Leukocyte adhesion and microvessel permeability

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Leukocyte adhesion and microvessel permeability. Am J Physiol Heart Circ Physiol 278: H1686–H1694, 2000.—To investigate the direct effect of leukocyte adherence to microvessel walls on microvascular permeability, we developed a method to measure changes in hydraulic conductivity ($L_p$) before and after leukocyte adhesion in individually perfused venular microvessels in frog mesentery. In 19 microvessels that were initially free of leukocyte sticking or rolling along the vessel wall, control $L_p$ was measured first with Ringer-albumin perfusate. Blood flow was then restored in each vessel with a reduced flow rate in the range of 30–116 µm/s to facilitate leukocyte adhesion. Each vessel was recannulated in 45 min. The mean number of leukocytes adhering to the vessel wall was 237 ± 22 leukocytes/mm². At the same time, $L_p$ increased to 4.7 ± 0.5 times the control value. Superfusion of isoproterenol (10 µM) after leukocyte adhesion brought the increased $L_p$ back to 1.1 ± 0.2 times the control in 5–10 min (n = 9). Superfusing isoproterenol before leukocyte adhesion prevented the increase in $L_p$ (n = 6). However, the number of leukocytes adhering to the vessel wall was not significantly affected. These results demonstrated that leukocyte adhesion caused an increase in microvessel permeability that could be prevented or reversed by increasing cAMP levels in endothelial cells using isoproterenol. Thus cAMP-dependent mechanisms that regulate inflammatory agent-induced increases in permeability also modulate leukocyte adhesion-induced increases in permeability but act independently of mechanisms that regulate leukocyte adhesion to the microvessel wall. Application of ivotifen, a mast cell stabilizer, and deferoxamine mesylate, an iron-chelating reagent, attenuated the increase in $L_p$ induced by leukocyte adhesion, suggesting the involvement of oxidants and the activation of mast cells in leukocyte adhesion-induced permeability increase. Furthermore, with the use of an in vivo silver stain technique, the locations of the adherent leukocytes on the microvessel wall were identified quantitatively in intact microvessels.

ACUTE INFLAMMATION is characterized by increased microvascular permeability to plasma proteins and leukocyte recruitment into inflammatory sites. A large increase in permeability of the microvessel wall is a critical event resulting in edema formation and loss of organ function. It is well established that exposure to inflammatory mediators alone causes a transient increase in microvessel permeability (30, 31). The activation of circulating leukocytes and their interaction with microvascular endothelial cells in response to various stimuli have also been demonstrated as processes resulting in an increase in microvessel permeability during acute inflammation (14, 37). In recent years, there has been growing interest in and information on the interaction of leukocytes and endothelial cells. Studies have been carried out in cultured endothelial monolayers, whole microvascular beds, isolated vessels, and organs in transgenic animals (7, 25, 28, 29, 39). Remarkable advances have been made in clarifying the molecular basis of leukocyte adherence to endothelial cells. In contrast, less attention has been given to identifying the critical process or factor contributing to the permeability increase and the regulatory mechanisms that can prevent the permeability increase induced by leukocyte adhesion. The relationship among leukocyte adhesion, emigration, and microvesSEL permeability has not been well defined.

Previously, we carried out a series of studies on how microvessel permeability was regulated in response to inflammatory agents using individually perfused intact microvessels (16, 18, 19, 22). Those experiments were conducted in the absence of leukocytes. We (16, 22) found that inflammatory mediators induced a transient increase in microvessel permeability, which was associated with a transient increase in endothelial cell cytoplasmic Ca²⁺ concentration ([Ca²⁺]i). The regulatory processes were identified either at the level of calcium influx or downstream from the calcium entry by measuring changes in [Ca²⁺]i in endothelial cells forming the microvessel wall under the same experimental conditions in which permeability was measured (16, 18–20, 22). Those studies created a bridge between studies of changes in permeability in intact microvascular beds and studies of endothelial cells in culture. Our present study extended this approach to the study of leukocyte adhesion and adhesion-associated changes in microvascular permeability. This experimental approach enabled paired experiments to be conducted in the same vessel, which allowed the changes in permeability after leukocyte adhesion to be evaluated based on its own control value, and the experimental conditions were precisely controlled.

