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Invited Review

Do adhesion molecules importantly regulate leukocyte kinetics within intraacinar microvessels of the lung?

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Summary. Precise assessment of blood cell kinetics in the pulmonary microcirculation is extremely difficult because pulmonary microvascular architecture contains arterioles, venules and capillaries in an exceedingly intricate and densely convoluted fashion. Conventional epiluminescence microscopy may not be suitable for investigation of blood cell kinetics in the pulmonary microcirculation, in which arterioles, venules and capillary networks are not located in the same plane. To overcome these impediments, we recently developed a real-time confocal laser luminescence microscope with a high-speed analysis component having the capacity to yield confocal-images of rapidly moving cells at a rate of 1,000 frames/sec and at sufficiently high magnification. In the current review, we will first introduce the details of our newly developed observation system constructed with a view to estimation of blood cell dynamics in the intraacinar microcirculation of the lung. Applying this novel method to isolated perfused rat lungs, we will secondly address the issue of whether or not leukocyte-endothelium interactions in the pulmonary microcirculation qualitatively differ from those serving in the systemic microcirculation. We will particularly shed light on possible roles of endothelial ICAM-1, endothelial P-selectin and leukocyte L-selectin in distorting leukocyte kinetics in the intraacinar microvessels under a variety of diseased conditions, including prolonged exposure to a hyperoxic environment inducing a significant upregulation of ICAM-1 as well as P-selectin on the pulmonary microvascular endothelium, and stimulation of leukocytes by an IL-8 analog causing downregulation of leukocyte L-selectin but inverse upregulation of CD18-related integrins.

Key words: Confocal microscope, Pulmonary microcirculation, Leukocyte, Adhesion molecules, ICAM-1, P-selectin, L-selectin

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Introduction

The microscopic data obtained from the systemic microcirculation, especially from mesenteric microvessels located in a plane, suggested that leukocytes, particularly polymorphonuclear cells roll slowly and subsequently adhere along the margins of postcapillary venules, thus forming a physiological marginated pool of leukocytes in systemic venules (Artherton and Born, 1973; Firrell and Lipowsky, 1989; Butcher, 1991). These vascular endothelium-leukocyte interactions have been considered to be elicited in connection with different classes of adhesion molecules in which selectin family induces the initial rolling and endothelial intercellular adhesion molecule-1 (ICAM-1) together with leukocyte CD18-related glycoproteins subsequently mediates the tight adherence of leukocytes to the venular endothelium (sequential multistep theory) (Butcher, 1991; von Andrian et al., 1991; Springer, 1994). Employing non-confocal luminescence microscopes which had been utilized for assessing leukocyte kinetics in the systemic microcirculation, several groups of investigators (Lien et al., 1987, 1990; Kuebler et al., 1994; Kuhnle et al., 1995) observed leukocyte kinetics in the pulmonary microvessels in which microvascular networks surround and conform to the shapes of alveoli. Quite unexpectedly, the leukocyte kinetics observed in pulmonary microvessels differed essentially among the studies done previously. The group in Munich (Kuebler et al., 1994; Kuhnle et al., 1995) reported that leukocytes labeled with rhodamin rolled not only on venular but also arteriolar endothelium in normal rabbit lungs. In addition, they showed that a large quantity of leukocytes adhered firmly to arterioles as well as venules, though their investigation involved only unstimulated leukocytes in intact lungs. On the other hand, Lien et al. (1987, 1990) demonstrated that leukocytes stained with fluorescein isothiocyanate (FITC) were delayed in neither arterioles nor venules and that site of cell sequestration was exclusively pulmonary capillaries. Applying monochromatic illumination videomicroscopy, Gebb et al. (1995) studied the subpleural microcirculation and demonstrated that many leukocytes

spontaneously rolled along, but did not adhere firmly to, the margins of both arterioles and venules, and that the majority of leukocytes stopped temporarily in capillary segments. We suspected that the discrepancy between the data obtained by these groups of investigators might be attributable to technical difficulties in assessing cell kinetics in the densely intertwined pulmonary microcirculation by means of classical non-confocal microscopy. With a conventional epi-fluorescence system, illumination of the entire field of view excites fluorescent emissions through the entire depth of the specimen, rather than just in the focal plane. Much of the emitted light coming from regions above and below the focal plane is collected by the objective lens thereby producing an out-of-focus blur in the final image of the specimen, markedly diminishing the contrast and sharpness of the image (Shotton, 1989). When the specimen is sufficiently thin and flat, as is the case with mesenteries, this drawback of non-confocal luminescence microscopy may have little impact on the measurement of blood cell kinetics in small vessels. However, the situation is distinctly different in pulmonary microvascular networks, in which numerous capillaries surrounding alveoli are densely intertwined among arterioles and/or venules within a depth which can be penetrated by an illuminating beam. Another issue warranting consideration is that previous studies focusing on leukocyte kinetics in the pulmonary microcirculation were conducted by recording image data at a routine video rate of 30 frames/sec. This recording speed may be unsuitable for a cell moving at high velocity in portions near the centerlines of microvessels (Ley and Gaehtgens, 1991). In order to overcome these obstacles, we have developed a real-time confocal laser scanning luminescence optical microscope incorporating a high-speed video analysis system, allowing precise discrimination of individual microvessels from neighboring vessels and measurement of axial velocities of various blood cells including leukocytes and erythrocytes in the highly complex pulmonary microcirculation. Utilizing this novel method, we have attempted to clarify the importance of various adhesion molecules expressed on the pulmonary microvascular endothelium and those on the leukocyte surface in regulating endothelium-leukocyte interactions in the intraacinar microvessels of the lung, including precapillary arterioles, postcapillary venules and capillaries. We have examined these interactions under a couple of experimental conditions, i.e., between intact endothelial cells and inactivated leukocytes (normal condition), between hyperoxia-activated endothelium and inactivated leukocytes (injury is located primarily in the lung and adhesion molecules are upregulated along vascular endothelial cells), as well as between intact endothelial cells and activated leukocytes by an interleukin-8 (IL-8) analog (primary injury exists somewhere in extrapulmonary sites and adhesion molecule expression is enhanced on the leukocyte surface). In addition, endothelium-leukocyte interactions have been investigated in mesenteric venules to answer the question of whether adhesion molecules would function in a qualitatively different manner between the pulmonary microcirculation and the systemic microcirculation. In the current review, we will extensively address the following issues on the basis of the experimental findings obtained from confocal observations: 1) whether adhesion molecules play a significant role in regulating pulmonary microvascular leukocyte kinetics under normal conditions in which only the constitutive adhesion molecules are expressed on both the endothelium and the leukocyte; 2) if so, what kinds of adhesion molecules importantly cause intervention in leukocyte kinetics in intact pulmonary microcirculation?; 3) whether participation of adhesion molecules in leukocyte movement in pulmonary microcirculation under conditions in which various adhesion molecules are upregulated either on the microvascular endothelium or on the leukocyte surface qualitatively differs from that under normal conditions; 4) if so, what kinds of adhesion molecules are of much importance in interference with microvascular leukocyte kinetics under diseased conditions?; and 5) is there any peculiar difference in adhesion molecule contribution to leukocyte kinetics between pulmonary and systemic microvessels?

