Time Course of Endothelial Cell Proliferation and Microvascular Remodeling in Chronic Inflammation

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Angiogenesis and vascular remodeling are features of many chronic inflammatory diseases. When diseases evolve slowly, the accompanying changes in the microvasculature would seem to be similarly gradual. Here we report that the rate of endothelial cell proliferation and the size of blood vessels increases rapidly after the onset of an infection that leads to chronic inflammatory airway disease. In C3H mice inoculated with *Mycoplasma pulmonis*, the tracheal microvasculature, made visible by perfusion of *Lycopersicon esculentum* lectin, rapidly enlarged from 4 to 7 days after infection and then plateaued. Diameters of arterioles, capillaries, and venules increased on average 148, 214, and 74%, respectively. Endothelial cell proliferation, measured by bromodeoxyuridine (BrdU) labeling, peaked at 5 days (18 times the pathogen-free value), declined sharply until day 9, but remained at ~3 times the pathogen-free value for at least 28 days. Remodeled capillaries and venules were sites of focal plasma leakage and extensive leukocyte adherence. Most systemic manifestations of the infection occurred well after the peak of endothelial proliferation, and the humoral immune response to *M. pulmonis* was among the latest, increasing after 14 days. These data show that endothelial cell proliferation and microvascular remodeling occur at an early stage of chronic airway disease and suggest that the vascular changes precede widespread tissue remodeling. (Am J Pathol 2001, 158:2043–2055)

Angiogenesis and vascular remodeling are important elements of the pathophysiology of cancer and chronic inflammatory diseases and may be essential for the persistence of these conditions.¹ The vasculature provides metabolic support for the diseased tissues and serves as the gatekeeper for incoming inflammatory cells and outgoing tumor cells that form metastases. The recognition

that newly formed and remodeled vessels are different from those normally present led to the identification of probes to target diseased vessels selectively^{2,3} and to the use of angiogenesis inhibitors in the treatment of cancer and chronic inflammation.^{1,4}

The properties and functional implications of angiogenic blood vessels have been examined in many animal models of cancer,^{5–8} but less is known about the vascular changes in chronic inflammation. One might expect the microvasculature to change gradually as protracted inflammatory diseases evolve, or it could change rapidly at the beginning and drive other aspects of the disease. Yet the time course of changes in the vasculature in chronic inflammation has not been addressed systematically, in part because of limited suitable animal models.

One animal model that has been useful for studying angiogenesis and vascular remodeling in chronic inflammation is the respiratory tract infection caused by *Mycoplasma pulmonis* in mice and rats. This infection causes severe, lifelong changes in the microvasculature of the airway mucosa,^{9–11} along with extensive tissue remodeling, leukocyte influx, epithelial and gland hyperplasia, and fibrosis in the airways and, in mice, pneumonitis.^{12–15} In rats infected with *M. pulmonis*, a familiar form of sprouting angiogenesis is the predominant change in the airway vasculature.^{9–11} In mice, two distinct types of changes occur in a strain-dependent manner.¹⁶ Sprouting angiogenesis occurs in C57BL/6 mice, but in C3H mice the airway microvasculature enlarges without increasing appreciably in length.¹⁶ This enlargement results from endothelial cell proliferation rather than vasodilatation.^{16,17} With the enlargement, endothelial cells of blood vessels in the anatomical position of capillaries acquire characteristics of venules, including leakiness, adherence of leukocytes, and expression of P-selectin and von Willebrand factor.¹⁸ These changes in endothelial cell phenotype could be key to the rapid influx of leukocytes that follows *M. pulmonis* infection and initiates the tissue remodeling.^{16,17}

Like other chronic inflammatory conditions, *M. pulmonis* infection is accompanied by progressive tissue remodeling. Without therapeutic intervention, the disease is

Supported in part by National Institutes of Health grants HL-24136 and HL-59157 from the National Heart, Lung, and Blood Institute (to D. M.).

Accepted for publication February 27, 2001.

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life-long. Interestingly, the enlargement of the microvasculature is evident as soon as 1 week after infection, before the full-blown disease develops.¹⁶ One possibility is that a sudden spurt of endothelial cell proliferation transforms the mucosal vasculature into a phenotype that supports the rapid influx of inflammatory cells and progression of the inflammatory response. One manifestation of such a phenotypic change would be the adherence of leukocytes to the endothelium. Alternatively, endothelial cells could proliferate at a uniform rate after the onset of the disease in parallel with the remodeling of other airway tissues. In either case, it is unclear how the rate of endothelial cell proliferation relates to the time course of vascular enlargement, disease progression, and overall severity. A further question is whether the proliferation of endothelial cells results in altered endothelial barrier function, consistent with the leakiness of angiogenic blood vessels in chronic inflammation and tumors.^{19–21}

In addressing these questions, we sought to: 1) compare the time course of the microvascular enlargement with the time course of endothelial cell proliferation in the airway mucosa after *M. pulmonis* infection and identify the affected segments of the microvasculature; 2) determine whether the airway microvasculature becomes leaky at the peak of endothelial cell proliferation; 3) compare the time course of leukocyte adhesion with the pattern of endothelial cell proliferation; and (4) assess the temporal relationship between the vascular enlargement and overall disease severity.

