Deep vein thrombosis resolution is not accelerated with increased neovascularization

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Introduction: **Deep venous thrombosis (DVT) resolution involves fibrinolysis, neovascularization, and fibrosis. We hypothesized that promoting neovascularization would accelerate DVT resolution.**
Methods: **A rat model of stasis DVT was produced with proximal ligation of the inferior vena cava (IVC) and all visible tributaries. One μg of interferon inducible protein (IP-10; angiostatic chemokine), basic fibroblast growth factor (bFGF; pro-angiogenic cytokine), epithelial neutrophil activating protein (ENA-78; pro-angiogenic chemokine), or saline solution control was injected into the IVC after ligation, and then via tail vein injection daily until sacrifice at either 4 or 8 days. Peripheral blood counts were measured, and thrombus weight was recorded at sacrifice. Laser Doppler in vivo imaging was used to estimate post-thrombotic IVC blood flow. Immunohistologic assessment of the thrombosed IVC for polymorphonuclear neutrophils (PMNs), monocytes (ED-1), and laminin (neovascular channels) was performed or the thrombus was separated from the IVC and assayed for keratinocyte cytokine (KC), monocyte chemotactic protein-1 (MCP-1), bFGF with enzyme-linked immunosorbent assay (ELISA), and total collagen with a direct colorimetric assay.**
Results: **Peripheral blood and intrathrombus PMNs and monocytes were not significantly different in the treated or control rats. There were no differences in any measure at 4 days. At 8 days, thrombus neovascularity, but not weight or collagen content, was increased in rats treated with bFGF or ENA-78 compared with control rats (17.6 ± 0.93, 16.2 ± 0.97 vs 13.2 ± 0.79; channels/5 high-power fields (hpf; n = 6-10; $P < .05$). Post DVT IVC blood flow was significantly increased in bFGF-treated rats but not in rats treated with IP-10 or ENA-78, as compared with control rats. Rats treated with ENA-78 had increased intrathrombus bFGF compared with control rats (85 ± 27 pg/mg protein vs 20 ± 6 pg/mg protein; n = 6; $P < .05$), but other mediators were not significantly different in treated rats compared with control rats.**
Conclusion: **Pro-angiogenic compounds increase thrombus neovascularization, but this does not correlate with smaller or less fibrotic DVT. Mechanisms other than neovascularization may be more important to hasten DVT dissolution. (J Vasc Surg 2004;40:536-42.)**

Clinical Relevance: **Improved therapy for deep venous thrombosis (DVT) will ideally increase the rate of thrombus dissolution and eliminate the bleeding risks of anticoagulation. This study evaluated promoting DVT neovascularization with angiogenic chemokines, and, while successful by experimental measures, this did not translate into smaller DVT. Solely promoting thrombus neovascularization will not likely speed resolution.**

Deep venous thrombosis (DVT) is a common condition, affecting approximately 250,000 patients per year and responsible for 50,000 deaths per year.[1] Anticoagulant therapies are effective for prophylaxis and treatment,[2] but the risk for anticoagulation-related hemorrhagic complications and development of chronic venous insufficiency (CVI) are significant over time. Angiogenesis, or neovascularization, is now recognized as an important process in many physiologic and disease states, including cancer, peripheral arterial vascular disease, and wound healing.[3] In the setting of a vascular occlusion such as DVT, it is logical to assume that angiogenesis may have a significant effect on the course of its resolution.[4] Previous reports by our group[5-7] have demonstrated a sustained neovascular response after experimental DVT and an apparent beneficial effect on its resolution.

Chemokines are chemotactic cytokines that direct leukocyte extravasation and activity.[8] They can be subdivided into cysteine-X-cysteine (CXC) and cysteine-cysteine families, members of which differ in their leukocyte specificities but often share angiogenic activity. Pro-angiogenic CXC chemokines have a specific peptide sequence, Glu-Leu-Arg (ELR), which confers the neovascular activity.[7] Growth factors are small peptides that stimulate endothelial cell proliferation and migration. Chemokines and growth factors are present during DVT resolution, and experimental studies have demonstrated beneficial effects of the CXC chemokine interleukin (IL)–8,[5] the CC chemokine monocyte chemotactic protein (MCP)–1,[9] and the growth factor vascular endothelial growth factor[10] on DVT resolution.

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However, whether increasing thrombus neovascularity increases thrombus dissolution is unknown.