The objectives of the present study were to determine the direct effect of leukocyte adhesion on permeability using individually perfused intact venular microvessels in frog mesenteries. Leukocyte adhesion was induced by reducing the rate of blood flow without the application of exogenous inflammatory stimuli. Changes...
in microvessel permeability were studied in the same vessel by measuring hydraulic conductivity ($L_p$) before and after leukocyte adherence. We hypothesized that the mechanisms that regulate the increases in permeability induced by inflammatory agents also apply to the increases in permeability induced by leukocyte adhesion. Therefore, we examined the CAMP-dependent mechanisms that have been shown to reduce inflammatory mediator-induced increases in permeability on leukocyte adhesion and on adhesion-associated inflammatory mediator-induced increases in permeability induced by leukocyte adherence. The involvement of increased production of oxidants and the activation of mast cells associated with leukocyte-endothelial cell interaction were also investigated. Furthermore, using an in vivo silver stain technique (15), the locations of the adherent leukocytes on the microvessel wall were identified quantitatively in intact microvessels.

**MATERIALS AND METHODS**

**Animal Preparation**

The experiments were carried out in frog mesenteries. Frogs (male Rana pipiens, 2.5–3.0 in.) were supplied by J. M. Hazen. The brain of the frog was destroyed by pithing while leaving the spinal cord intact. Preparation of frog mesenteries for measurement of $L_p$ has been well described (10). The upper surface of the mesentery was continuously superfused with frog Ringer solution during the preparation and experimentation. The temperature of the superfusate was maintained at 18–20°C. All of the experiments were carried out in venular microvessels, which were classified as segments where there is convergent flow, one or two branches distal to true capillaries. All of the vessels selected for the experiment had brisk blood flow and were initially free of leukocyte sticking or rolling along the vessel wall.

**Measurement of $L_p$ Before and After Leukocyte Adhesion**

Measurements of $L_p$ were based on the modified Landis technique, which measures the volume flux of water across the microvessel wall following occlusion of the vessel. Under certain circumstances, especially under high permeability states, measurements of $L_p$ have been consistent with measurements of solute permeability in individually perfused microvessels, in part because of the coupling of the initial increase in macromolecular flux to transcapillary water flows. The assumptions and limitations of the measurement have been evaluated in detail (10, 31). The initial transcapillary water flow per unit area of the capillary wall ($J_w/S_b$) was measured at a given pressure (20–30 cmH$_2$O) within each vessel. Microvessel $L_p$ was calculated as the slope of the relation between ($J_w/S_b$) and pressure.

In each experiment, control $L_p$ was measured first with Ringer-albumin perfusate (containing BSA 10 mg/ml). After leukocyte adhesion induced by reduced flow rate (details described in Reduced Shear Rate-Induced Leukocyte Adhesion in Frog Mesenteric Venular Microvessels), the same vessel was recannulated and perfused with Ringer-albumin perfusate, and changes in $L_p$ were measured in the presence of leukocyte adhesion. One experiment was carried out per frog mesentery.

**Reduced Shear Rate-Induced Leukocyte Adhesion in Frog Mesenteric Venular Microvessels**

To study the direct effect of leukocyte adhesion on microvessel permeability, we developed a method to induce leukocyte adhesion by reducing blood flow rate. After the measurement of baseline $L_p$, blood flow was resuspended in the same vessel. The central line red blood cell velocities ($V_{RBC}$) were then reduced from 700–800 µm/s to the range of 30–116 µm/s by gently pressing the adjacent branch vessel using a glass occluding rod to increase the resistance of upstream or downstream blood flow. The occluding rod was held by a micromanipulator. The mean bulk velocity ($V_{mean}$) was estimated as $V_{mean} = V_{RBC}/1.6$ (2), and the wall shear rate, calculated based on the Newtonian definition, $r = 8 (V_{mean}/D)$, was between 3 and 13 s$^{-1}$ (the mean vessel diameter, $D$, was 44 ± 2 µm). After 45–50 min of low shear rate flow, the same vessel was recannulated with Ringer-albumin perfusate, and changes in $L_p$ were measured in the presence of leukocyte adhesion. The number of leukocytes adhering on the same segment of the microvessel wall where $L_p$ had been measured was then counted under the microscope. We found that 45–50 min of reduced flow rate was necessary for a significant number of leukocytes to firmly attach to the vessel wall and was optimal for our experimental conditions. A longer period of reduced flow rate carried the risk that blood flow might stop in that vessel. Whenever that occurred, the experiment was discarded.

