Université de Montréal

Resolution of Deep Venous Thrombosis in Type 2 Diabetes Mice Model: Implication of Fibrinolytic and Matrix Metalloproteinase Systems

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SUMMARY

TYPE 2 DIABETES is a group of metabolic disorders that may result in a procoagulant and thrombogenic predisposition, which is related to the arterial complications. However it remains undetermined whether diabetic conditions may affect deep vein thrombosis (DVT) and its resolution. The objectives of this study were to determine the effect of diet-induced type 2 diabetes on the organization, resolution and recanalization of venous thrombi, the inflammatory response, and the fibrinolytic and MMP systems in a murine experimental model of venous stasis as assessed by angiography and molecular techniques.

The resolution and recanalization of DVT was decreased in type 2 diabetic mice as revealed by angiography, thrombus size and content and neovascular channel quantification by immunohistochemistry. Recruitment of monocyte/macrophages, detected by an anti-CD68 antibody, was increased, and a higher collagen deposition was found in the thrombosed inferior vena cava of diabetic mice. The plasminogen activators, u-PA and t-PA were downregulated, and their inhibitor, PAI-1 was upregulated conferring a relatively antifibrinolytic state in diabetic mice. The MMP system was enhanced in diabetic mice at one week post DVT followed by a decreased synthesis and activity at 2 weeks.

Diet-induced type 2 diabetes may impair the organization, resolution and recanalization of DVT through increased inflammatory response and disruption of fibrinolytic and MMP systems.

SOMMAIRE

LE DIABÈTE DE TYPE 2 est un groupe de désordres métaboliques dont l'impact est une atteinte artérielle, microvasculaire et macrovasculaire aboutissant à la dysfonction endothéliale avec pour conséquences : inflammation, hypercoagulabilité et thrombogénicité. Cependant, il n'est pas établi si ces désordres métaboliques ont la même répercussion sur le système veineux et notamment sur les mécanismes de l'organisation et la dissolution du thrombus veineux. Grâce à un modèle animal de diabète de type 2 par consommation de diète riche en lipides et un modèle de stase veineuse par ligature de la veine cave inférieure chez la souris, nous avons pu déterminer les effets de ces désordres sur la résolution du thrombus veineux. Les souris diabétiques présentent une diminution de la résolution et de la recanalisation du thrombus veineux comme l'indiquent les résultats angiographiques, la taille du thrombus et la quantification des néo-vaisseaux par immunohistochimie. La réponse inflammatoire détectée au niveau de l'expression génique du CD14 et par immunolocalisation d'un marqueur des macrophages le CD68, est fortement activée, accompagnée d'un dépôt de collagène au sein de la paroi veineuse. Le système fibrinolytique est également atteint par une réduction de l'expression des ARNm et des protéines des activateurs du plasminogène (u-PA et t-PA) et par une régulation à la hausse du profil d'expression du PAI-1. L'expression des métalloprotéinases MMP-2 et MMP-9 est temporellement affectée au cours de la thrombose veineuse. Après une induction/activation du système à une semaine après formation du thrombus, une réduction de la synthèse/activité est observée chez les animaux diabétiques.

Le diabète de type 2 induit par une diète enrichie chez la souris semble altérer l'évolution du thrombus veineux à travers une réponse inflammatoire amplifiée, une activité du système fibrinolytique diminuée couplée à un système des MMPs activé mais dont la modulation à la baisse semble durée-dépendante.

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α-SMA: α smooth muscle actin.

αvβ3 : integrin receptor.

B-FGF: basic fibroblast growth factor.

CAMs: cell adhesion molecules.

CD 14: a cell marker of inflammation (especially macrophages).

CD 31: a cluster of differentiation molecular

CHD: coronary heart disease.

CVD: cardiovascular disease.

CVI: chronic venous insufficiency.

DM: diabetes mellitus

ECM: extracellular matrix.

ECs: endothelial cells.

ENA-78: epithelial neutrophil activating protein.

eNOS: endothelial cell NOS.

EPCs: endothelial progenitor cells.

IL-1: interleukin-1.

IL-8: Interleukin-8.

IP-10: interferon inducible protein.

IVC : inferior vena cava

MC : monocyte

Mph : macrophages

MCP-1: Monocyte chemotactic protein-1.

MMPs: matrix metalloproteinases.

MT-MMPs: membrane type MMPs

NO: nitric oxide.

NOS: nitric oxide synthase.

PA: plasminogen activator

PAF: platelet activating factor.

PDGFs : platelet derived growth factor.

PE: pulmonary embolism

PGI2: prostacyclin.

PIGF : placental growth factor.

PMN: polymorphonuclear neutrophil.

SERPIN: serine proteinase inhibitor

TF: tissue factor.

TGF-β1: transforming growth factor beta 1.

Tie2: endothelial cell receptor tyrosine kinase.

TIMPs: inhibitors of matrix metalloproteinases.

TNF-α: tumor necrosis factor- α.

t-PA: tissue-type plasminogen activator.

TXA2: thromboxane A2.

UK: urokinase.

u-PA: urokinase-type plasminogen activator.

uPAR: u-PA receptor.

VCAM-1: vascular cell adhesion molecule-1

VEGF: vascular endothelial growth factor.

VN: vitronectin.

VTE: venous thromboembolism.

VWF: von Willebrand factor.

WPb: Weibel-Palade body.

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I. INTRODUCTION

I.1 DIABETES MELLITUS

I.1.1 Overview

Diabetes mellitus (DM) is a group of metabolic syndromes characterized by chronic hyperglycemia, disturbances of carbohydrate, fat and protein metabolism due to defects in insulin secretion, insulin resistance or both (1, 2). Chronic hyperglycemia is associated with multi-organ damage to the eyes, kidneys, nerves, heart, and blood vessels (1). Cardiovascular disease is the leading cause of premature death among patients with diabetes. The new classification system identifies four types of diabetes mellitus based on etiology: type 1, type 2, "other specific types" and gestational diabetes (1). The World Health Organization (WHO) predicts that between 1997 and 2025, the number of persons affected with diabetes will double from 143 to approximately 330 million (3).

Type 2 is the most common form of diabetes mellitus. It is a metabolic disorder that is primarily characterized by insulin resistance, relative insulin deficiency, and hyperglycemia and lead to function impairment of many organs, most importantly the cardiovascular system. Its prevalence is projected to rise in the future (5). Approximately 90-95% of diabetes is ascribed to Type 2 (4, 7), the development of which is attributed to both polygenetic and environmental factors.

I.1.2 Cardiovascular complications and pathogenesis

Diabetes mellitus causes considerable morbidity and mortality primarily due to microvascular (retinopathy, nephropathy, vasculopathy) and macrovascular

(ischemic heart disease, stroke, peripheral vascular disease) complications (6, 7), which can lead to considerable disability and premature death. Cardiovascular disease (CVD) is the major complication of type 2 diabetes and is responsible for more than 50% and up to 80% of deaths in people with diabetes as well as for substantial morbidity and loss of quality of life (3).

Although the pathogenesis of CVD in diabetes is not yet fully understood, multiple metabolic and endocrinologic factors are implicated (6, 7). Hyperglycemia and hyperinsulinemia due to insulin resistance are two metabolic abnormalities associated with type 2 diabetes mellitus and result in macrovascular and microvascular complications in multiple organ systems which accounts for the morbidity and mortality associated with this disease.

The phenotype associated with insulin resistance includes a dyslipidemia that is characterized by increased very low-density lipoprotein triglyceride levels, decreased high-density lipoprotein-cholesterol levels, and the presence of small, triglyceride-enriched, low-density lipoproteins (6, 8, 9). Clinical trials have shown that correcting the hyperglycemia can attenuate some of the microvascular complications of diabetes, such as retinopathy and nephropathy, but cannot suppress macrovascular complications, such as coronary heart disease (CHD) due to atherosclerosis.

Oxidant stress and inflammation accelerate CHD by activating the diacylglycerol-protein kinase C (DAG-PKC) signal transduction pathway, possibly by enhanced formation of glycosylated proteins and advanced glycation products and/or by increasing endothelial dysfunction (8). Recent studies have

shown possible biochemical mechanismsl by which hyperglycemia could cause its adverse effects on the vascular cells (**Figure 1**).

Insulin possesses anti-atherogenic properties. Insulin increases nitrous oxide (NO) production, which can cause vasodilatation and retard the migration and growth of arterial smooth muscle cells under physiological conditions (7). In pathological states of insulin-resistance, the enhancement of NO production from either acute activation of nitric oxide synthase (NOS) or endothelial cell NOS (eNOS) by insulin are blunted (9). Moreover, hyperinsulinemia may contribute to atherogenesis by stimulating the growth and production of the extracellular matrix (ECM) (10).

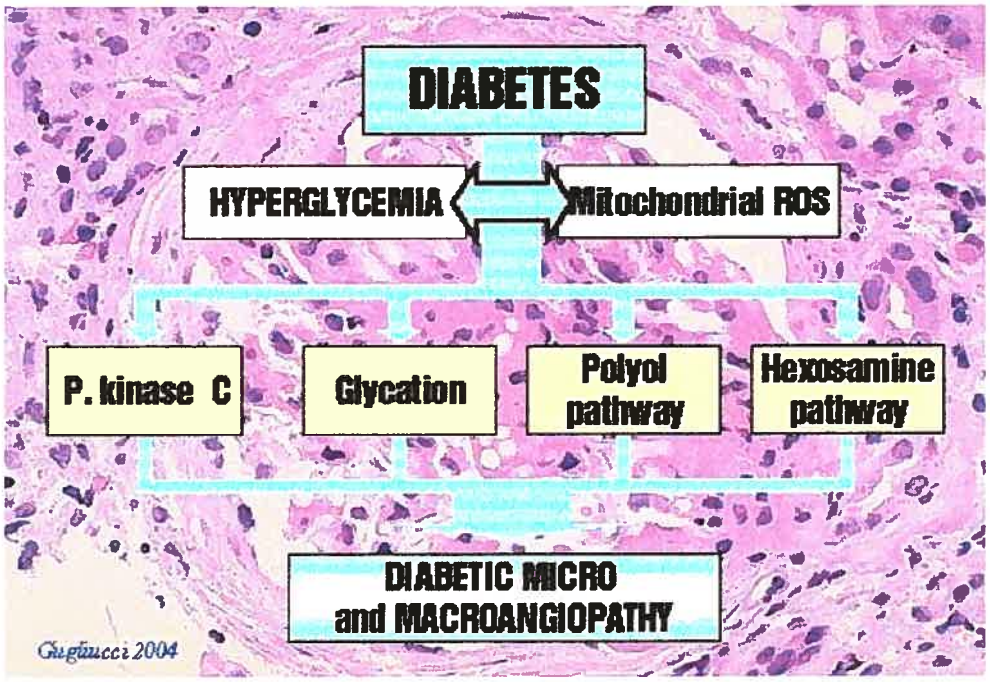


Figure 1: Four main pathways implicated in hyperglycemia-induced diabetic microvascular disease. (The Maillard reaction and diabetes mellitus. Dr Alejandro Gugliucci MD, PhD).

Metabolic risk factors include dyslipidemia, hypertension, glucose intolerance, and a prothrombotic state (11). The latter is a newly recognized factor in type 2 diabetes which manifests with increased fibrinogen levels, increased plasminogen activator inhibitor-1 (PAI-1), and platelet abnormalities (12-14).

I.1. 3 Diabetes and risk of thrombosis

There are several ways in which diabetes predisposes to a higher risk of thromboembolic events: alteration of the coagulopathic proteins, endothelial dysfunction, increased platelet adhesions, and altered fibrinolysis.

Diabetes can cause changes in the haemostatic system including increased concentration of fibrinogen, factor VII, von Willebrand factor (vWF), and plasminogen activator inhibitor 1 (PAI-1) (15), as well as decreased tissue plasminogen activator (tPa), eNOS, and NO production (16). These changes may result in endothelial dysfunction, increased adhesion of platelets and monocytes, abnormal fibrinolysis and hypercoagulation, and form a prothrombotic state. We will review them in details.

I.1.3.1 Endothelial dysfunction

Vascular endothelial cells maintain their vascular integrity through the release of a variety of paracrine factors such as NO, which regulates vasodilatation, anticoagulation, leukocyte adhesion, smooth muscle proliferation and the antioxidative capacity of endothelial cells (17). Endothelial dysfunction has been detected in patients with diabetes and now is a well recognized phenomenon. Hyperglycemia and insulin resistance are thought to be the primary reason, as

they change intracellular metabolism and produce excess superoxide radicals inside vascular cells (18, 19). In turn, these molecules impair release of NO, increase NO destruction, enhance release of endothelium-derived constricting factors and decrease sensitivity of the vascular smooth muscle to NO through mediators such as protein kinase C, the polyol pathway, non-enzymatic glycation and oxidative stress (18). As a result, this leads to an imbalance between smooth muscle cell growth, promotion and inhibition, thrombosis and fibrinolysis, inflammation, and cell adhesion (20).

I.1.3.2 Increased adhesion of platelets and monocytes

Platelets are small anucleate discoid cells that circulate in the bloodstream and participate in hemostasis and repair of vascular injury (19). The abnormal metabolic state that accompanies diabetes may activate platelets and alter their functional properties. Activated platelets interact with the endothelium and promote adhesion of platelets to monocytes (21). Many studies have demonstrated that platelet degranulation further increases platelet activation and diminishes the platelet's sensitivity to natural antiaggregating agents (21-23). Circulating platelet-monocyte aggregates may release procoagulant, oxidative and mitogenic factors (24). All these significantly contribute to the inflammatory and procoagulant response in diabetes.

I.1.3.3 Abnormal fibrinolysis and hypercoagulation

Defects in the coagulation and fibrinolytic cascade are important pathological mechanisms that can lead to thrombus formation (25). In healthy conditions, the endogenous fibrinolytic system represents an equilibrium between activators of

plasminogen (primarily tPA) and inhibitors of these activators such as PAI-1 (26). PAI-1 synthesis and release is regulated by insulin, proinsulin, VLDL cholesterol, and various cytokines. In the diabetic condition, the equilibrium between endogenous tissue plasminogen activator and PAI-1 is altered, as evidenced by decreased levels of tPa, increased tissue factor (TF) and PAI-1 (26). This is likely associated with increased production of proinflammatory cytokines such as interleukin (IL-6) from adipocytes. Elevated PAI-1 decreases local fibrinolysis and promotes hypercoagulation (27). Raised concentrations of fibrinogen, von Willebrand factor and other endothelium-derived mediators increase blood viscosity and promote platelet activation and adhesion (21).

Some evidence shows that diabetes mellitus may result in abnormal fibrinolysis and hypercoagulation predisposing to a procoagulant state. However, it is not known whether these abnormalities cause increased risk of venous thromboembolism and whether they affect thrombus recanalization. Data about this remains controversial. A recent retrospective study showed that the risk of VTE among diabetic patients is significantly increased as compared with the non-diabetic population (16).

In addition, diabetes may result in loss of balance in the production and the degradation of ECM proteins like fibronectin and collagen may lead to structural alterations such as basement membrane thickening and ECM protein deposition (28, 29). It is not known whether these changes cause an increased risk of venous thromboembolism.

I.1.4 Diabetic mouse models

Mouse models of type 2 diabetes are likely to be as complex and heterogeneous as the human condition. Strains of inbred mice and mice that spontaneously develop a type 2 diabetes-like phenotype through spontaneous mutations or induced mutations (*i.e.*, transgenic, targeted/"knockout", or chemically induced mutations) have been generated and are used in a wide variety of research areas including cardiovascular biology, developmental biology, diabetes and obesity, genetics, immunology, neurobiology, and sensorineural research (30, 31).

The genetically obese Zucker rat (32) is a spontaneous model of type 2 diabetes that has a missense mutation in the leptin receptor gene (33, 34). Other examples of spontaneous genetic mutations include the diabetic *db/db* mouse that contains a mutation in the leptin receptor gene (35) and the *ob/ob* mouse, a model for obesity that lacks the leptin protein (36). Genetically engineered models are now becoming the forefront of animal research. Another common way to develop a type 2 diabetic mouse model is by diet induction. To establish this model, an appropriate diabetogenic diet should be given to C57BL/6 mice for 10 weeks to induce obesity, hyperglycemia (with fasting blood glucose levels greater than 240 mg/dl), insulin resistance (with blood insulin levels of greater than 150 microU/ml), and increased plasma cholesterol concentrations. Thus, this model displays all the metabolic abnormalities of the human condition: obesity, hyperglycemia, and hyperinsulinemia (37).

Genetic factors may determine susceptibility to diabetes even with a standard high fat diet. Certain inbred mouse strains differ in their susceptibility to high fat diet-induced diabetes (39, 40) with C57BL/6J mice showing susceptibility to the weight gain and insulin resistance when fed with a high fat high sucrose diabetogenic diet (30, 41) These and other observations show that profound interactions between diet and genetic factors influence glucose homeostasis (38, 42, 43).

In this study, the C57BL/6J (B6) mouse strain was chosen as a model for studying diabetes mellitus, as this strain carries a genetic predisposition to develop non-insulin-dependent (type 2) diabetes.

I.2 DEEP VENOUS THROMBOSIS

Deep venous thrombosis (DVT) is a blood clot that forms in a vein deep in the body. Generally, most deep vein clots occur in the lower leg or thigh. They also can occur in other parts of the body such as in the lungs, resulting in pulmonary embolism (PE). They have a high prevalence both in the community and in hospitals, and are of considerable morbidity and mortality (44).

I.2.1 Prevalence and risk factors

DVT is a life-threatening and costly health problem (45). In young individuals, the incidence of DVT is of 1/100,000 people; at middle age it is approximately 1/1000, which is also the overall incidence; thereafter, it increases steeply and approaches 1%/year (46). DVT tends to be asymptomatic for long periods of time and difficult to detect by clinical examination unless it reaches a threshold for occlusion leading to symptoms or signs of venous insufficiency.

The pathogenesis of DVT invokes 'Virchow's triad' and is considered to be a combination of changes in stasis of blood within the veins, 'intimal injury' in the wall of the blood vessel, and 'hypercoagulability'. It is a serious problem because of its clinical sequelae including pulmonary embolism and chronic venous insufficiency (postphlebitic leg pain, swelling, chronic venous stasis ulcers, venous valvular incompetence, lipodermatosclerosis and claudication).

The formation of DVT is multifactorial. Many of the classic risk factors for arterial thrombosis are also risk factors for venous thromboembolism (47). Hereditary factors include gene mutations (such as factor V Leiden, the G20210A

prothrombin) and deficiencies in physiologic coagulation inhibitors (such as protein C, protein S its non-enzymatic cofactor, and antithrombin). In addition, increased levels of plasma factor VIII, fibrinogen, factor IX, factor XI, prothrombin, homocysteine, lupus anticoagulant, and antiphospholipid antibodies may also be implicated (48, 49). Acquired factors that can contribute to DVT include smoking, hypertension, varicose veins, cardiac dysfunctions, obesity, malignancy, hospitalization, surgery, venous trauma, immobilization, estrogen therapy and pregnancy (50). Recently, a retrospective study showed that the risk of venous thromboembolism among diabetic patients is greater than in the non-diabetic population (49). It is widely accepted that multiple risk factors interact which determines the risk of thrombosis (51).

I.2.2 Normal venous anatomy

Understanding venous pathophysiology requires some knowledge about venous anatomy and physiology. The primary function of the systemic veins is to return deoxygenated blood back to the right side of the heart and to act as a blood reservoir. Approximately 75% of the blood volume is contained within the venous system.

Veins differ from arteries. They possess the same 3 layers as arteries but the muscle layer is reduced. From the lumen to the periphery, the intima of the vein wall is a thin layer of smooth muscle cells (SMCs) covered by the endothelium. Underneath the intima, the media by which the appearance of a rudimentary internal elastica separates both layers consists of a thin inner layer of longitudinally oriented SMCs and a more prominent outer layer of

circular-oriented SMCs, both embedded in an extracellular matrix. The adventitia is composed of fibroblasts, bundles of collagen fibers, capillaries, and clusters of longitudinally oriented smooth muscle cells **(Figure 2).**

The thin and collapsable venous wall allows variations in shape with minimal changes in pressure (venous system is a low-pressure system) and is responsible for the capacitance function of the venous circulation. Veins are normally only partially filled with blood. They have three times the cross-sectional area of corresponding arteries.

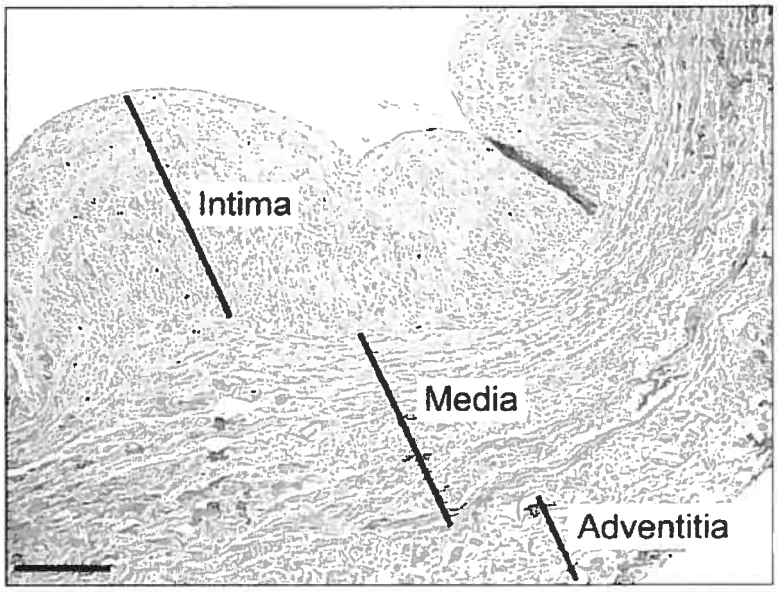


Figure 2: Vein wall with the intima underlying the endothelium, the media and the adventitia. Scale bar is 200 μm (44).

Veins of the extremities have valves. These are thin delicate bicuspid structures made of fibrous and elastic tissue lined with endothelium. At the site of each

valve the vein is dilated creating a sinus space around the valve which facilitates the opening and closing of the valve. Few valves are located in the femoral veins; the vena cava and common iliac veins are valveless (52). Two venous systems exist in the upper and lower extremities, the superficial and the deep venous system which are connected by perforating veins. The major superficial veins of the extremities have thicker walls than the deep veins. Under normal circumstances, two 'pumps' (foot and calf pumps) work together to propel venous flow against gravity and bicuspid valves direct flow from the superficial to the deep system. The purpose of the valves is to break up the column of blood in the vein and ensure unidirectional flow. Disease states interfere with these pumps leading to venous stasis and thrombosis which compromises valve function and results in venous hypertension (53).

In contrast to arterial thrombosis, which usually develops in association with vascular-wall injury leading to platelet-rich thrombus, venous thrombosis develops in regions of disturbed flow and relative stasis (as observed in the calf or venous sinuses), often in association with increased coagulability or endothelial damage. The thrombi are composed predominantly of fibrin and red blood cells (54). Deep vein thrombosis may lead to residual venous obstruction or reflux and result in post-thrombotic complications. Enhancing resolution of venous thrombi may preserve valve integrity and reduce the incidence of post-thrombotic complications (55).