Investigations of leukocyte kinetics by real-time confocal laser microscopy

In order to precisely estimate events in intraacinar microcirculation of the lung, we applied a real-time confocal luminescence microscope constructed by modifying a tandem scanning reflected light microscope. A detailed description of our system was given elsewhere (Yamaguchi et al., 1996a,b, 1997). Briefly, with this apparatus, scanning is achieved by high-speed rotation, in the primary image plane of the objective, of two Nipkow discs containing numerous pairs of diametrically opposed apertures arranged in a constant-pitch helical pattern. Each Nipkow disc has 20,000 pinhole apertures. In each of the apertures located on the upper disc, microlenses are installed, thus allowing 1,000 beams of light to be transmitted to an 8x8 mm visual field at any one time. Our confocal unit yields an approximately 1,000-fold greater resolution velocity than does a conventional confocal scanning optical microscope with a unitary beam such as the Galvanometer type. Furthermore, our system actually generates a continuous image at a rate of 1 msec/frame. A collimated beam from a light source impinging on the upper disc is focused, by microlenses, on the lower disc which has an array of apertures corresponding exactly to that of the upper disc. Subsequently, a light beam passing through the disc apertures is focused, by an objective lens, onto a single diffraction-limited point or in-focus volume element within a three-dimensional specimen. The same objective is then used to image the reflected light onto a high-sensitivity charge-coupled-

device (CCD) camera with an image intensifier. By incorporating an excitation wavelength of 488 nm emitted from a low-power air-cooled argon (Ar) ion laser with appropriate fluoresceins, the present confocal system allows us to obtain apparently instantaneous images at 1,000 frames/sec, with an optical sectioning depth of 0.75 μm, and to achieve a two-point spatial resolution of 0.2 μm. By combining the magnification achieved with the objective lens, the final magnifying power of our system attained about x1,000 on the video screen. Although our real-time confocal microscope has been found to be useful for studying the dynamic processe in living cells and analyzing the rapid movement of cytological or hematological specimens in a given organ, the issue of how to record real-time images obtained from the confocal system has required resolution. To overcome this difficulty, we have attempted to record all confocal information by means of a high-speed video analysis system connected to the image-intensified CCD camera. We have registered confocal images at a rate of 500 or 250 frames/sec.

Applying this sagacious method to isolated perfused rat lungs, we investigated microvascular kinetics of leukocytes stained with carboxyfluorescein diacetate succinimidyl ester (CFDASE) which diffuses into cells and forms a stable fluorochrome termed carboxyfluorescein succinimidyl ester, CFSE (Bronner-Fraser, 1985). Furthermore, we examined erythrocyte behaviors (stained with FITC) in exactly the same microvessels as used for assessing leukocyte kinetics. Diameters and architecture of microvessels (stained with FITC-dextran) were measured by processing a confocal video image with the computer-assisted digital image analyzing system (Fig. 1). The isolated lung was fixed on a microscopic stage in the supine position and perfused at a constant flow rate. Krebs-Henseleit solution with 3% bovine serum albumin and a small quantity of whole blood was used as the perfusate. To avoid the movement caused by artificial ventilation, the trachea was ligated at the end-inspiratory position and gas exchange was adequately maintained with an extracorporeal membrane oxygenator (ECMO). A gas mixture containing 21% O_2 and 5% CO_2 in N_2, was used as the gas flowing into the ECMO. A warmed and humidified gas mixture containing the same composition of gases as those used for the ECMO was supplied continuously to the lung surface.

Inactivated leukocyte kinetics in intact intraacinar microvessels

Although Kuebler et al. (1994) and Kuhnle et al. (1995) reported that a large portion of unstimulated leukocytes spontaneously adhered to intact arterioles as well as venules of the rabbit lung, other groups of investigators, including ourselves (Lien et al., 1990; Gebb et al., 1995; Yamaguchi et al., 1997; Aoki et al., 1997; Nishio et al., 1998), did not find the firm adhesion

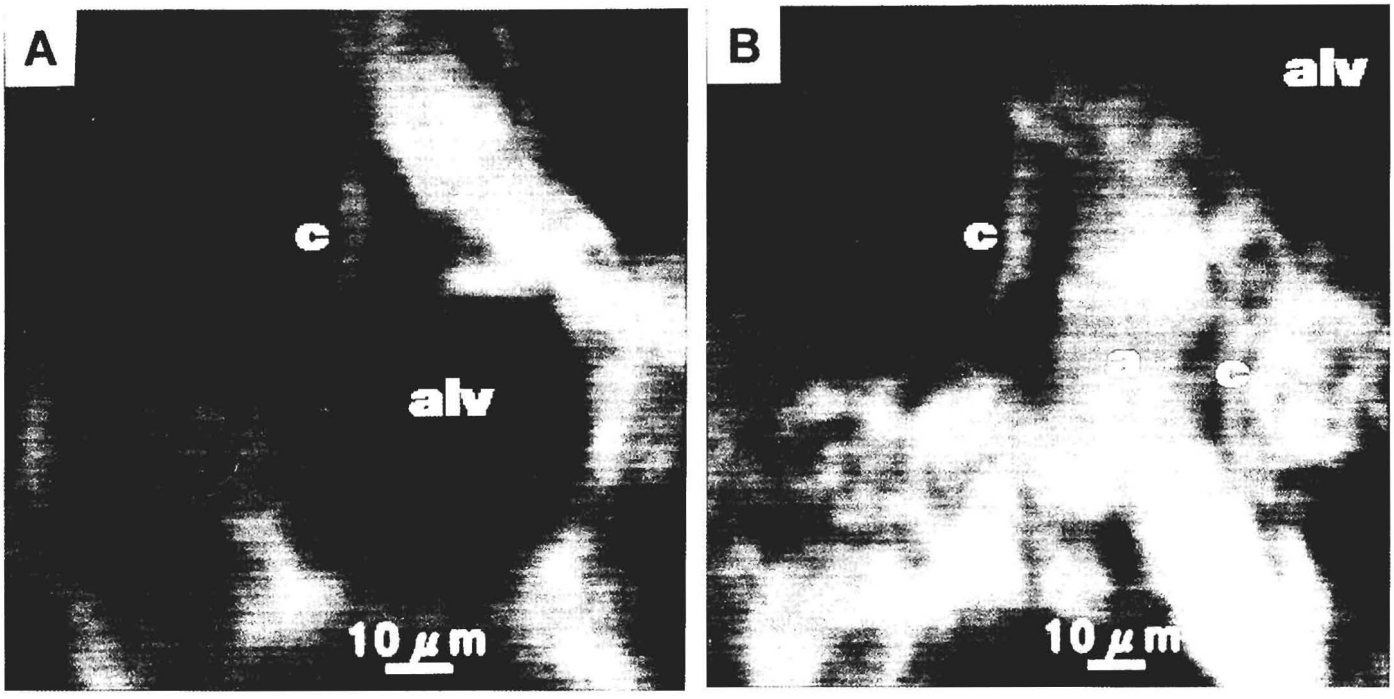

Fig. 1. Photographs of confocal images of intraacinar microvessels in the lung. **A.** Image of in-focus plane at a 5 μm depth from the surface (alveoli, a postcapillary venule running diagonally and capillaries meeting the venule are seen). **B.** Image of in-focus plane at a 25 μm from the surface (an arteriole running parallel to the capillaries is detected). alv: alveolus; a: arteriole; v: venule; c: capillary. Although photographs are taken at the same portion in the X-Y plane, vascular images are largely different when the depth changes. (Yamaguchi et al., 1997).

of leukocytes to either arteriolar or venular walls in normal lungs harvested from various animals. We consider that the inconsistent findings of Kuebler et al. (1994) and Kuhnle et al. (1995) with ours may be created from the difference in the observation system, i.e., the former groups used a conventional nonconfocal microscope, while we applied a confocal microscope with a high quality. In images obtained through non-confocal microscopic observations, the movement of blood cells in capillaries may be mistakenly interpreted as taking place in arterioles or venules because of the difficulty in exact discrimination of arterioles and venules from capillary networks. In fact, several groups of investigators demonstrated that unstimulated leukocytes were significantly entrapped within capillary segments having no injury (Lien et al., 1987, 1990; Downey et al., 1990, 1993; Aoki et al., 1997; Yamaguchi et al., 1997; Nishio et al., 1998). Considering the distinction in leukocyte adherence between pulmonary and systemic microvessels, we should pay attention to the fact that leukocytes do not adhere firmly to the vascular endothelium in pulmonary venules, though ICAM-1, which is the key integrin inducing firm leukocyte tethering in systemic venules (Butcher, 1991; von Andrian et al., 1991, 1992; Ley et al., 1993, Springer, 1994), is also constitutively expressed on venular endothelia of the lung (Fig. 2).

