%) and 15 min followed by 15 min in 100% dry ethanol before embedding with Spurr’s resin and dry ethanol. Sections 100 nm thick were cut using a Leica Ultracut microtome and stained with 2% lead citrate and 2% uranyl acetate before visualization with a JEOL electron microscope.

Mass spectrometry

Mass spectrometry was performed as described by Fuller et al. (19) with some modifications. Cell extraction. A total of 15 × 104 HBMECs (P24) were acclimated with 100 ng/ml CXCL8 for 0.5, 6, or 15 h at 37°C; unstimulated cells were used as a control. The cells were detached, centrifuged, washed with PBS, and incubated in 4 vol of 6 M urea, 2 M thiourea, 2% CHAPS, and 0.5% SDS, in water, then sonicated before being stored at −80°C overnight.

Protein precipitation. Four volumes of ice-cold acetone were added to each extract, then stored overnight at −20°C. The precipitated protein was then isolated by centrifugation of the extracts at 13,000 × g for 10 min at 4°C; insoluble cell debris was removed and discarded, and the pellets were air-dried and resuspended in 6 M urea in 50 mM TEAB. The protein concentration for each sample was determined using a Bradford assay.

Labeling and digestion of cell extracts. The cysteines were reduced and blocked using reagents provided with the iTRAQ kit (Applied Biosystems, Warrington, U.K.). The samples were diluted to <1 M urea with dissolution buffer, followed by incubation overnight at 37°C with trypsin (2 μg per 100-μg sample). The samples were then dried down in a vacuum centrifuge and labeled with iTRAQ reagents according to the protocol in the iTRAQ kit; the 114 tag represented the control, and 115, 116, and 117 tags were the 0.5, 6, and 16 h CXCL8-treated samples, respectively.

Ion exchange of iTRAQ-labeled peptides. iTRAQ peptides from the four samples were pooled and redissolved in 2.4 ml of 10 mM phosphate in 20% acetonitrile (buffer A). The whole sample was loaded on an SCX column (5-μm particles; 300-A pores; Fisher Scientific) at 400 μl/min. The column was washed at 800 μl/min with buffer A until the baseline returned to zero. A gradient was run at 400 μl/min from 0–50% 10 mM phosphate and 1 M NaCl in 20% acetonitrile (buffer B) over 35 min followed by a ramp up from 50 to 100% buffer B over 5 min. One hundred percent buffer B was held for 5 min (400 μl/min) before equilibrating the column for 10 min with buffer A (400 μl/min).

Protein identification by mass spectrometry. Fractions were redissolved in 25 μl 2% acetonitrile, 98% water, and 0.05% trifluoroacetic acid (buffer C) before being loaded onto an Acclaim Pepmap C18 trapping column ( Dionex, Surrey, U.K.); the fractions were then eluted onto an Acclaim Pepmap C18 analytical column (Dionex). The column was then washed in buffer A for 15 min before the peptides were eluted using a gradient from 0 to 30% of buffer B over 90 min, then 30–60% of buffer B over 35 min, followed by a final elution at 90% of buffer B for 10 min. The column was then washed and equilibrated in buffer A for 10 min (UltiMate 3000; Dionex). A blank sample (buffer A) was run in between each fraction to minimize any carryover of peptides and the loading of samples was randomized. Fractions were collected at 10-s intervals using a Probot microfraction collector ( Dionex Ltd) with 3 mg/ml o-cyanohydroxy cinnamic acid (Sigma-Aldrich) containing 70% acetonitrile and 0.1% trifluoroacetic acid at a flow rate of 1.2 μl/min. The fractionated peptides were then analyzed using a Bruker Ultraflex II MALDI TOF/TOF.

Mass spectrometry iTRAQ analysis. The peptide count is defined as the number of peptides with unique sequences matching the selected protein. The peptide count in 20% acetonitrile of protein in the control sample (numbered 114) was held for 5 min with the peptide in the control sample (numbered 114). The score is based on the total ion score, which is a score calculated by weighting ion scores for all individuals matched to a given protein, and is a measure of signal strength. The confidence interval is the confidence that the peptides found are part of the protein identified.

SDS-PAGE and Western blotting

Electrophoresis was performed using the BioRad Mini Protein III gel system. Slab 6–12% acrylamide gels (0.75 mm thick) were prepared from stock solutions. Protein samples from mass spectrometry cell extracts were used, protein concentrations determined, and 0.0001% (w/v) bromophenol blue added. Ten- to 20-μl samples were loaded onto gels, and electrophoresis was performed in electrophoresis buffer at 160 V for 1–2 h. After electrophoresis, gels were transferred to nitrocellulose membranes using a wet-blot system with transfer buffer. Proteins were transferred for 1 h at 90 V. After blotting, membranes were incubated in TBST containing 10% nonfat milk (dilution buffer) overnight at 4°C. The membranes were incubated, for 1 h at room temperature, in dilution buffer containing the following primary Abs: mouse monoclonal IgG1 to human tropomysin (1:1000; Sigma-Aldrich), rabbit polyclonal to human Rab7 (1:1000; Abcam, Cambridge, U.K.), mouse monoclonal IgG (1:200; Abcam), or rabbit polyclonal to human GAPDH (1:2000; 2B Scientific, Upper Heyford, U.K.). Positive Ab bands were visualized by development with either peroxidase-labeled goat anti-mouse Ig or peroxidase-labeled donkey anti-rabbit IgG (1:2000 in dilution buffer) and detected using a chemiluminescence system (GE Healthcare, Buckinghamshire, U.K.).

Intradermal injection of CXCL8 in rabbits

Human recombinant CXCL8 was injected intradermally, 1 μg/site, in Chimilla rabbits (n = 3; Charles River, Wilmington, MA); duplicate biopsies of the sites were taken 4, 2, 1, 0.5 h, and immediately after the injection (0 h). Vehicle-injected sites sampled at the same time points, and noninjected skin served as controls. Biopsies were processed for transmission electron microscopy as described earlier.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5. To determine statistical significance, because all the data showed a normal variation, and the ANOVAs were performed followed by Tukey posttest comparisons to establish significant variations between individual sample groups; a p value <0.05 was deemed as significant. For mass spectrometry, the iTRAQ data of CXCL8-treated and untreated HBMECs were compared by ANOVA using data for each peptide for a given protein.