Clinically, promoting DVT resolution with or without standard anticoagulation is attractive to decrease the risk for pulmonary embolism and to minimize the sequelae of CVI. Chemokines are attractive agents with pharmacologic potential in many disease states.[11] This study used therapeutic administration of interferon inducible protein (IP-10), a CXC ELR–negative angiostatic chemokine,[12] basic fibroblast growth factor (bFGF), a pro-angiogenic factor[10,13] and epithelial neutrophil activating protein (ENA-78), a CXC ELR–positive pro-angiogenic chemokine,[8,14] and measures of resolution of DVT. These agents were chosen because of their in vitro and in vivo activities, and the relatively narrow spectrum of leukocytes affected.

METHODS

Study design and animal model. Male Sprague-Dawley rats (350-450 g) were used in these studies. Thrombosis was induced with proximal ligation of the inferior vena cava (IVC) below the renal veins and ligation of all visible tributaries, as described.[5,6,15] A clot developed in more than 90% of rats. Rats were sacrificed at 4 or 8 days. At sacrifice the thrombosed IVC was exposed through the previous incision and removed en bloc. A small consistent portion was divided intact and preserved for histologic analysis, while most of the specimen was divided into wall and thrombus portions with blunt or sharp dissection. Only the thrombus was then weighed and snap-frozen for later biochemical analysis. All studies were approved by the University of Michigan Committee on the Use and Care of Animals and complied with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

Study agents. Control rats received normal saline solution, and 3 experimental groups at each time point received IP-10, bFGF, and ENA-78 (R&D). Six rats were assigned to each group at the 4-day time point, and 10 rats were assigned to each group at the 8-day time point. Rats were given 1 μg of the study agent daily in 0.1 mL of normal saline solution with 1:500 bovine serum albumin, with dosing extrapolated from other experimental studies.[10,12,13] No specific pharmacokinetic studies were performed. The study agent was first administered at the time of IVC ligation, with injection of the agent directly into the ligated IVC with a 30-gauge needle and cotton-tipped applicator pressure for hemostasis. Subsequent doses were given daily via tail vein injection until sacrifice.

Peripheral blood counts. A sample of arterial blood was taken from the dorsal tail artery before both laparotomies. Peripheral leukocyte quantification was determined by means of automated peripheral blood differential (Hemavet; CDC Technologies).

Laser Doppler scanning. A laser Doppler scanner (Lisca) was used to assess in vivo microvascular IVC blood flow, based on techniques as described.[16] At laparotomy the pre-IVC ligation, immediate post-ligation, and harvest of the exposed IVC region of interest were scanned (5-second scans with 30 seconds between, over 4 cycles). Depth was constant and adjusted for each rodent to ensure the best estimation of the mid-coronal IVC section. These scans were saved, and accompanying image software was used to estimate the mean color flow. This was summed and averaged for the 4 cycles. Mean intensity was determined for each rodent at the various time points, and represents intra-IVC blood flow velocity, not volume flow. The intensities were reported as percent of baseline flows specific to each animal to ensure consistency (each served as its own control).

Immunohistochemistry. The specimen harvested for histologic analysis was imbedded in paraffin, sliced into 10-μm sections, and stained as described.[5,6,17] In brief, the tissue was first deparaffinized and dehydrated through graded alcohols, and nonspecific binding sites were blocked with species-specific serum. The sections were then incubated with either 1:1000 anti-neutrophil (polymorphonuclear neutrophil [PMN]) antibody (Accurate Chemical), 1:100 ED-1 antibody (Serotec) for monocytes, or 1:500 anti-laminin (Santa Cruz Biotechnology) for neovascular channels.[5,6] Species-specific secondary antibodies and ABC kits (Vector Laboratories) were used according to the manufacturer's instructions to develop slides, and the slides were then counterstained with hematoxylin and coverslipped. In a blinded fashion, positive-staining cells or channels were counted in 5 randomly selected high-power fields (hpf) around the vein wall-thrombus interface.

Enzyme-linked immunosorbent assays. Thrombi were prepared for assays by homogenization, sonication (30 seconds), and centrifugation (15 minutes × 10,000g) in buffer solution. Enzyme-linked immunosorbent assays (ELISAs) were performed as described for keratinocyte cytokine (KC, a CXC rodent IL-8 analogue), MCP-1, and bFGF, with species-specific primary antibodies quantified by using a double-ligand technique.[5,15,17] Plates were read at 450 nm. Total protein was quantified to account for differing protein composition of samples. A modified Bradford assay (Pierce) was performed using the homogenate from ELISAs with serial dilutions of bovine serum albumin as standards. Results are expressed as nanograms or picograms per milligram of protein.

