Various adhesion molecules impair microvascular leukocyte kinetics in ventilator-induced lung injury

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MECHANICAL VENTILATION PLAYS a major role as a tool for basic life support, especially for treating patients with acute respiratory distress syndrome. However, many studies have ascertained that sustained artificial ventilation with a tidal volume set to maintain arterial Pco2 (Paco2) at a normal level leads to worsening of lung injury, which has been defined as ventilator-induced lung injury (VILI) (6). VILI has been shown to be caused by the augmented mechanical stress related to over-distension of lung units and, probably, cyclical airway closure and reopening, resulting in promotion of a neutrophil-evoked inflammatory response concomitant with significant neutrophil infiltration into the lung tissue and increased levels of various inflammatory cytokines in the bronchoalveolar lavage (BAL) fluid (15, 26, 28, 31).

The accumulation of neutrophils in the lung tissue is initially mediated through increased adhesion of neutrophils to endothelial cells of the inflamed pulmonary microvasculature, and this process requires the upregulation of various adhesion molecules expressed on the surface of pulmonary microvessel endothelial cells as well as circulating neutrophils (18, 22). Despite the importance of augmented expression of adhesion molecules for initiating neutrophil-evoked lung injury, there has been little study shedding light on this issue in lung models of VILI, except that reported by Yiming et al. (37). These authors demonstrate that P-selectin expression on endothelial cells of the pulmonary vasculature was enhanced at an early stage after commencing artificial ventilation with a high tidal volume, followed by neutrophil accumulation. Unfortunately, however, they did not analyze other important adhesion molecules mediating neutrophil adhesion to pulmonary endothelial cells, such as ICAM-1 and VCAM-1. Furthermore, the relative contribution of each hierarchy of microvessels, including arterioles, venules, and capillaries, to neutrophil sequestration to the pulmonary microcirculation has never been examined in a VILI model, although this approach is essential for deepening our understanding of the early pathogenesis of VILI.

In addition to neutrophil infiltration in the lung tissue, lymphocyte accumulation and activation may occur at sites of inflammation in VILI. However, there has been no reliable study analyzing the contribution of lymphocytes to the initiation and/or aggravation of VILI. Lymphocytes may have a number of functions, including immune response, release of various cytokines, and cytotoxicity (36), all of which are expected to play a pivotal role in the development and pathogenesis of VILI. Although the recruitment of lymphocytes to inflammatory sites in systemic organs and tissues has been shown to be mediated in part through an adhesive mechanism involving ICAM-1- and/or VCAM-1-dependent pathways (2, 34), the issue of whether the same holds true for lymphocyte recruitment to the microcirculation of the diseased lung has not been extensively addressed.

To elucidate the adhesive mechanism mediating the accumulation of neutrophils and lymphocytes in arterioles, venules, or capillaries in lungs injured by sustained artificial mechanical
ventilation, we analyzed the following issues: 1) differential expression of various classes of adhesion molecules, including ICAM-1, VCAM-1, and P-selection, along arteriolar, venular, and capillary walls in a rat model of VILI; 2) measurements of the dynamic behavior of polymorphonuclear cells (PMN: mainly composed of neutrophils) within arterioles, venules, and capillaries in the lung isolated from an animal exposed to prolonged ventilation by means of recently developed real-time confocal laser fluorescence microscopy in the presence or absence of MAb to each endothelial adhesion molecule; and 3) confocal microscopic measurements of the dynamic behavior of mononuclear cells (MN: mainly consisting of lymphocytes) in each microvessel of the isolated lung with VILI, with and without MAbs, to the respective adhesion molecules.

METHODS

Animals with VILI, fluorescein-labeled leukocytes, and isolated perfused lungs. All animals received care according to the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research. Specific pathogen-free Sprague-Dawley male rats, 8 wk of age, weighing 250–300 g, were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital followed by intermittent injection of 10 mg/kg pentobarbital every hour (n = 41). No muscle relaxant was used for anesthetization. To induce ventilator-associated lung injury, anesthetized Sprague-Dawley rats were artificially ventilated with a volume-controlled ventilator (KN-55 Natsume Sei-sakusho, Tokyo, Japan) with room air at a constant tidal volume of 15 ml/kg and respiratory rate of 70 breaths/min without PEEP for 6 h.

