Powerful Inflammatory Properties of Large Vein Endothelium In Vivo

Einar E. Eriksson, Eva Karlof, Karin Lundmark, Pierre Rotzius, Ulf Hedin, Xun Xie

Objective—Inflammatory responses of large vein endothelium are of importance in pathological processes such as venous thrombosis, chronic venous congestion, and vein graft atherosclerosis. However, the inflammatory properties of large vein endothelium are unclear.

Methods and Results—In this study, we used several microscopy techniques to investigate the inflammatory properties of large vein endothelium in vivo. We show that the endothelium in the mouse inferior vena cava (IVC) possesses powerful inflammatory properties that are distinct from the less inflammatory reactive aortic endothelium and virtually identical to endothelial responses in postcapillary venules. Inflammatory stimulation with tumor necrosis factor-α induced strong expression of cell adhesion molecules (CAMs) in the IVC. These CAMs promoted recruitment of leukocytes, platelets, and erythrocytes to the vein wall. The inflammatory responses altered endothelial structure and increased endothelial permeability in the IVC. Accumulation of blood cells and endothelial damage were markedly reduced in mice deficient in the endothelial leukocyte recruitment molecules E-selectin and P-selectin, indicating a central role for these molecules in driving structural and functional changes of IVC endothelium.

Conclusions—These findings provide the first comprehensive demonstration of the inflammatory capacity of large vein endothelium and emphasize the actions of endothelial cells as targets in large vein disease. (Arterioscler Thromb Vasc Biol. 2005;25:723-728.)

Key Words: endothelium ■ leukocyte ■ rolling ■ adhesion ■ vein ■ inflammation

The vascular endothelium functions as a barrier between tissue and blood and regulates diverse functions such as local and central hemodynamics, exchange of nutrients and metabolites, hemostasis, and immunity.1-3 The endothelium also plays important roles in inflammatory diseases, atherosclerosis, and thrombosis.4-7

The endothelial monolayer is a diverse family of cells that, depending on the location in the vascular tree, displays variable responses to a variety of stimuli. For instance, endothelial phenotype in arteries is characterized by the release of substances that influence vascular smooth muscle tone, vessel diameter, and local blood perfusion.8 Endothelium in postcapillary venules, on the other hand, is specialized at responding to local inflammatory stimuli by increasing its permeability toward macromolecules and by expression of molecules that mediate recruitment of leukocytes to sites of inflammation.9 The factors that guide endothelial cell phenotype are not fully understood. However, endothelial function is influenced by the developmental origin of the cells as well as by local blood flow dynamics.10,11

In contrast to the endothelium in arteries and venules, little is known about endothelial function in large veins. Nonetheless, the responses of large vein endothelium play important roles in clinical disease. For example, accumulation of leukocytes on venous endothelium is important in the initial stages of thrombosis.12 Moreover, destruction of venous valves leading to chronic venous congestion is imposed by inflammatory mechanisms.13 Furthermore, veins grafted into the arterial circulation in bypass surgery rapidly develop vein graft atherosclerosis, which is dependent on inflammatory responses of endothelial cells and subsequent accumulation of leukocytes in the vessel wall.14-16 Insights into the inflammatory properties of endothelial cells from various locations have been gained from cells in culture.17,18 However, because endothelial phenotype is influenced by factors in the local environment, endothelial cells rapidly alter their functional characteristics when isolated. Hence, complete understanding of endothelial responses cannot be gained from in vitro model systems. In in vivo models, it has been demonstrated previously that vein endothelium holds inflammatory capacity inasmuch as it can recruit leukocytes to the vein wall.19 However, these properties are shared by the endothelium in other vessels, and how the inflammatory properties of vein endothelium stand in relation to those of other vessels is unknown. Thus, data elucidating the inflammatory characteristics of large vein endothelium in a physiological situation would increase our knowledge of large vessel pathology.
Here, by use of several microscopy techniques, we show that the endothelium in the inferior vena cava (IVC), the largest vein in the mouse, holds powerful inflammatory properties that resemble those of the specialized inflammatory endothelium in postcapillary venules. In contrast, inflammatory responses in the aorta are modest compared with those in the IVC. These findings represent a comprehensive demonstration of the functional phenotype of endothelial cells in large veins and emphasize the importance of inflammatory responses in vein disease.

Methods
Detailed Materials and Methods are available online at http://atvb.ahajournals.org.

