Vascular endothelial growth factor and basic fibroblast growth factor are found in resolving venous thrombi

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Objective: **Resolution of venous thrombi is accompanied by an ingrowth of capillaries, which appears to be analogous to angiogenesis in other tissues. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are major regulators of angiogenesis. The aim of this study was to determine the temporal changes and the location of VEGF and bFGF expression in a rat model of venous thrombus resolution.**
Design and Methods: **Thrombi were induced in the inferior venae cavae of rats by creating a stenosis to reduce blood flow by 80% to 90%. Thrombi, adjacent inferior vena cava wall, and systemic blood were collected at 1, 3, 7, 14, 21, and 28 days after thrombus generation (n = 9). Sham operations were performed (n = 5), and blood was collected at 1, 3, and 7 days. VEGF and bFGF were measured with specific immunoassays, and levels in tissues were expressed as picogram per milligram soluble protein. Tissues from two animals that were humanely killed 7 days after thrombus formation were prepared for histological examination. Immunohistochemistry was performed by the use of antibodies against VEGF, bFGF, and ED-1 (a monocyte/macrophage marker).**
Results: **Laminated thrombi were reliably produced with a median weight at 1 day of 39 mg (range, 23-63 mg). There was a significant increase in thrombus VEGF concentration between 1 day (median, 247; range, 0-514) and 7 days (median, 556; range, 254-1741) ($P = .02$). There was no difference between the seventh day and subsequent days. There was a positive linear correlation between thrombus bFGF concentration and time ($R = 0.74$, $P < .0001$), with a more than 300-fold increase in bFGF concentration over the 28 days of the study. VEGF and bFGF concentrations in the adjacent vena caval wall did not change significantly with time. The serum VEGF was significantly raised at 1 day (median, 5520 pg/mL; range, 4040-7912 pg/mL) and 3 days (median, 3880 pg/mL; range, 2564-7232 pg/mL) compared with 7 days (median, 1790 pg/mL; range, 232-3228 pg/mL) ($P < .0001$). Similar changes in the serum VEGF also developed in the sham-operated animals. The serum bFGF (day 1 median, 15.5 pg/mL; range, 1-42 pg/mL) did not change with time. Immunohistochemistry showed that the VEGF antigen was localized to monocytes, endothelial cells, and spindle-shaped cells within the thrombus. The bFGF antigen was localized to mononuclear cells and spindle-shaped cells and was also present in the extracellular matrix.**
Conclusion: **VEGF and bFGF are found in organizing thrombi and have characteristic temporal expression patterns, which suggest that they have a role in thrombus resolution. Augmenting angiogenic growth factor expression may enhance thrombus recanalization, thus reducing long-term complications. (J Vasc Surg 2000;32:988-96.)**

The long-term complications of deep venous thrombosis are caused by a combination of valvular reflux and reduced venous outflow (relative obstruction).[1] Differences in the extent of thrombus resolution leave a variable degree of vein damage, which results in ambulatory venous hypertension. Valves

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are often damaged immediately,[2] and in addition, restricted outflow may cause valvular incompetence even at sites at a distance from the thrombus.[3]

The process of thrombus resolution was observed by Virchow[4] and subsequently studied in more detail by Cox[5] and Sevitt.[6] Thrombus is organized by a cellular infiltrate and ingrowth of capillaries (angiogenesis) in a manner similar to wound healing.[7] The complex process of angiogenesis involves the interaction of various cells, including monocytes, which secrete a spectrum of angiogenic and antiangiogenic growth factors.[8] The mechanisms responsible for thrombus resolution are now beginning to be investigated. Recent studies have shown that thrombus organization can be stimulated by the chemokines monocyte chemotactic protein-1 (MCP-1)[9] and interleukin-8,[7] both of which have angiogenic activity. Enhancing thrombus recanalization with specific angiogenic growth factors could result in more effective resolution, which would reduce the complications of post-thrombotic leg.

Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are major regulators of angiogenesis and are increased in wound healing, ischemic revascularization, and tumor growth. We hypothesize that they may also mediate thrombus recanalization. In this study we have attempted to define the molecular mechanism that controls thrombus organization by investigating the expression VEGF and bFGF during thrombus resolution in our animal model.

