Endothelial Cell Heterogeneity in Venules of Mouse Airways Induced by Polarized Inflammatory Stimulus

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We sought to determine whether the changes in microvascular endothelial cells (EC) caused by a polarized chronic inflammatory stimulus depend on proximity to the stimulus. C3H mice were infected with *Mycoplasma pulmonis*, which attaches to the airway epithelium and creates a polarized inflammatory stimulus across the airway wall. At 1, 2, or 4 weeks, the tracheal vasculature was stained by perfusion of silver nitrate to mark EC borders or biotinylated *Lycopersicon esculentum* lectin to label the EC surface and adherent leukocytes. E-selectin immunoreactivity and EC proliferation were also localized. We found that the size, shape, and immunoreactivity for adhesion molecules on EC nearest the airway lumen (subepithelial EC) were different from those on the opposite surface of the same vessels. Subepithelial EC were smaller, more irregular in shape, had greater E-selectin immunoreactivity, and had twice as many adherent leukocytes. In contrast, proliferating EC were uniformly distributed around the vessel circumference. We conclude that the polarized stimulus created by *M. pulmonis* infection differentially changes the size, shape, and function of EC nearest the airway epithelium. This heterogeneity may result from a gradient of inflammatory mediators that triggers the influx of leukocytes into the airway lumen. (*Am J Pathol* 1999, 155:93–103)

The endothelium of the microvasculature plays a key role in the inflammatory response. Importantly, endothelial cells (EC) regulate the local influx of select components of the blood to sites of tissue injury, while preserving overall blood flow. For example, histamine induces localized increases of plasma leakage¹ and leukocyte adhesion^{2,3} via changes in the endothelium.

Although localized EC responses to inflammatory stimuli have been described in many models of acute inflammation, less is known about such responses in chronic inflammation. In some cases of chronic inflammation, the inflammatory agent is confined to a particular tissue sur-

face. For example, respiratory pathogens that attach to the luminal surface of the airway epithelium induce an inflammatory response in the underlying airway mucosa and the influx of leukocytes into the airway lumen.^{4–7} Such a situation is likely to create a gradient of inflammatory signals across the airway wall. However, it is unknown whether such a gradient would produce graded changes in EC that depend on their proximity to the stimulus.

In normal tissues, the surface of vessels near epithelial cells may be specialized. For example, the endothelial fenestrae in intestinal capillaries are more abundant on the vessel surface nearest to the intestinal epithelium, and the EC nuclei tend to be located on the opposite surface.^{8,9} The fenestrae are believed to be induced by vascular endothelial growth factor (also called vascular permeability factor) secreted by the nearby epithelial cells.^{10,11} The polarized distribution of fenestrae in these vessels may facilitate the uptake of nutrients from the intestinal lumen. Similarly, polarized changes in EC in chronic inflammation could participate in the pathophysiology of the disease.

We sought to determine whether a polarized chronic inflammatory stimulus can induce heterogeneous changes in EC structure and function. *Mycoplasma pulmonis*, which attaches to the airway epithelium, induces chronic airway inflammation in mice.^{12,13} Associated with this inflammation is a large influx of leukocytes into the airway lumen.⁴

M. pulmonis infection also induces enlargement and proliferation of the airway microvasculature and changes in EC phenotype.⁷ In C3H mice, infection causes microvascular enlargement without an increase in the number of vessels.⁷ The enlargement is not due strictly to vasodilatation because the number of EC also increases. Because the stimulus in *M. pulmonis* infection is polarized, we asked whether the changes in the EC nearest the stimulus are more severe or different from those more distal, and, if so, whether these changes are associated with directed migration of leukocytes. We compared EC on the vessel surface nearest to the airway lumen to those on the opposite surface of the same vessels.

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We used *M. pulmonis* infection in C3H mice to determine 1) whether the size and shape of the EC on the surface of the venules nearest the airway epithelium (subepithelial EC) differ from those on the opposite surface (adventitial EC); 2) whether subepithelial EC preferentially express leukocyte adhesion molecules; 3) whether leukocytes adhere preferentially to subepithelial EC; and 4) whether EC proliferation is greater in subepithelial EC.

