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Serpins in venous thrombosis and venous thrombus resolution

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Abstract

Several serpins function as potent inhibitors of thrombolytic serine proteases. Venous thrombosis is a common and debilitating condition whose incidence is on the rise. Studies using genetically modified mice and inhibitors have shown that the plasminogen activator inhibitors (PAI), PAI-1 and PAI-2 are primary regulators of plasminogen activation and contribute to regulating the resolution of experimental venous thrombi, via inflammatory mechanisms, vascular remodeling and inhibition of fibrinolysis. Therapies to accelerate venous thrombus resolution would be beneficial, since delayed or incomplete clot resolution frequently leads to post-thrombotic syndrome, a long-term complication associated with debilitating limb swelling, pain and recurrent skin ulceration. Here we describe a useful and reproducible mouse model for the study of venous thrombus resolution involving ligation of the inferior vena cava and elucidation of the molecular and cellular determinants of venous thrombus formation and resolution.

Keywords

deep vein thrombosis; venous thrombus resolution; plasminogen activator; PAI-1; PAI-2; uPA; vena cava; post-thrombotic syndrome; mouse model

1. Introduction

Thrombolytic serine proteases are initiators of fibrinolysis, and also have direct and indirect crosstalk with immune and inflammatory pathways. The serine proteases in the thrombolytic cascade, urokinase- and tissue-type plasminogen activators (uPA and tPA, respectively), activate plasmin, which drives clot dissolution and inflammation including leukocyte infiltration, the activation of other proteases (such as MMP-9) and the regulation of coagulation factors. Both inflammatory mechanisms and fibrinolysis accelerate the dissolution of venous thrombi during resolution of deep vein thrombosis (DVT) (1-3), which is an exceedingly common and serious clinical condition associated with fatal pulmonary

embolism. Many patients with DVT develop a chronic condition of venous wall injury and venous hypertension that can cause debilitating edema and skin ulcers, known as post-thrombotic syndrome (PTS) (4-6).

Standard therapies for DVT rely on anticoagulation, which prevents subsequent formation and propagation of the thrombus as well as pulmonary embolism, but has little effect on the resolution of existing thrombi. Anticoagulation and thrombolytic therapies for DVT also have the added morbidity of hemorrhagic complications. Clinical studies show that a slower rate of endogenous thrombus resolution is correlated with the development of PTS (7). Therefore, methods to accelerate endogenous thrombus resolution would be of clinical benefit to patients with DVT. While the cellular and molecular mechanisms involved in venous thrombus resolution are poorly understood, it is clear that effective thrombus resolution requires inflammatory cells to sculpt immune responses and to mobilize fibrinolytic proteases to resolve the thrombus.

The activities of the thrombolytic proteases are modulated by serine protease inhibitors (serpins) which act as regulatory counterbalances in haemostasis and inflammation (8;9). Serpins that bind to and inhibit uPA and tPA, include the plasminogen activator inhibitors (PAI), PAI-1 and PAI-2. PAI-1 binds both tPA and uPA, and is considered the primary inhibitor of circulating tPA in the blood stream. PAI-2 is structurally similar to PAI-1, and was originally identified as possessing uPA inhibitory activity (10), but has also been shown to possess unique immunomodulatory and cell survival activities that are independent of uPA (11-15). Unlike PAI-1 deficiency, PAI-2 gene-deficient mice do not display any overt changes in fibrinolysis or spontaneous thrombosis (16).

Experimental mouse models of DVT strongly implicate uPA activity from bone marrow derived cells, specifically macrophages, as the major thrombolytic protease that mediates venous thrombus resolution (17). Mice with genetic uPA deficiency have markedly impaired resolution of venous thrombi, whereas genetic deficiency in tPA has no effect on thrombus resolution (18;19). Increased levels of PAI-1 activity are correlated with impaired fibrinolytic responses in patients with DVT, however reports on the role of elevated levels of PAI-1 in venous thrombosis have been contradictory (20;21). PAI-1 deficiency in mice has been shown to enhance both venous thrombus formation and resolution (22;23). Enhanced thrombus resolution in PAI-1 deficient mice occurs via altered MMP and uPA activities (22-24). Additionally the endothelial cell marker, CD31 and vein wall smooth muscle cell (VSMC) gene expression were enhanced in PAI-1 deficient models, suggesting a role for PAI-1 in endothelial integrity and vein wall remodeling (24). Consistent with this, transgenic mice overexpressing PAI-1 showed impaired thrombus resolution, although paradoxically, had significantly less collagen deposition in the vein wall, resulting in decreased vein wall fibrosis (25).

