The Effects of Vitronectin at Sites of Myocardial Ischemia and Infarction

by

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ABSTRACT

Myocardial infarction (MI) results from the thrombotic occlusion of an atherosclerotic coronary artery in patients with ischemic heart disease. The plasminogen activator (PA)/plasmin system plays significant roles in events leading to wound healing following MI. Vitronectin (VN) is an acute phase plasma protein which has a direct influence over the constituents of the plasminogen activator/plasmin system. However, the effects of VN on cardiac function and wound healing following ischemia and infarction remain to be elucidated. My dissertation addresses the investigation of the specific role of VN at sites of myocardial ischemia, infarction and fibrosis. More specifically, my work focused on the function of VN in modulation of cardiomyocyte contractility, its effects on cardiac function, and regulation of extracellular matrix degradation in ischemic and infarcted hearts. In vitro and in vivo models have been employed to study the detailed role of VN at sites of ischemia and infarction. Isolated neonatal and adult rat cardiomyocytes, as well as left anterior descending (LAD) coronary artery-ligated transgenic mice have been used to investigate post-MI function and left ventricular (LV) healing. The main findings in this work include the demonstration that during ischemia and infarction VN modulates post-MI myocardial repair/remodeling and cardiac function. VN enters ischemic cardiomyocytes and binds to sarcomeric desmin intermediate filaments apparently causing significant reduction in cardiomyocyte contractility. Further, following infarction VN affects myocardial wound healing by regulating the PA/plasmin and matrix metalloproteinase systems. As well, exercise training in a rat model of LAD ligation induced cardiomyocytes to synthesize and secrete tissue-type plasminogen activator, which may have a role in myocardial healing post-MI. My work on myocardial healing and repair in
experimental MI models indicates a potentially important role for plasma proteins such as VN on the PA/plasmin system.
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ABBREVIATIONS

Ab - antibody
ACE - angiotensin converting enzyme
ANF - atrial natriuretic factor
ATIII - antithrombin III

bp - base pairs
BSA - bovine serum albumin

CAD - coronary artery disease
CO - cardiac output
CVB3 - coxsackievirus B3
CVD - cardiovascular disease

ECM - extracellular matrix
EF - ejection fraction
eNOS - endothelial nitric oxide synthase
ERK - extracellular signal-regulated kinase

FBS - fetal bovine serum
FN - fibronectin

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

H9C2 - embryonic rat heart cell line
H&E - hematoxylin and eosin

IgG - immunoglobulin G
IGF-1 - insulin-like growth factor
IL-1 - interleukin 1
IL-6 - interleukin 6
IF - intermediate filaments
INF-γ - interferon gamma
ISO - isoproterenol
IVC - inferior vena cava

kb - kilobase
Kd - dissociation constant
kDa - kilo-Dalton

LAD - left anterior descending coronary artery
LV - left ventricle
LVEDP - left ventricular end diastolic pressure
LVESP - left ventricular end systolic pressure
MAC - membrane attack complex
MAP - mitogen-activated protein
MHC - major histocompatibility complex
MI - myocardial infarction
MMP - matrix metalloproteinase
MPO - myeloperoxidase
mRNA - messenger ribonucleic acid

PA - plasminogen activator
PAI-1 - plasminogen activator inhibitor type 1
PBS - phosphorous buffered saline
PECAM - platelet and endothelial cell adhesion molecule
PMSF - phenylmethylsulfonyl fluoride

RGD - arg-gly-asp
RT-PCR - reverse transcriptase polymerase chain reaction
RV - right ventricle

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMC - smooth muscle cells

T-system - transverse tubular system
TBS - tris-buffered saline
TGF-β - transforming growth factor beta
TIMP - tissue inhibitor of MMPs
TNF-α - tumor necrosis factor alpha
t-PA - tissue plasminogen activator
TUNEL - terminal deoxynucleotidyl transferase mediated nick-end labeling

u-PA - urokinase plasminogen activator
uPAR - urokinase plasminogen activator receptor

VEGF - vascular endothelial growth factor
vWF - von Willebrand factor
VIM - vimentin
VN - vitronectin

WHO - world health organization
WT - wild-type
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Throughout the years, there have been tremendous changes in the landscape with rapid advances in the field of research relating to remodeling following myocardial infarction. Novel mechanisms relating to breakthroughs in understanding the progression of wound healing post-MI are updated each month in a multitude of publications, advancing the field of cardiac pathogenesis. It would be impossible to keep up with this new knowledge and the technologies without the help of my mentors, collaborative scientists, laboratory technicians, fellow graduate students, computing and other laboratory support staff at the James Hogg iCAPTURE Centre for Cardiovascular and Pulmonary Research.

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DEDICATIONS

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CHAPTER I BACKGROUND

1.1 Myocardial ischemia and infarction

1.1.1 Incidence and epidemiology

Cardiovascular disease (CVD) is the leading cause of mortality and morbidity in men and woman in the industrialized world. The World Health Organization (WHO) estimates that in 2003 16.7 million people around the globe died of CVD, which is over 29 percent of all deaths globally [1]. Today, men, women and children are at risk, and 80 percent of the burden is in low- and middle-income countries. It is estimated that by 2020 heart disease and stroke will become the leading cause of both death and disability worldwide, with the number of fatalities projected to increase to more than 20 million per year and to more than 24 million a year by 2030 [2]. In Canada, it is estimated that a person dies of CVD every seven minutes, thus accounting for more deaths than any other disease. In the year 2000, this totaled 34 percent of male and 36 percent of female deaths in Canada. Moreover, CVD elicits a heavy burden on the Canadian economy costing about $18.4 billion annually [3].

Coronary artery disease (CAD) is the most prevalent type of CVD worldwide. Myocardial infarction (MI) is the most common consequence of CAD in both the developed and developing world. In Canada approximately eighty thousand people suffer a MI each year. Although the frequency of MI increases progressively with age, it may occur at any age. MIs affect only 5% of the population under the age of 40 and 45% occur in patients over the age of 65 [4]. Further, men are more likely to suffer an MI than women at an earlier age, however the incidence of CAD and MI
has doubled among women of all ages combined in the past decade. In the United States 49.4% of deaths caused by MI were women in the year 2001 [5]. Risk factors for MI are these associated with atherosclerosis being influenced by such factors as dyslipidemia, hypertension, tobacco use, diabetes mellitus, lipid rich diet and age. The final determination of risk from any of these factors involves genetic and hormonal interactions. However, over the past several years novel risk factors for CAD have been identified and include elevated homocysteine, C-reactive protein, and fibrinogen levels. Despite the fact that the worldwide incidence of MI is expected to increase over the next decades, some studies indicate that the incidence of heart failure has been gradually yet steadily declining over the years [6]. Such trends are encouraging and hopefully continue in the future as we strive to explore novel therapies.

1.1.2 Pathogenesis of myocardial infarction

Occlusion of a major coronary artery results in ischemia throughout the anatomic region supplied by that artery known as the area at risk, and is most pronounced in the subendocardium. Acutely ischemic myocardium undergoes progressive biochemical, functional, and morphologic changes, the outcome of which largely depends on the severity and duration of flow deprivation. The principal biochemical consequence of myocardial ischemia is the onset of anaerobic glycolysis within seconds, leading to inadequate production of high-energy phosphates (e.g., creatine phosphate and adenosine triphosphate [ATP]) and accumulation of potentially noxious breakdown products such as lactic acid [7]. Myocardial function is exceedingly sensitive to severe ischemia and a striking loss of contractility is evident within 60 seconds of onset. Ultrastructural changes
develop within minutes after the onset of ischemia and include sarcoplasmic reticulum and mitochondrial swelling, glycogen depletion and nuclear chromatic clumping and margination [8]. These early changes are reversible, and cell death is not immediate. Ultrastructural evidence of irreversible myocyte injury, primarily structural defects in the sarcolemmal membrane, develop after 20 to 40 minutes in severely ischemic myocardium (with blood flow of 10% or less of normal). As demonstrated experimentally in the dog, only severe ischemia lasting at least 20 to 40 minutes or longer leads to necrosis of affected cardiomyocytes [7]. Classic acute MI with extensive necrosis occurs when the perfusion of the myocardium is reduced severely below its needs for an extended interval (hours), causing profound, prolonged ischemia and resulting in wavy fibers and permanent loss of function through cell death by coagulation necrosis [9]. In contrast, if restoration of myocardial blood flow follows periods of deprivation less then 20 minutes, loss of cell viability generally does not result. Myocardial ischemia also contributes to arrhythmias. Sudden death, a leading cause of mortality in CAD patients occurs usually due to ventricular fibrillation caused by myocardial irritability induced by ischemia or infarction.