Spontaneous leukocyte rolling was identified by Gebb et al. (1995) along the margins of both arterioles and venules in the intact canine lung, whereas Lien et al. (1987, 1990) reported that FITC-stained leukocytes were not delayed in either arterioles or venules of the canine lung. The discrepancy between them may again be attributed to the fact that these investigators used nonconfocal microscopy to observe blood cell behaviors. Rolling leukocytes in arterioles and venules may be defined as a population of cells temporarily interacting with the vascular endothelium and thus travelling much more slowly than centerline erythrocytes (Schmid-Schoenbein et al., 1975, 1980, 1987). These cells can be identified by applying the velocity criterion proposed by Gaehtgens and colleagues (Gaehtgens et al., 1985, 1987; Ley and Gaehtgens, 1991), who calculated the critical velocity (Vcrit) of a freely flowing cell traveling close (i.e., hydrodynamic margination), but not adhering, to the vascular surface under a parabolic velocity profile in microvessels. Vcrit is expressed as $(Vmean)(2-\varepsilon)\varepsilon$, where Vmean and ε are the mean fluid velocity and the ratio of the leukocyte diameter to the microvessel diameter, respectively. It is likely that any cell flowing at a velocity below Vcrit is impeded by adhesive interaction with the vascular endothelium. Based on these criteria, our confocal measurements decisively exhibited that unstimulated leukocytes could tumble along both arteriolar and venular walls, though rolling phenomenon was much more frequently observed in arterioles (Fig. 3). The higher frequency in rolling leukocyte population in pulmonary arterioles, unique to

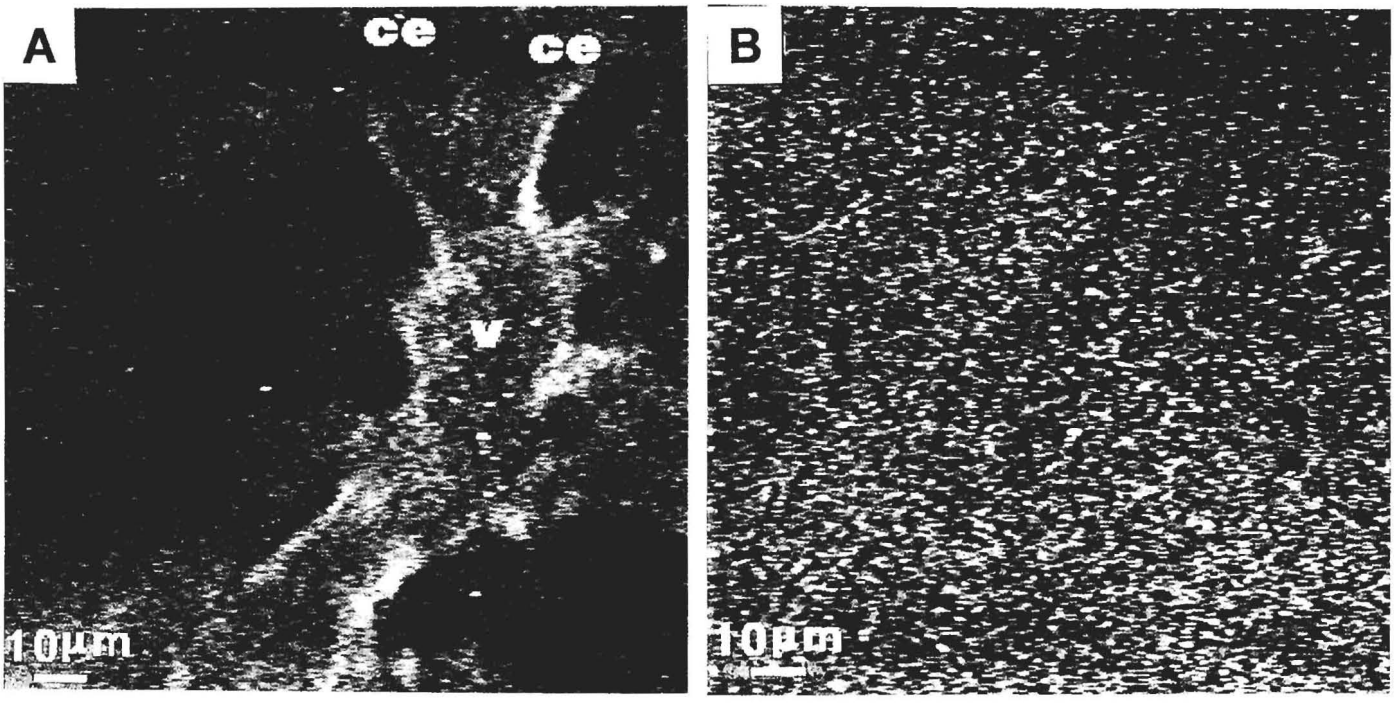

Fig. 2. Confocal views of ICAM-1 distribution along intact pulmonary microvessel walls. **A.** ICAM-1 expression along venular walls and entrances of capillaries connected to the venule. v: venule; ce: capillary entrance connected to venule. **B.** No ICAM-1 expression along arteriolar walls. An arteriole is running at the center of the image, which is not depicted in this figure but is confirmed by administering FITC-dextran. (Yamaguchi et al., 1997).

the pulmonary microcirculation, may be caused, in part, by a two-fold lower shear rate in arterioles than that in venules (Yamaguchi et al., 1997; Nishio et al., 1998). Supporting our findings, Staub et al. (1982) demonstrated, in a morphological study examining normal sheep lungs, that considerable numbers of leukocytes were sequestered in pulmonary arterioles. Interestingly, no rolling leukocytes qualitatively similar to those seen in systemic microvessels appear, i.e., in mesenteric venules, for instance, rolling cells show movements that may be described as "clumsy". However, in pulmonary microvessels, such a caterpillar-like movement could not be observed (Aoki et al., 1997). The experimental results obtained for the pulmonary microcirculation are at variance with those for the systemic microcirculation, in which leukocyte-endothelium interactions leading to rolling are largely confined to the venular segment (von Andrian et al., 1991, 1992; Ley et al., 1993; Suematsu et al., 1994, 1995; Aoki et al., 1997). There are several lines of evidence suggesting that interactions depending upon adhesion molecules including ICAM-1 and P-selectin may not account for spontaneous leukocyte rolling in pulmonary arterioles, because monoclonal antibodies against either ICAM-1 (1A29: Tamatani and Miyasaka, 1990) or P-selectin (ARP2-4: Tojo et al., 1996) did not reduce the relative population of spontaneously tumbling leukocytes along pulmonary arteriolar walls (Fig. 3). The most persuasive evidence for independence from ICAM-1 and P-selectin is that neither ICAM-1 nor P-selectin is detectable in normal arterioles, especially in those of the rat lung (Yamaguchi et al., 1997; Nishio et al., 1998). On the other hand, inhibition of L-selectin with fucoidin significantly, but not fully, reduced the rolling leukocyte frequency (Ley et al., 1993; Granert et al., 1994; Tuomanen, 1994; Yamaguchi et al., 1997), resulting in that adhesive pathway associated with L-selectin constitutively expressed on the unstimulated leukocyte plays an important role but does not explain the whole body of spontaneous rolling in pulmonary arterioles (Fig. 3). Relatively low shear force applied to pulmonary arterioles may fill up the gap. In systemic arterioles, mean blood flow is approximately three times that in venules with a comparable size, such that the arteriolar shear rate is 1,000 sec^{-1} or more (Ley and Gaehtgens, 1991; Perry and Granger, 1991). In contrast, pulmonary arterioles are exposed to half the shear rate of venules with nearly the same size (Yamaguchi et al., 1997; Nishio et al., 1998), indicating that low shear forces in pulmonary arterioles also contribute to the augmented leukocyte rolling in these arterioles. In pulmonary venules, inhibition of ICAM-1, P-selectin or L-selectin did not diminish the frequency of rolling leukocytes (Fig. 3), being sharply inconsistent with observations in the systemic venules, in which L-selectin was convincingly demonstrated to play a primary role in spontaneous leukocyte rolling (Picker et al., 1991; von Andrian et al., 1992).

In capillaries of systemic organs, leukocyte entrapment occurs only when the microcirculation is exposed to morbidly low shear conditions (Barroso-Aranda et al., 1988; Mori et al., 1992). Conversely, pulmonary capillaries can entrap leukocytes at physiological shear rates (Lien et al., 1987, 1990; Downey et al., 1990, 1993; Doerschuk et al., 1996; Aoki et al., 1997; Yamaguchi et al., 1997; Nishio et al., 1998).

