Inferior Vena Cava Ligation Rapidly Induces Tissue Factor Expression and Venous Thrombosis in Rats

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Objective—Although stasis is important in the pathogenesis of deep vein thrombosis (DVT), how it contributes to thrombogenesis is largely unknown. To gain mechanistic insight, we used a rat model of inferior vena cava (IVC) ligation.

Methods and Results—Rats were subjected to IVC ligation for 15 to 60 minutes. Ligation resulted in rapid IVC dilatation and by 60 minutes, thrombi were detected in all rats. Small thrombi were detected in the IVC of most rats after 15 minutes of ligation. Thrombi were rich in fibrin, contained aggregated platelets as well as trapped leukocytes and red cells, and most originated at sites of localized endothelial denudation. Immunohistochemical analysis revealed tissue factor (TF)-expressing leukocytes within the thrombi and adherent to the vessel wall. Despite a largely intact vessel wall, endothelial cells also stained for TF. The expression of TF colocalized with that of protein disulfide isomerase (PDI), an enzyme implicated in TF decryption.

Conclusions—These findings suggest that the rapid development of DVT after IVC ligation reflects a combination of stasis-induced vein wall injury and enhanced TF expression in endothelial cells and leukocytes. Because TF expression occurs so soon after ligation, new synthesis is unlikely. Instead, stasis-induced venous dilatation with or without exposure of subendothelial TF, may be responsible for vessel wall TF expression. Colocalization of TF and PDI raises the possibility that PDI-mediated TF decryption plays a role in the pathogenesis of DVT. (Arterioscler Thromb Vasc Biol. 2009;29:863-869.)

Key Words: deep vein thrombosis ■ inferior vena cava ligation ■ tissue factor ■ P-selectin ■ protein disulfide isomerase

Venous thromboembolism (VTE), which includes deep vein thrombosis (DVT) and pulmonary embolism (PE), is a major cause of morbidity and mortality. In the United States, it is estimated that up to 1 million people are afflicted with VTE each year. Of these, 300 000 develop a PE, which is fatal in one-third. VTE is the third most common vascular disease after heart attacks and strokes, and PE causes more deaths each year than breast cancer, AIDS, and traffic accidents combined. In addition to fatal PE, DVT also can lead to postthrombotic syndrome in up to 50% of patients. Thus, not only is VTE a major cause of mortality, but it also leads to significant morbidity.

Despite the burden of disease, little is known about the pathogenesis of VTE. Most DVT arise in the deep veins of the legs, and DVT is common after surgery, particularly orthopedic surgery. Thus, without vigorous perioperative thromboprophylaxis, there is venographic evidence of DVT in 50% to 70% of patients undergoing hip or knee arthroplasty or surgery for hip fracture. Furthermore, even with the best forms of prophylaxis, break-through DVT occurs in 25% to 35% of patients undergoing knee replacement surgery and in 10% to 15% of those having hip replacement surgery. Although direct injury to the vein wall may trigger the proximal DVT that is common after hip surgery, overt evidence of vein wall damage is lacking in most cases of secondary DVT. What causes DVT in the absence of vein wall injury, and why do patients develop DVT despite anticoagulant prophylaxis?

The risk factors for DVT include blood stasis, vessel wall injury, and hypercoagulability, as proposed by Virchow over 150 years ago. However, how these factors conspire to produce DVT is largely unknown. In vivo, thrombosis is triggered by tissue factor (TF), a normally cryptic protein that is exposed at sites of vessel wall injury. Although most arterial thrombi are caused by TF exposed at sites of atherosclerotic plaque disruption, gross vein wall disruption is not a common feature of DVT. Therefore, the source of TF that triggers DVT is uncertain. Although inflammatory mediators can induce TF expression in cultured endothelial cells, it is difficult to demonstrate endothelial cell TF expression in vivo. Instead, it has been suggested that monocyte-derived TF plays a role in the pathogenesis of DVT, and a growing body of evidence supports this hypothesis.

Under physiological conditions, monocyte TF is mostly encrypted and lacks the capacity to initiate coagulation. Cell damage, treatment with calcium ionophore, and induction of
apoptosis all increase TF activity. The explanations for TF decryption under these circumstances include phosphatidylserine exposure after cell activation, TF dimerization, and localization of TF to lipid rafts. More controversial is the role of protein disulfide isomerase (PDI) in TF decryption. A protein normally found in the endoplasmic reticulum, it has been postulated that cell surface PDI regulates the formation of a critical Cys186-Cys209 disulfide bond essential for TF activity. Whether PDI plays a role in TF decryption in DVT is currently unknown.