**Silver Staining of Boundaries of Endothelial Cells Forming Microvessel Walls**

To identify the locations of adherent leukocytes, silver stain was applied to individually perfused microvessels to illustrate the junctions of endothelial cells forming microvessel walls (15). At the end of the experiment, microvessels with leukocyte adhesion were recannulated and perfused with AgNO$_3$ (0.1 g/100 ml) in aqueous solution for 5–10 s ($n = 10$). The present pipette tip blocking by silver chloride precipitation at the moment when the pipette tip touched the tissue, the mesentery was flushed once with chloride-free Ringer solution right before the cannulation. After a brief AgNO$_3$ perfusion, each vessel was recannulated and perfused with Ringer-albumin perfusate to delineate the endothelial junctions. Details have been previously described (15). The numbers of adhering leukocytes located on the endothelial junctions versus off the junctions were then counted under the microscope in 10 microvessels. Photographs (see Fig. 7) for demonstration were taken with a CCD camera (ProgRes 3012, Kontron, Japen) and a Nikon Fluor ×60, numerical aperture 1.4, oil objective. The scan rate is 1,996 × 1,450 pixels, and photo output is 300 pixels/inch.

**Solutions**

Ringer solutions and Ringer-albumin perfusates. Frog Ringer solution was used for dissecting mesenteries, superfusing tissue, and preparing the perfusion solutions. The frog Ringer solution was prepared as described for all previous experiments (10). The pH of the Ringer solution was maintained at $7.40–7.45$ by adjusting the ratio of Na-HEPES to HEPES. A Ringer-albumin perfusate was prepared by adding 10 mg/ml BSA to Ringer solution. All perfusates used for control and test perfusion contained BSA (10 mg/ml). The chloride-free Ringer solution used for silver staining was prepared by replacing NaCl and KCl with sodium gluconate and potassium gluconate (Sigma) isoosmotically.

**Test solutions.** Deferoxamine mesylate (DFO, Sigma) at a concentration of 1 mM was applied to both the superfusate (Ringer solution) and perfusate (Ringer-albumin solution).
during the experiments. Ketotifen (Sigma) at 50 µM was applied to the superfusate only. Isoproterenol (Isuprel, Isoproternol HCl, UCDMC Pharmacy, Sanofi Winthrop Pharmaceutical) at a concentration of 10 µM was used in the superfusate. All test solutions were freshly prepared before each experiment.

Data Analysis and Statistics

All values in the text are means ± SE, except where noted otherwise. Changes in $L_p$ were expressed as the ratio of testing $L_p$ versus control $L_p$ ($L_{ptest}/L_{pcontrol}$). For statistical comparisons, the mean values of $L_p$ measured before and after leukocyte adhesion from the same vessel were used as paired data. The significance level at $P < 0.05$ was determined by a group of paired mean values of $L_p$ and was analyzed by paired $t$-test and nonparametric Wilcoxon Signed Rank test.

RESULTS

Leukocyte Adhesion-Induced Increases in $L_p$

The effects of leukocyte adhesion induced by a low flow rate on microvessel permeability were studied in 19 venular microvessels. The mean control $L_p$ of 19 microvessels that were initially free of leukocyte rolling and adhering was $4.9 ± 0.6 \times 10^{-7} \text{cm} \cdot \text{s}^{-1} \cdot \text{cmH}_2\text{O}^{-1}$. After 45–50 min of low shear rate flow, the mean number of adhering leukocytes in the same 19 microvessels was $237 ± 22 \text{leukocytes/mm}^2 \text{vessel wall}$. Figure 1 illustrates an individually perfused microvessel under control condition (left) and after leukocyte adhesion induced by low blood flow rate (right). Venular microvessel in frog mesentery was cannulated with a micropipette and perfused with Ringer-albumin perfusate.

![Control](image1.png) ![With Leukocyte Adhesion](image2.png)

Fig. 1. Photomicrographs of a microvessel under control condition (left) and after leukocyte adhesion induced by low blood flow rate (right). Venular microvessel in frog mesentery was cannulated with a micropipette and perfused with Ringer-albumin perfusate.

