Leucocyte/endothelium interactions and microvessel permeability: coupled or uncoupled?

Pingnian He*

Department of Physiology and Pharmacology, School of Medicine, West Virginia University, Morgantown, WV 26506-9229, USA

Received 11 January 2010; revised 4 May 2010; accepted 7 May 2010; online publish-ahead-of-print 13 May 2010

In response to infections or tissue injury, circulating leucocytes adhere to and migrate from the vessel lumen to interstitial inflammatory sites to combat invading pathogens. However, these defensive actions may also cause host tissue injury and microvascular dysfunction through oxidative bursts or enzyme release. For decades, the interaction between leucocytes and microvessel walls has been considered as a critical event leading to organ dysfunction. Extensive investigations have therefore focused on blocking specific adhesive ligands to prevent tissue injury. However, anti-adhesion therapies have shown limited success in preventing vascular dysfunction in clinical trials. Numerous studies have demonstrated temporal and spatial dissociations of leucocyte adhesion and/or emigration from permeability increases. The mechanisms that initiate the adhesion cascade have been found to be distinct from those that trigger the leucocyte oxidative burst responsible for increasing microvessel permeability. Recent studies demonstrated that endothelial activation by inflammatory mediators is critical for initiating platelet adhesion and platelet-dependent leucocyte recruitment resulting in augmented increases in microvessel permeability. These new developments suggest that targeting endothelial activation via directly enhancing endothelial barrier function might be a more efficient strategy than focusing on anti-adhesion or platelet/leucocyte depletion to prevent vascular damage during inflammation. Owing to space limitations and the wide range of studies in the field, this article will not serve as a comprehensive review. Instead, it will highlight the emerging evidence of adhesion-uncoupled permeability changes and establish a basis for re-evaluating the coupled relationship between leucocyte/platelet activation and microvessel permeability to achieve a better understanding of permeability regulation during inflammation.

Keywords
Microvessel permeability • Platelet adhesion • Platelet/leucocyte aggregation • Neutrophil ROS release • Leucocyte adhesion and migration

This article is part of the Spotlight Issue on: Microvascular Permeability.

1. Introduction
For decades, whether leucocyte/endothelium interactions directly cause increased microvessel permeability has been controversial. Certain studies suggested leucocyte adhesion and emigration to be the critical event leading to tissue and organ dysfunction during inflammation and ischaemia–reperfusion, as blocking antibodies or induction of neutropenia have been shown to prevent vascular damage. However, when vascular permeability was evaluated simultaneously with leucocyte/endothelium interactions, the sites of albumin leakage were often distinct from those of leucocyte adhesion and emigration. Several studies have also reported temporal dissociations of leucocyte adhesion and/or emigration from permeability increases. In addition, therapies targeting leucocyte adhesion to prevent vascular dysfunction have had limited success in clinical trials. These developments suggest that mechanisms other than leucocyte adhesion may be critical to the regulation of vascular leakage and barrier dysfunction during inflammation. This review will highlight adhesion-uncoupled changes in microvessel permeability as well as leucocyte/platelet-coupled permeability changes, while emphasizing mechanisms different from traditional views of adhesion-mediated coupling. By focusing on emerging evidence, this article attempts to clarify and consolidate some conflicting issues in the field, aiming for a better understanding of leucocyte/endothelium interactions and their effects on microvessel permeability during inflammation.

2. Leucocyte adhesion is uncoupled from increases in microvessel permeability
Integrin-mediated firm adhesion of neutrophils to cultured endothelial layers has been reported as the trigger for a cytokine-induced respiratory burst of neutrophils, which causes direct injury to endothelial cells. Although prevention of leucocyte adhesion and induction of neutropenia have exhibited some protection of the vascular barrier functions during

---

* Corresponding author. Tel: +1 304 293 1515, Fax: +1 304 293 3850, Email: phe@hsc.wvu.edu

Published on behalf of the European Society of Cardiology. All rights reserved. © The Author 2010. For permissions please email: journals.permissions@oxfordjournals.org.
reperfusion and acute inflammation,\textsuperscript{2,4–6,9,29} two lines of evidence challenge the view that leucocyte adhesion is directly associated with tissue damage during inflammation: (i) there are temporal and spatial dissociations of protein leakage from leucocyte adhesion and emigration in vivo\textsuperscript{19–22,30,31} and (ii) there are distinct mechanisms of initiating adhesion from that of inducing leucocyte oxidative burst.\textsuperscript{32–34}

2.1 Temporal and spatial uncoupling between leucocyte adhesion and protein leakage in the presence of inflammatory stimuli