C3H mice were inoculated intranasally with *M. pulmonis*, and 1, 2, or 4 weeks later the EC borders of the microvasculature were stained with silver nitrate to permit measurement of EC area and shape in tracheal whole mounts. The distribution of adherent leukocytes was determined by staining the vasculature with the lectin *Lycoopersicon esculentum*. The distribution of E-selectin immunoreactivity was determined by immunofluorescence, and proliferating EC were localized with BrdU labeling and anti-BrdU antibodies.

Materials and Methods

Chronic Airway Inflammation

Pathogen-free male C3H mice (C3H/HeNCrIBR, Charles River Laboratories, Hollister, CA) 7–9 weeks of age (17–21 g) were anesthetized with ketamine (87 mg/kg, Parke-Davis, Morris Plains, NJ) and xylazine (13 mg/kg, Ben Venue Laboratories, Bedford, OH) i.p. and given 10^5 CFU *M. pulmonis* UAB CT strain^{12,13} by nasal inoculation (25 μ l per nostril). Pathogen-free and *M. pulmonis*-infected mice were housed separately under barrier conditions. All experiments were performed in accordance with the guidelines of the Committee for Animal Research of the University of California, San Francisco.

Staining EC Borders with Silver Nitrate

At 1, 2, or 4 weeks, pathogen-free and *M. pulmonis*-infected mice were anesthetized (pentobarbital sodium 50 mg/kg, i.p., Abbott Laboratories, North Chicago, IL), and EC borders were stained with silver nitrate as described previously.^{7,14} Briefly, the mice were perfused via the ascending aorta with fixative (1% paraformaldehyde plus 0.5% glutaraldehyde in 75 mmol/L cacodylate buffer, pH 7.4, at a pressure of 120 mmHg) for 3 minutes followed by 0.9% NaCl for 2 minutes at 120 mmHg, 10 ml of 5% glucose in 10 seconds, 7 ml of 0.2% silver nitrate in 7 seconds, 10 ml of 5% glucose in 10 seconds, and fixative for 1 minute at 120 mmHg. After tracheas were incised along the ventral midline and removed, the silver halide was developed under bright light for 15 minutes. Tracheas were dehydrated (50%, 70%, 95%, 100% ethanol), flattened between two glass slides, cleared in toluene, and mounted in Permount (Fisher Scientific, San Francisco, CA).

Staining Vessels using *L. esculentum* Lectin

In some mice, the vasculature was stained with *L. esculentum* lectin as described previously.^{7,15} Briefly, after

perfusion fixation, mice were perfused with biotinylated lectin (5 μ g/ml, biotinylated *L. esculentum*, Vector Laboratories, Burlingame, CA), which binds uniformly to the luminal surface of EC and leukocytes. Tracheas were cut down the ventral midline, removed, and pinned luminal side up on petri dishes coated with Sylgard (Dow-Corning, Midland, MI). Tracheas were permeabilized with 0.3% Triton X-100 in phosphate buffered saline (PBS) overnight, incubated in avidin-peroxidase complex (Vector Laboratories) diluted 1:200 in 0.3% Triton X-100 in PBS overnight, and reacted with 0.5% 3,3'-diaminobenzidine (DAB, Sigma, St. Louis, MO) and hydrogen peroxide in 0.05 mol/L Tris buffer containing 1% Triton X-100. Tracheas were dehydrated and mounted as with silver nitrate staining. The rostral portion of some tracheas was removed before incision along the midline, then embedded in glycol methacrylate, cut into 2- μ m sections, and stained with toluidine blue for light microscopy.