Collagen assay. Thrombus collagen content was estimated with a commercially available kit (Bicolor LTD) according to the manufacturer's instructions. The Sircol collagen assay is a colorimetric assay in which Sirius red dye binds to the side chains of amino acids found in collagen. Plates were read at 590 nm. Specimen preparation and standardization of results were identical to those for ELISAs; units are micrograms per milligram of clot.

Statistical analysis. Data from all 4 treatment groups were compared with analysis of variance with the Bonferroni correction for multiple comparisons. $P < .05$ was considered significant, and all data are presented as mean ± SEM.

RESULTS

Early experimental DVT resolution was not affected by mediator administration.

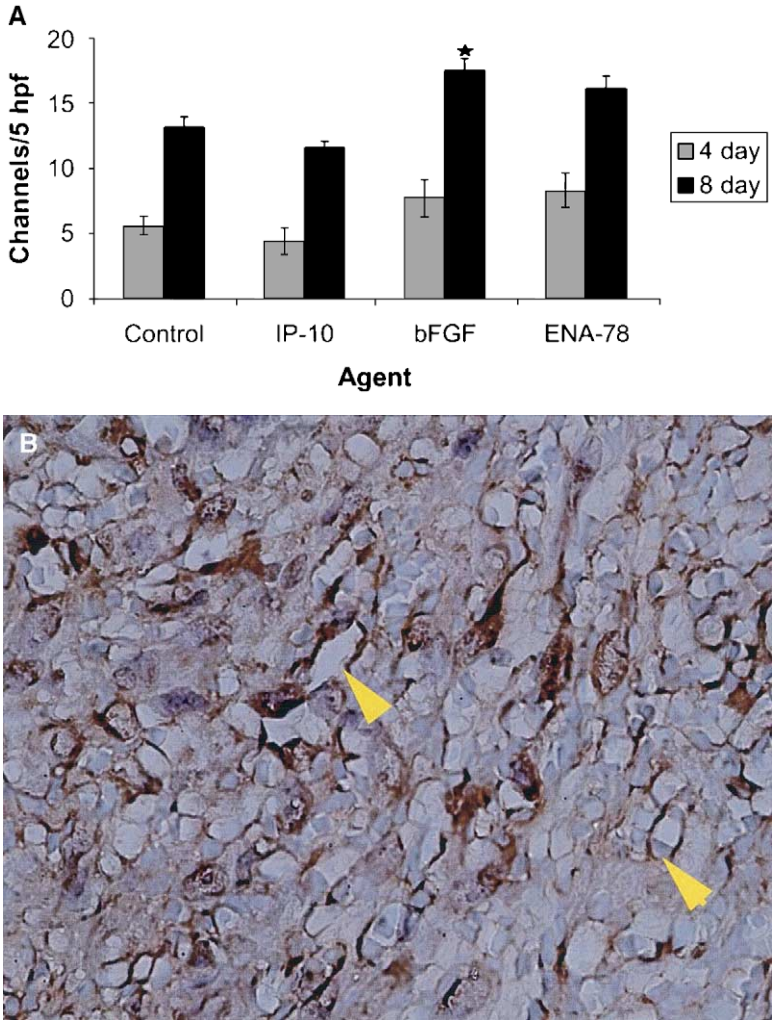

Fig 1. Neovascularization. **A,** Rats treated with pro-angiogenic basic fibroblast growth factor *(bFGF)* and epithelial neutrophil activating protein *(ENA-78)* had significantly more laminin-positive channels at 8 days (n = 6-8 per group; **P* < .05 treatment vs controls; *P* < .05, 8-day vs 4-day intergroup group). **B,** Representative section is shown with hematoxylin counterstaining at 1000×. *IP-10,* Interferon inducible protein; *hpf,* high-power field.

No outcome measurements revealed any significant differences between groups at 4 days, consistent with the notion that an angiogenic response develops later in the course of thrombus resolution.[6] The study agents were generally tolerated well, and animal deaths did not exceed 2 in any study group.