Increases in microvessel permeability in the presence of inflammatory mediators are often accompanied by increased leucocyte adhesion and migration. It has been well documented that inflammatory mediators can increase microvessel permeability in the absence of leucocyte adhesion.\textsuperscript{35–37} Thus, in the presence of blood cells, the direct effect of inflammatory mediators on endothelial cells and the contributions of leucocyte adhesion to microvessel permeability are easily confounded. In fact, several studies have shown that the timing and sites of leucocyte adhesion and migration are not well correlated with vascular leakages.\textsuperscript{19–22,38}

The spatial uncoupling of leucocyte adhesion and protein leakages has been demonstrated by a series of studies on rat trachea using combined silver nitrate staining with protein tracer to identify endothelial gaps and the sites of adherent or migrating leucocytes concurrent with protein leakages.\textsuperscript{19,20,30} After the venules were exposed to inflammatory stimuli, most of the adherent or migrating leucocytes were found at intercellular junctions but few were accompanied by protein leakage. The sites of leucocyte adhesion and migration were distinct from 94% of endothelial gaps. Similar observations were also reported in several early studies in vessels of rat skin and hamster cheek pouch using intravital microscopy.\textsuperscript{21,24}

The temporal uncoupling between leucocyte adhesion and vascular leakages has also been reported. When rat venules were exposed to histamine, both leakage and leucocyte adhesion occurred in the early phase of exposure but leakages lasted longer than the transient leucocyte adhesion. Moreover, blocking leucocyte adhesion showed no effect on leakage formation,\textsuperscript{22} indicating that the adhesion process is not the main contributor to increased permeability. Furthermore, although both platelet-activating factor (PAF) and leucotrine B4 (LTB4) induced leucocyte adhesion, increases in fluid filtration were observed only in PAF-stimulated tissues.\textsuperscript{29,31} Different effects of PAF and LTB4 on endothelial cells may explain the differences in fluid filtration. PAF can directly increase vascular permeability in the absence of blood cells,\textsuperscript{37} but not LTB4.\textsuperscript{37} Taken together, these studies suggest that leucocyte adhesion alone is not sufficient to increase microvessel permeability and that the activation of endothelial cells by specific mediators plays an essential role. Although these dissociations have been observed for decades, the underlying mechanisms remain obscure.

2.2 Differential mechanisms of initiating leucocyte adhesion and increases in microvessel permeability: leucocytes can adhere to microvessels without increased permeability

In addition to the established dissociations between leucocyte adhesion and protein leakage in the presence of inflammatory stimuli, recent studies suggest that adhesion is governed by mechanisms different from those inducing permeability increases and can thus occur in microvessels without increased permeability.\textsuperscript{34} Using combined autologous blood perfusion with permeability measurements in individually perfused microvessels, systemic administration of tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) in rats increased the expression of both neutrophil CD11/CD18 and endothelial intercellular adhesion molecule-1 (ICAM-1) and promoted leucocyte adhesion to venular walls (Figure 1). However, neither hydraulic conductivity, \(L_p\), nor solute permeability increased after leucocyte adhesion. Meanwhile, perfusion with the same concentration and duration of TNF-\(\alpha\) in a single mesenteric venule in the absence of blood showed no effect on basal permeability, indicating that despite its potency in inducing leucocyte adhesion, TNF-\(\alpha\) does not cause direct activation of endothelial cells under these experimental conditions. Thus, the differential actions of TNF-\(\alpha\) on leucocyte adhesion and microvessel permeability separate the direct effect of leucocyte adhesion on microvessel permeability from that of endothelial activation by inflammatory mediators. The absence of permeability increases with adherent leucocytes also indicates that the firm adhesion of leucocytes to the microvessel wall does not trigger neutrophil respiratory burst, as reactive oxygen species (ROS) release would have induced an increase in permeability. Consistent with these permeability coefficient measurements, silver staining of endothelial boundaries showed continuous silver lines without silver dot deposition beneath adherent leucocytes.\textsuperscript{34} These results also agree with studies conducted on rat trachea that showed no correlation between adhesion sites and endothelial gaps.\textsuperscript{30} Collectively, these results indicate that the stimuli required for endothelial cell activation resulting in permeability increases are different from those that elicit leucocyte adhesion. Although inflammatory mediator-induced increases in permeability may not lead to increased leucocyte adhesion in microvessels, the implications for clinical practice are significant. The ability to activate endothelial cells in the absence of leucocyte adhesion may have important therapeutic implications, particularly in conditions where leucocyte adhesion is undesirable, such as in sepsis or acute inflammation.