Irreversible injury of ischemic myocytes initially occurs in the subendocardial zone. Continuous ischemia causes a wavefront of cell death from the subendocardial region to the less ischemic subepicardial region involving progressively more of the transmural thickness of the ischemic zone. The precise location, size, and specific morphologic features of an acute MI depend on several factors, including (1) the location, severity, and rate of development of coronary atherosclerotic obstructions; (2) the size of the vascular bed perfused by the obstructed vessels; (3) the duration of the occlusion; (4) the extent of collateral
blood vessels; and (5) other factors, such as alterations in blood pressure, heart rate, and cardiac rhythm [4].

There are two types of MIs, each having differing morphology and clinical significance. The more common type is the transmural infarct, in which the ischemic necrosis involves the full or nearly full thickness of the ventricular wall in the distribution of a single coronary artery. The loss of the structural integrity of the collagen-rich ventricular extracellular matrix can result in cardiac rupture, and is one of the major life-threatening complications associated with the early (<72 hours) period after a transmural infarct [4]. In contrast, subendocardial (non-transmural) infarct constitutes an area of ischemic necrosis limited to the inner one-third or at most one-half of the ventricular wall, often extending laterally beyond the perfusion territory of a single coronary artery [4]. The two types of infarcts, however, are closely interrelated because, in experimental models and likely in humans, the transmural infarct begins with a zone of subendocardial necrosis that extends in a wavefront across the full thickness of the ventricular wall. Therefore a subendocardial infarct can occur as a result of a plaque rupture followed by coronary thrombus that becomes lysed before myocardial necrosis extends across the major thickness of the wall.

Thrombolytic therapy with various fibrinolytic agents such as streptokinase or tissue plasminogen activator (t-PA) is often used in an attempt to dissolve the thrombus to re-establish blood flow to the area at risk for infarction, and possibly to rescue the viable, ischemic heart muscle. Thrombolysis re-establishes the patency of the occluded coronary artery in about 70% of cases and significantly improves the survival rate. Thrombolysis, however, can at best remove a thrombus occluding a coronary artery; insignificant changes take place in the underlying atherosclerotic
plaque that initiated it. Emergency coronary angioplasty to open blocked coronary arteries may be performed instead of thrombolytic therapy, or in cases where thrombolytics should not be used. Stents are also commonly inserted into the artery to prevent re-occlusion of the vessel post-angioplasty. Emergency coronary artery bypass surgery may be required in cases where multiple vessels are occluded [10-12].

A partially completed infarct that is then reperfused can become hemorrhagic due to vascular injury that occurs during the period of ischemia as it turns leaky upon restoration of flow. Moreover, morphologic disintegration of cardiomyocytes that are critically damaged by the preceding ischemia becomes accentuated or accelerated by reperfusion [13, 14]. With early reperfusion of an area of ischemia, irreversibly injured myocytes often have necrosis with contraction bands which are intensely eosinophilic transverse bands that span the involved myofibers [15]. Contractile bands are produced by hypercontraction of myofibrils in the dying cell that has damaged membranes after ischemia due to exposure to plasma calcium ions. Thus, reperfusion has a dual effect salvaging reversibly injured cells, as well as in altering the morphology of lethally injured cells at the time of reflow [13, 16, 17].

1.1.3 Post-MI cardiac remodeling

To date, current therapies are able to restore blood flow and limit the area of infarction, however efforts to manage myocardial healing proved of diminutive benefit. Immediately following ischemic injury the heart undergoes a complex wound healing process also referred to as remodeling. Specifically, cardiac remodeling is a process by which the size, shape, and function of the heart are
regulated by mechanical, neurohormonal, and genetic factors. The remodeling is ongoing and extends for weeks to months depending on severity of injury sustained by the left ventricle (LV) [18-20] (Figure 1-1). In mammals cardiac wound healing is greatly hampered as virtually no regeneration of the myocardium occurs and dead cells are replaced by a scar. The remodeling process is highly regulated and involves invasion, transformation and death of various cell types. Remodeling of the infarcted LV involves genomic, molecular, cellular and interstitial modifications to the infarcted and non-infarcted myocardium as evident by structural and functional changes. The initial remodeling phase improves cardiac output (CO) and may actually serve a beneficial role by promoting survival, however progressive long-term remodeling results in reduction of ejection fraction (EF), a strong predictor of mortality.

Within hours following ischemia, cardiomyocytes undergo necrosis that initiates the activation of the complement cascade and cytokine-driven events, that in turn signal the inflammatory response necessary to clear away the debris and orchestrate downstream healing events. Similarly, myofibroblasts appear a short period following inflammatory cells and are essential for reconstruction of new collagen tissue and organization of the scar. In studies using a rat model of MI alterations to connective tissue were evident within the first hour and significant degradation in collagen was noted by 1 day [21]. Days to weeks following the injury, chronic inflammatory cells such as macrophages and mast cells secrete cytokines and growth factors which promote collagen synthesis by proliferating fibroblasts in order to replace myocyte loss. Additionally, neovascularization is also stimulated by the release of growth factors from the inflammatory cells. During
Myocardial Ischemia

Necrosis/Apoptosis  Cytokine Activation  Inflammatory Reaction

Myocardial Dysfunction

Fibroblast Proliferation  Provisional Matrix  Neovascularization

Healing/Scar Formation

Figure 1-1. Time course of myocardial response to ischemia. Hours following ischemia, cardiomyocytes undergo necrosis. Activation of the complement cascade and cytokines serves to recruit leukocytes and initiate the inflammatory response. Inflammation, while potentially detrimental to surviving cardiomyocytes is necessary to clear away the debris and orchestrate downstream healing events. Chronic inflammatory cells such as macrophages and mast cells secrete cytokines and growth factors, which in turn activate fibroblasts to proliferate and synthesize collagen to replace myocyte loss. Neovascularization is also stimulated by the release of growth factors from the inflammatory cells. Scar remodeling may continue for months to years, depending on the extent of the initial ischemic event.
the first week post-MI, the infarcted region may either contract or expand after loss of viable muscle is completed [22] and following several weeks a solid scar with a solid collagen structure is formed, depending on the extent of the myocardial injury. Cardiac fibrosis is reparative replacing areas of myocyte loss with structural scar or reactive involving diffuse increases in extracellular matrix (ECM) deposition at sites unrelated to focal injury. Furthermore, perivascular fibrosis surrounding intracoronary arterioles impairs myocyte oxygen availability, reduces coronary reserve, and exacerbates myocyte ischemia. Timing of control of post-MI fibrosis is critical since if prolonged it leads to progressive hypertrophy and dilation, eventually resulting in global fibrosis causing heart failure [23, 24]. Collagen-rich scar remodeling may however continue for up to months and even years post-MI.

Several factors have been implicating in having a substantial effect on infarct healing. Reperfusion promotes cardiomyocyte viability and thus limits infarct size along with other beneficial effects on healing. Furthermore, factors such as age, sex, drugs and neurohumoral response have also been suggested to contribute to infarct healing and expansion [25]. Thus, cardiac remodeling is an important aspect of cardiovascular disease progression and is therefore emerging as a therapeutic target in heart failure.

