Effect of early transient adherent leukocytes on venular permeability and endothelial actin cytoskeleton

Valeski, J. Edward, and Ann L. Baldwin. Effect of early transient adherent leukocytes on venular permeability and endothelial actin cytoskeleton. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H569–H575, 1999.—In a time course study of the development and recovery of venular leaks, it was shown that, after as early as 3 min of histamine application, venular leak formation was identified [Baldwin, A. L., and G. Thurston. Am. J. Physiol. 269 (Heart Circ. Physiol. 38): H1528–H1537, 1995]. This was accompanied by changes in the endothelial actin cytoskeleton and the presence of adherent leukocytes. The venular leaks remained elevated for at least 30 min, whereas the adherent leukocytes were decreased by 20 min. The present study was performed to determine the role that 3 min (early), transient histamine-associated adherent leukocytes play in the formation of venular leaks and changes in the endothelial actin cytoskeleton. In anesthetized rats, the microvasculature of a mesenteric window was perfused with buffered saline or fucoidin. FITC-BSA or FITC-BSA and 10^{-4} M histamine was added to the perfusate for the last 3 min. The vasculature was perfusion fixed, stained for filamentous actin, and viewed microscopically. Fucoidin pretreatment significantly reduced the number of early, transient histamine-associated adherent leukocytes (P < 0.01). The number of adherent leukocytes in leaky venules was significantly greater than that seen in nonleaky venules (P < 0.01); however, the reduction in the number of histamine-associated adherent leukocytes with fucoidin pretreatment had no significant effect on the number (P > 0.05) or area (P > 0.05) of FITC-BSA leaks or on the endothelial actin cytoskeleton.

rnat mesentery; confocal microscopy; fucoidin; histamine

ACUTE INFLAMMATION is characterized by hyperemia, increased microvascular permeability to fluids and plasma proteins, and the enhanced recruitment of leukocytes into the extravascular space. The recognition of injured sites is mediated by migration of leukocytes to the extracellular matrix, with a cascade involvement by several families of adhesion molecules (12, 17). These observations have led to the description of the now classical three-step inflammatory cell cascade: rolling, activation, and adhesion. This sequential multistep process is preceded by the initial capture of flowing leukocytes (via the selectin family of adhesion molecules) from the blood. It is followed by transendothelial migration and movement to the interstitium. The inflammatory mediator, histamine, causes a wide range of effects in the microvasculature. Histamine evokes vasodilation and increased local blood flow and interendothelial gap formation in postcapillary venules, resulting in plasma extravasation (15, 20, 21). Histamine also promotes leukocyte rolling through the expression of endothelial P-selectin in vitro (9) and in vivo (2, 11, 12). However, histamine alone causes little or no recruitment of leukocytes into human or animal tissues (3, 8).

In a time course study of the development and recovery of venular leaks, it was shown as early as 3 min after histamine application that venular leak formation occurred (4). It was also shown for the first time that the leaks were accompanied by changes in the endothelial peripheral actin rim (PAR) along with adherent leukocytes. The numbers of leaks did not vary significantly from 3 to 30 min. However, the number of adherent leukocytes was decreased by 20 min.

No one has specifically examined the early histamine-associated changes in vascular permeability and early, transient leukocyte adherence. For this reason, the overall objective of this study was to investigate the role of early (3-min), transient in vivo leukocyte adherence on vascular permeability in small venules of the rat mesentery. Five specific issues were addressed. 1) Can pretreatment with the selectin-binding carbohydrate fucoidin significantly reduce early, transient leukocyte adherence? 2) What is the effect of early, transient leukocyte adherence on the number of venular leaks? 3) What is the effect of early, transient leukocyte adherence on the area of venular leaks? 4) What is the effect of early, transient leukocyte adherence on endothelial actin cytoskeleton? 5) Is there a significant difference between the number of early, transient adherent leukocytes on leaky venules versus nonleaky venules?

MATERIALS AND METHODS

Cannulation and perfusion of rat mesentery. The animal procedure was similar to that previously described (4) and is summarized in this report. Twenty-two male Sprague-Dawley rats (350–400 g) were anesthetized with an intramuscular injection of pentabarbitone sodium (6 mg/100 g). The abdomen was opened, and a well-vascularized mesenteric window was selected and spread flat over a small Plexiglas platform. Approximately 10 min before cannulation, HEPES-buffered saline containing 0.5% bovine serum albumin (HBS)-BSA plus fucoidin (4 mg/kg; 15 animals) or HBS-BSA only (7 animals) was injected via the jugular vein. This dose was similar to that used by other investigators in a similar preparation (14). The superior mesenteric artery was cannulated close to the selected mesenteric window, and appropriate arterioles and venules bordering the window were ligated to allow perfusion of only the selected window.