Experimental protocols. To investigate the effects of various adhesion molecules on the microcirculatory behavior of leukocytes in the early stage of VILI, we designed six experiments for both CFSE-stained PMN and CFSE-stained MN: 1) Control group (PMN n = 6, MN n = 7): animals breathed spontaneously without a ventilator and anesthetizer. 2) Low tidal group (PMN n = 5, MN n = 5): animals were ventilated with a tidal volume of 7.5 ml/kg and respiratory rate of 70 breaths/min without PEEP for 6 h were assigned to the low tidal group (n = 10). These animals were divided into several groups depending on the leukocyte type observed (PMN or MN) and the MAbs to inhibit adhesion molecules (ICAM-1, VCAM-1, and P-selectin) expressed on the endothelial surface of pulmonary microvessels (see Experimental protocols). Spontaneously ventilated animals with room air without anesthesia were assigned to the control group (n = 13). Accumulation of PMN and MN in the alveolar septa and BALF was examined in the control, low tidal, and high tidal groups. The density of leukocytes in the septa was expressed as the number per single alveolus.

Preparation of fluorescein-labeled PMN and MN. We aspirated whole blood from the left ventricle of normal donor rats, in which 28 ± 9% of leukocytes were PMN and the remainder were MN (lymphocytes: 72 ± 9%). We isolated PMN and MN from whole blood aspirated from donor rats by applying a classic Ficoll density gradient procedure adapted for either rat PMN (specific gravity: 1.112) or MN (specific gravity: 1.090) (20). Both cell layers were labeled with carboxyfluorescein diacetate succinimidyl ester (CFDASE). This fluorescence precursor yields carboxyfluorescein succinimidyl ester (CFSE) in leukocytes but not in erythrocytes (27). After 30-min incubation of each leukocyte layer with CFDASE in vitro, the final solution was added to the perfusion reservoir connected to the isolated perfused lung system (see below). Preliminary experiments confirmed that the layer of PMN separated by the Ficoll method was mostly composed of neutrophils (96 ± 0.8%). In the layer of MN obtained by qualitatively the same method as applied for PMN, most of the blood cells were found to be lymphocytes (99 ± 0.5%). Leukocytes (PMN and MN) and platelets stained with CFSE were easily distinguishable during microscopic observation because of their distinctive difference in size.

Preparation of isolated perfused lungs. The animals with 6-h artificial ventilation were not anesthetized again, but the control animals were anesthetized with pentobarbital (50 mg/kg, intraperitoneally). These animals were artificially ventilated with room air for only the short time necessary to prepare the isolated perfused lung system. After median sternotomy was performed, animals were killed by exsanguination, and cannulas were inserted into the pulmonary artery and left atrium. Both cannulas were secured with ligatures. A ligature was placed around the aorta to prevent loss of perfusate into the systemic circulation. Isolated lungs prepared from the control, low tidal, and high tidal groups were fixed on a microscope stage in the supine position and perfused at a constant flow rate of 10 ml/min with physiological solution containing a small amount of whole blood ~10 min after exsanguination. Modified Krebs-Henseleit solution was used as the perfusate, and 3% bovine serum albumin was added to maintain isoosmotic pressure. Per fusate hematocrit was adjusted to 3.2 ± 0.8% by the addition of fresh blood obtained from donor rats, which were pretreated with 5 mg/kg heparin. Because the total volume of perfusate was adjusted to 100 ml, heparin concentration in the circulation system was ~6.5 μg/ml. To avoid movement caused by artificial ventilation, the trachea was ligated at the end-inspiratory point, and gas exchange was maintained with an extracorporeal membrane oxygenator (ECMO, Merasilo-X; Senko, Tokyo, Japan). A gas mixture containing 21% O2 and 5% CO2 in N2 was used as the gas flowing into the ECMO, resulting in perfusate PO2, PCO2, and pH of 142 ± 6 Torr, 37 ± 2 Torr, and 7.41 ± 0.03, respectively. A warmed and humidified gas mixture containing the same composition of gases as those used for the ECMO was continuously supplied to the lung surface to maintain a temperature of 37 ± 0.2°C and to avoid desiccation of the lung surface. Pulmonary arterial pressure was continuously monitored by force displacement of a pressure transducer (TP-400T; Nilohoden, Tokyo, Japan).