1.1.3.1 Structural and functional alterations

Progressive remodeling post-MI causes significant changes in heart geometry, mass and volume, which adversely affects cardiac function [26-29]. LV remodeling is an age dependant process [30] that is typically proportional to infarct size, and is directly proportional to decline in EF [31]. In the infarcted heart LV remodeling typically involves a combination of infarct expansion, pressure and
volume overload. Extensive studies using animal models of MI have been utilized to examine the relationship between infarct/remodeling myocardium and ventricular function [32-34]. Post-MI ventricular remodeling in a rat, for example, closely resembles what is observed in the human and is therefore a useful tool. In experimental models infarct expansion, dilation and thinning can occur as early as one day post-MI [35] and can progress for days to weeks post-infarction [34]. LV dysfunction associated with the structural changes is apparent as soon as several days after the insult [26, 34], however some studies report that normal muscle function is maintained for several weeks post-MI before a difference is noted [36]. Drastic changes are also noted in the function of the heart, which corresponds to the extent of myocardial loss. Interestingly, in small infarcts (less than 20%) no hemodynamic changes are noted in the rat hearts. On the other hand in animals with large infarcts (greater than 20%), post-MI LV enlargement has been characterized not as a result of progressive infarct expansion but, rather, is caused by the increase in length of the remaining viable cardiac tissue [37]. The non-infarcted myocardium hypertrophies as a compensatory mechanism in response to the increased stress and causes increased left ventricular end diastolic pressure (LVEDP) with progressive LV dilation occurring for several months and up to a year [38]. Over time myocardial fibrosis itself has important functional consequences for the remodeling heart. Progressive increases in fibrosis can cause systolic dysfunction and LV hypertrophy. Furthermore, fibrosis causes increases in the volume of the heart relative to the volume of muscle when compared to normal hearts [39]. Exaggerated mechanical stiffness caused by increased ECM content results in diastolic dysfunction. Moreover, at the myocyte level increased collagen content disrupts electrotonic connectivity between cells providing an electrical
substrate for reentrant arrhythmogenesis [40]. By impairing cardiomyocyte contractility, oxygenation and metabolism, fibrosis contributes significantly to post-MI ventricular dysfunction [41].

1.1.3.2 Cellular alterations

Following MI various cell types are involved in remodeling process. Cardiomyocytes are the most abundant cell type by volume and significantly contribute to overall heart mass and function. Post-MI, surviving cardiomyocytes become elongated or hypertrophied with the sarcolemma expanded as a compensatory response in order to maintain contractile function. These alterations are associated with the activation of hypertrophy-induced genes which regulate the assembly of contractile proteins and new sarcomeres [34, 42]. Increased levels of cardiomyocyte apoptosis are also evident following MI although the exact role of this mode of cell death as a part the remodeling process is poorly understood. Apoptosis may act as an important regulatory mechanism which is involved in the adaptive response to cardiac hypertrophy as previously indicated in a model of pressure-overload [43].

Cardiac fibroblasts also play a major role in post-MI remodeling. Fibroblasts play a central role as mediators of inflammatory and fibrotic myocardial remodeling in the injured and failing heart. Proliferating fibroblasts stimulate collagen synthesis to replace necrotic cardiomyocytes in the region of infarction, as well as in the non-infarcted region [44]. Within the infarct zone these cells first construct the immature scar of thin, loosely packed collagen fibers and subsequently produce a mature scar. The fibroblast phenotype and functional capabilities are regulated by cytokines and growth factors such as interleukin 1 (IL-1), tumor necrosis factor
alpha (TNF-α), Interferon-gamma (IFN-γ), and interleukin 6 (IL-6) that are released by various cell types including fibroblasts themselves. IL-1 is a robust and multipotent agonist that strongly inhibits proliferation and enhances chemotaxis [45], while TNF-α and IFN-γ are less effective by themselves but potentiate antiproliferative and pro-migratory responses of IL-1. Furthermore, fibroblasts serve as intermediate sensors and amplifiers of signals from immune cells and cardiomyocytes, through production of cytokines, growth factors, prostaglandins, and nitric oxide [46, 47]. Excessive remodeling and chronic injury ultimately overcomes the compensatory reactions of the myocardium and progresses to heart failure which is accompanied by persistent inflammation and fibrosis in which cardiac fibroblasts have an active role. However, to date the specific mechanisms of chronic activation of cardiac fibroblasts is poorly understood. Additionally, myofibroblasts are also involved in post-MI wound healing. This specialized phenotype of activated fibroblasts express contractile proteins and intermediate filaments (IF), including smooth muscle α-actin, vimentin and desmin; contract collagen gels in vitro; and may potentially play an important role in wound closure and structural integrity of healing scars. Cardiac myofibroblasts were shown to persist in mature infarct scars and drive the progression to fibrosis [48].

1.1.3.3 Strategies for preventing LV remodeling and progression to heart failure

It is evident that the neurohumoral system plays a major role in post-MI remodeling. Activation of the neurohumoral systems results in increased wall stress, thus causing LV dilation and progression to heart failure [49, 50]. Additionally, chronic activation promotes generation of reactive oxygen species (ROS) impairing cardiac function [51, 52]. Over the years interventions have been
used with favourable impact on the remodeling process in order to prevent progression to heart failure. Drugs such as statins have been shown to increase endothelial nitric oxide synthase (eNOS) production while reducing formation of reactive oxygen species [53, 54]. Angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers and beta blockers have been shown to alter LV remodeling [55, 56]. Additionally, inhibitors of matrix metalloproteinases (MMPs) can also be used as potential therapy as they have been shown to attenuate LV dilation. Furthermore, utilization of left ventricular assistance devices can lead to normalization of LV chamber geometry, regression of cardiomyocyte hypertrophy, and reduction in collagen [57].

In recent years regeneration of cardiac muscle has also received increased attention. It is thought that following MI the ideal treatment would not only induce angiogenesis and improve cardiac function, but also replace dead cardiomyocytes. To date there exists a great deal of controversy about the regenerative capability of cardiomyocytes and therefore other methods such as cell transplantation have been implemented. While smooth muscle cells (SMC), skeletal myocytes and bone marrow cells have been successfully engrafted into the infarcted myocardium with modest enhancement to cardiac function [58-60], engraftment of fetal cardiomyocytes provide contradicting results in various studies [61-64]. Increase in cardiac function following cell transplantation can be caused by a number of mechanisms which include; increasing wall thickness and decreasing dilation, secretion of growth factors which promote angiogenesis; decreasing apoptosis of non-infarcted cardiomyocytes; and the formation of new cardiomyocytes [42]. Despite the potential therapeutic benefit of stem cells for post-MI LV regeneration,
extensive knowledge is still required in order to understand and combat heart failure in a clinical setting.

1.2 Vitronectin

1.2.1 Introduction

Vitronectin (VN) is an acute phase plasma protein with multiple ligands that is produced primarily in the liver and is found mainly in the blood plasma and ECM [65]. VN is involved in numerous physiological processes such as fibrinolysis, coagulation, humoral immune response, angiogenesis and tumor metastasis [66]. In the extracellular matrix VN has multiple roles in cell adhesion, spreading, and migration by signaling Arg-Gly-Asp (RGD) sequence and, in particular, the regulation of plasminogen activator inhibitor type 1 (PAI-1) [67-69] by stabilizing the active, inhibitory conformation [70-72]. The diverse role of VN is dependent on its interactions with multiple ligands. VN binds glycosaminoglycans [73], collagen [74], heparin [75], plasminogen [76] and the urokinase-receptor [77], as well as various integrins including avb3, avb5, avb1, dllb3, avb6 and avb8 [78, 79]. In both normal and injured tissues VN binds IF such as vimentin and keratin [80, 81], while proteins such as factor XIII and PAI-1 promote conformational change in VN allowing for its self-association into multimeric structures which are involved in the control of different biological reaction systems [82, 83]. Additionally, VN interacts with membrane attack complex of C5b-9 [84], and to thrombin-antithrombin III (ATIII) complexes [85] implicating its participation in the immune response and in the regulation of clot formation.
1.2.2 Distribution and localization

VN was first discovered in 1967 by Holmes et al labeled as serum spreading factor or S-protein [86] and first purified from human serum in 1983 by Heymens et al [65], as well as Barnes et al [87]. The majority of VN is synthesized in the liver, however VN was present in ECM of other organs such as kidney, spleen, lung, brain and heart in lower concentrations [87] and has been purified from human, rabbit, mouse, rat, hamster, guinea pig, dog, horse, porcine, bovine, goat, sheep, chicken and goose tissues [88, 89]. Although VN biosynthesis is predominantly attributed to hepatocytes in vivo, a number of other cells are capable of its synthesis. Macrophages, monocytes, HUVECs and different hepatoma cells are all sources of VN production in vitro. Biosynthesis of VN is regulated by different factors including transforming growth factor beta (TGF-β), platelet-derived growth factor, epidermal growth factor and IL-6 [90, 91].