Stasis is a critical factor in the pathogenesis of DVT as highlighted by the fact that DVT is common after immobilization because of surgery or medical illness. How stasis promotes TF expression is unknown. Current thinking is that TF-bearing monocytes or monocyte-derived microparticles localize to activated endothelial cells where the TF can trigger clotting. Leukocytes attach to activated endothelial cells via adhesion molecules, such as ICAM or VCAM, on the surface of monocytes or leukocyte-derived microparticles, also can localize TF to the vessel wall.

The purpose of this study was to use a rat model to begin to explore how stasis induces DVT. Most animal models of DVT use prolonged stasis to induce clotting. When blood flow is impaired for many hours or days to create a mature thrombus, it is difficult to differentiate the triggers of thrombosis from the effects of prolonged vascular obstruction. To circumvent this problem, we induced DVT in rats by brief ligation of the inferior vena cava (IVC). Our studies reveal that (1) ligation induces rapid IVC dilatation and (2) thrombosis can be induced in most rats after as little as 15 minutes of IVC ligation and in all rats after 60 minutes ligation. Using a combination of histological and immunohistochemical analysis, we then used this model to begin to identify potential mechanisms for DVT.

Methods

Rat Model

Studies were done in Sprague-Dawley rats (250 to 300 g) obtained from Charles River (Quebec, Canada). The rats had free access to food and water. All procedures were approved by the Animal Research Ethics Board at McMaster University.

Rats were randomly divided into 3 groups: groups 1 (n=13) and 2 (n=15) underwent IVC ligation for 15 and 60 minutes, respectively, whereas the rats in group 3 (n=8), which served as a control, underwent sham surgery without ligation and were then followed for 60 minutes. Anesthesia was induced and maintained by inhalation of a combination of oxygen (0.5 L/min) and isoflurane (1% to 4%) delivered by face mask. The IVC was exposed via a midline laparotomy, dissected at the level of the renal veins, and then ligated below the renal veins. Control animals underwent the same surgical procedures except for IVC ligation (sham ligation). If the dissection was traumatic or the IVC was ruptured during surgery, rats were not studied further. To determine the extent of IVC dilatation, a caliper was used to measure its diameter before and 5, 15, or 60 minutes after ligation. After blood sampling from the left ventricle, rats were euthanized and the IVC was harvested and (1) fixed in 10% neutral buffered formalin either unopened or after being cut longitudinally for subsequent en face examination, or (2) placed in 2% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4, in preparation for scanning electron microscopy (SEM).

Histology and Immunohistochemistry

After formalin fixation, IVC specimens (9, 9 and 4 from groups 1, 2, and 3, respectively) were embedded in paraffin. Serial cross sections of the IVC were stained with hematoxylin and eosin (H&E). Giemsa or Fraser-Lendrum (MSB) staining was used to detect platelets or fibrin. For immunohistochemical staining, after antigen retrieval, sections were stained with a 1:250 dilution of a sheep antibody against human TF (Affinity Biologicals) that cross-reacts with rat TF as evidenced by immunoblot analysis of rat brain lysates demonstrating a 47-kDa band, a 1:50 dilution of a goat antibody against human P-selectin (Santa Cruz Biotechnology) that cross-reacts with rat P-selectin, and a 1:100 dilution of a rabbit antibody against rat protein disulfide isomerase (PDI, Cell Signaling). In all cases, sections without primary antibodies were used as negative controls.

RESULTS

Statistics

Results are expressed as mean±SD. Student t tests were used to compare the results in the experimental and control groups. A probability value <0.05 was considered statistically significant.
Results

Vein Wall Injury and Thrombus Formation
The IVC rapidly dilated after ligation with the diameter increasing from 3.12 ± 0.27 mm to 3.64 ± 0.25 mm within 5 minutes (P < 0.01). No further dilatation occurred thereafter (data not shown). Cross-sectional examination of the IVC revealed thrombi in all 9 rats after 60 minutes of ligation. Even after 15 minutes of ligation, small IVC thrombi were detected in 7 of 9 rats (Figure 1a). Based on morphological examination, thrombi consist mainly of fibrin, platelets, and erythrocytes (Figure 1b through 1d). Leukocytes (identified as monocytes and neutrophils, based on their nuclear morphology) were also seen within the thrombi. On careful analysis, focal areas of denuded endothelium were identified in all IVC after 15 minutes of ligation and overlying platelet aggregates were seen in specimens from 6 of the 9 rats in this group (Figure 1e). Even where the endothelium was intact, adherent leukocytes were found lining the IVC vessel wall in 6 of the 9 rats (Figure 1f). In some areas, fibrin strands and platelet aggregates appeared to be attached to a grossly intact vessel wall (Figure 1g). Results after 60 minutes ligation were similar to those after 15 minutes of ligation except that thrombi were found in the IVC of all 9 of the rats whose specimens were subjected to microscopic examination (data not shown). In contrast, no thrombi were found on cross-sectional analysis in the 4 control rats that underwent sham surgery without IVC ligation.