The mesenteric windows were flushed clear of blood with HBS (pH 7.4) containing 0.5% BSA and 1 U/ml heparin at 37°C. The preparations were then perfused for 3 min with either 0.5% FITC-BSA (Sigma, St. Louis, MO) plus 100...
µM histamine (14 animals) or FITC-BSA alone (8 animals). As soon as the vasculature of the window was filled with tracer, the pressure was adjusted to 40 mmHg, and the portal vein, which acted as the flow outlet, was clamped. After the clamp was removed, 1.5 ml of fixative (3% formaldehyde in HBS) was perfused via the cannula at a pressure of 100 mmHg. Next, the pressure was reduced to 40 mmHg, and the portal vein was clamped, and fixation was continued for 30 min. The vasculature of the mesenteric window was then perfused, via the cannula, with a cocktail of 3% formaldehyde, 0.1% Triton X-100, and rhodamine phalloidin (10 U/ml; Molecular Probes, Eugene, OR) in HBS for 30 min at 4°C to stain endothelial and leukocyte actin fibers. The vasculature was then perfusion fixed for 30 min with 3% formaldehyde and then flushed with HBS. The mesenteric tissue was excised and mounted between a glass slide and a coverslip using aqueous mounting medium ( Vectashield; Vector Laboratories, Burlingame, CA).

Assessment of venular leakage and leukocyte adherence. In this study, leakage site measurements were made on venules with the size range 8–70 µm diameter. An evaluation of overall vascular leakage was made by measuring the number and area of regions with extravascular FITC-BSA. Slides were examined using a Zeiss Axioplan microscope with a ×20 objective, numerical aperture 0.6, fitted for epifluorescence. The light source for epifluorescence was a 100-W mercury lamp. A video camera (Optronix VI 470) was mounted at the camera port of the microscope. Images of vessel networks (both leaky and nonleaky) from each slide (i.e., from each experimental animal), produced by epifluorescence with the appropriate FITC excitation and barrier filters (λ = 488 and 515 nm, respectively) and rhodamine excitation and barrier filters (λ = 568 and 610 nm, respectively) inserted into the light path, were viewed on a black and white monitor and were recorded on a video recorder. Videotaped images were later analyzed using an analog-to-digital converter and National Institutes of Health Image software to measure length and diameter of each venule (both leaky and nonleaky), the number of leaks per venule, the area of each leak, and the number of adherent leukocytes in both leaky and nonleaky venules. If one leak area was contributed by more than one venule, the leak area was divided by the number of vessels. Data were pooled for each experimental condition, and the following parameters were calculated: 1) average number of leaks per micrometer of venule length (venules without leaks were included in this measurement), 2) average leak area per micrometer of venule length, 3) average number of adherent leukocytes per micrometer of venule length (both leaky and nonleaky venules), 4) average number of adherent leukocytes per micrometer of venule length (nonleaky venules), and 5) average number of adherent leukocytes per micrometer of venule length (leaky venules). Each parameter was compared between different groups using one-way ANOVA. If a significant difference was found between groups, pairs of groups were compared using the Student’s t-test, with a P value <0.05 indicating statistical significance. All values are presented as means ± SE. The n used in these studies was the number of venules per group.

Actin cytoskeleton. The specimens were viewed with a confocal microscope (Zeiss LSM 410) equipped with a krypton-argon laser. With the use of a ×40 numerical aperture 1.0 oil immersion objective lens, a venule of interest was identified, and a series of images was collected at different focus levels (step size 1 µm) using the 568-nm line (for rhodamine phalloidin) and the 488-nm line (for FITC-BSA).

RESULTS

Effect of fucoidin treatment on venular leukocyte adherence. The effect of fucoidin treatment on the number of adherent leukocytes per micrometer of venule length in venules of the various preparations is shown in Fig. 1. Histamine control preparations showed 98.8 ± 10.2 × 10^-4 (SE; n = 117 venules) adherent leukocytes/µm. With fucoidin treatment, the number of histamine-associated adherent leukocytes [17.5 ± 3.2 × 10^-4 (SE; n = 128 venules)] was significantly less than histamine alone treatments (P < 0.01). Similarly, with the fucoidin only treatment, the number of adherent leukocytes [6.7 ± 1.4 × 10^-4 (SE; n = 146 venules)] was significantly less than the histamine treatment (P < 0.01). The mean number of histamine-associated adherent leukocytes was reduced >80% by fucoidin treatment.