Immunohistochemistry

Tissue was fixed by vascular perfusion of 1% paraformaldehyde in PBS, followed by perfusion of fluorescein-labeled *L. esculentum*. Tracheas were removed, infiltrated in 30% sucrose + 0.01% thimerosal in PBS overnight, embedded in OCT compound (Baxter Scientific Products, McGaw Park, IL), frozen in isopentane chilled by liquid nitrogen, and cut into 10- to 14- μ m sections. Tissue sections were air dried, then placed in 5% goat serum for 1–2 hours. After removing the blocking serum, the sections were incubated overnight in primary antibodies: i) rat anti-mouse E-Selectin (diluted 1:200, PharMingen, San Diego, CA), ii) biotinylated hamster anti-mouse ICAM-1 (diluted 1:200, PharMingen), or iii) rabbit polyclonal antibody to *M. pulmonis* (gift of Dr. Howard Watson, University of Alabama). Sections were incubated for 4 hours in secondary antibody (Cy3-labeled goat anti-rat, Amersham, Arlington Heights, IL; FITC-labeled streptavidin, Vector Laboratories; or Cy5-labeled goat anti-rabbit, Amersham) diluted 1:500. Sections were mounted in Vectashield (Vector Laboratories) and viewed on a Zeiss LSM 410 confocal microscope equipped with a krypton-argon laser.

Morphometric Measurements of EC

Subepithelial and Adventitial EC

EC on the vessel surface nearest to the airway epithelium (subepithelial EC) were compared to those on the opposite surface of the same vessels (adventitial EC). Subepithelial EC were located on the surface nearest the airway epithelium of collecting venules and adventitial EC on the opposite surface of the same vessels, nearest to the adventitial surface of the airway wall (Figure 1).

EC Area

The luminal surface area of EC was assessed in tracheal whole mounts of pathogen-free and infected mice

Figure 1. Diagram of cross-section of airway wall showing overview of airway (A) and position of subepithelial and adventitial EC in relation to airway epithelium, *M. pulmonis* organisms, and airway adventitial surface (B). *M. pulmonis* organisms attach to luminal surface of airway epithelium, which is located immediately above the remodeled blood vessels. Subepithelial and adventitial EC from the same vessels were compared. Measurements of EC size and shape, and number of adherent leukocytes, were made with central 60° of vessel axis, marked by lines.

after silver nitrate staining.^{7,14} Morphometric measurements were made on real-time digitized color video microscopic images with a Zeiss Axiophot microscope using a three-charge-coupled device color video camera (Sony Model DXC 755, Tokyo), a real-time color video digitizing card (Video-Logic DVA-4000, Cambridge, MA) in a Compaq SystemPro 486/33 computer (Houston, TX), a digitizing tablet (GTCO Digipad, model 1111A, Rockville, MD), and image analysis software developed in our laboratory. Using the digitizing pad, endothelial borders were traced and the area calculated for each cell. This

value represents the area of the cell cross-section at the level of the cell junctions. Measurements were made on 10 EC on the subepithelial and adventitial surfaces of collecting venules in the intercartilaginous regions of the trachea of 4 mice per group ($n = 4$).

Shape Parameters: Circularity and Elongation

Two parameters were used to characterize the shape of subepithelial and adventitial EC of the same vessels in silver nitrate-stained tracheal whole mounts. Microscopic images were acquired using IP Lab Spectrum 3.01 software (Signal Analytics Corporation, Vienna, VA) on a Power Macintosh, and shape parameters were calculated from the coordinates of the EC borders traced with a digitizing tablet. Circularity was calculated using the formula $4\pi A/P^2$, where A = area and P = perimeter, to yield an index of a cell's similarity to a circle. Circularity equals 1 for circular cells and decreases toward 0 as cells become elongated or irregular. Circularity is sensitive to overall cell shape and to local irregularities of the border. Elongation was calculated as $((a^2 - b^2)^{1/2}/a)$, where a is the length of the major axis of the cell and b is the length of the minor axis. Circular cells have an elongation of 0, whereas spindle-shaped cells have an elongation approaching 1. Elongation is an overall estimate of cell shape, and is not as sensitive to irregularities of the border as circularity.

Leukocyte Adherence

The number of leukocytes adherent on the subepithelial and adventitial surfaces of collecting venules was determined in tracheal whole mounts of mice infected for 4 weeks and stained with *L. esculentum* lectin. Measurements were made using a Multiple Oblique microscope (Edge Scientific, Santa Monica, CA) with a three-dimensional stereoscopic view that made it possible to identify adherent leukocytes on the subepithelial and adventitial surfaces.¹⁵ To avoid regions of ambiguity, we scored only cells bound to the central 60° of either the subepithelial or adventitial surface and adjusted the calculated surface area accordingly. The length and width of collecting venules were measured and, from these values, the luminal surface area was calculated ($=DL\pi/6$, where D = vessel diameter, L = vessel length, and the denominator corrects the surface area to the central 60°). Results were expressed as the mean number of leukocytes per square millimeter of vessel surface, calculated from 10 vessels per trachea and 4 mice per group ($n = 4$).