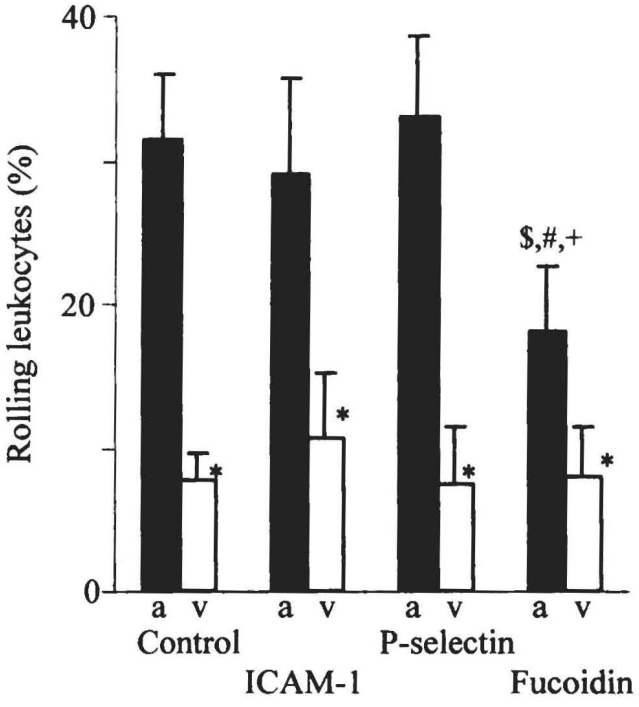

Fig. 3. Leukocyte rolling in intact pulmonary arterioles (a) and venules (v). Control: group without any medication; ICAM-1: group in which ICAM-1 was inhibited; P-selectin: group in which P-selectin was inhibited; Fucoidin: group in which P- and L-selectins were inhibited by fucoidin; *: smaller than the values obtained for arterioles; $: smaller than the values obtained for Control group; #: small as compared to the values observed for ICAM-1 group; +: small as compared to the values obtained for P-selectin group. (Yamaguchi et al., 1997).

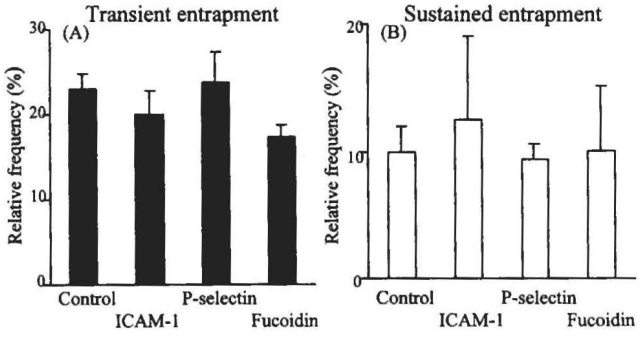

Fig. 4. Leukocyte behavior in intact pulmonary capillaries. **A.** Relative frequency of leukocytes transiently stopped. **B.** Relative frequency of leukocytes persistently stopped. No differences are observed among the groups. (Yamaguchi et al., 1997).

Although ICAM-1 is constitutively expressed in pulmonary capillaries (Fig. 2), its inhibition has little impact on temporary lodging and sustained arrest of unstimulated leukocytes (Fig. 4). Similarly, neither P-selectin nor L-selectin inhibition exerts any influence upon leukocyte sequestration in capillaries. Supporting this view, P-selectin was not detectable along capillary walls of the intact rat lung (Yamaguchi et al, 1997; Nishio et al., 1998). These findings may indicate that adhesion molecules are not involved in entrapment of inactivated leukocytes in normal pulmonary capillaries. Instead, it may be attributable to less deformability in leukocytes (Worthen et al., 1989; Downey et al., 1990, 1993; Wiggs et al., 1994) and to the geometry unique to the pulmonary capillary networks where a great fraction of capillary bed contains narrow segments for leukocyte transit (Wiggs et al., 1994; Doerschuk et al., 1996).

Summarizing endothelium-leukocyte interactions in normal pulmonary microvessels, they seem to qualitatively differ from those of the systemic microcirculation for several reasons: (1) leukocytes do not adhere firmly to venular endothelium; (2) leukocyte rolling occurs preferentially in arterioles and may be mediated, in part, by an ICAM-1- and P-selectin-independent but L-selectin-dependent mechanism; (3) low shear forces may be one of the important factors

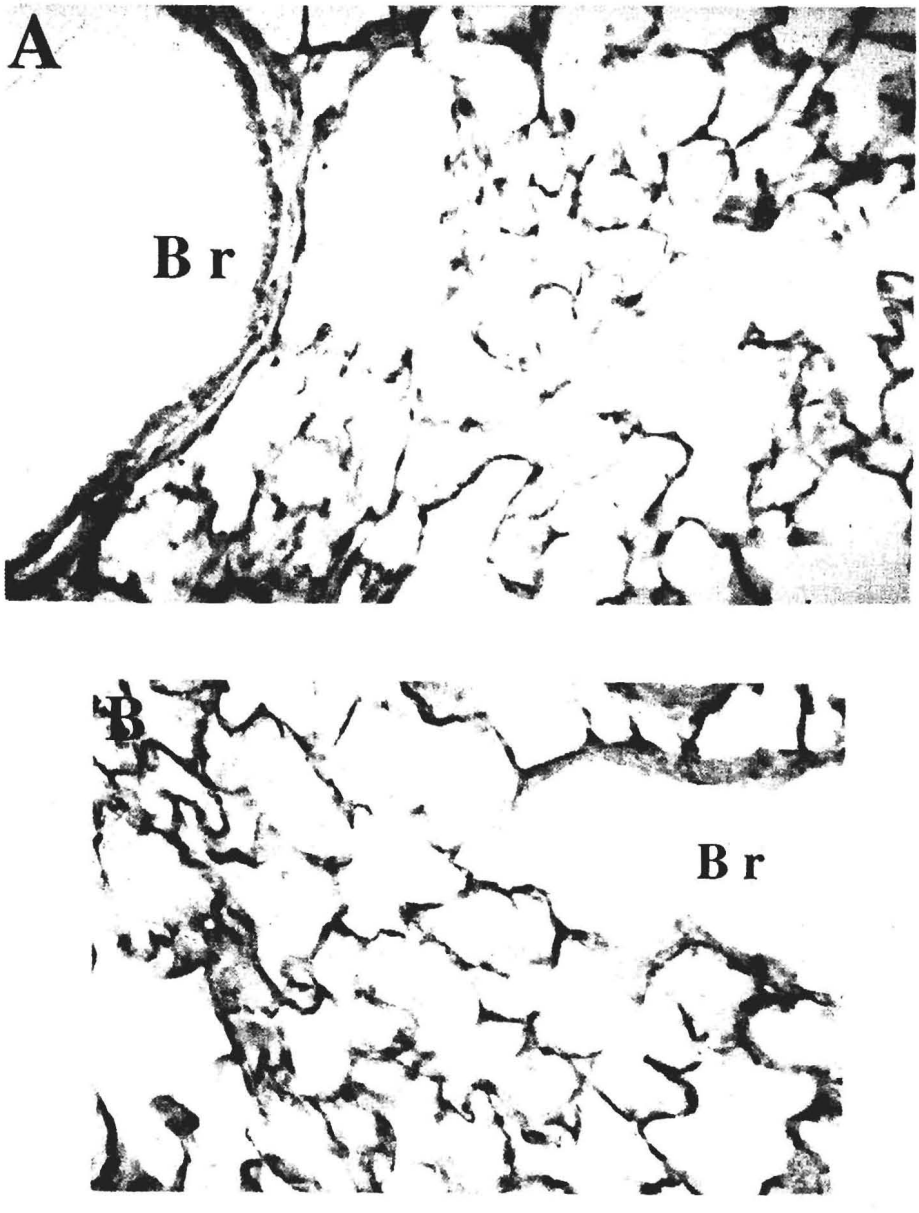

Fig. 5. Histochemical examination of ICAM-1 expression. **A.** ICAM-1 expression in the normoxia-exposed lung. **B.** ICAM-1 expression in the hyperoxia-exposed lung. Brown color: positive immunoreactivity for ICAM-1, which is much stronger in the hyperoxia-exposed lung than that in the normoxia-exposed lung. Br: bronchiole (no ICAM-1 expression). (Nishio et al., 1998). x 200

inducing leukocyte rolling in pulmonary arterioles; (4) spontaneous rolling in pulmonary venules may not be associated with adhesion molecules; (5) pulmonary leukocyte sequestration occurs mainly in alveolar capillaries rather than in postcapillary venules, and (6) adhesion molecules may not be involved in the tethering of leukocytes to intact capillary endothelium.