Mice with PAI-2 deficiency also demonstrate enhanced venous thrombus resolution, although, unlike PAI-1 deficiency, thrombus formation is unaffected (22). In addition, the enhanced venous thrombus resolution observed in PAI-2 deficient mice is associated with increased uPA activity and reduced levels of PAI-1, with no significant effect on MMP-2 and -9 activities (22;26). Flow cytometric quantitation of the relative cell populations within

venous thrombi showed that PAI-2 and PAI-1 deficiencies result in different inflammatory cell populations within venous thrombi, indicating differential sculpting of the inflammatory environment during venous thrombus resolution. These data suggest that specific serpin antagonism may represent a novel approach to accelerate venous thrombus resolution via both inhibition of fibrinolysis and inflammatory vascular remodeling.

Molecular studies of venous thrombus resolution have benefited significantly from the application of clinically relevant experimental mouse models of DVT. In humans, venous thrombi arise from vascular endothelial injury, venous stasis, and/or alterations in blood hypercoagulability (Virchow's triad). The thrombi generally are found in both the vein valve pockets and dilated sinuses of the lower limbs, and form on the surface of activated endothelium (27). They are fibrin and red blood cell rich, and have a laminar structure consisting of layers of platelets, leukocytes and fibrin, that encompass the main erythrocyte mass. They differ from arterial thrombi in being relatively platelet poor and red blood cell rich.

Murine experimental models of venous thrombosis accurately mimic many of the clinical and pathophysiological features observed in human DVT, and have yielded important insights into the molecular and cellular processes that drive venous thrombosis and its resolution (22;28-30). Venous thrombosis models mimic different aspects of DVT and its resolution, each having unique advantages and limitations (31;32). Importantly they offer the advantage of genetic manipulation to dissect molecular mechanisms. We find the stasis inferior vena cava (IVC) ligation model (22;28-30;33), to be a robust, reproducible murine model that accurately mimics many of the clinical and pathophysiological features observed in human DVT (32). This well established model involves laparotomy, division of the lumbar veins from the renal veins to the caval bifurcation, and complete ligation of the IVC immediately below the renal veins. Acute DVT thrombi are formed beginning from 3 hours and reach maximal size by 3-4 days after IVC ligation. The thrombi are associated with significant neutrophil influx and accumulation, and there is a tipping of the balance of prothrombotic and fibrinolytic activities. Beyond this time, subacute and chronic phases of DVT occur which are associated with predominant monocyte influx from days 6-12, with maximal intra-thrombus macrophage levels typically observed around day 8. Fibrinolytic and fibrotic tendencies predominate at this time, thrombus resolution occurs and thrombus weight decreases. Experimental thrombus weight measurements at day 12 or later are taken as an indicator of thrombus resolution (22;33). Infiltration of multiple myeloid cells, collagen deposition and tissue remodeling occur during the chronic stages in both the vein wall and thrombus, similar to human chronic DVT and PTS (31).

While the stasis model does not reproduce the clinical scenario where a thrombus is nonocclusive, it does reproducibly mimic complete occlusion, which is pathologically significant since human DVTs are initially occlusive in 88% of cases (34). To overcome the potential limitation of lack of blood flow in the stasis IVC model, the stenosis IVC model of venous thrombosis results in incomplete ligation and allows restricted blood flow, rather than complete occlusion (29). The stenosis model is less reproducible than the stasis model with a larger variation in the size of the thrombus and sometimes an absence of thrombi in some mice; however, it offers continuous minimal venous flow (29), as well as access for

therapeutic drug delivery. Both the stasis and stenosis models enable recovery of thrombi at specified time points that are analyzed for molecular and cellular indicators of inflammation and thrombus resolution (immunohistochemistry, qPCR, immunoblotting, flow cytometry). Injury to the vein wall as a consequence of DVT is typically measured by histological analysis of the vein wall structure and thickening (collagen deposition), and inflammation (measured by inflammatory cell infiltrate and cytokines/chemokines).