Our strategy was to infect mice with *M. pulmonis* by intranasal inoculation and then to measure the time course of changes in blood vessel diameters, the number of 5-bromo-2'-deoxyuridine (BrdU)-labeled endothelial cells, and the amount of leukocyte adhesion in the microvasculature of the tracheal mucosa. We also examined the amount and location of blood vessel leakiness and assessed the time course and severity of the systemic immune response.

Materials and Methods

Infection of Mice with *M. pulmonis*

Specific pathogen-free, 7- to 8-week-old male C3H/HeN mice (average body weight, 24 g) were purchased from Charles River Laboratories (Hollister, CA) and anesthetized intramuscularly with ketamine (100 mg/kg; Parke-Davis, Morris Plains, NJ) and xylazine (5 mg/kg; Ben Venue Laboratories, Bedford, OH). Mice were inoculated intranasally with 10⁵ colony-forming units of *M. pulmonis* (strain UAB CT7) in a volume of 50 μ l (25 μ l per nostril). Infected and pathogen-free control mice were caged and 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 of Airway Microvasculature

To make it easier to measure the size of blood vessels and to quantify intravascular leukocytes, the vasculature was stained by perfusion of a lectin that binds uniformly to the luminal surface of endothelial cells and leukocytes.²² Briefly, after anesthesia, mice were injected intravenously with biotinylated *Lycopersicon esculentum* lectin [100 μ g lectin per 100 μ l of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered saline; Vector Laboratories, Burlingame, CA]. After 2 to 3 minutes, the chest was opened and the vasculature was perfused with 1% paraformaldehyde and 0.5% glutaraldehyde in phosphate-buffered saline (PBS) for 3 minutes, followed by PBS for 2 minutes at a pressure of 120 mm Hg via a blunt 13-gauge needle inserted through the left ventricle into the ascending aorta. Tracheas were cut along the ventral midline, removed, and pinned luminal surface up on Petri dishes coated with Sylgard (Dow-Corning, Midland, MI). The specimens were permeabilized with 0.3% Triton X-100 in PBS overnight and then incubated in avidin-biotin-peroxidase complex (1:200; Vector) in 0.3% Triton X-100 in PBS overnight at room temperature. After washing with PBS for at least 2 hours, tracheas were then reacted for 10 to 15 minutes with 0.05% diaminobenzidine (Sigma Chemical Co., St. Louis, MO) and hydrogen peroxide in PBS. The tissues were washed thoroughly with deionized water, dehydrated with ethanol, removed from the Sylgard, flattened between two glass slides in absolute ethanol overnight, cleared in toluene, and mounted in Permount (Fisher Scientific, San Francisco, CA).

In other mice, the borders of endothelial cells were stained by vascular perfusion of silver nitrate.^{16,23} Briefly, the vasculature was perfused in succession with 1% paraformaldehyde and 0.5% glutaraldehyde in 75 mmol/L of cacodylate buffer (pH 7.4) at a pressure of 120 mm Hg for 3 minutes, followed by 0.9% NaCl for 2 minutes, 10 ml of 5% glucose delivered with a hand syringe at a rate of 1 ml per second, 7 ml of 0.2% silver nitrate for 7 seconds, 10 ml of 5% glucose for 10 seconds, and fixative again for 1 minute. The tracheas were incised along the midline, removed, and the silver halide was developed under a bright light for 15 minutes. Finally, the tracheas were dehydrated in alcohol, cleared in toluene, and mounted in Permount and observed as whole-mount preparations.