On SEM, the endothelial surface of the IVC appeared intact in the control rat; only occasional fibrin strands were detected (Figure 2a). In contrast, an extensive fibrin network and platelet aggregates were found on the IVC surface after 15 minutes of ligation (Figure 2b and 2c); fibrin strands without leukocytes were seen in some areas (Figure 2b). After 60 minutes ligation, endothelial cell denudation, platelet aggregates, and adherent leukocytes also were seen (Figure 2d).

TF and PDI Expression
In vivo coagulation is initiated by TF. There is some evidence suggesting that PDI plays a role in TF decryption at sites of arterial injury. To explore the possibility that PDI plays a similar role in DVT, we set out to determine the extent to which TF and PDI colocalize after IVC ligation. TF staining was detected in leukocytes, which were adherent to the vein wall or trapped within the thrombus (Figure 3a and 3b). After 15 and 60 minutes ligation, TF staining was evident in endothelial cells lining the IVC (Figure 3c); such staining was minimal in the 60 minutes controls (Figure 3d) and absent in the negative control (Figure 3e). TF staining also was evident in the subendothelium at sites of endothelial denudation (Figure 3f). The fluorescence intensity of TF staining in

Figure 1. Histological evaluation of the ligated IVC. A representative IVC specimen after 15 minutes ligation is shown (a). Under higher magnification, thrombi were primarily composed of fibrin, platelets, and erythrocytes (b through d). Occasional leukocytes were found within the thrombus (b, d). Focal areas of endothelial denudation were seen with attached platelet aggregations (e), leukocytes were found adherent to the vessel wall (f), and, in some areas, fibrin strands with attached platelets were seen (g). E indicates erythrocytes; EC, endothelial cell; F, fibrin; L, leukocyte; P, platelets; bars represent 100 μm in a, 50 μm in b through d, and 20 μm in e through g.
endothelium was quantified and expressed as the fold increase over that measured in the 60 minutes controls. TF expression in endothelial cells increased 1.5±0.3- and 1.5±0.2-fold after 15 and 60 minutes ligation, respectively (P<0.01). Confocal microscopic examination of en face specimens revealed increased TF staining in the endothelium after 15 or 60 minutes ligation (Figure 3g) compared with that seen in the controls (Figure 3h and Table).

As summarized in the Table, PDI staining in the endothelium also increased after IVC ligation (Figure 3i) compared with that in the controls (Figure 3j). Confocal microscopic examination of double-stained sections obtained after 15 minutes ligation localized the majority of TF and PDI to the leukocytes. The endothelium was also positive for both TF and PDI staining (Figure 3k through 3n), whereas no fluorescence was observed in the negative controls (Figure 3o and 3p). The Mander overlap coefficients (R) of TF and PDI staining are 0.98±0.01, 0.96±0.02, and 0.93±0.04 in endothelial cells, leukocytes adherent to the vessel wall, and leukocytes in the IVC lumen, respectively, consistent with a high degree of overlap.

### P-Selectin Expression

P-selectin plays an important role in leukocyte rolling, adhesion, and transmigration on the vessel wall. Immunostaining of 60 minutes control samples demonstrated P-selectin expression in endothelial cell granules, likely reflecting Weibel-Palade bodies (Figure 4a). The majority of the endothelial cell granules disappeared after 15 minutes ligation (Figure 4b), even though the intensity of P-selectin staining increased (Table), particularly in areas where adherent leukocytes were found (Figure 4c). No fluorescence was observed in the negative control (Figure 4d).

The percentage of vein wall expressing P-selectin is higher where there are adherent leukocytes than it is where leukocytes are absent (36.2±13.0% and 5.0±3.8%, respectively; P<0.01). These findings support the concept that P-selectin plays a role in leukocyte adhesion to the vessel wall after IVC ligation.