Inactivated leukocyte kinetics in activated intraacinar microvessels

ICAM-1 expression is significantly upregulated along pulmonary venular and capillary endothelia (Fig. 5), while P-selectin expression is sparsely enhanced only in arterioles (Fig. 6), by an exposure to a high oxygen environment for a couple of days, indicating that hyperoxia-exposed lungs are exceedingly pertinent to know fundamental roles of various endothelial adhesion molecules for interference with leukocyte kinetics in the pulmonary microvasculature. These experimental conditions mimic the primary lung injury caused by a certain insult and are thought to be devoted to estimating possible effects of upregulated endothelial ICAM-1 and P-selectin upon cell kinetics of unstimulated leukocytes with constitutive L-selectin but no upregulation of $ß_2$ integrins (CD11/CD18 components).

Upregulation of ICAM-1 and P-selectin induced no resolute attachment of unstimulated leukocytes to any microvessels, including precapillary arterioles and postcapillary venules, again indicating that neither ICAM-1 nor P-selectin is a decisive factor for firm leukocyte tethering in the pulmonary microcirculation (Nishio et al., 1998). Meanwhile, relative frequency of rolling leukocytes is significantly increased, especially in venules, and rolling leukocyte population in venules exceeds that in arterioles (Fig. 7). Augmented number of venular leukocytes with rolling was certainly reduced by administrating either anti-ICAM-1 monoclonal antibody or fucoidin, but not monoclonal antibody to P-selectin, strongly suggesting that endothelial ICAM-1, if upregulated, tends to generate a significant adhesive force mediating venular rolling of leukocytes (Nishio et al., 1998). We suppose that leukocyte rolling related to the ICAM-1 pathway is unique to the pulmonary venule, despite the same phenomenon having been qualitatively observed in systemic venules under the condition with extremely low shear force (Gaboury and Kubes, 1993). In contrast to normal lungs, constitutive leukocyte L-selectin appears to be of equal importance for inducing venular rolling in hyperoxia-injured lungs. This may be explained if hyperoxia exposure significantly upregulates sialyl Lewis X carbohydrates (SLeX), a counterpart ligand for L-selectin on the vascular endothelium. However, this should require clarification because no studies have convincingly demonstrated that expression of SLeX is reliably augmented during hyperoxia exposure. In arterioles, situations qualitatively differ from those in venules, i.e., ICAM-1 was not upregulated and ICAM-1 inhibition exerted no influence upon arteriolar leukocyte rolling (Fig. 7). Furthermore, monoclonal antibody against P-selectin did not improve the arteriolar rolling, despite P-selectin being somewhat upregulated along arteriolar walls in hyperoxia-injured lungs (Fig. 6). On the other hand, L-selectin inhibition remarkably restored rolling phenomenon in arterioles. Taken together, these observations may indicate that, similar to normal lungs, arteriolar leukocyte rolling in hyperoxia-activated lungs is preferentially mediated via an L-selectin-dependent but ICAM-1- and P-selectin-independent mechanism (Nishio et al., 1998).

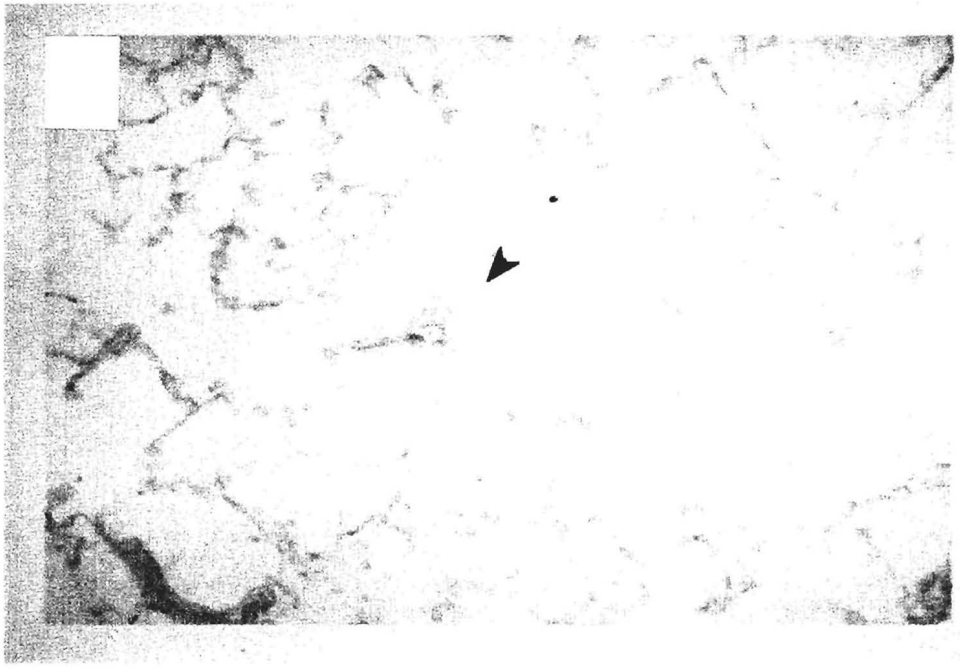

Fig. 6. Histochemical examination of P-selectin expression. Scattering induction of P-selectin along the arteriolar wall is seen (brown color indicated by the arrow). (Nishio et al., 1998). x 200

Capillary entrapment of leukocytes was considerably enhanced in lungs activated by hyperoxia exposure (Fig. 8). In opposition to the findings observed for intact lungs, however, this enhancement was almost fully diminished by suppressing ICAM-1, but not by inhibiting P- and L-selectins (Fig. 8). These findings may be supported by the fact that ICAM-1, but not P-selectin, was evidently upregulated along capillary walls in lungs treated with hyperoxia (Figs. 5, 6). In intact lungs, leukocyte sequestration in capillary segments may be caused solely by mechanical obstacles (see above), while that in hyperoxia-activated lungs may be additionally induced by an ICAM-1/CD18-associated adhesive pathway. Keeney et al. (1994) reported that leukocyte infiltration into the airspace was not ameliorated by the treatment of monoclonal antibody to leukocyte CD18, the counterpart ligand for endothelial ICAM-1, in injured lungs prepared from guinea pigs exposed to a hyperoxic environment. Joining the results obtained by Nishio et al. (1998) with those by Keeney et al. (1994), we may infer that ICAM-1/CD18-related adhesive pathway is of primary importance for eliciting an early-stage leukocyte accumulation within pulmonary capillaries, but not for leukocyte transmigration into the airspace in a relatively late stage after exposure to hyperoxia. ICAM-1/CD18-independent mechanisms, including a variety of chemoattractants and other adhesion molecules, appear to be responsible for leukocyte transmigration from capillaries into the airspace (Fox et al., 1981; Doerschuk et al., 1990; Bullard et al., 1995). The importance of ICAM-1/CD18-independent mechanisms has recently been confirmed in leukocyte invasion upon the airspace in the case of severe pneumonia caused by gram-positive organisms (Doerschuk et al., 1990; Bullard et al., 1995).