Measurement of Vessel Size

The diameter of arterioles, capillaries, and venules (10 vessels of each type, as determined by previously described criteria¹⁶) was measured in the mucosa of lectin-stained tracheal whole mounts from pathogen-free mice and mice infected for 4, 5, 7, and 14 days ($n = 4$ per group). Blood vessels in whole mounts of tracheas stained with lectin were measured with a Zeiss Axiophot microscope coupled to a color charge-coupled device video camera (model DXC-755; Sony, Tokyo, Japan), a real-time color video digitizing card (Video-Logic DVA-4000; Video-Logic, Cambridge, MA) in a personal computer, a digitizing tablet (model 1111A; GTCO Digipad, Rockville, MD), and image analysis software developed for this purpose in our laboratory.²³

BrdU Labeling and Measurement of Endothelial Cell Proliferation

The thymidine analogue, BrdU (Sigma), was injected intravenously (1 mg in 100 μ l of PBS per mouse) into pathogen-free mice and mice infected with *M. pulmonis* for 1, 3, 5, 7, 9, or 11 days or 2, 3, or 4 weeks ($n = 3$ to 6 per group). Three hours later, tissues were fixed by perfusion of 1% paraformaldehyde and 0.25% glutaraldehyde in PBS (pH 7.4; Sigma) for 3 minutes and with PBS for 2 minutes via the left ventricle at a pressure of 120 mm Hg. Preliminary experiments demonstrated that the combination of this dose of BrdU and the 3-hour interval between injection and vascular perfusion resulted in distinct labeling of proliferating endothelial cells in pathogen-free and infected tracheas. Tracheas and lungs were removed, washed with PBS, infiltrated with 30% sucrose in PBS at 4°C overnight, and embedded in OCT compound (Sakura Finetek USA Inc., Torrance, CA); 10- μ m-thick sections were then cut on a cryostat.

BrdU-labeled cells were detected immunohistochemically in cryostat sections as described previously.²⁴ Briefly, after air-drying, sections were digested with 0.005% pepsin (Sigma) in 0.01 N HCl at 37°C for 10 minutes and then 4 N HCl for 30 minutes at room temperature. Background staining was blocked by incubation in 5% normal goat serum for 30 minutes; then the sections were incubated with a mouse monoclonal antibody to BrdU (1:200; DAKO, Carpinteria, CA) at room temperature for 2 hours or at 4°C overnight, followed by an alkaline phosphatase-conjugated goat anti-mouse IgG (1:200; Jackson ImmunoResearch, West Grove, PA) at room temperature for 1 hour. The alkaline phosphatase-conjugated antibody was visualized as a red or dark blue reaction product using substrate kits I or IV (Vector Red or BCIP/NBT; Vector) in 0.1 mol/L of Tris-HCl buffer (pH 8.2 or 9.5, respectively) at room temperature. Sections were fixed again with 1% glutaraldehyde in PBS at room temperature for 5 minutes, washed in distilled water, counterstained with hematoxylin, and mounted in Aquatex (Merck, Darmstadt, Germany).

BrdU-labeled endothelial cell nuclei, identified as elongated oval regions of immunoreactivity in the lining of lectin-stained blood vessels, were counted in 10 to 12 sequential sections from the rostral trachea of each mouse. The percentage of BrdU-labeled blood vessels was expressed as the ratio of vessels having BrdU-labeled endothelial cells to the total number of vessel profiles per cross-section of trachea. In addition, the endothelial cell-labeling index was calculated as the ratio of number of BrdU-labeled endothelial nuclei to total number of endothelial nuclei stained with hematoxylin.

Some tracheal whole mounts were double-stained for lectin histochemistry and BrdU immunohistochemistry. After injection of *L. esculentum* lectin and perfusion fixation as above, tracheas were washed in PBS for at least 1 hour and then three times in deionized water for 5 minutes each. The tracheal whole mounts were digested with 0.003% pepsin (Sigma) in 0.01 N HCl (pH 2) at 37°C for 50 minutes, and then the luminal surface of the tra-

chea was stroked gently with a fine camelhair brush to remove loosened epithelial cells, some of which were labeled with BrdU. The tissues were treated with 4 N HCl at room temperature for 50 minutes and then washed 7 times for 5 minutes each in deionized water and then 7 times in PBS. Background staining was blocked by incubation in 5% normal goat serum for 1 hour, then incubated with the mouse monoclonal antibody to BrdU (1:200, DAKO) at room temperature overnight, followed by an alkaline phosphatase-conjugated goat anti-mouse IgG (1:200, Jackson) at room temperature for 2 hours. The dark-blue alkaline-phosphatase reaction product was visualized with the BCIP kit for 5 to 10 minutes at room temperature as described above. The tracheas were incubated with avidin-biotin-peroxidase complex for visualizing the biotinylated lectin staining, dehydrated with ethanol, cleared with toluene, and mounted in Permount.

Quantification of Adherent Intravascular Leukocytes

Leukocytes adherent to the luminal surface of blood vessels were counted in lectin-stained tracheal whole mounts from pathogen-free mice and mice infected for 3, 4, 5, 7, or 14 days ($n = 4$ to 6 per group). In these preparations, adherent leukocytes were easily identified as lectin-stained, brown spheres within blood vessels. In each trachea, leukocytes were counted at a magnification of 400 in 20 regions of mucosa, each measuring 0.096 mm² (total area 1.92 mm² per trachea), located between cartilage rings. Values are expressed as number of adherent intravascular leukocytes per square millimeter of mucosal surface.