Observation of microvascular cell kinetics by confocal fluorescence microscopy. We used a real-time confocal laser scanning fluorescence optical microscope (CSU10; Yokokawa, Tokyo, Japan) incorporating a high-speed video analysis system (35). The reflected light or fluorescence emission from the specimen was imaged onto a high-sensitivity charge-coupled device (CCD) camera with an image intensifier (VSG; Kodak, San Diego, CA). By incorporating an excitation wavelength of 488 nm emitted from a low-power air-cooled argon laser (532-BSA04; Omnicrome, Cino, CA), the present confocal system allowed us to obtain apparently instantaneous images at 1,000 frames/s. The final magnifying power of our system reached...
The highest value of $V_r$ was assumed to be equal to the capillaries. The microvessel into which blood cells flowed from the capillary network entered the capillary network was defined as the arteriole, whereas the reservoir. The microvessel from which fluorescein-labeled blood cells and erythrocyte behavior had been analyzed, 200 min determined, as well.

To examine the architecture of microvessels in which leucocyte and erythrocyte behavior had been analyzed, 200 $\mu$l of 5% FITC-dextran with a molecular weight of 145,000 (Sigma) were added to the reservoir. The microvessel from which fluorescein-labeled blood cells entered the capillary network was defined as the arteriole, whereas the microvessel into which blood cells flowed from the capillary network was taken to be the venule.

Quantitative analysis of cell kinetics in arterioles, venules, and capillaries. The highest value of $V_r$ was assumed to be equal to the centerline velocity ($V_{\text{max}}$) in a given arteriole or venule. $V_{\text{max}}$ was used to estimate mean fluid flow ($V_{\text{mean}}$) in each microvessel, where $V_{\text{mean}} = V_{\text{max}}/1.6$ (8). Wall shear rate ($\gamma$) was estimated from the equation: $\gamma = 8 (V_{\text{mean}}/D)$, where $D$ represents vessel diameter (23).

An adherent leucocyte in an arteriole or venule was defined as a cell that was firmly attached to the vascular endothelium and did not move during the observation period. Rolling leucocytes in arterioles and venules were defined as cells transiently interacting with the vascular endothelium and thus traveling much more slowly than centerline erythrocytes. We identified these cells by applying the velocity criterion proposed by Gaethtgens and colleagues (8), who calculated the critical velocity ($V_{\text{crit}}$) of a freely flowing cell traveling close to, but not adhering to, the microvascular wall. $V_{\text{crit}}$ is defined as $V_{\text{mean}}(2-6)e$, where $e$ is the ratio of the leucocyte diameter to the microvessel diameter. Any cell flowing at a velocity below $V_{\text{crit}}$ was assumed to be slowed by the interaction with the vascular endothelium and defined as a rolling cell.

To quantify the cell transit in capillaries, we classified the observed leucocytes into two categories: 1) cells moving smoothly without interruption and 2) impeded cells stopping for $>20$ ms within a capillary segment, thus including the cells with transient entrapment and those with sustained arrest in capillaries during the observation period.

Intravital determination of ICAM-1, VCAM-1, and P-selectin expression along microvessel walls. We examined the expression of ICAM-1, VCAM-1, and P-selectin along microvessel walls by applying the intravital fluorescence microscopic method ($n = 3$ for each).

This method allows reliable separation of arterioles from venules in association with quantitative determination of each adhesion molecule expressed along microvessel walls. In brief, 4 $\mu$g/g body wt, Sigma) and FITC-labeled anti-mouse IgG antibody were used as negative controls. Arterioles, venules, and capillaries were distinguished by administering both FITC-dextran and FITC-labeled erythrocytes at the end of each measurement. We measured the complex-elicited fluorescence intensity along microvessel walls by processing a confocal image with a digital image-analyzing system (Quadra 840AV/Image 1.58; Apple, Cupertino, CA).

Determination of ICAM-1, VCAM-1, and P-selectin expression by immunohistochemical staining. The lungs with high tidal volume ventilation were fixed by administration of periodate-lysine-parafomaldehyde solution through the trachea, embedded in optimum cutting temperature compound (Miles, Elkhart, IN), and then frozen in dry ice and acetone ($n = 5$). Frozen sections 4 $\mu$m thick were cut on a cryostat and fixed on slides in cold acetone for 10 min and overnight at $-20^\circ$C. These sections were washed with PBS, and nonspecific staining was inhibited by incubation with 10% normal pig serum (COSMO BIO, Tokyo, Japan) in PBS for 20 min at room temperature. The sections were then incubated with anti-ICAM-1 MAb (MR106; Santa Cruz Biotechnology, Santa Cruz, CA), anti-VCAM-1 (C-19, Santa Cruz), or anti-P-selectin antibody (C-20, Santa Cruz) diluted 10-fold with PBS, for 1 h at room temperature. After these sections were rinsed with PBS, they were incubated with a 100-fold dilution of secondary antibody (anti-goat IgG-horseradish peroxidase, Santa Cruz) for 1 h at room temperature. After they were rinsed with PBS, they were incubated with 20 mg 3,3′-diaminobenzidine tetrachloride and 20 $\mu$l of 30% hydrogen peroxide in 100 ml of PBS for 2 min at room temperature. After washing them in water, we performed counterstaining of nuclei with methyl green. Immunoreactivity for ICAM-1, VCAM-1, or P-selectin in each lung section was determined with a light microscope. In total, 30 sections obtained from each of the upper, middle, and lower lung fields of five different animals were used for immunohistochemical assessment of ICAM-1, VCAM-1, and P-selectin expression in the control, low tidal volume-ventilated and VILI lungs, respectively.

