Adenovirus-Mediated VEGF Gene Therapy Enhances Venous Thrombus Recanalization and Resolution

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Objective—Rapid thrombus recanalization reduces the incidence of post–thrombotic complications. This study aimed to discover whether adenovirus-mediated transfection of the vascular endothelial growth factor gene (ad.VEGF) enhanced thrombus recanalization and resolution.

Methods and Results—In rats, thrombi were directly injected with either ad.VEGF (n=40) or ad.GFP (n=37). Thrombi in SCID mice (n=12) were injected with human macrophages transfected with ad.VEGF or ad.GFP. Thrombi were analyzed at 1 to 14 days. GFP was found mainly in the vein wall and adventitia by 3 days, but was predominantly found in cells within the body of thrombus by day 7. VEGF levels peaked at 4 days (376±299 pg/mg protein). Ad.VEGF treatment reduced thrombus size by >50% (47.7±5.1 mm² to 22.0±4.0 mm², P=0.0003) and increased recanalization by >3-fold (3.9±6.69% to 13.6±4.1%, P=0.024) compared with controls. Ad.VEGF treatment increased macrophage recruitment into the thrombus by more than 50% (P=0.002). Ad.VEGF-transfected macrophages reduced thrombus size by 30% compared with controls (12.3±0.89 mm² to 8.7±1.4 mm², P=0.04) and enhanced vein lumen recanalization (3.39±0.34% to 5.07±0.57%, P=0.02).

Conclusion—Treatment with ad.VEGF enhanced thrombus recanalization and resolution, probably as a consequence of an increase in macrophage recruitment. (Arterioscler Thromb Vasc Biol. 2008;28:1753-1759)

Key Words: thrombosis □ angiogenesis □ gene therapy

Deep vein thrombosis (DVT), which develops in the deep veins of the calf muscles, is associated with outflow restriction, valvular damage, valvular reflux, and ambulatory venous hypertension. This can lead to the postthrombotic syndrome, which occurs in approximately a third of affected patients and is characterized by leg swelling, pain, and chronic ulceration.¹²

The majority of patients who develop a DVT are treated by anticoagulation with low-molecular-weight heparin or unfractionated heparin, and warfarin.⁴ Such treatment prevents thrombus extension, death by pulmonary embolism, and recurrence of venous thromboembolism, but does not accelerate thrombus resolution, and is associated with a risk of bleeding.⁴ Rapid restoration of vein patency using thrombolytic agents may reduce the incidence of postthrombotic syndrome⁵ but has a greater risk of bleeding, and patients must be treated within a few days of thrombus development. Rapid natural recanalization is associated with improved valve preservation and a lower incidence of reflux, while patients with shorter recanalization times are reported to have a better clinical outcome.⁶⁷ Valve damage and subsequent incompetence is therefore not an inevitable consequence of thrombosis, and it is reasonable to expect that enhancing natural thrombus resolution might reduce the incidence of postthrombotic complications.

Thrombosis resolution occurs by a process of organization that is similar to that found in wound healing.⁸⁹ Thrombus organization begins immediately and is accompanied by the formation of vascular channels,⁸¹⁰¹¹ which appear within and around the thrombus as resolution proceeds. The development of the recanalizing channels may be regulated by the recruitment of a variety of cells, including endothelial cells, macrophages, and bone marrow–derived progenitors.⁸¹²¹³ The invading proangiogenic cells express growth and chemotactic factors such as vascular endothelial growth factor (VEGF), which may promote the formation of the vascular channels within the thrombus.⁸¹⁴¹⁵

VEGF is a potent angiogenic cytokine and has been delivered as recombinant protein, using plasmids or viral gene constructs to encourage blood vessel formation in ischemic tissues.¹⁶¹⁷ We have previously shown that VEGF is expressed in a temporal pattern by cells recruited into the thrombus during natural resolution,¹⁵ and that treating thrombi with recombinant VEGF protein or plasmid containing the VEGF gene accelerates thrombus recanalization and resolution.¹⁸¹⁹
This study aimed to determine whether more efficient transfer of the VEGF gene using an adenoviral vector would have a greater effect on thrombus recanalization and resolution. The mechanisms by which VEGF enhanced thrombus resolution were also investigated.