Figure 1 Video images of a rat mesenteric venule before and after leucocyte adhesion induced by systemic application of TNF-\(\alpha\). TNF-\(\alpha\) induced significant leucocyte adhesion (19 leucocytes/100 \(\mu\)m of vessel length, right image) without increasing microvessel permeability. The hydraulic conductivity (\(L_p\)) measured before (control, left image) and after leucocyte adhesion (right image) was 3.5 \(\pm\) 0.3 and 3.6 \(\pm\) 0.5 (SD) \(\times\) 10\(^{-7}\) cm/s/cmH2O, respectively. With a similar number of adherent leucocytes to that shown in the right image, the measured permeability coefficient to \(\alpha\)-lactalbumin was also not altered from that of the control (from Zeng et al.\textsuperscript{34} and used with permission).
promote leucocyte adhesion, TNF-α-induced leucocyte adhesion clearly demonstrates that leucocytes can adhere to microvessels in the absence of increased permeability. The types and threshold of stimuli required to trigger ROS release from adherent leucocytes are different from those that regulate leucocyte adhesion. The conditions required for inducing neutrophil oxidative burst and ROS-induced increases in microvessel permeability are further discussed in Section 4.

3. Leucocyte migration is uncoupled from increases in microvessel permeability

Leucocyte migration is a critical step in white cell mobilization from vessel lumen to sites of tissue infection. Openings at endothelial junctions and/or damage to vascular walls during leucocyte migration were assumed to cause increased transport of fluid and large molecules. Thus, leucocyte adhesion and migration have been proposed as a prerequisite for permeability increases. However, in the absence of endothelial activation by inflammatory mediators, leucocyte transmigration has been shown to involve only a transient opening of adherent junctions and the quick reassembly of adherent junctions results in minimal or no apparent macromolecule leakage during the migration process. 

3.1 Uncoupling of leucocyte migration and permeability increases at the level of single vessels and tissues

The uncoupling of leucocyte migration and permeability increases have been demonstrated with direct measurements of permeability coefficients during leucocyte transmigration in individually perfused microvessels. Systemic injection of TNF-α increased leucocyte adhesion but not emigration and permeability. When a low concentration of the chemotactic peptide formyl-Met-Leu-Phe-OH (fMLP, 1 μM) was applied to the surrounding tissue in the presence of adherent leucocytes, leucocyte emigration increased but Lp measured during leucocyte emigration was unchanged, suggesting that the leucocyte emigration is not sufficient to cause a measurable permeability increase at the level of single vessels. The observed uncoupling between leucocyte migration and permeability increases may be explained by a quickly reversible disruption of endothelial junction integrity during leucocyte migration, particularly if emigration is asynchronous.

A recent study using an aseptic cutaneous wound model provided additional evidence of the temporal and spatial uncoupling between neutrophil extravasation and increases in vascular permeability. Maximum increases in permeability occurred 6 h ahead of the peak neutrophil influx. Additionally, extravasated EGFP-PMNs were visualized from the wound centre than that of accumulated fluorescence-labelled albumin. Most importantly, neutrophil depletion did not affect the magnitude of permeability increases 24 h after wounding, indicating that the endothelium is the primary regulator of fluid and protein transport. Application of Rho-kinase inhibitor to the wound reduced permeability increases by 70% but had no effect on neutrophil accumulation. These results suggest that the increased permeability was mainly mediated via direct activation of endothelial cells through Rho-dependent signalling and unaltered by neutrophil recruitment.

3.2 Uncoupling of leucocyte migration and macromolecule leakage at migration sites

Structural confirmation of the global uncoupling of permeability increases and transmigration observed in single vessels and whole tissues are provided by ultrastructural evidence at the migration site. Application of LTB4 to hamster cheek pouch induced significant neutrophil adhesion to and emigration across microvessel walls in 15 min, but albumin leakage was limited. Electron micrographs shown in Figure 2 illustrate the multistep process of emigration and its influence on macromolecule transport. Emigration was initiated by neutrophil pseudopod insertion, mostly through inter-endothelial junctions. During emigration, endothelial cells maintained intimate contact with the emigrating cells. Following emigration, endothelial cells quickly resealed the barrier by forming endothelial bridges around neutrophils. Most interestingly, after penetrating endothelial cells and endothelial basal lamina, emigrating neutrophils crawl along the abluminal space between the endothelial basal lamina and the luminal side of the surrounding pericytes before reaching the interstitium. The intimate contact between neutrophils and endothelial cells during the entire emigration process provides an ultrastructural explanation for the limited transendothelial escape of macromolecules. An experiment conducted in rabbit skeletal muscle also support these findings, as application of LTB4 did not result in endothelial gap formation and LTB4-induced leucocyte diapedesis showed no damage to the vasculature. It is worth noting that like most cytokines, LTB4 has been reported to only prime neutrophils and does not directly trigger respiratory burst from adherent or emigrating leucocytes. This may explain the discrepancy in barrier function changes when using other stimuli, such as fMLP, which are capable of directly triggering ROS release.