VN is highly conserved among species sharing significant sequence homology, with some species-specific variations [89]. In the plasma VN circulates in concentrations of 200-400 μg/mL or 3-5 μM and constitutes 0.2-0.5% of total plasma protein [92]. Concentrations of VN in the plasma do not differ from that in serum as encountered with fibronectin (FN) and fibrinogen. Platelets contain approximately 0.2% of VN which is stored primarily in alpha-granules [93, 94]. VN is also found in urine, amniotic fluid and bronchoalveolar lavage fluid with no age dependent difference in concentrations. In the circulation VN is primarily found in the “closed” confirmation, while a small fraction (~ 2%) is in the “open” form [95]. Moreover, “open” VN occurs in a monomeric form which typically binds to other VN molecules forming multimers and ternary complexes, or other associated factors such as ATIII, C5b-9 complex. In the ECM VN is predominately multimeric and
bound to the matrix, heparin or forms multimers where it serves a function in adhesion, spreading, migration and stabilization of PAI-1 [96]. Accumulation of VN has been identified at sites of tissue damage particularly in areas of sclerosis, fibrosis and necrosis of various tissues including the liver, skin, kidney, nerve, vessels, bone marrow, lymphoid tissue and heart [97-101].

1.2.3 Structure and function

VN is composed of several structural domains as detailed by Suzuki et al [102]. The first 45 (amino acids 1-44) form the amino terminal segment of VN which is also know as somatomedin B due to its identical structure. The somatomedin B domain harbors functional groups which are involved in the binding of PAI-1 [69]. The Arg-Gly-Asp (RGD) sequence (residues 45-47) follows the somatomedin B domain and is responsible for mediating the attachment and spreading of cells to the ECM via its specific integrin receptors. Residues 53-64 are involved in the binding of the ATIII complex, and in neutralizing at least part of VN's polycationic domain (residues 348-379), present at its carboxyl-terminal end. An ionic interaction between the above mentioned acidic and basic stretches is probably involved in stabilizing the 3-dimensional (3-D) VN structure as well as in the formation of its multimers. There are two collagen binding domains on VN. A sequence adjacent to the RGD sequence as well as a site adjacent to the heparin binding domain. A region spanning the majority of the VN molecule (residues 132-459) accommodates six hemopexin repeats which also anchors a disulfide bridge (Cys274-Cys453). Cleavage of this bond results in 10 and 65 kDa fragments of VN.

In the carboxyl terminal of VN two distinct domains are found. Residues 332-348 is the binding site for plasminogen, which is directly followed by heparin
binding sequence, in which the glycosaminoglycan binding sequence is also found (residues 348-379) [103]. Also found in the carboxyterminal region is an additional binding site for PAI-1 found between residues 348-370 [104]. VN also contains numerous sites for proteolytic cleavage and phosphorylation sites. VN becomes phosphorylated in the amino terminus by casein kinase II Thr 50 and Thr 57 [105], as well as in the carboxyl terminus edge of VN by protein kinase C at Ser 362 and by cAMP-dependent protein kinase at Ser 378 [106]. Degradation of VN occurs by thrombin, elastase, plasmin and Granzyme B as demonstrated by in vitro experiments. Furthermore, in blood it appears that VN is also cleaved endogenously at Arg379-Ala380, by an unidentified protease. Linear organization of VN is described in Figure 1-2.

The functions of VN are both diverse and complex in nature. As previously mentioned, VN participates in pericellular proteolysis of the ECM through its localization and binding to PAI-1, participates in the immune response by binding to complement, as well as in regulation of clot formation by heparin and ATIII (Figure 1-3). One the most essential functions of VN is in regulating hemostasis. Following vascular injury the coagulation cascade is initiated in order to prevent excessive loss of blood. A series of steps take place which eventually results in the generation of thrombin, a serine protease which in turn cleaves soluble fibrinogen into insoluble fibrin. It is fibrin in conjunction with aggregating platelets that establish a haemostatic plug at the site of injury. In the circulation thrombin is bound and inhibited by ATIII, a serpin which forms a tight complex. VN acts as a pro-coagulant through its interactions with heparin. By binding heparin, VN neutralizes it and together they are cleared from the circulation. The absence of heparin prevents the facilitation of ATIII binding, allowing for interactions with
Figure 1-2. Linear organization of VN. Upper panel: localization of the binding domains of VN towards various ligands; the binding sites for plasminogen activator inhibitor-1 (PAI-1), urokinase receptor (uPAR), integrins, thrombin ± antithrombin III complex (TAT) and collagen are located in the N-terminus of the molecule, while the binding domains for plasminogen, heparin and PAI-1 are located in the carboxyl terminal edge. Lower panel: localization of the proteolytic cleavage and phosphorylation sites in VN. In vitro, VN undergoes phosphorylation by casein kinase II (CKII) at threonine50 and threonine57 adjacent to the RGD domain, by protein kinase C (PKC) at serine362 and by cAMP-dependent protein kinase (PKA) at serine378, both in the carboxyl terminus edge of VN. In vitro, VN is susceptible to degradation by thrombin, elastase and plasmin at the indicated cleavage sites. The endogenous cleavage site of VN isolated from human blood is also indicated. Adapted from Int J Biochem Cell Biol. 1999 May;31(5):539-44.
Figure 1-3. Major biological functions of VN. VN is anchored to the extracellular matrix via its collagen binding domain, and its glycosaminoglycan (GAG) binding-domain. It promotes cell adhesion, spreading and migration by interaction with specific integrins, as well as with the urokinase receptor (uPAR). The interaction of VN with integrins, in the presence of growth factors (insulin, basic fibroblast growth factor), has been also implicated in cell proliferation. VN is involved in fibrinolysis, due to its ability to stabilize the active conformation of plasminogen activator inhibitor-1 (PAI-1), and to bind the uPAR. It is also involved in the immune defense through its interaction with the terminal complex of complement and in hemostasis through its binding to heparin and thereby neutralizing the antithrombin III inhibition of thrombin and factor Xa. VN also exhibits a key role in cell contractility. Adapted from *Int J Biochem Cell Biol.* 1999 May;31(5):539-44.
thrombin and therefore preventing fibrin formation [107, 108]. VN also binds directly to the ATIII complex and clears the inactivated pair from circulation [85].

Furthermore, through its interaction with PAI-1, VN stabilizes its conformation and promotes its inhibitory activity. Prevention of plasminogen activation exerts changes in a number of physiological and pathological processes. The binding of VN to PAI-1 inhibits fibrinolysis, interferes with cell adhesion and migration, and promotes thrombus formation. Moreover, this interaction affects vital pathological processes including tissue remodeling, angiogenesis, tumor growth and metastasis. Through specific interactions with various integrins, VN is involved in cell adhesion, regulation of cytoskeletal reorganization, intracellular ion transport, lipid metabolism, activation of signaling pathways and gene expression. As previously stated numerous VN receptors have been identified which come from the integrin family consisting of non-covalently linked α and β subunits. VN receptors share either the αv or β3 subunits and include αvβ1, αvβ3, αvβ5, αvβ6, αvβ8, and αIIbβ3 [78, 79, 109]. All these receptors use the RGD sequence as a common binding motif within their ligands to bind to VN. Integrin αvβ5 exhibits selective affinity binding strictly to VN in the ECM. However, not all of these receptors are specific for binding VN; some bind other proteins containing the RGD sequence such as fibrinogen, FN, von Willebrand factor and thrombospondin. αvβ3, which binds VN with high affinity, is primarily responsible for cell migration onto various substrates and ECM of tissues.