**Materials and Methods**

### Adenoviral Vectors

Delivery of VEGF was achieved using an adenovirus vector containing cDNA for the 165 splice variant of human VEGF (ad.VEGF). Virus containing the gene for green fluorescent protein (ad.GFP) was used as a control. (See supplemental material for details of construct growth and purification, available online at http://atvb.ahajournals.org).

### Adenovirus-Mediated VEGF Gene Transfer In Vitro

Production of VEGF by ad.VEGF-transfected HEK293 cells was assessed by ELISA (R&D Systems). Ad.GFP-infected cells were used as a control.

The biological activity of the VEGF produced by ad.VEGF-transfected HEK293 cells was assessed by measuring human umbilical vein endothelial cell proliferation (see online supplemental material).

### Rat and Mouse Models of Thrombosis

Laminated thrombus was produced in the inferior vena cava (IVC) of rats and mice using a flow model of thrombosis previously described (See online supplemental material).

### Adenovirus-Mediated Gene Transfer Into Thrombus: Tracking Studies

Thrombus, formed 24 hours earlier in adult male Wistar rats (n=9), was injected with 10⁵ PFU ad.GFP (10 µL). Three animals were euthanized at 3, 7, and 14-days after gene transfer to assess viral transfection and GFP protein expression. These were the only group of animals in which the thrombus was perfus-fused with 4% paraformaldehyde. Sections (10 µm), taken at intervals through each thrombus, were mounted with DAPI (Vectorshield, Vector Laboratories). Fluorescent images were captured and processed as shown in online supplemental material.

### VEGF Production in Thrombus Transfected With ad.VEGF

Thrombi were induced in 28 adult male Wistar rats. Thrombi were injected with 10 µL sterile PBS containing 10⁸ pfu ad.VEGF (n=20, 5 per group) or 10⁶ pfu ad.GFP (n=8, 2 per group) 24 hours after induction. Animals were killed 1, 2, 4, and 7 days later. Blood was collected and the IVC harvested from the ligature to the confluence of the iliac veins and snap frozen in liquid nitrogen (LN). Human VEGF protein concentration was measured in tissue homogenates and plasmas by ELISA (Quantikine, R&D Systems).

### Thrombus Resolution After VEGF Gene Therapy

#### Treatment With Naked ad.VEGF

Thrombi were induced in 48 adult male Wistar rats (supplemental Figure I). Thrombi were injected with 10 µL of sterile PBS containing 10⁷ pfu (n=20) or 10⁵ pfu (n=8) ad.VEGF or 10⁷ pfu ad.GFP (n=20) 24 hours after induction.

#### Treatment With ad.VEGF Transfected Macrophages

Human macrophages (10⁵/mL) were transfected with ad.VEGF or ad.GFP (500-MOI) after MCSF stimulation. Thrombi were formed in 22 mice with severe combined immunodeficiency (SCID) and injected 24 hours later with 10⁵ (10 µL) macrophages transfected in vitro with either ad.VEGF (n=11) or ad.GFP (n=11, supplemental Figure II).

Animals were killed 7 days after gene transfer, and IVC, from the ligature to the confluence of the iliac veins, harvested and processed for paraffin sectioning. Sections were stained with Martius Scarlet Blue (MSB). Confirmation of endothelial cell phenotype of cells lining channels was carried out by immunohistochemistry (IHC) with VEGF (A-20, Santa Cruz) and VEGF receptor-2 (VEGFR2, A3, Santa Cruz).

Six thrombi were randomly chosen from each of the 10⁵ pfu ad.VEGF and ad.GFP-treated animals and analyzed for macrophage and endothelial cell content. Cells were located in the thrombus using IHC with a monoclonal mouse antirat macrophage antibody (ED1, Serotec) and polyclonal rabbit anti VEGFR2 antibody (A3), respectively.

### Image Analysis

Thrombus area was expressed as a percentage of the total vein lumen area, while the areas of recanalization and macrophage and endothelial cell staining were calculated as a percentage of the area of the vein lumen and thrombus, respectively. Full details of this analysis are given in the online supplemental material.