![A](image3.png) ![B](image4.png)

Fig. 2. Single experiments to show changes in hydraulic conductivity ($L_p$) as a function of time with and without leukocyte adhesion. A: mean control $L_p$ ($L_{pcontrol}$) of this vessel was $2.7 ± 0.5 (SD) \times 10^{-7} \text{cm} \cdot \text{s}^{-1} \cdot \text{cmH}_2\text{O}^{-1}$. After 45 min of low blood flow, the adhered leukocytes were $160 \text{per mm}^2 \text{vessel wall}$, which was accompanied with a sustained increase in $L_p$ ($L_{ptest}$). B: control experiment in another microvessel that followed same time course and procedure without reducing flow rate and no leukocyte adhesion. Mean control $L_p$ in this vessel was $2.5 ± 0.3 (SD) \times 10^{-7} \text{cm} \cdot \text{s}^{-1} \cdot \text{cmH}_2\text{O}^{-1}$. When same vessel was recannulated, after normal blood flow was resumed for 45 min without leukocyte adhesion, $L_p$ showed a small transient increase in 1 min after recannulation and then fell back to the control level.

The mean control $L_p$ of 5 microvessels was $2.37 ± 0.22 \text{leukocytes/mm}^2 \text{vessel wall}$. Figure 1 illustrates an individually perfused microvessel under control conditions and after leukocyte adhesion. Leukocyte adhesion, at a mean density of $237 ± 22 \text{leukocytes/mm}^2 \text{vessel wall}$, caused a significant, sustained increase in $L_p$. The mean of the ratio $L_p$ with leukocyte adhesion relative to control was $4.7 ± 0.5 (n = 19)$. The time course of changes in $L_p$ after leukocyte adhesion measured in a single experiment is shown in Fig. 2A.

To investigate whether increases in $L_p$ were due to the experimental procedures, control experiments were carried out in another five microvessels following the same time course and procedures but with a faster flow rate ($>500 \mu\text{m/s}$) and no leukocyte adhering to the wall. The mean control $L_p$ of 5 microvessels was $2.8 ± 0.7 \times 10^{-7} \text{cm} \cdot \text{s}^{-1} \cdot \text{cmH}_2\text{O}^{-1}$. We found no significant increases in $L_p$ in the absence of leukocyte adhesion after 45 min of resumed blood flow. A single experiment is
Effect of Isoproterenol on Leukocyte Adhesion-Induced Increases in $L_p$

We demonstrated previously that cAMP-enhancing agents prevented increases in microvessel permeability in response to inflammatory mediators such as ATP or bradykinin (18, 20). We, therefore, investigated the effect of the $\beta$-adrenergic agonist isoproterenol on leukocyte adhesion-induced increases in permeability. We first tested the action of isoproterenol on another sustained high-permeability state caused by the removal of albumin from the perfusate. The mean control $L_p$ in a group of four microvessels was $4.0 \pm 0.9 \times 10^{-7} \text{cm\cdot s}^{-1}\cdot\text{cmH}_2\text{O}^{-1}$. Ringer perfusion caused a 2.4 ± 0.1-fold sustained increase in $L_p$. Superfusion of isoproterenol (10 µM) on the mesentery brought the increased $L_p$ back to control level. The mean $L_p$ was only $0.9 \pm 0.2$ times the control in 2–5 min after the superfusion of isoproterenol.

The effect of isoproterenol on leukocyte adhesion-induced increases in $L_p$ was then tested in 9 of 19 microvessels. The mean control $L_p$ of 9 microvessels was $6.1 \pm 0.8 \times 10^{-7} \text{cm\cdot s}^{-1}\cdot\text{cmH}_2\text{O}^{-1}$. After 45–50 min of resuming blood flow with a low shear rate, we found the mean density of leukocyte adhesion to be $220 \pm 43$ leukocytes/mm$^2$ vessel wall. The mean $L_p$ after leukocyte adhesion was $4.9 \pm 0.9$ times the control value. Adding isoproterenol (10 µM) to the superfusate brought the increased $L_p$ back to 1.1 ± 0.2 times control level within 5–10 min. Figure 4A shows a single experiment, and Fig. 4B shows summarized results.

Effect of Presuperfusion of Isoproterenol on Leukocyte Adhesion and Adhesion-Induced Increases in $L_p$

To test whether superfusion of isoproterenol (10 µM) before reducing blood flow rate affected the adhesion process and prevented the adhesion-induced increases in $L_p$, experiments were carried out in six microvessels. The mean control $L_p$ of six microvessels was $5.7 \pm 0.8 \times 10^{-7} \text{cm\cdot s}^{-1}\cdot\text{cmH}_2\text{O}^{-1}$. After measurement of control $L_p$, superfusion of isoproterenol started at the same time as low rate of blood flow was resumed and continued to the end of the experiment. The mean density of leukocyte adhesion with reduced rate of blood flow and continuous superfusion of isoproterenol was $168 \pm 54$ leukocytes/mm$^2$ of the vessel wall, which was lower, but not statistically significant, than the value without presuperfusion of isoproterenol. However, the increased $L_p$ associated with leukocyte adhesion was completely abolished. The mean of the ratio of $L_p$ with leukocyte adhesion relative to control with superfusion of isoproterenol was $0.9 \pm 0.2$. A single experiment is shown in Fig. 5A. Figure 5B shows the summarized results.