VN also has a strong function in linking urokinase-dependent events, particularly in directing the localization of urokinase plasminogen activator receptors (uPAR) into focal adhesions in order to promote urokinase plasminogen activator (u-PA) mediated proteolysis. Binding of ECM VN to uPAR is regulated by u-PA which increases VN-uPAR association and thus promotes cell adhesion. However,
the binding of uPAR to VN can be inhibited by PAI-1, as both uPAR and PAI-1 bind overlapping regions on VN that is located close to the integrin binding site [110]. Moreover, through its binding to uPAR, VN promotes interactions with integrins αv, α5, β1 and β3 [111] thereby regulating actin cytoskeleton reorganization and induction of cell motility [112]. VN-uPAR interactions have also been implicated in uPAR-mediated signaling as demonstrated by SMC chemotaxis and cytoskeletal rearrangements [113]. Extensive research has shown that VN induces uPAR-dependent signaling primarily through p130Cas/Crk/DOCK180/Rac and is independent of MEK/ERK activity [114]. Therefore, through its interactions with uPAR VN plays a key role in regulating cell adhesion, migration as well as cytoskeletal reorganization which are important to both physiological and pathological processes. Thus, VN encompasses multiple functions which promote wound healing and contribute to a variety of mechanisms controlling and minimizing tissue injury during persistent inflammation in various tissues including the heart (Figure 1-4). However, despite clear evidence of VN in both health and disease VN knockout mice (VN-/−) mice exhibit normal mammalian development, fertility and survival [115].

1.3 Plasminogen activator (PA)/plasmin and matrix metalloproteinase (MMP) systems

1.3.1 Components

1.3.1.1 PA/plasmin system

The fibrinolytic system also referred to as the plasminogen activator (PA)/plasmin system has many diverse physiological and pathological functions. Its primary role is in the dissolution of fibrin however its role has also been implicated
Figure 1-4. Potential effects of VN at sites of myocardial injury. Following myocardial ischemia inflammatory cells and plasma proteins influx into the ECM through permeabilized vessels. VN may potentially effect fibroblasts, inflammatory and endothelial cells by mediating adhesion, migration and proliferation, which in turn influence ECM remodeling. Influx of VN into cardiomyocytes may reduce contractility and potentially affect structural integrity.
in regulation of cellular activity, tissue development and ECM remodeling. Activation of the liver derived proenzyme plasminogen into the active enzyme plasmin causes plasmin-dependent cleavage of ECM proteins, as well as activation of proteinases and growth factors. Plasminogen activators t-PA and u-PA belong to the family of serine proteases, which are the primary initiators of the conversion of pro-form plasminogen into the active plasmin through enzymatic cleavage. Typically t-PA is associated with maintaining vascular patency [116] while u-PA mediates tissue remodeling [117, 118] however both PAs exhibit a high functional overlap. The activation of plasminogen is regulated by the balance between PAs and PAI-1.

T-PA has a primary role in the lysis of fibrin clots in the circulation. t-PA is continuously secreted in the active form by endothelial cells, mast cells, keratinocytes, melanocytes, neurons, SMC, fibroblasts, as well as cardiomyocytes [119-123]. In the absence of fibrin, t-PA activates plasminogen at a low rate. As a 70 kDa glycoprotein, t-PA exists primarily in the single-chain form but is cleaved by plasmin to a two-chain form similar to u-PA. The protein consists of 5 distinct domains including the finger domain in the N-terminus followed by a growth factor domain and two kringle domains [124]. The C-terminal region contains the serine protease domain. Symbolic of all serine proteases t-PA also contains a catalytic triad (His322/Asp371/Ser463) which is involved in substrate cleavage. The finger and kringle domains are important in binding of t-PA to fibrin [125].

u-PA is a single-chain glycoprotein with a molecular mass of 53 kDa which is converted to a two-chain form by plasmin cleavage. The structure of u-PA protein is composed of three domains. In the N-terminus the growth factor domain is followed by the kringle domain, together known as the amino-terminal fragment.
The serine protease domain is found in the carboxyl-terminal region. Like t-PA, u-PA is secreted by various types of cells including numerous inflammatory and cancer cells (breast, colon, ovarian, gastric, cervix, endometrium, bladder, kidney, and brain [126]. Furthermore, u-PA has a high-affinity binding site on the cell surface for its receptor, u-PAR. u-PAR is a heavily glycosylated 55 kDa protein that is composed of three domains D1, D2 and D3 and contains a glycosyl-phosphatidylinositol or GPI anchor in the C-terminus which is integrated into the outer leaflet of cell membrane [127]. u-PAR is responsible for the primary activation of plasminogen in tissues via u-PA binding, however its role extends further than local proteolysis participating in intracellular signal transduction [128].

The diverse functions of PAI-1 have been demonstrated in regulating hemostasis, wound healing, atherosclerosis, metabolic disturbances such as obesity and insulin resistance, tumor angiogenesis, chronic stress, bone remodeling, asthma, rheumatoid arthritis, fibrosis, glomerulonephritis and sepsis [129]. These functions are attributed to a combination of factors including the antiproteolytic activity of PAI-1, as well as to the binding interactions with VN. PAI-1 is a 54 kDa glycoprotein which belongs to a family of serine protease inhibitors (serpins) and is primarily found in blood, stored in α-granules of platelets. PAI-1 is expressed and secreted by a variety of cells including endothelial cells, adipocytes and cardiomyocytes, SMC, fibroblasts, monocytes and macrophages [129-131] and is significantly increased in the acute phase of injury which is influenced by a large number of factors including: cytokines, thrombin, insulin and atherogenic lipoproteins [132]. PAI-1 inhibits plasminogen activation preventing t-PA and u-PA activity by forming a 1:1 stoichiometric reversible complex, hence decreasing proteolytic activity and promoting fibrin matrix stability. Additionally, active PAI-1
interacts specifically with VN which alters VN's structural confirmation by binding to the reactive centre loop of PAI-1, thus making it more stable [133]. Binding of PAI-1 to VN also competes with uPAR-dependent or integrin dependent binding of cells to the ECM which are crucial to numerous physiological and pathological processes [134]. PAI-1 may therefore play a role in cell adhesion and/or migration via a mechanism independent of its antiproteolytic activity. The binding of u-PA to uPAR stimulates intracellular signaling [114] and induces conformational changes in uPAR [135] which increase its affinity for VN and promote its interaction with a variety of integrins [114, 136]. By disrupting uPAR–VN and integrin–VN interactions, PAI-1 can detach cells from ECM as a result of binding to u-PA present in u-PA–uPAR–integrin complexes [134, 137]. Moreover, the anti-adhesive effect of PAI-1 on cells adherent to VN have been associated with apoptosis mediated through the caspase 3 pathway [138].

1.3.1.2 MMP system

MMPs are proteases which hydrolyze the ECM and have a diverse role in both physiological and pathological functions. All MMPs consist of a pro domain, catalytic domain, a hinge region and hemopexin domain [139], and are either anchored to cell plasma membrane or secreted. All MMPs possess common functional features including: 1) they are secreted in a latent proform and require activation for proteolytic activity, 2) they contain zinc at their active site, 3) they need calcium for stability, 4) they function at neutral pH, and 5) their function is inhibited by tissue inhibitors of MMPs (TIMPs) [140]. MMPs are categorized into six groups based on their substrate specificity, domain organization and sequence specificity. The groups are collagenases (MMP-1, 8 and 13), gelatinases (MMP-2
and 9), stromelysins (MMP-3 and 10), metrilsins (MMP-7 and 26), membrane-type MMPs or MT-MMPs (MMP-14, 15, 16, 17, 24 and 25) and other (MMP-12, 19, 20, 21, 23, 27 and 28). All collagenases cleave fibrillar collagens (types I, II, and III) which are tightly bound and cross-linked. Gelatinases are capable of degrading gelatin, but also type IV collagen in basement membranes. Stromelysins are active against a broad spectrum of ECM components including proteoglycans, laminins, FN, VN, and collagens, while MT-MMPs activate some latent MMPs, having the capacity to cleave many of the ECM components [139]. Although MMPs are best known for their role in the proteolysis of extracellular protein targets, some MMPs are also localized to the cells [141]. For example, MMP-2 is also localized to the sarcomere within cardiomyocytes where it proteolyzes contractile proteins causing acute, reversible contractile dysfunction [142].