Results

Chemokine binding and the microstructure of ECs

ECs were grown on plastic in the presence of serum until ~70% confluence and this enabled the visualization of the cell periphery. They were then incubated with or without chemokines in serum-free media. In the presence of chemokines using phase contrast, filopodial protrusions (also named “filopodia”) were seen at the cell periphery, occurring within 30 min (Fig. 1A, 1B). Time-lapse video imaging showed that these were dynamic remodeling structures, extending and retracting rapidly after addition of the chemokine (data not shown). The inclusion criteria for these microstructures were: one or more finger-like processes that extended from the cell, measuring up to 10 μm in length and 0.2–1 μm in width, and were not lamellipodia. These filopodial protrusions frequently contacted adjacent cells even extending over their surfaces. Cells were excluded that had a normal rounded or spindle-like morphology and had no extending cell processes. When the ECs were treated with chemokines for 6 and 16 h, filopodia were still present. The cell lines used in this model were HBMECs, HMVEC-ECs, and HUVECs. The ECs were treated with CXCL8, CXCL10, and CCL5 at concentrations varying from 0 to 500 ng/ml. For each chemokine treatment in all three cell lines, there were significant changes in the percentage of cells that formed filopodia with increasing concentrations of chemokine (p ≤ 0.0001; Fig. 1C).

HBMECs. The percentage of HBMECs that formed filopodial protrusions in the absence of chemokines ranged from 3.8 ± 2.8 to 11.2 ± 0.8%. All concentrations of CXCL8, CXCL10, and 1–200 ng/ml CCL5 caused a significant increase in the percentage...
of cells with filopodia compared with the control \((p \leq 0.0001; \text{Fig. 1C})\). The percentage of cells that formed these microstructures ranged from 24.7 ± 6.1% with 1 ng/ml CXCL8 up to 51.6 ± 1.8% with 200 ng/ml CXCL8. With CXCL10, the percentage of cells with filopodia increased from 21.1 ± 1.3% (1 ng/ml) up to 46.5 ± 2.7% (200 ng/ml). In the presence of CCL5, the percentage of cells with filopodia ranged from 23.6 ± 6.1% (1 ng/ml) to 28.0 ± 3.6% (100 ng/ml). At higher chemokine concentrations, the amount of these microstructures reduced such that at 500 ng/ml, CCL5 had no significant effect (Fig. 1C). There were significant reductions in the percentage of filopodia at higher chemokine concentrations. CXCL10 caused the percentage to decrease from 46.5 ± 2.7% (200 ng/ml) to 37.3 ± 1.26% with 500 ng/ml \((p \leq 0.0001)\). In the presence of CCL5, there were significant reductions in the percentage of filopodia at higher chemokine concentrations. CXCL10 caused the percentage to decrease from 46.5 ± 2.7% (200 ng/ml) to 37.3 ± 1.26% with 500 ng/ml \((p \leq 0.0001)\). With CXCL8 stimulation, there was no significant reduction in the percentage of these structures formed at higher chemokine concentrations, although at 500 ng/ml, the percentage was slightly lower (51.5 ± 1.6% with 200 ng/ml to 43.6 ± 2.1% with 500 ng/ml).

Cells were also treated with inflammatory cytokines to activate them to examine their effects on filopodial protrusion formation. HBMECs treated with TNF-α and IFN-γ (both at 100 ng/ml) for 16 h promoted filopodia formation compared with no cytokine control \((p < 0.0001)\), amounting to 45 ± 3.3% of HBMECs forming these structures. Addition of CXCL8 (100 ng/ml) for 30 min after TNF-α/IFN-γ activation further enhanced filopodia formation to 53 ± 3.5%, although not significantly. Overall, TNF-α/IFN-γ activation of HBMECs did not significantly stimulate filopodia formation compared with use of CXCL8 alone (100 ng/ml for 30 min), which amounted to 49 ± 2.6% (Fig. 1C).

The effects of the presence of serum on microvilli formation was examined. Control cells that were incubated in the presence of serum (10% FBS) and no chemokine had low levels of filopodia (9 ± 1.8%), being similar to those obtained in the absence of serum and no chemokine (range, 3.8–11.2%; Fig. 1C). Addition of 100 ng/ml CXCL8 for 30 min in the presence of serum stimulated filopodia formation to 30 ± 3.3% \((p = 0.0003)\). Therefore, filopodia formation does not appear to occur because of exposure of ECs to a serum-free environment in combination with chemokine.

HMVEC. The percentage of HMVEC that formed filopodia in the absence of chemokines ranged from 4.2 ± 0.2 to 4.5 ± 0.3%. All concentrations of CXCL10 and 10–500 ng/ml CXCL8 and CCL5 caused a statistically significant increase in the percentage of cells with filopodia \((p \leq 0.0001 \text{ to } p < 0.01)\); 1 ng/ml CXCL8 and CCL5 had no significant effect (Fig. 1C). The percentage of cells that formed filopodia increased from 6.3 ± 10.3% with 1 ng/ml CXCL8 up to 18.1 ± 1.0% with 200 ng/ml. With CXCL10, the percentage of cells with these microstructures ranged from 9.9 ± 0.5% (1 ng/ml) up to 16.3 ± 0.5% (10 ng/ml). In the presence of CCL5, the percentage of cells with filopodia increased slightly from 5.5 ± 0.3% (1 ng/ml) up to 13.1 ± 0.7% (200 ng/ml).
percentage of filopodia formed showed changes at higher chemokine concentrations. In the presence of 500 ng/ml CXCL8, the filopodia percentage was significantly reduced from 18.1 ± 1.0% (200 ng/ml) to 13.0 ± 0.7% (p = 0.0001). Similarly, with 500 ng/ml CCL5, the percentage of filopodia reduced significantly from 13.1 ± 0.7% (200 ng/ml) to 7.4 ± 0.5% (p = 0.0001), almost a 2-fold reduction in these structures. CXCL10 showed significant reductions in filopodia when the chemokine concentrations increased to >10 ng/ml. There were significant differences between 10 and 200 ng/ml (16.3 ± 0.5 to 10.6 ± 0.4%), 10 and 500 ng/ml (16.3 ± 0.5 to 11.5 ± 0.7%), 100 and 200 ng/ml (14.5 ± 0.6 to 10.6 ± 0.4%), and 100 and 500 ng/ml (14.5 ± 0.6 to 11.5 ± 0.7%; all p < 0.0001).