3.3 Molecular basis of the uncoupling between leucocyte migration and permeability increases

Recent studies using more advanced genetic and immunofluorescence approaches have revealed the molecular basis for earlier observations on leucocyte migration. Live cell imaging of transduced vascular endothelial-cadherin/GFP fusion construct during leucocyte migration across endothelial monolayers demonstrated that paracellular gaps formed only during leucocyte transmigration and were not associated with leucocyte adhesion. Altered vascular endothelial-cadherin during migration quickly diffused back to reseal the gap. The kinetics of these local structural changes is inconsistent with proteolysis. Confocal microscopy studies in cultured cells found that leucocyte transmigration through either transcellular or paracellular pathways were associated with the formation of endothelial ‘cuplike’ structures enriched in ICAM-1. These structures may provide directional guidance for transmigration, as disruption of cup formation inhibited the transmigration. An in vivo study in chemokine-treated mice cremaster vasculature revealed that emigrating neutrophils were completely encapsulated by endothelial dome structures. This intimate contact between neutrophils and endothelial cells may function to limit plasma protein leakage. Moreover, a recent confocal immuno-fluorescence study in mice cremaster venules demonstrated that
emigrating neutrophils preferentially exited in vessel regions with low matrix protein expressions between pericyte coverage. \(^{45}\) This finding is well correlated with the observations shown in Figure 2, as neutrophils crawling along in the space between endothelial basal lamina and surrounding pericytes could be searching for a low matrix protein region for egress. This final step of transmigration can also be predicted with no large local leakage because the endothelial cells may have quickly resealed the barrier, whereas the emigrated cell starts to penetrate through low matrix protein regions between pericyte coverage.

### 3.4 Leucocyte emigration through activated endothelial cells

In most of the studies addressed above, endothelial cells were exposed to certain cytokines or chemokines that promote the expression of adhesion molecules and leucocyte/endothelium interactions without directly activating endothelial cells and without increasing permeability. These studies effectively isolate the effect of leucocyte adhesion and emigration on endothelial barrier function from the direct effect of inflammatory mediators on endothelial cells.

Although under ischaemia and reperfusion\(^{1,9}\) or other inflammatory conditions,\(^{23,43,46}\) a significant increase in neutrophil adhesion and emigration was usually observed with vascular leakages, the magnitude of permeability increases was not correlated with the number of adherent and emigrated leucocytes.\(^{22,23}\) Furthermore, inhibiting leucocyte adhesion and emigration with anti-adhesion antibodies or neutrophil depletion could not completely prevent venular leakages.\(^{1,22,23,43}\) Under these conditions, endothelial cell activation and/or ROS release from adherent or emigrating neutrophils may all contribute to barrier dysfunction and permeability increases. Interestingly, under these conditions, endothelial bridges forming around emigrating neutrophils were shown as incomplete and junctional contacts were either unable to be reestablished or were achieved at a slower pace.\(^{1,43}\

These observations suggest that endothelial cells activated by agonists or by ROS release from activated leucocytes have altered junctional structures that may act differently from inactivated endothelial cells during leucocyte emigration. Under these conditions, emigrating leucocytes may aggravate already weakened endothelial junctions, resulting in fluid and protein leakages at emigration sites.
4. Leucocyte-dependent permeability increases: role of released ROS from primed neutrophils

In response to a variety of stimuli, neutrophils release large quantities of ROS through respiratory burst, which is essential for killing invading microorganisms but can also cause tissue damage. Although this process has long been studied, the stimuli required for full neutrophil activation remain controversial. Currently, most studies have been conducted in vitro and little information is available on how neutrophil respiratory burst is regulated in vivo and how it relates to microvessel permeability.

