Chapter 15

Molecular characterization of post-thrombotic syndrome

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The post-thrombotic syndrome represents a poorly understood and significant vascular health problem. This review focuses on our current understanding of the pathogenesis of post-thrombotic syndrome. We emphasize the cellular and molecular mechanisms that are responsible for the critical components of post-thrombotic syndrome. These include the initiation of deep venous thrombosis, the pathogenesis of elevated venous pressure, and the factors responsible for nonhealing of venous stasis ulcers. (J Vasc Surg 2007;45:116A-122A.)

A significant and common long-term complication after deep venous thrombosis (DVT) is the occurrence of post-thrombotic syndrome, which consists of pain, edema, and varying degrees of skin changes up to and including chronic venous stasis ulcers. Estimates of the incidence of post-thrombotic syndrome after DVT are variable and range from 25% to 75%. Fortunately the severe manifestations of post-thrombotic syndrome are less common and occur in 5% to 10% of patients after DVT. Post-thrombotic syndrome is responsible for substantial disability, discomfort, and health care costs. In the United States, the annual direct cost has been estimated to be at least $200 million. Because those affected are often of working age, the indirect costs are also quite large, leading to 2 million workdays lost annually in the United States. Thus post-thrombotic syndrome, although not lethal, is a debilitating chronic condition that deserves a clearer understanding to allow the development of targeted therapy.

Several studies have examined the potential factors that predict the onset and severity of post-thrombotic syndrome after DVT. Recurrent DVT increases the risk of post-thrombotic syndrome sixfold, and thus, the risks for development of post-thrombotic syndrome are higher in patients with persistent prothrombotic risk factors such as cancer or hypercoagulable states, most likely because of recurrent or persistent DVT. A substantial fraction of DVT patients do not go on to develop post-thrombotic syndrome, which suggests that alterations or derangements in the process of thrombus resolution are critical in the pathogenesis of post-thrombotic syndrome. This review describes our current knowledge regarding the pathogenesis

FACTORS PREDISPOSING TO POST-THROMBOTIC SYNDROME

Venous thrombosis is a result of at least one of three known predisposing factors (Virchow’s triad): hypercoagulability, venous stasis, and endothelial damage. The clinical risk factors for DVT development include hypercoagulable states (both familial and acquired), trauma, pregnancy, lower extremity fractures, malignancy, and surgery involving the lower extremities, pelvis, or abdomen.

Two basic pathologic mechanisms are involved in the initiation of DVT. In patients without direct trauma or manipulation of the veins, stasis and hypercoagulability induce thrombosis, usually starting in the calf veins. The process often begins on the upper aspect of the valve cusps, where localized stasis and turbulence create a nidus for thrombus initiation. The second mechanism involves direct trauma, manipulation, or compression of a segment of vein causing thrombus formation at the site of endothelial damage. Common clinical examples of this mechanism include DVT secondary to vein compression from popliteal or femoral aneurysms, thrombosis of iatric veins manipulated during pelvic surgery, and thrombosis in vein segments adjacent to displaced fractures.

Once the thrombus forms in the vein, endogenous lysis results in recanalization and reabsorption of the mass. Extension of the thrombus can occur simultaneously with thrombolysis, and the overall progression of the initial DVT towards propagation or resolution depends on the balance between these two processes. If conditions favoring thrombosis are present in adjacent venous segments, then extension or propagation of the thrombus is likely. This is illustrated clinically by the higher incidence of persistent DVT in patients with ongoing hypercoagulable conditions. Concurrently, the resultant venous obstruction induces enlargement of collateral veins around the region of throm-
bosis. Thrombus resolution can also induce destruction of the venous valves and lead to venous reflux.

ROLE OF CHEMOKINES AND GROWTH FACTORS

The initial thrombotic event involves the generation of a cross-linked fibrin matrix containing platelets and minimal inflammatory cells. The activated platelets initially release angiostatic chemokines such as platelet factor-4, but as the thrombus matures and becomes more cellular (24 to 72 hours), the chemokines released are neutrophil and monocyte chemotactants such as interleukin-8 (IL-8), which is a proinflammatory, proangiogenic chemokine known to attract polymorphonuclear neutrophils (PMNs). Intravenous administration of IL-8 accelerated thrombus resolution as measured by thrombus weight in a rat model of stasis induced venous thrombosis. The peak effect of IL-8 was noted upon administration between day 4 and day 8, when the cellular composition of the resolving thrombus begins to change from neutrophils, the predominant early cell type, to monocytes.