HUVECs. The percentage of HUVECs that formed filopodia in the absence of chemokines ranged from 3.5 ± 0.6 to 4.1 ± 0.7%. All concentrations of CXCL10 and 10–500 ng/ml CXCL8 and CCL5 caused a significant increase in the percentage of cells with these structures (p ≤ 0.0001); 1 ng/ml CXCL8 and CCL5 had no significant effect (Fig. 1C). The percentage of cells that formed filopodia ranged from 4.9 ± 0.4% with 1 ng/ml CXCL8 up to 18.9 ± 0.8% with 500 ng/ml CXCL8. With CXCL10, the percentage of cells with filopodia increased from 14.7 ± 0.6% (1 ng/ml) to 21.9 ± 1.1% (200 ng/ml). In the presence of CCL5, the percentage of the cells with the structures changed from 5.1 ± 0.3% (1 ng/ml) to 11.9 ± 1.0% (200 ng/ml).

There were significant differences in filopodia formation at higher concentrations of CXCL10 and CCL5; CXCL8 caused no significant reduction at higher concentrations. In the presence of CCL5, there was a reduction in filopodia formation, from 19.8 ± 1.6% with 100 ng/ml and 19.8 ± 1.0% with 200 ng/ml to 12.5 ± 0.8% with 500 ng/ml (p ≤ 0.0001). CXCL10 showed a similar pattern, reducing from 21.4 ± 0.9% with 100 ng/ml and 21.9 ± 1.1% with 200 ng/ml to 15.6 ± 0.7% with 500 ng/ml (p ≤ 0.0001).

Receptors involved in filopodial protrusion formation

To determine which receptors were involved in the formation of filopodial protrusions, we pretreated CXCL8-stimulated HBMECs with blocking Abs to chemokine receptors or enzymes that cleave the GAG HS (Fig. 2A). With no blocking treatment and no chemokine stimulation, the percentage of cells that formed filopodia was 14 ± 0.9%. Treating the cells with CXCL8, in the presence of IgG control, caused an increase in the percentage of ECs with these structures up to 54 ± 2.4%. When the cells were treated with heparanase I, the percentage of cells with filopodia was significantly reduced to 33 ± 2.4% (p ≤ 0.0001) when compared with the CXCL8-treated control; with heparanase III, the percentage of cells with filopodia was 38 ± 2.8%, again significantly different from the control (p ≤ 0.0001); with anti-CXCR1, the percentage was 37 ± 1.8% (p ≤ 0.0001); with anti-CXCR2, the percentage decreased to 29 ± 2.4% (p ≤ 0.0001). When heparanase I and anti-CXCR2 were used together, the percentage of cells with filopodia reduced further to 9 ± 0.9% (p ≤ 0.0001).

After treatment with anti-DARC, the proportion of cells that formed filopodia was decreased to 13 ± 2.1% (p ≤ 0.0001), which was not significantly different from that of the negative control (no blocking treatment and no chemokine stimulation). All the other single treatments were significantly higher than the negative control (p ≤ 0.0001). Similarly, there were differences between the percentages of filopodia formed after blocking with anti-DARC compared with the other treatments used (heparanase I, heparanase III, anti-CXCR1, or anti-CXCR2; p ≤ 0.0001).

Major HS proteoglycans (HSPGs) found to be expressed by HBMECs were syndecan-3 and -4 (data not shown). HBMECs were incubated with blocking Abs to syndecan-3 and -4 at temperatures of either 4°C or 37°C before incubation with CXCL8, to determine whether these HSPGs were being internalized upon binding (Fig. 2B). When incubated at 4°C, the percentage of ECs that formed filopodia with no CXCL8 or blocking treatment was 11.1 ± 0.7%; in the presence of CXCL8 and the IgG control, the percentage was 29.8 ± 1.1%. When the cells were treated with anti–syndecan-3 at 4°C, the percentage (compared with the isotype control) decreased significantly to 15.0 ± 1.3% (p ≤ 0.0001); with anti–syndecan-4, the percentage decreased but not significantly (25.4 ± 2.2%). At 37°C, the number of HBMECs that showed filopodia formation with no CXCL8 or blocking treatment was 4.6 ± 0.3%; with the isotype control (in the presence of CXCL8), the percentage was 30.0 ± 0.8%. There was a decrease in filopodia formation after treatment with both anti–syndecan-3 (8.9 ± 0.7%; p ≤ 0.0001) and anti–syndecan-4 (3.7 ± 0.3%; p ≤ 0.0001) when compared with the isotype control. In control ECs in the absence of CXCL8, cold exposure at 4°C increased filopodia formation compared with 37°C, although these values did not significantly differ, suggesting that physical stress such as cold temperatures may upregulate filo-
podia abundance, although the effect was less than that seen with chemokines.

**HS epitope expression**

HBMECs were activated with CXCL8 as described earlier, then labeled with Abs AO4BO8 and HS4C3 specific for 2,6-0– and 3-0–sulfated epitopes of HS, respectively (20, 21), to determine whether HS with these sulphation patterns was expressed on filopodial protrusions shown in Figs. 1 and 2 by phase-contrast imaging. Analysis of AO4BO8 immunofluorescence after treatment with CXCL8 revealed an abundance of 2,6-0–sulfated HS expression on filopodial protrusions that extended between cells and over the cell surface, appearing as a meshwork (Fig. 3A, 3B, 3E, 3G; see Supplemental Fig. 1). The width of this meshwork was ~200–500 nm. HS on the filopodial meshwork colocalized with CXCL8 (Fig. 3E, 3F). With Ab HS4C3, HS was also present on filopodial protrusions (Fig. 3C, 3D), which were often less extensive than with AO4BO8 and needed higher magnifications to image them (Fig. 3G, left panels). There was no fluorescence when isotype control Ig or PBS was used instead of primary HS or CXCL8 Abs.