4.1 Additional stimuli are necessary to trigger respiratory burst from adherent leucocytes resulting in ROS-induced increases in microvessel permeability

Leucocyte adhesion induced by certain mediators, such as LTB4 or TNF-α, has been shown to cause neither increases in permeability coefficients nor vascular leakages in whole vascular beds, indicating that adhesion may not directly trigger leucocyte respiratory burst. Otherwise, ROS released either locally or to the blood stream might have increased permeability. Chemiluminescence (CL) measurements in isolated neutrophils, an assessment of oxidative activity of the cells, showed no ROS production in either LTB4 or TNF-α stimulated neutrophils, suggesting that mediators promoting adhesion may not necessarily be sufficient to induce neutrophil respiratory burst. Studies in intact microvessels further evidenced that adhesion and the respiratory burst activity are independent processes requiring different stimuli. Following systemic application of TNF-α to induce leucocyte adhesion without increases in permeability, application of 10 μM fMLP, which can trigger neutrophil ROS release without direct effects on endothelial cells, induced increases in microvessel permeability within 5 min of fMLP addition. The time course was well correlated with that of fMLP-induced CL responses in isolated neutrophils. More importantly, the magnitudes of peak $I_p$ increases upon fMLP stimulation linearly correlated with the number of adherent leucocytes at venular walls. The application of superoxide scavenger, SOD, completely inhibited the permeability increases, indicating that superoxide and superoxide-derived oxidant radicals released from adherent leucocytes are directly responsible for the permeability increases. The fMLP-induced adherent leucocyte-mediated permeability increases are different in time course and concentration requirements than those for inducing leucocyte migration. A significant number of transmigrated leucocytes was only observed after 1–2 h of fMLP application to the surrounding tissues at a low dose of 1 μM that did not increase permeability. These results indicate that TNF-α-induced adherent leucocytes can be further activated by additional stimuli, such as fMLP, resulting in ROS-induced increases in microvessel permeability. These observations are also supported by in vitro studies, reporting that the ligation of integrins alone was insufficient for full activation of neutrophils without initiating a second activation signal by another inflammatory stimulus. Taken together, these studies suggest that leucocyte adhesion and mediators that promote leucocyte adhesion can be independent from those that trigger ROS production. Therefore, under certain conditions, additional stimuli are required for NADPH oxidase activation from adherent leucocytes and increased permeability.

4.2 Priming roles of cytokines in leucocyte ROS production

Although neither exposure of isolated neutrophils to TNF-α alone nor TNF-α-induced leucocyte adhesion in intact microvessels is sufficient to induce a leucocyte respiratory burst and permeability increases, the actions of TNF-α on leucocytes are not completely independent from their oxidative responses. It has been reported that full oxidative neutrophil response requires pre-exposure to a priming agent. Priming agents do not elicit effector function on their own; instead, they potentiate other stimulus-induced cellular responses. Many cytokines and pro-inflammatory mediators have been reported as effective neutrophil primers, but the phenomenon of priming in vivo has not been well characterized. One recent study showed that pre-incubation of isolated neutrophils with TNF-α did not induce ROS release as measured by CL activity, but markedly augmented superoxide production in response to other mediators, such as fMLP, in a dose and incubation time-dependent manner. Application of anti-TNF MoAb blocked TNF-α-mediated neutrophil priming. The in vivo priming effect was further demonstrated with neutrophils isolated from TNF-α-treated animals. Whereas neutrophils exposed to TNF-α in vivo did not show increased ROS production without additional stimuli, this pre-treatment potentiated fMLP-stimulated ROS production by three-fold. These results indicate that systemically applied TNF-α not only promotes leucocyte adhesion, but also primes neutrophils regardless of their adhesion status, thus increasing their potential for augmented ROS release when additional stimuli are present. Priming-induced enhanced neutrophil respiratory burst in the presence of other stimuli in vivo provides a mechanistic explanation for the clinical observations that high plasma levels of cytokines and/or circulating endotoxin are associated with adult respiratory distress syndrome, organ failure, and mortality. Together, these studies indicate that enhanced ROS release upon exposure of primed adherent leucocytes to additional stimuli may increase the antimicrobial activity of neutrophils but can also aggragate tissue damage.