The effect of fucoidin treatment on the number of adherent leukocytes per micrometer of leaky venule length and on the number of adherent leukocytes per micrometer nonleaky venule length in venules of the various preparations is shown in Fig. 2. In leaky venules, the number of adherent leukocytes of both the fucoidin only treatment [26.4 ± 5.0 × 10^-4 (SE; n = 28 venules)] and the histamine-fucoidin treatment [24.7 ± 4.7 × 10^-4 (SE; n = 79 venules)] were significantly less than the histamine treatment [149.3 ± 18.2 × 10^-4 (SE; n = 78 venules; P < 0.01)]. Similarly, in nonleaky venules, the number of adherent leukocytes of both the fucoidin only treatment [2.0 ± 0.8 × 10^-4 (SE; n = 118 venules; P < 0.01) and the histamine-fucoidin treatment [5.9 ± 2.9 × 10^-4 (SE; n = 49 venules)] was significantly less than the histamine treatment [26.2 ± 9.4 × 10^-4 (SE; n = 39 venules; P < 0.01) and P < 0.05, respectively]. The numbers of adherent leukocytes per micrometer of venular length in nonleaky venules of all the individual treatments were significantly less than the respective leaky venule value (P < 0.01). Also, the mean values for both the leaky fucoidin treatment and

![Fig. 1. Average number (no.) of adherent leukocytes per length of venule (includes both leaky and nonleaky venules) for different treatments. Error bars indicate SE. *Fucoidin and histamine-fucoidin values are significantly less than histamine value (P > 0.01).](http://ajpheart.physiology.org/DownloadedFrom)
the leaky histamine-fucoidin treatment were similar to each other and to the histamine nonleaky value.

Effect of leukocyte adherence on the number of venular leaks and leak area

The effect of leukocyte adherence on the average number of leaks per micrometer of venule length for the different treatments is shown in Fig. 3. Although the average number of leaks of the fucoidin treatment \[10.3 \pm 2.2 \times 10^{-2} \text{ (SE); } n = 146 \text{ venules}\] was significantly less than the histamine treatment \[65.2 \pm 7.9 \times 10^{-2} \text{ (SE); } n = 117 \text{ venules; } P < 0.01\], the histamine-fucoidin treatment \[76.6 \pm 10.4 \text{ (SE); } n = 128 \text{ venules}\] was not significantly different from the histamine treatment.

The effect of leukocyte adherence on average leak area (µm²) per micrometer of venule length for the different treatments is shown in Fig. 4. The average leak area of the fucoidin treatment \[7.2 \pm 2.6 \times 10^{-2} \text{ (SE); } n = 146 \text{ venules}\] was significantly less than the histamine treatment \[54.1 \pm 14.1 \times 10^{-2} \text{ (SE); } n = 117 \text{ venules; } P > 0.01\]. The histamine-fucoidin treatment \[72.8 \pm 22.8 \text{ (SE); } n = 128 \text{ venules}\] was not significantly different from the histamine treatment.

Figure 5 demonstrates low-power views of the typical distribution of FITC-BSA leaks (A, C, and E) with rhodamine phalloidin-stained adherent leukocytes (B, D, and F). Both the histamine (A) and histamine-fucoidin (C) preparations contained numerous FITC-BSA leaks in contrast to infrequent leaks in the fucoidin preparations (E). In the histamine preparations (B), adherent leukocytes were identified in most, but not all, leaky vessels. Occasionally, adherent leukocytes were identified in nonleaky vessels in all three preparations. Adherent leukocytes were, typically, not identified in leaky histamine- and fucoidin-treated venules (D).

Effect of leukocyte adherence on endothelial actin cytoskeleton

When examined by confocal microscopy, the histamine and histamine-fucoidin preparations demonstrated similar actin cytoskeleton staining patterns, i.e., patterns that were similar to findings previously reported for 3-min histamine preparations (4). Likewise, the fucoidin preparations demonstrated an actin cytoskeleton staining pattern similar to control preparations, as reported previously (4). Briefly, histamine (Fig. 6A) and histamine-fucoidin preparations (Fig. 6C) demonstrated a similar leak distribution, with leaks coinciding with disruptions in the PAR (Fig. 6, B and D). Adherent leukocytes were frequently identified near, or within, leaks in the histamine preparations (Fig. 6B) yet were infrequently localized with venules in the histamine-fucoidin (Fig. 6D) or fucoidin only preparations. Fucoidin preparations (not shown) revealed endothelial cells with a prominent PAR surrounding the cell and apparently decorating the cell borders. In the fucoidin preparations, the PARs were smooth and continuous, as indicated by the normal actin pattern located between two histamine-induced disruptions in Fig. 6B.
DISCUSSION