Animals
Wild-type (WT) C57BL/6 mice and E-selectin−-, P-selectin−-, and EP-selectin−/− deficient mice (E−/−, P−/−, and EP−/− mice, respectively) back-crossed to a C57BL/6 background for at least 6 generations were used.

Cytokine Stimulation
Two to 3 hours before experiments, mice were treated with 0.5 μg tumor necrosis factor-α (TNF-α) in PBS either by local injections or by intravenous injection as depicted. TNF-α stimulation ex vivo was performed on vessels that were excised from anesthetized mice and incubated with 50 μg/mL TNF-α in HEPES-buffered RPMI 1640 culture medium supplemented with 1% FCS at 37°C.

Intravital Microscopy
IVC and Aorta
Under anesthesia, the abdomen was opened by a midline incision, and the intestines were retracted to expose the IVC and aorta. Intravital fluorescence microscopy was performed directly on the exposed vessels after an intravenous injection of Rhodamine 6G (0.3 mg/mL, 0.67 mg/kg).

Cremaster Muscle
Intravital microscopy on the cremaster muscle was performed according to standard protocols. Intravital microscopy experiments were recorded and analyzed off-line.

Measurement of Endothelial Permeability
Parallel to injection of TNF-α, mice were treated with a jugular vein injection of fluorescein isothiocyanate (FITC)-dextran (250 mg/kg). Two to 3 hours later, mice were anesthetized, and the abdominal IVC and aorta were excised en bloc, separated, mounted on face on glass slides, and investigated in a confocal microscope. Scanning was performed in z-sections at 5-μm intervals and the images were analyzed.

Results
TNF-α Induces Expression of Inflammatory Cell Adhesion Molecules on IVC Endothelium In Vivo
To investigate the inflammatory properties of large vein endothelium, we first investigated whether TNF-α can induce endothelial expression of endothelial cell adhesion molecules (CAMs) in the mouse IVC and aorta/iliac arteries. Treatment with TNF-α was chosen because this cytokine is a key molecule in the inflammatory response, and the characteristics of TNF-α-induced inflammation has previously been extensively investigated in microvessels in peripheral tissues.

In untreated IVCs and arteries, weak expression of intercellular adhesion molecule-1 (ICAM-1) was demonstrated, whereas staining for vascular CAM-1 (VCAM-1), P-selectin, and E-selectin was negative with exception for irregular staining for VCAM-1 on arterial endothelium (n=3; Figure 1A1 through 1D1 and 1A3 through 1D3). However, perivascular treatment with TNF-α in vivo induced expression of all investigated CAMs on endothelial cells in the IVC (n=3; Figure 1A2 through 1D2 and 1A4 through 1D4). In contrast, the response to TNF-α in the aorta and iliac arteries was moderate, with consistent expression of only ICAM-1 and VCAM-1, whereas selectin expression was less prominent than in the IVC. Interestingly, CAM expression in arterioles and venules adjacent to the aorta and IVC was similar to that in seen in the respective type of large vessel for each respective type of CAM. Stainings for VCAM-1 and P-selectin in microvessels are shown in Figure 1E and 1F, whereas stainings for ICAM-1 and E-selectin that further confirmed this notion are not shown. The data indicate that CAM expression on aortic and IVC endothelium in response to TNF-α is not very different from that in the respective type of microvessels.

It is possible that the differences in CAM expression between the aorta and the IVC are influenced by differences in the capacity for TNF-α to diffuse through the vessel walls of the respective types of vessels. However, the distinct responses of aortic and IVC endothelium were also evident in experiments in which vessels were treated with TNF-α ex vivo, where the cytokine acts directly on endothelial cells. Stainings for VCAM-1 and P-selectin in vessels stimulated ex vivo are displayed online in supplemental Figure I (available online at http://atvb.ahajournals.org). Stainings for ICAM-1 and E-selectin that gave equivalent results are not shown.