I.2.3 Resolution of thrombus

DVT resolves by a the development of venous collaterals (vasculogenesis), clot retraction, organization and recanalization (angiogenesis) that is similar to the formation of granulation tissue in healing wounds (56). These processes may occur simultaneously and are influenced by the fibrinolytic and matrix metalloproteinases (MMPs) systems through a series of cellular and molecular events (**Figure 3**) (57).

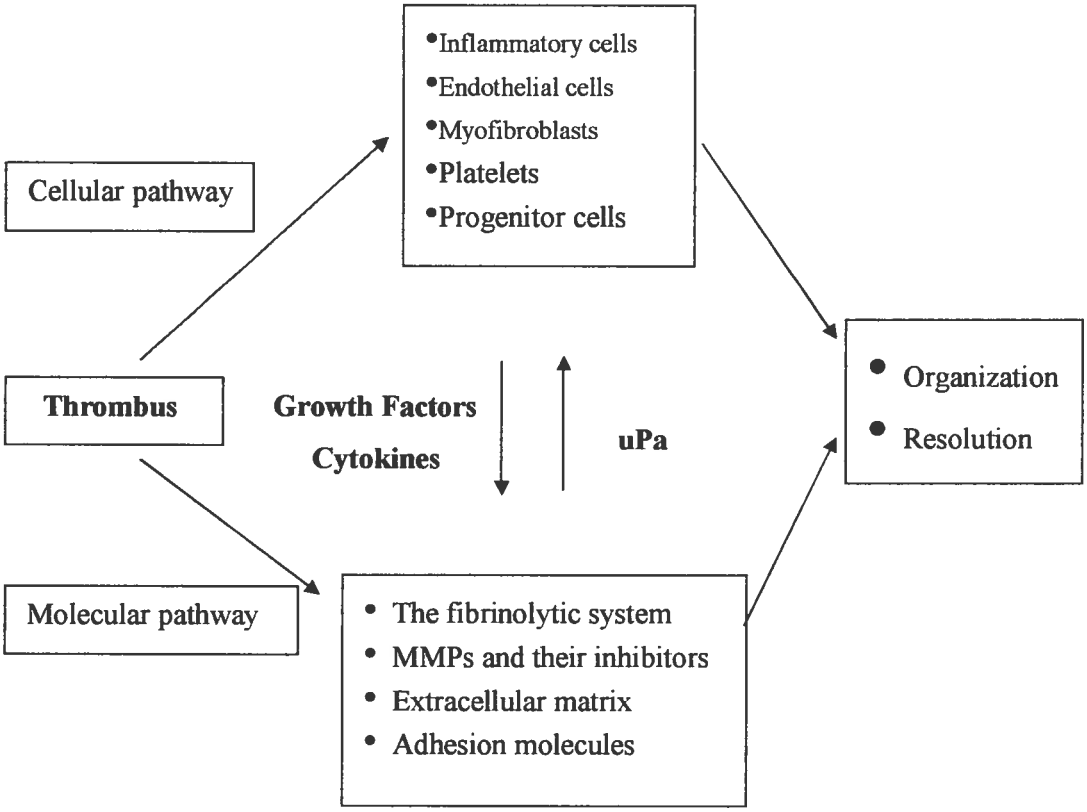


Figure 3: Cellular and molecular pathways during resolution of thrombus

I.2.3.1 Cellular pathway

The resolution of thrombus occurs through recruitment of inflammatory cells (mainly monocytes), invasion of endothelial cells (ECs), vascular smooth muscle cells (VSMCs) and myofibroblasts leading to recanalization (58). Several cellular processes occur: (a) covering of the surface of thrombus with neutrophils, monocytes (MCs) and an endothelial layer; (b) penetration by neutrophils and monocytic cells; (c) development of myofibroblasts and new capillaries; and (d) recanalization—the formation of one or more channels inside and parallel to the original vessel (59).

I.2.3.1.1 Inflammatory cells

Thrombus formation and its resolution are both strongly associated with a subacute inflammatory reaction (60) with the release of fibrinolytic, chemotactic and growth mediators. Early after venous thrombosis, circulating neutrophils migrate through the vein wall, possibly via the vasa vasora and respond to chemokines by invading the thrombus, which causes thrombus retraction and lysis. Subsequently, monocytes, macrophages and lymphocytes are the predominant leukocyte subpopulations which promote tissue remodeling, recanalization and also retraction of the thrombus (61, 62).

The thrombus contains trapped thrombin and fibrin that are potent monocyte chemoattractants (17). The process of recanalization of thrombus is based on the ability of MCs/Mphs (monocytes/macrophages) to penetrate the extracellular matrices and create tubular spaces ("tunnels") of lower density (60, 63). Recanalization is induced by expression of a variety of cytokines, angiogenic factors, proteases and their inhibitors that regulate cell migration, extracellular

matrix turnover and tissue remodeling (4, 6, 24, 25). It is also plausible, however, that once monocytes convert to the macrophage phenotype in the thrombus, their fibrinolytic activity increases, which causes lysis of the thrombus (64). Currently, proteolytic activity of MCs/Mphs on vasculogenesis based on the engraftment of circulating EPCs is thought as an important mechanism of recanalization of thrombus (59, 60).

Whether the inflammatory response after DVT is affected by hyperglycemia remains to be determined.

I.2.3.1.2 Endothelial cells

As a unique multifunctional cell with critical basal and inducible metabolic and synthetic functions, ECs may react to physical and chemical stimuli within the circulation and regulate haemostasis, vascular remodeling, vasomotor tone, and immune and inflammatory responses. In addition, ECs play a pivotal role in angiogenesis and vasculogenesis (65, 66). Endothelium in resting state is both an anticoagulant and antithrombotic by secretion of a variety of molecules important for the regulation of blood coagulation and platelet function, such as nitric oxide, prostacyclin and thrombomodulin. Vessel exposure to cytokines or proinflammatory molecules may shift the balance towards a procoagulant/prothrombotic phenotype of the ECs (**Figure 4**) (65) and inhibit fibrinolysis by reducing the component of the fibrinolytic system. The balance of endothelial properties can be tipped to favor platelet aggregation and clot formation (67, 68). Operating in coordination, these changes can allow fibrin formation and platelet activation.

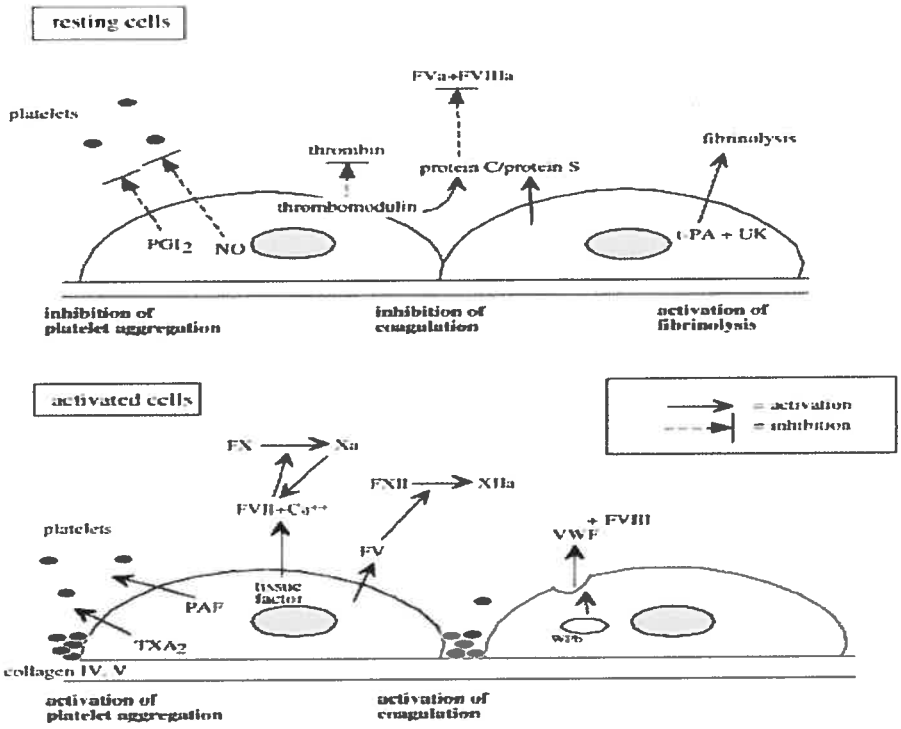


Figure 4: Schematic representation of the role played by endothelial cells in coagulation and fibrinolysis pathways. NO, nitric oxide; PAF, platelet activating factor; PGI_2, prostacyclin; tPa, tissue plasminogen activator; TXA2, thromboxane A2; UK, urokinase; vWF, von Willebrand factor; WPb, Weibel-Palade body (65).

Moreover, ECs coordinate the recruitment of inflammatory cells to sites of thrombus. These cells produce and release cytokines and growth factors serving as communication signals to leukocytes. Cytokines induce a proinflammatory phenotype of endothelial cells. Upon activation of these cells with tumor necrosis factor- α (TNF-α) or interleukin-1 (IL-1), platelet-activating factor (PAF) is secreted and stimulates platelet aggregation and neutrophil adhesion to regulate vascular remodeling (69).

Recanalization of thrombus is similar to angiogenesis, because recanalization may be conceived as the formation of endothelialized channels lined by "endothelial-like" cells, which express many of the endothelial markers (e.g. CD31, VCAM-1 and ICAM-1) (70). Recanalization is also marked by new formation of vessels which stain for laminin, a basement membrane protein that is known to promote early EC migration and capillary tubule formation within the organizing thrombus. vWF, thrombomodulin and tissue factor are expressed in the larger, more prominent channels that appear in older thrombi suggesting that they are lined by more mature ECs (71). This process requires different sequential steps including the release of proteases from activated ECs with subsequent degradation of the basement membrane, ECs migration, proliferation, and differentiation of mature blood vessels into the interstitial space (69, 72).

Some studies performed in our laboratory on coil embolization of intracranial aneurysms showed that early endothelial invasion of the clot leads to recanalization and recurrence of aneurysms. Moreover, this process can be prevented by endothelial denudation (73), which may prove that the endothelium plays an important role in recanalization of thrombus.

However, whether the process of recanalization and neovascularization of venous thrombi is affected by type 2 diabetes is unknown.

I.2.3.1.3 Myofibroblasts

Myofibroblasts are highly specialized mesenchymal cells derived from fibroblasts. These cells populate the adventitia tunica (74) and participate in vessel injury repair as well as thrombus recanalization. In the injured vessel,

numerous cytokines and growth factors (i.e., TGF-β) can influence the proliferation level of fibroblasts as well as their transition to myofibroblasts (74).

Several lines of evidence indicate that uPA appears to be an important determinant influencing adventitial cell proliferation and myofibroblastic modulation. In injured adventitia, exogenous uPA stimulated myofibroblast proliferation and in vitro, upregulated the content of α-SM actin in fibroblastic cell culture. Moreover, uPA neutralizing antibody attenuated α-SM actin expression by adventitial cells after injury of vessel (75).

During organization and recanalization of thrombus, the balance between uPA-dependent development of endothelialized channels and MMP-9-dependent contraction by myofibroblasts of the residual provisional fibrin/collagen matrix between recanalized channels, would result in progressive enlargement of the recanalized spaces (59).

I.2.3.1.4 Platelets

Platelets are cell fragments released from the bone marrow into the bloodstream and involved in the cellular mechanisms of primary haemostasis. When a blood vessel injury occurs, platelets exhibit a sequence of events: 1) adhesion of platelets to the injury site, 2) spreading of adherent platelets over the exposed subendothelial surface, 3) secretion of platelet granule constituents, 4) platelet aggregation, and 5) platelet coagulant activity (22, 76). Endothelium disruption provides binding sites for adhesive proteins such as von Willebrand factor (vWF) in the subendothelial matrix (which binds to the platelet glycoprotein Ib/IX complex) and fibrinogen, as well as fibronectin through integrin receptors. These

adhesive proteins are thought to form a bridge between platelets and subendothelial connective tissue. Once they adhere to the subendothelium, platelets spread out on the exposed surface and additional platelets from the circulation adhere, first to the basal layer of adherent platelets and eventually to one another. Fibrinogen mediates platelet aggregation to form a mass of aggregated platelets through building bridges from platelet to platelet (22, 76). Initially, the platelets involved in the thrombus formation favour angiostatic chemokines, such as platelet factor-4 and subsequently secrete angiogenic cytokines such as vascular endothelial growth factor (VEGF) which complexes with fibronectin resulting in ECs migration and proliferation. This complex is more potent than VEGF alone (77) which may regulate the revascularization of thrombus.

I.2.3.1.5 Progenitor cells

Progenitor cells arise from division of stem cells. A subset of these cells such as endothelial progenitor cells (EPCs), along with the properties of hemangioblasts that express the leukocyte antigen, CD45 (78), have been implicated in revascularization, vascular repair, and myocardial regeneration. In addition, mesenchymal stem cells also can differentiate into both VSMCs and endothelial cells, and reveal a high degree of plasticity to participate in the development of vascular systems, including angiogenic sprouting and vessel enlargement (79, 80).

Recent reports suggest that following thrombus formation, circulating EPCs derived from bone marrow stem cells may arrest at the site of thrombus, infiltrate through thrombus, and differentiate into endothelial cells to contribute to

endothelialization/recanalization of thrombus, or into α-SMA positive cells that participate in neointima formation (81, 82). Their angiogenic effects are most likely mediated by secretion of growth factors (82). Significant numbers of bone marrow-derived progenitor cells have also been found in naturally resolving thrombus (59). As shown by Singh and coworkers, thrombus resolution is markedly delayed in urokinase gene deleted animals which can be rescued by bone marrow transplantation (83).

I.2.3.2 Molecular pathway

The fibrinolytic system and matrix metalloproteinases (MMPs) are major components of the molecular pathway and play a pleiotropic role in resolution of thrombosis. The fibrinolytic system may regulate endothelial cell infiltration by degrading fibrin matrices (84). MMPs expressed by ECs, neutrophils, monocytes/macrophages can degrade the extracellular matrix to promote the migration of ECs (85). In addition, chemotactic agents and growth factors (including angiogenic cytokines) expressed or secreted by inflammatory cells and platelets also participate in the interaction of molecular and cellular pathways (86). All of these may affect tissue remodeling and revascularisation of thrombus.

I.2.3.2.1 The fibrinolytic system and major components

The fibrinolytic system constitutes a critical response mechanism to thrombus formation and evolution. The central components comprise an inactive proenzyme, plasminogen that can be converted to the active enzyme, plasmin, which in turn degrades fibrin into soluble fibrin degradation products. Two immunologically distinct physiologic plasminogen activators (PA) have been

identified: the tissue-type PA (tPa) and the urokinase type PA (uPa) together with the major inhibitors of PA, plasminogen activator inhibitor-1 and -2 (PAI-1, PAI-2), while plasmin is inhibited mainly by α2-antiplasmin. tPa, a serine protease, is responsible for the removal of fibrin from the vascular tree (87, 88), whereas, uPa bound to its receptor uPAR, is regarded as the critical trigger for plasmin generation during cell migration and invasion, and may be responsible for regulating the activation of other proteases, such as the MMPs (eg, procollagenases and macrophage elastase) (**Figure 5**) (88). Plasmin can also activate or liberate growth factors from the ECM including latent TGF-β1, bFGF and VEGF (89).

PAI-1, a member of the serine proteinase inhibitor (SERPIN) family is the primary inhibitor of plasminogen activators in plasma and in the pericellular matrices, which bonds two-chain active uPA or tPA to reduce activity of uPA or tPA through covalent complex formation (88). Vitronectin (VN) binds to uPAR,, an abundant plasma and matrix glycoprotein; whereas PAI-1 controls recognition of VN by uPAR or the αvβ3 integrin receptor, and is stabilized by binding to a plasminogen activator inhibitor binding protein identified as S-protein, suggesting a role in coordinating cell adhesion and migration (90). Moreover, PAI-1 detaches cells from extracellular matrices; vitronectin, fibronectin and type I collagen through an uPA/uPAR-dependent mechanism by inactivating integrins (91). Thus, PAI-1 could be considered as a deadhesion molecule (e.g., thrombospondin, tenasin) disrupting the link between the cytoskeleton and the focal adhesion plaque and resulting in the loss of stress fibers and a decrease in the strength of integrin–ligand interactions (182).

During natural resolution of venous thrombi, there is an increase in the activity of the fibrinolytic mediators, tissue-type plasminogen activator (tPa) and urokinase-type plasminogen activator (uPa) and this activity is expressed by invading monocytes (182)

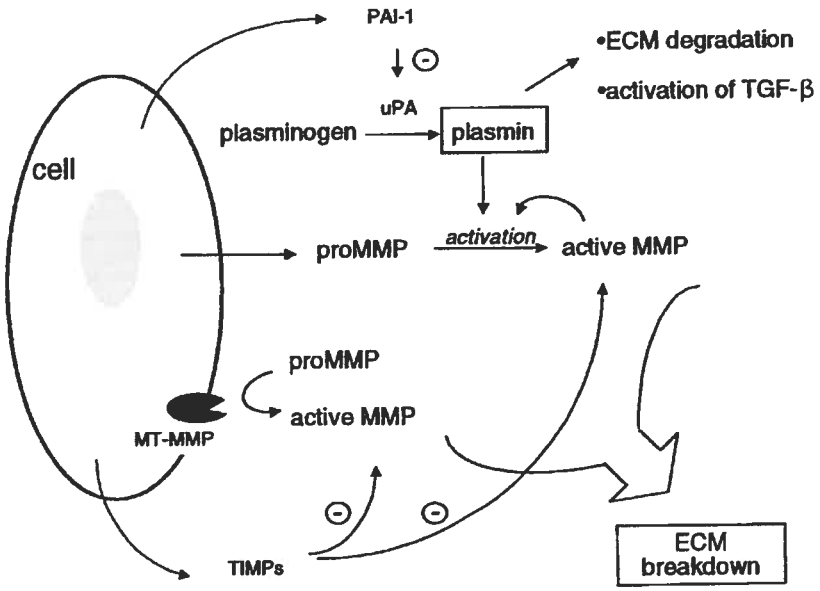


Figure 5: An extensive network of additional proteases, inhibitors, receptors and modulators are directly associated with and are influenced by the PA system. The largest group is the matrix metalloproteinases (MMPs) and their respective inhibitors, the tissue inhibitors of MMPs (TIMPS) (94).

The levels of uPA were usually found to be greater than those of tPa. Subsequent gene knockout studies have shown that deletion of the gene encoding for uPA markedly inhibited normal thrombus resolution, but the tPa gene knockout had no effect. Absence of uPA is also associated with delayed monocyte recruitment into the thrombus (83).

uPa may have a dual function; one related to proteolytic matrix breakdown, the other related to matrix production via proteolytic activation of growth factors, such as the fibrogenic TGF-ß1. This dual role of uPa may explain why ECM degradation and collagen deposition were both attenuated in the absence of uPa (95).

PAI-1 plays a determining role in controlling thrombus formation. PAI-1 activity was increased in patients with deep vein thrombosis (DVT) and pulmonary embolism (96). Further evidence for the role of PAI-1 in venous thrombosis comes from animal models. Transgenic mice, that were genetically engineered to synthesize PAI-1 in excess, had higher rates of venous thrombosis than mice with normal PAI-1 levels (97). In a model of venous stasis-induced DVT after ligation of the inferior vena cava, Deatrick and coworkers demonstrated an alteration in the normal profibrinolytic to antifibrinolytic state of the vessel by a decrease in the ratio of uPA to PAI-1 (60).

Clinical and experimental studies have suggested an important role of PAI-1 in arterial and venous thrombosis and the maintenance of systemic vascular hemostasis (98). Moreover, experiments with transgenic mice deficient in PAI-1 support a role for this serpin in both vascular remodeling after arterial injury (99) and the formation of pulmonary fibrosis that occurs after inflammatory injury (100). Upregulation of PAI-1 in endothelial cells and smooth muscle cells after acute vessel injury (101) and thrombus formation (102) suggest that elevated PAI-1 may play a role in vascular remodeling after deposition of a thromboembolus.

There is increasing evidence that diabetes mellitus is associated with several defects in coagulation and fibrinolysis that may lead to a procoagulant, thrombogenic predisposition (103). However, it is not known whether these perturbations cause a decrease in thrombus recanalization.

I.2.3.2.2 Matrix metalloproteinases and their inhibitors

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases, which can degrade essentially all ECM components in physiological and pathological conditions, but also participate in cell migration, angiogenesis, and tissue remodeling during organ development, wound healing, inflammation, and cancer (104, 105, 106). Currently more than 24 members of the MMP family have been identified and classified into subgroups of collagenases, gelatinases, stromelysins, and membrane types (MT-MMPs) based on their structure and substrate specificity (107, 108). MMPs are produced by secretion of both vascular and inflammatory cells. Their activity is regulated at multiple levels: gene transcription and synthesis of inactive zymogens, posttranslational activation of zymogens, and interactions of secreted MMPs with tissue inhibitors of metalloproteinases (TIMPs) (104, 105). The TIMP family known at present consists of four distinct members (TIMPs 1 to 4), and is expressed in most tissues and body fluids. Except for TIMP-dependent inhibition of MMPs, these proteinases have been recently recognized to stimulate cell proliferation participating in mitosis and tissue differentiation, to regulate cell survival and apoptosis, and to inhibit angiogenesis (105). The balance of MMPs and TIMPs activity controls the diffusion of substances and the migration of cells through ECM. These proteinases also modulate signal transduction pathways by various

substrates, including inflammatory mediators, growth factors, and growth factor receptors (105).

MMP-2 and MMP-9 (gelatinases A and B) are the main enzymes able to degrade non natural ECM gelatin and type IV collagen (109), which is the major structural protein of the ECM, but also an integral part of endothelium basement membranes. ECM degradation and basement membrane disruption are the key steps in thrombus organization and recanalization. These enzymes play a critical role in vascular remodeling induced by altered arterial flow (110, 111), tissue ischemia and aortic aneurysms (112). Moreover, MMP-2 activity is critical for migration of endothelial cells (113) and monocytes/macrophages (114). Targeted deletion of MMP-2 abolishes angiogenesis in vivo (115, 116). Thrombin treatment of endothelial cells induces MMP-2 activity (117) and pro-MMP-2 can also be proteolytically activated in vitro by the coagulation proteins activated protein C (APC) (118) and factor Xa (119).

During experimental venous thrombosis, the expression of MMP-2 and MMP-9 is increased (60, 120). But their involvement in thrombus resolution and vein wall fibrosis in diet–induced type 2 diabetic mice remains undefined.

I.2.3.2.3 Extracellular matrix

The thin extracellular matrix (ECM) underlying the endothelium is termed the basement membrane. It is made up of structural interacting glycoproteins produced by VSMCs and fibroblasts (121). The most abundant components are laminin, entactin, collagen IV and glycosaminoglycans (heparan sulfate) (122).

These ECM components act as structural support promoting cell adhesion and barriers between tissue compartments regulating cellular migration. Basement membrane components contain the RGD (arg-gly-asp) sequence that control cell shape, migration, proliferation, differentiation, morphogenesis, and survival (123). Cells use a series of receptors (integrins, cell surface proteoglycans, and a newly described class of cell-surface-expressed tyrosine kinase receptors) to form linkages with ECM. In this cellular behavior, cells can be provided with directional guidance clues for migration (124). When blood vessel is damaged or presents a platelet-fibrin thrombus, the ECM may act as a ligand to adhere to in areas of exposed basement membrane on the endothelial monolayer by circulating blood cells and neointimal cells (123). The phenomena of the cell-matrix interaction can be modulated by the balance of activity of a class of proteases known as MMPs and their inhibitors.