METHODS

Animal model of thrombus resolution. The resolution of venous thrombi was studied with a previously described rat model of inferior vena caval thrombosis.[10] Adult male Wistar rats were anesthetized with halothane and buprenorphine, and laparotomy was performed. The inferior vena cava was exposed below the renal veins, and flow was measured with an ultrasonic flow probe (Linon Instrumentation, Diss, UK). A 4-0 silk ligature was then placed around the cava and progressively tightened until flow was reduced by 80% to 90% of the original flow. The ligature was left in place, the wound was closed, and the animal recovered. The study was performed under the Animals (Scientific Procedures) Act 1986.

Collection and processing of tissue samples. Thrombi were induced in six cohorts of nine animals. The inferior vena cava and the contained thrombus were harvested from the confluence of the iliac veins up to the renal veins at time intervals of 1, 3, 7, 14, 21, and 28 days after thrombus formation. Blood was collected by direct cardiac puncture into vials that contained serum separation gel and clot activator (Vacutainer).

Tissues samples were separated, weighed, and snap frozen at –80°C. Tissues were then cryohomogenized in liquid nitrogen by means of a Mikro-Dismembrator II (B. Braun Biotech International, Melsungen, Germany) and resuspended in Dulbecco's phosphate-buffered saline (Sigma, UK). Homogenates were spun at 14,000*g* for 5 minutes at room temperature. The supernatants were decanted, snap frozen, and stored at –80°C before assay. Serum was separated from the collected blood by centrifugation at 3000*g* for 10 minutes at room temperature, snap frozen, and stored at –80°C before assay.

In addition, three cohorts of five animals underwent sham operations. The procedure was identical in all respects, including the placement of a ligature around the cava, but the ligature was tied loosely so as not to constrict the caval blood flow. Blood was collected from these animals at 1, 3, and 7 days after operation.

VEGF and bFGF assay. VEGF and bFGF were measured in tissue homogenates and serum samples with a human VEGF competitive enzyme immunoassay (Accucyte; Cytimmune, College Park, Md) and a human bFGF sandwich enzyme immunoassay (Quantikine HS; R+D Systems, Abingdon, UK). Both assays cross-react with the rat antigens. Thrombus protein concentration was measured by means of the Coomassie colorimetric method with a bovine serum albumin standard (Pierce, Rockford, Ill).

Immunohistochemical studies. Thrombi were formed in two animals and harvested after 7 days. Tissues were fixed in 10% formal saline and embedded in paraffin wax. Sections of 5 μm were taken at 0.5-mm intervals through each thrombus and stained by the use of a monoclonal antibody against VEGF and a polyclonal antibody against bFGF (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif). Sections were also stained with a monoclonal antibody against rat monocytes (ED-1; Serotec, Oxford, UK). Primary antibody binding was localized with a biotinylated second antibody in conjunction with a streptavidin-peroxidase complex. Sections were counterstained with Nuclear Fast Red (Vector Laboratories, Peterborough, UK).

Statistical analysis was performed with the Mann-Whitney test for nonparametric data. All tissue results are expressed as picogram per milligram soluble protein.

RESULTS

Thrombi were initially occlusive with a median weight at 1 day of 39 mg (range, 23-63 mg). By 4

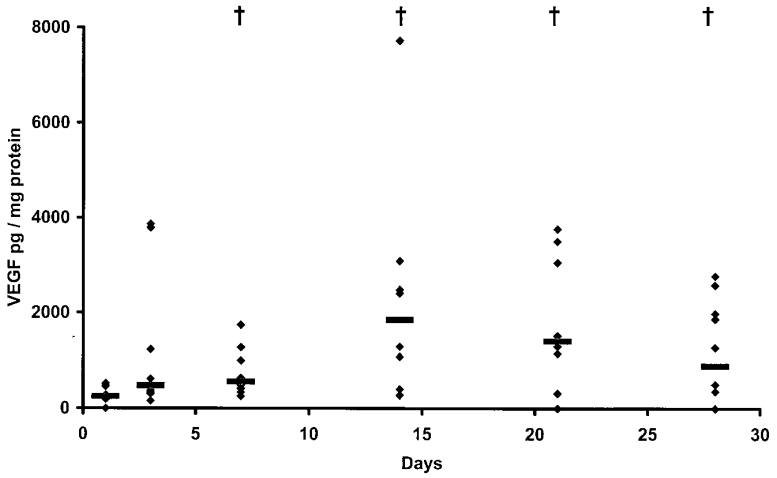

Fig 1. Thrombus VEGF content during resolution. *Horizontal line* represents median value. There was significant increase in thrombus concentration of VEGF from 7 days and thereafter compared with first day. †$P < .05$ (Mann-Whitney test).