HBMECs were pretreated with heparanase I and III to degrade this GAG (Fig. 3G) and the effects quantitated to verify that HS was present on the filopodial meshwork (Fig. 3H). An EC was scored positive when the HS-positive filopodia/meshwork was associated with it. Under normal conditions (no CXCL8 or enzymatic treatment), 10.8 ± 2.8% of the ECs showed expression of AO4BO8 on these structures and 3.2 ± 1.0% with expression of HS4C3 (Fig. 3H). When the cells were treated with 100 ng/ml CXCL8, there was a significant increase in AO4BO8 expression (29.0 ± 4.7%; p < 0.001) and HS4C3 expression (23.2 ± 1.7%; p < 0.0001). When CXCL8-stimulated HBMECs were pretreated with heparinase I, the percentage of cells expressing either 2,6-0–sulfated HS (2.6 ± 0.8%; p < 0.0001) or 3-0–sulfated HS (3.8 ± 1.6%; p < 0.0001) on the microstructures was significantly decreased. No difference on the expression of the AO4BO8 epitope (31.8 ± 0.4%) was observed when the cells were pretreated with heparinase III (Fig. 3G, 3H). However, there was a significant re-

**FIGURE 3.** Localization of HS 2,6–0– and 3–0–sulfated motifs on endothelial filopodia. (A) CXCL8-stimulated HBMECs analyzed by phase contrast showing a filopodial protrusion. (B) Same image as (A) using immunofluorescence with an Ab against the 2,6–0–sulfated HS epitope (AO4BO8). Arrows show the localization of HS to the filopodium. (C) CXCL8–stimulated HBMECs analyzed by phase contrast showing a filopodial extension. (D) Same image as (B) using immunofluorescence with an Ab against the 3–0–sulfated HS epitope (HS4C3). Arrows show the localization of HS to the filopodium. Double labeling using Abs to HS (E) and CXCL8 (F) showing colocalization (arrows). Scale bar, 5 μm (A–F). (G) CXCL8–stimulated HBMECs pretreated with and without heparinase I (10 U/ml) and heparinase III (2U/ml), and analyzed by immunofluorescence with Abs against 3–0–sulfated epitopes (HS4C3) and 2,6–0–sulfated epitopes (AO4BO8; both green) with DAPI showing nuclear staining (blue). With HS4C3 Ab, arrows show the filopodia. Scale bar, 5 μm (HS4C3) and 20 μm (AO4BO8). (H) Analysis of immunofluorescence shown in (G) with the Abs described; the percentage of cells with positive staining on the filopodia was quantified. Data show the means ± SE of the percentage of cells with filopodia in 10 fields of view (at original magnification ×200) from one experiment representative of two independent experiments. **p ≤ 0.001, ***p ≤ 0.0001.
FIGURE 4. Endothelial filopodial and microvillous protrusions and leukocyte interaction. (A–F) HBMECs were cultured on 3-μm pore transwell filters and treated with 100 ng/ml CXCL8 for 60 min in the basal compartment of transwells. Leukocytes were added to the apical compartment for the final 30 min before fixation and analysis using transmission electron microscopy. (A) An unstimulated HBMEC monolayer (E) grown on a transwell filter (F). (B) Microvillus protrusion (M) formed by the EC (E) in response to CXCL8 stimulation. (C) A CXCL8-stimulated EC layer (E) with a leukocyte (L) in close proximity to endothelial microvilli (M). (D) A leukocyte (L) interacting with a filopodial protrusion (F) of HBMECs (E) stimulated with CXCL8. This microstructure is extending laterally over the EC surface, whereas in (B) and (C), microvilli extend more vertically. (E) A leukocyte (L) interacting with CXCL8-stimulated HBMECs (E). Arrows indicate leukocyte podosomes. (F) A CXCL8-treated layer of HBMECs (E) in the presence of leukocytes (L); there is an absence of large intercellular gaps between ECs (arrow). (G) Rabbit skin was injected with CXCL8 in vivo, and after 30 min biopsies were taken and processed for electron microscopy. Note the presence of a protrusion similar to those seen in vitro (B–D). (H) Same as (G) except that skin was vehicle injected instead of CXCL8. Scale bar, 2 μm.

Reduction in the proportion of cells positive for the HS4C3 epitope (2.2 ± 0.4%: p ≤ 0.0001) after heparanase III treatment. With HS4C3, cell nuclei were also positive (Fig. 3D, 3G) in the presence or absence of CXCL8 and in the presence of heparanases; the significance of these results is as yet unknown.

Leukocyte interaction with HBMEC microstructures and transendothelial migration

The effect of chemokine stimulation on ECs was analyzed using transmission electron microscopy to examine filopodia/microvilli formation and their interaction with leukocytes. In these experiments, HBMECs were grown to confluence on the filters of transwells and 100 ng/ml CXCL8 was added to the lower well, adding the chemokine to the basal EC surface. When the ECs were unstimulated, the membranes were relatively smooth and featureless (Fig. 4A). When stimulated with CXCL8 for 30 min, microvillous protrusions had formed that extended upward from the apical cell surface (Fig. 4B, 4C). These measured ~200 nm in width and were up to 1 μm in length. In addition, there were longer filopodia-like protrusions, measuring around 200–500 nm in width. These extended laterally over the surface of the cell (Fig. 4D) and even over the adjacent cell, measuring micrometers in length (Supplemental Fig. 2). These filopodial and microvillous protrusions were associated with leukocytes (Fig. 4C, 4D). Quantitation of filopodial protrusions revealed that in the absence of CXCL8, 8 ± 8% (mean ± SE) of cells (n = 12) formed filopodial protrusions, and this increased significantly to 50 ± 14% (n = 14 cells) in the presence of the chemokine (p = 0.02). For the microvilli-like structures, values were 15 ± 10% (n = 13 cells) without CXCL8, increasing to 57 ± 14% (n = 14 cells) with the chemokine (p = 0.02). Leukocytes also formed podosomes that were interacting with the endothelial layer (Fig. 4E).