Overview of methods:

All procedures are performed in accordance with the Institutional Animal Care and Use Committee approved guidelines. Mice are placed under anesthesia, receive pre-emptive analgesics and an incision made on the abdomen to expose the major vein in the abdomen, the caudal vena cava (or IVC in humans). The vena cava is dissected free from the surrounding tissue and a tie placed around the vein and tied to cause the clot to form immediately below it. Sham control surgeries are performed, wherein mice will have anesthesia and be operated on in the same manner however no tie will be placed around the vena cava. The sham operation is required to control for any inflammatory effects that result from opening and closing the abdomen as well as dissecting the surrounding tissue free to allow for the vena cava to be ligated. The abdomen is then closed and the mouse are allowed to recover from surgery. Following surgery, mice are euthanized at various time points for analysis of changes in thrombus formation (e.g. 2, 4 days post operatively) or thrombus resolution (e.g. 8, 12, and 21 days post operatively). Physical and molecular characteristics such as thrombus weights, gene expression, protein expression, enzyme activities, cytokine profiles, inflammatory cell infiltration and immune activation, and immunohistological characteristics are studied.

2. Materials

2.1 Vena cava ligation

1. A variety of inbred and mixed breeds of mice have been used to study venous thrombosis and resolution. C57Bl/6 mice and genetic mutants derived from this strain have been used extensively in this regard (see **Note 1**). Reproducibility can be enhanced by consideration of a number of animal related aspects (see **Note 2**).
2. Isoflurane (Piramal Critical Care Inc., PA).
3. Oxygen (in 100% O₂ tanks).
4. Bleach or other disinfectants (MB10, Roccal-D).
5. Electrical clipper with a #40 clipper blade.
6. Glass bead sterilizer.
7. Chemical sterilizer (Cidex).
8. Commercial depilatory reagent (Nair).
9. Sterile cotton-tips.
10. Sterile gauze.

11. Sterile drape.
12. Sterile saline solution.
13. Betadine surgical scrub.
14. 70% ethanol solution.
15. Ocular lubricant (Puralube, Lacri-lube).
16. Sterile water.
17. Thermal cautery unit (Bovie).
18. Isothermal heating pad (Braintree Scientific Deltaphase Isothermal Pad).
19. Sterile (7-0) silk suture (Ethicon).
20. Sterile absorbable (4-0 to 6-0) Vicryl suture (Ethicon).
21. Sterile non-absorbable (4-0 to 6-0) Prolene suture (Ethicon).

2.2 Thrombus collection and histological analysis

1. Neutral buffered formalin solution (Fisher).
2. Zinc fixative (BD Biosciences).
3. O.C.T. solution (VWR).
4. Primary and secondary antibodies.

2.3 RNA and protein analysis

1. Trizol solution (Invitrogen).
2. cDNA synthesis kit.
3. Real-time quantitative PCR analyzer.
4. SYBR Green PCR master-mix.
5. Tissue Protein Extraction Reagent (T-Per)(ThermoScientific)
6. Immunoblotting instrument and reagents

2.4 Flow Cytometry

1. collagenase II (Worthington).
2. tPA (EMD Millipore).
3. anti-CD11b (ABD Serotec)
4. anti-Ly-6B.2 (ABD Serotec)
5. Draq5 (Cell Signaling Technology)

3. Methods

3.1 Surgical Instrument Preparation

1. For each survival surgery, sterilize instruments by steam sterilization at 121°C for 30 minutes.
2. If performing multiple surgeries in one session, remove any organic debris from the surgical instruments using soap and water and sterilize the distal 1/3 of instruments between animal surgeries using a Glass Bead sterilizer at 230°C for 15 sec.
3. Place the instrument tips on a rolled up sterile gauze to allow air cooling or rinse with sterile water prior to use on the next animal to prevent possible thermal trauma.
4. For instruments that cannot withstand high temperature, cold sterilize using either Cidex for 10 hours or Sporicidin for 7 hours.
5. If chemicals are used for sterilization purpose, rinse the instruments with sterilized water before surgical procedure.

3.2 Surgical Area Preparation

1. Dedicated small animal surgery room, secluded from high foot traffic should be used for all animal surgical procedures.
2. Surgery suite should be used only by trained individuals.
3. Disinfect the surgical area with an approximate 10% dilution of household bleach or similar disinfectant.
4. Once dry, place a clean pad/drape on the surgical surface.