Localization and Measurement of Plasma Leakage

Sites of leakage were identified microscopically by using the particulate tracer Monastral blue pigment, which was injected intravenously (30 mg/kg in saline) 5 to 10 minutes before the vasculature was perfused with fixative.²⁵ Tracheas were then removed and prepared as whole mounts.^{9,26} Sites of leakage were identified as patches of Monastral blue pigment trapped in vessel walls. For quantitative studies of leakage, Evans blue dye (30 mg/kg in saline; EM Sciences, Cherry Hill, NJ) was injected intravenously into anesthetized mice in a volume of 100 μ l, as described previously.²⁷ Thirty minutes later, the chest was opened, both atria were cut, and the vasculature was perfused with 1% paraformaldehyde in 0.05 mol/L citrate buffer (pH 3.5) for 2 minutes at a pressure of 120 to 140 mm Hg via a cannula in the left ventricle. Tracheas and bronchi were removed, gently blotted with filter paper, and weighed. Evans blue dye was extracted from the tissues using formamide and measured by spectrophotometry.²⁷ Measurements were expressed as ng dye/mg wet weight of trachea.

Pathogen-free

M. pulmonis-infected

Assessment of Disease Severity

Blood (0.2 to 0.4 ml) was removed from anesthetized mice into heparinized syringes and centrifuged at 8000 rpm for 6 minutes for measurement of *M. pulmonis* antibody titers. Serum was diluted 1:5 with sterile saline, heat-inactivated at 56°C for 30 minutes, and then frozen until analyzed by indirect enzyme-linked immunosorbent assay (BioReliance Corporation, Rockville, MD). The enzyme-linked immunosorbent assay had a threshold sensitivity of 1 ng immunoglobulin per well. The lungs, bronchial lymph nodes, thymus, and spleen were removed, blotted dry, and weighed after vascular perfusion of fixative.

Statistics

Values are presented as means $\pm$ SE of data for three to six mice per group unless otherwise indicated. The significance of differences between groups was determined by Student's *t*-test or by analysis of variance followed by the Dunn-Bonferroni test for multiple comparisons. Differences were considered significant when $P < 0.05$.

Results

Time Course of Microvascular Enlargement and Endothelial Cell Proliferation

Microvascular Enlargement

Major changes in the tracheal microvasculature were evident in lectin-stained tracheas of *M. pulmonis*-infected mice throughout the 28-day period of the study (Figure 1, A and B). All segments of the microvasculature became significantly larger within a few days after infection (Figure 1, C to H). The main arterioles in pathogen-free mice were relatively straight tubes of uniform $\sim 13\text{-}\mu\text{m}$ diameter running parallel to the cartilage rings (Figure 1C); after infection they had the same general location but enlarged to a diameter of $\sim 31\text{ }\mu\text{m}$ (Figure 1D). Capillaries, which included most of the mucosal vessels overlying the cartilage rings, changed from $\sim 8\text{-}\mu\text{m}$ tubes without adherent leukocytes (Figure 1E) into $\sim 26\text{-}\mu\text{m}$ venule-like vessels with abundant leukocytes adherent to their endothelium (Figure 1F). These remodeled vessels had the normal anatomical location of capillaries but had a venular phenotype. Similarly, venules, which were normally the largest (average diameter, $36\text{ }\mu\text{m}$) and most numerous vessels in the region of mucosa between cartilage rings (Figure 1G), increased in diameter to $\sim 62\text{ }\mu\text{m}$ (Figure 1H). The endothelium of remodeled capillaries

Figure 2. Time course of enlargement of arterioles (open triangles), capillaries (open squares), and venules (open circles) in trachea of C3H/HeN mice after *M. pulmonis* infection. Values are means $\pm$ SE. *, Significantly different ($P < 0.05$) from pathogen-free group (value at 0 days); $n = 4$ mice per group.

and venules was covered with leukocytes (Figure 1, F and H).

The enlargement of arteriole diameters was statistically significant on day 4 after infection (63% increase, Figure 2). Capillary diameters (131% increase) and venule diameters (48% increase) were significantly larger on day 5 (Figure 2). Vessels reached their maximal size at 7 days, when on average the diameters of arterioles, capillaries, and venules were 148, 214, and 74% larger, respectively. The size of all types of vessels tended to plateau after 7 days (Figure 2). The enlargement at 7 days represented average diameter increases of $18\text{ }\mu\text{m}$ for arterioles and capillaries and $26\text{ }\mu\text{m}$ for venules.