Although neutrophil priming has long been recognized, the underlying mechanisms are not well understood. Several studies have indicated that both receptor and post-receptor regulations are involved in the priming process. Specifically, phosphatidylinositol 3-kinase signalling has been implicated as a key regulator of C5a-mediated priming effects on neutrophil and monocyte oxidative burst activity. Phosphorylation-induced conformational changes of p47phox appear to be essential for mobilizing cytosolic components to the plasma membrane to interact with membrane components of NADPH oxidase, which results in the generation of large quantities of ROS in response to additional stimuli. A better understanding of the mechanisms that regulate neutrophil NADPH oxidase is crucial for attempts to modulate neutrophil activation and prevent tissue injury caused by enhanced ROS release during acute inflammation. Details about potential signalling pathways can be found in related articles.
4.3 Roles of activated circulating blood cells in microvessel permeability

For decades, neutrophil/endothelium interactions have been considered a hallmark of acute inflammatory responses. However, a significant portion of responsive leukocytes remains in the peripheral circulation during inflammation. On the basis of the isolated neutrophil studies, oxidant burst can be independent of adhesion. Therefore, as long as the appropriate stimuli are present in the vasculature, ROS can be released from activated non-adherent neutrophils and the increased ROS in the plasma may play important roles in activating endothelial cells, resulting in increased microvessel permeability. This mechanism has been validated in vivo with single-vessel perfusion experiments. Perfusing each vessel with fMLP-stimulated neutrophils in suspension elicited neutrophil concentration-dependent increases in endothelial [Ca2+]i and microvessel Lp. The increased Lp was abolished by ROS scavenger or antioxidant agents, indicating that increases in microvessel permeability are directly associated with the released ROS from activated neutrophils and that a physical interaction between neutrophils and endothelium is not necessary. C3a-activated neutrophils have also been reported to increase permeability in isolated coronary venules in the absence of adhesion. The enhanced fMLP-induced ROS production from neutrophils isolated from TNF-α-treated animals further supports the hypothesis that cytokine priming-induced enhanced ROS production occurs in circulating leukocytes. Linking these observations with the neutropenia studies, it is speculated that the observed beneficial effect of neutropenia on vascular leakages may not be exclusively due to the absence of adherent leukocytes. Instead, it may result from the absence of activated leukocytes in the circulating pool and the reduced ROS level in the plasma. This may also explain the dissociation between leukocyte adhesion and fluid filtration observed in neutropenic rats exposed to PAF. In the aforementioned study, PAF-induced increases in fluid filtration were observed mainly in capillaries, whereas PAF-induced neutrophil adhesion occurred in venules. Interestingly, neutrophil depletion attenuated PAF-induced capillary filtration, which may suggest that the neutrophilic effect on fluid filtration may possibly be attributed to a reduced circulating level of oxidants.

5. Roles of platelets in leucocyte recruitment and microvessel permeability during inflammation

The essential function of platelets is haemostasis. Under normal physiological conditions, circulating platelets do not adhere to vascular walls. However, at sites of endothelial damage, haemostasis is initiated by the interaction of platelets with the exposed subendothelial matrix. The platelet/subendothelial matrix interaction has also been recognized as critical for initiating the development of atherosclerosis, in which endothelial cells damaged by lipid oxidation trigger platelet adhesion, resulting in subsequent thrombosis and atherosclerotic plaque formation in inflamed arterial walls. There is increasing evidence that platelets also play important roles in leucocyte recruitment and ROS-induced permeability increases in venules under a variety pathological conditions, such as ischaemia/reperfusion, stroke, and cigarette smoke-induced inflammation. However, the mechanisms of platelet adhesion and their impact on permeability in inflamed venules remain to be elucidated.

5.1 Endothelial gap formation is the initiating step for platelet interaction with venular endothelial cells

As discussed previously, leucocyte/endothelium interactions in the absence of additional stimuli that trigger oxidative burst are not associated with endothelial gap formation and subsequent increases in microvessel permeability. The molecular basis of platelet activation apparently differs from that of leucocytes. Although platelets can be activated by certain agonists such as ADP, thrombin, or activated leucocytes in the circulation, the firm adhesion of platelets to large vessels during haemostasis or atherosclerosis relies on the interaction of platelet membrane glycoprotein with matrix ligands such as collagen and laminin at damaged sites of the subendothelial lamina. However, in inflamed venules, platelet/endothelium interactions have been reported to be mediated by P-selectin.

By dissecting the individual steps resulting in platelet/leucocyte aggregate adhesion in individual vessels, recent studies indicate that the mechanisms that initiate platelet adhesion during haemostasis or atherosclerosis in large vessels also apply to inflamed venules. The results demonstrated that unlike leucocyte adhesion that can occur in vessels without increased permeability, platelet adhesion requires an increase in microvessel permeability. As illustrated in Figure 1, systemic application of TNF-α that did not increase microvessel permeability induced only non-aggregated leucocyte adhesion without the involvement of platelets. In contrast, adding PAF to TNF-α-exposed vessels changed the adhesion pattern from single leucocytes to leucocyte/platelet aggregates. As illustrated in Figure 3, PAF is known to induce transient endothelial gap formation that exposes endothelial basal lamina. The causal relationship between endothelial gap formation and platelet adhesion is supported by the observation that inhibition of the initial permeability increases by applying a cAMP-enhancing agent prior to the application of PAF.