3.3 Animal Evaluation

1. Record evaluations of animal alertness and hydration status.
2. Record the animal body weight prior to anesthesia.

3.4 Anesthesia

1. Induce anesthesia with isoflurane delivered using a precision vaporizer (see **Note 3**), 3-4.5% in 100% O₂ into an induction chamber with an attached scavenger.
2. Post loss of consciousness, stop the isoflurane flow and flush the induction chamber with 100 % O₂ prior to removing the animal.
3. Continue anesthesia via face mask with 1.5-3% isoflurane in 1L/min O₂ by inhalation delivered via precision vaporizer with a charcoal scavenger for waste gas (see **Note 4**).
4. Monitor the depth of anesthesia by testing of rear foot reflexes before any incision is made, and continue observation (at least every 15 minutes) of

respiratory pattern, mucous membrane color and responsiveness to manipulations throughout the procedure.

5. Record the description of anesthesia, periodic evaluation of anesthetic depth and surgery start and stop times for each animal.
6. In the event of light anesthesia, temporarily stop the surgery and follow up with anesthesia supplementation.

3.5 Animal preparation

1. Place the animal in a ventro-dorsal position on the surgical prep area.
2. Using a 23 or smaller gauge needle, inject appropriate analgesics subcutaneously on the back or shoulder region for pre-emptive analgesia. (see **Note 5**).
3. Wipe the injection site with 70% alcohol on a sterile cotton tip or prep pad.
4. Remove the hair from the abdominal area of the animal using a depilatory agent or using an electrical clipper. Hair should be removed from a region greater than 1cm from the planned incision site (see **Notes 6 and 7**).
5. Transfer the animal to the surgery table with thermal support provided by scientific grade heating pads placed on an insulated surface (towel or cardboard).
6. Apply ocular lubricant to the eyes of the animal to reduce the chance of corneal desiccation.
7. Prepare the skin area with an approximate 10% dilution of Betadine surgical scrub using a clean cotton applicator or 2×2 cotton gauze. Rinse the skin with 70% alcohol using a cotton applicator, and repeat both steps three times.
8. During surgery, place a sterile 4×4 cotton gauze unfolded with a fenestration cut in the center to prevent contamination.

3.6 Surgeon Prep

1. Wear a clean lab coat or a surgical scrub top and a face mask during the surgical procedure.
2. Wash hands with surgical scrub using antibacterial soap, dry the hands on a clean towel (paper or cotton), then don sterile gloves.
3. Maintain aseptic practices throughout the surgical procedures.
4. Change gloves between animals or if grossly contaminated.

3.7 Surgical Procedure

1. Make a midline incision of approximately 3-4 cm (depending on the size of the mice) on the abdomen.
2. Expose the aorta and the vena cava in the retroperitoneal area.

3. With care, cauterize the lateral side-branches of the vena cava and posterior branches of the vena cava between the renal veins and iliac bifurcation using a cautery tool (see **Note 8**).
4. Make a small window between the vena cava and the abdominal aorta immediately caudal to the renal veins (see **Note 9**).
5. For the stasis DVT model, place a 7-0 non-absorbable silk suture underneath the vena cava immediately below the renal veins and then tie it to stop blood flow through the vessel.
6. For the stenosis DVT model, prior to tying the silk ligature in step 5, place a 4-0 Prolene suture longitudinally over the IVC (29)(see **Note 10**). Then gently remove the Prolene suture by sliding it out from the ligature to restore a small amount of blood flow through the vena cava.
7. For sham surgery, follow the same procedure as described above except do not ligate the vena cava (see **Note 11**).
8. Close the abdominal fascia with a continuous absorbable Vicryl suture (4-0 to 6-0).
9. Close the skin incision with a continuous monofilament non-absorbable Prolene suture (4-0 to 6-0) (see **Notes 12 and 13**).

3.8 Animal recovery and post-surgery monitoring

1. Allow the animals to recover from surgery in a separate cage with fresh bedding and placed with $\frac{1}{2}$ the cage bottom on a thick towel and the other $\frac{1}{2}$ of the cage bottom on a scientific grade thermal supportive device/pad until starting to ambulate.
2. Monitor the animals every hour for the first four hours and then move the animals back to their cages (see **Note 14**).
3. Following this, monitor the animals at least 2 times daily for the first 72 hours post-surgery and then daily until day 5. Follow monitoring of the animals at least 3 times weekly until euthanasia after day 5.
4. Continue the administration of analgesics daily to provide 72 hours of coverage postoperatively (see **Notes 5 and 15**).