A variety of growth factors have been found to be active within the resolving thrombus. Vascular endothelial growth factor (VEGF) increases after day 1 within the thrombus and peaks between day 14 and 21 in a flow constriction model of thrombosis. However, basic fibroblast growth factor (bFGF) increases in the thrombus in a linear fashion over time, with a 300-fold increase in expression found between day 1 and 28. No change in serum or vein wall concentration was noted for either VEGF or bFGF. VEGF was expressed by endothelium and the monocyte infiltrate within the thrombus, although the endothelial expression may be constitutive and unrelated to thrombus resolution. Mononuclear cells and spindle shaped cells within the thrombus were found to express bFGF.

VEGF and bFGF are regulators of angiogenesis and may regulate the formation of the neovascular channels that connect with both lumen and vasa vasaorum. A later study used plasmid-mediated gene transfer of VEGF to attempt to accelerate thrombus resolution. There was a 48% reduction in mean thrombus area and 31% increase in the percentage of thrombus area that was recanalized. This suggests that the recanalization process is positively influenced by cytokines and the cells that they recruit, rather than being a passive mechanical retraction of the fibrin clot during maturation.

The possibility that angiogenic chemokines and growth factors present in DVT could be used to accelerate thrombus resolution has spurred several additional studies. Administration of IL-8, monocyte chemotactic protein-1 (MCP-1), and VEGF was beneficial in experimental thrombus resolution. More mixed results were noted with injection of angiogenic cytokines such as the angiostatic chemokine interferon-inducible protein (IP10), bFGF, and epithelial neutrophil–activating protein (ENA-78) directly into experimental DVT. At 8 days, control and IP10-treated rats had similar number of intrathrombus channels, and animals treated with ENA-78 and bFGF showed a small increase in number of channels, but only bFGF induced an actual increase in blood flow within the resolving thrombus as measured by Doppler flow probes. Because these experiments involved complete surgical ligation of the rodent infrarenal vena cava, it is not clear what the physiologic significance of intrathrombus flow is. This model has a different pattern of recanalization and thrombus remodeling than clinical DVT and flow restriction models of thrombus resolution.

Channel formation within the thrombus, which has also been described as neovascularization, was originally thought to be secondary to clot retraction. It is now postulated to be an active and directed component of thrombus resolution. It has been shown that the cells lining the vascular channels express the endothelial marker von Willebrand factor and the potent angiogenic chemokine IL-8. Exogenous IL-8 increased early neovascularization and promoted leukocyte influx during thrombus resolution after infrarenal vena caval ligation. IL-8 administration also increased intrathrombus PMN on day 4, monocyte infiltration on day 8, and fibroblasts within the thrombus at all time points. Total intrathrombus channel volume, as determined by a fluorescent high-molecular-weight marker, was also increased in animals treated with IL-8. It has been proposed that intrathrombotic channels play a critical role by allowing leukocyte influx and subsequent fibrinolytic protease release.

Plasmin lyases clot by breaking down fibrinogen and fibrin within the clot. The major direct activator is tissue plasminogen activator (tPA), a molecule synthesized as a single-chain molecule in endothelial cells. It promotes conversion of plasminogen to plasmin, in presence of fibrin. The resulting complex can then convert additional plasminogen to plasmin. Elastase is one of the primary proteases released by PMNs and acts with tPA to lyse thrombus. Interestingly, studies of the urokinase-type plasminogen activator (uPA) and tPA knockout mice, demonstrate that the uPA gene, but not the tPA gene, is critical to thrombus resolution.