Role of Oxidative Stress and Mast Cell Activation in Leukocyte Adhesion-Induced Increases in Microvessel Permeability

To further investigate the mechanisms of leukocyte adhesion-induced increases in $L_p$, we tested the potential involvement of oxidants and the activation of mast cells.

The role of mast cells in leukocyte adhesion-induced increases in $L_p$ was tested in 12 microvessels using the mast cell stabilizer ketotifen. In 5 of the 12 vessels, low-
Flow-induced leukocyte adhesion was 130 ± 44 leukocytes/mm² vessel wall, with an associated increase in $L_p$ of 3.7 ± 0.6-fold ($P < 0.05$). When ketotifen (50 µM) was applied to the superfusate, the increased $L_p$ was reduced to 1.8 ± 0.5 times the control in 5–10 min ($P > 0.05$). In another seven microvessels, ketotifen was continuously superfused on the mesentery after control measurements. The mean density of leukocyte adhesion was 185 ± 33 leukocytes/mm² vessel wall, but the mean $L_p$ measured with leukocyte adhesion was only 1.7 ± 0.3 times the control value ($P > 0.05$). Results are shown in Fig. 6A.

To further test that ketotifen caused permeability reduction associated with leukocyte adhesion was not due to its action on endothelial cells, the effect of ketotifen on albumin removal–caused increases in $L_p$ was studied in another six microvessels. We have demonstrated previously that removing albumin from the perfusate caused a sustained increase in $L_p$, which was associated with a transient increase in endothelial cell [Ca$^{2+}$] ($17$). We found that the mean $L_p$ of six vessels increased to 2.7 ± 0.4 times the control after removing albumin from the perfusate. The elevated $L_p$ remained at 2.5 ± 0.4 times the control after ketotifen (50 µM) was superfused for 5–10 min. These results do not support that ketotifen may stabilize endothelial cells resulting in the reduction of $L_p$.

The involvement of oxidants was tested in another five microvessels using DFO (1 mM), an intracellular ion chelator that interrupts the iron-catalyzed oxidative process. Results are shown in Fig. 6B. The mean control $L_p$ was $2.9 \pm 0.6 \times 10^{-7}$ cm·s$^{-1}$·cmH$_2$O$^{-1}$. Superfusion and perfusion of DFO (1 mM) slightly increased basal $L_p$ to $1.5 \pm 0.2$ times the control value. The mean density of leukocyte adhesion was $123 \pm 19$ per mm² vessel wall, and $L_p$ measured with leukocyte adhesion was only $2.4 \pm 0.6$ times the control ($P > 0.05$). The mean number of adherent leukocytes in this group was lower than in the group of 19 vessels ($237 \pm 22$ leukocytes/mm² vessel wall) but was not significantly different from the group of five vessels ($130 \pm 44$ leukocytes/mm² vessel wall) shown above, in which $L_p$ increased 3.7 ± 0.6-fold. In comparison with this magnitude of $L_p$ increase, DFO significantly attenuated the $L_p$ increase induced by leukocyte adhesion.

**Fig. 5.** Effect of superfusion of isoproterenol before reducing flow rate on leukocyte adhesion and adhesion-induced increases in $L_p$. A: single experiment showing that presuperfusion of isoproterenol (10 µM) prevented leukocyte adhesion–induced increases in $L_p$ but did not significantly affect leukocyte adhesion ($n = 6$). Right, mean numbers of leukocyte adhesion with ($n = 6$) and without ($n = 19$) superfusion of isoproterenol.