The regulation of MMP activity is under a tight control and occurs at three levels; transcription, activation of latent proform, and inhibition by TIMPs. In normal adult mammalian tissues MMPs expression is generally low, however it is substantially upregulated under various conditions. Induction and stimulation of MMP at the transcriptional level is regulated by a variety of mediators including cytokines, hormones and growth factors [140]. Latent MMPs are activated by three different mechanisms which include 1) stepwise activation, 2) activation on the cell surface by MT-MMPs and by 3) intracellular activation [143]. Pro-MMP are activated through the cleavage of the pro-peptide domain by proteinases including trypsin, chymase, elastase, and plasmin, the most potent proteinases *in vivo*. Cleavage of the propeptide region induces a conformational change in MMP structure, rendering the activation site exposed. MT-MMPs also activate latent MMP particularly on the surface of cells.
Inhibition of active MMPs occurs by the binding interactions with TIMPs. There are four members in the TIMP family; 1, 2, 3 and 4 that bind to MMPs in a 1:1 molar ratio and inhibit its activity by interacting with its zinc-binding site within the catalytic domain. TIMPs generally vary in the degree of specificity towards different MMPs. TIMPs 1 and 2 exhibit more diverse biological functions potently inhibiting the majority of MMPs with exception of MMP-2 and 9. TIMP-3 specifically inhibits MMP-1, 2, 3, 9 and 13, while TIMP-4 is highly expressed in cardiac tissue and inhibits MMP-1, 3, 7 and 9 [144]. Furthermore, other naturally occurring inhibitors such as α2-macroglobulin prevents activity of all four MMPs along with other proteases.

1.3.2 Role of PA/plasmin and MMP systems in cardiac health and disease

The PA/plasmin system has been implicated in a variety of physiological and pathological processes such as fibrinolysis, cell migration, cell-cell signaling, inflammation, tumor invasion and metastasis and wound repair [145]. Elevated levels of PAI-1 associated with vascular thrombosis, MI [146, 147], and cardiac fibrosis [131] occur as a direct consequence of PAI-1 binding with PAs to form complexes inhibiting plasmin generation and activation of MMPs [140] preventing pericellular proteolysis. Furthermore, the PA/plasmin system also exhibits essential roles in hemostasis by regulating cell adhesion, migration, expression of growth factors and angiogenesis [148-150].

MMPs are produced in a number of myocardial cells including fibroblasts, inflammatory cells and cardiomyocytes. Involvement of MMPs is clearly noted in the heart, particularly in the wound healing/repair process. During post-MI cardiac
remodeling a number of MMPs including MMP-1, 2, 3 and 9 are expressed and elevated in both human and animal myocardium [151, 152]. During the remodeling process following MI, MMPs have a significant role in ECM degradation, cell migration, angiogenesis and regulation of growth factors. Furthermore, increased MMP activity has also been associated with dilated cardiomyopathy [118, 153] and vascular remodeling [154, 155]. The activity of TIMPs has also been implicated in numerous cardiac diseases. Reduced levels of TIMPs were noted in ischemic cardiomyopathy, as well as at sites of MI [156, 157]. Reduction of TIMPs following MI severely affects myocardial structure and ventricular performance [158].

1.3.3 Role of PA/plasmin and MMP systems in coronary artery disease and thrombosis

Hemostasis plays an important role in the progression of atherosclerosis and development of cardiac complications of acute coronary syndromes. In most cases of acute coronary syndromes, the pathogenesis is formation of a mural thrombus on a ruptured or eroded atherosclerotic plaque. t-PA plays a key role in physiological plasminogen activation to plasmin [159]. In the presence of a fibrin clot, t-PA has a 100-fold greater affinity for plasminogen. Digestion of fibrin clots via plasmin-dependent cleavage results in soluble fibrin degradation products. Deficiency in t-PA greatly reduces the capacity to remove fibrin clots potentially resulting in thromboembolic disease [160]. t-PA deficient mice (t-PA^{-/-}) exhibit abnormal accumulation of fibrin due to impaired fibrinolysis, resulting in a severe thrombotic phenotype with a global decrease in cardiac function [161-165]. Animal models of MI demonstrate that infusion of t-PA elicits myocardial salvage by restoring blood
flow and intermediary metabolism in the region of risk by prompting thrombolysis [166].

Furthermore, endogenous fibrinolytic capacity is predictive of future rates of morbidity and mortality associated with MI, stroke, angina pectoris, and post-operative thrombosis [167-172]. The role of PAI-1 in stabilization of thrombi and post-MI coagulative necrosis has been recognized and received much consideration. Studies using overexpression of human PAI-1 in mice demonstrate the development of spontaneous venous thrombosis [173]. Also, enhanced thrombosis in atherosclerosis-prone mice was associated with increased arterial expression of PAI-1 [174]. Hence, at sites of vascular damage the tightly regulated fibrinolytic balance is offset, favouring fibrin clot formation due to overwhelming inhibitory activity of PAI-1. Such imbalance results in thrombosis leading to myocardial ischemia with progression to infarction. Moreover, increased levels of PAI-1 are observed in patients with occluded arteries and are associated with higher incidence of MI.

Although contribution of MMPs to vascular fibrinolysis are not substantial, they have also been implicated at sites of vascular remodeling and thrombosis. Increased levels of MMP activation, particularly caused by increased inflammation contribute to pathological matrix destruction and atherosclerotic plaque rupture. Plaque rupture represents a failure of intimal remodeling, where the fibrous cap overlying an atheromatous core of lipid undergoes catastrophic mechanical breakdown. Plaque rupture promotes coronary thrombosis leading to MI. Fibrin can be directly solubilized by MMPs, as demonstrated by Bini et al [175], yielding fibrin degradation products. These are either different from that of plasmin,
resulting in a D-like monomer fragment [175] or of similar molecular mass to that of the plasmin product depending on the specific MMPs involved [176].

Moreover, the MMP and PA/plasmin systems act in synchrony to degrade the major components of the vascular extracellular matrix. Activation of MMPs contributes to intimal growth and vessel wall remodeling in response to injury by promoting migration of vascular SMC. Inhibiting the activity of specific MMPs or preventing their upregulation could ameliorate intimal thickening and prevent MI.

1.3.4 Role of PA/plasmin and MMP systems in myocardial wound healing

Reduced fibrinolytic activity by virtue of low PA activity and enhanced levels of PAI-1 not only promotes thrombosis, but is critical in the fibrotic reorganization and post-MI tissue remodelling. Cardiac ECM is a dynamic structure, which has a prominent role in a variety of disease processes. The ECM is composed of numerous constituents and cells which produce them [177-179]; all essential for maintenance of proper cardiac geometry and function [180]. The PA/plasmin and MMP systems modulate wound healing by regulating ECM breakdown and synthesis [150, 181] (Figure 1-5). Post-MI the heart undergoes a complex healing process that involves a series of phases including inflammation, granulation, removal of necrotic tissue and tissue repair [116, 182].

Activation of plasmin regulates degradation of fibrin and other matrix proteins involved in migration of inflammatory cells, MMP and growth factor activation, angiogenesis and collagen remodeling [117, 183-189]. There is functional overlap of the activation of plasmin with the activation of the MMP system [190], as well as with stimulation of growth factors (i.e. TGF-β), both crucial in the remodeling phase.
Studies in human and animal models of MI and heart failure suggest that impaired PA/MMP activity attenuates revascularization and promotes cardiac fibrosis [117, 185, 192-194]. Infarct healing was virtually absent 5 weeks following surgically-induced MI in plasminogen knockout mice in which inflammatory cells did not migrate into the infarcted myocardium, necrotic cardiomyocytes were not removed and the formation of granulation tissue did not occur [185]. However, these mice also showed a complete absence of fibrotic scar formation and no LV dilation was observed. Moreover, plasminogen knockout mice also had depressed MMP-2 and 9 activity, suggestive of plasmin specific effects [185].

Mice with u-PA deficiency were protected against early cardiac rupture. Similar results were also observed in wild-type (WT) mice by injection of adenoviral vector overexpressing PAI-1 prior to MI. However subsequent long-term inhibition of PA activity impaired LV remodeling and prevented therapeutic myocardial angiogenesis [117]. It was also evident that MMP activity was significantly reduced in both plasminogen and u-PA deficient mice. Together these findings indicate that the effects of plasminogen and/or u-PA deficiency are at least in part mediated by activation of MMPs.