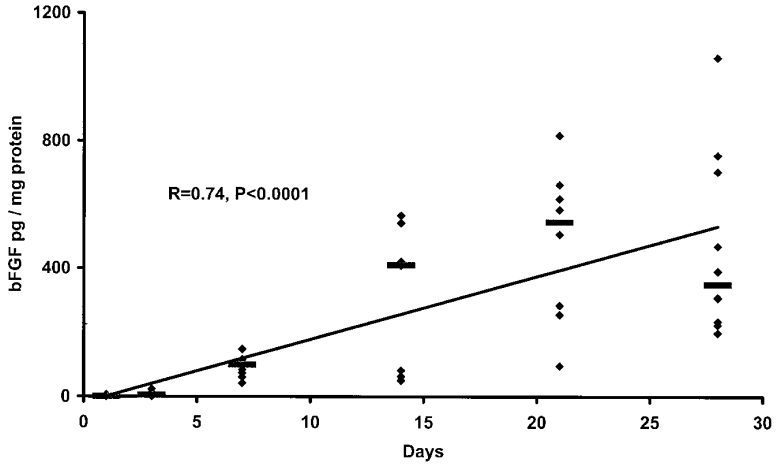

Fig 2. Thrombus bFGF content during resolution. *Horizontal line* represents median value. There was positive correlation between thrombus bFGF concentration and time ($R = 0.74$, $P < .0001$).

weeks the thrombi had resolved to a remnant in the vein wall.

Thrombus VEGF. There was a significant increase in thrombus VEGF concentration in the thrombus between 1 day (median, 247; range, 0-514) and 7 days (median, 556; range, 254-1741) ($U = 11.0$, $P = .02$) (Fig 1). After 7 days there was considerable variation in VEGF concentration (range, 0-7726), although it was always significantly higher than the first day ($P < .05$). There was no significant difference between the seventh day and all subsequent days. Three of the thrombi that had VEGF levels greater than three SDs of the mean appeared more organized and smaller.

Thrombus bFGF. There was a positive correlation between thrombus bFGF concentration and time ($R = 0.74$, $P < .0001$) (Fig 2). There was a greater than 300-fold increase in bFGF concentration from the first day (median, 1.1; range, 0.38-5.37) to 28 days (median, 349; range, 198-1059).

Inferior vena cava VEGF and bFGF. VEGF concentration (median, 159; range, 0-831) and bFGF concentration (median, 311; range, 116-884) in the adjacent inferior vena cava did not change with time (Figs 3 and 4).

Serum VEGF. Serum VEGF was significantly raised at 1 day (median, 5520 pg/mL; range, 4040-

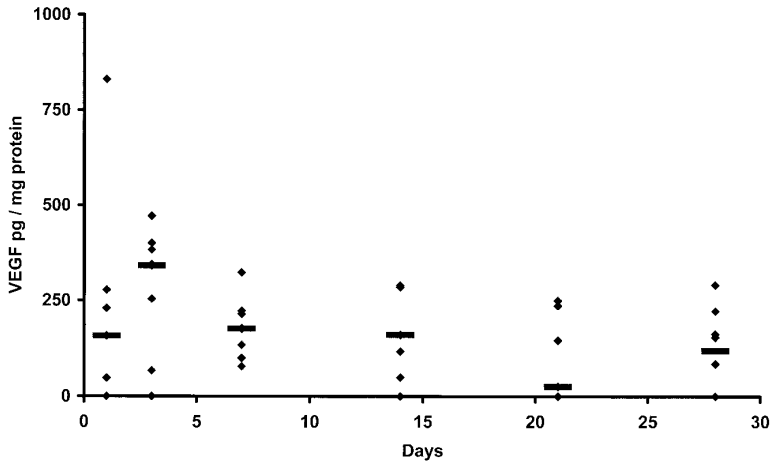

Fig 3. VEGF content in adjacent inferior vena cava during thrombus resolution. *Horizontal line* represents median value. There was no change in VEGF concentration with time.