ROLE OF CELL TYPES

What is the role of the various cell types in the resolving thrombus? Is it analogous to wound healing? The primary focus in the literature has been on the role of neutrophils and monocytes on thrombus resolution. One experiment that investigated the role of the neutrophils used a neutropenic rat model on the basis of the observation that multiple episodes of neutropenia were a potential clinical risk factor for a venous thromboembolic event. The rats were treated with antineutrophil serum and then underwent vein ligation to generate experimental DVT. Intrathrombus monocyte numbers remained unchanged, but the rats were neutropenic throughout the experimental time points. The thrombus in these rats was larger by both weight/length ratio and in vivo area. The thrombi of neutropenic rats were also increasingly fibrotic, with a 3.3-fold increase in collagen noted. Although colla-
genase content was not directly measured in this experiment, it is plausible that the lack of collagen turnover was due to decreases in neutrophil-derived matrix metalloproteinases (MMPs) and elastase.

Most of the chemokines tested were similar between groups, but keratinocyte cytokine was elevated by 20-fold in the neutropenic group. Macrophage inflammatory protein 1α and monocyte chemoattractant protein-1 were lower in neutropenic animals, indicating a role for neutrophils in affecting the cytokine pattern during thrombus resolution.

Monocytes are predominant in the venous thrombus at day 8, where they help orchestrate resolution of the thrombus by fibrinolytic, chemotactic, and angiogenic mechanisms. Monocytes can degrade fibrin in the absence of plasmin.17 Monocytes that infiltrate experimental thrombi express plasminogen activators.18 When exogenous macrophages generated by peritoneal lavage were injected into experimentally induced thrombi, thrombus size decreased fivefold and recanalization increased more than fourfold.19

MCP-1 is produced by fibroblasts, smooth muscle, endothelium, and monocytes and serves to attract and activate monocytes and basophils. The vein wall demonstrates increased amounts of MCP-1 in experimental DVT, which plays a role in thrombus and vein wall monocyte infiltration.20 Endogenous MCP-1 expression increases from 1 to 7 days after DVT formation and remains elevated for 14 days. Injection of exogenous MCP-1 into experimentally produced thrombi resulted in increased thrombus organization, decreased cross-sectional area, and lower thrombus weights. This treatment, surprisingly, did not affect numbers of monocytes within the thrombus, but they were significantly increased in the vein wall. The monocytes have been shown previously to move into the thrombus after vein wall infiltration, rather than direct deposition from flowing blood into the thrombus.20 Mice deficient in the cysteine-cysteine chemokine receptor 2 (CCR2), which mediates chemotactic movement of monocytes in response to MCP-1, had thrombi with significantly lower macrophage content. Thrombus resolution was also impaired in these mice.19

MECHANISMS FOR SUBSEQUENT POST-THROMBOTIC SYNDROME

If the DVT completely resolves, what leads to subsequent post-thrombotic syndrome? Numerous mechanisms have been postulated, and the increased fibrotic nature of the vein wall is thought to be one cause (Fig 1). DVT induces a paracrine-like scarring process in the adjacent vein wall and subsequent fibrosis.21 In a mouse model of DVT, the presence of thrombus leads to an increase in total vein wall collagen over time, peaking at 12 days after thrombus formation in the model. The authors also demonstrated a corresponding increase in procollagen I and III messenger RNA (mRNA).21

The fibrosis contributes to valve failure. This, in turn, appears to promote venous hypertension, a critical factor in the development and persistence of chronic venous ulcers in humans.22 Prolonged approximation of the delicate venous valve leaflet against the vein wall or entrapment within the thrombus during the intense inflammatory process leads to leaflet destruction. Loss of valve leaflets results in deep venous reflux, which allows postural changes in the static venous pressure to be directly transmitted to the lower leg. The elevated venous pressure in the lower aspect of the limb leads to dysfunction of the microcirculation, which can then lead to reflex, varicose veins, and finally, ulcers. Inflammatory changes have been noted in the valve leaflets, with infiltration by monocytes and macrophages greatest in the valve sinus and proximal venous wall.23

The changes in collagen composition may be due to the localized action of leukocytes. Peripheral blood samples of patients with chronic venous insufficiency have shown activated leukocytes accumulate in the lower extremity in patients with venous hypertension.24 Leukocytes are known to express multiple matrix metalloproteinases (MMPs), which play a significant role in collagen degradation. The MMPs are a family of soluble and membrane-bound zinc-dependent endopeptidases that participate in tissue remodeling during morphogenesis, tumor progression, cell migration, angiogenesis, and wound healing. Released in zymogen form, they can be extracellularly activated by cleavage of the aminoterminal propeptide. MMP-8 and MMP-9 have been shown to be secreted by neutrophils and macrophages, and interstitial macrophages have been shown to express MMP-1 and MMP-3. The macrophages are thought to use these proteinases to degrade the extracellular matrix and to assist digestion of stromal collagen fibers.