The integrity of the EC layer was examined because CXCL8 may have induced the formation of interendothelial gaps and leukocytes may have migrated through them. Transmission electron microscopy of the EC layer 15 and 30 min after the addition of CXCL8 did not reveal the formation of large interendothelial gaps or openings in the presence or absence of leukocytes (Fig. 4F) (22). This suggests that CXCL8 did not cause EC disruption leading to impairment of the EC barrier in relation to leukocyte migration.

In the same experiments, leukocyte transendothelial migration was examined. Under control conditions, in the absence of CXCL8 and minimal filopodia/microvilli formation, only 6% of neutrophils migrated (Fig. 5). With the addition of CXCL8 (100 ng/ml for 30 min) and increase in filopodia/microvilli formation there was elevated neutrophil migration (5-fold, p ≤ 0.0001). Because HS was shown to be present on filopodial structures (Fig. 3), the involvement of this GAG was examined. Under control conditions, in the absence of CXCL8, neutrophils that migrated through the CXCL8-stimulated endothelial monolayer was significantly decreased after EC pretreatment with

![Graph showing percentage of neutrophils migrated](https://example.com/graph.png)

**FIGURE 5.** Neutrophil transendothelial migration. Same in vitro experiments as in Fig. 4. HBMECs were cultured on 3-μm pore transwell filters and treated with 100 ng/ml CXCL8 for 60 min in the basal compartment of transwells. Leukocytes were added to the apical compartment for the final 30 min to allow for transendothelial migration. The percentage of neutrophils that migrated through an unstimulated endothelial monolayer (control) or a CXCL8-stimulated endothelial monolayer is shown. The graph indicates significant reductions when HS was cleaved with heparinase I or III. Data represent percentage means ± SE (n = 3 endothelial monolayers) and are representative of two individual experiments; ***p ≤ 0.0001, **p ≤ 0.01, comparing treatments indicated.
heparinase I (4.0 ± 2.0%; p ≤ 0.0001) and heparinase III (8.0 ± 1.2%; p ≤ 0.001).

Because a mixture of neutrophils and mononuclear cells was added in transendothelial migration assays, the migration of monocytes and lymphocytes across the ECs was also analyzed. Gating on monocytes and lymphocytes, it was found that there was insignificant migration in response to CXCL8 (100 ng/ml for 30 min), amounting to <0.5% of these cells that migrated, whereas neutrophil migration amounted to ~30% (Fig. 5).

**Endothelial filopodia/microvilli formation in vivo**

CXCL8 was injected into the skin of rabbits and biopsies were taken at time points up to 4 h and processed for transmission electron microscopy to examine whether the microstructures described earlier in vitro also occur in vivo. Filopodial and microvillous protrusions occurred on the ECs of venules in the dermis from 30 min after injection; these were variable in length and were villous protrusions occurred on the ECs of venules in the dermis.

**Effect of CXCL8 stimulation on differential endothelial protein expression**

Because a functional role for CXCL8 had been determined in the formation of EC surface protrusions, the changes that were occurring within the cell after CXCL8 stimulation were examined by mass spectrometry.