### Discussion

IVC ligation induces rapid dilatation of the IVC and subsequent thrombosis. Microscopically, focal areas of endothelial cell denudation and platelet aggregation at the sites of injury appear to be early events. Leukocyte adherence to the vessel wall also occurs soon after IVC ligation. TF expression is increased in leukocytes and endothelial cells, and P-selectin is expressed on the endothelial cells. These results suggest that the rapid development of DVT after IVC ligation reflects a combination of stasis-induced vein wall injury and enhanced TF expression.

### IVC Ligation: The Potential Role of Stasis and Hypoxemia

Although IVC ligation is commonly used as a stimulus for DVT in animals, most of these models use prolonged stasis for DVT induction. When blood flow is blocked for many hours or days to create a mature thrombus, it is difficult to differentiate the triggers of thrombosis from the effects of prolonged vascular obstruction. Although Stewart proposed a 4-stage model for the initiation and propagation of DVT, the detailed morphological changes that coincide with thrombus formation have not been determined. To address this issue, we examined the IVC of rats after 15 or 60 minutes of ligation. Because surgical trauma to blood vessels can trigger thrombosis, we restricted our dissection to the IVC segment immediately below the renal veins.

DVT occurred soon after IVC ligation in our model; most rats had small thrombi after 15 minutes ligation, and all had DVT after 60 minutes ligation. Complete ligation of the IVC restricts venous return and induces rapid dilatation. Vessel stretching may expose TF-expressing subendothelial cells, which could trigger coagulation. In addition, platelets may adhere to exposed subendothelial von Willebrand factor, collagen, or other matrix proteins. Clinical observations support a role for venous dilatation in the initiation of DVT. Thus, in patients undergoing hip replacement surgery, venous dilatation has been correlated with subsequent development of DVT. Such studies prompted the use of dihydroergotamine, an agent that prevents venodilatation, in combination with low-dose heparin for perioperative thromboprophylaxis; this is an approach that yielded mixed results.

Ligation also has the potential to induce hypoxemia, which can upregulate adhesion molecule expression by endothelial cells and TF expression by monocytes. Therefore, the rapid thrombosis that occurs after IVC ligation in our model may reflect stretch-induced vessel wall injury or hypoxemia-induced TF expression.

### Initiation of DVT: Potential Sources of TF

DVT is initiated by TF. Where does this TF come from? Potential sources include the vessel wall, leukocytes, and leukocyte-derived microparticles. Vessel wall TF can be expressed by subendothelial cells exposed at sites of denud-
ing injury, intact endothelial cells, or by leukocytes adherent to the vessel wall. All of these sources appear to contribute to TF expression in our model. Focal areas of endothelial denudation with adherent fibrin strands are observed. Vein wall TF expression in these areas likely reflects exposed subendothelial cells. However, TF expression also is observed in apparently intact endothelial cells. It has been difficult to demonstrate endothelial cell TF expression in vivo. Although ligation-induced hypoxemia may induce TF expression, it is unlikely that de novo TF synthesis could occur within 15 to 60 minutes of occlusion. Therefore, where does this endothelial cell TF come from? One possibility is that it reflects transfer of TF from leukocytes or leukocyte-derived microparticles. TF-expressing leukocytes are found adherent to the vessel wall. These adherent cells could transfer TF to the adjacent endothelium. Although not evident in our study, TF transferred from microparticles also could contribute to endothelial cell TF.

We detected TF expression in both monocytes and neutrophils after IVC ligation. The rapid TF expression in monocytes is more likely to reflect decryption of endogenous TF expression by leukocytes, which are TF-positive and adhere to the vessel wall after ligation. However, TF expression also is observed in apparently intact endothelial cells. It has been difficult to demonstrate endothelial cell TF expression in vivo. Although ligation-induced hypoxemia may induce TF expression, it is unlikely that de novo TF synthesis could occur within 15 to 60 minutes of occlusion. Therefore, where does this endothelial cell TF come from? One possibility is that it reflects transfer of TF from leukocytes or leukocyte-derived microparticles. TF-expressing leukocytes are found adherent to the vessel wall. These adherent cells could transfer TF to the adjacent endothelium. Although not evident in our study, TF transferred from microparticles also could contribute to endothelial cell TF.