### The Effect of ad.VEGF Treatment on Circulating Cell Numbers

Thrombus was formed in a cohort of 20 8-week-old FVB/N mice (supplemental Figure III). A sham-operated group (n=10) and untreated animals (n=10) were used as controls. The thrombi were injected after 24 hours with either 10⁵ pfu ad.VEGF (n=10) or with 10⁶ pfu ad.GFP (n=10). The sham operated group underwent relaparotomy and closure, without an injection. Blood was analyzed for expression of haemotopoietic (CD45²⁺), progenitor (Sca1²⁺), and endothelial (VEGFR2²⁺) markers at 3 days (see online supplemental material). Expression of VEGFR2 distinguished endothelial progenitor cells present both inside the thrombus and in the periphery of the wall (Figure 1a).

### Statistics

Results are expressed as mean±SE, or median and range for small sample sizes. Statistical significance was determined by using parametric or nonparametric tests as appropriate.

### Results

#### Adenovirus-Mediated VEGF Gene Transfer In Vitro

Conditioned-medium from ad.VEGF-infected HEK293 cells contained more than 5-fold more VEGF protein than cells infected with ad.GFP (500 pg/mL [450 to 600] versus 96 pg/mL [60 to 105]). Macrophages transfected with ad.VEGF produced 1445 pg/mL (1350 to 1602) of VEGF protein compared with no detectable VEGF in the conditioned-media from ad.GFP transfected cells. Incubation with neat and a 1/10 dilution of conditioned medium from ad.VEGF-transfected HEK293 cells increased HUVEC proliferation by ~75% and 15%, respectively, compared with 2% induced by basal-medium (P<0.001 ANOVA) and ~30% by VEGF standard (5 µg/mL, P<0.01; supplemental Figure IV).

#### Adenovirus-Mediated Gene Transfer Into Thrombus

GFP expression was predominantly seen in the innermost aspect of the vein wall at 3 days, with a small number of transfected cells present both inside the thrombus and in the periphery of the wall (Figure 1a). More GFP-expressing cells were seen within the thrombus at day 7 (Figure 1b and 1c). The pattern of
transfection at 2 weeks was similar to that seen at 1 week, albeit with fewer GFP-expressing cells in the thrombus.

Human VEGF was detected in ad.VEGF-treated thrombi at all time points with the highest amounts after 2 days (300±125 pg/mg protein) and 4 days (376±299 pg/mg protein). VEGF was not detectable in ad.GFP transfected thrombi (Figure 2). Human VEGF was not detected at any time point in the plasma from animals treated with either ad.VEGF or ad.GFP.

Thrombus Resolution After Treatment With Naked ad.VEGF

All animals developed thrombi. Treatment with 10⁸ pfu ad.VEGF resulted in a greater than 50% reduction in thrombus size (47.7±5.1 mm² versus 22.0±4.0 mm², P=0.0003; Figure 3a) and more than 3-fold increase in recanalization area (3.9±0.69% versus 13.6±4.1%, P=0.024; Figure 3b) compared with thrombi injected with ad.GFP (high-powered micrographs of recanalizing channels are shown in supplemental Figure V). The amount of thrombus, expressed as a percentage of the vein lumen, was significantly reduced in animals treated with 10⁸ pfu ad.VEGF (96.1±0.69% versus 86.4±4.1%, P=0.024; Figure 3c). Representative sections of thrombi from treated and control animals are shown in Figure 4 (and supplemental Figure VI). Thrombi treated with the 10⁹ pfu ad.VEGF dose were also smaller and better recanalized than those treated with ad.GFP, but the 10⁹ pfu did not confer any advantage compared with 10⁸ pfu ad.VEGF.

Macrophage and VEGFR2⁺ Cell Content of ad.VEGF-Treated Thrombi

Thrombi injected with ad.VEGF at a 10⁸ pfu dose had a higher macrophage cell content than thrombi treated with ad.GFP (19.9±1.6% versus 12.8±1.17%, P=0.002; Figure 5a, staining with ED1 is shown in Figure 5c through 5e). There was no difference between the number of VEGFR2⁺ cells in sections of thrombus from ad.GFP and ad.VEGF treated groups (Figure 5b).