Thrombosis and inflammation that occur in DVT result in valve destruction and chronic vein wall changes that lead to venous reflux and the syndrome of chronic venous insufficiency marked by thickened, noncompliant vein walls and incompetent valves.

After the development of a DVT, a late fibrotic response, similar to a healing wound, occurs in vein walls (60), involving the progression of the normally thin and compliant vein wall to a relatively thick and fibrotic state. There is deposition and accumulation of collagen procollagen I and procollagen III, and loss of normal vessel ECM such as heparin sulphate which has antifibrotic properties and is important for mediating normal vessel physiologic responses

deposition and accumulation of collagen procollagen I and procollagen III, and loss of normal vessel ECM such as heparin sulphate which has antifibrotic properties and is important for mediating normal vessel physiologic responses (125). Extracellular matrix molecules such as fibronectin and vitronectin enhance endothelial cell migration and tubule formation by binding to $\alpha v\beta 3$ (126).

The presence of these molecules has yet to be confirmed in naturally resolving thrombi, and moreover in hyperglycaemic conditions, but it seems likely that they have a role in this process.

I.2.3.2.4 Selectins

The selectins are a small family of lectin-like adhesion receptors. There are three family members, L-, E-, and P-selectin. Their major physiological role is thought to be largely responsible for the initial attachment and rolling of leukocytes on stimulated vascular endothelium. This family consists of P-selectin expressed on activated platelets and activated endothelium, E-selectin expressed on activated vascular endothelium, and L-selectin expressed on the surface of neutrophils. P-selectin is present in the granules of platelets and the Weibel–Palade bodies of ECs. It is first translocated to the plasma membrane of these cells, mediating the initial inflammatory response (127, 128). P-selectin plays a role in perithrombotic inflammation and in mediating leukocyte influx into areas of inflammation. Previous studies have shown that decreases in thrombogenesis and increases in thrombolysis can be achieved in primate, porcine, and rat models of arterial and venous thrombosis in which P-selectin is antagonized (58, 129, 130). These P-selectin driven interactions lead to activation of the coagulation cascade with

leukocyte tissue factor (TF) upregulation, which further potentiates vein wall inflammatory events.

I.2.3.2.5 Proangiogenic factors

Monocytes express a variety of proangiogenic factors such as VEGF, bFGF and interleukin-8 (IL-8), a prototypic cysteine-X-cysteine (CXC) chemokine with polymorphonuclear neutrophil (PMN)-activating and chemoattractant properties, and confers proangiogenic activity (134), which may generate an 'angiogenic drive' within the thrombus (65, 135). Both VEGF and bFGF are expressed in resolving thrombi, and are associated with the appearance of channels within and around the thrombus (136). VEGF expression was localized to several cells in the thrombus, including endothelial cells and the monocyte infiltrate. Expression of bFGF was found on mononuclear cells and spindle-shaped cells within the thrombus (136).

Monocyte chemotactic protein-1 (MCP-1) is a potent and specific activator of monocytes and basophils. In a rat model of venous thrombosis, the vein wall adjacent to the thrombus was found to contain increasing amounts of MCP-1 and when this cytokine is directly injected into venous thrombus, it alters its organization (137). Part of the effect produced by injecting MCP-1 may have been as a consequence of its angiogenic properties (**Table 1**).

Factor	Pro-AngiogenicActivity*	Reference
VEGF	↑ EC chemotaxis ↑ EC mitosis Mobilize EPC's	(135)
bFGF	↑ EC mitosis ↑ EC migration ↑ αVβ3 integrin	(135)
MCP1	↑ EC chemotaxis ↑ capillary sprouting	(54)
IL8 ↑	EC chemotaxis ↑ EC mitosis	(58, 65)
MMP2 MMP9	↑ EC migration	(6)

*These are generally known proangiogenic activities associated with these factors

Table 1: Proangiogenic factors expressed within resolving thrombus (70).

I.2.4 Treatment of deep vein thrombosis

Treatment costs to the U.S. health care system are in the range of billions of dollars per year just for the acute treatment of venous thrombosis, without even considering the amount of money spent on the treatment of the sequelae of DVT (chronic venous insufficiency) and PE (chronic pulmonary hypertension) (138).

I.2.4.1 Standard treatments

Prophylaxis and treatment of DVT aim to prevent propagation of the fractured thrombi as emboli leading to death from pulmonary embolism. Another goal is to minimize the sequelae of CVI known as the post-thrombotic syndrome. Enhanced

thrombus resolution is associated with reduced valvular damage and venous hypertension and fewer long term complications (6, 139). Standard treatments include anticoagulants (low-molecular-weight heparin that acts on both thrombin and factor Xa or warfarin), thrombolytics agents (for example, streptokinase, recombinant urokinase and tissue-plasminogen activator that lyse the thrombi), surgical thrombectomy and compression stockings as prophylactic measures. These agents are often considered alternatives by inhibiting thrombus extension and do not accelerate natural thrombus resolution. Thrombolytics are less used because of a small but significant risk of severe hemorrhage (140) and in patients with stroke or with a recent operation (6, 139, 140). Compression stockings are sometimes recommended to relieve pain and swelling, (141). However, these treatments do not seem to effectively reduce the incidence of the post-thrombotic venous insufficiency (142, 143).

I.2.4.2 New approaches

Although acute therapy for DVT is well established, potential new therapies have emerged to promote thrombus vascularization and resolution without altering the normal hemostatic mechanisms, such as delivery of angiogenic growth factors and cell-based therapy. The application of these methods may enhance rapid resolution of DVT, but also provide other perspectives on treatment.

I.2.4.3 Angiogenic therapy

A number of pro-angiogenic factors, including VEGF, IL-8, bFGF, have been reported to enhance neovascularization of ischemic tissues (6, 61). Currently the concept of stimulating therapeutic angiogenesis has also been applied to the

recanalization of venous thrombus (**Table 2**). Treatment with recombinant IL-8 in a rat model of IVC thrombosis resulted in increased recruitment of neutrophils, monocytes and markedly promoted early neovascularization enhancing the resolution of thrombosis (61).

Varma and coworkers (144) used therapeutic administration of pro-angiogenic compounds promoting DVT neovascularization, such as interferon inducible protein (IP-10), an angiostatic chemokine, basic (bFGF), a pro-angiogenic factor and epithelial neutrophil activating protein (ENA-78), a pro-angiogenic cytokine. These angiogenic chemokines 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. VEGF administration (136) enhance thrombus resolution by a variety of mechanisms (6).

VEGF, bFGF, platelet derived growth factor (PDGFs), placental growth factor (PIGF), the angiopoietins and their receptors, which are mediators associated with angiogenesis, have also been proved to promote the formation of blood vessel function (145, 146). These factors could provide more choice in treatment of thrombosis.

I.2.4.4 Cell-based approach

Thrombus resolution depends on the interaction of an assortment of cells. Bone marrow-derived progenitor cells are known to participate in revascularization, provide the necessary precursors as these immature cells can differentiate into a diversity of phenotypes, including macrophage, lymphocyte and endothelial cells (147). Recent studies have shown that thrombus resolution is markedly delayed in

uPA knock out animals, but rescued by bone marrow transplantation (83). These progenitor cells have been made to improve thrombus resolution as a cell-based approach and showed some benefit in small clinical trials (147-149). Endothelial progenitor cells, delivered locally or injected into the circulation, incorporate into newly formed vessels to enhance local angiogenesis by secreting a variety of pro-angiogenic cytokines (150).

Modulation of monocyte fibrinolytic and growth factor production in vitro, with subsequent reinjection of these cells, may provide an alternative treatment for venous thrombosis. This therapy might also be useful to recanalize mature thrombi when fibrinolytic treatment is ineffective (151).

I.2.4.5 Gene therapies

Gene therapy is the insertion of genes into an individual's cells and tissues to treat a disease. Endothelial progenitor cells are also used as vectors to deliver pro-angiogenic genes. It has been confirmed that transplantation of endothelial progenitor cells transfected with VEGF are more effective than unmodified cells in the angiogenesis and revascularization of ischemic tissues (152, 153). However, this therapy is limited by vector toxicity. Current studies of gene therapy focus on reducing toxicity and improving vectors by looking into mechanisms retargeting vector to the tissue of interest, minimizing or eliminating viral gene expression (**Table 2**) (135).

Therapy	Actions inThrombus	Reference
Single chemokines		
IL8	↑ neovascularisation, ↑ blood flow ↓ distal venous pressure, ↑ resolution	(61)
VEGF	↑ neovascularisation, ↑ MØ recruitment, ↑ organization, ↑ tPA/uPA, ↑ EPC mobilisation	(88)
bFGF	↑ neovascularisation, ↑ blood flow	(56)
Multiple chemokines		
VEGF isoforms (121/165/189)	100x more potent angiogenic effect in ischaemic tissue	(102)
VEGF + PlGF	Robust neovascularisation	(101, 103)
Cell based		
EPCs	Incorporate into neovessels, secrete proangiogenic cytokines, known benefits in ischaemic tissue	(107,110, 111)
Pluripotent stem cells/bone marrow	Supply progenitors for multiple cell types (Monocyte, lymphocyte, endothelial) into thrombus	(74, 107, 112)
EPC as vector (EPC +Ad.angiogenic l gene construct)	More effective than EPC alone, greater cell longevity	(113, 115)

Table 2: Potential new therapies promoting recanalization and resolution of venous thrombi (6).

I.2.5 Treatment of complication

Proangiogenic therapy can cause complications such as inflammation with an immunogenic response to viral vectors (154). Stimulation of therapy to angiogenesis may result in rupture of atherosclerotic plaques, development and growth of vascular malformations (155). Enhanced angiogenesis therapy also carries risks of neoplasia and tumour growth. In addition, administration of bone

marrow cells can carry a theoretical possibility for malignant transformation. The selection of single types of stem cell may reduce treatment complication (6, 155).

I.2.6 Animal models of venous thrombosis

If an optimal animal model exists, it would have a natural propensity for venous thrombosis, a similar clotting cascade and platelet interaction to that of the human, a lower extremity that closely resembles the human with a functional calf musculature, ability to walk upright and sufficiently tall (when standing) to allow hemodynamic study and sufficiently large to allow surgical intervention (164). Overall, large animals like pigs and monkeys have been better suited to study thrombosis as they are more similar to human physiology than smaller species such as mice, rats, rabbits and dogs (39). However, the study of thrombosis with these animals is restricted by cost and ethical considerations. Nevertheless, numerous studies have used rodent models taking advantage of low cost, availability, practical breeding, technical feasibility and the availability of transgenic knockout mice. Some limitations may limit extrapolation to human thrombi as the relatively rapid (3-4 weeks) rate of thrombus resolution in rodent models and the difference in hemodynamics in the smaller diameter of the vena cava may affect thrombus revascularisation (6).

Murine in vivo models are appealing because of their well-defined genetic background, and the possibility of using syngeneic "knockout" and mutant mice producing a variety of metabolic settings.

Animal models of venous thrombosis have been classified as non-genetic and genetic models. Non-genetic models of thrombosis have been produced by a

combination of blood-flow stasis with either increased coagulability or endothelial damage (46).

Several injury models applied to the arterial systems have also been used in the venous system. Injection of endotoxin (156), collagen and epinephrine, Factor Xa, or tissue factor (157), thrombin (158) as well as hyperoxia (159) or hypoxia (160) all served to induce a hypercoagulable state and fibrin deposition in the mouse. Other methods included application of 70 % ferric chloride (161), or sodium morrhuate or long-term nitric oxide synthase inhibition using L-NAME (162). In addition, thrombosis can be induced using ^{125}I-labeled fibrinogen mixed with thromboplastin (163). After administration of the thrombogenic stimulus systemically or directly into the stasis region, this model can be performed with or without mechanical vena cava stasis (2). They include vein ligation (164), or vein interruption by means of a silicone band or an intraluminal balloon catheter (165), or by the intra-stent stenosis (166). Flow stasis was also induced by a combination of devascularization, electric injury (167), or photochemical injury (23). These models represent useful tools for the better understanding of the venous thromboembolic events under conditions similar to those seen in humans (54). Other genetic models (such as transgenic models of thrombosis) are a number of spontaneous or genetically engineered mouse strains with overexpression or deletion of various elements in the lipid transporters, coagulation, platelet, and fibrinolysis pathways.

As our objectives are focused on the pathogenesis of venous thrombosis in diabetic mice, we used a reproducible model of venous thrombosis (168). The inferior vena cava (IVC) stenosis model, with a 94.4% ± 0.5% reduction in IVC

diameter (138), leading to a stasis-induced venous thrombosis is more adapted to our goals, as shown by earlier studies that venous thrombi produced in this model were formed in flowing blood and were morphologically similar to human thrombi (151). Moreover, this model has more availability, improved technical feasibility, standardization of local thrombosis and lower maintenance costs (169). Thus, the ability to study thrombus recanalization in diabetic mice should be appropriate in this model.

II RESEARCH PROPOSAL

There is increasing evidence that cardiovascular complications in type 2 diabetes mellitus can, in part, be explained by several defects of coagulation and fibrinolysis (47), promoting adhesion of platelets and monocytes, and endothelial dysfunction increasing the risk of thrombosis and vascular damage (170).

Venous thrombosis develops in regions of disturbed flow and relative stasis often in association with increased coagulability or endothelial damage which should predispose diabetic subjects to development of venous thrombosis. (47). Recently, a retrospective study showed that a higher incidence of VTE i.e. deep vein thrombosis (DVT) and pulmonary embolism (PE) was associated with diabetes (47) and obesity, independent of age, race and sex. (171).

Furthermore, the concept that thromboembolic arterial diseases and VTE are two distinct entities has been revised with numerous case control studies demonstrating the potential link between venous and arterial thrombosis (172).

These finding led us to speculate that thrombus organization and resolution are affected by the metabolic perturbations that arise in association with type 2 diabetes, such as hyperglycemia, insulin resistance, and increased release of free fatty acids that engender a cascade of endothelium-mediated dysfunctions that potentiate inflammation, abnormal fibrinolysis and thrombosis (7, 38, 47).

Because the majority of patients with type 2 diabetes have diet induced obesity, we sought to study the effects of diabetes on venous disease using an experimental modal of DVT in a mouse model of diet induced obesity/diabetes.

In this diabetic model, endothelial dysfunction and hypercoagulation have been confirmed (121).

II. 1. HYPOTHESES

Our hypotheses are that diet-induced type 2 diabetes will:

1) Impair venous thrombi resolution and recanalization.

2) Affect monocyte/macrophage recruitment in venous thrombi by activating the inflammatory response which would be expected to enhance thrombus resolution as reported by numerous studies in normal mice.

3) Result in prolonged exposure of vein wall to thrombus which allows vein wall remodeling through ECM deposition.

4) Downregulate the molecular factors involved in the regulation of fibrinolysis: the fibrinolytic system, especially uPa, the main protease implicated in thrombus resolution.

5) Affect the plasminogen activators, the MMP system, particularly MMP-2 and MMP-9 will be enhanced.

II. 2. RESEARCH GOALS

II. 2. 1. Main objective

The aim of the study was to determine whether the metabolic perturbations that characterize type 2 diabetes induced in fat diet fed mice would impair thrombus organization, resolution and recanalization in an experimental mouse model of DVT using angiography analysis and molecular techniques.

II. 2. 2. Specific objectives

Many specific objectives arise from the main objective to achieve this research project (summary in **Table 3**).

1) The first goal was to assess thrombus resolution and recanalization to see whether type 2 diabetes alters angiographic and angiogenic responses through analysis of angiographic scores, thrombus size and weight, and neovascular channels detected by immunohistochemistry.

2) Second, to confirm the elevated inflammatory response through the examination of the involvement of the monocyte/macrophage recruitment in diabetes-related impairment of resolution in vivo by immunohistochemistry.

3) Additional goal includes insight into the fibrotic response to evaluate whether diet-induced type 2 diabetes mediates vein wall damage by comparing ECM deposition in thrombosed IVC sections from normo- and hyperglycemic mice.

4) Venous thrombolysis in vivo is mediated primarily by activation of the plasminogen-plasmin axis. We thus examined the relation of the fibrinolytic system to DVT resolution in high fat diet mice, through the quantification of the mRNA and protein levels of the plasminogen activators, uPa and tPa and their major inhibitor, PAI-1.

5) Finally, we investigated MMP-2 and MMP-9 gene and protein expression and their gelatinolytic activities in IVC/thrombus specimens obtained from control and diabetic mice and their immunolocalization and expression in IVC/thrombus sections.

Experimental design	
10 week-diet-induced type 2 diabetes	
Inferior vena cava thrombosis model **1 week 2 weeks**	
I - Thrombus resolution **Recanalization/Neovascularization**	a) Angiographic score b) Thrombus area and mass c) Neovascular channel quantification
II - Inflammation	a) CD 14 gene expression b) Immunohistochemical staining
III - Fibrosis	Picrosirius red staining
IV - Fibrinolytic system (uPA, tPA and PAI-1) **MMP system** (MMP-2 and MMP-9)	a) Gene expression (RT-PCR) b) Protein expression (Western blotting, zymography and immunohistochemistry)

Table 3: Summary of specific objectives

III. MATERIAL AND METHODS

All experimental procedures were approved by the institutional animal care committee in accordance with guidelines of the Canadian Council on Animal Care.

III.1 Diet-induced type 2 diabetic mouse models

The C57BL/6 mouse strain was used as a model of diet-induced type 2 diabetes. Only male mice were used to avoid the potentially confounding effects of sex. The C57BL/6 strain was chosen as it has provided the background for previous experiments (173) and has the availability of various genetic knockouts. Forty mice, 5 weeks of age, purchased from Charles River (St-Constant, Quebec) were maintained in a temperature-controlled barrier facility with a 12-hour light/dark cycle and were given free access to food and water. Mice were fed rodent chow pellets for a 1-week acclimatation period prior to initiation of diet studies.

Thereafter, the mice, 20 per group, were assigned to 1 of 2 dietary treatments for 10 weeks: the low-fat diet D12450B (control group) and the high-fat diet D12451 (diabetes group) fed ad libitum. D12450B (10 kcal% fat)/ D12451 (45 kcal% fat) have been used widely to develop diet-induced type 2 diabetes mice. Furthermore D12450B was also used as diet of control group (203). Research Diets (New Brunswick, NJ) manufactured the diets. The composition of the diets is listed in **Table 4.**

Body weight and plasma glucose of each animal were measured weekly throughout the study until age of 16 weeks. Blood was drawn after an 8-hour fast

via saphenous vein in non-anesthetized mice. Plasma glucose concentration was determined with ONETOUCH Ultra (LifeScan, Inc.). A successful type 2 diabetic C57BL/6J mouse was defined as having average fasting blood glucose levels of greater than 12mmol/L (174).

Table 4: Composition of the diets (Formulated by Research Diets, Inc.,)

Product #	**D12451**		**D12450B**	
	gm%	**kcal%**	**gm%**	**kcal%**
Protein	24	20	19.2	20
Carbohydrate	41	35	67.3	70
Fat	24	45	4.3	10
Total		**100**		**100**
kcal/gm		**4.73**		**3.85**
Ingredient	**gm**	**kcal**		
Casein, 80 Mesh	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	72.8	291	31.5	1260
Maltodextrin 10	100	400		
Sucrose	172.8	691	350	1400
Cellulose, BW200	50	0	50	0
Soybean Oil	25	225	25	225
Lard	177.5	1598	20	180
Mineral Mix S10026	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	505	0
Potassium Citrate, 1 H2O	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
FD&C Red Dye #40	0.05	0	0.05	0
Total	**858.15**	**4057**	**1055.0**	**4057**

III.2 Animal model of venous thrombogenesis: a mouse inferior vena cava stasis model.

An established rodent model of DVT by a combination of low flow and endothelial damage was used to induce the formation of venous thrombi in the vena cava of normal and diet-induced diabetic mice **(Figure 6)** (175). Mice were anesthetized with isoflurane (Baxter Health care Ltd), and the inferior vena cava was exposed below the renal veins through a midline laparotomy incision. The intestines were retracted, and retroperitoneal blunt dissection of the infrarenal vena cava was performed to mobilize a 5 mm segment distal to the left renal vein. A 5-0 Prolene suture was placed alongside the vena cava. A severe stenosis was produced in the vein by tying a 5-0 silk suture around the vena cava to include the Prolene suture. The Prolene was then pulled out to allow blood to continue to pass up the vein. A neurosurgical vascular clip was applied to the dissected vena cava for 15 seconds on 2 separate positions, 30 seconds at a time to induce endothelial damage. The intestines were replaced, and the abdominal wall was sutured. The animals were then allowed to recover from the anaesthesia. The mice tolerated the procedure well and were recovered in the laboratory before being returned to the animal housing facility. All operative deaths occurred in the immediate perioperative period and were related to anaesthetic administration. No remote deaths secondary to infection occurred, and the mice continued to feed well and had access to water and chow.

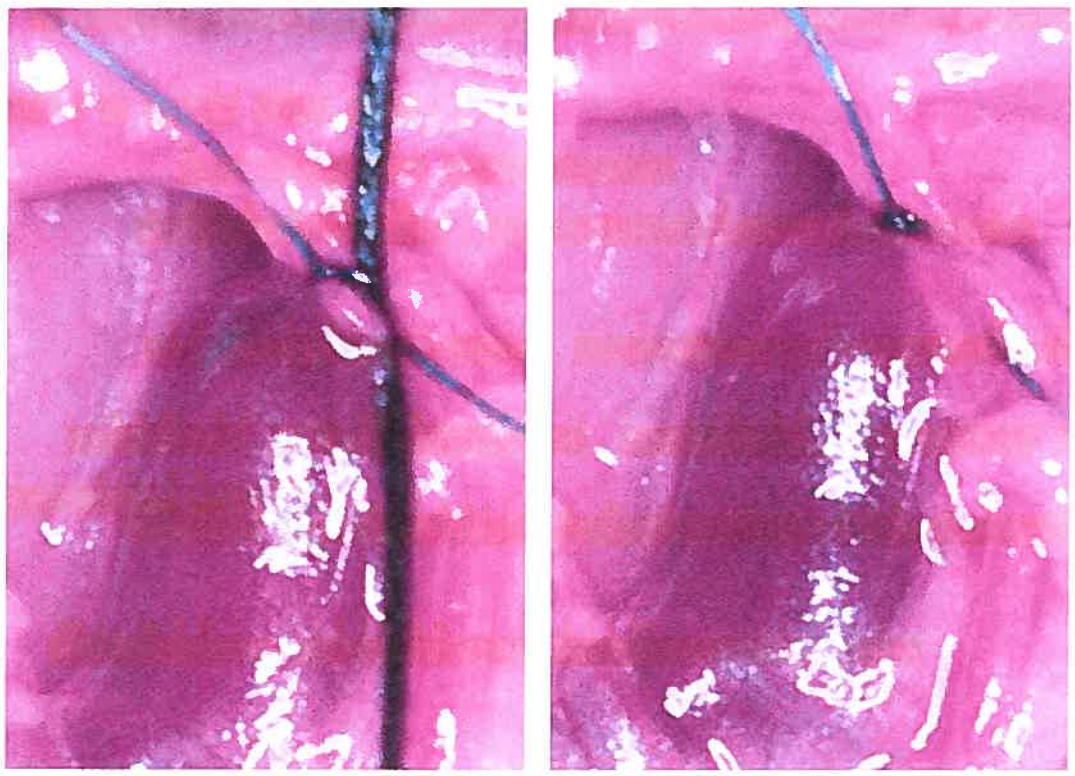


Figure 6: Rat inferior vena cava (IVC) stenosis model of venous thrombosis (58). A severe stenosis was produced in the vein by tying a 5-0 silk suture around the vena cava to include the prolene suture in the right panel. The prolene was then pulled out to allow blood to continue to pass up the vein in the left panel.