3.9 Measurement of the thrombus (see **Note 16**)

1. At specific time-points post-surgery, euthanize the animals by administration of isoflurane (5% in 100% O₂ into an induction chamber with an attached scavenger), followed by cervical dislocation to ensure death.
2. Make an incision in the abdominal to expose the retroperitoneal area.
3. Remove the intestine to gain proper access to the formed thrombus (see **Note 17**).

4. Excise the vena cava containing the thrombus by cutting above the silk ligature and at the iliac bifurcation of the vena cava (see **Notes 18** and **19**).
5. Carefully weigh the thrombus using a scientific weighing scale and record the measurements.
6. The length of the thrombus may be measured using a slide caliper.

3.10 Analysis of the thrombus

The recovered thrombus tissue is analyzed for cellular infiltrates as well as expression of genes and proteins using various standard techniques as described below.

1. For immunohistochemical staining and quantification of cellular infiltrates into the thrombus, fix the thrombus in tissue fixative (see **Note 20**), embed in paraffin and cut tissue sections (5 μ m) using standard techniques. Tissue sections may be analyzed by using Hematoxylin & Eosin staining for morphology and Masson's Trichrome staining for collagen, a marker of fibrosis. Thrombus sections may be immunostained for the presence of cellular infiltrates using cell specific antibodies (*e.g.* anti-Lys-6G for neutrophils and anti-F4/80 for macrophages (33) (see **Note 21**).
2. For measurement of the cellular infiltrates into the thrombus using flow cytometry (22;29), cut the thrombus into 1 mm pieces, process into a single cell suspension using collagenase II (1 mg/ml) and tPA (20 micrograms/ml). Count the cells, and stain the cell suspension for macrophages and activated neutrophils with anti-CD11b and for neutrophils with anti-Ly-6B.2 using standard protocols according to the manufacturer's instructions. Draq5 is used to identify nucleated cells and to subtract debris from the cell suspension. We perform flow cytometry analyses using a Becton-Dickinson FACS Calibur flow cytometer and process post-collection data with FlowJo analysis software (Tree Star, Inc).
3. To measure gene expression, extract total RNA from homogenized thrombus samples with TRIzol reagent, reverse transcribe into cDNA according to the manufacturer's instructions, and perform real-time PCR amplifications using Sybr Green reagents. To normalize expression data, use an internal control, such as the ribosomal gene 36B4, for each sample.
4. To measure expression of proteins, extract proteins from tissues using a lysis buffer (we use T-Per) and perform immunoblotting analysis using standard techniques. ImageJ software may be used for quantitation of band intensity by densitometric analysis relative to a standard loading control protein.
5. Protease activities present in the thrombus may be analyzed by zymography or by ELISA assay of extracted lysates. Gelatin zymography may be performed to measure MMP-2 and MMP-9 activities (29;33).

4. Notes

1. The stasis DVT mouse model produces a consistent thrombus within the vena cava that is fibrin, leukocyte, platelet and red cell rich, and forms on the surface of the endothelium.
2. Variability in this model can be introduced by a number of factors. Each laboratory should determine the sensitivity of their mouse strain (inbred, transgenic or outbred) to the development of venous thrombi by IVC ligation. We prefer to use pathogen-free, 8-12 week old mice, in groups of 8-10 animals for these experiments. Studies using genetically modified mice should be littermate controlled using mice generated by crossing heterozygous males and females. Many investigators might also confine analyses to male mice, to avoid possible effects of inter-gender differences.
3. Isoflurane is best used with an anesthetic chamber fitted with a precision vaporizer to deliver controlled amounts of anesthetic and oxygen. The vaporizer should be calibrated at least annually. Mice may also be anesthetized by administration of pentobarbital (50 mg/kg) or combination of Ketamine/Xylazine (Ketamine 80-100 mg/kg and Xylazine 10-12.5 mg/kg) via intraperitoneal injection.
4. If a chemical fume hood is available in the indicated procedure room, waste gas should be vented via tubing to this fume hood.
5. Common analgesics [Buprenorphine (0.05-0.1 mg/kg) or Carprofen (5 mg/kg)] are administered subcutaneously using a 23 or smaller gauge needle.
6. Perform the hair removal away from the surgery area and remove any loose hair prior to transferring the animal to the surgery table.
7. In case of depilatory agent usage, use a commercial product such as Nair (*e.g.* from a local drugstore) applied to the surgical area for 2-3 minutes followed by removal using a cotton tip. Rinse the skin area with mild soapy water and then twice with sterile saline or water for the removal of the residual depilatory agent from the surrounding skin/hair coat.
8. Practice is required to become proficient in reproducibly isolating, manipulating and carefully cauterizing the side branches of the vena cava without injuring the vessel or the aorta.
9. It is helpful to run two sterile cotton tips along the long axis of the aorta-vena cava bundle for the separation of the aorta from the vena cava.
10. The Prolene suture can be replaced with a 0.36 mm guide wire (35;36) or a blunted 30 gauge needle (37;38). The degree of partial obstruction of blood flow can be adjusted using needles or ligatures of different diameter.
11. This control (Sham surgery) is necessary for the evaluation of any normal inflammation that arises from the surgical procedure.