PAI-1 and t-PA also play a role in post-MI wound healing, as expression levels for both genes were upregulated for weeks in the infarcted rat cardiac tissue [195-197]. Immunoperoxidase staining for PAI-1 and t-PA distribution in human and rat [198] samples of acute and healed myocardial infarcts demonstrated that high levels of both antigens localized within the healing infarct days post-MI. This suggests that the PA/plasmin system plays a key role in myocardial wound healing, and elevated activation of t-PA is suggestive of its imperative function post-MI. Although the role of t-PA and the PA/plasmin system have been described with
regard to many diverse pathologies [199-203], the specific role of t-PA in myocardial repair post-MI remains to be elucidated. Recently more emphasis has also been given to the function of t-PA in remodeling of ECM by activation of plasmin and augmentation of MMPs at sites of injury [204-208]. Preliminary observations from our laboratory further uphold the notion that t-PA plays a key role in remodeling as expression and secretion levels of t-PA were increased in cultured rat cardiomyocytes and healing myocardium of male adult rats for weeks post-MI.
Figure 1-5. Regulation of post-MI ECM remodeling by PA/plasmin and MMP systems. In the ECM t-PA and u-PA stimulate plasmin generation which initiates fibrin lysis into degradation products, and activation of latent MMPs. Active MMPs degrade ECM unless inhibited by TIMPs. At sites of infarction plasma VN enters both reversibly and irreversibly injured cardiomyocytes and binds to PAI-1 in the ECM forming steady complexes to enhance PAI-1 stability and activity. PAI-1 inhibits plasmin activation preventing ECM degradation and promotes fibrosis.
Thus, the PA/plasmin and MMP systems are essential in regulating wound healing following MI. Interactions between the two systems regulate the ECM remodeling particularly in the post-MI myocardium. The mechanisms involved in infarct healing are primarily based on the proteolytic activity of plasmin, promotes ECM degradation and activation of MMPs, and hence promote cell migration, activates growth factors, and prevents the inhibitory activity of TIMPs [209].

1.3.5 Effects of hypertrophic stimuli

Cardiac tissue levels of t-PA and u-PA increase when subjected to a variety of stimuli. Exercise training is known to induce cardiac hypertrophy and increase in systemic fibrinolytic activity [210, 211] with enhanced t-PA and u-PA plasma levels [212, 213] suggesting an increased fibrinolytic reserve. Similarly, exercise training also enhances MMPs 2 and 9 levels in muscle and connective tissue [214, 215]. Following MI, the beneficial effect of exercise training is also evident. Exercise training exhibits favourable effects on post-MI ventricular remodeling by increasing the wall thickness of the non-infarcted myocardium, limiting wall tension and attenuating LV dilation when compared with sedentary control animals [198]. These effects may be in-part related to increases in PAs and/or MMP activity [216, 217] which may influence myocardial hypertrophy.

Pathological hypertrophy has also been associated with altered cardiac fibrinolytic activity. Hypoxia-induced ventricular hypertrophy stimulates synthesis of cardiac mRNA levels of both t-PA and PAI-1, with increased secretion of t-PA into the vasculature of perfused hearts [218]. Pressure overload-induced LV hypertrophy in a porcine model demonstrated increases in ventricular mRNA for PA and PAI-1 which preceded morphological changes in the early stages prior to
development of heart failure [219]. Conversely, loss or inhibition of u-PA or MMP-9 attenuated LV remodeling and preserved cardiac function after acute pressure overload in mice [118].

The PA/plasmin and MMP systems are also activated in response to activation with beta-adrenergic stimuli. Levels of t-PA and u-PA secretion were increased in plasma from patients treated with isoproterenol (ISO) [212]. Moreover, when induced with ISO, activities of MMP-1 and -2 increased in hypertrophied rat cardiac tissues and enhanced cardiomyocyte MT1-MMP synthesis and release was noted in the remodeling porcine LVs [220, 221].

1.4 Intermediate Filaments

1.4.1 Structure and function

Intermediate filaments (IF) are a major component of the cytoskeleton, and of the nuclear lamina in most eukaryotic cells. The organization of IFs and their association with plasma membranes suggests that the principal function of IFs is mainly structural, to reinforce cells and organize them into tissues [222, 223]. The most important function of the IFs however, is to provide mechanical support for the plasma membrane where it comes in contact with other cells or with the ECM. Also, IF proteins exhibit a strong specificity of expression for particular cell types, for example desmin is expressed in all muscle cells [222].

In contrast to the other two major cytoskeletal systems of eukaryotes, microfilaments and microtubules, IFs appear to be much simpler in their basic properties. The basic subunit of IF assembly is an anti-parallel tetramer. IFs are formed of a dimers composed of two α-helical chains interwound in a coiled-coil rod [223]. Most IF proteins assemble under suitable conditions to form uniform 8-12 nm
wide filaments. However, several types of IF proteins are obligate heteropolymers and require presence of heterologous IF proteins in order to assemble in the copolymer form. IFs are classified into classes based on their sequence similarities, including: (a) keratins (types I and II), (b) vimentin-like proteins (type III), (c) neuronal proteins (type IV), and (d) lens IF proteins (type V) [222, 224].

Vimentin and desmin belong to type III IF proteins. Vimentin is typically expressed in blood vessel endothelial cells, other mesenchymal cells such as fibroblasts and in some epithelial cells [222]. Vimentin fibers terminate at the nuclear membrane, and at the cell-surface desmosomes and hemidesmosomes. In those locations vimentin IFs keep the nucleus and other organelles in defined places within the cell. Vimentin tends to form radial networks, but can also form a ring and wrap around a cellular component like a lipid droplet. Vimentin is also frequently cross-linked with microtubules [225].

Desmin is another type III IF that is expressed in all types of muscle and functions in linking myofibrils into bundles [222]. Desmin is associated with Z-lines and sites of muscle-tendon and muscle-muscle cell attachment. Desmin filaments encircle the Z-disks and make connections between neighboring Z-disks or with the overlying plasma membrane. Through the inter-myofibrillar connections, desmin filaments align Z-disks in neighboring cells. Thus, desmin is responsible mainly for stabilizing sarcomeres in contracting muscle. Desmin also copolymerizes with vimentin, and various other proteins. As a major component of cardiomyocytes, desmin IFs also forms physical links with the nucleus, contractile proteins, sarcolemma, ECM as well as, other organelles [226]. Additionally, the cellular distribution of desmin IFs in the cardiomyocyte makes it an ideal candidate for cellular signaling.
In most myogenic systems, synthesis of the IF protein vimentin precedes that of muscle specific desmin. It was illustrated that after the initial synthesis of vimentin myogenic differentiation begins and cells synthesize desmin [227]. Thereafter, vimentin and desmin readily coassemble with one another both *in vitro* and *in vivo*. Newly synthesized desmin integrates into the preexisting vimentin filament network. As myogenic differentiation proceeds, relative levels of vimentin and desmin interchange where vimentin synthesis ceases and desmin becomes the major, and in most cases the only IF protein in the mature muscle cell. Change in the composition of the IF is accompanied by an alteration of IF organization in which the longitudinal system is transformed into a transverse system associated with Z-lines, the sarcolemma and the myotendinous junction. The pattern of desmin organization during myotome differentiation differs markedly from systems in which vimentin is present. Desmin fails to form the longitudinal IF system characteristic of vimentin-containing myotubes and it is initially concentrated at the intersomite junction and the lateral sarcolemma. As myogenic differentiation proceeds, a reticular network of desmin filaments appears followed by the association of desmin with Z-lines. Thus, vimentin and desmin differ significantly in the types of IF networks they form *in vivo* [227].

1.4.2 Desmin-associated cardiomyopathies

Desmin IFs are critical mechanical integrators of the cytoskeleton that protect the cell from repeated mechanical stress. There have been increasing reports of human cardiomyopathies associated with abnormal accumulation and aggregation of desmin IFs. Recently identified desmin mutations in humans suffering from skeletal muscle myopathy and cardiomyopathy suggest that these diseases might
arise as a consequence of impaired function of desmin filaments. Studies with null mice for desmin demonstrate that desmin is essential for normal cardiac function and suggest that the absence of an intact desmin filament system, rather than accumulation of the protein, may be responsible for the pathology seen in some of the desmin-associated cardiomyopathies. It is also apparent that mutant desmin interferes with the normal assembly of IFs, resulting in fragility of the myofibrils and severe dysfunction of skeletal and cardiac muscles [228]. Skeletal and cardiac myopathies develop in mice that lack desmin, suggesting that mutations in the desmin gene may be pathogenic [228]. Mice lacking desmin have hypertrophied cardiomyocytes arising from increase in cross-sectional cell area. Moreover, desmin null mice exhibit increased ventricular free wall thickness, and also experience wall thinning, dilation and impaired function at 12 months [229]. Ultrastructurally, desmin null mice exhibit sub-sarcolemmal clumping and proliferation of mitochondria that is associated with reduced mitochondrial respiration. It has also been suggested that by promoting anchorage of mitochondria to areas of high energy demands, desmin regulates cellular energy production [230]. This insinuates that although desmin is not required for the differentiation of skeletal, cardiac, and smooth muscle, it is essential to strengthen and maintain the integrity of these tissues.