III.3 Angiography

Angiography was used to assess blood flow through thrombus recanalization. The mice were sacrificed at 1 (DM n=10, control n=10) and 2 weeks (DM n=10, control n=10) after angiography was performed. Mice were injected with lethal dose of pentobarbital (Somnotol; MTC Pharmaceuticals, Cambridge, Ontario, Canada) (65 mg/mL; 120 mg/kg intraperitoneally) just before angiography. A 1 ml syringe filled with radiopaque contrast medium (iodixanol [Visipaque], 320

mg/mL) and a 261/2G needle were used in the femoral vein for injection. Occlusion was defined as the absence of anterograde blood flow through the thrombosed venous segment; any antegrade opacification was sufficient to label the vena cava as recanalized.

III.4 Tissue harvest / Measurement of thrombus size and infrarenal vena cava weight

The inferior vena cava to the iliac bifurcation was harvested and weighed, and the weight was normalized to vein length (mg/cm) to calculate the mass of thrombus that formed during the period of venous stasis. This technique is an indirect measure of thrombus content but a reliable measure of thrombus resolution (61).

The samples were then immediately snap-frozen by immersion in liquid nitrogen and store at -80^0C until further processing for biochemical study or were fixed overnight in Zn-Tris solution (176), dehydrated, and embedded in paraffin using a routine histological procedure for immunohistochemistry. Each group (n=10) of samples was processed for mRNA extraction (n=4), zymography and western blotting (n=3), and immunohistochemistry (n=3).

The sizes of the thrombi were marked by using the area of thrombus in each section measured with image analysis software Vision PE, Clemex Technologies Inc., (Longueuil, QC, Canada). These measurements were expressed in square millimeters.

III.5 Histopathologic and immunohistochemical analysis

Histological and immunohistochemical studies were performed on paraffin-embedded venous tissue sections. Five-micrometer thick samples were deparaffinized in xylene and rehydrated in decreasing concentrations of alcohol. Endogenous peroxidase was quenched by incubation of sections in 0.3% H_2O_2 in methanol for 15 min. The slides were washed twice with 0.05 mol/l Tris·HCl, 0.15 mol/l NaCl, and 0.03% Tween 20 (TBT) pH = 7.4, between each step throughout the experiment. Nonspecific binding was blocked by 30-min incubation with 10% normal goat serum (NGS) or 5% dry milk in TBT (TBB). Immunoreactivity was detected for all primary antibodies listed in **Table 5** by overnight incubation in TBB in a humidified chamber and revealed by peroxidase (Vectastain ABC KIT, Vector Laboratories, Burlingame, CA). Peroxidase activity was detected with 1 mg/ml diaminobenzidine tetrahydrochloride (Pierce Biotechnology, Rockford, IL) as chromogen and 0.1% H_2O_2 as substrate. Sections were counterstained with haematoxylin solution (Vector, Burlingane, ON) and subsequently dehydrated in graded ethanol solutions, cleared in xylene, and mounted in Permount (Fisher Scientific, Montreal, QC). Slides were visualized with a computerized imaging system (Vision PE, Clemex Technologies Inc., Longueuil, QC, Canada). Nonspecific staining was verified by omission of the primary antibody or by using nonimmune normal serum. Perivascular collagen fibrils were detected histologically by Picrosirius red staining (177) and examined by polarized light microscopy. Collagen volume fractions were evaluated by analyzing staining intensity per pixel in multiple fields mentioned ART Recherches et Technologies Avancees Inc that only vein wall collagen has been quantified (15–20 fields/section, 2 sections/animal, and 3 mice/group

Antibodies Anti-	Source	Dilution	Antigen retrieval	Specificity
Biotinylated **GSL-1**	Vector Labs., Burlingame, CA	1:300	None	Endothelial cells
-Integrin αvβ3 Clone LM609	Chemicon	1:50	10 minutes at 95°C in 10 mM sodium citrate (pH 6.0)	ECs, Macrophages, VSMCs and Fibroblasts
-CD68 (H-2550): sc-9139	Santa Cruz Biotechnology, Inc.	1:20	10 minutes at 95°C in 10 mM sodium citrate (pH 6.0)	Macrophages
-MMP-9 Clone VIIC2	Neomarkers Lab vision Corp		10 minutes at 95°C EDTA 1mM pH 8.0	Myofibroblasts, ECs and Macrophages
-MMP-2 Clone CA-4001	Neomarkers Lab vision Corp	1:20	10 minutes at 95°C EDTA 1mM pH 8.0	VSMCs, ECs and Macrophages
-tPA lone GMA-043	Chemicon			
-uPA (H-140): sc-14019	Santa Cruz Biotechnology, Inc.	1:20	10 minutes at 95°C in 10 mM sodium citrate (pH 6.0)	
-PAI-1 (H-135): sc-8979	Santa Cruz Biotechnology, Inc.	1:100	12 minutes in 0.1% Trypsin) in TBS at room temperature.	

Table 5: List of antibodies

III.5.1 Macrophage content

Paraffin sections of the thrombus taken at 7 and 14 days after surgery from groups of control mice and diabetic mice (n=3 per group). They were processed for macrophage immunoreactivity using the monoclonal antibody against the CD 68 antigen. The percentage area of the thrombus containing the stained CD 68 antigen (macrophage density) was measured with the image analysis software described above. In a blinded fashion, positively stained cells in ten high-power fields radially around the thrombus were counted and totalled and then indexed to total thrombus area. The average macrophage density in each group was calculated.

III.5.2 Neovascular channel quantification

Neovascularization was defined by Griffonia Simplicifolia Lectin 1 (GSL-1) or αvβ3 positive stained channels. GSL-1 is a biotinylated lectin that identifies ECs that specifically display α-methyl-D-galactopyranosyl groups (178). The specific αvβ3 integrin represents also a marker of neovascularization as it has been identified as a critical modulator of angiogenesis (179).

These channels were counted in the entire thrombus section and totaled. Total channel counts were then indexed to total thrombus area to account for differences in section technique, IVC sample location, and size of thrombus.

III.6 Western blot analysis

Proteins were extracted from IVC samples by homogenating with lysis buffer (1M Tris pH 7, 1M NaCl, 100mM Sodium Fluoride, 200mM Sodium ortho-vanadate, NP-40 100ul/10ml, 1 tablet of Complete mini, 100mM PMSF) and protein concentration determined by the Bradford method (BIO-RAD protein assay, BIO-RAD). Tissue samples were denaturated and equal amounts of protein extracts (20 μg) were loaded onto a 10% SDS-polyacrylamide gel according to Laemlli (180). Immediately after electrophoresis, the proteins were transferred to Pure Nitrocellulose Membrane (BIO-RAD Trans-blot Transfer Medium) in transfer buffer (Tris–glycine, pH 8.3 and 20% methanol). The nitrocellulose membrane was saturated with 5% skimmed milk powder in blotting buffer (0.05M NaPO4 PH 7.4, 0.154M NaCl, 0.1% Tween 20) for 1 hour at room temperature. The membranes were washed in TBS-T, and then incubated with MMP-2 or MMP-9 antibody (1:400 dilution), and uPa and tPa antibody (1: 200 dilution) overnight at 4°C with gentle shaking. Antibody binding was visualized either with goat anti-mouse or anti-rabbit IgG antibody conjugated to horseradish peroxidase (Calbiochem, San Diego, Calif) (1:5000) for 90 min at room temperature. Peroxidase activity was revealed by chemiluminescence with SuperSignal West Pico (Pierce, Rockford, IL). The membranes were exposed to x-ray film and developed. Quantification of the bands was assessed with AlphaEase software (Alpha Innotech Corporation, San Leandro, CA) and corrected per ug of protein.

III.7 Zymographic activities

Gelatinolytic activities of MMP-2, MMP-9 were determined by zymography. This technique is a modification of SDS-PAGE, based on the incorporation of an

enzymatic substrate (gelatin) into the electrophoretic gel and the incubation of the gel in appropriate buffers. It allows visualization of gelatin-degrading enzymes at picogram levels. Electrophoresis of equivalent amounts of total soluble protein prepared as one part sample with one part Tris-Glycine SDS sample buffer (2X), was performed under non-reducing conditions in a 7.5% SDS polyacrylamide gel containing 1 mg/mL gelatin (Sigma Chemical). The gel was then washed three times for 20 min in 2.5% Triton X-100 to remove the sodium dodecylsulfate. The gel was developed for 18 hours at 37° C in a solution containing 50 mmol/L Tris-HCL (pH 7.9) and 5 mmol/L $CaCl_2$. The solution was removed, and the gel was stained with coomassie blue and then destained in 10% acetic acid/40% methanol. MMP activities were then observed in the gel as light bands against a dark background. Control zymograms were performed in a similar fashion with samples known to express both MMP-2 and MMP-9 (melanoma cell line). Gels were photographed and densitometric analysis of lysis areas were quantified using AlphaEase software (Alpha Innotech Corporation, San Leandro, CA).

III.8 RNA isolation and RT-PCR analysis

After sacrifice, tissues were subjected to mechanical homogenization in TRIzol reagent (Molecular Research Center, Inc, Cincinnati, Ohio) and combined with chloroform. Samples were centrifuged, and nucleic acids (RNA) were extracted from the aqueous layer. The RNA was subjected to reverse transcription to produce cDNA. Aliquots of total thrombus RNA (500 ng) were used for first strand cDNA synthesis in 20µl reaction volume using Superscript II reverse transcriptase (Invitrogen). After determining the linear range of RT-PCR for each of the target genes, amplification of the gene under investigation was carried out

using the primers summarized in **Table 6** and chosen in two different exons to distinguish genomic contamination. PCR amplifications were performed with Platinum Taq DNA polymerase (Invitrogen) according to manufacturer's instruction on an Eppendorf Mastercycler gradient using following program: step 1, 94°C for 1 min; step 2, between 52 and 64°C for 1 min; and step 3, 72°C for 1 min. 40 cycles were performed for the amplification of genes of interest and 30 cyclers for β-actin. The amplification for each gene was in the linear curve. PCR products were visualized on 1.5% agarose gel stained with ethidium bromide and UV trans-illumination. Quantitative analysis was carried out using a computerized densitometric imager (ImageQuant; Amersham Biosciences, Canada) to obtain gene/β-actin ratios. Genes that were studied included metalloproteinases (MMP-2 and 9), uPa and PAI-1 classically involved in clot fibrinolysis and thrombus organization and CD14 for monocyte/macrophage activity. The list of primer sequences are summerized in **Table 6.**

Gene	Forward primer	Reverse primer
MMP-2	5'-CTTTGCAGGAGACAAGTTCTGG-3'	5'-TTAAGGTGGTGCAGGTATCTGG-3'
MMP-9	5'-CCATGAGTCCCTGGCAG-3'	5'-AGTATGGATGTTATGATG-3'
CD14	5'-GGAAGCCAGAGAACACCATCG-3'	5'-GCAGGGCTCCGAATAGAATCC-3'
uPa	5'- GGAGAGCTCCTATAATCCTG-3'	5'-CCAGCTCACAATCCCACTCA-3'
PAI-1	5'-AGGGCTTCATGCCCCACTTCTTCA-3'	5'-AGTAGAGGGCATTCACCAGCACCA-3'
β-actin	5'-CATGGATGACGATATCGCTGCGC-3'	5'-GCTGTCGCCACGCTCGGTCAGGATC-3'

Table 6: Sequences of primers of selected genes for RT-PCR

III.9 Statistical analysis

Descriptive statistics were collected and data reported as mean ± SEM. Graphic representations were created using Sigma Plot (v 3.5). Statistical analysis was performed with Sigma Stat (v 3.5). Angiographic occlusion rates at different times were analyzed by Fisher s Exact test. For comparison between DM group and control group, all data, blood glucose, body weight, thrombus size and weight, mRNA expression levels and protein levels (western blot and zymography), were subjected to statistical analysis by a non-parametric Mann–Whitney test or by two-way analysis of variance (if normally distributed). For all statistic tests, a P value of less than 0.05 was considered significant.

IV RESULTS

There was no surgical mortality in the operating process. Deep vein thrombosis, with or without recanalization, did not lead to loss of function of limb in any mouse.

IV.1 Successul development of type 2 diabetes in mice

Adult C57BL/6 male mice were fed during 10 weeks with a high-fat diet to induce obesity and diabetes. The body weight increased faster (+17.5%) in high fat-diet fed mice than that in chow-diet fed mice, and reached to the significantly different point by 10 weeks (37.5 ± 2.2 g versus 31.9 ± 2.1 g, n=20 in each group, $P< 0.001$) and maintained thereafter (**Figure 7**). The same happened to plasma glucose level, and the significant increase showed in 10 weeks of feeding reached 55.5% (12.6±1.1 mmol/L versus 8.1±0.9 mmol/L, n=20 in each group, $P < 0.001$), which indicated the successful development of mild type 2 diabetes in these mice (**Figure 8**).

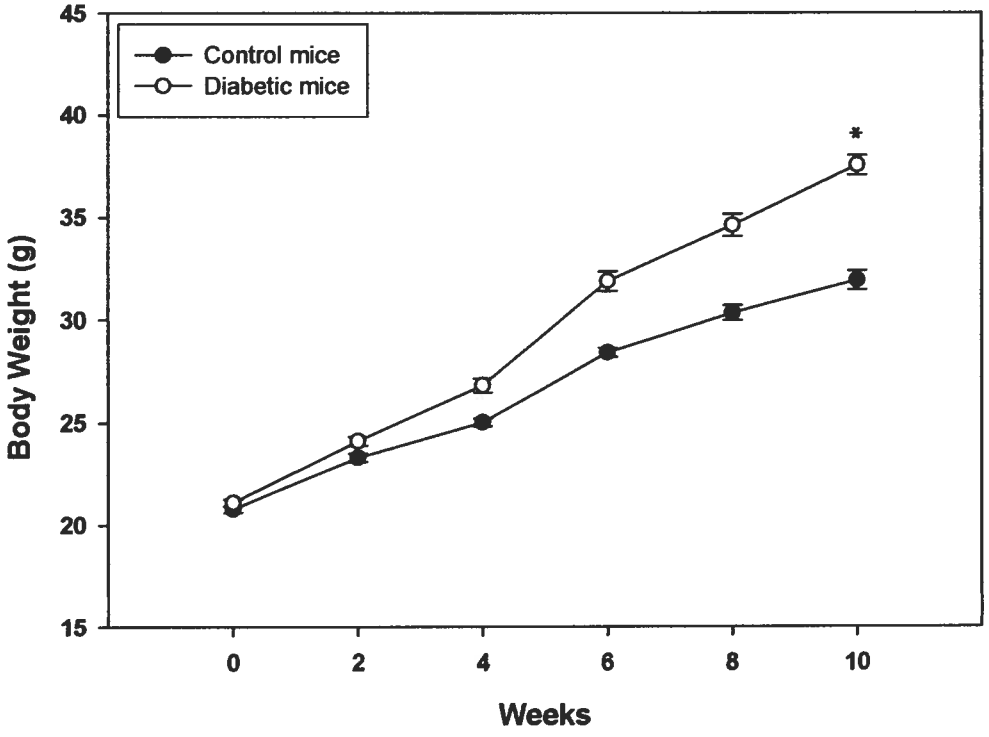


Figure 7: Body weight growth in control and diabetic mice (*P< 0.001 vs control mice, n=20).

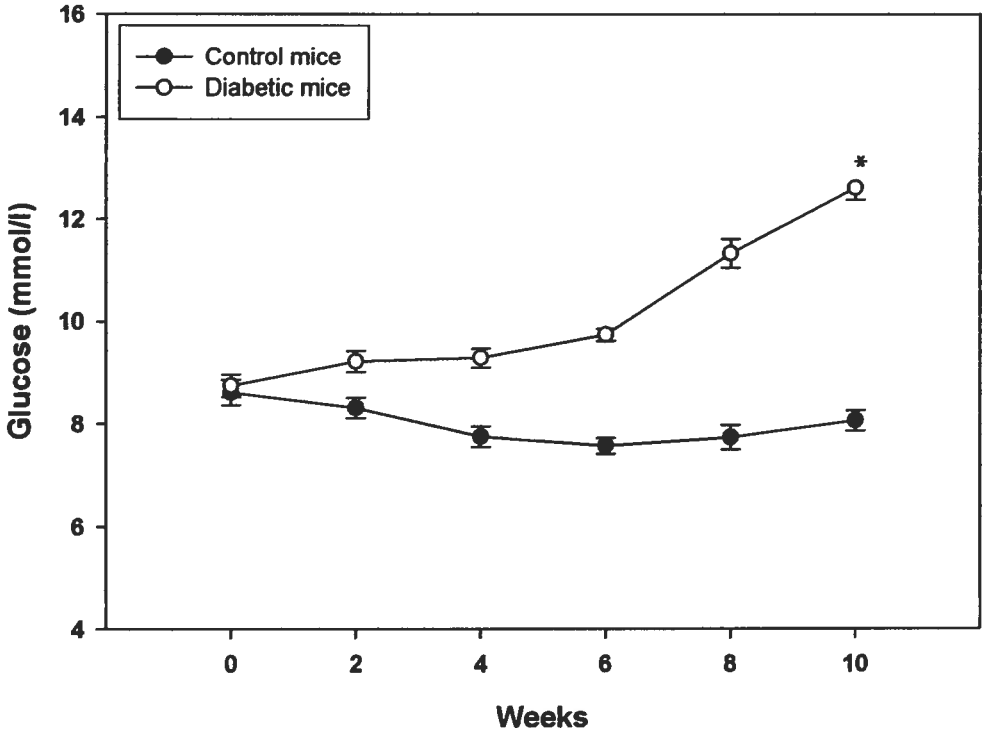


Figure 8: Blood glucose levels in control and diabetic mice (*P< 0.001 vs control mice, n=20).

IV.2 Diabetic mice have less thrombus resolution

The 8-day time point of thrombi was chosen from previous studies done by Wakefield et al. (56), who reported that no outcome measurements revealed any significant difference between groups at 4 days, consistent with the notion that an angiogenic response develops later in the course of thrombus resolution.

Thrombus resolution was assessed by two separate methods, thrombus area and thrombus mass.

IV.2.1 Thrombus area

At 1 week, the thrombus area was 13.69 ± 0.49 mm^2 in control group and 24.23 ± 1.66 mm^2 in the diabetic group, which was statistically different ($P<0.05$) **(Figure 9)**. Two weeks after DVT, a higher significant difference was also observed between these two groups (2.68 ± 1.34 vs 12.41 ± 0.71 mm^2, $P<0.05$). Furthermore, 2 weeks after the surgery, the thrombus area in both groups became smaller, especially in the control group, where the reduction was 80% for the normoglycemic mice, compared to 49% for diabetic mice ($P<0.05$).

IV.2.2 Thrombus mass

As a gross but reliable measure of thrombus dissolution, diabetic thrombosed IVC weight normalized by length was significantly higher at 1 and 2 week-DVT than control mice **(Figure 10)**. At these time points, the increase was 85% and 56% respectively ($P<0.05$). Similarly as in thrombus area, the decrease in thrombus weight 2 weeks after surgery was statistically significant in the 2 groups and more

important in the control (40%) than diabetic group (18%) reflecting a lesser ability of diet-induced type 2 diabetic mice to resolve venous thrombi.

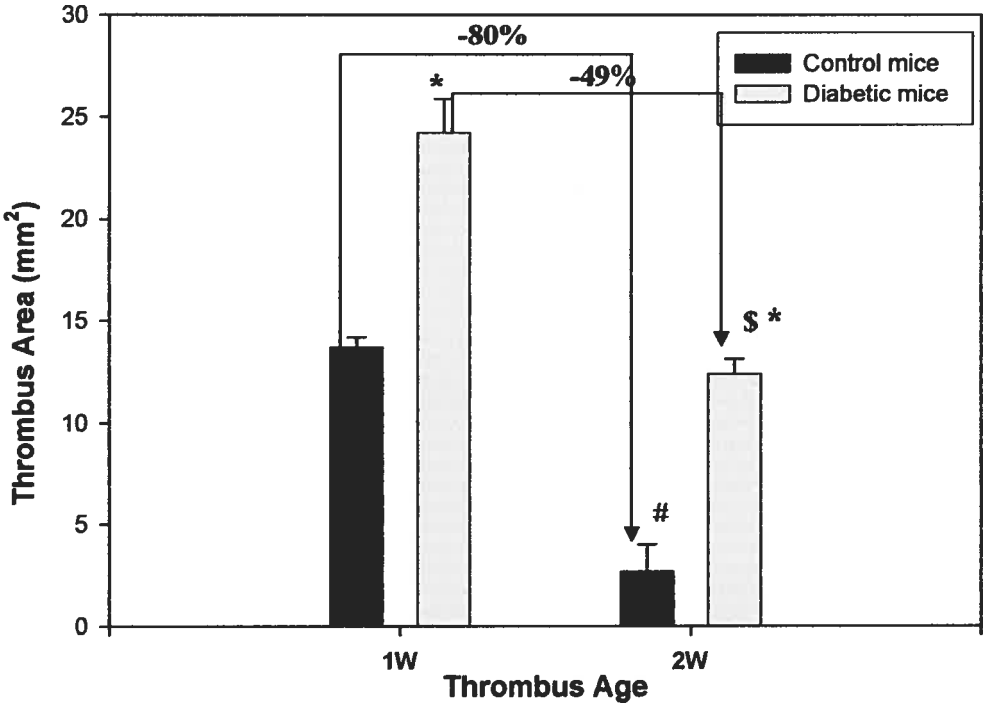


Figure 9: Thrombus areas in control and diabetic groups at 1 week or 2 weeks after surgery. Thrombus area in the diabetic group was greater than in the control group with 1.8-fold and 4.6-fold increase at 1 and 2 weeks respectively. At 2 weeks, there was a significant decrease in both groups, which was more conspicuous in the control group (80% vs 49% in diabetic mice) (*P< 0.05 compared with respective control; # P < 0.05 compared within control mice $ P < 0.05 compared within diabetic mice, n=3).