BrdU Staining of Proliferating Cells

A thymidine analogue, 5-bromo-2'-deoxyuridine (BrdU) (Sigma) was injected intravenously (1 mg in 100 μ l PBS) into pathogen-free mice and mice infected with *M. pulmonis* for 1 week. One hour later, mice were fixed by vascular perfusion of 1% paraformaldehyde in PBS. Tracheas were removed, washed in PBS, frozen in liquid nitrogen, and cut into 10- μ m-thick cryosections. Cells

incorporating BrdU were identified using an indirect immunoenzymatic staining method.¹⁶ Briefly, sections were digested with 0.005% pepsin (Sigma) in 0.01 N HCl at 37°C for 10 minutes and then immersed in 4 N HCl for 30 minutes at room temperature. Sections were incubated with mouse monoclonal antibody to BrdU (diluted 1:200, Dako) for 2 hours, followed by incubation with alkaline phosphatase-conjugated goat anti-mouse (Jackson Immuno Research, 1:200) for 30 minutes. The alkaline phosphatase reaction was colored red with ALP substrate kit I (Vector Red, Vector Laboratories). Some sections were lightly counterstained with hematoxylin and then mounted in Aquatex (Merck, Darmstadt, Germany). The distribution of BrdU-labeled EC was assessed in 10 tracheal sections from 2 mice. BrdU-labeled EC were scored as being on the subepithelial aspect (more than half the cell nucleus on the vessel surface nearest the airway lumen) or adventitial aspect of a vessel.

Transmission Electron Microscopy

Transmission electron microscopy was used to localize the site of attachment of *M. pulmonis* organisms. Tracheas of pathogen-free and *M. pulmonis*-infected mice (4 weeks) were fixed by vascular perfusion of 3% glutaraldehyde in cacodylate buffer, pH 7.1, for 10 minutes at 120 mmHg, removed, and fixed overnight at 4°C.¹⁷ Specimens of intercartilaginous and posterior membrane regions of tracheas were isolated with a razor blade and postfixed in 2% osmium tetroxide for 14 to 18 hours at 4°C. After treatment with uranyl acetate (2% for 48 hours at 37°C), tissues were embedded in epoxy resin (LX112, Ladd Research, Burlington, VT), thin-sectioned (Ultracut, Leica, Deerfield, IL), and stained with lead citrate. Sections 80 nm in thickness were examined with a Zeiss EM-10C electron microscope.

Statistical Analysis

Measurements are shown as means ($\pm$ SE) from 4 mice per group. The significance of differences for multiple groups (cell area, circularity, and elongation) were tested by analysis of variance and Fisher's test. Differences in number of adherent leukocytes were tested by Student's *t*-test. *P* < 0.05 was considered significant.

Results

As has been reported previously,⁷ infection of C3H mice with *M. pulmonis* induced prominent remodeling of the tracheal vasculature. The vascular remodeling consisted of increased microvascular diameter and increased number of EC, without an increase in vessel number or length (Figure 2, A-C). The attachment of *M. pulmonis* organisms to the apical surface of the airway epithelial cells (Figure 2D) created a polarized stimulus whereby subepithelial EC were closer to the stimulus than EC on the opposite surface (Figure 1).

Heterogeneity of EC Area and Shape

As revealed by silver nitrate staining of the EC borders, there were no differences in morphology between subepithelial EC and adventitial EC in venules of pathogen-free mice (Figure 3A). Subepithelial EC and adventitial EC in pathogen-free mice had the same cell surface area (Figure 4) and shape, as revealed by the measurements of circularity (Figure 5A) and elongation (Figure 5B). There were very few adherent leukocytes in airway vessels of pathogen-free mice.