Unstimulated leukocyte kinetics in lungs with endothelial injury by hyperoxia exposure can be summarized as follows: (1) prolonged exposure to hyperoxia causes pulmonary endothelial injury in association with upregulated ICAM-1 in both venules and capillaries but with scattering augmentation of P-selectin expression in arterioles; (2) nevertheless, firm adherence of leukocytes is not seen in any venules or arterioles in hyperoxia-injured lungs; (3) hyperoxic injury significantly enhances leukocyte rolling in venules; (4) similar to unstimulated leukocyte kinetics in intact lungs, arteriolar rolling is regulated via L-selectin-dependent but P-selectin- and ICAM-1-independent mechanisms; (5) dissimilar from unstimulated leukocyte behavior in intact lungs, venular rolling is predominantly mediated via ICAM-1- and L-selectin-dependent but P-selectin-independent pathways, indicating that leukocyte kinetics in pre- and post-capillary microvessels are governed by different classes of adhesion molecules; and (6) in contrast to the accumulation of unstimulated leukocytes in intact capillaries, that in lungs with endothelial injury is importantly mediated via upregulated ICAM-1. These findings again suggest that leukocyte

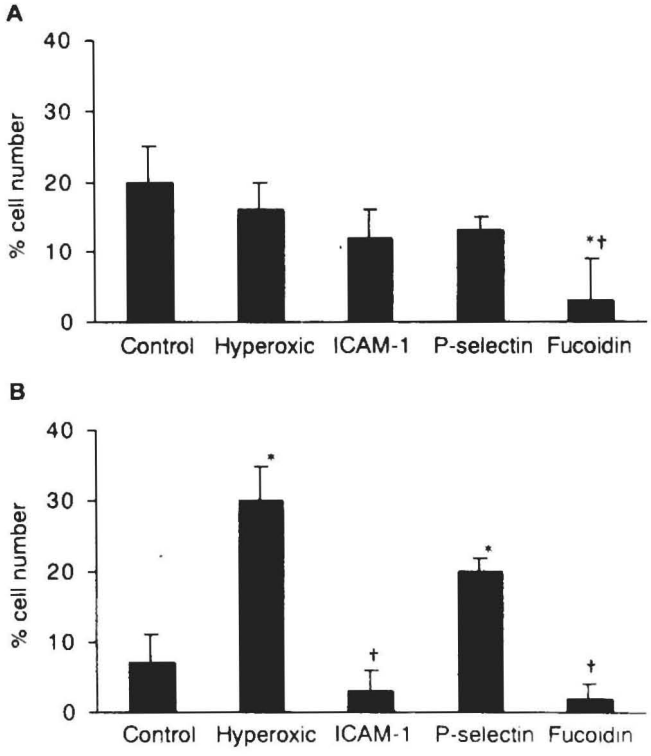

Fig. 7. Relative frequency of leukocytes rolling along arteriolar **(A)** or venular **(B)** walls in hyperoxia-exposed lungs. Control: normoxia-exposed lungs. Hyperoxic: hyperoxia-exposed lungs with no medication. ICAM-1: hyperoxia-exposed lungs treated with anti-ICAM-1 antibody. P-selectin: hyperoxia-exposed lungs treated with anti-P-selectin antibody. Fucoidin: administration of fucoidin-treated leukocytes into hyperoxia-exposed lungs. *: differing from the Control group. +: smaller than the value for the Hyperoxic group. (Nishio et al., 1998).

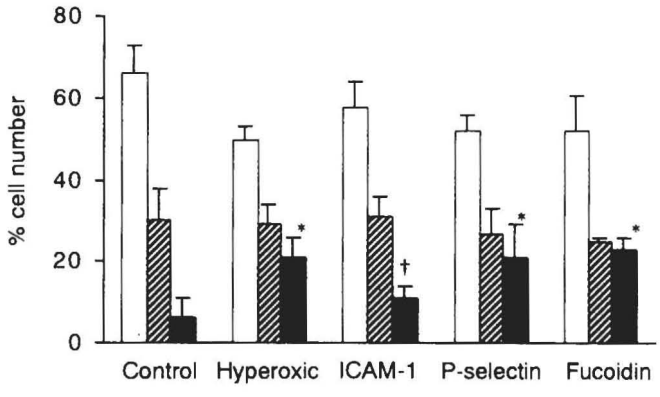

Fig. 8. Leukocyte entrapment in pulmonary capillaries exposed to hyperoxic environment. Groups studied are the same as those defined in Fig. 7. From the left, relative frequency of leukocytes moving smoothly without any interruption, that stopped transiently, and that stopped persistently. *: larger than the value obtained for the Control; +: smaller than the value observed for the Hyperoxic group. (Nishio et al., 1998).

kinetics are quite different between the pulmonary and systemic microcirculation.

Leukocyte kinetics activated with IL-8 in intact intraacinar microvessels

To simulate a variety of diseased conditions in which inflammatory leukocytes are largely activated, leukocytes were stimulated with rat cytokine-induced neutrophil chemoattractant (CINC/gro), a peptide possessing biological activities analogous to those of the human IL-8 (Watanabe et al., 1992; Aoki et al., 1997). FACScan flow cytometric examinations revealed that, in response to CINC/gro, L-selectin and SLeX were markedly downregulated, whereas CD18 was inversely upregulated on the surface of neutrophils (Fig. 9). These changes elicited by CINC/gro occur specifically on neutrophils but not on lymphocytes (Watanabe et al., 1992). Thus, the experimental conditions defined here mimic systemic inflammatory response syndrome (SIRS), represented by sepsis in which infectious foci are located in extrapulmonary sites. These experimental conditions allow a precise estimation of interactions between microvessels with inactivated endothelium and activated leukocytes with shedding L-selectin and SLeX but upregulated CD18 integrins.

Firm cell adhesion was not visible in pulmonary arterioles and venules (Aoki et al., 1997). Rolling fractions of CINC/gro-stimulated leukocytes were convincingly increased in venules, whereas arteriolar rolling did not differ from that observed for unstimulated leukocytes (Fig. 10). Although monoclonal antibodies against CD18 (WT-3) and ICAM-1 (1A29) exerted little impact upon pulmonary arteriolar rolling, venular rolling of CINC/gro-treated leukocytes in the lung was conspicuously abolished by anti-CD18 as well as anti-ICAM-1 monoclonal antibodies (Fig. 10), indicating that arteriolar rolling of cytokine-stimulated leukocytes in the lung is not regulated, but venular rolling is extensively mediated, via ICAM-1/CD18-associated mechanisms. The arteriolar findings are at variance with those observed for normal and hyperoxia-activated lungs with unstimulated leukocytes, in which constitutive L-selectin on the leukocyte surface is found to be the essential factor distorting leukocyte behavior in pulmonary arterioles (Yamaguchi et al., 1997; Nishio et al., 1998). Furthermore, venular findings are also not strictly consistent with those obtained for unstimulated leukocyte kinetics in hyperoxia-activated lungs, in which, in addition to upregulated ICAM-1 glycoprotein, L-selectin is found to be undoubtedly important for leukocyte rolling. These inconsistencies may be explained by the fact that CINC/gro stimulation alters leukocyte deformability through the intracellular actin polymerization and thereby increases cell rigidity (Westlin et al., 1992). The leukocyte with less deformability may not allow extension of its surface area at a point of contact with the vascular endothelium, thus hindering the efficiency of adhesive force produced by L-selectin-dependent pathway.

Although a majority of leukocytes stimulated upon CINC/gro were entrapped within pulmonary capillary segments with a discontinuous motion, they were almost completely improved by treatment with either anti-ICAM-1 or anti-CD18 monoclonal antibodies (Fig. 11), being highly consistent with the findings obtained for unstimulated leukocyte kinetics in lungs with ICAM-1

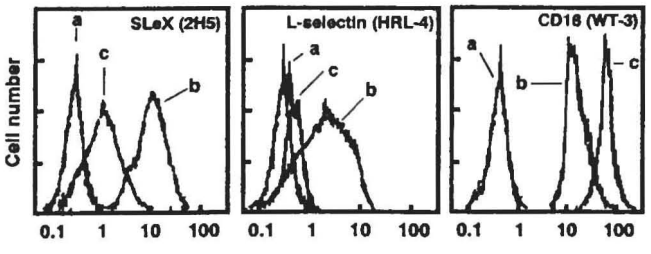

Fig. 9. Effects of CINC/gro stimulation on expressions of SLeX, L-selectin, and CD18 on rat neutrophils. a: background fluorescence; b and c: fluorescence intensities before and after CINC/gro stimulation, respectively; 2H5: monoclonal antibody (MAb) against SLeX; HRL-4: MAb against L-selectin; WT-3: MAb against CD18. Note that CINC/gro stimulation downregulates surface expression of SLeX and L-selectin, whereas CD18 is upregulated. (Aoki et al., 1997).