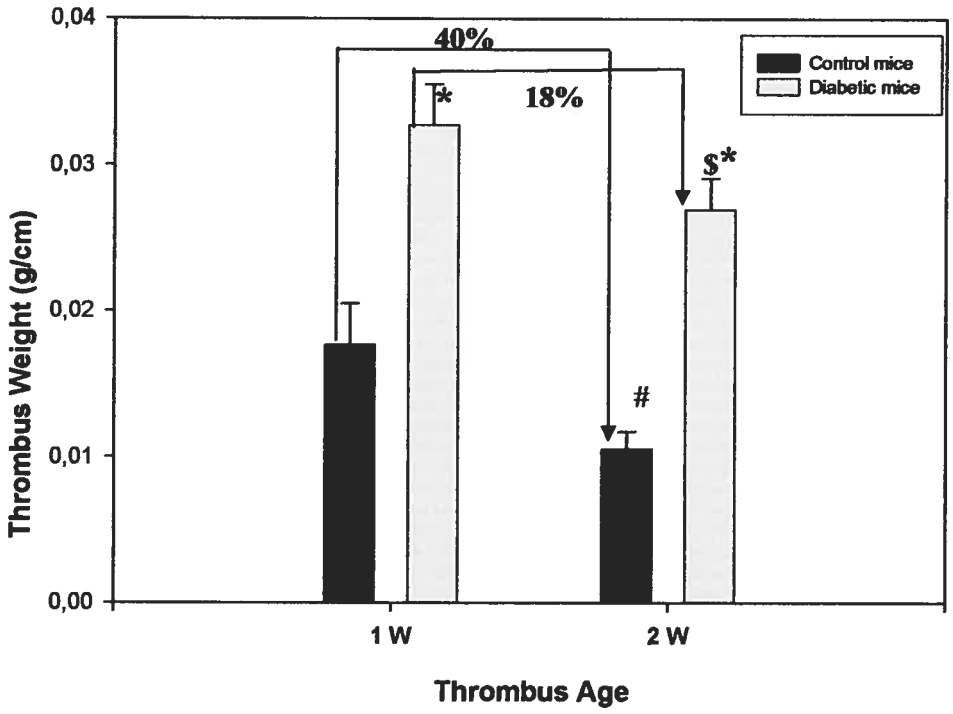


Figure 10: Thrombosed IVC mass/length in control and diabetic groups at 1 week or 2 weeks after surgery. There was a statistically significant difference between control and diabetic group at both 1 week and 2 weeks after surgery. (*P< 0.05 compared with respective control; # P < 0.05 compared within control mice $ P < 0.05 compared within diabetic mice, n=3).

IV.3 Thrombus recanalization is impaired in diabetic mice

To determine whether diabetes would impair natural thrombus recanalization, angiography analysis of thrombus blood flow and immunohistological methods were used.

IV.3.1 Angiography

Angiography analysis enables detection of in vivo intra and perithrombus blood flow through thin plane in the thrombosed IVC, and is used in a similar manner to that described for models of experimental intracranial aneurysms elaborated in our laboratory (73). Restoration of blood flow after IVC ligation was scored 0 for occluded veins and 1 for recanalized, as shown in **Figure 11** with representative angiographies. Both 1 week-control and 1 week-diabetic mice presented with occluded IVC. Two week-control mice showed blood flow through side collaterals and within the thrombus body, whereas blood flow in 2-week diabetic mice was mainly via side collaterals.

As shown in **Table 7**, at 1 week after the surgery, 80% (8/10) mice in control group occurred complete IVC occlusion, meanwhile 90% (9/10) mice in diabetic group did. No significant differences in flow restoration between them.

At 2 weeks after the surgery, it was documented in 90% (9/10) of control mice, meanwhile it was efficient in 10% (1/10) of diabetic mice ($P=0.001$, $n=10$).

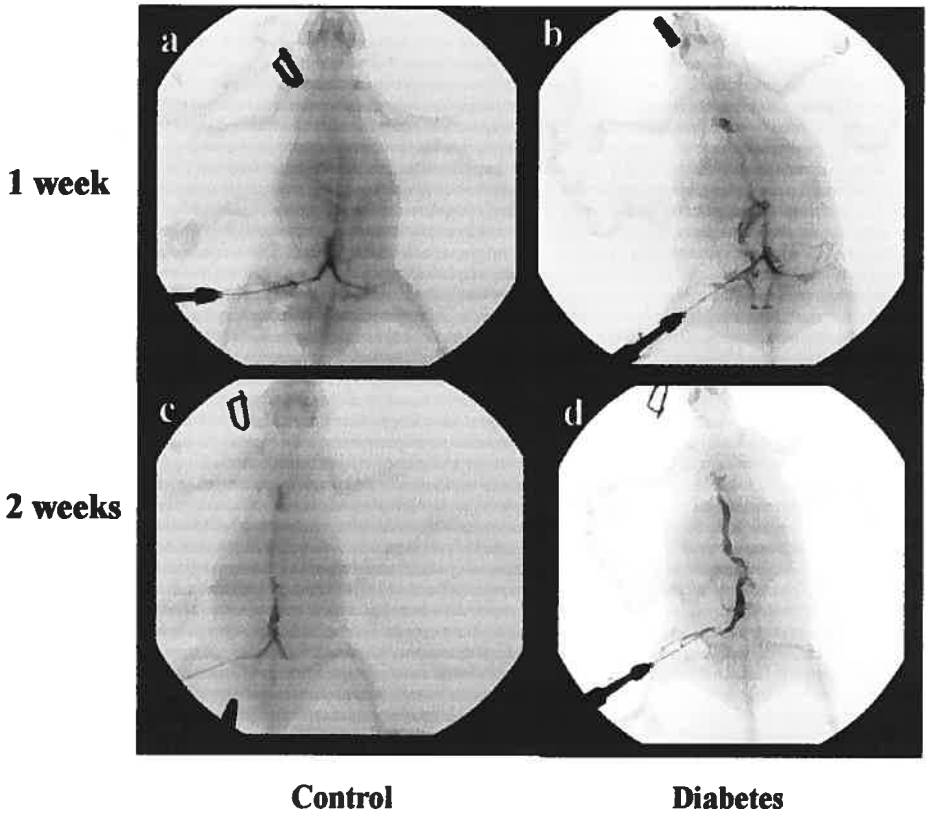


Figure 11: Angiograms in control and diabetic groups at 1 week or 2 weeks. A and B show two representative mice which presented with complete IVC occlusion at 1 week. C shows thrombus recanalization in control group at 2 weeks. D reveals maintained complete IVC occlusion in the diabetic group at 2 weeks (The venous blood recirculated through by-pass vein).

Time	Group	Amount	Recanalization		
			Yes	No	Rate
1 week	Control	10	2	8	20%
	DM	10	1	9	10%
2 week	Control	10	9	1	90%
	DM	10	1	9	10%

Table 7: Angiography scores in control and diabetic groups

IV.3.2 Histological and immunohistological analysis of neovascular channels

In many studies, thrombus neovascularization identified by histological and immunohistoligical methods confirmed that channels communicate with the systemic circulation (56, 61). In the current study, thrombus neovascularization was assessed by immunohistochemistry and was estimated by counting GSL-1 or αVβ3 integrin-positive channels. The correlation between αvβ3 positive channels and GSL-1staining is essentially identical (data not shown). As shown in **Figure 12a and b** in both control and diabetic mice, recanalizing channels were identified as spaces, lined by flat GSL-1 positive endothelial-like cells. These channels were identical in morphology and develop in the periphery of the thrombus as part of the normal course of resolution but varied in proportion between the two groups. Fewer channels were present in 2 weeks of diabetic mice thrombi as compared with controls despite an important immunostaining in the margin of the thrombus (**Figure 12d**). Some GSL-1 positive cells were also

detected invading the thrombus. In the 2 week-control mice, thrombus had been reduced to a longitudinal, endothelialized subintimal streak (arrow in **Figure 12c**).

Consistent with these observations was channel quantification **(Figure 13)**. At 1 week after DVT, there was no difference in the number of neovascular channels between control and diabetic mice. Control mice had 6.3 ± 0.3 channels/thrombus area (mm^2), and diabetic mice had a similar number of channels (5.6 ± 0.4 channels/thrombus area (mm^2). Of note, 2 week-diet induced type 2 diabetic mice had 3-fold fewer GSL-1 positive channels compared with controls (n=3; $P<0.05$).

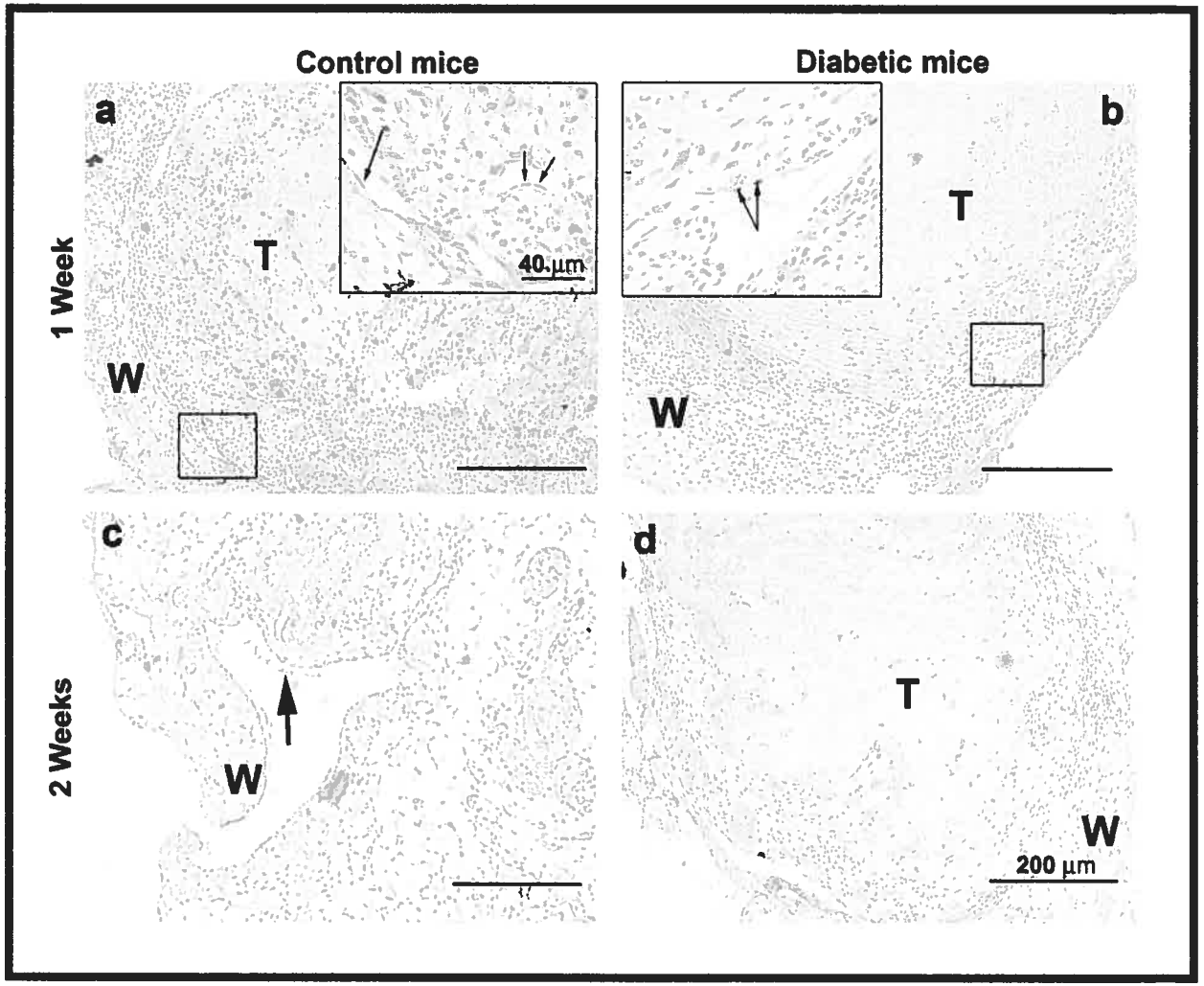


Figure 12: Neovasculization in 1 and 2 week-control and diabetic groups. Channels are surrounded by GSL-1-positive staining cells (brown staining and demarcated with black arrows). a: 1 week-control mice IVC section. b: IVC section from 1 week-diabetic mice. c: 2 week-control mice recanalized IVC section. d: A diabetic 2-week throbosed IVC section with few GSL-1 positive channels. Sections were counterstained with hematoxylin – the microphotographs are representative of results obtained from three to five sections per mouse with n=3 mice per group. Scale bar: 200 µm. Arrows in higher magnification indicate channels; W, wall and T, thrombus.

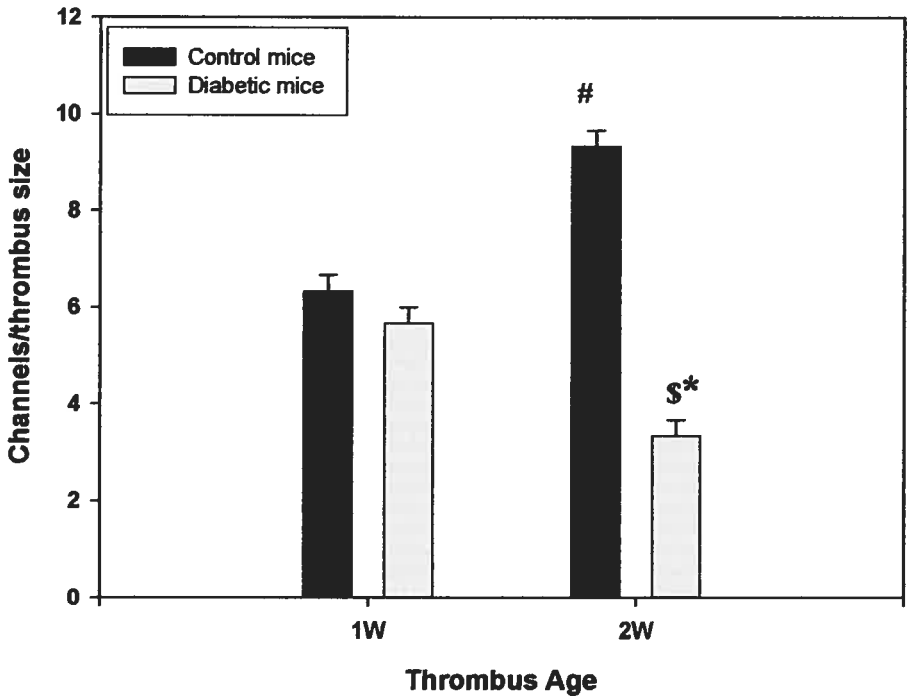


Figure 13: Quantification of thrombus neovascular channels by positive GSL-1 staining. No significant difference was observed between the number of channels in diabetic and control mice thrombi at 1 week after surgery. Note significantly fewer channels in 2 week-diabetic thrombi as compared with controls. (*P< 0.05 compared with respective control; # P < 0.05 compared within control mice $ P < 0.05 compared within diabetic mice, n=3).

IV.4 Diabetic mice have a higher inflammatory response

IV.4.1 Expression of CD14 mRNA

Thrombus macrophage content was investigated by CD14 mRNA experession at surface of macrophages. As compared with control mice, the expression of CD14 mRNA was upregulated in diabetic mice. This difference was statistically significant in 1 and 2-week-old thrombi with a 1.3 fold ($P<0.05$) and 9 fold ($P<0.05$) increase respectively. Interestingly, CD14 mRNA expression decreased by 7 fold at 2 weeks in control group ($P<0.0001$), while contrarily it showed a higher increase at 2 weeks in diabetic group. However, the difference of CD14 mRNA expression level between 1 and 2 weeks in diabetic group did not reach significance; due to the high variability of the values **(Figure 14).**

IV.4.2 Immunohistochemical staining of CD68

The inflammatory response in each thrombus cross-section was investigated by an immunohistochemical technique using a monoclonal CD68 antibody to identify macrophages. At 1 week post-ligation, venous thrombi in diet-induced type 2 diabetic animals exhibited more CD68 positive cells **(Figure 15b)** than normoglycemic mice **(Figure 15a)**. At this time point, the macrophages were mainly found within the periphery of the thrombus in both control and diabetic mice. In contrast, in 2-week-old venous thrombi, the distribution was different in diabetic mice **(Figure 15d)**; macrophage recruitment invaded the totality of the thrombus and the staining was significantly enhanced. No apparent macrophage

infiltration was observed in 2-week-old venous thrombi of control mice which were all without any significant staining **(Figure 15c)**.

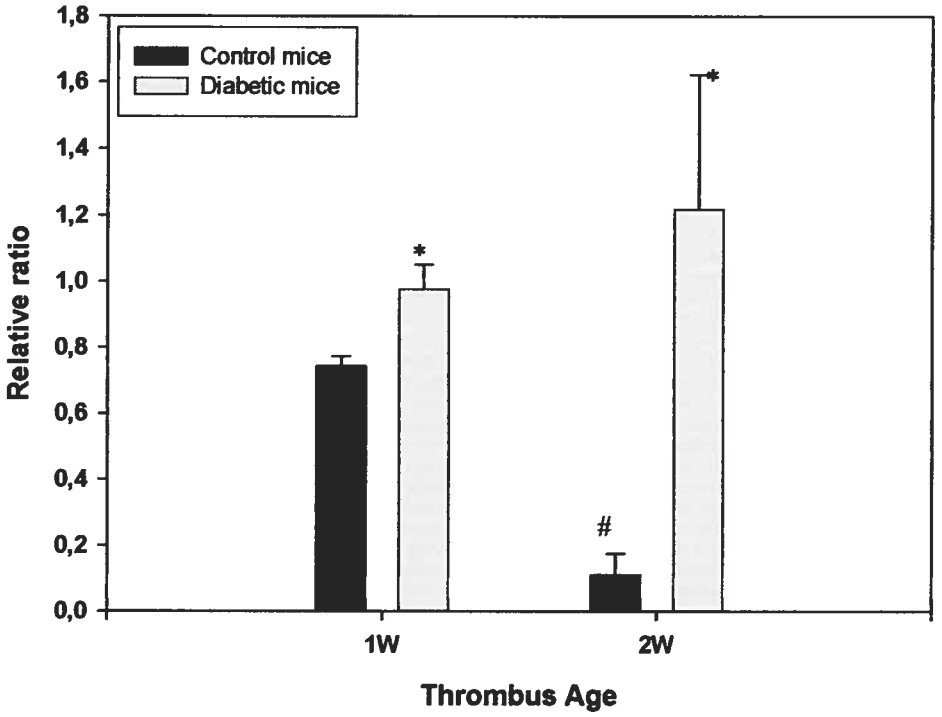


Figure 14: CD14 mRNA expression in control and diabetic mice. At 1 week, CD14 mRNA was upregulated in diabetic group compared to control group (0.977 ± 0.0741 vs 0.745 ± 0.028). At 2 weeks, CD14 mRNA was continuously upregulated in diabetic group (0.977 ± 0.0741 vs 1.217 ± 0.405), while it was downregulated in control group (0.745 ± 0.028 vs 0.105 ± 0.065). (*P<0.05 compared with respective control; # P < 0.05 compared within control mice n=3).

infiltration was observed in 2-week-old venous thrombi of control mice which were all without any significant staining **(Figure 15c)**.

The quantification of the inflammatory response in diet-induced type 2 diabetes mice as seen in **Figure 16**, showed approximately a two fold increase at 1 week as compared to control animals ($P<0.01$). At 2 week-post ligation, intrathrombus macrophages in diabetic sections were 35 times greater than in control sections ($P<0.001$).

Moreover, thrombus macrophage content decreased significantly as the thrombus ages between 1 and 2 weeks in control mice (six-fold lesser, $P<0.01$); while it amplified in 2-week-diabetic mice within the same period (three fold greater than one-week-diabetic mice, $P<0.01$).

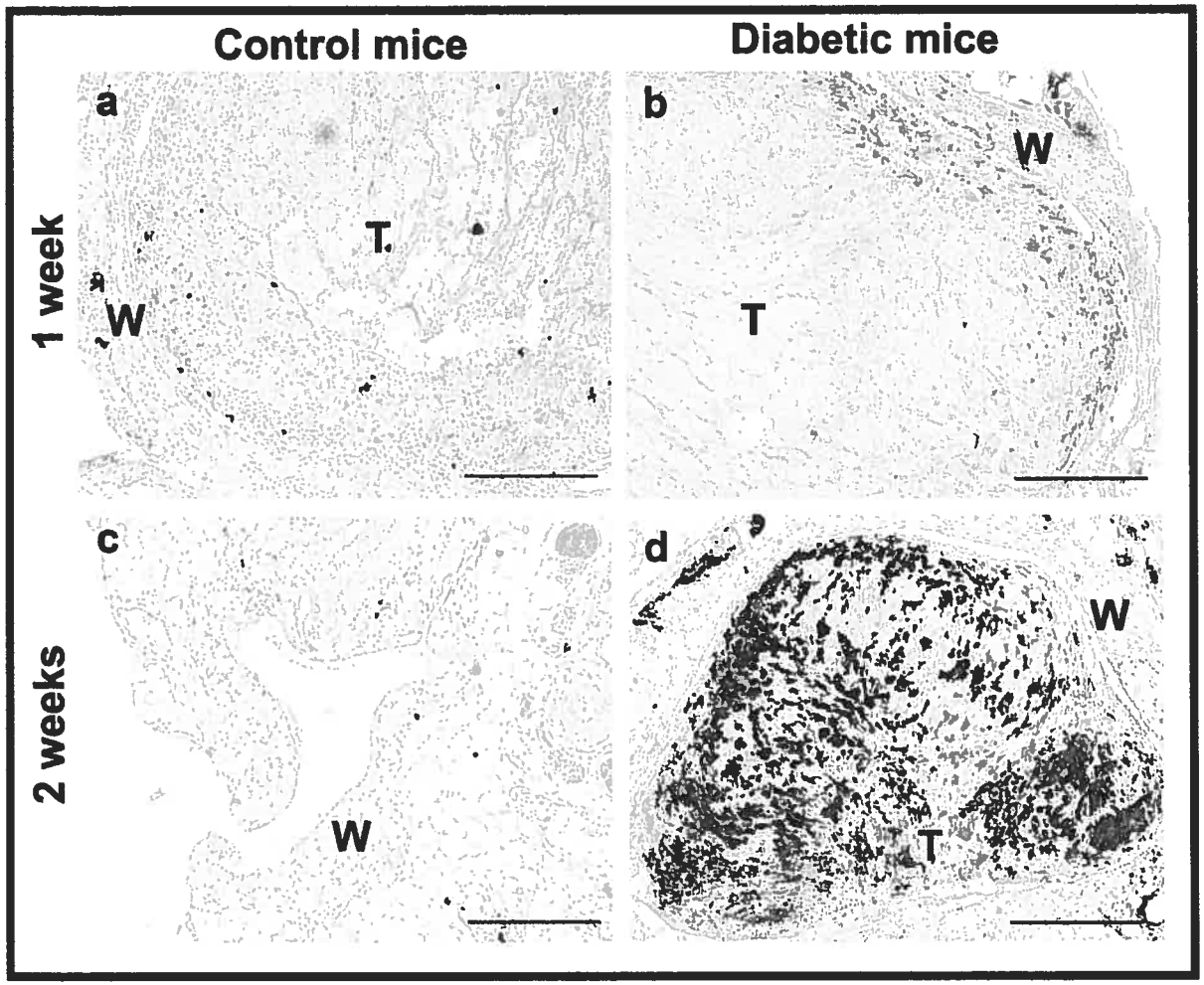


Figure 15: Macrophage content labeled by anti-CD68 antibody in the thrombus of control and diabetic mice at 1 or 2 weeks after IVC thrombosis. Note – more diffuse inflammation in the periphery of thrombus in 1-week old thrombus diabetic mice (b) than control (a) and– a strong immunostaining of CD68 was detected entirely in thrombus cross section of 2-week old thrombus in diabetic mice (d) – no significant staining was seen in 2 week-old thrombus in control mice. Sections were counterstained with hematoxylin the microphotographs are representative of results obtained from three to five sections per mouse with n=3 mice per group. Scale bar: 200 μm. W, wall and T, thrombus.