Histological examination and BAL. The lungs harvested from control and low and high tidal volume-ventilated rats were inflated with formalin at a pressure of 30 cmH$_2$O for 48 h ($n = 5$ for each).

Six sections, cut at identical intervals from the apex to the base of the left lung, were embedded in paraffin and stained with hematoxylin-eosin. In each section, 10 fields were randomly chosen, and leucocytes localized within the alveolar septa in which the capillary network is located were counted at a magnification of $\times 1,000$ with an oil immersion lens. The density of leucocytes in the septa was expressed as the number per single alveolus. The same lung sections were used for estimating leucocyte differential counts, i.e., the distinction between PMN and MN. The histological samples obtained from each animal were evaluated in a double-blinded fashion by two doctors.

BAL was carried out by washing the lung with 3 ml phosphate-buffered saline twice. The number of total cells and the differential cell counts were determined on Diff-Quick-stained preparations (Kokusai Shiyaku, Kobe, Japan).

Statistical analysis. The results are presented as means ± SD. Significant differences in the frequency of rolling cells (PMN and MN), their capillary entrapment, and their accumulation in alveolar septa among experimental groups (i.e., Control, Low, High, High+anti-ICAM-Ab, High+VCAM-Ab, and High+P-selectin-Ab groups) were determined by applying one-way analysis of variance (ANOVA) followed by Scheffe’s multiple-comparison analysis. The values obtained for arterioles, venules, and capillaries in respective experimental conditions were statistically examined by two-way ANOVA together with Scheffe’s analysis for multiple comparisons. A $P$ value <0.05 was taken to be statistically significant.

RESULTS

Basic characteristics of lungs with VILI. The BALF concentrations of LDH and protein in the high tidal group were...
markedly higher than those in the control and low tidal groups (Fig. 1, A and B, respectively). In addition, the numbers of PMN and MN in BALF collected from the high tidal group were much higher than those in the control and low tidal groups (Fig. 1C). The inspiratory peak airway pressure was \(8.6 \pm 0.7\) cmH\(_2\)O in the high tidal group and \(6.3 \pm 0.7\) cmH\(_2\)O in the low tidal group. Pulmonary arterial pressure was \(10.6 \pm 1.6\) cmH\(_2\)O in the high tidal group, \(8.6 \pm 1.8\) cmH\(_2\)O in the low tidal group (\(P < 0.001\) vs. High), and \(8.4 \pm 1.7\) cmH\(_2\)O in the control group (\(P < 0.001\) vs. High). In the histological analysis, the high tidal group showed no serious tissue injury but slightly thickened alveolar walls and significant leukocyte infiltration into perivascular and peribronchiolar regions as well as alveolar spaces. The number of PMN accumulated within the alveolar septa in the high tidal group averaged \(0.32 \pm 0.19/\)alveolus, being much higher than that in the control group \((0.11 \pm 0.09/\)alveolus\) and the low tidal group.

Fig. 1. Biochemical and morphological data observed for control lungs (Control), low tidal volume-ventilated lungs (Low), and high tidal volume-ventilated lungs (High). A: LDH concentration in bronchoalveolar lavage fluid (BALF). B: protein concentration in BALF. C: number of polymorphonuclear cells (PMN) and mononuclear cells (MN) in BALF. D: number of PMN and MN within alveolar septa estimated from lung tissue sections stained with hematoxylin-eosin. The density of leukocytes in the septa is expressed as the number per single alveolus. *Significantly different from value in control lungs; $significantly different from value in low tidal volume-ventilated lungs.

(0.15 ± 0.12/alveolus) (Fig. 1D). Similarly, the number of MN accumulated within the alveolar septa in the high tidal group (0.09 ± 0.01) was significantly higher than that in the control (0.04 ± 0.10, P < 0.05) and low tidal groups (0.03 ± 0.05, P < 0.05) (Fig. 1D).