In infected mice, the size and shape of venular EC differed from those in pathogen-free mice, and furthermore, subepithelial EC differed from adventitial EC. Subepithelial EC were smaller and more irregular in shape than adventitial EC (Figure 3, A-C). The borders of subepithelial EC were tortuous and irregular (Figure 3B), whereas those of adventitial EC were much smoother (Figure 3C). The differences in size and shape of EC were readily apparent on the different surfaces of individual venules (Figure 3, E and F). At 1 week after inoculation with *M. pulmonis*, the surface area of subepithelial and adventitial EC were significantly different from each other: the area of adventitial EC was 56% greater than pathogen-free values and had increased much more than subepithelial EC (Figure 4). The area of subepithelial EC tended to decrease thereafter, so that the difference between the two surfaces became even more pronounced. At 4 weeks the adventitial EC were 72% larger than the subepithelial EC (Figure 4).

The circularity of subepithelial EC tended to decrease at 1 and 2 weeks after inoculation and was significantly less than the pathogen-free value at 4 weeks (Figure 5A). In contrast, the circularity of adventitial EC tended to increase at 1 and 2 weeks and was significantly larger than the pathogen-free value at 4 weeks (Figure 5A), as well as significantly larger than that of subepithelial EC (Figure 5A). The elongation of both subepithelial and adventitial EC decreased in infected mice (Figure 5B); however, the elongation of subepithelial EC did not differ from that of adventitial EC.

The decreased values of circularity of subepithelial EC, together with lower values of elongation, reflect greater irregularity of the borders combined with overall rounding of cell shape. The increased circularity and decreased elongation of adventitial EC reflect the smoother borders and rounding of cell shape. The changes in cell shape are summarized in diagrammatic form (Figure 6).

Heterogeneity of Immunoreactivity for Cell Adhesion Molecules

To learn more about the preferential adhesion of leukocytes to subepithelial EC in tracheal vessels of infected mice, we examined the distribution of the EC adhesion molecules E-selectin (CD-62e) and ICAM-1 (CD-54). E-selectin immunoreactivity was not found in any tracheal vessels in pathogen-free mice (Figure 7A) but was present in some tracheal venules of infected mice at 4

Figure 2. A, B: Biotinylated *Lycopersicon esculentum* lectin staining of vessels (arrows) in tracheal whole mounts of (A) pathogen-free and (B) *M. pulmonis*-infected (2 weeks) C3H mice. Vessels in the infected mouse trachea are enlarged and contain abundant adherent leukocytes. C: Glycolmethacrylate section of infected mouse trachea stained with toluidine blue, showing airway epithelial cells (arrowheads), an enlarged blood vessel, and location of subepithelial (Su) and adventitial (Ad) endothelial cells (arrows). D: Transmission electron micrograph revealing numerous mycoplasmal organisms attached to the luminal surface of the airway epithelium (arrowheads). Scale bars, 100 μ m (A, B); 25 μ m; (C); 1 μ m (D).

weeks. Of the venules that had E-selectin immunoreactivity, 45% had greater immunoreactivity on subepithelial EC (Figure 7B), 48% had uniform E-selectin reactivity, and 7% had greater immunoreactivity on adventitial EC. ICAM-1 immunoreactivity was also greater in subepithelial EC of some vessels after infection (data not shown) but, unlike E-selectin, was present on other cell types including leukocytes, making quantitation more difficult.

Heterogeneity of Leukocyte Adherence

Infection with *M. pulmonis* induced an influx of leukocytes in the airways (Figure 2, C and D), and numerous leukocytes adhered to the endothelium of tracheal vessels. However, the adhesion of leukocytes to the endothelium was not uniform: more leukocytes adhered to subepithelial EC than to adventitial EC (Figure 3, E and F). Subepithelial EC of collecting venules in infected mice had twice as many adherent leukocytes as adventitial EC at 4 weeks after infection (Figure 8).