Thrombi Treated With ad.VEGF-Transfected Macrophages

Mouse thrombi injected with macrophages transfected with ad.VEGF were 30% smaller than thrombi treated with ad.GFP transfected macrophages (12.3±0.89 mm² to 8.7±1.4 mm², P=0.04; Figure 6a). Lumen recanalization was also enhanced by more than 30% after treatment with ad.VEGF-transfected macrophages (3.39±0.34% to 5.07±0.57%, P=0.02; Figure 6b). There was a small but significant reduction in thrombus size expressed as a percentage

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** GFP expression in ad.GFP-treated thrombi showing fluorescence predominantly associated with the vein wall and its surrounding tissue at 3 days (a; bar=50 μm). b, By day 7, GFP expression can be seen within the thrombus (bar=200 μm). c, GFP is cell-associated (bar=10 μm). d and e, Negative controls (bar=30 μm). Sections b, c, and e have been counterstained with DAPI (blue).

![Figure 2](http://atvb.ahajournals.org/)

**Figure 2.** Human VEGF protein concentration measured in ad.VEGF-treated thrombi at days 1, 2, 4, and 7. VEGF protein peaked between 2 days and 4 days after injection. VEGF protein was not detectable in thrombi treated with the ad.GFP construct.
of vein lumen in ad.VEGF-transfected macrophage-treated animals (96.6±0.34% versus 94.9±0.57%, P=0.02; Figure 6c).

The Effect of VEGF Gene Transfer on Circulating Cells

There were no significant differences in the number of circulating CD45+/ScaI+/VEGFR2+ cells between any of the groups of animals studied (supplemental Figure VIIa). There were, however, twice as many ScaI+ cells after ad.VEGF-treatment compared with no intervention (75230±11696 versus 34888±7235, P=0.014), or the sham-operated group (75230±11696 versus 36795±6266, P=0.0096; supplemental Figure VIIb). The mean number of circulating ScaI+ cells in the ad.VEGF-treated group was 33% higher than that of the ad.GFP-treated mice (75230±11696 versus 56617±8067), but this difference did not reach statistical significance (P>0.05). There was 41% more ScaI+ cells in Ad.GFP-treated animals compared with sham controls, but this also did not reach statistical significance (P>0.05). No difference was found in circulating ScaI+ cell numbers between sham controls and animals having no intervention (P>0.1).

Discussion

The resolution of experimental thrombi was markedly enhanced within 7 days of treatment with ad.VEGF. Thrombus size was halved, and vein lumen recanalization was increased by more than 3-fold. This was a more potent effect than the
differences observed 14 days after delivery of either recombinant human VEGF protein or a plasmid expressing human VEGF in our previous studies.\textsuperscript{18,19} This was not unexpected as adenoviral vectors are more efficient transfectants of both dividing and nondividing cells than expression plasmids.\textsuperscript{27} The VEGF concentrations in the thrombus were more than 20-fold higher than that obtained after VEGF-plasmid transfection.\textsuperscript{19} This transient elevation of VEGF expression is in keeping with other studies that have used adenovirus-mediated VEGF gene delivery and reported increased revascularization.\textsuperscript{28,29} Our study supports these findings, showing that an increase in VEGF in the first 4 days after transfection was sufficient to enhance resolution by day 7. It appears that early transient VEGF expression is sufficient to drive the remodeling processes needed for resolution and that these processes continue after the VEGF levels have decreased. The mechanism for the effect of VEGF on thrombus resolution requires elucidation.

Angiogenic compounds have previously been used to enhance resolution in experimental thrombi, with modest results. Administration of basic fibroblast growth factor (bFGF) protein increased recanalization of thrombi but had no effect on thrombus volume or organization.\textsuperscript{30} Injection of interleukin (IL)-8 and monocyte chemotactic protein 1 (MCP1), proteins with known angiogenic properties, has been shown to improve blood flow through resolving experimental thrombi and accelerated their dissolution.\textsuperscript{14,31} VEGF has been used extensively to promote neovascularization in ischemic tissues. VEGF has been delivered either as recombinant protein, or using plasmid and viral gene constructs, to human subjects in an attempt to improve lower limb perfusion.\textsuperscript{16,32} So called “therapeutic angiogenesis” has also been used to treat ischemic heart disease.\textsuperscript{33,34}

