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

Ji Zhou, Linda May, Peng Liao, Peter L. Gross, Jeffrey I. Weitz

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;29:00-00.)

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.1,2 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.3 In addition to fatal PE, DVT also can lead to postphlebitic syndrome in up to 50% of patients.4 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.5 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.6 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.6 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 thrombosis are caused by TF exposed at sites of atherosclerotic plaque disruption, gross vein wall disruption is not a common feature of DVT.6,7 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.8 Instead, it has been suggested that monocyte-derived TF plays a role in the pathogenesis of DVT,6 and a growing body of evidence supports this hypothesis.9 Under physiological conditions, monocyte TF is mostly encrypted and lacks the capacity to initiate coagulation.10 Cell damage, treatment with calcium ionophore, and induction of

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apoptosis all increase TF activity. The explanations for TF
decryption under these circumstances include phosphatidyl-
serylne 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 immobili-
zation 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,
which tether leukocytes to the endothelial cell surface.
Activated endothelial cells express P-selectin on their surface,
which, by binding P-selectin glycoprotein ligand-1 (PSGL-1)
on the surface of monocytes or leukocyte-derived MPs, 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 impeded for many hours or days to create a mature
thrombus, it is difficult to differentiate the triggers of throm-
bois 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) throm-
bois 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 Spraque-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, respec-
tively, 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
between 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% glutaralde-
hyde 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 demon-
strating 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.
Sections were washed once with phosphate buffer saline (PBS),
icubated with 1:200 dilutions of the respective Alexa488-labeled antiserum,
Alexa594-labeled antigen, or rabbit secondary antibodies
(Molecular Probes), and mounted on slides with DAPI medium
(DAKO). Slides were examined using a light microscope equipped with
fluorescence (Zeiss Axioskop 2), and images captured with a
CCD color video camera (Sony) were analyzed using Northern Exposure
(Empix), SigmaScan (SPSS), or ImageJ (National Insti-
tutes of Health). Sections were also examined using a confocal
microscope (Leica DMI 6000 B and Leica TCS SP5 Scanner). Images were captured under glycerol using a 63× objective and
processed using Leica Application Suite Advanced Fluorescence
software. To determine the extent to which TF and PDI colocal-
ized, images were analyzed using MacBiophotonics ImageJ and
the extent of colocalization was quantified using the Mander
overlap coefficient.

En Face

IVC specimens (3, 5, and 3 from groups 1, 2, and 3, respectively)
were washed once with PBS containing 0.1% triton X-100, blocked
with normal serum, and then incubated overnight with the primary
antibodies listed above at the same concentrations. After another
PBS wash, samples were incubated overnight at 4°C with 1:300
dilutions of the same secondary antibodies, washed with PBS and
then with 0.05 mol/L TBS, and then incubated for 5 minutes with a
1:20 000 dilution of Invitrogen Sytox green. Samples were washed with
0.05 mol/L TBS and then mounted in aqueous medium
containing an antifade agent. En face IVC specimens were visualized
with a confocal microscope (Yokogawa CSU22 on an Olympus
BX61WI base) fitted with a 60× water-immersion objective. Images were captured with a CCD camera (C9300, Hamamatsu) and
analyzed using Slidebook 4.2 (Intelligent Imaging Innovations), and
intensity of staining was quantified using ImageJ.

SEM

To visualize the intimal surface, IVC specimens (1 from each group)
were examined using SEM. Briefly, after fixation, the IVC was
opened and glued to coverslips with the adventitia facing down.
Specimens were postfixed in 1% osmium tetroxide in 0.1 mol/L
sodium cacodylate buffer, dehydrated using ascending concentra-
tions of ethanol, critical-point dried (Critical Point Drier, Ladd
Industries), mounted on stubs, sputter coated with gold (SEM
Coating Unit E5100, Polaron Instruments, Inc), and then sub-
jected to SEM examination using a JEOL JSM-840 Scanning
Electron Microscope.

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 $3.12 \pm 0.27$ mm to $3.64 \pm 0.25$ 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 2 days).

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.19 To explore the possibility that PDI plays a similar role in DVT, we set out to determine whether 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 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$\pm$$0.3$- and 1.5$\pm$$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 staining to the leukocytes (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 $0.98 \pm 0.01$, $0.96 \pm 0.02$, and $0.93 \pm 0.04$ in endothelial cells, leukocytes adherent to the vessel wall, and leukocytes in the IVC lumen, respectively, consistent with a high degree of overlap.

### 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>
</tr>
</thead>
<tbody>
<tr>
<td>TF</td>
<td>$1.45 \pm 0.38^*$</td>
<td>$1.45 \pm 0.24^†$</td>
</tr>
<tr>
<td>PDI</td>
<td>$2.60 \pm 0.41^†$</td>
<td>$2.89 \pm 0.43^†$</td>
</tr>
<tr>
<td>P-selectin</td>
<td>$1.94 \pm 0.29^†$</td>
<td>$1.64 \pm 0.29^†$</td>
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*P<0.05 vs control; †P<0.01 vs control.

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 ($36.2 \pm 13.0\%$ and $5.0 \pm 3.8\%$, 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 induced 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 venodilation, in combination with low-dose heparin for peroperative 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 at sites of denuding 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. Vessel 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 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.

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. 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. 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. 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.

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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Disclosures
None.

References


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