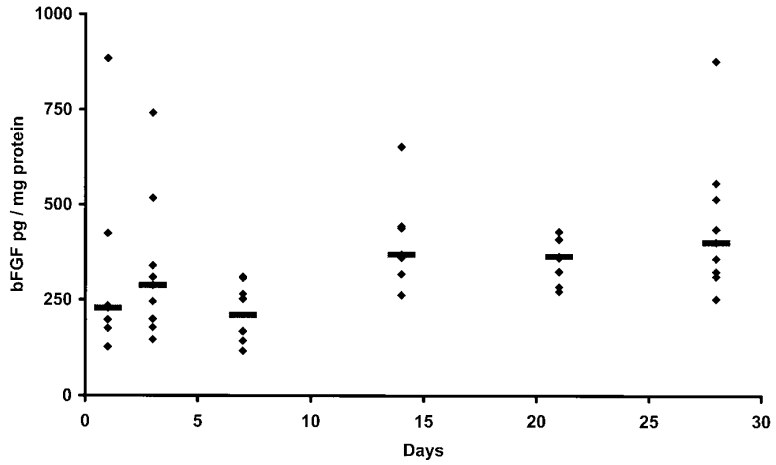

Fig 4. bFGF content in adjacent inferior vena cava during thrombus resolution. *Horizontal line* represents median value. There was no change in bFGF concentration with time.

7912 pg/mL) and 3 days (median, 3880 pg/mL; range, 2564-7232 pg/mL) compared with 7 days (median, 1790 pg/mL, range 232-3228 pg/mL) (both $P < .0001$) (Fig 5). There was no change after 7 days. The sham-operated animals had the same high levels at 1 and 3 days. There was no difference between VEGF levels in the experimental and sham animals at days 1, 3, and 7.

Serum bFGF. Serum bFGF did not change with time (median, 15 pg/mL; range, 1-46 pg/mL) (Fig 6). There was no difference to sham-operated serum bFGF.

Histology. VEGF expression was localized to the periphery of the thrombus at 7 days (Fig 7). High-power microscopy (Fig 8, *A*) and comparison with ED-1–stained sections (Fig 8, *B*) demonstrated that VEGF was localized to endothelial cells, monocytes, and spindle-shaped cells in the thrombus. Cells with endothelial morphology staining for VEGF appeared to be forming tubular structures near the edge of the thrombus (Fig 7). The bFGF was expressed by mononuclear cells and spindle-shaped cells in the thrombus and was also present in the extracellular matrix (Fig 9). Immunoglobulin G controls showed no staining.

DISCUSSION

In this study both VEGF and bFGF were found in resolving thrombi and had distinctive temporal

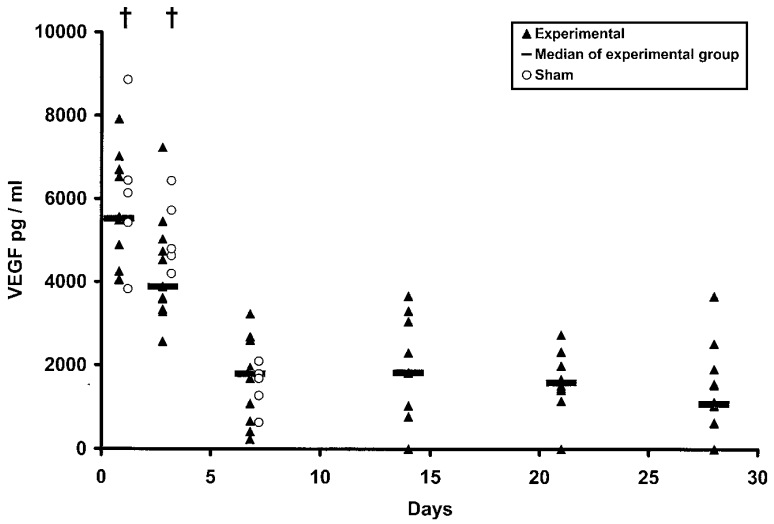

Fig 5. Serum VEGF. There was significant increase in serum VEGF concentration at days 1 and 3 compared with day 7. This was also seen in sham-operated animals. †Values compared with day 7, $P < .0001$ (Mann-Whitney test).