Figure 3 Electron micrographs demonstrating PAF-induced endothelial gap formation and the exposed endothelial basal lamina in rat mesenteric venules. The top graph illustrates an intact endothelial junction from a microvessel perfused with albumin-Ringer’s solution. The vessel shown in the bottom graph was perfused with PAF plus fluorescence microspheres (FM, 100 nm) for 10 min before fixation and demonstrates PAF-induced endothelial gap formation. The accumulated FMs at inter-endothelial junctions (arrows with a solid line) were retained by intact basal membrane underneath the gaps (arrows with a dotted line) that may be the initiation sites for platelet interaction (from Jiang et al. and used with permission).
was sufficient to prevent the adhesion pattern of aggregated platelet/leucocyte (Figure 4A) and the prolonged permeability increase. Instead single adherent leucocytes were observed (Figure 4B) and no changes in $L_p$ occurred. In these studies, PAF was thought to act mainly on endothelial cells rather than platelets, since rat platelets have low affinity for PAF binding. Furthermore, no platelet adhesion was observed when activated platelets were placed in normal recipient mice, indicating that activated platelets alone are not sufficient for platelet/endothelial interactions.

In addition to subendothelial matrix-mediated platelet adhesion, both platelet and leucocyte recruitment in venules have been reported as a P-selectin-mediated process in colitis and hypercholesterolaemia mice, based on the results of blocking antibodies and the use of P-selectin-deficient mice. It is important to further investigate the relative contributions of P-selectin-mediated and subendothelial matrix-mediated platelet adhesion when endothelial gaps form during inflammation.

5.2 Platelet endothelial interactions recruit additional leucocytes to the inflamed vessels

Platelets can interact with vascular walls via exposed subendothelial matrices and also with neutrophils through a P-selectin-mediated process. Although some studies reported leucocyte-dependent platelet adhesion in postischaemic venules, more recent experimental evidence indicates that the adhesive properties of adherent platelets to inflamed venules is crucial for the recruitment of additional leucocytes, resulting in platelet/leucocyte aggregates and amplified ROS-induced vascular injury.

In an aseptic cutaneous wound study, neutrophil depletion showed no effect on the magnitude of permeability increases 24 h after wounding. However, depletion of circulating platelets reduced permeability increases by 40% and also reduced neutrophil accumulation by 50% 24 h after wounding. These findings suggest that the initial permeability increase-induced platelet activation is essential for leucocyte recruitment and that platelet-dependent neutrophil recruitment contributes to the late-phase increases in microvessel permeability. In inflamed colonic venules, anti-platelet serum treatment dramatically reduced leucocyte adhesion, whereas neutropenia, induced by anti-neutrophil serum, did not prevent platelet adhesion. Thus, neutrophil recruitment depends on platelets, whereas platelet adhesion can be independent from the presence of neutrophils. Platelet-dependent leucocyte recruitment has also been reported in inflamed brain microvessels, lymphocyte delivery to high endothelial venules (leucocyte homing), and atherosclerosis development. In vitro experiments have also demonstrated platelet-dependent leucocyte recruitment. After perfusing blood through a chamber coated with cultured endothelial cells, platelets were found to mainly adhere to partially exposed subendothelial matrix, whereas neutrophils preferentially attached to matrix-adhered platelets rather than cultured endothelial cells. Thus, subendothelial matrix-mediated platelet adhesion may serve as a bridge for the endothelium to recruit circulating neutrophils through selectin and integrin-dependent adhesive interactions. Detailed molecular mechanisms of platelet-mediated leucocyte recruitments are further described in related articles.