Expression of ICAM-1, VCAM-1, and P-selectin in lungs evaluated by immunohistochemical staining. Alveolar walls in the lungs of the control and low tidal groups showed faint but positive immunoreactivity for ICAM-1. ICAM-1 expression...
ICAM-1 expression was upregulated and discernible along the expression was appreciably enhanced along the alveolar walls 2, arterioles, in the lungs of the control and low tidal groups (Fig. was also detected along venules and capillaries, but not along microvessels in the control and low tidal groups, they were sparsely but perceptibly enhanced along arteriolar, venular, and capillaries in the high tidal group (Fig. 2, H–J and N–P).

Expression of ICAM-1, VCAM-1, and P-selectin along microvessels evaluated by intravital microscopy. In negative control experiments involving the administration of mouse IgG and FITC-labeled anti-mouse IgG antibody, no fluorescence was detectable along microvascular walls in any experimental group. Intravital confocal fluorescence analysis confirmed that ICAM-1 was expressed along venular and capillary walls, but not along arteriolar walls, in the control group (Fig. 2, D and E). On the other hand, ICAM-1 was significantly upregulated not only along venular and capillary walls, but also along arteriolar walls, during the development of VILI (Fig. 2, F and G).

VCAM-1 was not detectable along any microvessels in the control group (Fig. 2K). VCAM-1 was upregulated in both venular and capillary walls, but not in arteriolar walls, in the high tidal group (Fig. 2, L and M).

In the control group, P-selectin was not detectable in any microvessels (Fig. 2Q). Although P-selectin was appreciably upregulated along venular walls in the high tidal group, P-selectin expression was not evident along arteriolar and capillary walls (Fig. 2, R and S).

Compared with immunohistochemical study, intravital fluorescence microscopy has some advantages, especially in the identification of microvessels along which a given adhesion molecule is expressed. The intravital microscopic method allows examination of adhesion molecule expression under conditions in which microcirculatory flow is maintained. Therefore, arterioles and venules are easily identified from the difference in their flow directions. However, the sensitivity in detecting the expression of each adhesion molecule is expected to be relatively low with the intravital microscopic method compared with immunohistochemical staining. On the basis of these facts, we classified the degree of expression of each adhesion molecule into two categories: strong and faint enhancement. Strong enhancement was defined as upregulation of the adhesion molecule confirmed by both intravital microscopy and immunohistochemical study, whereas faint enhancement was defined as upregulation detected by immunohistochemical study only (Table 1). According to this classification, ICAM-1 expression was shown to be strongly enhanced along all microvessels, including arterioles, venules, and capillaries in high tidal volume-ventilated lungs. On the other hand, VCAM-1 expression was strongly enhanced along venules and capillaries but faintly enhanced along arterioles in VILI lungs. P-selectin expression in high tidal volume-ventilated lungs was strongly enhanced only in venules but faintly enhanced in both arterioles and capillaries.

Kinetics of PMN in pulmonary microcirculation. There were no significant differences in the vascular diameter including those of precapillary arterioles, postcapillary venules, and capillaries between the control, low tidal, and high tidal groups. The arteriolar diameter analyzed averaged 20.2 ± 3.4 μm in the control group, 20.7 ± 4.1 μm in the low tidal group and 19.9 ± 3.7 μm in the high tidal group. The mean venular diameter was nearly the same as that of arterioles, i.e., 22.4 ± 5.2 μm in the control group, 20.1 ± 3.9 μm in the low tidal group, and 21.8 ± 5.2 μm in the high tidal group, with no difference among the three groups. Addition of MAb against any adhesion molecule exerted little influence on microvascular diameter.

Vmean in arterioles was 0.92 ± 0.36 mm/s in the control group, 1.08 ± 0.75 mm/s in the low tidal group, and 0.93 ± 0.57 mm/s in the high tidal group, corresponding to a wall shear rate of 395, 419, and 394 s⁻¹, respectively. Venular Vmean was 1.03 ± 0.48 mm/s in the control group, 1.12 ± 0.57 mm/s in the low tidal group, and 1.37 ± 0.61 mm/s in the high tidal group, yielding a wall shear rate of 379, 464, and 523 s⁻¹, respectively. The values of Vmean and shear rates in arterioles were not significantly different among the control group, the low tidal group, and the high tidal group. Similarly, both values in venules among three groups were not significantly different. Vmean and wall shear rate were not influenced by the addition of any MAb. Vmean as well as wall shear rates tended to be lower in arterioles than those in venules, but statistically not significant.