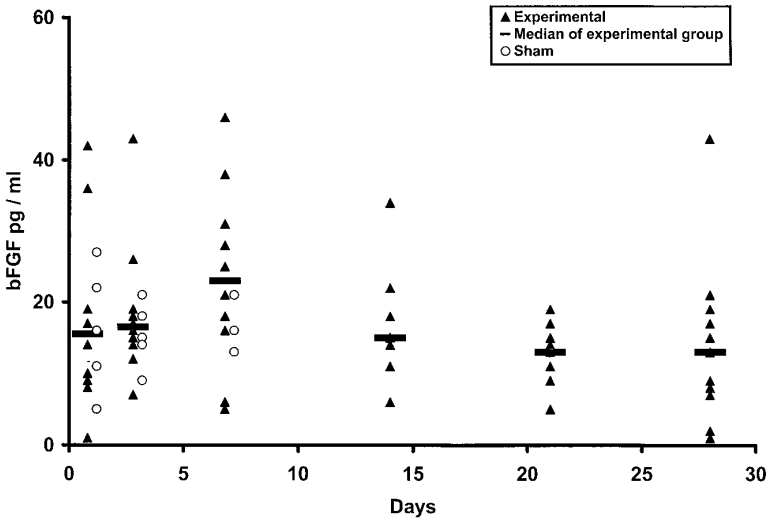

Fig 6. Serum bFGF. There was no change in serum bFGF with time and no difference between animals with thrombus and sham-operated animals.

patterns of expression. This expression of angiogenic growth factors within a resolving thrombus is a novel finding. There was a rise in VEGF concentration in the thrombus, which appeared to peak at about 14 to 21 days, and there was a strong linear correlation between bFGF concentration in the thrombus and time. No change was found in the levels of either angiogenic growth factor in the adjacent vein wall. The initial rise in VEGF serum concentration after thrombus formation was also found in sham animals and was presumably related to surgical dissection and postoperative wound healing. A rise in serum VEGF as a consequence of thrombus resolution might have been overwhelmed by the increase in its concentration after surgery. VEGF expression was histologically localized to several cells in the thrombus, including endothelial cells and the monocyte infiltrate. Some of the cells expressing VEGF

appeared to be forming lumens and were possibly migrating endothelial cells beginning to recanalize the thrombus. Expression of bFGF was found on mononuclear cells and spindle-shaped cells within the thrombus.

In this study we have demonstrated expression of angiogenic proteins in resolving thrombi, but we have not confirmed synthesis of these proteins by any specific cells. It is possible that the angiogenic factors found are synthesized elsewhere and sequestered into the thrombus. Both VEGF and bFGF are synthesized by many cell types, including monocytes, fibroblasts, and endothelial cells.[11] The strong localization of the proteins to these latter three cell types suggests that production within the thrombus is more likely. We are currently attempting to confirm synthesis of angiogenic growth factors within resolving thrombi using in situ hybridization and reverse transcription polymerase chain reaction.

The process of thrombus generation and resolution in rats is not totally comparable to humans, but this type of study could not be ethically performed in humans. In our model, thrombus is formed in flowing blood, and it is therefore superior to models involving simple venous occlusions that produce a "clot" rather than thrombus. It also has the potential advantages that endothelium is maintained and the hemostatic balance is unchanged. Laminated thrombi formed in this model closely resemble human venous thrombi.

Recanalization with new vascular channels occurs within all organizing thrombi that we have examined, partly as a consequence of thrombus contraction and partly as a result of endothelial cell proliferation, endothelial cell migration, and new vessel formation. The vascular spaces formed within the thrombus have been shown to communicate with both the vessel lumen[4] and the vasa vasorum.[12] Thrombus recanalization is therefore similar to angiogenesis, and the same mechanisms that regulate new vessel formation elsewhere may affect the process of thrombus resolution. The origin of the endothelial cells that are found within new channels in the thrombus is unknown. They may be derived from proliferation of local venous endothelial cells or circulating endothelial progenitor cells. Alternatively, it has been suggested that monocytes or other cells may be able to change their phenotype into endothelial cells.[13]