**Fig. 6.** Effects of mast cell stabilizer ketotifen and iron-chelating reagent desferrioxamine mesylate (DFO) on leukocyte adhesion–induced increase in $L_p$. A: superfusion of ketotifen (50 µM) reduced $L_p$ increase associated with low flow rate–induced leukocyte adhesion (left, $n = 5$). Superfusion of ketotifen before reducing flow rate abolished higher magnitude increase in $L_p$ associated with leukocyte adhesion (right, $n = 7$). B: summary showing that superfusion and perfusion of DFO significantly attenuated $L_p$ increase induced by leukocyte adhesion ($n = 5$). WBC, white blood cells. *$P < 0.05$ from control. **$P < 0.05$ from $L_p$ values measured after leukocyte adhesion.
Location of Leukocyte Adhesion in Intact Microvessels

To identify the location of leukocyte adhesion on microvessel walls, we delineated the boundaries of endothelial cells forming microvessel walls in 10 of the microvessels after leukocyte adhesion, using the in vivo silver stain technique we developed previously (15). We found that the majority of adherent leukocytes were selectively located on the endothelial clefts. There were a total of 227 leukocytes adhering on the walls of 10 microvessels, of which 210 were overlapping with endothelial junctions (92%). The remaining 8% were mostly located close to the side of the vessel, which made their locations difficult to determine with a two-dimensional view. Figure 7 illustrates the endothelial cell boundaries and adhering leukocytes from a segment of a single perfused venular microvessel.

DISCUSSION

This paper introduces new methods to study leukocyte adhesion and its effect on microvessel permeability using individually perfused intact venular microvessels and to demonstrate quantitatively the location of leukocyte adhesion with silver staining in live vessels. The unique feature of using individually perfused microvessels to study leukocyte adhesion is that paired experiments can be conducted in the same vessel to precisely measure the changes in microvessel permeability before and after leukocyte adhesion under well-controlled experimental conditions.

Our results demonstrate that reduced shear rate-induced leukocyte adhesion resulted in a sustained increase in \( L_p \) in individually perfused frog venular mesenteric microvessels. The inhibitory effects of ketotifen, the mast cell stabilizer, and DFO, an ion chelator, on increased \( L_p \) suggest that increased oxidant stress and mast cell activation might be involved in the \( L_p \) increase associated with leukocyte adhesion. Superfusion of isoproterenol to increase intracellular cAMP levels prevented or restored the increases in \( L_p \) mediated by leukocyte adhesion but did not significantly affect the leukocyte adherence. The application of the in vivo silver stain technique to individually perfused venular microvessels with leukocyte adherence demonstrated that the adherent leukocytes in intact microvessels were preferentially located at the junctions of endothelial cells.

Reducing Shear Rate-Induced Leukocyte Adhesion

To identify the role of leukocyte adhesion on microvessel permeability during acute inflammation, we attempted to separate the effect of cytokines or inflammatory mediators from the effect of leukocyte adhesion on microvessel permeability as much as possible. Therefore, we chose to induce leukocyte adhesion by reducing flow rate without the application of exogenous inflammatory stimuli. The shear rate-dependent leukocyte adhesion has been observed both in the presence of inflammatory stimuli and in noninflamed tissues (4, 32, 33). Our experiments demonstrated that reducing flow rate from 700–800 µm/s to 30–116 µm/s for 45 to 50 min induced a significant increase in leukocyte adhesion (237 ± 22 leukocytes/mm² of vessel wall). The potential mechanisms of low flow rate-induced leukocyte adhesion in noninflamed tissues have been pro-

![Fig. 7. Photomicrographs to illustrate preferential location of leukocyte adhesion on microvessel wall in frog mesentery. Photographs show upper (A) and lower (B) surface of same vessel. Endothelial cell borders were outlined with silver stain. Leukocyte attachment sites are indicated with arrows.](http://ajpheart.physiology.org/)

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posed. A hydrodynamic interaction between erythrocytes and leukocytes might be the major physical force contributing to the leukocyte margination and rolling (34). It was demonstrated in vitro that a reduction of shear stress leads to pseudopod projection and spreading of leukocytes on the endothelium by a shear-sensitive membrane ion exchange mechanism, which was suppressed by K⁺ channel blockers and chelation of external Ca²⁺ (32). Our preliminary immunofluorescent staining in individually perfused microvessels, and other in vivo systemic staining, showed some constitutive expression of intercellular adhesion molecule-1 on endothelial cells under basal conditions in rat mesenteric venular microvessels (21, 24), which may also contribute to reduced shear-facilitated leukocyte rolling and subsequent firm adherence. In addition, reduced flow rate may result in the accumulation of locally produced inflammatory mediators that may elicit the activation and upregulation of adhesion molecules on leukocytes and endothelial cells (14). Therefore, our method to study the effect of low-shear-induced leukocyte adhesion on microvessel permeability avoided the involvement of exogenous inflammatory stimuli but might not exclude the possibility of activating endothelial cells by local endogenous inflammatory mediators.