Inflammatory CAMs Mediate Interactions Between Leukocytes and Endothelium in the IVC
To determine whether CAMs expressed on IVC endothelium are capable of mediating inflammatory interactions with leukocytes, we developed a novel intravital fluorescence microscopy model that allows for direct observations of interactions between blood cells and endothelial cells in the IVC. The interactions in the IVC were then compared with interactions in the aorta and in cremaster muscle venules. Video clips from the IVC are displayed in supplemental Videos I and II (available online at http://atvb.ahajournals.org). In untreated vessels, rolling of leukocytes was observed in the IVC and in venules, whereas rolling was virtually absent in the aorta, as shown previously23 (Figure 2A1; n=4 mice for all groups; venules n=19). However, treatment with TNF-α induced rolling of leukocytes in the aorta (n=4; P<0.05), whereas rolling flux in the IVC and in venules was not affected (P=0.247; n=8 and P=0.151; n=4 mice; 23 venules, in the IVC and in venules, respectively; Figure 2A2). The characteristics of leukocyte rolling in the IVC and in venules was altered by treatment with TNF-α inasmuch as rolling velocity decreased from 66±5.9 and 48±3.0 to 22±1.9 and 12±2.1 μm/s (P<0.05) in the IVC and in venules, respectively. Rolling velocity in the TNF-α-treated aorta was higher compared with rolling velocity in the TNF-α-treated IVC (49±5.3 μm/s, P<0.05 compared with the IVC), which is in agreement with higher shear stress and lower CAM expression in the aorta.
Because leukocyte firm adhesion represents the next step in the adhesion cascade, we investigated firm adhesion of leukocytes in various vessels. The number of adherent leukocytes was low in untreated IVCs and venules, which is parallel to what has been shown previously in the microcirculation. Firm adhesion was upregulated in the IVC and in venules after treatment with TNF-α, demonstrating that IVC endothelium is able to recruit leukocytes (P < 0.05 compared with the untreated IVC; Figure 2A3). Firm adhesion was also induced by TNF-α in the aorta, although at a much lower density (P < 0.05 compared with the untreated aorta; Figure 2A3).

Apparently, local treatment with TNF-α induces a stronger leukocyte adhesion response in the IVC compared with the aorta. However, as is the case for expression of CAMs, it is possible that these responses are influenced by differences in the capacity for the cytokine to penetrate the aortic and IVC walls.

Figure 1. Top, Immunohistochemical staining for ICAM-1 (A), VCAM-1 (B), P-selectin (C), and E-selectin (D) in the mouse IVC and aorta/iliac arteries. Images labeled 1 show an overview of untreated vessels; 2 shows overview of vessels treated with TNF-α; 3 shows close-up of the endothelium in the IVC of untreated animals; 4 shows close-up of the IVC in TNF-α-treated animals; and 5 shows TNF-α-treated IVCs where primary antibody was excluded from the staining protocol. RIA indicates right iliac artery; LIA, left iliac artery. Bars in 1 and 2 = 100 μm. Bars in 3 through 5 = 20 μm.

Bottom, Staining for VCAM-1 (E) and P-selectin (F) in arterioles (1) and venules (2) from the same TNF-α–treated sections as in (B) and (C). Relevant isotype controls are thus shown in 1B5 and 1C5. Bars = 25 μm.

Figure 2. Leukocyte rolling and adhesion in the mouse aorta, IVC, and cremaster muscle venules. The used stimuli are depicted in the illustrations. A1 through A3, Leukocyte rolling and adhesion in the mouse aorta, IVC, and cremaster muscle venules after perivascular treatment with TNF-α. B1 and B2, Leukocyte rolling and adhesion in mouse vessels after treatment with TNF-α. Leukocyte rolling is expressed as the average number of leukocytes that rolled across a 0.1-mm-long reference line perpendicular to blood flow during 30 s. The number of adherent leukocytes is expressed as the average number of cells that adhered per square millimeter endothelial surface area. *Significant difference compared with the aorta for rolling; †significant difference compared with the aorta for adhesion.
To control for this, we performed experiments in which TNF-α was administered intravenously through the tail vein where the barrier function of the vessel wall should not influence endothelial exposure to TNF-α. As shown in Figure 2B1 and 2B2, intravenous injection of TNF-α evoked similar responses of leukocyte rolling and adhesion to those induced by local administration of cytokine, with adhesive interactions between leukocytes and endothelium being more prominent in the IVC compared with the aorta (P<0.05 between the aorta and IVC for leukocyte rolling and adhesion). In parallel, rolling velocity was not significantly different in the IVC and aorta treated with intravenous TNF-α compared with vessels treated with perivascular injection of cytokine (P=0.150 and P=0.727 for the aorta and IVC, respectively). Firm adhesion of leukocytes was lower in the IVC treated with intravenous TNF-α compared with those vessels that received local treatment (P<0.05), indicating a local effect of the cytokine on IVC endothelium.