It is difficult to determine whether the channels that developed around the thrombus were purely associated with angiogenesis or whether there is a contribution from thrombus retraction. Our previous studies suggest that the small vascular channels that develop between the thrombus and the vessel wall may influence the formation of large vascular spaces that are important for the patency of the vein lumen.\textsuperscript{11} Thrombus appears to resolve through mechanisms similar to those found during wound healing, and it is likely that a combination of neovascularization and thrombus retraction is important in this process.
A variety of cells including monocytes, endothelial cells, and bone marrow–derived progenitor cells infiltrate the thrombus during its resolution. Monocytes are naturally recruited into resolving thrombi over time, and thrombus resolution is inhibited when their recruitment is restricted. VEGF has a number of properties that affect the recruitment, migration, and proliferation of many cells, including the monocytes. VEGF also activates macrophages to produce a number of proteases (including plasminogen activators and matrix metalloproteinases), growth factors, and chemokines that regulate recruitment of cells and turnover of extracellular matrix. In the present study, treatment with adenovirus increased the numbers of thrombus macrophages (ED1+ cells). The adenovirus could, therefore, have enhanced thrombus recanalization and resolution by stimulating the recruitment and activation of macrophages.

VEGF mobilizes and recruits EPCs from the bone marrow and may, therefore, have contributed to de novo formation of vascular spaces within and around the thrombus (vasculogenesis). The number of circulating EPCs (VEGFR2+/Sca1+/CD45−) was, however, similar in all groups of animals, suggesting that EPC mobilization was not the mechanism by which adenovirus affected thrombus recanalization. The total number of progenitors (Sca1+ cells) was elevated in the adenovirus-treated animals. This may have been a consequence of the inflammatory response induced in the host by the adenovirus rather a direct effect of VEGF.

It was hoped that treating the thrombus with adenovirus-transfected macrophages would be as effective as injecting the naked construct, as these cells are normally recruited into the thrombus in large numbers. This would have given the impetus for the development of a less invasive treatment, whereby adenovirus-transfected macrophages are injected peripherally, or alternatively the adenovirus construct could be targeted to circulating monocytes. The increase in thrombus organization and recanalization that occurred after direct injection of transfected monocytes was, however, more modest than that observed with adenovirus treatment. This may have been for a number of reasons. The direct injection of naked adenovirus into the thrombus probably resulted in the transfection of a variety of cells (endothelial, macrophage, smooth muscle cells, and neutrophils) found within the resolving thrombus. In contrast, adenovirus “packaged” in a relatively small number (10^5) of injected cells, all of which may not have been retained in the thrombus and may have limited the total amount of VEGF protein expressed. Another plausible explanation is that differences between the speed of natural resolution/recanalization in the rat and mouse models may have accounted for this discrepancy. The use of human macrophages may have been another confounding factor, as their migration and activity in the thrombus may not have had the same impact on resolution as native macrophages. Targeting rat or mouse monocytes with adenovirus-gene construct in vivo could address these problems.

The acceleration in experimental thrombus resolution obtained in the present study was substantial. Similar benefits have, however, been reported in experimental models of tissue ischemia, which were not borne out in the double blind, randomized, controlled clinical trials. The lack of therapeutic benefit in man is thought to be because the blood vessels that grow after short-term treatment with proangiogenic agents are unstable and of small caliber. The processes that regulate resolution in young healthy animals are also radically different from those of older human populations. Thrombus resolution may, however, only require a short-term stimulus for the rapid formation of cell-lined vascular spaces within and around the fresh thrombus to establish a patent lumen. Although thrombus resolution in the rodent vena cava is a more rapid and almost complete process compared with human thrombosis, the recanalization process and cellular changes that occur are remarkably similar.

The long-term aim of our work is to develop treatments that will enhance natural resolution, and in the context of the human literature this should reduce postthrombotic complications. Our models of thrombus resolution clearly do not develop postthrombotic syndrome (PTS), and our outcome measures are therefore restricted to recanalization and organization. It will have to wait for clinical studies before outcome measures regarding PTS can be assessed.