Endothelial Cell Proliferation

Using BrdU to define the time course of endothelial cell proliferation, we found that labeled nuclei were rare in the trachea and bronchi of pathogen-free mice (Figure 3; A, C, and E) but were common in *M. pulmonis*-infected mice (Figure 3; B, D, and F). After infection, BrdU-labeled nuclei were most abundant in the airway epithelium (Figure 3B), but labeled endothelial nuclei were easily identified around the perimeter of the enlarged blood vessels in the trachea (Figure 3D) and bronchi (Figure 3F). Measurements showed that 1.1% of vessel profiles in sections of tracheas from pathogen-free mice had BrdU-labeled endothelial cell nuclei, whereas after infection the propor-

Figure 1. Micrographs of *L. esculentum* lectin-stained microvasculature in tracheal whole mounts of pathogen-free (A, C, E, and G) and *M. pulmonis*-infected (B, D, F, and H) mice. Low magnification overviews (A and B) show regular arrangement of normal vessels in trachea of pathogen-free mouse (A) compared to enlarged vessels in trachea of infected mouse (B). Enlarged vessels, shown here at 7 days after infection, included arterioles (C and D), capillaries (E and F), and venules (G and H). Arrows mark examples of corresponding segments of the normal and remodeled microvasculature. Granular brown staining between blood vessels in B is because of the presence of abundant extravascular neutrophils made visible by the avidin-biotin-peroxidase histochemistry. Adherent intravascular leukocytes were present in remodeled capillaries (F) and venules (H) but not arterioles (D). Scale bar in H applies to all figures; bar length represents $160\text{ }\mu\text{m}$ in A and B; $50\text{ }\mu\text{m}$ in C–H.

Pathogen-free

M. pulmonis-infected

Figure 4. Time course of endothelial cell proliferation assessed by BrdU labeling of cells in the tracheal mucosa of C3H/HeN mice after *M. pulmonis* infection. Values are means $\pm$ SE of the proportion of blood vessels with one or more BrdU-labeled endothelial cell nucleus in sections of trachea. *, Significantly different ($P < 0.05$) from pathogen-free group (value at 0 days). Pathogen-free group and 5-day infection group contained six mice each; other groups had three mice each.

tion was 2.4% at 1 day, 7.3% at 3 days, and 19.4% at 5 days (Figure 4). After the peak at 5 days, labeling declined to 12.2% at day 7, 5.9% at day 9, and gradually decreased to 2.8% at 28 days (Figure 4).

Estimates of labeling index indicated that only $1.4 \pm 0.4\%$ of endothelial cell nuclei had BrdU labeling in the trachea of pathogen-free mice, but $10.9 \pm 0.6\%$ were labeled at 5 days after infection, an increase of approximately eightfold ($P < 0.0001$, $n = 6$ per group).

Examination of BrdU-labeled whole mounts revealed that labeled endothelial cell nuclei were numerous in all segments of the tracheal microvasculature at 3 to 7 days after infection but were uncommon there in pathogen-free mice (Figure 3, G and H). After infection, BrdU-labeled nuclei were abundant in the epithelium and were also found in the lamina propria and smooth muscle.

Endothelial Cell Enlargement

Endothelial cells outlined by silver nitrate staining of tracheal venules in pathogen-free mice had a polygonal shape and a uniform size (Figure 5A). By comparison, during the period of greatest endothelial cell proliferation (5 days after infection), giant endothelial cells were scattered among endothelial cells of normal size (Figure 5B). Although constituting only a small minority of endothelial cells, giant cells were at least twice the size of endothelial cells in normal venules and were very conspicuous. Sites of leukocyte adherence and migration, appearing as tiny

rings at endothelial cell borders, were visible in venules of infected mice (Figure 5B).

Plasma Leakage from Remodeled Blood Vessels

Monastral blue did not leak from any blood vessel in the trachea of pathogen-free mice (Figure 5C), but focal regions of extravasated Monastral blue were in the wall of some tracheal vessels at the peak of endothelial cell proliferation 5 days after infection (Figure 5D). The distribution of leakage was not uniform. Some vessels had intense Monastral blue labeling, but many had none. Most leaky sites were in venules. Arterioles had none. Some sites of Monastral blue leakage were near silver dots (ie, endothelial gaps²³) at endothelial cell borders in silver nitrate-stained specimens (Figure 5D). However, when the overall amount of plasma leakage in the trachea and bronchi was measured with Evans blue, the values for infected mice were not significantly different from those for pathogen-free mice at any time point (Figure 6). Leakage expressed per microgram of tissue was consistently greater in bronchi than in tracheas and there was a trend toward higher values at 5 days, but the large variability among animals obscured any differences because of the infection.