### Table I. Differential endothelial protein expression after CXCL8 stimulation

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>CI (%)</th>
<th>Peptide Count</th>
<th>Score</th>
<th>0.5 h</th>
<th>6 h</th>
<th>16 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tropomyosin-1</td>
<td>100</td>
<td>6</td>
<td>271</td>
<td>0.81 ± 0.10</td>
<td>0.79 ± 0.12</td>
<td>0.63 ± 0.14</td>
</tr>
<tr>
<td>Fascin</td>
<td>100</td>
<td>7</td>
<td>521</td>
<td>0.88 ± 0.09</td>
<td>0.99 ± 0.05</td>
<td>1.00 ± 0.16</td>
</tr>
<tr>
<td>Myotrophin</td>
<td>100</td>
<td>3</td>
<td>214</td>
<td>1.50 ± 0.17</td>
<td>1.05 ± 0.20</td>
<td>1.18 ± 0.09</td>
</tr>
<tr>
<td>Myosin isoform 2</td>
<td>94</td>
<td>1</td>
<td>36</td>
<td>0.86 ± 0.10</td>
<td>0.65 ± 0</td>
<td>0.65 ± 0</td>
</tr>
<tr>
<td>Myosin IE</td>
<td>100</td>
<td>1</td>
<td>71</td>
<td>0.74 ± 0</td>
<td>0.82 ± 0</td>
<td>0.76 ± 0</td>
</tr>
<tr>
<td>Perlecain (HSPG2)</td>
<td>100</td>
<td>12</td>
<td>644</td>
<td>1.01 ± 0.21</td>
<td>1.27 ± 0.32</td>
<td>1.28 ± 0.30</td>
</tr>
<tr>
<td>Type XVIII collagen (endostatin)</td>
<td>100</td>
<td>3</td>
<td>171</td>
<td>0.75 ± 0.11</td>
<td>0.54 ± 0.38</td>
<td>0.97 ± 0.23</td>
</tr>
<tr>
<td>Type XII collagen</td>
<td>100</td>
<td>7</td>
<td>514</td>
<td>0.82 ± 0.11</td>
<td>0.75 ± 0.07</td>
<td>0.84 ± 0.13</td>
</tr>
<tr>
<td>Caveolin</td>
<td>99</td>
<td>1</td>
<td>43</td>
<td>0.78 ± 0</td>
<td>0.86 ± 0</td>
<td>0.88 ± 0</td>
</tr>
<tr>
<td>Rab 7</td>
<td>100</td>
<td>3</td>
<td>96</td>
<td>1.13 ± 0.05</td>
<td>1.08 ± 0.12</td>
<td>1.15 ± 0.27</td>
</tr>
<tr>
<td>Rac 1</td>
<td>99.92</td>
<td>1</td>
<td>50</td>
<td>1.58 ± 0</td>
<td>1.64 ± 0</td>
<td>1.53 ± 0</td>
</tr>
<tr>
<td>Cytoskeletal and adhesion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myosin 9</td>
<td>100</td>
<td>95</td>
<td>7635</td>
<td>0.88 ± 0.2</td>
<td>0.92 ± 0.24</td>
<td>0.94 ± 0.23</td>
</tr>
<tr>
<td>Cadherin-13</td>
<td>99.9</td>
<td>1</td>
<td>56</td>
<td>0.64 ± 0</td>
<td>0.70 ± 0</td>
<td>1.05 ± 0</td>
</tr>
<tr>
<td>Myosin VI</td>
<td>100</td>
<td>5</td>
<td>297</td>
<td>0.88 ± 0.22</td>
<td>0.97 ± 0.33</td>
<td>1.1 ± 0.14</td>
</tr>
<tr>
<td>Tropomyosin-2</td>
<td>100</td>
<td>8</td>
<td>75</td>
<td>0.84 ± 0.12</td>
<td>0.80 ± 0.12</td>
<td>0.62 ± 0.15</td>
</tr>
<tr>
<td>Signaling</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rab1B</td>
<td>100</td>
<td>2</td>
<td>130</td>
<td>0.98 ± 0.03</td>
<td>0.97 ± 0.1</td>
<td>0.96 ± 0.04</td>
</tr>
<tr>
<td>Rab2A</td>
<td>97</td>
<td>3</td>
<td>35</td>
<td>0.90 ± 0.071</td>
<td>1.00 ± 0.5</td>
<td>0.97 ± 0.1</td>
</tr>
<tr>
<td>Rab4A</td>
<td>100</td>
<td>2</td>
<td>84</td>
<td>1.01 ± 0.01</td>
<td>1.10 ± 0.02</td>
<td>1.13 ± 0.14</td>
</tr>
<tr>
<td>Rab4B</td>
<td>100</td>
<td>2</td>
<td>110</td>
<td>0.96 ± 0.04</td>
<td>0.96 ± 0.12</td>
<td>1.03 ± 0.02</td>
</tr>
<tr>
<td>Rab11A</td>
<td>100</td>
<td>2</td>
<td>83</td>
<td>1.10 ± 0.09</td>
<td>1.02 ± 0.02</td>
<td>1.03 ± 0.01</td>
</tr>
<tr>
<td>Rab35</td>
<td>100</td>
<td>2</td>
<td>84</td>
<td>0.92 ± 0.08</td>
<td>0.95 ± 0.12</td>
<td>0.95 ± 0.05</td>
</tr>
<tr>
<td>Rab37</td>
<td>100</td>
<td>2</td>
<td>90</td>
<td>0.89 ± 0.01</td>
<td>1.04 ± 0.03</td>
<td>0.97 ± 0.03</td>
</tr>
<tr>
<td>Rab33B</td>
<td>100</td>
<td>1</td>
<td>77</td>
<td>1.01 ± 0</td>
<td>1.08 ± 0</td>
<td>1.01 ± 0</td>
</tr>
<tr>
<td>Rab6A</td>
<td>100</td>
<td>1</td>
<td>77</td>
<td>1.01 ± 0</td>
<td>1.08 ± 0</td>
<td>1.01 ± 0</td>
</tr>
<tr>
<td>Rab6B</td>
<td>100</td>
<td>2</td>
<td>79</td>
<td>0.99 ± 0.01</td>
<td>0.97 ± 0.10</td>
<td>0.98 ± 0.02</td>
</tr>
<tr>
<td>Rab39</td>
<td>100</td>
<td>2</td>
<td>77</td>
<td>1.00 ± 0.004</td>
<td>1.00 ± 0.05</td>
<td>1.02 ± 0.004</td>
</tr>
</tbody>
</table>

*The total ion score confidence interval (CI %) for the protein identification.

The total ion score of CXCL8-treated (115, 116, and 117) and untreated (114) HBMEC samples (±SD from mean).

The average iTRAQ ratios of CXCL8-treated (115, 116, and 117) and untreated (114) HBMEC samples (±SD from mean).

Significantly changed proteins comparing CXCL8-treated and untreated HBMEC (p < 0.05; apart from where SD = 0 with 1 peptide count).

Cytoskeletal, adhesion, and signaling proteins that changed but not significantly. Data are representative of three individual experiments.
compared with the control (1.13 ± 0.05 at 0.5 h, 1.08 ± 0.12 at 6 h, and 1.15 ± 0.27 at 16 h), and Rac1, which also increased (1.58 at 0.5 h, 1.64 at 6 h, and 1.53 at 16 h).

There were other cytoskeletal and adhesion proteins of interest that showed changes compared with the control at various time points, although they were not significant (Table I). These included myosin 9, cadherin-13, myosin VI, and tropomyosin-2. In terms of signaling, numerous additional Rab GTPases showed changes after CXCL8 stimulation, although changes were not significant; these included Rab1B, Rab2A, Rab4A, Rab4B, Rab11A, Rab35, Rab37, Rab33B, Rab6A, Rab6B, and Rab39.

Western blot analysis, using the same cell digests, was performed using Abs against tropomyosin, fascin, and Rab7 to confirm the changes observed in the mass spectrometry; band densities at each time point were measured and the log data presented in graphical format (Fig. 6). The band densities for tropomyosin decreased with increasing time when compared with the no chemokine control (Fig. 6A); fascin decreased at 0.5–6 h followed by a slight increase at 16 h (Fig. 6B); the Rab7 showed an increase at 0.5 h with a gradual decrease from 6–16 h (Fig. 6C). These data are in general agreement with the iTRAQ ratios.

The results of the study led to the development of a model of EC filopodial and microvillous protrusion formation by chemokines (Fig. 7).