Adenoviral delivery of VEGF was chosen for this proof of concept study as it provided a greater sustained production of VEGF than the expression plasmid or VEGF protein used in our previous studies. Administration of adenovirus gene constructs given locally is reported to be relatively safe. There are, however, problems, such as activation of the immune system, which limit the use of adenoviruses as gene delivery vehicles. These may be overcome through advances in gene therapy, whereas the development of cell specific or tissue targeting techniques could also provide a means of directing gene transfer and improve the efficacy of VEGF gene delivery. There are also methods of sustained or targeted VEGF protein and naked gene delivery that could potentially be used to circumvent many of the real and perceived problems associated with the use of viral constructs and are worthy of further investigation. These include encapsulation of VEGF protein in biodegradable microspheres, which could be administered into the thrombus by image-guided (eg, ultrasound) injection; or encapsulation in microbubbles, delivered systemically and targeted to thrombus by ultrasound-induced release.

Although thrombolysis is a therapeutic option for the rapid removal of a venous thrombus when it is relatively fresh, it does not remove organizing thrombus and there is also a significant risk (up to 16%) of pathological bleeding. It is clear, therefore, that the development of alternative strategies that enhance the natural process of resolution are worth pursuing. Increasing VEGF levels in the thrombus might provide a useful adjunct to current conventional anticoagulation.

Disclosures
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References
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In the article that appeared on page 1753 of the 28.10 issue, author A. Wadoodi’s name should be moved prior to G.S. Kanaganayagam in the author byline. Additionally, K.G. Burnand should move to the third place in the byline, prior to J.A. Gossage. The error has been noted in the online version of the article, which is available at http://atvb.ahajournals.org.

Reference:
Supplemental Materials

Adenovirus construct growth and purification

The adenoviral constructs were grown in human embryonic kidney cells (HEK-293, Stratagene, UK). These cells were infected with ad.VEGF or ad.GFP at 1x10^8, adenovirus plaque forming units (pfu) per ml. The adenoviral infection was allowed to progress until maximum cytopathic effect was seen. The adenoviral constructs were purified using double caesium chloride density gradient centrifugation, desalted and passed through a 0.22µm filter. The caesium chloride gradient was prepared in injectable sterile water. Sterile plastic ware and equipment was used throughout. The viral titres were estimated in semi-confluent HEK293 cell monolayers using the Adeno X Rapid Titre kit (Becton Dickinson, UK). The batches of virus used for these experiments were purified to ~10^{11} pfu/ml and stored.

HUVEC proliferation assay

Human umbilical vein endothelial cells (HUVECs, 10^3 cells) were suspended in 100µl of endothelial cell basal medium-2 (EBM-2, Cambrex Biosciences, UK), seeded into individual wells of a 96-well plate. Neat or 1 in 10 dilution of supernatant (10µl) from ad.VEGF transfected HEK293 cells was added to triplicate wells. A VEGF standard solution (10µl) containing 5µg/ml VEGF was used for comparison. The cells were incubated at 37°C in 5% CO₂ and their proliferation quantified using the XTT method at 4-days.
Monocyte isolation, macrophage transfection and VEGF determination

Monocytes, isolated from blood using Optiprep, were seeded onto low adherence petri-dishes (Corning, UK) and incubated with 100ng/ml of monocyte colony stimulating factor (MCSF, R&D Systems, UK). The viable macrophages were harvested after 3-days and incubated in a 96 well plate with ad.VEGF at 500 MOI (multiplicity of infection). Our studies have shown that MOIs as high as 2500 do not affect macrophage viability (unpublished data). Cells infected in exactly the same way with ad.GFP were used as matched controls. Cell viability was assessed by lactate dehydrogenase release (Promega, UK) and caspase-3 activity (Sigma, UK).

Macrophage transfection for injection into thrombus

Macrophages were suspended in RPMI medium containing 10% foetal bovine serum (FBS), 2mM glutamine, 100units/ml penicillin and 0.1mg/ml streptomycin. The cells were placed in 12-well low adherence culture dishes and at a density of $10^6$ / ml, pre-treated for 72 hours with 100ng/ml human monocyte colony stimulating factor (MCSF). The cells were then infected with either ad.VEGF or ad.GFP. The culture plate was immediately centrifuged for 2 hours at 1850g and 20°C and 1.5 ml of supplemented RPMI containing 100units/ml penicillin, 0.1mg/ml streptomycin and 20% FBS was then added. The transfected macrophages were harvested after 4-days in culture. Cell viability was assessed by lactate dehydrogenase release and caspase-3 activity.
Rat and mouse models of venous thrombogenesis

A midline laparotomy was performed and a 5mm segment of the IVC just below the left renal vein was dissected. The technique used for thrombus induction in the isolated segment of IVC differed in the rat and the mouse:

**In the rat** - a 4/0 silk ligature was passed behind the IVC just below the left renal vein. A 3mm ultrasonic flow probe (Linton Instrumentation, UK) was then placed around the IVC, distal to the ligature. The ligature was tightened to reduce blood flow by 80-90% and tied in place.