Angiogenesis in wound healing is orchestrated by monocytes, and these cells may also have an important role in thrombus organization and resolution.[14] They infiltrate the thrombus where they produce and release tissue plasminogen activator

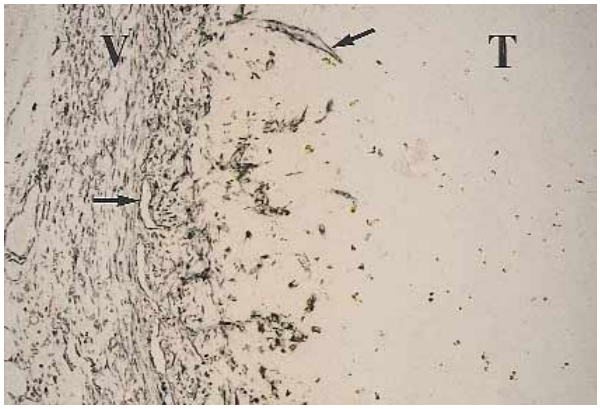

Fig 7. Thrombus 1 week after formation (original magnification ×100) stained for VEGF (*black*). *T,* Thrombus; *V,* vein wall; →, cells with endothelial morphology appearing to form tubules near periphery of thrombus and lining retraction spaces between thrombus and vein wall.

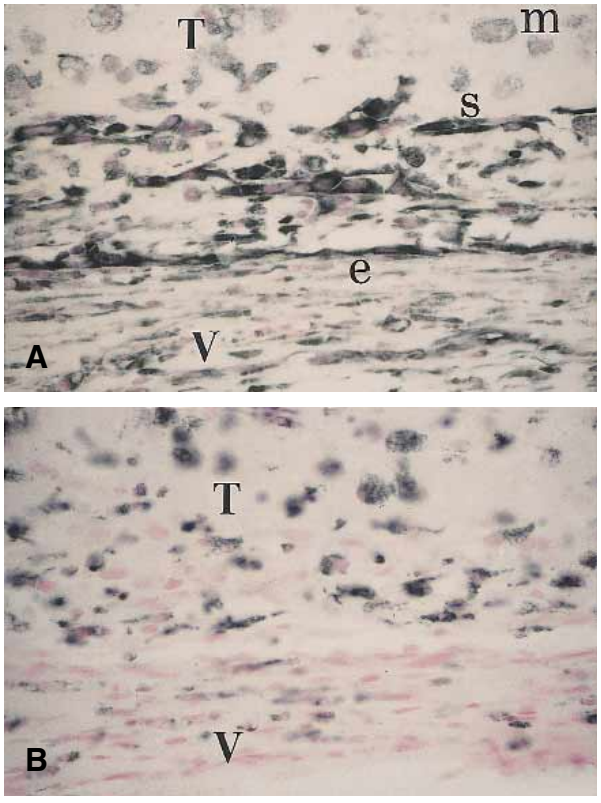

Fig 8. A, High-power micrograph (original magnification ×400) of thrombus edge (*T*) and vein wall (*V*) stained for VEGF (*black*). VEGF is localized to mononuclear cells (*m*), spindle-shaped cells (*s*), and endothelium (*e*). **B,** High-power micrograph (original magnification ×400) of thrombus edge (*T*) and vein wall (*V*) stained for monocyte marker ED-1.