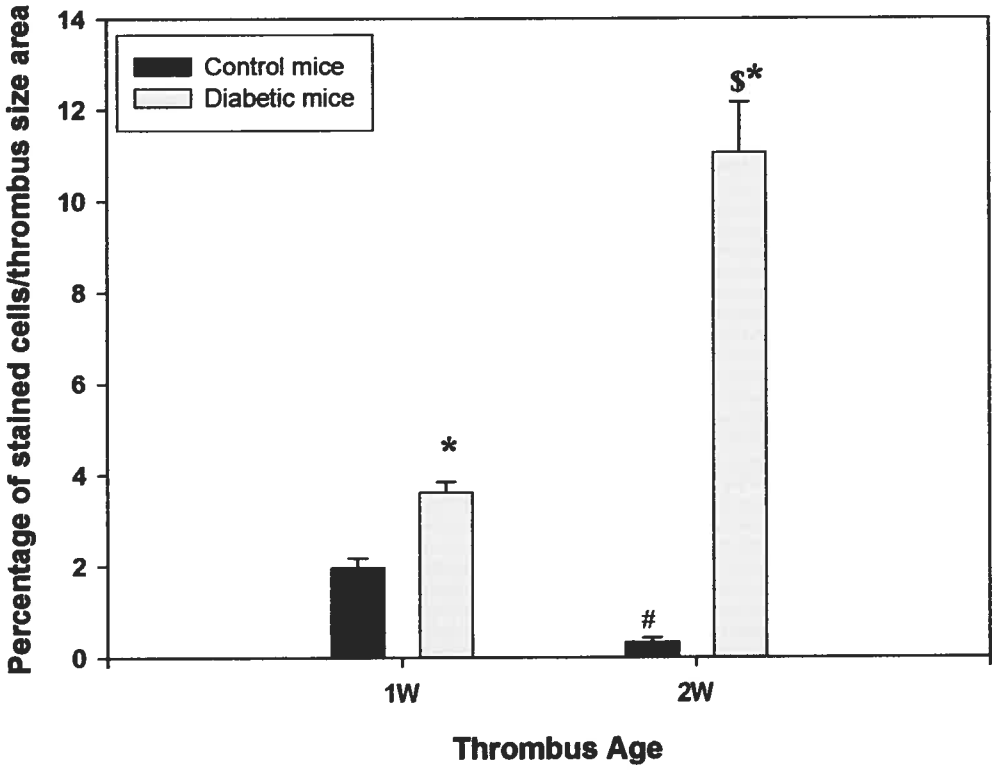


Figure 16: Thrombus macrophage content in control and diabetic mice at 1 or 2 weeks after IVC ligation. By image analysis, the percentage of CD68 stained cells per thrombus size area was evaluated for three mice in each group. (*P<0.05 compared with respective control; #P<0.05 compared within control mice $ P<0.05 compared within diabetic mice, n=3).

IV.5 Vein wall fibrosis was elevated in diabetic mice

To assess if the altered DVT resolution in the diabetic mice affected the normal thrombus fibrotic process, histochemical staining for total collagen was investigated. Picrosirius red staining showed the intensity of fibrosis to be concentrated in the thrombus periphery and in the vein wall at 1-week time point in both control and diabetic mice **(Figure 17a and 17b)**.

At 2 weeks, a strong staining was documented in both groups **(Figure 17c and 17d)**. Collagen level quantification of the vein wall was reported in **Figure 18**, and indicated at 1 week-post DVT, two-fold less collagen deposition in diabetic mice than control mice. While the fibrotic response showed a trend toward an increase in diabetic mice at two weeks, this difference did not reach statistical significance. Both groups showed an increase in collagen deposition between 1 and 2 weeks after surgery. However, the increase in control mice was 25.7% whereas it was 183% in the diet fat-fed mice, reflecting higher alteration in the vein wall remodelling.

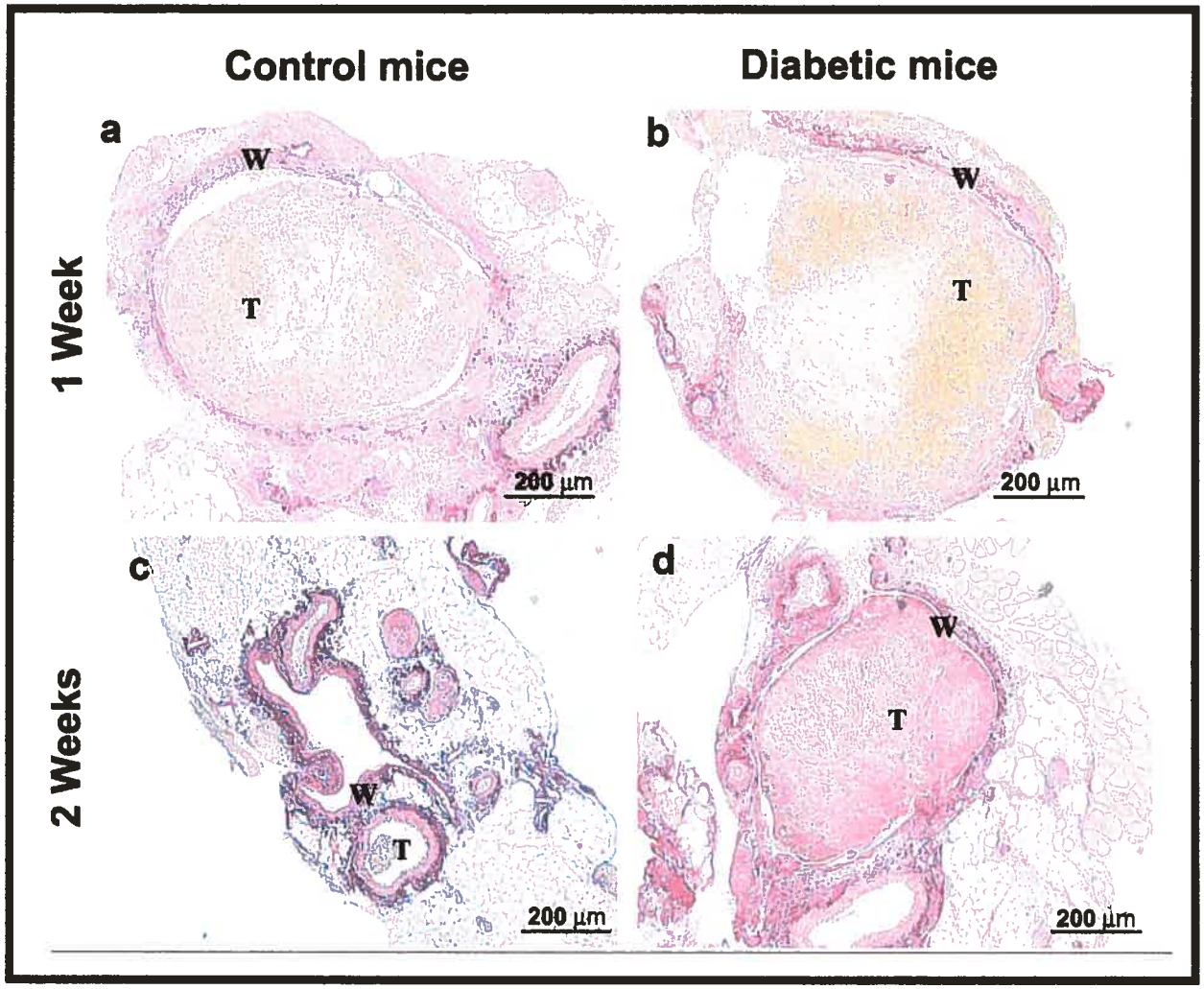


Figure 17: Picrosirius red staining of total collagen in control (a, c) and diet-induced diabetic mice (b, d) at 1 week (a, b) and 2 weeks (c, d) after surgery. Compared to control mice, much more collagen fibrils were found in the margin of the thrombus and the vein wall in the diabetic mice at 2 weeks after the surgery. Scale bar: 200 µm. W, wall and T, thrombus.

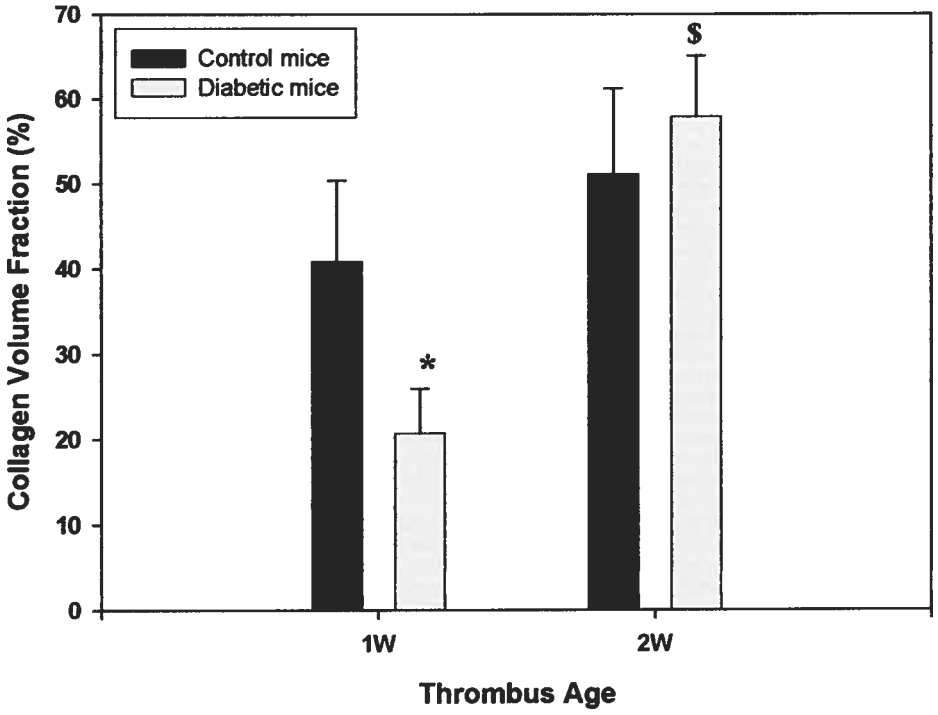


Figure 18: Collagen quantification of the vein wall in control and diabetic mice at 1 or 2 weeks. Data are derived from quantification of the intensity of staining per pixel in each IVC section and represent the mean of 15-20 fields per section, two sections per mouse, n=3 per group. (*P< 0.05 compared with respective control and $P < 0.05 compared within diabetic mice, n=3).

IV.6 The fibrinolytic system was altered in diabetic mice

As other investigators have shown, the fibrinolytic system is probably the primary mechanism for DVT resolution (83, 181, 182). Gene expression was analyzed and detection of protein expression was performed for plasminogen activators uPA and tPA, and for their main inhibitor PAI-1.

IV.6.1 uPa and PAI-1 mRNA levels

uPA mRNA levels trended to lesser expression in diabetic mice at 1 week (-21%) and 2 weeks (-12%) after thrombus induction, but these decreases did not reach statistical significance **(Figure 19)**. However, analysis of the thrombosed IVC homogenate showed a significant downregulation in uPA gene expression between 1 and 2 weeks in both groups (-57%, $P = 0.013$ in control group and –52% $P = 0.021$ in diabetic group).

PAI-1 and uPA mRNA expressions trend in opposite directions in the thrombosed IVC during DVT resolution. One week after IVC ligation, mRNA levels of PAI-1 increased in diabetic mice by 20% **(Figure 20)**. Although this did not reach statistical significance, the up-regulation was greatest (+55%) in 2-week-old thrombosed IVC (control mice 0.74 ± 0.06 vs diabetic mice 1.15 ± 0.02, $P = 0.002$, n=4).

As thrombus ages and resolves, gene expression of PAI-1 was less downregulated in diabetic mice (-14%, $P = 0.023$) than in control mice (-34%, $P = 0.061$) reflecting less fibrinolytic activity leading to reduced thrombus resolution with diet-induced type 2 diabetes.

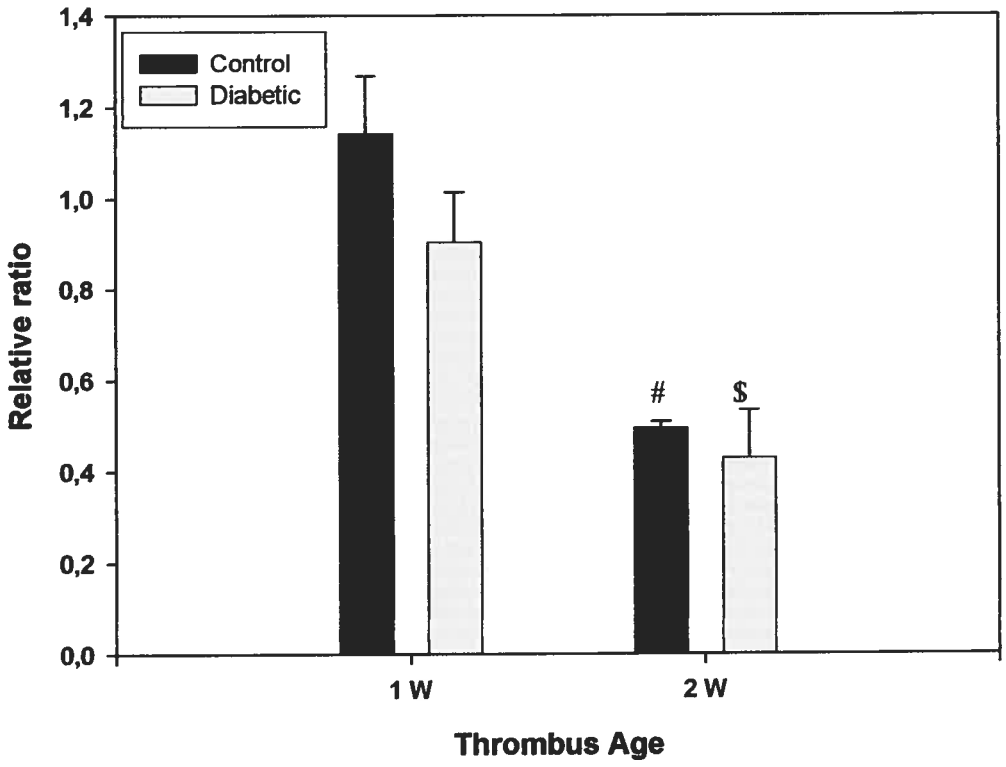


Figure 19: Expression of uPa mRNA in control and diabetic mice. uPA mRNA levels normalized with β-actin mRNA levels showed a twofold decrease at 1 and 2 week after surgery in both control and diabetic mice. (#P<0.05 compared within control mice $P < 0.05 compared within diabetic mice, n=4).

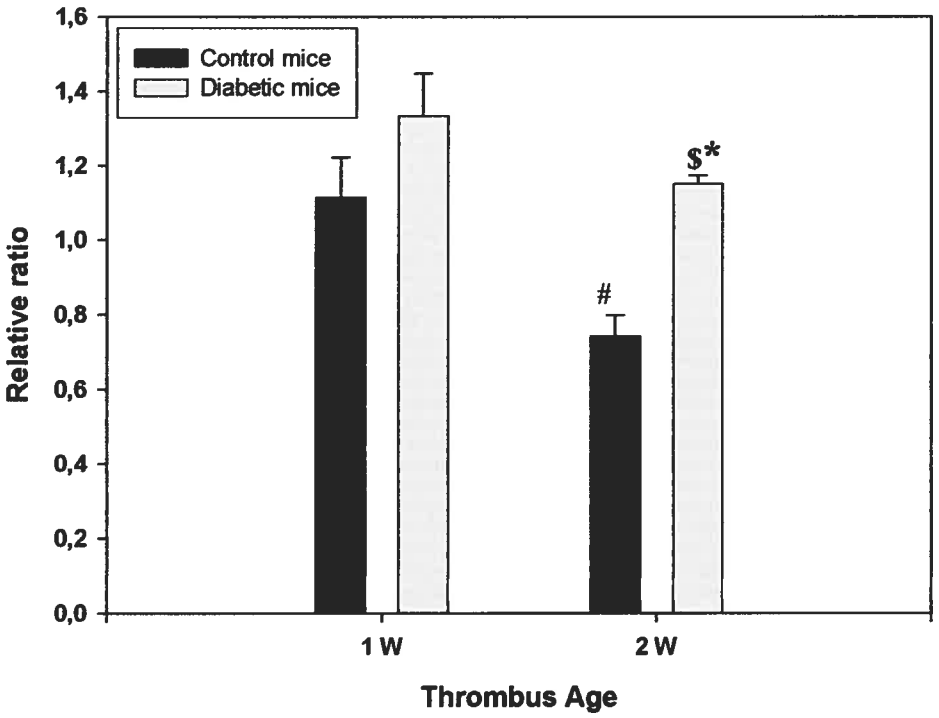


Figure 20: **Expression of PAI-1 mRNA in control and diabetic mice**. PAI-1 mRNA expression showed higher levels in diabetic IVC/thrombus samples than in controls. A significant downregulation followed as the thrombus matured in both groups (-14% vs -34%) (*$P<0.05$: compared with respective control; #P<0.05 compared within control mice and $P<0.05 compared within diabetic mice, n=4).

IV.6.2 Expression of uPa, tPa and PAI-1 protein (Western blot analysis)

At 1 week, there was no significant difference of uPa protein expression between control and diabetic groups (P=0.098) **(Figure 21)**. At 2 weeks, uPa protein expression was down-regulated compared to control (18% less than control P=0.0098). In both groups, uPa expression was decreased with time by 30% at 2 weeks when compared to 1 week (P<0.05).

The expression of tPa in diabetic mice was down-regulated compared to the control either at 1 week (P=0.0269) or at 2 weeks (P=0.0369). The tPa level at 2 weeks was lower than that at 1 week in both control and diabetic groups but this decrease did not reach statistical significance **(Figure 22)**.

In contrast, the expression of PAI-1 protein was up-regulated in diabetic mice compared to the control either at 1 week (P=0.0033) or at 2 weeks (P=0.0466). However, as the thrombus ages, there was no significant difference between the PAI-1 level at 1 week or at 2 weeks in diabetic group **(Figure 23)**

Serial thrombosed IVC sections from control and diabetic mice were immunostained with an antibody against uPa and PAI-1 **(Figure 24)**. In organized thrombus, uPa and PAI-1 were mainly found in the ingrowing subendothelium. uPa was accumulated in the subendothelium under microthrombi and was strongly positive in control group at 1 week **(Figure 24a)** and 2 weeks **(Figure 24c),** as compared with DM group **(Figure 24b and 24d).** PAI-1 was slightly stronger positive in DM group at 1, 2 weeks, as compared with control group..

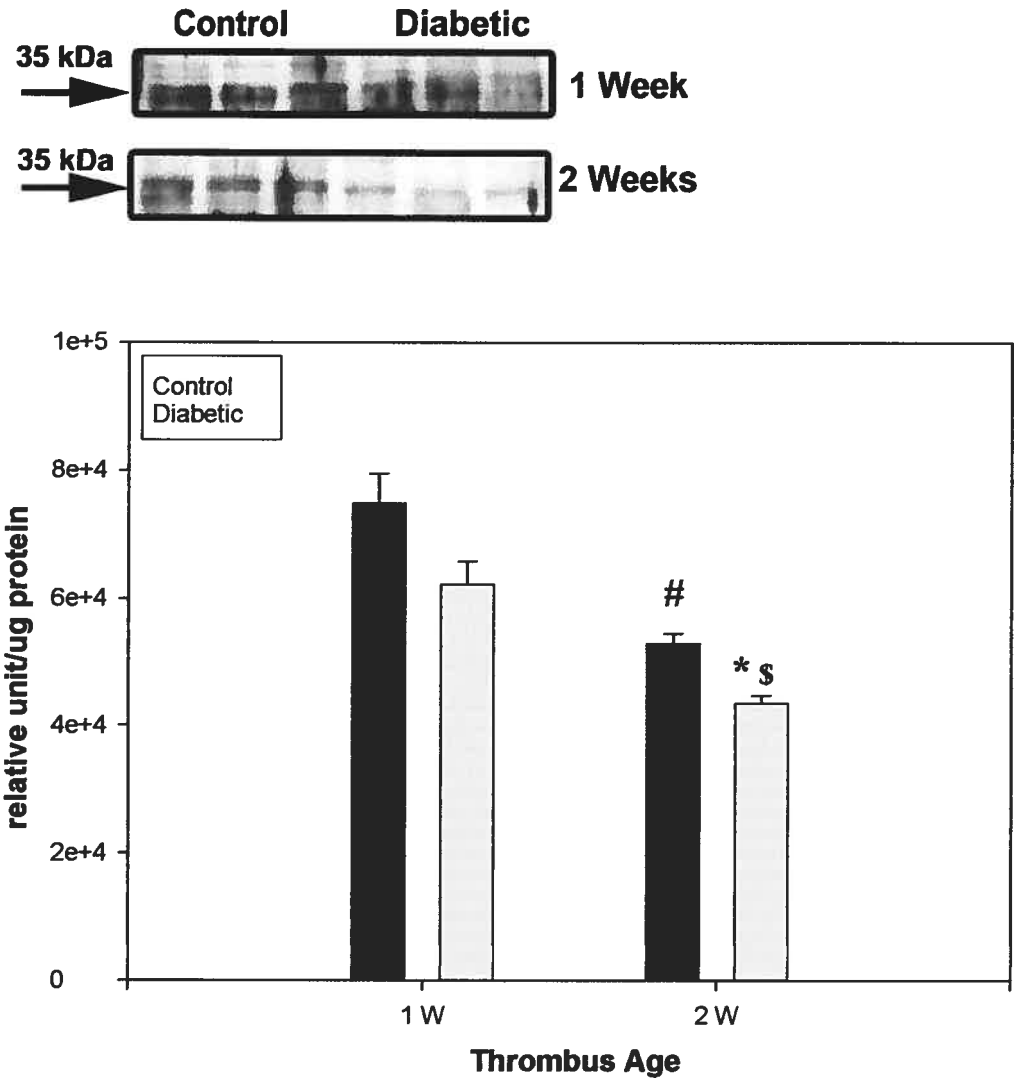
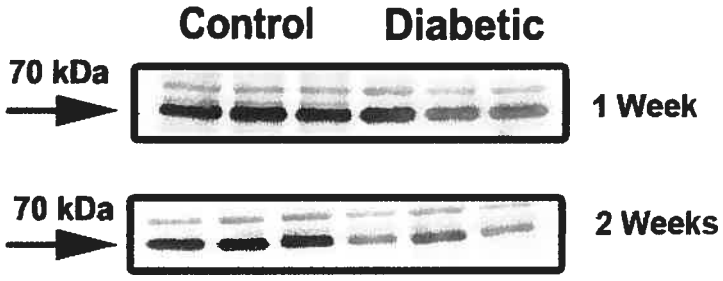


Figure 21: Western blot analysis of uPa in control and diabetic protein extracts. At 2 weeks, uPa protein expression in diabetic mice was statistically different from control group. (*P< 0.05 compared with respective control; #P<0.05 compared within control mice $P<0.05 compared within diabetic mice, n=3).