Pro-angiogenic compounds increase thrombus neovascularization. Thrombus neovascularization was estimated by counting laminin-positive channels, as described.[5] Neovascular channels develop in the periphery of the thrombus as part of the normal course of resolution in this model, and laminin staining reliably identifies these channels. The channels do not appear to invade the adventitia; rather, they restrict themselves to within the thrombus. After 8 days the correlation between laminin and von Willebrand factor staining is essentially identical.[6] Similar sections of thrombosed IVC were selected for consistency between groups. At 4 days there were no differences in the number of neovascular channels between any groups. At 8 days the number of channels increased significantly in all groups (n = 5 per group; *P* < .05 for all). Control rats had 13.2 ± 0.8 channels/5 hpf, and IP-10–treated rats had a similar number of channels (11.6 ± 0.5 channels/5 hpf). However, rats treated with ENA-78 and bFGF demonstrated a 1.2-fold and 1.3-fold increase, respectively, in neovascular channels, compared with control rats (n = 5 per group; *P* < .05 for both; Fig 1). Only exogenous bFGF increased thrombus microvascular blood flow. Laser Doppler ultrasound enables detection of in vivo intrathrombus and perithrombus blood flow through a thin plane in the thrombosed IVC, and is used in a similar manner to that described for models of hind limb ischemia.[16] Restoration of blood flow after IVC ligation was quantified as a percentage of baseline (pre-ligation) flow for that rat, enabling each rat to

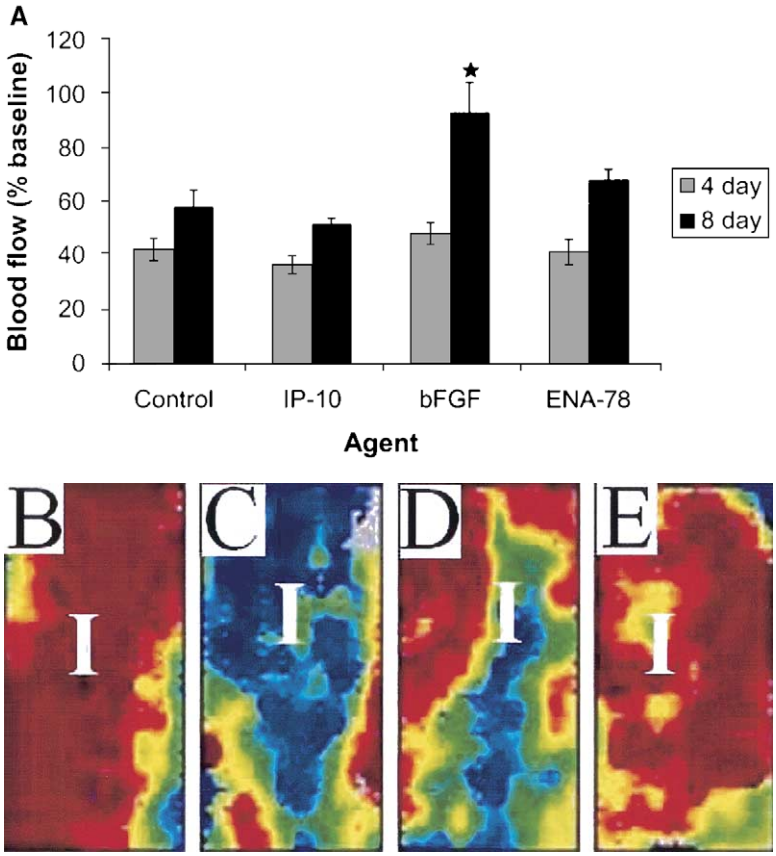

Fig 2. Laser Doppler scan. Intrathrombus blood flow (**A**) was greater in rats treated with basic fibroblast growth factor *(bFGF)*, but was not affected by other compounds (n = 6-8 per group; **P* < .05, treatment vs controls; *P* < .05, 8-day vs 4-day intergroup group). Representative laser Doppler images are shown before ligation **(B)**, after ligation **(C)**, at 8-day sacrifice in a control animal **(D)**, and at 8-day sacrifice in a bFGF-treated animal **(E)**. *IP-10,* Interferon inducible protein; *ENA-78,* epithelial neutrophil activating protein; *IVC,* inferior vena cava (IVC)

serve as its own control. At 4 days there were no differences between groups. As with the neovascular channel counts, all groups increased their percentage of blood flow from 4 days to 8 days (n = 5-8; $P < .05$). In rats treated with intravenous bFGF 93.5% ± 11.1% of pre-ligation blood flow was restored at sacrifice, compared with 58.2% ± 6.2% flow restoration in control rats (n = 5-8; $P < .05$). There were no significant differences in flow restoration among rats treated with IP-10 (51.6% ± 2.3%) or ENA-78 (68.5% ± 3.8%), compared with control rats (Fig 2).