Adhesion of Intravascular Leukocytes

Adherent intravascular leukocytes, which were prominently stained by the biotinylated *L. esculentum* lectin, were scarce in all segments of the tracheal microvasculature in pathogen-free mice (Figure 1; A, C, E, and G) but were abundant in infected mice (Figure 1; B, F, and H; and Figure 7). Adherent leukocytes were numerous in remodeled capillaries and venules (Figure 1, F and H) but were rare in arterioles (Figure 1D). At 4 days after infection, adherent leukocytes were ~ 40 times as numerous as in pathogen-free mice (1286 ± 388 versus 32 ± 8 intravascular leukocytes per mm^2 of mucosal surface area, $n = 4$ mice per group, $P < 0.0001$) and remained abundant at later time points (Figure 7). Extravascular neutrophils, made visible by the reaction of myeloperoxidase in the lectin histochemistry, were also abundant in the tracheal mucosa of infected mice (Figure 1, B and H).

Systemic Responses to *M. pulmonis* Infection

The mice became lethargic and lost weight after inoculation with *M. pulmonis* and from day 5 onward were significantly lighter than the pathogen-free controls (Figure 8A). The weight of infected mice gradually fell

Figure 3. A–F: Micrographs showing BrdU-stained nuclei (red) in sections of tracheas of pathogen-free (A, C, and E) and *M. pulmonis*-infected (B, D, and F) mice. BrdU-labeled nuclei are scarce in pathogen-free tracheas (A and C) and bronchi (E) but are abundant (arrows) at 5 days after infection (B, D, and F). BrdU-labeled cells include endothelial cells (arrows), epithelial cells (arrowheads), and scattered cells in the connective tissue. Whole mounts, in which the vasculature was made visible by staining with *L. esculentum* lectin, show that few BrdU-labeled nuclei (blue) are present in pathogen-free trachea (G) but many are present 3 days after infection (H). The presence of labeled epithelial cells and other cells makes labeled endothelial cells more difficult to identify in whole mounts than in sections. Scale bar in H applies to all figures; bar length represents 100 μm in A and B, 25 μm in C–F, and 50 μm in G and H.

Pathogen-free

M. pulmonis-infected

Figure 6. Amount of Evans blue leakage (ng/mg wet weight tissue) from vasculature of trachea (filled circles) and bronchi (open circles) of pathogen-free and *M. pulmonis*-infected C3H/HeN mice. Values are means $\pm$ SE. No values for infected rats were significantly different from corresponding values for the pathogen-free group (value at 0 days); $n = 6$ to 18 mice per group for tracheas and 4 to 5 mice per group for bronchi.

through day 21 and then stabilized, but pathogen-free mice continued to gain weight during this period and at day 28 were 50% heavier than the infected mice.

Serum antibody titers to *M. pulmonis* were undetectable until day 14 and rapidly increased between days 21 and 28 (Figure 8B). The weight of the bronchial lymph nodes reflected the strong local immunological response to *M. pulmonis* infection and was a sensitive indicator of disease progression. Bronchial lymph nodes increased almost linearly in size from day 3 after infection: the mean weight increased nearly 40-fold, from ~ 2 mg at the outset to 77 mg at 28 days (Figure 8C). Lung weight gradually increased with the influx of inflammatory cells and edema fluid and nearly doubled throughout the first 28 days after infection (Figure 8D). The spleen enlarged gradually and at 28 days was $\sim 70\%$ heavier than in pathogen-free mice (Figure 8E), mainly because of expansion of the white pulp (data not shown). In contrast, thymus weight rapidly decreased after infection. The thymus was significantly smaller on day 5 and by day 7 had lost 70% of its mass in comparison to corresponding pathogen-free mice (Figure 8F).

Overall, these measurements emphasize the slow, progressive infiltration of the lungs after *M. pulmonis* infection, accompanied by gradual but continuous enlargement of draining lymph nodes and spleen. The humoral immune response, as reflected by serological antibody titers to the infectious organisms, also occurred relatively late. By comparison, endothelial cell proliferation occurred rapidly after infection and the peak was transient, but the changes were long lasting.

Figure 7. Adherent leukocytes within tracheal blood vessels in pathogen-free and *M. pulmonis*-infected C3H/HeN mice. Values are means $\pm$ SE expressed as number of intravascular leukocytes per square mm of tracheal mucosal surface. *, Significantly different ($P < 0.05$) from pathogen-free group (value at 0 days); $n = 4$ to 6 mice per group.

The rapid onset of vascular remodeling was accompanied by a reduction in body weight and shrinkage of the thymus.