It is well established that histamine triggers in vivo leukocyte rolling, via P-selectin, in postcapillary venules (2, 11, 12) and the potentiation of chemoattractant-induced leukocyte adhesion (14). Lindbom et al. (14) demonstrated a close relationship between the extent of rolling and the magnitude of firm adhesion in vivo, and they also demonstrated that rolling is a precondition for firm adhesion. After adhesion, leukocytes are activated by various chemoattractants. The $\beta_2$-integrins and immunoglobulin superfamily members, such as intercellular adhesion molecule-1 (ICAM-1), then

Fig. 5. Three representative low-power epifluorescence image pairs of FITC-BSA (A, C, and E)- and rhodamine phalloidin (B, D, and F)-stained histamine (A and B), histamine-fucoidin (C and D), and fucoidin (E and F) preparations. Histamine (A) and histamine-fucoidin (C) preparations demonstrate similar distribution of numerous FITC-BSA leaks (arrows), in contrast to fucoidin preparation (E). Adherent leukocytes (arrowheads) are typically identified in leaky venules of histamine preparation (B); however, some leaky venules (asterisk) are devoid of adherent leukocytes. Leaky venule (asterisk) in the histamine-fucoidin (D) and nonleaky venule (asterisk) in the fucoidin preparation (F) are devoid of adherent leukocytes. Scale bar = 20 µm.
interact to arrest leukocyte rolling and mediate adhesion between activated leukocytes and vascular endothelium. This eventually leads to diapedesis between endothelial cells (13).

In a previous study by Baldwin and Thurston (4), the time course effect of histamine on endothelial actin cytoskeleton and leak formation was investigated. It was shown for the first time that at 3 min of histamine application adherent leukocytes were identified, coincident with leak formation and endothelial actin cytoskeleton changes. The number of adherent leukocytes was decreased by 20 min. In the present study, we investigated the relationship of early (3-min), transient leukocyte adhesion with the coincident formation of venular leaks.

It is not surprising that we blocked early leukocyte adhesion with the selectin-binding carbohydrate fucoidin. P-selectin surface expression is virtually the earliest inducible event in the inflammatory cascade and, thus, most likely coincides with early leukocyte adhesion. This molecule is rapidly (within minutes) translocated to the endothelial surface by histamine from a preformed state in Weibel-Palade bodies (7). Similarly, fucoidin has been used to block both in vivo leukocyte rolling and leukocyte adhesion (3, 11).

The approach we used to examine the effect on leukocyte adhesion was to examine our preparations only after they had been fixed. The technique allows for a precise concentration of histamine to be perfused throughout the microcirculation of a small region of intestine. This ensures that the endothelia of all vessels in a given mesenteric network receive the same histamine dosage, almost simultaneously. In most other studies, histamine is added to the suffusate, possibly allowing for irregularities in delivery because of differences in vascular wall thickness. Kubes and Kanwar (11) added histamine to the suffusate in their stabilized mast cell leukocyte rolling model. It is interesting that they observed leukocyte adhesion only at 60 min of histamine application and not at earlier time points. They reasoned that histamine possibly stimulated the synthesis and expression of platelet-activating factor (PAF), allowing for the activation of leukocytes. Their reasoning was based on recent reports that histamine can stimulate the rapid synthesis and expression of endothelial PAF (24). However, recent studies clearly demonstrated that PAF-induced leukocyte adherence could be attenuated by pretreatment with monoclonal antibodies against CD18, CD11b, ICAM-1, and E-selectin but not with P-selectin antibodies (23). Be-
Also, in other in vivo studies using preparations similar to our study (2, 11, 12), the effects of histamine on leukocyte rolling and adhesion focused on time points after 15–20 min of histamine application, using either suffused histamine (2, 11) or intraperitoneal histamine (12). Again, our previous study clearly demonstrated that early histamine-induced leukocyte adhesion is transient and is reduced by 10–20 min (4), at time points not specifically addressed in other studies. Kanwar (11) reported that, due to spontaneous rolling, fucoidin treatment had no effect on histamine-induced vascular leak formation. They also focused on time points after 15 min of histamine application. Using a rabbit skin model, Arfors et al. (1) demonstrated that prevention of leukocyte adhesion with fucoidin had no effect on histamine-induced vascular leakage, however, after 4 h of histamine application.