Distribution of EC Proliferation in Infected Mice

To determine whether the small size and irregular shape of subepithelial EC were a result of selective proliferation

of these cells, we labeled proliferating cells with BrdU. No proliferating EC were observed in 10 sections of tracheas from pathogen-free mice. At 1 week after infection, numerous cells in the tracheal mucosa were labeled with BrdU (Figure 7C), including EC (Figure 7, C and D). The proliferating EC were distributed approximately symmetrically around the circumference of the vessels: 11 of 25 (44%) of BrdU-labeled EC in 10 tracheal cross-sections from 2 infected mice were on the subepithelial surface of the vessels, while 14 (56%) were on the adventitial surface.

Discussion

Our findings show that the EC nearest the airway epithelium differed from those on the opposite surface of remodeled blood vessels in mice infected with *M. pulmonis*. Subepithelial EC in collecting venules were smaller and more irregular in shape than adventitial EC. Both subepithelial and adventitial EC in infected mice differed from EC in corresponding vessels of pathogen-free mice. In addition, subepithelial EC had twice as many adherent leukocytes as adventitial EC, as well as greater E-selectin and ICAM-1 immunoreactivity. In contrast, EC proliferation occurred on both surfaces of vessels. These results

Figure 3. Silver nitrate staining of EC borders visualized by Nomarski DIC microscopy in tracheal whole mounts of C3H mice. **A:** Smooth borders (arrow) of subepithelial EC in pathogen-free mouse. One adherent leukocyte (arrowhead) is present. **B:** Irregular, scalloped borders (arrows) of subepithelial EC nearest the tracheal lumen in mouse infected for 4 weeks. Numerous adherent leukocytes (arrowheads) and extravasating leukocytes (double arrowhead) are present. **C:** Smooth borders (arrow) of adventitial EC in tracheal venule of mouse infected for 4 weeks. One adherent leukocyte (arrowhead) is present. **D, E:** Comparison of subepithelial and adventitial surfaces of the same venule in infected C3H mouse (4 weeks). **D:** Subepithelial EC have irregular, scalloped cell borders, relatively small size, and numerous adherent leukocytes (arrowheads). **E:** Adventitial EC of the same venule have smooth borders, larger size, and fewer adherent leukocytes (arrowheads). Scale bars, 10 μ m (A-C); 25 μ m (D, E).

Figure 4. Surface area of subepithelial and adventitial EC in tracheal venules of C3H mice after infection with *M. pulmonis*. Values represent mean $\pm$ SE of 10 endothelial cells per trachea and 4 tracheas per group ($n = 4$) measured in venules stained with silver nitrate. Statistically significant difference ($P < 0.05$) from corresponding value for: * corresponding adventitial EC in infected mice, † adventitial, and ‡ subepithelial EC in pathogen-free mice.

show that EC nearest an inflammatory stimulus can undergo different changes than EC on the opposite surface of the same vessels.

Heterogeneous Response of the Microvascular Endothelium

The heterogeneous changes in the endothelium of infected mice accompanied other changes in the microcirculation such as increases in vessel diameter, the number of EC,⁷ and amount of uptake of intravascular cationic liposomes.¹⁷ The increase in vessel diameter appears to be due to vasodilatation in the first few days after infection, but thereafter the enlargement is accompanied by an increase in the number of EC. The rounding of EC shape may be associated with the increase in vessel diameter. However, the heterogeneity of EC around the circumference of venules cannot be explained by the increase in vessel diameter. Further, EC proliferation was approximately uniform around the circumference of vessels; thus, shape changes in subepithelial EC were not solely a result of cell proliferation.

What factors induced the EC to become smaller and more irregular in shape on one surface of the vessels? The irregular shape of the subepithelial EC may be a result of repeated physical distortion of the cell junctions during leukocyte migration between these cells. In other vessels with high rates of leukocyte transmigration, the EC are also very irregular. For example, the EC borders are irregular in tracheal venules during the late-phase inflammatory reaction following antigen challenge¹⁸ and

Figure 5. Circularity (A) and elongation (B) of subepithelial and adventitial EC in tracheal venules of pathogen-free and infected C3H mice. Values represent mean $\pm$ SE of 10 endothelial cells per trachea and 4 tracheas per group ($n = 4$) measured in tracheal venules stained with silver nitrate. Statistically significant difference ($P < 0.05$) from corresponding value for: * corresponding adventitial EC in infected mice, † adventitial, and ‡ subepithelial EC in pathogen-free mice.