The hypothesis of valve failure as a possible cause of MMP activation, leading to perpetuation of an inflammatory cascade, has been tested (Fig 2). When venous blood pressure was elevated experimentally by creation of a femoral arteriovenous fistula in rats, venous reflux increased fivefold during a period of 6 weeks.26 The valves were dilated and the vein walls appeared to weaken. The activity of MMP-2 in the vein was increased, with a peak at 7 days, and remained elevated at 42 days. MMP-9 activity was also significantly above controls at day 7, but this effect did not last through the time course.

Fig 1. A resolving thrombus formed by vein constriction in the mouse, 7 days postprocedure.
What are the mechanisms by which elevated venous pressure induces nonhealing skin ulcers? Ambulatory venous hypertension is caused by superficial and deep venous incompetence, and subsequent alteration of the skin microcirculation is thought to lead to ulceration. Capillaries become dilated and tortuous as they respond to increased hydrostatic pressure. They then begin to leak plasma, proteins, and erythrocytes. Chronic leakage of fibrinogen leads to formation of extravascular cross-linked fibrin around the capillaries, noted histologically as fibrin cuffs. This pericapillary protein acts as a barrier to decrease oxygen diffusion from the capillaries and thus contributes to tissue hypoxia, despite adequate arterial supply. The blood flow in the dysfunctional capillaries is hampered to the point where some become occluded (Fig 3).

The findings of increased MMP activity and their potential role are supported in human subjects by the finding of an imbalance between type I collagen and type III collagen in the proximal segments of varicose veins. That same group found that type III collagen was degraded extracellularly in cultures of smooth muscle cells taken from varicose veins compared with controls, and that the production of type III collagen was partially restored in the presence of a synthetic MMP inhibitor. These studies indicate that varicose veins have particular differences in their protease expression profile that may play a role in the pathogenesis of venous insufficiency.

MMPs have been implicated in the excessive extracellular matrix degradation within the chronic venous ulcers that may contribute to the failure to heal. Venous ulcer patients have been categorized into healing and nonhealing groups. Nonhealing ulcers were defined as >20% reduction in ulcer surface area at 8 weeks. There was a trend (although not statistically significant) toward higher levels of MMP-2 in the nonhealing ulcers, with a concomitant decrease in tissue inhibitor of metalloproteinase-2 (TIMP-2). Other groups have seen MMP activity in both lipodermatosclerosis and frank ulceration, so it is likely that this result was more the effect of a small sample size.

TIMPs are believed to regulate proteolytic activity of MMPs by binding to them. Decrease of TIMP can lead to imbalance in TIMP/MMP ratio, with a net excess of activated MMPs within the chronic wound. There was a correlation ($r = 0.94$) noted between higher MMP-2 and lower TIMP-2 in the nonhealing group, suggesting an increase in proteolytic activity was due to the balance between these two factors.

Platelet derived growth factor-AA (PDGF-AA) was also found to be increased in the healing group of this study. This growth factor stimulates other growth factors and production of matrix components collagen and hyaluronic acid. It is also known to stimulate smooth muscle cell migration, angiogenesis, and collagenase production. Is impaired healing associated with decrease in PDGF? PDGF AA is up-regulated in capillaries and fibroblasts of acute and chronic wounds, but not in nonhealing dermal wounds or normal skin. PDGF has been found to stimulate MMP production in fibroblasts.