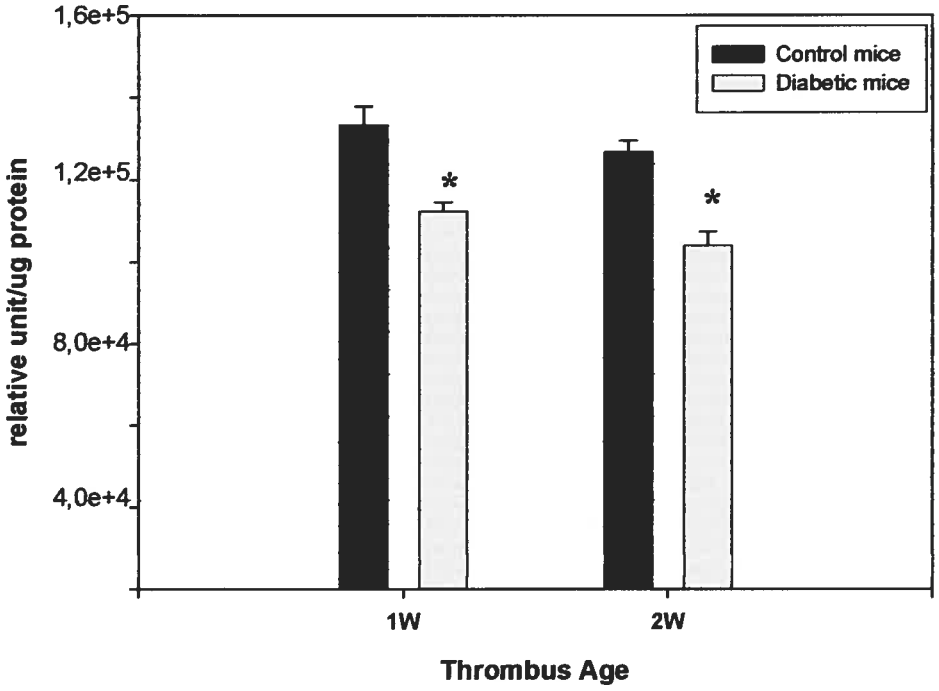
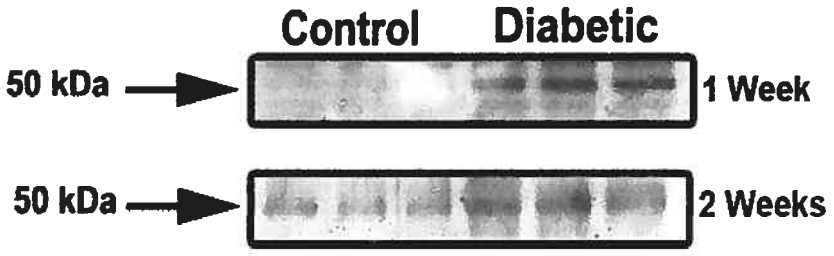


Figure 22: Western blot analysis of tPa in protein extracts of control and diabetic mice. The expression of tPa in diabetic mice was down-regulated compared to the control either at 1 week (-19% P=0.0269) or at 2 weeks (-21% P=0.0369). (*P<0.05: vs control; n=3).



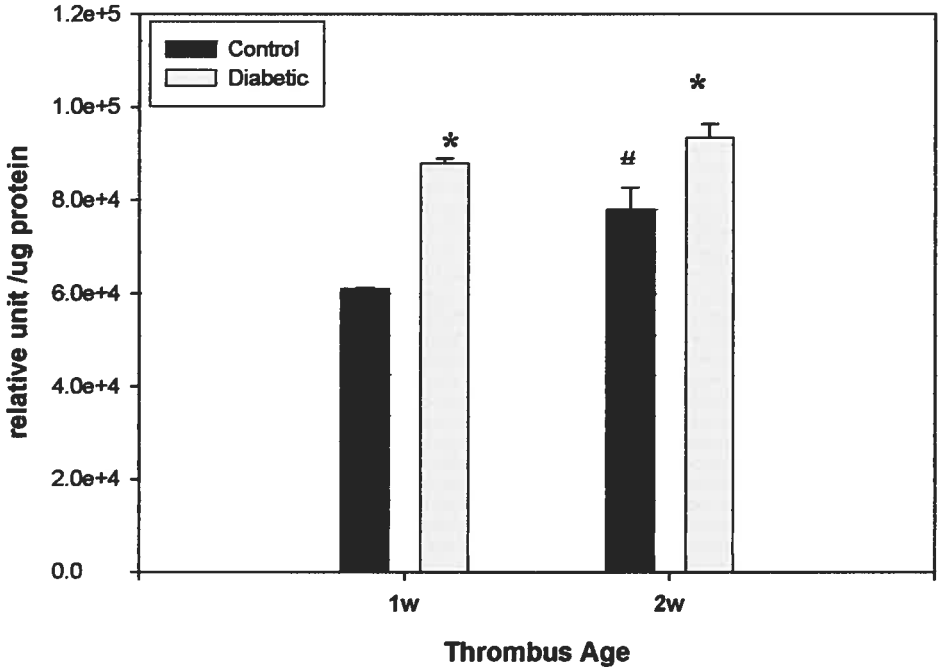


Figure 23: Western blot analysis of PAI-1 in protein extracts of control and diabetic mice. The level of PAI-1 protein expression is 1.5 and 1.2-fold higher in diabetic mice than their respective controls. (*P<0.05: vs control; # P<0.05: vs 1-week control mice, n=3)

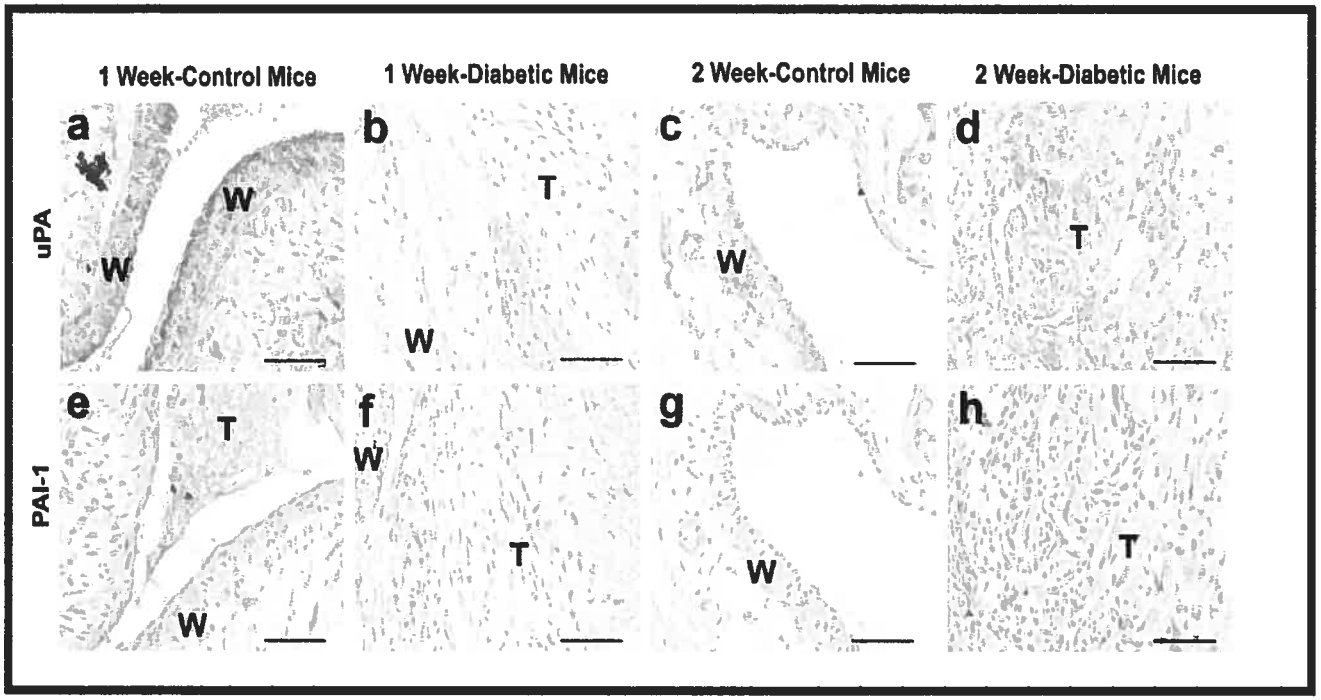


Figure 24: Changes in uPa and PAI-1 immunoreactivity after 1 and 2-week DVT in control and diabetic mice. Diabetic mice at 1 week (b) and 2 weeks (d) showed less uPa labeling in vein wall (W) and thrombus section (T) compared to control mice at 1 week (a) and 2 weeks (c). In contrast, a stronger immunostaining of PAI-1 was detected in diabetic mice at 1 (f) and 2 weeks (h)-DVT compared to control mice (e and g). Tissue slices were counterstained with hematoxylin. The microphotographs are representative of results obtained from three to five sections per mouse with n = 3 mice per group. Scale bar: 50μm.

IV.7 The MMP system is enhanced in Diabetic mice followed by a decreased synthesis and activity

IV.7.1 Expression of MMP-2 and MMP-9 mRNA

Compared to the control mice, the MMP-2 mRNA levels of diabetic mice were up-regulated at 1 week (P=0.018) but down-regulated at 2 weeks (P=0.002) **(Figure 25)**. The mRNA level was increased at 2 weeks in the control group (P=0.0014), and decreased in the diabetic group (P=0.0021).

The MMP-9 mRNA expression of diabetic mice was up-regulated at 1 week (2.36-fold increase compared to control group, P = 0.028) **(Figure 26)**, while decreased to the control level at 2 weeks. The difference between the mRNA level at 2 weeks and at 1 week in diabetic mice was statistically significant (P = 0.013), whereas the control group showed an upregulation of MMP-9 mRNA level by 2 weeks after IVC ligation.

IV.7.2 Gelatinolytic activities of MMP-2 and MMP-9

1 week- MMP-2 total gelatinolytic activity in diabetic mice was 1.7-fold greater than in control mice (P<0.05) and became 1.2-fold increased by 2 weeks (P<0.05) **(Figure 27)**. As thrombus matured, MMP-2 activity decreased by 20% in diabetic mice contrary to control mice where the MMP-2 activity augmented by 12.5% (P<0.05). Similarly, MMP-9 activity was greater in diabetic thrombi 1.5-fold at 1 week and 1.3-fold at 2 weeks as compared with controls (P<0.05). However the decrease by 2 weeks was not statistically significant in diabetic mice.

IV.7.3 Expression of MMP-2, MMP-9 protein

Densitometric analysis of MMP-2 and MMP-9 levels by Western immunoblotting showed significantly increased levels in the thrombosed vein homogenate at 1 and 2 weeks in diabetic mice as compared with controls (**Figure 28 and 29**).

Two-fold greater protein level of MMP-2 and 1.7 fold greater protein level of MMP-9 were present in the diabetic ligated vein at 1 week, as compared with control thrombi ($P<0.05$). By 2 weeks, less MMP-2 (-30%) and MMP-9 (-18%) activities were present in the diabetic groups ($P<0.05$) although, still remaining more elevated than controls groups.

Thrombosed IVC sections from control and diabetic mice were immunostained with an antibody against MMP-2 and MMP-9 **(Figure 30).** Staining was present in both the vein wall and cellular components of the thrombus. Both at 1 week and 2 week-DVT, the diabetic mice showed more MMP-2 and MMP-9 staining compared to control mice. Two weeks after surgery, the immunoreactivity of both MMP-2 and MMP-9 in diabetic mice was less pronounced as compared to the immunostaining 1 week after DVT.

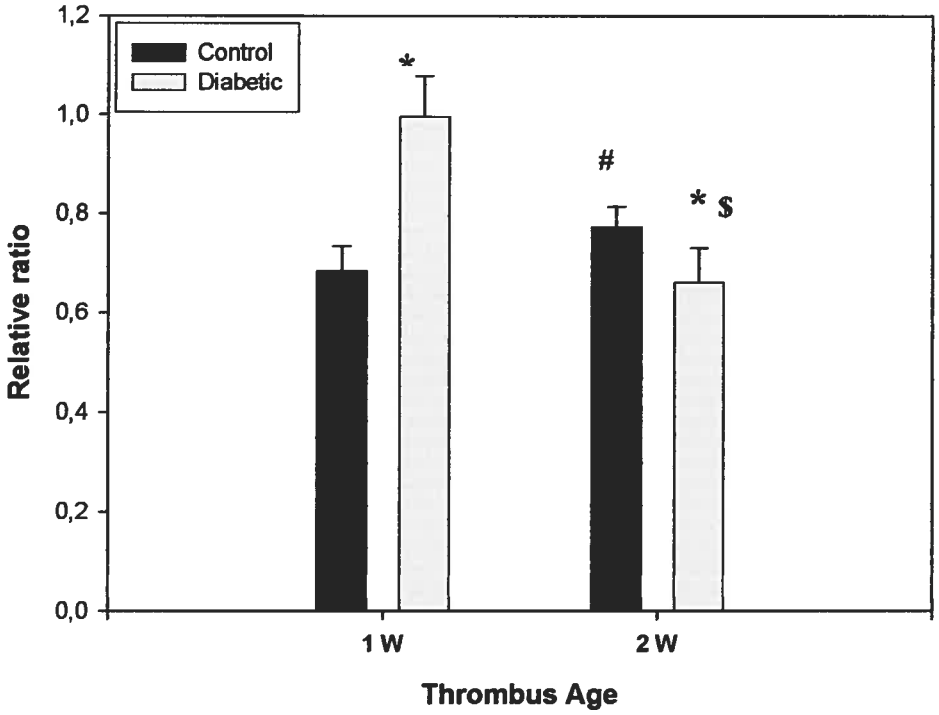


Figure 25: Expression of MMP-2 mRNA in control and diabetic mice. Diabetic mice showed an upregulation of MMP-2 mRNA levels by 1 week whereas, a dowregulation occurred by 2 weeks after thrombus induction (*P<0.05: vs control; \$$P$<0.05: vs 1-week diabetic mice, #P<0.05: vs 1-week control mice, n=3).

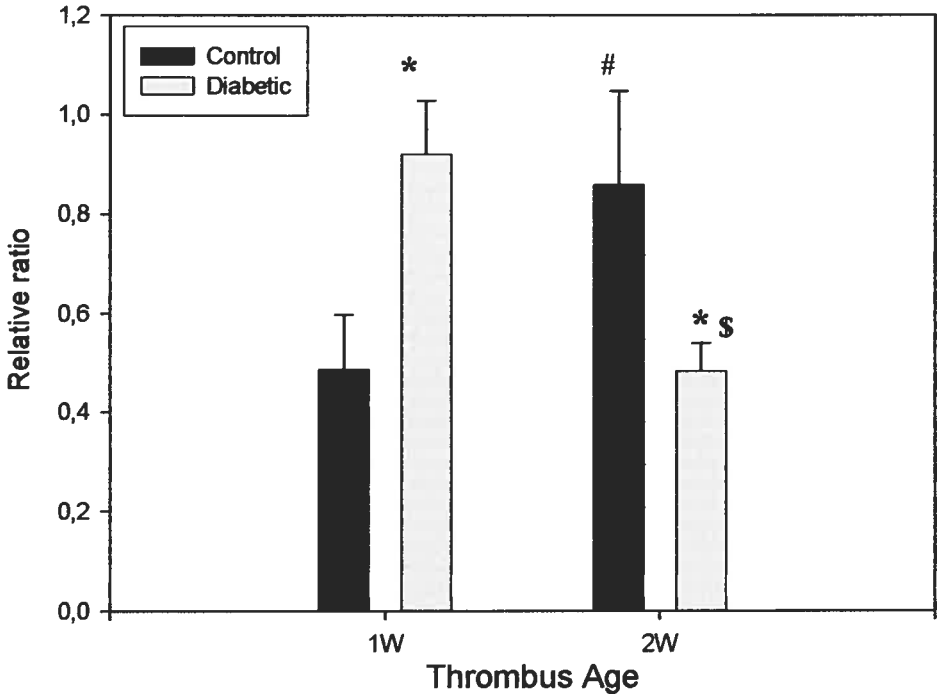


Figure 26: Expression of MMP-9 mRNA in control and diabetic mice. An upregulation of MMP-9 mRNA level was detected by 1 week after IVC ligation followed by a downregulation by 2 weeks in diabetic mice, in opposite, control group showed a higher level by 2 weeks. (**P*<0.05: vs control; $*P*<0.05: vs 1-week diabetic mice, # *P*<0.05: vs 1-week control mice, n=3).

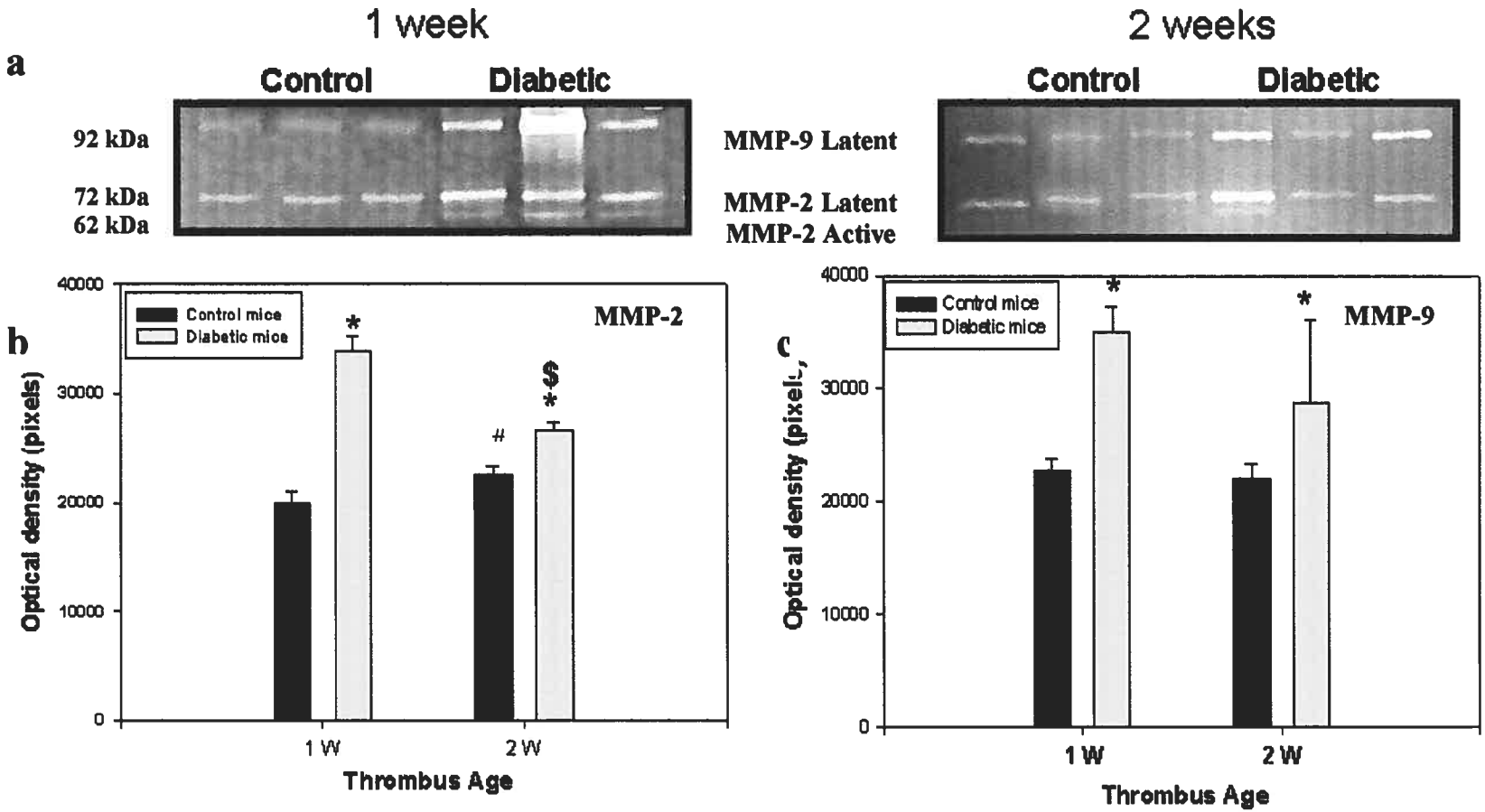


Figure 27: Thrombosed IVC MMP-2 and MMP-9 activities. a: Representative zymogram showing most activity in the latent form in 1- and 2- week control and diabetic mice. b and c: Densitometric evaluation of 72-kDa MMP-2 and 92-kDa MMP-9 activity in control and diabetic mice. (*P<0.05 compared with respective control; #P<0.05 compared within control mice; $P<0.05 compared within diabetic mice, n=3).

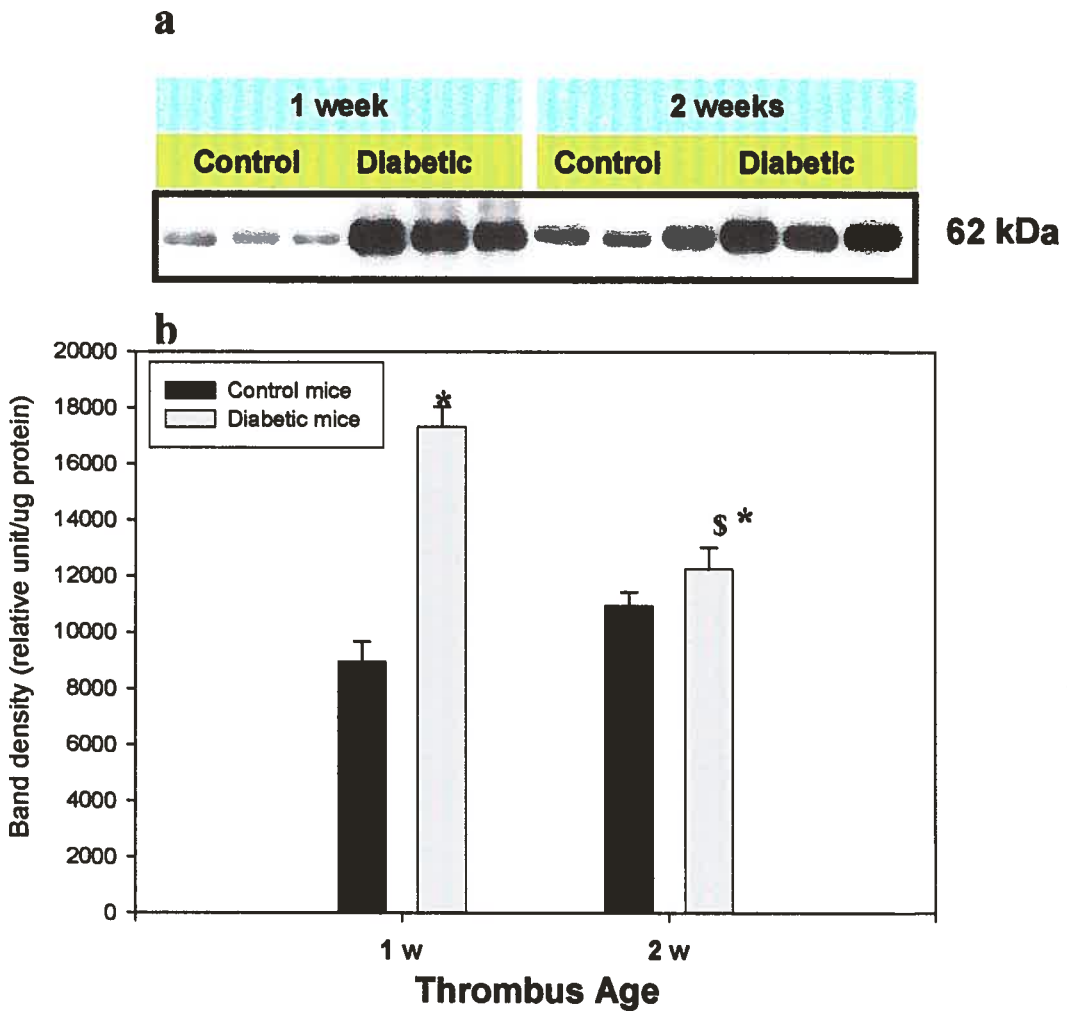


Figure 28: Expression of MMP-2 in control and diabetic thrombosed IVC.