Figure 6. Diagram summarizing shape changes in subepithelial and adventitial EC in tracheal venules after infection with *M. pulmonis*.

Figure 7. A, B: E-selectin immunoreactivity in trachea of pathogen-free (A) and *M. pulmonis*-infected (B) (4 weeks) C3H mice. E-selectin (red), *M. pulmonis* (blue), and lectin-stained blood vessels (green) visualized by three-color confocal microscopy. A: Little or no E-selectin immunoreactivity in blood vessel and no *M. pulmonis* immunoreactivity on airway epithelium (arrowheads) in pathogen-free trachea. B: Vessel in infected trachea with more E-selectin immunoreactivity (red, arrows) on surface nearest the airway lumen. *M. pulmonis* immunoreactivity on the luminal surface of airway epithelium (blue, arrowheads). C, D: BrdU-labeled cells localized by immunohistochemistry with alkaline phosphatase reaction product (red) in infected C3H mice (1 week). BrdU injected i.v. 1 hour before perfusion. C: BrdU-labeled nuclei in trachea from infected mouse are present at the base of the airway epithelium, in lamina propria, and in blood vessel (arrow). D: Higher magnification of BrdU-labeled EC nuclei (arrows) located on subepithelial and adventitial surfaces of blood vessel. Scale bars, 25 μ m (A, B); 10 μ m (C); 10 μ m (D).

in normal high endothelial venules of lymph nodes (G Thurston, P Baluk, DM McDonald, unpublished observations). Consistent with this possibility, subepithelial EC had twice as many adherent leukocytes as adventitial EC.

The circumferential heterogeneity of EC was apparent at 1 week after infection and became more pronounced at 2 and 4 weeks. In comparison, vessel diameter was maximally increased at 1 week and did not further increase thereafter.⁷ Thus the increase in EC heterogeneity occurred while the vessels remained constant in diameter. In addition, the *M. pulmonis* organisms remained adherent to the luminal epithelial surface throughout the

infection, so the increased EC heterogeneity is not a result of redistribution of the inflammatory stimuli. The gradual increase in heterogeneity may reflect a gradual increase in the gradient of inflammatory mediators that mediate changes in the EC.

The mediators that induce the shape changes in EC may come from several sources. First, factors from the *M. pulmonis* organisms may act directly on EC, as they do on macrophages¹⁹ and lymphocytes.²⁰ Second, infected epithelial cells may produce factors that act on EC. Third, leukocytes migrating to the epithelium may contribute to heterogeneous changes in the endothelium. Examination

Figure 8. Number of adherent leukocytes on subepithelial and adventitial EC in C3H mice infected with *M. pulmonis* for 4 weeks. Values represent mean $\pm$ SE of 17–23 vessels per trachea and 4 tracheas per group ($n = 4$). *, significantly different from subepithelial EC ($P < 0.05$).

of *M. pulmonis* infection in mice deficient in selected cytokines or immune effector cells may be helpful to sort out these possible mechanisms.

Endothelial Heterogeneity in Normal Tissue

Clues to the possible mechanisms of EC heterogeneity may come from studies of normal tissues. The capillaries in the intestinal villi are specialized for exchange of nutrients: the capillary surface nearest the intestinal lumen is thin and fenestrated, and the nucleus is located the opposite surface of the capillary.⁹ Similarly, in the lung alveoli, the surface of capillaries nearest the airway lumen is specialized for gas-exchange: the cytoplasm is thin, the basal lamina of the capillary and epithelium fuse, and the endothelial nucleus is located on the opposite surface of the capillary. In another example, the capillary surface nearest the synovium of the rat knee joint is fenestrated and attenuated, whereas the opposite surface is thicker and contains the endothelial nucleus.²¹ Indeed, the different surfaces of tracheal vessels in rodents that are not infected with *M. pulmonis* may have different numbers of fenestrae,^{22,23} although the particular features of EC which we examined in the present study were uniformly distributed in pathogen-free mice. The development of EC specializations appears to be mediated by signals from the epithelium. In the case of the development of fenestrae, vascular endothelial growth factor may be the epithelial-derived mediator.^{10,11}