Extracellular matrix metalloproteinase inducer (EMMPRIN) is another potentially important protein noted to have higher expression perivascularly in biopsies of venous leg ulcers than biopsies of the skin of healthy controls. EMMPRIN is a membrane-bound glycoprotein produced by normal keratinocytes. It has been shown to stimulate synthesis of MMP-1, MMP-2, and MMP-3 de novo in cultures of human fibroblasts. MMP-1 and MMP-2 are secreted as zymogens and are then activated by proteolytic cleavage, mostly by serine proteases such as plasmin. The activity of these proteins then results in enhanced turnover of the extracellular matrix. Plasmin and the plasminogen-activating cascade components were not found in the dermal structures, and therefore, it is...
possible that activation of these MMPs is stimulated by EMMPRIN.

Plasminogen activation in both venous ulcers and lipodermatosclerosis is enhanced by uPA and its receptor. Analysis of human ulcer biopsies showed mRNA and protein levels of uPA and its receptor to be elevated in venous ulcers. Immunohistochemical staining of venous leg ulcers showed that the plasminogen activator is enhanced perivascularly. Tissue-type plasminogen activator was not elevated. Elevated plasminogen activation implies that maintenance of proteolytic activity is an important component of the pathophysiology of venous leg ulcers, and this may be a response to the well-described presence of perivascular fibrin cuffs, deposits of fibrin and collagen around the microvasculature.

Lipodermatosclerosis is a precursor state to venous ulceration characterized primarily by skin induration and scleroderma-like hardening. Histologic analysis demonstrates the loss of cellular components and formation of fibrous scar tissue of the reticular dermis composed of collagen bundles and degraded elastic fibers. In addition, fibrin cuffs, which contain I and III collagen and fibronectin in addition to fibrin, are found around the dermal capillary vessels. Elevated MMP-1, MMP-2, and TIMP-1 gene expression (but not MMP-9 or TIMP-2) has been identified in skin biopsy specimens of patients with lipodermatosclerosis. They were able to determine increased collagenolytic activity as well. The MMP-1 protein was found diffusely in epidermis and dermis, whereas MMP-2 was centered perivascularly and around collagen bundles. MMP-2 cleaves collagen I and III, as well as elastin, type IV collagen, fibronectin, and laminin.

It is tempting to speculate that the increased extracellular matrix turnover found in lipodermatosclerosis might be a result of increased MMP-2 activity. TIMPs control the activity of MMP-2 by forming one-to-one complexes with MMP-2. TIMP-2 is more than sevenfold effective than TIMP-1. No concomitant increase in TIMP-2 was found, so the activity of MMP-2 is likely enhanced because of decreased inhibition in addition to increased expression.

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a potent cytokine that has been used to treat chronic venous skin ulcers. Multiple studies that used GM-CSF noted success for chronic wounds, but it does not enhance repair of acute wounds in healthy individuals. The possible indirect actions of GM-CSF on healing of chronic ulcers have been investigated. After the wounds of human subjects were injected with GM-CSF, VEGF expression within the ulcer in multiple cell types (mainly macrophages) was increased compared with preinjection levels in the same subject. Blood vessel density was also increased in these specimens. These authors postulated that the mechanism of action was to increase vascularization by induction of angiogenic factors released by hematopoietic cells.

VEGF is known to be important in angiogenesis as a mitogen and chemoattractant for endothelial cells. Secreted VEGF is degraded by proteolytic activity of the wound exudates of chronic venous ulcers. It is possible that the effect of GM-CSF on venous ulcers is to increase expression of VEGF, leading to improved angiogenesis and wound resolution. Culturing PMA-differentiated monocytes indirectly tested one aspect of this hypothesis, and a two-fold increase in VEGF expression was observed 12 hours after treatment with GM-CSF.

Transforming growth factor-β1 (TGF-β1) is a cytokine associated with both tissue remodeling and regulation of MMP activity. Previous studies have shown a correlation between TGF-β1 level in venous ulcer and disease severity. Because TGF-β1 is known to transcriptionally regulate MMP-1, MMP-2, and MMP-9, Siato et al hypothesized that the increased activity of TGF would be associated with differences in mRNA and protein levels of these MMPs. Patients were grouped by CEAP class, and the control group consisted of patients with arterial disease but no clinical evidence of chronic venous insufficiency. Their analysis of lower extremity venous ulcer biopsy specimens showed that MMP-1 mRNA was decreased and TIMP-1 mRNA was increased. However, the protein levels of MMP-1 and TIMP-1 were no different from controls. One way to interpret this dissociation is the possibility that post-transcriptional modification is also an important regulatory mechanism of synthesis and activity of these two proteins.