Table. Intensity of Fluorescence (Fold Increase Over the Control, Mean ± SD) Detected in En Face IVC Specimens Examined Using Confocal Microscopy

<table>
<thead>
<tr>
<th>Staining</th>
<th>15-min Ligation</th>
<th>60-min Ligation</th>
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<tbody>
<tr>
<td>TF</td>
<td>1.45 ± 0.38*</td>
<td>1.45 ± 0.24†</td>
</tr>
<tr>
<td>PDI</td>
<td>2.60 ± 0.41†</td>
<td>2.89 ± 0.43†</td>
</tr>
<tr>
<td>P-selectin</td>
<td>1.94 ± 0.29†</td>
<td>1.64 ± 0.29†</td>
</tr>
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*P < 0.05 vs control; †P < 0.01 vs control.
rather than de novo synthesis. We also found TF expression in neutrophils. This finding is in keeping with the results of other investigators who detected rapid neutrophil TF expression in a mouse model of sepsis. However, TF mRNA was not detected in neutrophils in that model, raising the possibility that the TF was transferred to neutrophils from other TF-expressing cells or microparticles.25

The relative importance of circulating versus vessel wall TF in the pathogenesis of thrombosis is unclear. A recent study in mice expressing low levels of TF suggested that vessel wall TF is more important than circulating TF as the initiator of thrombosis.26 However, TF-expressing microparticles are found in the blood of patients with DVT and such microparticles have been implicated in the pathogenesis of DVT in patients.6,27,28 Additional work is needed to address this controversy.

PDI Expression
In our model, PDI colocalized with TF in leukocytes and endothelial cells. The source of PDI is unknown, but platelets are a possibility because these cells release PDI on activation.29 Although the role of PDI in TF decryption remains controversial, the rapid colocalization of PDI and TF after IVC ligation positions them for interaction. Additional studies are needed to investigate the potential role of PDI-mediated TF decryption in the pathogenesis of both venous and arterial thrombosis.

P-Selectin
Because they express PSGL-1 on their surface, leukocytes and microparticles can be tethered to P-selectin expressed on activated platelets or to P- or E-selectin expressed on activated endothelial cells. Such interactions can promote the transfer of TF from 1 cell type to another.6 Recent studies have shown that mice deficient in P- or E-selectin exhibit a reduced thrombus mass at sites of venous injury.30 Furthermore, agents that block the P-selectin/PSGL-1 interaction inhibit venous thrombosis in baboon or rat DVT models.31,32

Adhesion molecules also play a role in leukocyte rolling and adhesion to the vessel wall. Such adhesion molecules can recruit leukocytes to sites where the vein wall is activated. Recent studies have shown that hypoxia induces P-selectin expression in cultured endothelial cells. P-selectin is expressed within minutes and does not require de novo protein synthesis.33 Our en face data demonstrate that P-selectin in the endothelial cells lining the IVC of controls is concentrated in granules that likely reflect Weibel-Palade bodies. After 15 minutes ligation, P-selectin expression in these granules has disappeared, which may reflect its release from these intracellular storage depots. This finding is consistent with in vitro studies, which have shown Weibel-Palade body exocytosis within 5 minutes of endothelial cell exposure to hypoxia.34 These observations raise the possibility that P-selectin contributes to leukocyte adhesion.

Role of Platelets in DVT
Although platelets predominate in arterial thrombi, their role in venous thrombogenesis is less clear. In our study, platelets appear to be the first cells to deposit at sites of endothelial cell denudation. Furthermore, platelet aggregates are found within the thrombi and on the surface of fibrin strands. These results raise the possibility that platelets play a role in the pathogenesis of DVT.

Limitations of Our Study
Although ligation-induced rodent models of DVT are commonly used, the relevance of findings in animals to the pathogenesis of DVT in humans is uncertain. Nonetheless,
stasis is an important determinant of thrombosis in humans that helps explain why immobilization is a major risk factor for DVT. Although the high platelet count in rats may exaggerate the importance of platelets in the pathogenesis of DVT, the fact that aspirin reduces the risk of postoperative VTE, albeit to a lesser degree than that produced by anticoagulants, suggests that platelets also play a part in the pathogenesis of DVT in humans. In summary, we used a rat IVC ligation model to study the pathogenesis of DVT. Thrombosis occurs rapidly after IVC ligation reflecting a combination of stasis-induced vein wall injury and enhanced TF expression. TF expression is detected in endothelial cells and in leukocytes adherent to the vein wall where it colocalizes with PDI, raising the possibility that PDI-induced TF decryption plays a role in DVT. Further studies are needed to test this hypothesis and to identify the source of PDI.

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