We found no PMN adhering firmly to the arteriolar and venular walls under any experimental condition. In the high tidal group, the frequency of rolling PMN was much higher not only in venules (25.4 ± 14.8%), but also in arterioles (34.6 ± 15.2%), than in the control group (venule: 8.1 ± 11.6% vs. VILI, P < 0.05, arteriole: 11.8 ± 13.1% vs. High, P < 0.05) and in the low tidal group (venule: 5.5 ± 11.1% vs. High, P < 0.05, arteriole: 15.5 ± 8.7% vs. High, P < 0.05) (Fig. 3A). The augmentation of PMN rolling along the venular wall in high tidal volume-ventilated lungs was clearly inhibited by MAb against ICAM-1, VCAM-1, or P-selectin (Fig. 3A). Although PMN rolling along the arteriolar wall in the high tidal group was significantly inhibited by either anti-ICAM-1 MAb or anti-VCAM-1 MAb, it was not inhibited by the administration of anti-P-selectin MAb (Fig. 3A).

The frequency of PMN entrapped within capillary segments for at least 20 ms (temporal and sustained entrapment) was enhanced in the high tidal group (45.2 ± 10.4%) compared with the control group (25.3 ± 1.4% vs. High, P < 0.05), and the low tidal group (17.4 ± 6.1% vs. High, P < 0.05). This enhancement was significantly suppressed by anti-ICAM-1 MAb but not by anti-VCAM-1 or anti-P-selectin MAb (Fig. 4A). In our experimental systems, the isolated lungs were perfused with the constant flow, and the leukocyte kinetics was measured under steady state and confirmed by confocal laser

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++, Strong; +, faint.

Table 1. Upregulation of various classes of adhesion molecules in high tidal-ventilated lung
microscopy. Therefore, the capillary switching could not be observed (1).

**Kinetics of MN in pulmonary microcirculation.** MN did not show firm adhesion to microvessel walls, including arterioles and venules, under any experimental condition. The frequency of rolling MN in venules was significantly higher in the high tidal group (24.7 ± 19.3%) than in both the control (27.3 ± 12.2% vs. High, *P* < 0.05) and low tidal groups (24.1 ± 18.3% vs. High, *P* < 0.05). The enhanced entrapment of MN in capillary segments was effectively inhibited by MAb against ICAM-1 or VCAM-1 (Fig. 4 B).

![Fig. 3. Ratio of rolling leukocytes to whole leukocytes in arterioles and venules. Control, control lungs; Low, low tidal volume-ventilated lungs without addition of any MAb; High, high tidal volume-ventilated lung without addition of any MAb; Anti-ICAM-Ab, addition of anti-ICAM-1 MAb in high tidal volume-ventilated lung; Anti-VCAM-Ab, addition of anti-VCAM-1 MAb in high tidal volume-ventilated lung; Anti-P-selectin-Ab, addition of anti-P-selectin MAb in high tidal volume-ventilated lung. A: relative frequency of rolling PMN in arterioles and venules of high tidal volume-ventilated lung. B: relative frequency of rolling MN in arterioles and venules of high tidal volume-ventilated lung. *Significantly higher than value obtained in control lungs; $significantly higher than value obtained in low tidal volume-ventilated lungs; #significantly lower than value in high tidal volume-ventilated lung in the absence of any MAb.

![Fig. 4. Number of entrapped leukocytes in capillary segments. A: relative frequency of entrapped PMN in capillaries of high tidal volume-ventilated lung. B: relative frequency of entrapped MN in capillaries of high tidal volume-ventilated lung. See Fig. 3 legend for bar definitions. *Significantly different from value obtained in control lungs; Significantly higher than value obtained in low tidal volume-ventilated lungs; #significantly lower than value in high tidal volume-ventilated lung in the absence of any MAb.]
 DISCUSSION