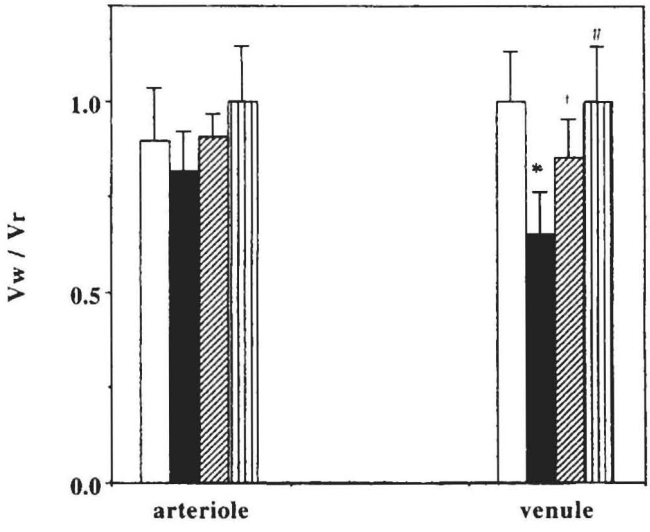

Fig. 10. Rolling leukocytes activated upon CINC/gro in intact pulmonary arterioles and venules. Relative flowing velocity (Vw/Vr), defined as leukocyte velocity (Vw) against centerline erythrocyte velocity (Vr), is substituted for representing rolling phenomenon. Decreased Vw/Vr indicates increase in adhesive interactions between the vascular endothelium and the leukocyte. From the left, Vw/Vr values in the Control (without CINC/gro stimulation), those in the group in which leukocytes are activated with CINC/gro, those under conditions where CINC/gro-leukocyte kinetics are observed in the presence of MAb to CD18, and those under conditions where CINC/gro-leukocyte kinetics are obtained in the presence of MAb to ICAM-1, respectively. *: smaller than the values obtained for the Control; +: greater than the value of the CINC/gro group but smaller than that of the Control; #: greater than the value obtained in the CINC/gro group and not different from that in the Control. (Aoki et al., 1997).

upregulation (Nishio et al., 1998), in which ICAM-1 inhibition fully restored leukocyte accumulation in pulmonary capillaries (Fig. 8). Although changes in leukocyte deformability inducing mismatch of leukocyte-capillary diameters have been postulated to serve as an alternative mechanism underlying ICAM-1/CD18-independent sequestration of leukocytes in pulmonary capillaries (Worthen et al., 1989; Doerschuk et al., 1992; Wiggs et al., 1994), experimental findings collected from our studies suggest that relative contribution of cell deformability to eliciting leukocyte entrapment in capillaries may be blunted under conditions of endothelial or leukocyte adhesion molecule expression being significantly enhanced. Involvement of ICAM-1/CD18-associated pathway in the mechanism underlying pulmonary leukocyte accumulation and in the subsequent lung injury was reported in other models of acute lung injury induced by tumor necrosis factor, gram-negative sepsis including *Pseudomonas aeruginosa* and *E. coli*, zymosan-activated plasma, IL-1, phorbol myristate acetate (PMA), and IgG or IgA immune complexes, as well (Doerschuk et al., 1990, 1996; Walsh et al., 1991; Doerschuk, 1992; Lo et al., 1992; Mulligan et al., 1992, 1993; Hellewell et al., 1994). These diseased states, especially gram-negative sepsis, are known to upregulate ICAM-1 expression on endothelial cells with no increase in the expression of leukocyte CD18-related proteins and L-selectin (Rothlein et al., 1986; Smith et al., 1988; Diamond et al., 1990; Hogg et al., 1991; Argenbright et al., 1991; Burns et al., 1994; Doerschuk et al., 1996). On the other hand, blockade of CD18 was found to effectively inhibit leukocyte sequestration in the gut, but not in the lung, in ischemia-reperfusion injury (Vedder et al., 1988). Furthermore, recent data obtained from studies utilizing P-selectin/ICAM-1 double mutant mice showed that peritoneal leukocyte emigration induced by *Streptococcus* pneumoniae was attenuated in mutant mice, whereas pulmonary leukocyte emigration occurred as observed in the wild-type mice (i.e., P-selectin and ICAM-1 are not knocked out), suggesting that ICAM-1/CD18-related pathway would not be of importance for leukocyte accumulation in a severe infection of *Streptococcus pneumoniae* (Bullard et al., 1995). In contrast to gram-negative sepsis, infection caused by *Streptococcus pneumoniae* enhances the expression of leukocyte CD18-associated integrins with no upregulation of endothelial ICAM-1 (Burns et al., 1994; Doerschuk et al., 1996). In addition, hydrochloric acid and C5a complement elicit leukocyte emigration in the lung that is ICAM-1/CD18-independent (Doerschuk et al., 1996). These findings, including our own, may consistently indicate that participation of ICAM-1/CD18 adhesive mechanism in inducing leukocyte accumulation in the lung (mainly in alveolar capillaries) is insult-specific. Under conditions in which the insult administered exceedingly influences the cell deformability, leukocyte accumulation in pulmonary capillaries may be mainly mediated via a mechanism independent of adhesion molecules. Meanwhile, if the insult significantly upregulates the expression of ICAM-1 (as in hyperoxia-exposed lungs) and/or CD18-related integrins (as in CINC/gro stimulation), ICAM-1/CD18-associated mechanisms may become important for leukocyte accumulation in pulmonary capillaries. The details of which kinds of cytokines predominantly upregulate which kinds of adhesion molecules require clarification. Furthermore, the issue of which cytokines specifically distort the cellular deformability should also be extensively addressed.

Leukocyte kinetics activated by an IL-8 analog (downregulation of L-selectin but inverse upregulation

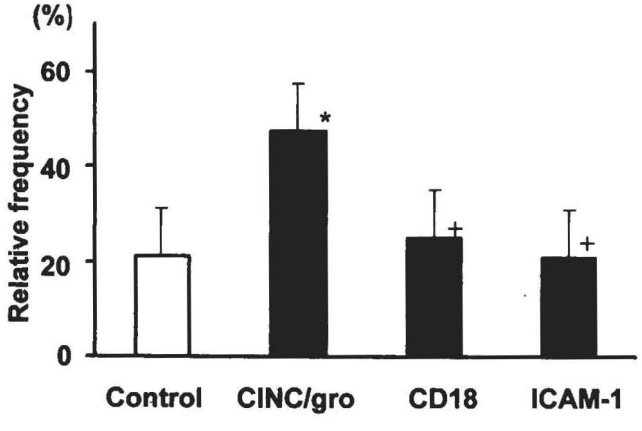

Fig. 11. Entrapment of CINC/gro-activated leukocytes in intact pulmonary capillaries. Control: cells without stimulation of CINC/gro. CINC/gro: cells with CINC/gro pretreatment. CD18: CINC/gro-treated leukocyte kinetics in the presence of MAb against CD18. ICAM-1: CINC/gro-stimulated leukocyte kinetics in the presence of MAb against ICAM-1. *: significantly greater than the value obtained for the Control group. +: smaller than the value obtained for the CINC/gro group and not different from that for the Control. (Aoki et al., 1997).

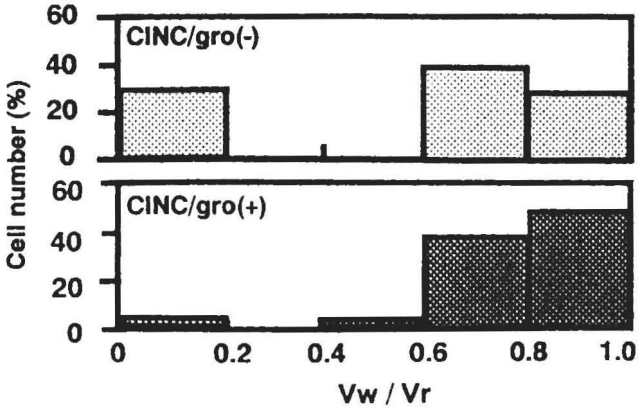

Fig. 12. Distribution of relative flowing velocity of leukocytes (Vw/Vr) in intact mesenteric venules. CINC/gro(-): leukocytes without CINC/gro stimulation. CINC/gro(+): cells stimulated with CINC/gro. There is a significant difference between the two groups. Note that cells with extremely low flowing velocity are observed only in the group without CINC/gro stimulation, i.e., CINC/gro(-). (Aoki et al., 1997).

of CD18 integrins) in intact lungs is briefly summarized as follows: (1) activated leukocytes exhibit no firm adhesion along arteriolar and venular walls; (2) rolling leukocyte frequency is enhanced especially in venules; (3) dissimilar to other experimental conditions, arteriolar rolling is not mediated via L-selectin-dependent adhesion mechanism, probably due to increased cell rigidity masking the adhesive force generated by L-selectin-related pathway; (4) in partial contradiction to other conditions, venular rolling appears not to be regulated by L-selectin-dependent pathway but is predominantly mediated via both ICAM-1/CD18-related mechanism and increased cell stiffness; and (5) leukocyte entrapment in capillaries is significantly augmented via ICAM-1/CD18-associated adhesion, but, in this case, decreased cell deformability may not be important.