Increase in Permeability Associated With Leukocyte Adhesion

Most of the in vitro and in vivo studies have demonstrated that leukocyte adhesion, emigration, and increased protein leakage are closely related events. However, some studies have reported that increased vascular permeability is independent from leukocyte emigration (23). Studies in cat mesentery demonstrated that both platelet-activating factor (PAF) and leukotriene B₄ promoted leukocyte adherence in postcapillary venules, but only PAF-induced leukocyte adherence increased vascular protein leakage (25). The investigators proposed that the differences of the oxidant production induced by these two inflammatory mediators might be attributed to these distinctive permeability responses. Those data suggested that the attachment of leukocytes to endothelial cells lining microvessel walls does not always result in an increase in microvessel permeability. Our preliminary study on tumor necrosis factor-α-induced leukocyte adhesion in rat venular microvessel also showed that the leukocyte adherence was not associated with an increase in venular hydraulic permeability (21). However, the low flow rate-induced leukocyte adhesion in frog mesenteric venular microvessels showed a sustained significant increase in permeability, which was different from the transient increase in permeability we observed when the microvessel was exposed to inflammatory mediators (16, 22).

Increased production of oxidants by activated neutrophils and endothelial cells and the activation of mast cells have been proposed as important initiating events contributing to the tissue injury associated with leukocyte adhesion (11–13, 26). Our results with using ketotifen and DFO suggest that the elevated oxygen radicals and factors released from activated mast cells might be attributed, in part, to the permeability increase associated with leukocyte adhesion under our experimental conditions. Ketotifen has been reported both in vitro and in vivo to stabilize mast cells by inhibiting the degranulation and releases of cytokines and chemical factors (9, 14). Our experiments showing that ketotifen significantly attenuated the Lₚ increase associated with leukocyte adhesion, but had no effect on albumin-removal-induced increases in Lₚ, indicated that the Lₚ reduction effect of ketotifen was not due to its action on endothelial cells. We have demonstrated previously that increases in Lₚ caused by removing albumin from the perfusate was an endothelial cell-involved event, which was accompanied by a transient increase in endothelial cell [Ca²⁺] (17).

Our results also suggested that the activated mast cells and increased oxidant stress are not the initial cause of leukocyte adhesion, because pretreating vessels with ketotifen or DFO attenuated the Lₚ increase without affecting leukocyte adhesion, indicating that their primary effects were mainly exerted on endothelial cells to increase permeability, rather than the process of leukocyte adherence to the vessel wall. Although we do not completely exclude the possibility that local inflammatory mediators may activate endothelial cells and mast cells during low flow periods, resulting in increased Lₚ, it is more likely that activated leukocytes contributed to the increased oxidant stress, which further activated mast cells and then endothelial cells to increase Lₚ. These results are consistent with and supported by an in vivo study (38), which demonstrated that elevated hydroperoxide levels were concurrent with an increase in the number of adherent neutrophils during superfusion of PAF. The application of ion chelator or hydroxyl radical scavenger significantly attenuated the PAF-induced oxidative changes in the venule but had little effect on neutrophil adhesion (38). Quantitative measurements of the time course of the changes in the levels of oxygen radicals and the degranulation rate of mast cells associated with leukocyte adhesion under our experimental conditions are needed to further elucidate underlying mechanisms and signal transduction pathways.

Differential Actions of cAMP on Permeability Increase Associated With Leukocyte Adhesion and Adhesion Process

Previously, we and others (18, 20, 31) have demonstrated that increased cAMP levels in endothelial cells prevent or abolish inflammatory mediator-induced increases in permeability. The action of elevated cAMP levels on microvessel permeability has been demonstrated with electron microscopy as the result of an increase in tight-junction strands between endothelial cells under resting conditions (1) or a reduction in the number of gaps between endothelial cells in venular vessels in response to inflammatory mediators (3). The effect of increased cAMP levels in endothelial cells on leukocyte adhesion and adhesion-mediated increases...
in permeability has not been well defined, especially in intact microvessels. Studies in cultured endothelial cells demonstrated that increasing cAMP levels attenuated the increase in endothelial permeability mediated by neutrophils or hydrogen peroxide but did not alter the activation and attachment of neutrophils (36). Our present study in intact microvessels demonstrated that increasing cAMP levels in endothelial cells before and after leukocyte adhesion prevented or restored the increases in $L_p$ associated with leukocyte adhesion but had no effect on the adhesion process. These results conform to the hypothesis that increased cAMP levels in endothelial cells not only regulate inflammatory mediator-induced increases in permeability but also adhesion-induced increases in permeability. The results also indicated that the mechanisms regulating the adhesion process differ from those regulating microvessel permeability.