12. Other monofilament 4-0 to 6-0 non-absorbable sutures, such as PDS and Nylon, can be used instead of Prolene for closing the skin. The skin sutures remain in place for animals euthanized prior to day 14 and removed from animals surviving longer than 14 days.
13. In our experience, the maximum duration of surgery is about 40 minutes.
14. In our experience with vena cava ligation performed on mice, the animals do not show significant impairment of activity after recovery period.
15. A Procedural card indicating the description of the surgical procedure (abbreviated), and record of analgesic administration should be placed on the animal cages.
16. We routinely use the weight of the thrombus as a measurement of the extent of thrombus formation as well as resolution (22;33). Others have used thrombus mass (thrombus weight/thrombus length) as the measurement of the formed thrombus (39;40) where the length of the thrombus can be measured using a slide caliper. Measurement of the thrombus can also be performed using high-frequency ultrasonography (36) which also provides the ability to measure blood flow through the formed thrombus. Being non-invasive, high-frequency ultrasonography is particularly useful for measurement of developing and resolving thrombi in live animals. This technique is also helpful for identifying animals with a thrombus in the stenosis model, where not all animals that have undergone surgery develop a thrombus (41).
17. A relatively large range of thrombus sizes may be expected depending on the mouse strain used.
18. In our experience, to separate the surrounding tissue from the formed thrombus, use of a pair of cotton-tips is helpful.
19. By consistently cutting the vena cava above the ligature and at the distal end of the iliac bifurcation to keep a constant length of the vena cava containing the thrombus, variability in the thrombus weights is minimized during collection. In addition, up to day 8, the clot may be separated from the vein wall by gently teasing open the vein wall using two forceps and removing the formed clot.
20. In our experience, tissue fixed in Zn fixative provides optimal staining for endothelial cells, whereas tissue fixed in 10% Neutral Buffered Formalin (NBF) works well for staining of most other markers.
21. A minimum of three animals should be analyzed at each time point.

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Figure 1. Mouse model of venous thrombus resolution.

Representative images of (A) Separated vena cava with the silk ligature passed through the space between the vena cava and the aorta, (B) A tie is formed around the vena cava, (C) The ligature is tightened to stop the blood flow through the vena cava, (D) Typical thrombus formed at day 2 after surgery. (E) Flow diagram of surgical procedures. Adapted from (28).

Table 1.

Advantages and disadvantages of mouse models of deep vein thrombosis:

Stasis Model	Stenosis Model
<p>Stasis is achieved by complete ligation of the inferior vena cava immediately caudal to the renal veins using a silk or monofilament suture.</p> <p>Advantages:</p> <ul style="list-style-type: none"> • Consistently results in formation of a thrombus. • Resulting thrombus is relatively constant in size, with very limited variability. • Represents acute deep vein thrombosis scenario. • This model can be used to study the pathophysiology of acute to chronic deep vein thrombosis process. <p>Disadvantages:</p> <ul style="list-style-type: none"> • Endothelial damage due to the use of ligature. • Lack of flow limits the analysis of effects of systemic drugs and cellular kinetics. 	<p>Stenosis is achieved by placing a prolene suture (or 30-gauge needle) onto the vena cava longitudinally before ligation, and subsequently taken out after ligation to allow for limited blood flow.</p> <p>Advantages:</p> <ul style="list-style-type: none"> • Thrombus is formed in presence of laminar flow, representing clinical scenarios where vein is not completely occluded. • A good model for thrombus formation analysis. • Flow through the forming thrombus allows for effect of systemic drug and cellular kinetics analysis. <p>Disadvantages:</p> <ul style="list-style-type: none"> • Does not consistently result in formation of a thrombus in all animals. • Resulting thrombus is highly variable in size. • Some resulting thrombi are completely occluded.