Discussion

This study of the time course of changes in airway blood vessels after *M. pulmonis* infection in C3H mice revealed a rapid, transient spurt of microvascular remodeling. Endothelial cell proliferation reached a peak at 5 days, declined sharply until day 9, and plateaued at ~ 3 times baseline. Tracheal blood vessels doubled in size during the first 7 days after infection and then vessel caliber plateaued. All segments of the microvasculature were affected. In addition, remodeled capillaries and venules, but not arterioles, were sites of leukocyte adhesion. Sites of plasma leakage through endothelial gaps were also present, but the overall amount of leakage in the airways was not significantly increased, so the leaks seem to be focal. Changes in the airway microvasculature were among the earliest detected. Most systemic manifestations of the infection, including the production of antibodies to *M. pulmonis*, followed these changes.

Endothelial Cell Proliferation

One of the aims of the present study was to determine the time course of endothelial cell proliferation in C3H mice after *M. pulmonis* infection. We were surprised to find that the peak in endothelial cell division, as reflected by BrdU labeling, was brief and occurred quite early, at only 5 days after the onset of the disease, whereas tissue re-

Figure 5. Blood vessels with endothelial cells outlined by silver nitrate staining in tracheas of pathogen-free (A and C) and *M. pulmonis*-infected C3H/HeN mice (B and D) after injection of Monastral blue. Endothelial cells in venule (A) and capillary (C) of pathogen-free mouse have regular borders, a relatively uniform size, and no extravasated Monastral blue. Endothelial cells in venule (B) and capillary (D) of a mouse infected for 5 days have irregular borders punctuated by silver rings (attached leukocytes, arrows), heterogeneity of size and shape (B), scattered silver dots (endothelial gaps, arrowheads) (D), and extravasated Monastral blue (D). A giant endothelial cell (asterisk) is present in the remodeled venule (B). Scale bar in D applies to all figures; bar length represents 25 μ m in A and B, 15 μ m in C and D.

Figure 8. Systemic changes in C3H/HeN mice after *M. pulmonis* infection, including change in body weight (A), serological antibody titer to *M. pulmonis* (B), weight of bronchial lymph nodes (C), lung weight (D), spleen weight (E), and thymus weight (F). Values are means $\pm$ S. E. *, Significantly different ($P < 0.05$) from pathogen-free group (value at 0 days); $n = 6$ to 18 mice per group.

modeling continued to evolve for weeks. At 5 days $\sim 11\%$ of the endothelial cells took up BrdU during the 3-hour period of labeling, compared to a baseline value of $\sim 1\%$. This labeling index is within the range found in some other conditions accompanied by microvascular remod-

eling and angiogenesis, such as wound healing, delayed hypersensitivity reaction, and certain types of tumors.²⁸⁻³⁰ After 5 days, the rate of endothelial cell proliferation fell sharply but remained above baseline as the chronic airway disease evolved.

Growth factors and other cytokines that stimulate endothelial cell proliferation after *M. pulmonis* infection may come from several sources. Mitogens from *M. pulmonis* organisms may directly affect endothelial cells as they do lymphocytes.^{31–33} Endothelial cell mitogens may also come from activated epithelial cells. *M. pulmonis* organisms adhere to the luminal surface of the airway epithelium, creating a polarized stimulus that produces phenotypic and functional changes in endothelial cells on the surface of blood vessels facing the epithelium.¹⁷ Endothelial cells on the opposite side of the same vessels are not similarly affected. *M. pulmonis* infection also caused the proliferation of epithelial cells, as it did endothelial cells, as indicated by increased BrdU labeling. In contrast to the polarized changes presumably mediated by activated epithelial cells, proliferating endothelial cells were uniformly distributed around the vessel circumference, suggesting that the mitogenic stimulus does not come from one direction.¹⁷ Therefore, *M. pulmonis* organisms attached to airway epithelial cells are unlikely to be the sole source of endothelial cell mitogens. Another obvious source of growth factors is the inflammatory cells that infiltrate the airway mucosa in abundance after *M. pulmonis* infection. Neutrophils, macrophages, and lymphocytes are well-documented sources of endothelial cell mitogens.^{11,34–36}

Relation of Endothelial Cell Proliferation to Inflammatory Response

The present study provides clear evidence that the peak of the endothelial cell proliferation at 5 days is a relatively early component of the inflammatory response to *M. pulmonis* infection, coinciding with the initial influx of leukocytes and preceding the humoral immune response by more than a week. The triggering stimulus for endothelial cell proliferation must be activated even earlier. Biochemical changes, as indicated by increased expression of tumor necrosis factor- α , interleukin-1, interleukin-6, and interferon- γ , are known to occur within a few hours after infection.³⁷ However, the identity and source of the growth factors responsible for the proliferation of endothelial cells are still under investigation.