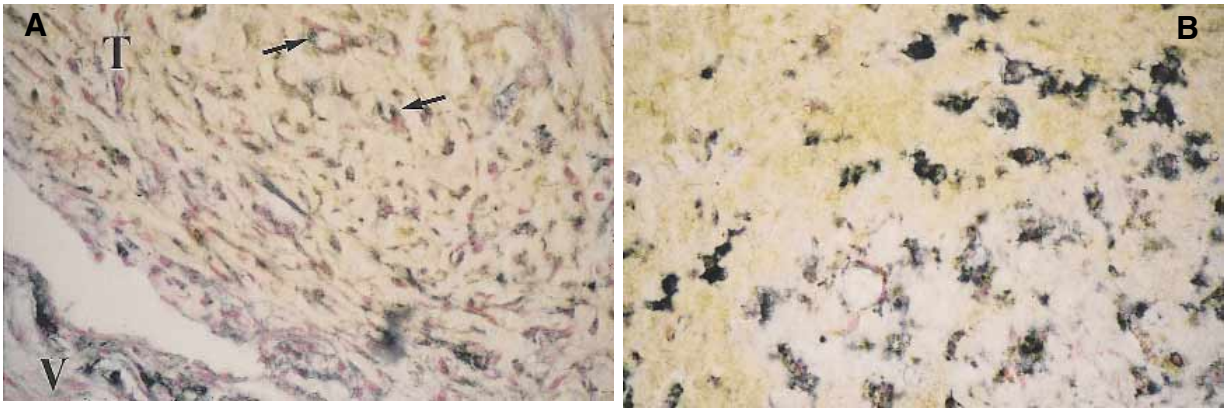

Fig 9. A, Thrombus 1 week after formation (original magnification ×200) stained for bFGF (*black*). *T,* Thrombus; *V,* vein wall. bFGF is localized to a variety of cell types including mononuclear cells, spindle-shaped cells, and cells lining channels forming within the thrombus →. **B,** High-power micrograph (original magnification ×400) of thrombus stained for bFGF (*black*) showing mononuclear cells expressing bFGF.

and urokinase.[15] They can also actively degrade fibrin in the absence of plasmin[16] and produce inhibitors of fibrinolytic activity. Monocytes are capable of synthesizing and secreting a number of proteolytic and angiogenic factors that direct tissue remodeling.[17] Little, however, is known of the mechanisms by which monocytes are recruited into the thrombus or how they are activated, but modulation of these processes may have a profound effect on thrombus resolution. Damaged endothelial cells or platelets within the thrombus may release many cytokines and growth factors that influence monocyte migration and function. We have already shown that levels of the cytokine MCP-1 are raised during thrombus resolution, and when this cytokine is directly injected into venous thrombus, it alters organization.[9] Part of the effect produced by injecting MCP-1 may have been as a consequence of its angiogenic properties.

Most patients with a deep vein thrombosis eventually have long-term complications of pain, swelling, lipodermatosclerosis, and ulceration,[18] which have a large social and economic impact. These symptoms are caused by a combination of valvular incompetence and persistent outflow restriction. Thrombus may damage the valves by direct involvement and may also produce valvular incompetence in the distal venous system.[3] Current treatment with anticoagulants has little, if any, effect on thrombus resolution[19] and does not preserve valves. Early natural recanalization[20] and rapid pharmacologic thrombolysis[21] preserve valve integrity and may improve clinical outcome.[22,23] Many, however, believe that the risk of hemorrhagic complications may not justify the use of thrombolytic agents,[24] and better treatments are needed for patients who present at a late stage.

Treatment of ischemic heart disease and peripheral vascular disease with angiogenic growth factors has produced promising results.[25] Similar treatments could be used to produce rapid resolution of venous thrombosis. Promoting thrombus recanalization with angiogenic growth factors might improve venous outflow and preserve valvular competence. Wakefield et al have already shown that the use of interleukin-8, a CXC chemokine and potent inducer of angiogenesis, can promote the migration of inflammatory cells into a thrombus and enhance early neovascularization. VEGF and bFGF are well-recognized angiogenic growth factors and have a synergistic action.[26] VEGF is a potent and specific endothelial mitogen and chemotactic factor, whereas bFGF also has significant angiogenic activity.[27] Angiogenic growth factors may have a local action within the thrombus, but in addition, VEGF mobilizes endothelial progenitor cells from the bone marrow that circulate and contribute to the formation of new channels in different circumstances.[28] These cells may not only help to recanalize the thrombus but may also encourage collateral development. We are now undertaking further studies to determine whether thrombus recanalization or venous collateral development can be modified by augmenting the expression of angiogenic factors.

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DISCUSSION

Dr Edmund J. Harris (Stanford, Calif). Dr Wakefield, Dr Ehrenburg, members and guests. Mr Waltham comes to us today with the promise of clarifying the relationship between angiogenesis and thrombus recanalization in deep venous thrombosis. The author's group has extensive experience with a rat model of deep venous thrombosis involving the inferior vena cava. The group proposes to measure the expression of VEGF and bFGF in this well-developed model to help us gain insight into the role of these angiogenic factors and recanalization of venous thrombosis.