Expression of adhesion molecules in lungs with VILI. The present study demonstrated that ICAM-1 is constitutively expressed along venular and capillary walls, but not along arteriolar walls, in intact rat lungs (Fig. 2). This is consistent with our previous work (18, 22) and also with the work reported by Shen et al. (25), who demonstrated constitutive ICAM-1 expression on unstimulated cultured human pulmonary microvascular endothelial cells (HPMEC). Nishio et al. (18) reported that VLA-4 could be mobilized from intracellular granules to the plasma membrane in human blood PMN upon cell activation or during transendothelial migration. In addition, Shang and Issekutz (24) reported that VLA-4 was expressed on the surface of C5a-activated PMN, thus mediating ICAM-1-independent migration of PMN through the vascular endothelium to lung connective tissue. The integrin VLA-9, also expressed on neutrophils, is an alternative receptor for VCAM-1 (30). Though we could not directly measure the levels of VLA-4 and VLA-9 expression on the PMN surface, we believe that the interactions between endothelial VCAM-1 and PMN VLA-4 and VLA-9 are important pathways causing the abnormal microvascular kinetics of leukocytes, including MN and PMN in high tidal volume-ventilated lungs. This is because inhibition of VCAM-1 improved the microvascular rolling of MN and PMN in high tidal volume-ventilated lungs (Fig. 3).

There is no doubt about the importance of endothelial P-selectin in the mediation of abnormal leukocyte kinetics (PMN and MN) in pulmonary microvessels, especially in venules and capillaries, in diseased lungs. Bless et al. (4) demonstrated that, in the cobra venom factor model, P-selectin was upregulated along venular and capillary walls within 1 h, followed by its sustained expression for the next 7 h. Furthermore, P-selectin was confirmed to play a vital role in the development and pathogenesis of various types of lung injury (12, 16, 17). However, to our knowledge, no significant role of enhanced arteriolar P-selectin in evoking lung injury has been conclusively demonstrated. In the present study, we therefore attempted to clarify the importance of P-selectin expressed along arteriolar walls in addition to venular walls and capillary segments in the initial phase of lung injury caused by sustained artificial ventilation.

Importance of adhesion molecules modulating PMN kinetics in high tidal volume-ventilated lungs. Augmented PMN rolling along postcapillary venules in high tidal volume-ventilated lungs was significantly suppressed by MAb against ICAM-1, VCAM-1, or P-selectin (Fig. 3), indicating that these adhesion molecules are equally important for inducing venular PMN rolling in ventilator-associated lung injury. The direct effect of VILI on the adhesion molecule expressions of white blood cells is unclear. Ohta and coworkers (19) reported that in rat lungs receiving high tidal ventilation, the expression of Mac-1 and ICAM-1 on neutrophils and macrophages increased significantly more than in the low tidal ventilation. On the other hand, Choudhury and coworkers (7) showed in an in vivo mouse model of VILI that PMN sequestration in injurious ventilation was markedly inhibited by administration of anti-L-selectin antibody, but not by anti-CD18 antibody, suggesting that this phenomenon is mediated by L-selectin-dependent but CD18-independent mechanisms. Interestingly, however, no rolling PMN qualitatively similar to those seen in systemic venules appeared; i.e., rolling leukocytes in systemic venules frequently show caterpillar-like movement (32), whereas rolling PMN observed in pulmonary venules revealed smooth movement with a significant reduction in velocity compared with that of erythrocytes flowing in the central part of the microvessel. Furthermore, we should emphasize the fact that firm tethering of PMN along venular walls was not observed under any experimental condition in both intact and VILI preparations of our experimental system. These findings are in radical contradiction to the sequential two-step theory of en-
dothelium-leukocyte interaction developed to explain leukocyte rolling and subsequent firm adhesion to vessel walls in the systemic microcirculation (6, 13, 32). Several lines of evidence based on observations in the systemic microcirculation showed that the selectin family such as P-selectin (endothelial surface) and/or L-selectin (leukocyte surface) would initiate cell rolling along microvessels (especially venules) as the first step, leading to firm adhesion mediated by the immunoglobulin superfamily such as ICAM-1 expressed on the microvascular endothelium as the second step (6). Our findings suggest that the second step of firm adhesion of PMN to endothelial cells is lacking or very weak in pulmonary venules, and only the rolling step of PMN plays a role in promoting the venular sequestration of these cells in diseased lungs. A further interesting point is that, besides P-selectin, both ICAM-1 and VCAM-1, which are adhesion molecules inducing firm adhesion between PMN and endothelial cells in systemic venules, act to cause PMN rolling in pulmonary venules.

In arterioles, augmented PMN rolling in high tidal volume-ventilated lungs was appreciably improved by the inhibition of endothelial ICAM-1 or VCAM-1 but not by the inhibition of P-selectin (Fig. 3). Although these findings are somewhat different from those observed for venules, in which all three adhesion molecules contribute equally to PMN rolling, they suggest that rolling in arterioles is as important as that in venules for PMN sequestration into the pulmonary microcirculation in high tidal volume-ventilated lungs. Again, we would like to emphasize the fact that ICAM-1- and VCAM-1-dependent, P-selectin-independent, arteriolar PMN rolling, which is expected to reflect the initial stage of tissue injury, should be taken as a phenomenon unique to ventilator-associated lung injury.