5.3 Platelet/leucocyte aggregate adhesion induces amplified ROS production and sustained increases in microvessel permeability

Under a variety of pathological conditions, including cigarette-induced inflammation, ischaemic stroke, and reperfusion models, leucocytes did not adhere to the endothelium as single cells, but instead as aggregates held together by activated platelets. Platelet/leucocyte aggregate adhesion and aggregate formation in peripheral blood has been considered a biomarker for determining the severity of tissue injury. However, correlative permeability changes under those conditions have not been measured, especially in comparison to other inflammatory conditions in the absence of platelet/leucocyte aggregate adhesion. It is well known that inflammatory mediators such as histamine, bradykinin, serotonin, and PAF only induce transient increases in endothelial gap formation and microvessel permeability.
in intact microvessels.\textsuperscript{30,69,79} In contrast, sustained permeability increases are observed in venules with TNF-\(
\alpha\) plus PAF-induced platelet/leucocyte aggregate adhesion.\textsuperscript{46}

Studies using autologous blood perfusion combined with single-vessel permeability measurements effectively distinguished permeability increases mediated by inflammatory mediators from those induced by platelet/leucocyte aggregate adhesion.\textsuperscript{46} In these studies, microvessels experience an early transient and late sustained phases of permeability increase upon application of TNF-\(\alpha\) and PAF. Immediate permeability increases induced by PAF alone peaked after 10 min of application and returned to baseline in an hour. However, in the presence of platelet/leucocyte adhesion, permeability remained elevated at six times the control level 3 h after the start of TNF-\(\alpha\) and PAF application. Thus, late-phase increases in microvessel permeability must be attributed to the adherent leucocyte/platelet aggregates. It is known that exposure of isolated rat neutrophils to either TNF-\(\alpha\) or PAF alone does not directly trigger ROS release. However, these mediators enhance ROS production from platelet and leucocytes when additional stimuli are available.\textsuperscript{32,33,51,80} Thus, under inflammatory conditions, mediators released from activated endothelial cells and platelets may trigger enhanced ROS production from circulating and adherent leucocytes. Collagen-induced platelet adhesion is reported to generate H\(_2\)O\(_2\),\textsuperscript{81} that may also serve as the secondary stimuli to trigger amplified ROS production from TNF-\(\alpha\) and PAF-primed neutrophils. On the basis of a recent study, superoxide, despite its potency, acts as an inflammatory mediator inducing only transient increases in microvessel permeability, whereas the more stable H\(_2\)O\(_2\) is responsible for prolonged increases in microvessel permeability and tissue damage.\textsuperscript{82}

6. Summary

For decades, studies on protecting vascular barrier function have focused on the specific ligands and receptors responsible for the adhesive interactions between leucocytes and endothelial cells, as well as the development of anti-adhesion therapeutics. Emerging evidence suggests that adhesive interactions between leucocytes and endothelial cells can be independent from the potential endothelial damage caused by leucocyte ROS release and are therefore dissociated from increases in microvessel permeability. The stimuli required for initiating leucocyte adhesion to vascular walls are different from those that trigger oxidative burst for ROS-mediated permeability increases. Although increased permeability by inflammatory mediator-induced direct activation of endothelial cells may promote leucocyte/platelet/endothelial cell interactions resulting in amplified ROS production and exacerbating increased permeability,\textsuperscript{46,83} the adhesion and emigration process in the absence of already activated endothelial cells does not cause structural changes sufficient for vascular leakages. However, in the presence of appropriate stimuli, the released oxygen metabolites and proteolytic enzymes from adherent and circulating leucocytes may also play important roles in vascular dysfunction. Furthermore, the initial activation of endothelial cells with exposed subendothelial matrix at sites of endothelial gaps has been implicated as an essential step for platelet adhesion-mediated leucocyte recruitment and prolonged permeability increases. Thus, endothelial cells activated by inflammatory mediators not only directly increase permeability in the absence of leucocyte adhesion, but may also initiate platelet-dependent leucocyte recruitment and amplified ROS-mediated permeability increases. In developing therapies to prevent vascular damage, instead of focusing on anti-adhesion or platelet/leucocyte depletion, it may be more efficient to directly target endothelial activation through mechanisms that enhance endothelial barrier functions, thereby preventing both the direct effect of inflammatory mediators on endothelial cells and activated blood cell-mediated increases in microvessel permeability during inflammation. Recently, there are excellent reviews about the endothelial signalling events and molecular regulation associated with endothelium/leucocyte or different leucocyte subset interactions,\textsuperscript{74,85} which will guide the future investigations of signalling-mediated changes in vascular functions.

Acknowledgements

The author would like to thank Professor Robert Goodman for his invaluable suggestions and proofreading this manuscript.

Conflict of interest: none declared.

Funding

This work was supported by the National Institutes of Health, Heart, Lung, and Blood Institute, Grant numbers HL56237 and HL084338 to P.H.

References

14. Rumbaut RE, Harris NR, Saj AI, Huxley VH, Granger DN. Leakage responses to \(\text{N}^\text{\text{\u02c7}}\)-NAME differ with the fluorescent dye used to label albumin. Am J Physiol 1999;276:H333–H339.