This is one of several groups of authors who have seen increased active and latent forms of MMP-2 in the chronic venous wound. Increased MMP-2 activity was not observed, reflecting previous results. TGF-β1 is proposed to suppress MMP gene expression by binding to an inhibitory element of the MMP promoter. MMP-2 lacks this element, which would explain its continued increased expression despite increased TGF-β1 levels.

Another intriguing action of MMPs that may affect healing of chronic venous ulcers is the inhibition of angiogenesis. Although MMP proteins are generally associated with angiogenesis because they are involved in endothelial cell and vascular smooth muscle cell migration, MMPs are also responsible for generation of angiostatic (antiangiogenic) molecules such as angiostatin by the cleavage of plasminogen to generate an NH3 terminal fragment that inhibits endothelial cell proliferation. MMPs can also proteolytically generate endostatin, a proteolytic fragment of collagen XVIII, which also has antiangiogenic activity.

Granulation tissue is an essential feature of resolution of wounds and is dependent on the development of new capillaries from the wound edge. Chronic ulcers typically do not form granulation tissue. Could it be an increased angiostatic response that is mediating this observation? Ulrich et al used an in vitro model of angiogenesis to investigate this question. A coculture of human umbilical vein endothelial cells and diploid fibroblasts was treated with the wound exudates of patients with chronic venous ulcers and acute wound fluid from skin grafts for partial thickness burns. The length of tubes that formed after treatment with chronic wound exudates was almost one third the length of those that were treated with the exu-
dates of acute wounds or a VEGF solution and similar to the length of those treated with suramin, a known angiogenesis inhibitor. However, when a synthetic MMP-2/MMP-9 inhibitor was added to the chronic wound fluid, the tubule length significantly increased. This study did not definitively explain the role of MMP-2 or MMP-9 in angiostasis, but it did demonstrate that the antiangiogenic milieu in chronic wound fluid is MMP-dependent, and this may contribute to the chronic nonhealing state characteristic of venous ulcers.

Examination of the growth potential of fibroblasts taken from nonhealing venous ulcers reveals another potential contributing factor to ulcer persistence. The reduction in proliferative capacity of differentiated mammalian cells after a certain number of proliferative cycles is known as cellular senescence. Decreased growth in cell culture and other characteristics of cellular senescence have been noted in fibroblasts isolated from nonhealing venous ulcers, but not from thigh biopsy specimens of the same patients.

This suggests that the prolonged nonhealing noted in chronic venous stasis ulcers may be due to exhaustion of the proliferative capacity of skin fibroblasts. It remains to be determined if molecular therapy targeting the cellular mechanisms responsible for senescence, such as telomerase activity, will be applicable for this most severe complication of post-thrombotic syndrome.

**CONCLUSION**

The post-thrombotic syndrome is a chronic and debilitating complication of DVT. The thrombus can create elevated venous pressure by mechanical obstruction. Even when the thrombus is resolved, the resultant fibrosis can cause valve damage, which leads to reflux and subsequent localized venous hypertension. The vein walls become dilated and valve leaflets fail to approximate, only furthering the venous hypertension. Capillary leak is a dire result of this elevated hydrostatic pressure that induces edema and subsequent chronic inflammation. Venous stasis within the microcirculation accumulates leukocytes, which, in addition to erythrocytes, fibrinogen, and other macromolecules, leak out into the interstitium and further contribute to persistent localized inflammation.

Enhanced extracellular matrix turnover by increased MMP activity can lead to fibrin and collagen deposition around the dermal capillaries and the generation of fibrin cuffs that impair oxygenation and nutrient perfusion. Loss of epidermal integrity then follows, and the resultant ulcer demonstrates increased proliferation of the fibroblasts, which may become senescent. In addition, the imbalance of MMP/TIMP within the wound impairs angiogenesis and inhibits granulation tissue formation. The synergy between these complex relationships leads to a dermal environment that is unable to heal and allows the persistence of the venous ulcer, which is the most serious manifestation of the post-thrombotic syndrome.

**REFERENCES**


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