The importance of various adhesion molecules expressed along capillary segments seems to differ qualitatively from that along venular or arteriolar walls, because we found that only ICAM-1 contributed to the enhanced capillary entrapment of PMN in high tidal volume-ventilated lungs (Fig. 4). The pathological significance of either VCAM-1 or P-selectin expression in abnormal PMN kinetics in capillaries with high tidal volume ventilation is not clear at present. Further study is necessary to demonstrate their exact role. Combining the findings on PMN kinetics observed for arterioles, venules, and capillaries, we can conclude that the contribution of different classes of adhesion molecules to PMN kinetics in the pulmonary microcirculation is vessel type specific (Table 2). Furthermore, besides the cell entrapment in capillary segments, PMN accumulation in the pulmonary microcirculation in ventilator-associated lung injury appears to be preferentially mediated via rolling, but not via firm adhesion, in both arterioles and venules.

**Importance of adhesion molecules modulating MN kinetics in high tidal volume-ventilated lungs.** MN, especially lymphocytes, are also expected to be of importance in the pathogenesis of VILI, though there has been no detailed analysis concerning the contribution of MN to VILI. Indeed, we found that the number of MN in BALF harvested from lungs injured by sustained artificial ventilation was greatly increased compared with that in BALF both from control and low tidal volume-ventilated lungs (Fig. 1). Furthermore, the accumulation of MN within the alveolar septa was significantly enhanced in high tidal volume-ventilated lungs (Fig. 1), suggesting that analysis of the microcirculatory kinetics of MN is as pivotal as that of PMN for understanding the entire pathological aspect of VILI. On the basis of this background, we investigated the dynamic behavior of fluorescein-labeled MN in pulmonary microvessels of isolated lungs with high tidal volume ventilation by adding a solution mainly consisting of lymphocytes to the perfusion circuit. Contrary to the findings obtained for PMN, P-selectin had no effect on the behavior of MN in any microvessels in high tidal volume-ventilated lungs (Figs. 3 and 4). Similar to PMN, however, anti-ICAM-1 or anti-VCAM-1 MAb improved the abnormal MN behavior in arterioles and venules (Fig. 3). Differing from PMN, VCAM-1 appeared to significantly enhance MN entrapment in capillaries (Fig. 4). These findings indicate that the relative contribution of endothelial adhesion molecules to the initiation of abnormal leukocyte kinetics in the pulmonary microcirculation differs significantly between PMN and MN, although ICAM-1 is universally important for the kinetics of both cells in any microvessels of lungs with high tidal volume ventilation.

To summarize the findings obtained from lungs with VILI (Tables 1 and 2): the expression of endothelial adhesion molecules differs quantitatively between different microvessels; i.e., ICAM-1 expression is strongly enhanced along all microvascular walls, including arterioles, venules, and capillaries, whereas VCAM-1 is strongly upregulated in venules and capillaries but weakly upregulated in arterioles. P-selectin expression is significantly enhanced along venular walls but weakly enhanced along arteriolar and capillary walls. Consistent with the findings of adhesion molecule expression, the abnormal cell kinetics of both PMN and MN is importantly mediated via an ICAM-1-dependent pathway in all microvessels, suggesting that ICAM-1 inhibition could play a pivotal role as an effective therapeutic strategy in preventing the development of VILI. Furthermore, we would like to stress the fact that ICAM-1- and VCAM-1-dependent arteriolar rolling of PMN and MN appears to function as a specific mechanism for the accumulation of these inflammatory cells in the pulmonary microcirculation in the early stage of VILI.

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**Table 2. Adhesion molecules influencing abnormal leukocyte (PMN or MN) behavior in microvessels in high tidal-ventilated lung**

<table>
<thead>
<tr>
<th>Rolling or Capillary Entrapment</th>
<th>PMN</th>
<th>MN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arteriole</td>
<td>ICAM-1</td>
<td>ICAM-1</td>
</tr>
<tr>
<td>Venule</td>
<td>ICAM-1</td>
<td>ICAM-1</td>
</tr>
<tr>
<td>Capillary</td>
<td>ICAM-1</td>
<td>ICAM-1</td>
</tr>
</tbody>
</table>

PMN, polymorphonuclear cell; MN, mononuclear cell.
REFERENCES