The group did not measure local expression of these angiogenic proteins, but rather quantified protein levels in the excised segments of inferior vena cava at serial timepoints, and compared these protein levels with sham-operated animals and serum protein levels acquired at sacrifice. Histological evaluation of specimens was performed at 7 days post-thrombosis, with localization of the protein performed by immunohistochemical analysis.

The group noticed a significant increase in VEGF in the thrombus between 1 and 7 days, which was not noticed in the sham-operated group. Yet both experimental and sham-operated groups showed significant elevation in the serum VEGF at 1 and 3 days and less so by 7 days. Is it possible that the thrombus is merely trapping VEGF and the thrombus is not actively producing VEGF as the authors suggest? I would be more comfortable with in situ hybridization data localizing VEGF mRNA to the endothelial cells, monocytes, and spindle-shaped cells observed in the immunohistochemical localization of VEGF protein. Do the authors have any in situ hybridiza-

tion studies confirming the presence of increased VEGF transcript in this model?

Similarly, bFGF mRNA was not characterized in the thrombus or the vessel wall. Protein levels of bFGF increased over time, but the initial levels were significantly lower than those in the adjacent vena cava. Even as the thrombus levels of bFGF increased over time, these peak levels do not appear significantly different than the bFGF levels in the adjacent nonthrombosed vena cava, admittedly, values that had a wide variability. Although serum bFGF levels remain low throughout the study period in both experimental and control animals, the thrombus bFGF levels begin lower than adjacent vena caval levels and then approach parity by 28 days when the thrombus has resolved. This would suggest to me that thrombus suppresses bFGF expression and bFGF is not involved in thrombus recanalization. What are your thoughts on this alternative interpretation of your data?

I too have an interest in the process of thrombus resolution in deep venous thrombosis, and my work has previously been presented at the American Venous Forum. My rat model is one of chronic iliac venous obstruction rather than one of venous stenosis, as in this model. In my studies, thrombus recanalization occurred by 4 weeks, even with complete ligation of the iliac vein. The unexpected finding was the development of neovascular channels around the area of ligation, again suggesting angiogenesis as a mediator of thrombus recanalization. Did you notice any extravascular channels in your model as well? Thrombin is a potent stimulator for expression of angiogenic proteins, mediated via its stimulation of proteinase-activated receptors found in mesenchymal, smooth muscle, and endothelial cells. In our model, the thrombin receptor is significantly upregulated during thrombus resolution. Clot-bound thrombin being released by the process of thrombolysis is the presumed stimulus. Did you measure thrombin activity in the resolving thrombus in your model? I would bet that thrombin levels would correlate with the rise in the VEGF levels, both in thrombus and in the adjacent vena cava, due to washout of the released thrombin.

I enjoyed reading your clear manuscript and thank you for your efforts to provide me the manuscript in a timely manner. I thank the forum for the opportunity to discuss this work.

Mr Matthew Waltham. I thank Dr Harris for his constructive comments.

In this study we have shown that the concentrations of VEGF and bFGF in resolving thrombi increase with time and that these factors are localized to cells within the thrombus. It is not possible to say whether this is the result of production within the thrombus or trapping by these cells, but we did find that thrombus concentration of these growth factors was independent of the inferior vena cava concentration adjacent to the thrombus. We are now performing both quantification of mRNA expression in the thrombus RNA extracts and in situ hybridization analysis. We have not specifically studied extravascular channels produced during recanalization in this model. The strengths of our model are that true laminated thrombi are formed within flowing blood and that recanalization is studied in a nonoccluded vessel. This closely resembles the true clinical scenario. Your observation, Dr Harris, of extravascular channels is very interesting, and their development may involve a mechanism related to that of collateral formation elsewhere. Treatment of venous thrombosis with angiogenic growth factors may also promote development of such collaterals. We have not measured thrombin within our model. The list of factors with angiogenic activities seems to grow longer by the week, and we have decided to look at two that may have therapeutic benefit. We particularly picked VEGF and bFGF because they are the two best characterized factors and are known to play a central role in controlling angiogenesis in other tissues. Of course, the true test of which factors are important in thrombus recanalization and resolution is whether recanalization can be enhanced or blocked by stimulating or inhibiting the particular factor.

Thank you again for your comments and thank you to the American Venous Forum for giving me the pleasure of presenting this paper.