Role of EC Heterogeneity in Leukocyte Migration

Are the fates of leukocytes that migrate through the subepithelial EC different from those that migrate through the

adventitial EC? Because of the higher immunoreactivity for the adhesion molecules E-selectin and ICAM-1 on subepithelial EC, and the preferential adhesion of leukocytes, it is tempting to speculate that the heterogeneity of EC facilitates directed migration of leukocytes and thereby focuses the inflammatory response. Indeed, the preferential adhesion to subepithelial EC may account in part for the accumulation of leukocytes, particularly neutrophils, in the tracheal epithelium and airway lumen of mice infected with *M. pulmonis*.⁴ Techniques for continuously monitoring the adhesion and migration of leukocytes in tissues exposed to polarized inflammatory stimuli will be necessary to further address this question.

EC Distribution of E-Selectin

Interestingly, much of the E-selectin immunoreactivity in tracheal vessels of mice infected for 4 weeks appeared to be in intracellular granules. Some studies of E-selectin immunoreactivity *in vivo* have observed fairly uniform staining of the EC surface and not granular staining (for example,²⁴). However, E-selectin immunoreactivity has typically been examined 4 to 6 hours after stimulus with lipopolysaccharide or other agents, during the peak of E-selectin expression. We also observed more uniform E-selectin immunoreactivity on the endothelial surface of pulmonary microvessels after treatment with lipopolysaccharide using the current staining procedure (G Thurston, TJ Murphy, DM McDonald, unpublished results). However, after the lipopolysaccharide-induced peak, E-selectin surface expression decreases in most vascular beds,²⁵ probably through internalization and degradation in lysosomes.^{26,27} Thus, the granular E-selectin immunoreactivity in tracheal vessels of mice infected for 4 weeks may be due to the prolonged and persistent inflammatory stimulus, with internalization and turnover of the receptor. Because we also observed adherent leukocytes at sites with very little E-selectin immunoreactivity, additional adhesion molecules are likely to be involved.

Uniform Distribution of EC Proliferation

In infected mice, proliferating EC were distributed uniformly around the circumference of tracheal vessels, whereas EC shape, E-selectin immunoreactivity, and leukocyte adhesion were polarized. This difference suggests that the changes may be induced by separate mechanisms. For example, the mediators that induce proliferation may have a uniform distribution, and the mediators that induce E-selectin, etc., may have a polarized distribution. Another possibility is that EC proliferation is a response to prolonged vasodilatation, which affects all EC, whereas the induction of E-selectin is driven by a gradient of mediators across the airway wall.

Polarized Stimuli in Chronic Inflammation

After nasal inoculation, *M. pulmonis* organisms adhere to the luminal surface of the airway epithelium. As far as we observed by immunofluorescence or electron microscopy, organisms did not invade the airway wall (but see below). The concentration of organisms on the epithelial surface creates a polarized inflammatory stimulus, and blood vessels and other structures within the airway wall are exposed to a gradient of substances from the organisms and epithelial cells. The magnitude of the difference in stimulus across a blood vessel would depend on the location and size of the vessel, with large vessels just beneath the epithelium having the greatest difference between subepithelial and adventitial EC.

Several factors determine the distribution of the *M. pulmonis* organisms. First, specific adhesion molecules enable the organisms to attach to the airway epithelial cells.²⁸ Second, an intact immune system is apparently necessary to confine the organisms to the airway lumen, because the organisms can spread to extrapulmonary sites including the joints in immunodeficient mice.^{29,30}

A polarized inflammatory stimulus is not unique to infection with *M. pulmonis*. Other airway pathogens, such as Sendai virus, infect the airway epithelium.⁶ Also, inhaled smoke and other particulate irritants are confined to the airway lumen. It is unknown whether heterogeneous changes in the microvascular endothelium also occur in these conditions.

Conclusions

We conclude that some airway microvessels of *M. pulmonis*-infected mice are circumferentially heterogeneous in terms of EC size, shape, phenotype, and function. This heterogeneity may result from a gradient of inflammatory mediators and substances from the organisms that produce a directional migration of leukocytes. These findings suggest a mechanism by which EC can focus an inflammatory response toward the stimulus.

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