Only bFGF was altered with angiogenic compound administration. There were no significant differences between any treatment group at 4 days. However, at 8 days rats treated with ENA-78 had an intrathrombus bFGF concentration greater than that in control rats (85 ± 27 vs 20 ± 6 pg/mg protein; n = 8; $P < .05$) and ENA-78–treated rats at 4 days (27 ± 4 pg/mg protein; n = 5; $P < .05$). Rats treated with bFGF had significantly greater intrathrombus bFGF at 8 days than at 4 days (26 ± 11 pg/mg protein; n = 5; $P < .05$), and showed a trend toward increased intrathrombus bFGF concentration, compared with control rats at 8 days (68 ± 12 pg/mg protein; n = 9; $P = .12$). There was no significant difference between control and IP-10–treated rats (24 ± 6 pg/mg protein; n = 6; P = NS; Fig 3). Similar levels of intrathrombus KC and MCP-1 were found between the groups at 8 days (KC: control, 0.62 ± 0.49 ng/mg protein; IP-10, 0.52 ± 0.15 ng/mg protein; bFGF, 1.52 ± 0.53 ng/mg protein; ENA-78, 0.16 ± 0.12 ng/mg protein. MCP-1: control, 8.38 ± 5.1 ng/mg protein; IP-10, 5.74 ± 3.49 ng/mg protein; bFGF, 12.7 ± 6.6 ng/mg protein; ENA-78, 10.5 ± 7.3 ng/mg protein; n = 5; P = NS). No significant levels of any exogenous growth factor or chemokines were found in serum samples at harvest (data not shown).

Pro-angiogenic compounds did not affect intrathrombus leukocyte differential. Intrathrombus neutrophils were quantified at immunohistochemistry, and were not different between control rats (14.8 ± 1.5) or rats treated with IP-10 (15.6 ± 1.0), bFGF (16 ± 1.5), or ENA-78 (18.4 ± 0.9) at 8 days. Intrathrombus monocytes (ED-1–positive) were also not significantly different between any of the groups at either time point (4 days: control, 46.7 ± 2.7; IP-10, 50.2 ± 2.2; bFGF, 49.7 ± 1.9; ENA-78, 45.7 ±

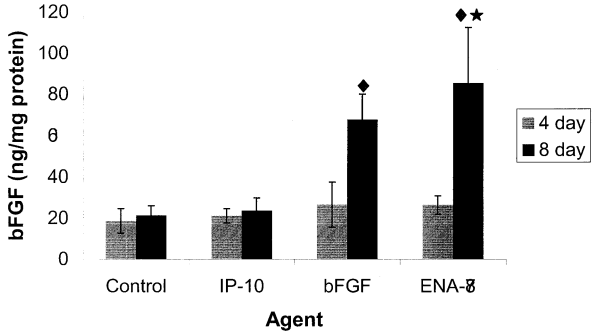

Fig 3. Basic fibroblast growth factor *(bFGF)* concentration. bFGF was quantified with an enzyme-linked immunosorbent assay (ELISA) and normalized to total protein. Rats treated with epithelial neutrophil activating protein *(ENA-78)* had significantly greater intrathrombus bFGF at ELISA than control rats did at 8 days, whereas rats treated with bFGF showed a trend toward higher intrathrombus bFGF (n = 6-10 per group; $*P < .05$ vs 8-day controls; $P < .05$, 8-day vs 4-day intergroup group comparison). *IP-10,* Interferon inducible protein.