An earlier study (5), using the hamster cheek pouch model, demonstrated that anti-neutrophil serum (ANS) depletion of circulating neutrophils had no effect on vascular permeability after as early as 3 min of topical histamine application. However, there are several significant differences between this study and ours. The study by Bjork et al. (5) demonstrated a significant decrease in the number of vascular leaks after 5–10 min of histamine application, whereas our previous study (4) demonstrated an increase in the number of vascular leaks at 3 min, which persisted for at least 30 min. They used FITC-dextran 150 (molecular weight 150,000) to detect vascular leaks, whereas we used FITC-BSA, potentially a more sensitive indicator of vascular leakage. Also, the study by Bjork et al. (5) was complicated by the topical application of leukotriene B₄ (LTB₄) ~30 min before the histamine application. LTB₄ is a powerful chemoattractant. Although Bjork et al. clearly demonstrated a depletion of neutrophils with ANS in the vascular pool, it is possible that LTB₄ attracted neutrophils to the endothelium from adjacent tissue sites, where neutrophils were not subjected to ANS. Finally, differences in vascular permeability between the hamster cheek pouch model and the rat mesenteric model have been documented (10).

Various techniques have been developed to overcome the effects of spontaneous leukocyte rolling. This refers to rolling caused by the manipulation of tissue during preparation, making the assessment of experimentally induced rolling and later events difficult. There are reported differences in the kinetics of spontaneous rolling and the methodologies for its control. Kubes and Kanwar (11) reported that, due to spontaneous rolling, histamine-induced rolling could be demonstrated only by stabilizing the mast cell population with chromomycin before manipulation. Because, in our previous study (4), early histamine-associated adhesion was not observed in the untreated preparations, it is unlikely that spontaneous rolling was a factor. Ley (12) demonstrated that spontaneous leukocyte rolling could be minimized for the first several minutes, suggesting that, even without mast cell stabilization, P-selectin had not become overly expressed on the cell surface. Similar to our earlier study, leukocyte adhesion was not identified at 15–30 min of histamine application. Other investigators were able to reduce spontaneous rolling for at least several minutes without stabilizing the mast cell population (12, 22).

In this study, we demonstrated that, in the same preparation, the number of adherent leukocytes in leaky vessels was significantly greater than the number of adherent leukocytes in nonleaky vessels. Although fucoidin pretreatment significantly decreased histamine-associated leukocyte adhesion, this resulted in no significant change in either the number of leaks or the leak area, suggesting that histamine-associated adhesion had no effect on leak formation. Indeed, the numbers of adherent leukocytes in leaky vessels in both the fucoidin only and histamine-fucoidin treatments were similar to the number of adherent leukocytes in nonleaky histamine-treated preparations. Also, leukocyte adhesion resulted in no visible alterations in the endothelial actin cytoskeleton.

It is highly likely that P-selectin expression is responsible for the early transient histamine-associated leukocyte adherence. After histamine application, P-selectin is rapidly expressed (maximally at 3 min) on the endothelial cell surface. Using immunofluorescence microscopy at this time point, P-selectin expression has a punctate, focal surface distribution, rather than a diffuse surface distribution (7). Immunoelectron microscopy studies, after 1 min of histamine application, demonstrated increased microvilli on the endothelial surface, with P-selectin expression frequently located on the microvilli surface (16). This relatively focal concentrated surface distribution may facilitate the firm early adhesion of a population of circulating leukocytes, as the leukocytes sample the endothelial surface. It has also been reported that the surface expression of P-selectin declines to control levels by 20 min (7), coincident with low levels of adhesion described in our earlier publication (4) and in other publications (2, 11, 12). P-selectin surface expression declines without the chemoattractant stimulation of other surface factors, and leukocytes are released.

In the present study, we examined, for the first time, the relationship of early, transient leukocyte adherence, coincident with the formation of venular leaks and endothelial actin cytoskeleton changes. We demonstrated that the selectin-binding carbohydrate fucoidin significantly reduced the number of early 3-min histamine-associated adherent leukocytes. The reduction in leukocyte adherence was not associated with a reduction in the number of leaks, the size of the leaks, or changes in endothelial actin cytoskeleton. There were significantly greater numbers of adherent leukocytes identified in leaky venules compared with nonleaky venules. Although it appears that leukocyte adherence does not cause vascular leakage, it is possible that compromised endothelial cell barrier function can influ-
ence leukocyte adhesion. Further studies to investigate this alternate scenario would be interesting. In summary, although coinciding with the appearance of 3-min histamine-associated vascular leaks and endothelial actin cytoskeleton changes, the changes were not influenced by leukocyte adherence.

This work was in part supported by the American Heart Association, Arizona Affiliate, and the Arizona Disease Control Research Commission.

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Received 1 October 1998; accepted in final form 19 March 1999.

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