**Discussion**

There have been some reports on the formation of filopodial and microvillous processes by ECs under inflammatory conditions and in response to chemokine and chemoattractant (2–11). This study extended these previous reports, examining formation of these structures in more detail, their composition, the receptors involved in their formation, and intracellular changes. Chemokine-stimulated filopodia formation was not specific to CXCL8; the same effects were observed when the cells were incubated with CXCL10 and CCL5. However, the magnitude of response was different between the chemokines and cell types. The greatest response was for CXCL8 on HBMECs (13-fold increase), and the lowest was CCL5 on HMVECs (3-fold increase). In addition, chemokines stimulated filopodia formation by both microvascular (HBMEC and HMVEC) and macrovascular ECs (HUVEC).

Several chemokine binding molecules were shown to be involved in the formation of endothelial filopodial protrusions after CXCL8 treatment. These were CXCR1 and CXCR2, DARC, and HS and its proteoglycans syndecan-3 and syndecan-4. All the receptors and binding molecules caused a significant reduction in the percentage of cells that formed filopodia. The involvement of CXCR1 and CXCR2 suggests that signaling via these receptors is involved in creating these EC microstructures. DARC in particular played a role in their formation. Although high endothelial

---

**FIGURE 6.** Western blot analysis of unstimulated and CXCL8-stimulated HBMECs. Western blot analysis of HBMEC cell extracts in the absence or after addition of CXCL8 for the indicated time; total cell extracts were analyzed using the indicated Abs. Graphical data represent the log average band intensities from the Western blots (y-axis) for each of the time points (x-axis) normalized for equal loading: (A) tropomyosin (band A 37 kDa, band B 35 kDa isoforms), representative blot of three individual experiments; (B) fascin, representative of two individual experiments; and (C) Rab7, the lower of the two bands was quantitated, which corresponded to the expected m.w. of the protein. Data are representative of two individual experiments. (D) Representative blot probed for GAPDH, which was used a loading control.
An effect on filopodial protrusion formation through syndecan-4 may be dependent on internalization because a significant reduction of cells with these structures was only seen at 37°C; at 4°C, there was no effect. Zimmerman et al. (26) showed syndecan-4 and fibroblast growth factor receptor internalization and their colocalization in recycling endosomes; their study provides evidence that syndecan-4 can internalize ligands and recycle them. Therefore, in this study, it is possible that syndecan-4 is internalizing upon binding to CXCL8 and is recycling the chemokine via the endocytic pathway.

Using Abs to specific HS epitopes, we found 2,6-O− and 3-O− sulfated HS to be present on filopodial protrusions, occurring as a meshlike structure. Confirmation of their expression was achieved with the use of heparanases; when the cells were incubated with heparanase I, the expression of both HS epitopes on the meshwork was reduced to background. In contrast, heparanase III degraded the 3-O but not the 2,6-O epitope. Heparanase III cleaves 1→4 linkages between hexosamine and glucuronic acid; however, it has weak activity with disaccharides containing iduronic acid, and its action is blocked if iduronic acid is sulfated at C2 (IdoA-2SO₃). Therefore, because the 2,6-O HS epitope contains sulfated idurionate at C2, this may explain the lack action of heparanase III. Spillmann et al. (27) reported that HS binding to CXCL8 correlates with the occurrence of 2,6-O− sulfated disaccharides, and CXCL8 has been shown to bind to 6-O− sulfated glucosamine (28). Therefore, the presence of 2,6-O− sulfated HS on the filopodial mesh may be involved in CXCL8 binding and sequestration and presentation of the chemokine to leukocytes. In this respect, HS on ECs has been widely reported to bind, concentrate, and present chemokines to leukocytes during extravasation (9, 29–32). These chemokine binding sites on HS may be located on syndecan-3 and -4 because these are major HSPGs expressed on HBMECs in this study. Such sites may be relevant in inflammatory diseases because in rheumatoid arthritis, there is induction of a CXCL8 binding site on the HS chains of syndecan-3 by synovial ECs (25).

Transmission electron microscopy showed that leukocytes interacted with EC protrusions in transwell experiments, suggesting a role for them in leukocyte transmigration. Two types of structure were observed: microvillous protrusions that extended vertically and were short, and filopodial protrusions that were longer, extending laterally over the cell surface; leukocytes interacted with both types. Carman et al. (13) showed that ECs formed ICAM-1−enriched microvilli-like projections that embraced leukocytes and extended up their sides to form cuplike structures. It is possible that the microvilli observed in this study are similar to those described by Carman et al., because the observed microvilli showed a positive expression of ICAM-1 (data not shown); however, it is currently unknown whether this is the case because cuplike structures were not seen. Previous studies (12, 33) suggest a role for podosomes in leukocyte extravasation. Podosomes have been shown to form on highly migratory cells and in leukocytes adhering to endothelium. The podosomes cause local displacement of cytoplasm, cytoskeleton, and other organelles by forcing podo-pints into the surface of the EC. The work presented in this article shows that the leukocytes formed podosomes and they interacted with EC protrusions, possibly before leukocyte transendothelial migration through that area of the EC.

Heparanases were used to degrade HS and these reduced neutrophil transendothelial migration. This could have been caused by the removal of HS from filopodia/microvilli, thus altering the ability of the ECs to bind and present CXCL8 to the neutrophils. This agrees with an earlier study (9) reporting that when CXCL8 was injected in the skin, it localized to EC microvillous processes and longer projections; however, a modified version of CXCL8...
with impaired binding to HS showed reduced localization to these structures and reduced neutrophil recruitment. In addition, heparanase reduces the formation of filopodial protrusions (Fig. 2A); therefore, the inhibited neutrophil migration may have also been caused by this factor. Therefore, the heparanase effect may have been caused by a combination of reduced chemokine presentation and filopodia formation.

In transwell experiments, it is conceivable that CXCL8 may have induced the formation of large gaps or openings in the EC layer, creating a chemotactic gradient without an EC barrier, thereby allowing neutrophils to migrate through them. However, electron microscopy did not reveal the existence of such gaps that have been seen by this technique in the presence of mediators such as histamine (22). In this study, CXCL8 was added to the basal EC surfaces in transwell chemotaxis experiments in Figs. 4 and 5, and to the apical surfaces in Figs. 1–3 and Table I. Both approaches involved an identical time point (30 min) and chemokine concentration (100 ng/ml) to generate filopodial protrusions. These microstructures were generated whether the chemokine was applied apically or basally, although it is as yet unknown whether the mechanism of their formation is identical depending on where the EC encounters the chemokine first.