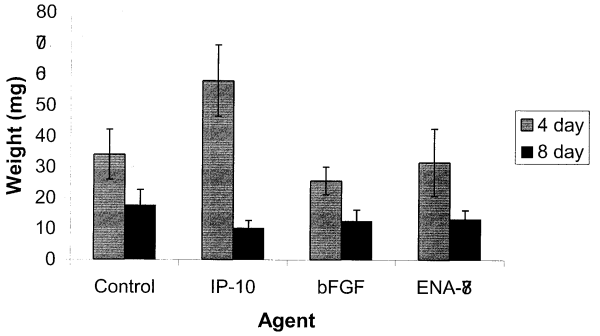

Fig 4. Thrombus weight. Thrombus was weighed and measured immediately after sacrifice. There were no significant differences between any groups at the 4-day or 8-day time point. However, in all groups thrombi were smaller at the 8-day time point compared with the 4-day time point (n = 8-10 per group; $*P < .05$, 8-day vs 4-day intergroup group comparison). *IP-10,* Interferon inducible protein; *bFGF,* basic fibroblast growth factor; *ENA-78,* epithelial neutrophil activating protein.

2.3. 8 days: control, 44.3 ± 1.3; IP-10, 40.6 ± 1.3; bFGF, 40.4 ± 3.8; ENA-78, 43.6 ± 1.4). Similarly, though intrathrombus PMNs were approximately 3-fold greater at 4 days than at 8 days, no significant difference was found between the treatment groups at 4 days (control, 42.7 ± 2.2; IP-10, 4.08 ± 3.9; bFGF, 37 ± 3.8; ENA-78, 46.7 ± 5.8; all cells/5 hpf; n = 6-8; P = NS).

Pro-angiogenic compounds did not affect overall DVT dissolution or fibrosis. Thrombus weight is a gross but reliable measure of thrombus dissolution.[5,17] Though the thrombus weight of control rats (17.7 ± 5.0 mg) trended larger than in treated rats, these differences did not reach significance (Fig 4). Thrombus collagen turnover occurs as the thrombus ages, and is influenced by clot duration and leukocytes present. Thrombus collagen content did not differ between control rats (2.25 ± 1.2 μg/mg clot) and rats treated with IP-10 (1.5 ± 0.53 μg/mg clot; P = .58), bFGF (1.83 ± 1.6 μg/mg clot; P = .83), or ENA-78 (2.2 ± 2.2 μg/mg clot; P = .98).

DISCUSSION

Neovascularization is a well-established event in most wound healing processes, of which DVT resolution is a typical example.[3,4] Restoration of blood flow through a clot has been identified in the earliest descriptions of venous thrombus resolution[4,18] and other mechanisms of thrombus resolution, such as leukocyte-derived plasminogen activation, have been linked to this neovascular response.[19-21] This report suggests that, although increasing thrombus neovascularization is possible with pro-angiogenic compounds such as ELR-positive CXC chemokine ENA-78 or the growth factor bFGF, no increase in DVT dissolution was coincidentally observed. Furthermore, that only bFGF increased physiologic blood flow (at laser Doppler scanning) suggests a disconnect between thrombus neovascular channel number and intrathrombus and perithrombus blood flow. It may be that the channel size is more important than the absolute number, but this is difficult to quantify. From previous studies, most resumption of blood flow after DVT occurs between the thrombus–vein wall interface as it retracts, and not within the thrombus itself.[5,6,15] Thus bFGF may cause greater functional thrombus contraction, consistent with its extra-angiogenic activities,[22] despite thrombus collagen not being significantly increased. Furthermore, altered neovascularization was probably related to the direct activity of these agents on leukocytes or matrix cells, not by chemoattractant activities, inasmuch as no difference in leukocyte type or number, or increase in common PMN-associated (KC) or monocyte-associated (MCP-1) mediators was observed.

Neutrophils are the predominant early leukocytes in DVT,[23] and mediate angiogenesis in a variety of settings. The PMN chemokine IL-8 has been associated with angiogenesis in diseases in human beings.[5,24] In these studies PMN recruitment and angiogenesis were both mediated by IL-8, and angiogenesis was seen after an increase in leukocytes had taken place. The specific contributions of neutrophil influx and angiogenesis are undefined, but have recently been partially elucidated in studies of neutrophil depletion.[15] For example, PMN depletion is associated with larger thrombi and greater thrombus collagen content, whereas IL-8 supplementation is associated with smaller thrombi coincident with increased neovascularization.[5] While adversely affecting thrombus resolution, PMN depletion does not appear to have any effect on development on neovascular channels or in vitro angiogenic activity (Henke, unpublished data). Indeed, other investigators have noted that neutropenia does not impair wound healing.[25] As shown in the current study, ENA-78 increased intrathrombus bFGF levels significantly, possibly through stimulation of PMN vascular growth factor production.[26]