The early onset of vascular remodeling after *M. pulmonis* infection is consistent with the time course of angiogenesis in the delayed hypersensitivity and graft-versus-host reactions, where blood vessel proliferation peaks at day 2 to 3 and lasts through day 6.^{29,38} Similarly, the peak of angiogenesis occurs at 3 to 5 days in fractured bones.³⁰

Relation of Endothelial Cell Proliferation to Vascular Leakiness

The airway vasculature in rats infected with *M. pulmonis* for 4 weeks is abnormally leaky under baseline conditions.²¹ However, under the same conditions the overall amount of plasma leakage in the airways of mice infected with *M. pulmonis* for 4 weeks is within the range found in

pathogen-free mice.¹⁶ In an earlier study in mice,¹⁶ we were unaware of the time course of endothelial cell proliferation and did not ask whether leakage occurs in the first few days after infection; we also did not look for focal regions of leakage that may be undetectable by Evans blue measurements of entire tracheas. In the present study, having learned about the peak of endothelial cell proliferation at 5 days, we measured Evans blue leakage at multiple times during the first 2 weeks after infection. Furthermore, we used the particulate tracer Monastral blue as a complementary test for focal sites of leakage.^{23,25} This approach revealed scattered sites of Monastral blue extravasation in remodeled capillaries and venules during the phase of rapid endothelial cell proliferation. Considering that the endothelial cells of arterioles also underwent proliferation, but no leakage was observed there, the leakage in remodeled vessels was not a necessary consequence of endothelial cell proliferation, although this may indicate that the media of arterioles opposes extravasation because of loss of endothelial barrier function. Although the experiments did not detect a significant increase in Evans blue accumulation after infection, even at the peak of endothelial cell proliferation, there was a trend toward more leakage around day 5, yet the interanimal variability precluded any meaningful interpretation.

The observations of Monastral blue extravasation are consistent with evidence that venules are the primary site of plasma leakage, as well as leukocyte migration, in a variety of pathological conditions, including the response to specific inflammatory mediators.^{39–46} Capillary-like vessels leak under some conditions, but the leaky vessels are typically newly formed by angiogenesis.^{47–49} Capillary-like vessels are the principal sites of leakage in the airways of *M. pulmonis*-infected rats, which have extensive angiogenesis,^{11,21} but these tiny new vessels do not form in C3H mice where microvascular enlargement is the dominant change.¹⁶

Change in Endothelial Cell Phenotype

Enlargement of the airway microvasculature in C3H mice after *M. pulmonis* infection is accompanied by distinctive changes in endothelial cells.¹⁶ In addition to undergoing proliferation, some endothelial cells enlarge to nearly twice normal size and resemble multinucleated giant endothelial cells located over atherosclerotic plaques.^{50,51} Giant endothelial cells in the airway microvasculature may be a manifestation of rapid proliferation, but they are unlikely to play a major role in increased vessel caliber because they were infrequent and their presence was limited to the period of most rapid endothelial cell proliferation.

M. pulmonis infection increases the expression of the leukocyte adhesion molecule P-selectin on remodeled capillaries and venules.¹⁸ P-selectin-dependent rolling is one of the earliest observable events in the recruitment of leukocytes to inflamed tissues.⁵² Our studies revealed that leukocyte adhesion to remodeled vessels occurred early in the disease in C3H mice, beginning during the

period of rapid vascular remodeling and continuing thereafter. Sustained leukocyte migration is one of the most prominent features of *M. pulmonis* airway disease.^{12,13} Similar microvascular remodeling and leukocyte influx occur in many other inflammatory conditions.^{42,43} Rapid changes in endothelial cell phenotype may play a key role in the evolution of *M. pulmonis* disease by controlling leukocyte traffic into airway tissues. Thus, the early onset of microvascular remodeling may be a gatekeeper in the development of the chronic inflammatory airway disease.

Conclusions

Remodeling of the airway microvasculature occurs early in the evolution of chronic respiratory disease after *M. pulmonis* infection in C3H mice. Endothelial cell proliferation peaks during the first few days after infection, and the phenotype of endothelial cells changes concurrently to support leukocyte adherence and migration. Generalized remodeling of the airway mucosa and systemic manifestations of mycoplasma disease become prominent later. The remodeled microvasculature, with extensive leukocyte adhesion and sites of leakage, may be key to sustaining the inflammatory response in the airways.

Acknowledgments

We thank Ms. Julie Gibbs-Erwin and Dr. J. Russell Lindsey at the University of Alabama at Birmingham for providing the *M. pulmonis* organisms and Ms. Nicole Glazer at UCSF for help with the photography.

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