ITRAQ mass spectrometry showed that upon CXCL8 binding to ECs, there were changes in cytoskeletal, extracellular matrix, and signaling proteins at all the time points examined. The changes in the cytoskeletal proteins, such as tropomyosin, suggest a reorganization of the EC cytoskeleton upon CXCL8 binding; no changes were detected in the levels of actin. Tropomyosin provides structural stability and modulates actin filament function (34); therefore, this protein may be associated with changes in the stability of the cytoskeleton that may occur during chemokine-stimulated filopodia/microvillus formation. Fascins are actin-binding proteins that cross-link F-actin (35); they have been shown to be involved in filopodial protrusion formation in several cell types. Alteration in the levels of myosin was apparent, and this molecule is also known to interact with actin filaments (36). Therefore, fascin and myosin may both be involved in EC microstructure formation seen in this study through their cytoskeletal roles. Changes in extracellular matrix proteins, such as perlecan and endostatin (type XVIII collagen), which are associated with the basement membrane, suggest that the CXCL8-stimulated formation of filopodia/microvilli may be associated with changes in basement membrane–EC interactions. There were differences between the basement membrane proteins, perlecan expression increased after 6 h, whereas endostatin decreased at 0.5 and 6 h before increasing again after 16 h. Changes in signaling molecules such as the small GTPases Rab7 and Rab1c are associated with chemokine-stimulated filopodia formation. In this connection, chemokine receptors are associated with Rab and Rac signaling, leading to changes in the organization of the actin cytoskeleton and intracellular vesicular trafficking (37, 38). Because CXCR1 and CXCR2 were found to be involved in filopodia formation in response to CXCL8, it is possible that these receptors may be acting via Rab and Rac, leading to changes to the cytoskeleton and intracellular trafficking. Indeed, both CXCR1 and CXCR2 have been shown to be involved in the reorganization of the cytoskeleton when microvascular ECs are stimulated with CXCL8 (39), and enhanced vesicle formation occurs in ECs when stimulated with this chemokine (8). Concerning the formation of the filopodial and microvillus structures in response to chemokines, these happened rapidly, being already formed at 30 min. Therefore, their formation is unlikely to be protein synthesis dependent but reflects rapid changes in the levels of cytoskeletal, signaling, and extracellular matrix proteins. However, at later time points of 6 and 16 h, altered protein synthesis is more likely to occur.

This study used an in vitro assay of endothelial protrusion formation using phase-contrast microscopy to determine dose responses of EC types to different chemokines. The assay detected filopodia-like structures extending horizontally from the periphery of cells grown on plastic. These structures were also positive for HS by immunofluorescence microscopy, which showed a filopodial meshwork that bound CXCL8. Similar long filopodial structures that extended horizontally were also visible on the endothelial surface using electron microscopy, which probably represents the meshwork in thin section. The size of the filopodial structures were in general agreement considering the various experimental approaches and processing methods. By electron microscopy, they measured around 200–500 nm in width. The meshwork by immunofluorescence also measured around 200–500 nm in width. These were within the range of filopodial protrusions seen by phase contrast, being 200–1000 nm in width. The formation of these filopodial structures seen by all three techniques was stimulated by chemokines. In addition, vertically extending microvilli-like structures were apparent on the apical EC surface using electron microscopy and were less obvious using the other imaging techniques. Furthermore, endothelial filopodial/microvilli-like structures were observed on ECs in vivo, after CXCL8 injection into rabbit skin. These structures extended laterally, as well as more vertically, and measured up to micrometers in length at the luminal endothelial surface. These have also been seen in earlier studies in human and rabbit skin (8, 9). Therefore, our in vitro assay appears to reflect similar structures formed in vivo. In this study, we have used the terms “filopodia” and “microvilli” to describe various endothelial structures observed by different techniques. These have also been described in the literature, and similar structures have also been termed membrane wrinkles, projections, or simply protrusions.

The findings of this study led to the development of a model of EC filopodial and microvillus protrusion formation by CXCL8 (Fig. 7). When ECs encounter CXCL8 generated under inflammatory conditions, the chemokine binds to the CXCR1/2 receptor and to the chemokine binding molecules HS, syndecan-3, syndecan-4, and DARC, each of which may be involved in the formation of filopodia. Upon CXCL8 binding (Fig. 7B) there is an alteration in signaling pathways involving small GTPases. The actin filaments of the cytoskeleton begin to reorganize, via changes in molecules such as tropomyosin and fascin, which reduce in levels, and this leads to one of two possible events: either the actin filaments begin to move outward, causing the filopodia to form, or the action of the filopodia forming pulls the actin filaments toward them. Similarly, changes in the cytoskeleton are associated with microvillus protrusion formation. In addition, a disruption of the basement membrane allows the cells to adapt to their surroundings via changes in endostatin and perlecan. On the filopodia and microvilli (9), CXCL8 binds to HS motifs, which may be located on syndecan-3/4 proteoglycans, and is presented to blood leukocytes (Fig. 7C). Sequestration and clustering of the chemokine on the HSPGs located on the EC microstructures increases the surface concentration of chemokine to activate blood leukocytes leading to firm adhesion, crawling, and leukocyte transendothelial migration. This study provides evidence for a novel mechanism by which chemokines may stimulate their own presentation at sites of inflammation by creating EC filopodial and microvillus protrusions.

Acknowledgments
We thank Dr. Ian Holt for assistance with the confocal microscopy; Drs. Heidi Fuller and Emma Wilson, Wolfson Centre for Inherited Neuromuscular Disease, Robert Jones and Agnes Hunt Orthopaedic Hospital, Keele University, Oswestry, U.K., for the mass spectrometry work; Profs. Guido David (University of Nantes) and Toin Van Kuppevelt (University of Leuven).
for donating the syndecan and HS phage display Abs, including helpful discussions; and Karen Walker from the transmission electron microscopy unit at Keele University.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