However, secondary elevation did not seem to convey the same increase in perithrombus blood flow that exogenous bFGF did. Similarly, that ENA-78 increased thrombus neovascularization may have been secondary to its effect of increasing local bFGF rather than to its primary pro-angiogenic activity, because most data regarding this compound are from pathologic disease states, such as neoplasia.[3,14]

Monocyte and macrophage influx has also been described as essential for angiogenesis.[27,28] Indeed, some investigators have hypothesized that macrophage influx may be significantly more important than neutrophil influx in mediating angiogenesis,[29] which further supports the notion that the detrimental effects of neutropenia are not due to inhibition of angiogenesis. The agents administered in our studies did not affect monocyte or macrophage influx, but do have monocyte activating properties.[14] Other experimental studies of DVT have noted that monocyte influx specifically is associated with improved thrombus resolution[30] and that exogenous MCP-1 improves thrombus resolution.[9] Neither of these findings is contradicted by our studies, and the notion that these effects are due to monocyte influx stimulated with chemokines or growth factors is further supported.

Thrombus dissolution occurs via fibrinolysis and fibrosis to produce intraluminal scar. While bFGF may stimulate collagen production, that no total increase in thrombus collagen was found argues that it may promote collagen maturation, increase cross-linking, or increase myofibroblast influx.[3,22] Neither IP-10 nor ENA-78 have specific pro-fibrotic properties, unlike other chemokines such as MCP-1, which was not significantly altered with the treatments, and is consistent with the findings of the current study.[27] Also consistent with the lack of difference in thrombus collagen was the lack of difference in DVT weights, though this interrelation has not been proportionate in other studies with the same basic model.[17,31] In assessing these studies our group has considered thrombus weight to be the most important measure of resolution, inferring that decreased thrombus burden will diminish the risk for sequelae such as pulmonary embolism or CVI. The interrelation of fibrosis and neovascularization is conflicting. Studies of pulmonary fibrosis[32] suggest a positive association, while inhibiting neovascularization in a wound-healing model was not associated with decreased local collagen levels.[33] Of note, surgical trauma from IVC ligation appears to make little contribution to this fibrotic process (unpublished data).

Limitations of the current study are that the angiogenic compounds tested, while having documented strong activities in vitro and in vivo, have other leukocyte activation properties that may confound the overall angiogenic balance. For example, ENA-78 is a PMN activator that causes release of proteases that may break down natural fibrinolytic proteins, and IP-10, primarily a lymphocyte chemoattractant, exerts its angiostatic effect via CXCR3 receptors,[14] which may not have been present on intrathrombus cells. Furthermore, lymphocytes appear to be important in thrombus resolution after 14 days. Current studies in a mouse model indicate that the early response is primarily of the Th1 response, which supports the generalized inflammatory response to thrombosis, and a later Th2 response, which has a less clear role (unpublished data). No attempt was made to label the infused agents to confirm thrombus distribution, because previous venography data suggested the primary route of tail vein is to the IVC, even with an occlusion present.[15] While increased intrathrombus PMN would be empirically expected in the ENA-78–treated group, it may be that this increased PMN influx occurred before harvest (eg, at around 2 days) and was not detected at these later time points. Consistent with this observation is our previous study that showed no significant increase in 8-day PMN influx with exogenous IL-8 administration.[5] This study also cannot conclude the converse, that is, that neovascularization inhibition does not impair DVT resolution, because no isolated angiostatic agent was tested. Endostatin has powerful angiogenic inhibitory effects, but in settings other than neoplasia, such as wound healing,[33] it has had mixed results and is costly to procure experimentally. Last, vascular endothelial growth factor was not measured in this model system, because previous experiments suggest that this is a less important mediator than bFGF.[5]

Clinically, the more complete the DVT resolution the less likelihood of development of CVI.[34] Our study has shown that venous thrombus neovascularization can be increased by pro-angiogenic compounds that do not affect leukocyte influx or inflammatory activity, as measured by prototypical chemokine levels, but do not appear to decrease thrombus size. Further work is needed to clarify the basic mechanisms of thrombus resolution, with the aim of accelerating thrombolysis with minimal systemic bleeding risk, and countered against the need for firm adhesion of the residual thrombus to prevent pulmonary embolism.

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