Leukocyte kinetics activated with IL-8 in intact mesenteric venules

Interestingly, the behavior of CINC/gro-stimulated leukocytes exhibited quite distinct pictures in mesenteric venules as compared with those observed in the pulmonary microcirculation (Aoki et al., 1997). Although time history of the leukocyte influx in the mesenteric venules showed no significant difference between leukocytes with, and those without, CINC/gro activation during the initial 10 minutes after the CFSE-labeled leukocyte injection, the difference became evident in a time-dependent manner. The disappearance rate of the labeled leukocytes stimulated upon CINC/gro turned out to be greater than that of unstimulated leukocytes, presumably because of entrapment of these cells in other organs (e.g., lung). About 30% of inactivated leukocytes showed rolling movements with typical caterpillar-like behavior, while the rolling flux was significantly diminished when the cells were pretreated with CINC/gro (Fig. 12), resulting in that CINC/gro treatment induced marked reduction of the baseline leukocyte rolling in mesenteric venules. These results sharply contradict those obtained in pulmonary venules (see above). Differences in the leukocyte behavior became more evident when the mesentery was superfused with proadhesive stimuli of fMLP (Aoki et al., 1997). Although, in the absence of fLMP, leukocytes treated either with or without CINC/gro exhibited no firm adherence to mesenteric venular walls, fMLP superfusion markedly induced firm adherence of unstimulated leukocytes, but not of CINC/gro-stimulated leukocytes, to the venular endothelium. These experimental findings strongly suggest that L-selectin-dependent mechanisms are essentially required to evoke venular rolling and subsequent firm adhesion of leukocytes in the mesenteric microcirculation. On the other hand, ICAM-1/CD18 pathway cannot be involved in the sequential multistep processes of endothelium-leukocyte interactions in the mesenteric venules so far as leukocytes are lacking in L-selectin.

Combination of the results in the pulmonary microcirculation with those in the systemic microcirculation (e.g., mesenteric microvessels) provides extremely important implications for understanding the reason why the lung is a primary target of organ damage during SIRS, in which a variety of cytokines including IL-8 are released into the circulation (Solomkin et al., 1994; Meduri et al., 1995). Under a septic circumstance, locally released cytokines upregulate leukocyte CD18 components but inversely shed L-selectin, leading to peeling leukocytes attached faintly (i.e., rolling) or firmly (i.e., tethering) to venular walls in the systemic microcirculation and to shifting these detached leukocytes to pulmonary postcapillary venules (i.e., rolling) and alveolar capillaries (i.e., entrapment) via ICAM-1/CD18-dependent mechanism and, to a lesser extent, via decreased cell deformability. Excessive accumulation into alveolar capillaries and subsequent transendothelial migration of these activated leukocytes will cause serious parenchyma damage corresponding to the situation of acute respiratory distress syndrome, ARDS. Although the full picture of the leukocyte accumulation and transmigration mechanisms operating in the pulmonary microcirculation has yet to be elucidated, the findings presented in the current study remind us of effectiveness and limitations of the anti-adhesion therapy for extinguishing ARDS. For instance, anti-L-selectin therapy may make sense in case of a dangerous insult being confined in the lung (i.e., localized pneumonia), but it may be very risky if the invasive insult exists in the extrapulmonary organs, because decrease in L-selectin functions causes a further accumulation of activated leukocytes in the pulmonary microcirculation, thus aggravating the lung injury. Anti-ICAM-1/CD18 therapy may be helpful, regardless of the original portion of the insult yielding ARDS, under certain diseased conditions in which ICAM-1 and/or CD18 integrins are convincingly upregulated. These conditions may break out in disorders such as hyperoxic lung injury (Nishio et al., 1998) and gram-negative sepsis (Doerschuk et al., 1990, 1996; Walsh et al., 1991; Doerschuk, 1992). Under these circumstances, intrapulmonary and extrapulmonary causes for ARDS may together augment leukocyte accumulation in the lung via ICAM-1/CD18-related pathway, therefore anti-integrin therapy may successfully attenuate leukocyte margination in the pulmonary microcirculation. On the other hand, anti-ICAM-1/CD18 therapy may not be effective in ischemia-reperfusion injury (Vedder et al., 1988) and gram-positive sepsis caused by, for instance, *Streptococcus pneumoniae* infection, in which no increase in ICAM-1 expression on endothelial cells was observed (Bullard et al., 1995; Doerschuk et al., 1996). These considerations suggest that we should select the adequate anti-adhesion therapy considering the respective diseased state.

In conclusion, physiological and biological characteristics of pulmonary microvessels appear to be quite different from those of the systemic micro-

circulation, in which sequential multistep processes of rolling, tight adhesion and migration involving different classes of adhesion molecules are thought to serve as a central mechanism for endothelium-leukocyte interactions (Butcher, 1991; von Andrian et al., 1991, 1992; Springer, 1994). Several lines of evidence suggest that, in the systemic microvessels, especially in post-capillary venules, the initial interaction, i.e. rolling, is mediated by a reversible selectin-dependent binding of the leukocyte to the endothelium. Subsequently, tight adhesion between the leukocyte and the endothelium occurs via an integrin-dependent mechanism. Trans-endothelial migration of leukocytes into tissues may be mediated, in part, by the pathway associated with platelet endothelial cell adhesion molecule-1 (PECAM-1) (Newman et al., 1990; Albelda et al., 1991; Tanaka et al., 1992; Schimmenti et al., 1992; Muller et al., 1993; Xie et al., 1993; Vaporciyan et al., 1993). The important role of PECAM-1 in transendothelial migration has recently been studied in IgG immune complex-induced injury, which involves ICAM-1/CD18-dependent adhesion pathways. Anti-PECAM-1 antibodies have been reported to attenuate neutrophil emigration by about 80% (Vaporciyan et al., 1993), though the role of PECAM-1 in ICAM-1/CD18-independent adhesion pathways has not been critically evaluated. Extensive studies done in our laboratory strongly indicate that the sequential multistep theory obtained in the systemic microcirculation cannot be strictly applied to leukocyte kinetics in the pulmonary microcirculation, in which rolling occurs but is lacking in firm adhesion. Under normal conditions, pulmonary leukocyte rolling, one of the important mechanisms constituting marginated pool of leukocytes, predo-minantly occurs in arterioles via L-selectin-dependent process in addition to lower shear force in arterioles. In intact pulmonary venules, little conspicuous rolling is observed, probably due to a much higher shear force in venules than in arterioles. Meanwhile, pulmonary capillary networks are able to trap leukocytes even at a physiological shear rate, the corresponding mechanism being seemingly independent of adhesion molecules. Firm adherence of leukocytes to the pulmonary arteriole as well as venule endothelium does not take place under any diseased conditions including those with up-regulation of endothelial ICAM-1, P-selectin and leukocyte CD18-related integrins. Although ICAM-1/CD18-associated pathway is a key process inducing firm adherence of leukocytes in the systemic venules, it appears to mediate rolling rather than firm adhesion of leukocytes in pulmonary venules under pathological conditions. Furthermore, ICAM-1/CD18-related pathway becomes important for causing enhanced leukocyte accumulation in alveolar capillaries under conditions in which expression of ICAM-1- and/or CD18-associated proteins are significantly upregulated.

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