The molecular mechanisms responsible for interactions between leukocytes and endothelium in the IVC were also investigated. In the untreated IVC, leukocyte rolling was absent in P-/- mice (n=5; P<0.05; Figure 3A) and in WT mice treated by an intravenous injection of a function-blocking antibody against this CAM (n=3; P<0.05). However, no change in the flux or velocity of rolling leukocytes was observed in untreated veins in E-/- mice (n=3; P=0.311; Figure 3A). In IVCs treated with TNF-α, rolling was decreased but not abolished in P-/- mice and in WT mice treated with P-selectin antibody (n=5 and n=9, respectively; P<0.05; Figure 3A; and data not shown). In E-/- mice, rolling flux and rolling velocity were increased compared with WT mice (n=3; P<0.05; Figure 3A, velocity data not shown). Corresponding to the changes in rolling flux and velocity, leukocyte adhesion was attenuated in P-/- and E-/- mice (P<0.05; Figure 3B). Moreover, the changes in rolling and adhesion in P-/- and E-/- mice were reflected in EP-/- mice, in which rolling and adhesion were almost absent (n=4; P<0.05; Figure 3A and 3B). Interestingly, all data on the molecules that mediate leukocyte rolling in the IVC are virtually identical to what has been shown previously in postcapillary venules, emphasizing a remarkable similarity between the inflammatory responses in these vessel types.24–27

**Inflammation in the IVC Induces Vein Edema**

A major sign of inflammation in peripheral tissues is the development of inflammatory edema, which is dependent on the opening of intercellular gaps between venular endothelial cells, which subsequently leads to an increase in macromolecular permeability. To determine the properties of endothelial permeability in the IVC, we investigated changes in permeability induced by TNF-α by studying the accumulation of the plasma tracer molecule FITC–dextran in the walls of the IVC and the aorta. As seen in Figure 4, increased fluorescence from accumulation of FITC–dextran in the IVC wall was detected after treatment with TNF-α compared with control (n=4 and n=8 for control and TNF-α–treated vessels, respectively; P<0.05). However, no increase in fluorescence was demonstrated in the aorta, showing that the aorta does not respond to TNF-α as strongly as the IVC. Moreover, the data indicate that FITC–dextran that accumulated in the IVC had leaked through the IVC endothelium and not through the endothelium in perivascular microvessels, which would have also stained the aorta. Thus, the data indicate that inflamed IVC endothelium increases its permeability to macromolecules.

**Large Vein Inflammation Triggers Accumulation of Platelets and Erythrocytes and Secondary Endothelial Injury**

To further elucidate the consequences of interactions between leukocytes and large vessel endothelium, we performed scanning electron microscopy (SEM) on the luminal surface of the IVC and the aorta after perivascular stimulation with TNF-α (Figure 5; Table I available online at http://atvb.ahajournals.org). In untreated veins, the endothelial surface was smooth and confluent, and minimal adhesion of blood components was detected (Figure 5A). However, in IVCs treated with TNF-α, leukocyte adhesion was substantial, similar to what was seen in experiments with intravital microscopy (Figure 5B). Furthermore, the endothelium displayed protrusions that were apparently formed by transmigrated leukocytes (Figure 5B), and leukocytes captured in the process of transmigration were abundant (Figure 5C). Moreover, endothelial integrity was severely compromised with irregularly shaped cells and intercellular gap formation (Figure 5D). These gaps are likely responsible, at least in part, for the increased endothelial permeability detected by confocal microscopy. Moreover, stimulated IVCs displayed an increased number of platelets and erythrocytes adherent to the
endothelium (Figure 5B, 5D, and 5E). Aggregates of blood cells and amorphous material that may represent coagulation were also observed, suggesting that treatment with TNF-α may induce the initial stages of thrombosis (Figure 5B and 5F). Interestingly, accumulation of blood cells and aggregates, as well as endothelial damage, was reduced in EP–/– mice in which leukocyte recruitment to the vein wall is restricted (Table I), indicating an important role for inflammatory responses and the endothelial selectins in these processes. Concurrently, endothelial injury was not observed in the TNF-α–treated aorta, in which leukocyte recruitment is not as prominent as in the IVC. Thus, the data support that accumulation of leukocytes is a key event in generating endothelial damage in the IVC.