A: representative immunoblot demonstrating increased MMP-2 (62 kDa) levels in the diabetic tissue at 1 and 2 weeks after thrombus induction as compared to control. B: densitometric analysis of immunoreactive bands further indicates that MMP-2 proteins are decreased in diabetic group after 2 weeks post DVT. (*$P<0.05$: compared with respective control, $P< 0.05 compared within diabetic mice, n=3)

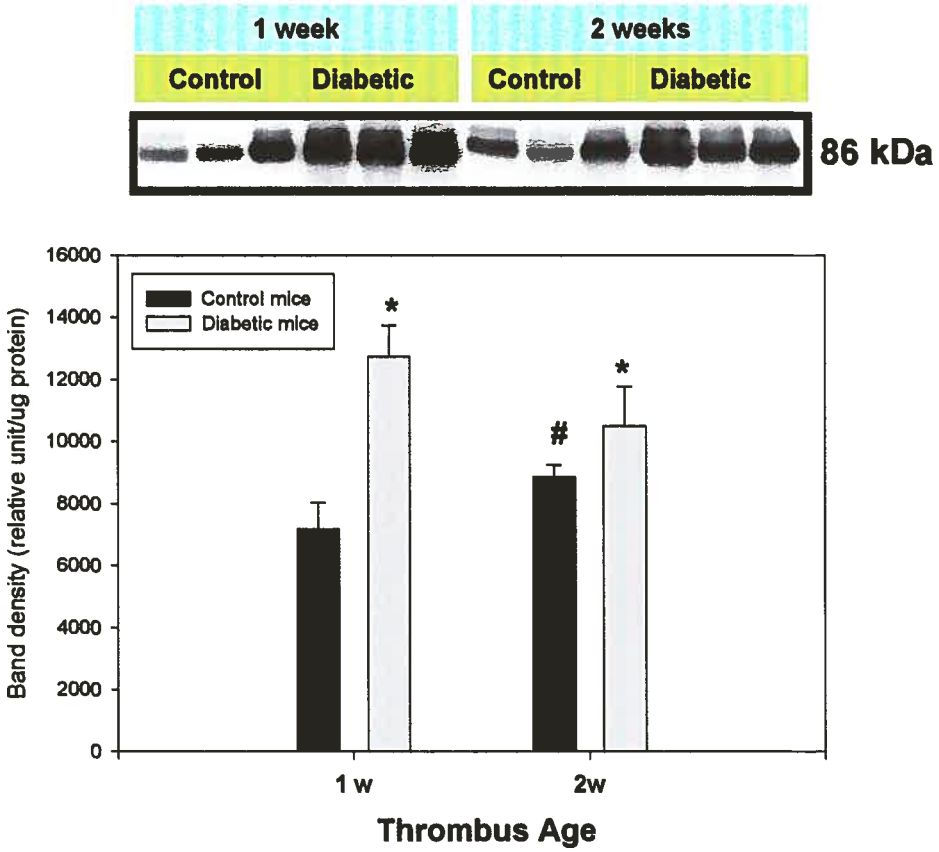


Figure 29: Expression of MMP-9 in control and diabetic thrombosed IVC.

A: representative immunoblot demonstrating increased MMP-9 (86 kDa) levels in the diabetic tissue at 1 and 2 weeks after thrombus inductuion as compared to control. B: densitometric analysis of immunoreactive bands further indicates that MMP-9 proteins are decreased in diabetic group after 2 weeks post DVT. (**P*<0.05: compared with respective control, # P<0.05 compared within control mice, n=3).

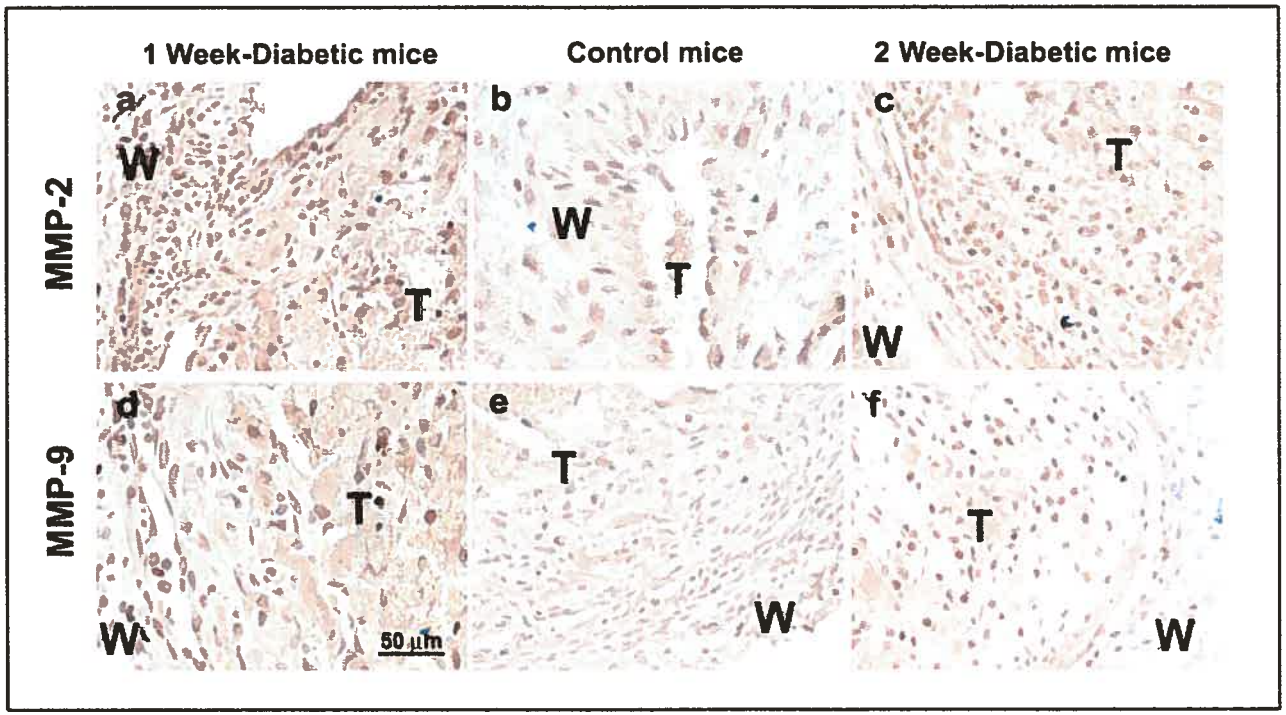


Figure 30: Changes in MMP-2 and MMP-9 immunoreactivity after 1 and 2-week DVT in control and diabetic mice. Diabetic mice at 1 week (b) and 2 weeks (d) showed more MMP-2 immunostaining in vein wall (W) and thrombus section (T) than control mice (c). Note a reduced expression of MMP-2 was detected in 2-week diabetic mice compared with 1-week diabetic thrombosed IVC section. Similarly, MMP-9 expression was greater in diabetic mice compared to controls with diminished expression at 2 weeks after DVT. Tissue slices were counterstained with hematoxylin. The microphotographs are representative of results obtained from three to five sections per mouse with n=3 mice per group. Scale bar: 50µm.

V. DISCUSSION

In the first part of the thesis, molecular and cellular mechanisms associated with venous thrombus organization and resolution in normoglycemic mice and those related to the arterial complications of type 2 diabetes were reviewed. However, arterial and venous thromboses are separate pathological disorders, different in their pathology, pathophysiology, epidemiology and treatments. Arterial thrombosis could be considered as a chronic disease related to a slowly increasing severity of atherosclerosis, although its symptoms are usually acute due to blocking of the vital blood flow to an organ. In contrast, venous thrombosis is an acute disorder with chronic symptoms and occurs from a sudden clot. Arterial and venous thrombosis share and differ vastly in some etiologies. For example, the atherogenic factors for arterial thrombosis such as smoking, hypertension and hyperlipidemia do not appear to affect the risk of venous thrombosis (183). Therefore, the current study associates these two pathologies, DVT and diet-induced type 2 diabetes, to determine whether there is an adverse hemostatic impact of type 2 diabetes on the formation and resolution of DVT in mice.

In addition to promoting obesity, high-fat diets have been associated with an increased risk for developing type 2 DM in epidemiological studies, and notably this association has been found independent of obesity (43).

Several murine models of type 2 diabetes to study vascular complications have been reported. One animal model that is particularly susceptible to the effects of dietary fat is the C57BL/6J (B6) mouse. This animal will develop severe obesity, hyperglycemia, hyperinsulinemia, insulin resistance and endothelial dysfunction (36) when weaned to high-fat diets, but will remain lean and euglycemic if the fat

content of the diet is limited (184). As a model of type 2 diabetes, B6 mice seem to closely resemble common forms of the human disease in that they will only manifest the disease after developing obesity and in which the disease results from the interaction between environmental factors and genetic predisposition.

Animal models of stasis-induced DVT after ligation of the inferior vena cava (IVC) have been widely used to investigate the mechanisms responsible for thrombus resolution (61, 131 and 181). Our mouse model design is inspired by combination of reduced flow and endothelial disturbance, two components of Virchow's Triad. The venous thrombi produced in this model have a laminar structure and were morphologically similar to human thrombi (56, 61).

Although type 2 diabetes has been long demonstrated as a high risk factor of thrombosis, to the author's knowledge, no study has shown its role in the formation and evolution of the deep vein thrombus (DVT).

DVT resolution involves dissolution of the thrombus matrix by fibrinolysis, cellular influx and neovascularization. Our current data (results of western blot were normalized by per ug of protein) show that all these parameters are affected in diabetic mice, revealing that DVT resolution is impaired and might suggest that the metabolic disorders, such hyperglycemia, insulin resistance, dyslipidemia and advanced glycation end product might be implicated in the pathogenesis of DVT.

The data herein support several mechanisms associated with type 2 diabetes that may directly affect thrombus resolution. Five interrelated conclusions can be drawn from the data where diet induced type 2 diabetes is associated with: 1) decreased venous thrombus resolution and neovascularization; 2) a higher inflammatory response in the thrombosed IVC, 3) a more pronounced vein wall

remodeling; 4) an altered fibrinolytic balance with a trend towards an antifibrinolytic state; and 5) an upregulation followed by a dowregulation in the expression profile of the MMP system. These findings may present plausible molecular mechanisms for impaired venous thrombi resolution which could contribute to more risk of post-phlebitic syndrome in type 2 diabetes.

V.1 Type 2 diabetes decreases the resolution and recanalization of DVT

Venous thrombi resolution and recanalization involves thrombus retraction and neovascularization. Retraction results in the formation of cell-lined clefts between the body of the thrombus and intima of the vein wall. This is combined with new vascular channels within the thrombus as a result of endothelial cell proliferation and migration. Therefore similar to angiogenesis, thrombus recanalization is affected by the same mechanisms that regulate new vessel formation.

Our diet-induced diabetic mice showed a significant reduction in venous thrombi resolution and recanalization as evidenced by a larger thrombus size and content, by unfavorable angiographic evolution and, by a reduced number of neovascular channels identified and quantified through the use of immunohistochemistry.

Diabetes is associated with abnormal angiogenesis. The insufficient angiogenesis contributes to impaired wound healing, and impaired new vessel growth development (185). Diabetes-induced impairment of collateral formation has been demonstrated in animal models. Hindlimb ischemia created by ligation of the femoral artery is associated with a reduced formation of capillaries and a reduction in blood flow to the ischemic limb in diabetic (NOD) versus non-diabetic (C57) mice (186). A possible mechanism by which diabetics are

affected in their ability to forms collaterals was reported by the study of Tepper et al., which suggests that type 2 diabetes may alter circulating endothelial progenitor cells (EPCs) biology exhibiting impaired proliferation, adhesion and incorporation into vascular structures. Interestingly, EPCs are recruited into venous thrombi and may play an important part in the resolution of thrombi (71). These findings may suggest a similar alteration in EPC functions during DVT resolution in diabetic mice.

In addition, the abnormal metabolic state of type 2 diabetes increases adhesion of platelets and monocytes and results in abnormal fibrinolysis and hypercoagulation. Activated platelets interact with the endothelium and promote adhesion of platelets to monocytes. Circulating platelet-monocyte aggregates may release procoagulant, oxidative and mitogenic factors (24). The equilibrium between endogenous tissue plasminogen activator and PAI-1 is altered. All these significantly contribute to the inflammatory and procoagulant response in diabetes.

V.2 Type 2 diabetes increased inflammatory response in DVT

Diabetes associated vascular disease affects multiple vascular beds. There is a significant inflammatory component with activation of the transcription factors such as nuclear factor-κB and activator protein 1, increased endothelial and leukocyte expression of adhesion molecules and release of chemokines that attract monocytes and inflammatory cytokines such as IL-1, TNF-α (187).

The interrelation between venous thrombosis and inflammation has been well characterized. Previous studies have shown that monocytes are recruited in large

numbers into maturing human and experimental venous thrombi (62). Moreover, a role for the thrombus in directing the inflammatory reaction has been suggested and thrombus resolution did not occur if their recruitment is restricted (60-62).

Our data showed markedly upregulated CD14 gene expression in thrombosed IVC from diabetic mice and intense macrophage invasion initially around thrombus edge which became entirely distributed within the thrombus. This temporal and spatial pattern of monocyte movement was reported previously in non diabetic mice by McGuinness et al. (151).

The concept that a proinflammatory environment is crucial for venous thrombi resolution has been documented in many laboratories (128, 164). It is possible as it has been shown in normoglycemic mice, that monocytes/macrophages orchestrate thrombus resolution by producing a variety of cytokines, chemotactic factors, angiogenic factors, proteases and their inhibitors that regulate cell migration, ECM turnover and revascularisation. However, in diabetic mice this increasing macrophage recruitment into the thrombus did not improve its resolution. It is likely that once monocytes convert to macrophage phenotype in the thrombus, their fibrinolytic activity decreases (down-regulation of uPA/tPA and up-regulation of PAI-1) which will impair lysis of thrombus from within.

Thus, it is reasonable to speculate that a mechanism which is macrophage dependent may contribute to altered DVT resolution in diabetic mice and these inflammatory cells may be prothrombotic as shown in many circumstances via the production of tissue factor (188), PAI-1 (189) and thrombin activation (190).

V.3 Type 2 diabetes elevates the vein wall fibrosis in DVT

ECM displays a very dynamic equilibrium with constant synthesis, degradation and reorganization. Vascular ECM proteins such as collagen 1 and 3, fibronectin and thrombospondins not only function as scaffolding proteins but are also involved in matrix signaling by interacting with the integrin family of protein and trigger growth-promoting signals (191).

Many studies have provided evidence for diabetes–induced alteration in ECM turnover and regulation. There is a particular impact on the ECM component, the basement membrane whose thickening is an ultrastructural hallmark in diabetic patients (192). In veins from human diabetic patients and an experimental diabetic animal model, patches of thickening were observed and could be related to endothelial cell dysfunction (19).

Prior study has suggested that a late fibrosis that occurs in vein walls after the development of a DVT is the result of a loss of the dynamic equilibrium. This involves the progression of vein walls from normally thin and compliant to a relatively thick and fibriotic state, with the deposition and accumulation of collagen and the loss of normal vessel ECM (194). In this study, after two-week-DVT, the elevated vein wall fibrosis was found in both control and DM; however, the increase collagen deposition in diabetic mice within 1 and 2W, is by far more important than in CM, which indicated a higher alteration in the vein wall remodeling. Thus it is likely that type 2 diabetes contribute to vein wall damage with further enhancement of thrombus collagen deposition, which thickens the venous wall. This is known to compromise valvular function and leads to post thrombotic venous insufficiency (193).

V.4 Type 2 diabetes alters the fibrinolytic and MMPs system in DVT

Fibrinolytic systemic activity is known to be diminished in diabetic and insulin-resistant human subjects, which might be attributable to several mechanisms, including PAI-1 production (16). Hyperglycemia directly or indirectly (eg, via oxidative stress or advanced glycation products) increases MMP expression and activity (194).

DVT resolution is known to be an inflammatory process and involves fibrinolytic and MMP systems through a series of cellular and molecular events. Previous studies have shown that the activities of the fibrinolytic system are altered and those of MMPs (MMP-2, MMP-9) are markedly increased (60, 95) after formation of venous thrombi.

V.4.1 Type 2 diabetes inhibits the fibrinolytic system

Prior experimental studies suggest the abnormality of the fibrinolytic system is one of the major etiologic factors in DVT (195, 196). The plasmin system, by activation of uPA, is a primary mechanism of venous thrombolysis (164). Urokinase, with its dominant role in cell invasion, is thought to be an important mediator of pericellular proteolysis, whereas, tPA is responsible for the dissolution of fibrin from the vascular tree. Both are inhibited by PAI-1. In normoglycemic conditions, fibrinolytic activity is enhanced by upregulation of expression and activities of uPA and tPA after DVT (95, 197), but is impaired in arterial vessels in type 2 diabetes (7, 198).

Our data show that diet-induced type 2 diabetic mice is associated with a decrease in uPA and tPA expressions and an increase in their PAI-1 expression at the gene and protein levels in the thrombosed IVC. These variations demonstrate an alteration in the fibrinolytic state in the venous bed with a trend towards an anti-fibrinolytic state. Increased PAI-1 production and reduced uPA and tPA levels seemed to be an important contributor to the development of venous thrombosis and the failure of resolution and recanalization in diabetic mice as evidenced from impaired venous thrombus resolution in uPA-/- knockout mice (164).

In addition to fibrin degradation, the fibrinolytic system also plays a role in other biological processes including angiogenesis. One of the functions of plasmin in the vascular wall is the activation of MMPs.

V.4.2 Type 2 diabetes enhances MMPs system

The MMP family is essential for cell migration, matrix remodeling, and angiogenesis. Their proteolytic activity must be precisely regulated by TIMPs through complexes of the catalytic MMP domains with various TIMPs (7, 104). In concert, MMPs are able to degrade a wide spectrum of matrix proteins and therefore, they are considered to be the primary class of proteases involved in degradation of the endothelial basement membrane and interstitial matrix degradation. Quiescent ECs produce little or no MMPs, whereas the expression of several MMPs is strongly up-regulated in activated ECs in vitro and in endothelium of vessels in wound healing, inflammation and tumors (199). MMPs stimulate angiogenesis primarly by matrix degradation and also may include the

activation of growth factors and cytokines, the recruitment of EPCs and the degradation of inhibitors.

In Type 2 diabetes, it is well documented that gene expression and activity of MMPs are altered in arterial vessel wall. However, there are still controversies regarding the effects of type 2 diabetes on vascular MMPs. These changes result in an imbalance in vascular matrix homeostasis and contribute to damage of a gel-like form and scaffolding structure. Some of studies have shown increased mRNA levels and activity of MMPs, independent of the presence or absence of tPA or uPA in type 2 diabetes (191, 200).

The expression of the principal collagenases (MMP-2 and MMP-9) is increased in the DVT model (7, 57, 60). Circumstantial evidence has suggested an important role of MMPs systems in molecular pathway involved in the resolution of the thrombus (7, 57).

Our data showed that mRNA expression and activity of MMP-2 and MMP-9 significantly increased at 1 and 2 week-post DVT compared to normoglycemic mice. However, their expression was decreased at 2 weeks, after formation of DVT in type 2 diabetic mice. These results may suggest that gene expression of MMP-2 and MMP-9 were stimulated by type 2 diabetes and formation of DVT, but stimulation of type 2 diabetes dominated in late resolution of thrombus and revealed time-dependent differential regulation of MMPs (60, 105, 109). Our data are in agreement with results of the arterial vasculature reported by Portik-Dobos and colleagues (201). They showed that changes in MMP synthesis and activity might be time dependent and speculate that in the early phase of diabetes, the

MMP system may be upregulated to allow the vascular cells to migrate and contribute to intimal hyperplasia. However, with the progression of diabetes, the MMP system is suppressed, causing ECM deposition and fibrosis.

In addition, the pathogenesis of impaired angiogenesis in diabetes may be explained by an up-regulation of MMP-2 and MMP-9 resulting in an increased formation of potent angiogenic factor such as tumstatin and angiostatin generated by theses proteases through proteolytic cleavage of plasminogen as reported by Chug et al., 2006 (202) in the arterial vasculature. Furtheremore, in our study the downregulation of MMP-2 and MMP-9 at 2-weeks was correlated with increased collagen deposition suggesting impaired collagenolysis.

DVT formation and Type 2 diabetes could increase activities of MMP-2 and MMP-9 respectively. Their increased activities observed in diabetic venous thrombi are probably the combinations of the effects of the two pathologies.

V.5 CONCLUSIONS

Using a deep venous thrombosis model in diabetic mice, this study demonstrated a higher risk of thrombosis and less thrombus resolution in the diabetic environment as illustrated by higher thrombus weight and size, and decreased recanalization. An increase of late macrophage infiltration to the thrombosis site under the diabetic condition may contribute to the increased fibrosis during the thrombosis pathogenesis. The upregulated PAI-1, and downregulated uPA and tPA protein level in diabetic DVT mice may contribute to the higher risk of thrombosis. Interestingly, the upregulation followed by a downregulation of MMP-2 and MMP-9 is associated with the increase of thrombosis in diabetic mice which may be explained by the *in situ* abnormal degradation of ECM induced by MMPs followed by the exposure of thrombogenic tissue to the peripheral blood in hypercoagulatic condition.

It is likely that type 2 diabetes amplified the risk of venous thrombosis and further impaired its resolution through a higher inflammatory response and alteration of the fibrinolytic and MMP systems. However, it would be necessary to confirm these results in other experimental models of type 2 diabetes.

Finally, this study may suggest that thromboembolic arterial diseases share some similarities with venous thrombosis.

Alteration of endogenous fibrinolytic balance through modulation of PAI-1, uPA, and tPA might be a useful therapeutic target for the prevention of thrombosis in diabetic patients.

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