**In the mouse** - a neurosurgical vascular clip (Braun Medical, UK) was applied to the 5mm portion of IVC below the left renal vein for 15 seconds on 2 occasions, 30 seconds apart to induce endothelial damage. A length of 5-0 polypropylene suture was then placed alongside the IVC. A 4-0 silk ligature (Ethicon Ltd, UK), passed around the IVC just below the left renal vein, was tightened and tied, incorporating the polypropylene suture. The polypropylene suture was then withdrawn leaving an approximately 90% stenosis in the IVC. Controls included sham operated animals that underwent the same procedure where a silk ligature was passed around the IVC, but left untied. This study was carried out under the Animals (Scientific Procedures) Act 1986.

**Thrombus processing for sectioning and image analysis**

All paraffin embedded thrombi were sectioned (5\(\mu\)m) throughout their entire length and sections taken at defined intervals (500\(\mu\)m in the rat and 300\(\mu\)m in the mouse). Digital images were captured at high magnification (x400) as described above. Images of the whole section area were tiled to make a single composite. The perimeter of the thrombus and any peri- or intra-
thrombus cell-lined recanalisation channels, or cell staining, was delineated on the digitised sections and their area measured by a “blinded” observer using computer-assisted image analysis (Image-Pro Plus). Digital image analysis was calibrated by capturing an image of a standard 1mm graticule slide. Thrombus area was also expressed as a percentage of the total vein lumen area. The areas of recanalisation, macrophage and endothelial cell staining were calculated as a percentage of the area of the vein lumen and thrombus respectively.

Images were captured using a microscope mounted CoolSnap-Pro CF colour digital camera and ProScan motorised stage (Datacell, Berkshire, UK). High magnification (x400) images were obtained of the whole section area and tiled to make a single composite using image analysis software (Image-Pro Plus, Media Cybernetics, USA).

**Flow cytometry**

Fluorescence activated cell sorting (FACS) analysis was carried out on blood samples labelled using PerCP-CY5.5-conjugated rat anti-mouse CD45 antibody (30-F11, Beckton & Dickenson, a pan leukocyte marker), fluorescein-isothiocyanate (FITC)-conjugated rat anti-mouse Sca1 antibody (a pan progenitor cell marker); and R-phycoerytherin (R-PE)-conjugated anti-VEGFR2 (Avas 12α1, Beckton & Dickenson, an endothelial cell marker). The CD45 receptor is expressed on haematopoietic cells (mature and progenitor)\(^{30}\) and the Sca1 receptor can be used to differentiate multipotent progenitor cells.\(^{31}\) The endothelial cell marker, VEGFR2 \(^{32,33}\), was used to distinguish endothelial progenitors from the Sca1+/CD45+ circulating stem
cell population. The stained cells were enumerated using three-colour flow cytometry on a FACScan flow cytometer (Beckton & Dickenson, UK). Gates were used to exclude debris, platelets and dead cells before acquiring one million events per sample.

**Adenovirus-mediated gene transfer into thrombus**

Thrombus was injected with adenovirus adenovirus (10 µl) through the anterior wall of the IVC using a 10 µl Hamilton syringe with 36 gauge needle to minimise wall damage and blood loss.

**Perfusion-fixation**

Mice were perfuse-fixed transcardially with 4% paraformaldehyde following a saline flush. Perfusion was at low pressure to avoid disrupting the thrombus. The IVC was harvested from the ligature to the confluence of the common iliac veins and placed in 4% paraformaldehyde overnight. The specimen was then placed in 18% sucrose for 24 hours. Samples were embedded in OCT freezing compound (Tissue Tek, USA), snap frozen in isopentane, pre-cooled in liquid nitrogen, and stored at -80°C for subsequent sectioning.