In contrast to our results, a study of leukocyte adhesion in rat airway mucosa demonstrated that formoterol, the $\beta_2$-adrenergic receptor agonist, inhibited the adhesion of neutrophils to the venular endothelium (6). In that study, formoterol was given intravenously, and it is possible that the antiadhesion effect of formoterol was due to the action of increased cAMP levels in leukocytes rather than that in endothelium. An in vitro study reported that increasing cAMP levels in neutrophils inhibits RhoA activation and integrin-dependent leukocyte adhesion induced by chemotactic agents (5, 27). Under our experimental conditions, superfusion of isoproterenol on mesentery appears to have its main effect on endothelial cells forming microvessel walls and a minimal effect on leukocytes. Because of the potential involvement of mast cells in increased permeability, it is possible that superfusion of isoproterenol may have an action on mast cells as well. The increased cAMP levels in mast cells may suppress the release of chemical mediators and the production of cytokines (35).

Preferential Location of Leukocyte Adhesion in Intact Venular Microvessels

Efforts have been made to identify the locations of leukocyte migration as intercellular versus transcellular, although it has not been a clear issue. There has not been much effort to identify the location of the initial capturing of leukocytes on microvessel walls. Recently, Burns et al. (7, 8) reported that the initial capture and transmigration of neutrophils in cultured endothelial monolayers occur preferentially at endothelial borders. McDonald's study (30) in venules of rat trachea reported that the appearance of silver rings at leukocyte attachment sites were usually located at endothelial cell borders after inflammatory stimuli. There has been no previous quantified evidence demonstrating how significantly the leukocyte adhesion occurs at preferential locations from a random distribution in intact microvessels. Our experiments provided a simple, novel method to illustrate the location of leukocyte adhesion in vivo by applying the in vivo silver stain technique (15) to the leukocyte adhesion study using individually perfused microvessels. We found that over 92% of the adherent leukocytes had a definite position at the junctions of endothelial cells, and the remaining 8% were at uncertain positions due to their location close to the side of the vessel. These results quantitatively demonstrate that adherent leukocytes preferentially attach to the junctions of endothelial cells forming microvessel walls.

Because a relatively large portion of the venular vessel wall is occupied by the junctions of endothelial cells, we conducted a calculation to determine whether 92% of leukocytes attached to the junction is significantly different from a random distribution on the vessel wall. First, we measured the cleft length per unit area in a frog venular microvessel by tracing the silver line at endothelial cell borders from a two-dimensional projected image using a digitizer. It was 120 ± 5 mm/mm². If we expand this cleft length to a band with a 3-µm width, which equals half of the contact length of leukocytes, the calculated band area is 36% of the total area of the vessel wall without the exclusion of the overlap at the tricellular corner. From this calculation, leukocytes have less than a 36% probability of attaching to the junctions by a random process. The fact that >92% of the leukocytes adhered at the junctions is significantly distinguishable from a random distribution. The preferential adhesion indicates that certain adhesion molecules responsible for leukocyte adhesion are located at the endothelial junctions. A study by Burns et al. (7) further demonstrated that P-selectin was distributed on borders of cultured endothelial cells, which might be responsible for this preferential location of the initial capturing of leukocytes. Future studies using intact microvessels to identify the distribution of adhesion molecules on endothelial cells and the molecular basis responsible for the preferential capture of leukocytes will provide new insight for a better understanding of the adhesion process in vivo.

In summary, our study introduced a valid in vivo method to study leukocyte adhesion and its effect on microvessel permeability. Leukocyte adhesion induced by low flow rate caused a sustained increase in $L_p$ in frog mesenteric venular microvessels. Increasing intracellular cAMP levels prevented the permeability increase associated with leukocyte adhesion without affecting the adhesion process. These observations indicate that cAMP-dependent mechanisms that regulate inflammatory agent-induced increases in permeability also modulate leukocyte adhesion-induced increases in permeability but act independently of mechanisms that regulate leukocyte adhesion to the microvessel wall. The preferential location of adherent leukocytes demonstrated with in vivo silver staining in intact microvessels suggests that the adhesion molecules responsible for leukocyte adhesion are located at endothelial clefts in intact microvessels.

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