Discussion

The inflammatory characteristics of large vein endothelium are of importance in the understanding of large vein diseases, their prophylaxis, and their treatments. In this study, we show that endothelial cells in the IVC hold powerful inflammatory properties that are equally as potent as those in postcapillary venules and distinct from endothelial responses in arteries and arterioles. Differences in how endothelial cells from different locations respond to inflammatory stimulation have been suggested previously, primarily from in vitro models.17,18 However, the present data provide a comprehensive demonstration of the physiological phenotype of large vein endothelium in vivo. Together with previous data on the inflammatory characteristics of the endothelium in other locations, the findings indicate that endothelial cells in the systemic vasculature hold either of 2 basic phenotypes: 1 arterial and 1 venular, the properties of which may be adjusted by flow patterns or by local molecular stimuli.8,10 This is in accordance with the observation that arterial and venous endothelial cells are derived from separate sets of embryonic precursor cells with distinct gene expression profiles.11 Evidently, such differences in gene expression maintained throughout development lead to marked functional diversities between endothelium in arteries and veins.

The differences in endothelial responses are, with no doubt, important for the respective disease panoramas seen in different types of blood vessels. For instance, the proinflammatory properties of vein endothelium likely influence the development of venous thrombosis and vein graft atherosclerosis in which inflammatory responses play major roles.7,12,14,16 In parallel, the development of thromboembolic complications in diseases such as infections, inflammatory disease, or malignancy may be influenced by the reactive venous endothelium that can respond to systemic inflammatory stimuli released in these syndromes. Such notion is supported by our data in which strong adhesion of leukocytes was induced by intravenous treatment with TNF-α. Moreover, the fact that IVC endothelium responds to the minimal manipulation associated with intravital microscopy by rapid upregulation of P-selectin and subsequent rolling of leukocytes may also help explain why veins grafted to the arterial circulation are prone to rapid occlusion if exposed to excessive surgical trauma.28 In contrast, although accumulation of leukocytes derived from the arterial bloodstream is important in the development of atherosclerosis, aortic endothelium is to blood cells not nearly as adhesive as endothelium in veins. Consequently, the mechanisms that direct the formation of atherosclerotic lesions to arteries but not veins appear not to be related to the capacity of endothelial cells to recruit leukocytes and should thus be influenced by other factors such as vessel structure or flow and pressure dynamics.

The present study also demonstrates that leukocytes inflict damage to inflamed venous endothelium. This has been suggested previously from experiments on veins exposed to toxic agents;29 however, we now demonstrate that this is true also after stimulation with the key inflammatory mediator TNF-α. Moreover, platelets and erythrocytes also accumulate on TNF-α–treated IVC endothelium. It is possible that the accumulation of platelets is influenced by adherent leukocytes, as suggested previously in microvessels.30 However, the roles for platelets
and erythrocytes in large veins and in tissue inflammation are still obscure, which calls for further study.

This study presents a novel intravital microscopy technique allowing for study of pathological events in the mouse IVC. Several reports previously used intravital microscopy on microvessels as model systems for direct observation of the formation of thrombi. By use of the technique presented here, venous pathology can be observed directly in the IVC, which represents a relevant system for study of venous thrombosis and its complications. The data presented also have technical implications in studies on inflammatory responses of endothelial cells in vitro. Because venous endothelium appears to be similar to that in postcapillary venules, endothelial responses in relation to tissue inflammation should be performed preferably with cells harvested from large veins or their equivalent. In contrast, inflammatory responses related to arterial pathology such as early atherosclerosis or arterial injury are probably best studied by use of arterial cells.

In conclusion, we demonstrated that large vein endothelium holds powerful inflammatory properties. The data give insights into the determinants of endothelial phenotype and in the understanding of venous thrombosis, pulmonary embolism, and vein graft atherosclerosis. The data also provide important information on the distinct pathological profiles in large arteries and veins and emphasize the importance of careful selection of endothelial cells for experimental studies in vitro. Future intravital microscopy studies on veins may provide further data on pathological events of large clinical significance.

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References

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**Video Legends**

Video clips from intravital microscopy on the IVC. Intravital microscopy was performed using a Leitz Biomed microscope with a Lietz Wetzlar SW25/0.60 water immersion objective after intravenous labeling of leukocytes with rhodamine 6G. Images were recorded with a VNC-703 CCD camera. Flow is from top to bottom. Movements are related to pulsations in the aorta and to changes in venous flow related to breathing. **Video I** and **Video II** show interactions between leukocytes and endothelium in untreated and TNFα-treated mice, respectively.
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All data represent average number of events in individual vessels and expressed as number of events per mm$^2$. Exp.# = Experiment number.