Functional studies provide compelling evidence that mutations in the human desmin gene are pathogenic and interfere with the assembly of IFs [228, 231-233]. Functional abnormalities caused by the mutant desmin can be reversed by the insertion of WT desmin. In skeletal and cardiac muscles, normal desmin encircles the Z bands that hold together the actin filaments and help transmit tension along the myofibrils, protecting their structural integrity during repeated muscle
contractures over time [228]. Defects in the function of desmin as well as desmin-associated proteins may therefore cause fragility of the myofibrils and impair contraction. In both mice and humans that lack desmin, cardiomyopathy, skeletal myopathy and metabolic myopathy develop with advancing age as mechanical stress disrupts myofibrils and affects the Z bands [228]. Moreover, myocardial tissue of patients with end-stage heart failure of ischemic origin showed a decreased number of desmin-positive myocytes which is associated with reduced cardiac function [234]. This deficiency in myocardial desmin could potentially lead to increase in LV diastolic pressure due to reduced LV contractility. Although cytoskeletal desmin is clearly involved in numerous genetic and non-genetic forms of cardiac diseases, recent study of infarcted rat hearts show no changes in desmin cardiac levels in post-MI remodeling [235]. Thus, despite the extensive knowledge regarding the role of desmin deficiency and/or accumulation in various pathologic states, further work is required in order to investigate its effects in many unidentified cardiac disease states.

1.4.3 Ischemia, stunning and hibernation

Following a period of ischemia, regional mechanical function of the myocardium can remain depressed acutely for several hours to days (stunning), or chronically for days to weeks (hibernation) (Figure 1-6). Myocardial stunning is a mechanical process that occurs following ischemia and reperfusion despite absence of irreversible damage or restoration of coronary flow [236]. At sites of myocardial stunning, myocardial contractility remains depressed for variable
Figure 1-6. Fate of myocardium following acute and chronic ischemia injury. Following a period of ischemia and reperfusion, regional mechanical function of the myocardium can remain depressed acutely for several hours to days (stunning), or chronically for days to weeks (hibernation) despite absence of irreversible damage. Prolonged ischemia without reperfusion results in cell death.
intervals. There are a number of potential mechanisms involved in the pathogenesis of myocardial stunning by which contractile dysfunction can be explained. It has been suggested that contractile dysfunction in stunned myocardium is strongly correlated with the disappearance of cytoskeletal proteins which could be mediated by a calcium-dependent intracellular protease activated during reperfusion [237]. Disruption of cytoskeletal proteins may be a possible mechanism for stunning, although it may be a secondary effect of protease activation. In the stunned myocardium, regions of reduced desmin IF are evident which likely contribute to a reduction of contractility.

Chronic myocardial dysfunction which results in long-term oxygen consumption and contractile depression is termed hibernation. Hibernation is a reversible condition in which the dysfunctional, viable myocardium recovers following reperfusion [238]. Although hibernating myocardium is considered independent of stunned myocardium, both conditions involve reduced expression of cytoskeletal proteins, including desmin IF. In hibernating myocardium structural degeneration is commonly characterized by reduced mRNA and protein expression and disorganization of cytoskeletal proteins, including desmin [239]. Structural changes occur in which both temporal and spatial variations of structural proteins are evident. The expression of these proteins is continuously changing from stunned to hibernating myocardium occurring globally and independent of regional reduction of coronary flow [240]. Spatial reorganization of desmin in the cytoplasm and a loss of colocalization with desmoplakin at the intercalated disks are evident in stunned myocardium after 24 hours. With progression to hibernating myocardium, desmin expression is lost at the intercalated disks [240].
Thus, desmin along with other contractile and cytoskeletal proteins exhibit an important function in myocardial stunning and hibernation following an ischemic episode. Although the precise role of desmin IF in mechanical regulation of cardiomyocyte contractility remains to be investigated, it is clear that desmin plays an essential role in structural stability and may promote survival of the myocytes following ischemia.
CHAPTER II  HYPOTHESIS AND SPECIFIC AIMS

The central hypothesis of this work is that vitronectin 1) modulates cardiac function by impairing cardiomyocyte contractility 2) modulates post-MI remodeling in by regulating extracellular matrix proteolysis and cell-matrix interactions

The following specific aims to be addressed are the following:

Aim 1  To determine the effects of vitronectin in wound healing following myocardial infarction

Sub aim 1  Investigate the effects of vitronectin in regulation of post-MI extracellular matrix remodeling

Sub aim 2  Examine the effects of vitronectin on cardiac structure and function

Aim 2  To investigate the distribution and function of vitronectin at sites of hypoxic and ischemic injury

Sub aim 1  Examine the binding interactions of vitronectin with cytoskeletal desmin intermediate filaments
Sub aim 2 Investigate the effects of vitronectin on regulation of cell contractility

Aim 3 To investigate the effects of hypertrophic stimuli on the localization of plasminogen activator/plasmin systems components at sites of acute myocardial infarction
CHAPTER III RESEARCH QUESTIONS AND STRATEGIES

The *questions* relevant to the hypothesis and specific aims are:

1) How does accumulation of VN affect the expression, distribution, levels and activity of proteins responsible for ECM degradation/synthesis? Does VN cause specific differences in enzymatic activity post-MI? Does post-MI VN accumulation affect levels of post-MI fibrosis? Is VN involved in post-MI remodeling by regulating the adhesion/migration of inflammatory cells, endothelial cells and fibroblast. Does VN exhibit an effect on post-MI hypertrophy of non-infarcted myocardium? Do VN-/- mice exhibit differences in cardiac architecture post-MI? Does post-MI VN accumulation have an effect on cardiac function?

2) Does VN enter ischemic cardiomyocytes? Does this occur by active or passive transport? Does VN bind to sarcomeric desmin in ischemic cardiomyocytes? What is/are the specific region(s) responsible for binding of VN to desmin? Does VN have an effect on cardiomyocyte function?

3) Does exercise modulate the fibrinolytic system (i.e. t-PA, u-PA and PAI-1) and VN following MI? Are cardiomyocytes capable of t-PA expression, storage and secretion? Is cardiomyocyte t-PA expression induced by hypertrophic stimuli?
To answer these questions, I have conducted research with a strategy described in the materials and methods, these approaches and the results and discussion of the experiments are detailed in the following four chapters:

Chapter IV, “Effects of vitronectin on cardiac structure and function post-myocardial infarction”. Chapter V, “Effects of vitronectin on regulation of the plasminogen activator/plasmin and matrix metalloproteinase systems in wound healing post-myocardial infarction”. Chapter VI, “Effects of vitronectin at sites hypoxia and myocardial ischemia”, and Chapters VII, “Expression of cardiomyocyte tissue plasminogen activator following myocardial infarction”.

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CHAPTER IV  EFFECTS OF VITRONECTIN ON CARDIAC STRUCTURE AND FUNCTION POST-MYOCARDIAL INFARCTION

4.1 Rationale

Following MI there is extensive cardiac remodeling resulting in LV dilation, a major cause of congestive heart failure and sudden cardiac death. VN is a diverse glycoprotein which has numerous biological functions many important to tissue healing process. In the ECM, VN exhibits multiple functions including cell adhesion, migration, and spreading. VN has multiple ligands and accumulates at various sites of inflammatory cell-induced damage and necrosis. Additionally, VN localizes at sites of MI, and prolonged accumulation of VN at sites of damage of various tissues reflects chronic injury associated with fibrosis. Preliminary studies from our laboratory demonstrated an accumulation of VN at sites of ischemic myocardial damage and post-MI fibrosis. Thus, we were interested in investigating the effects of VN on the post-MI remodeling, particularly with respect on cardiac structure and function.
4.2 Experimental Methodology

4.2.1 Genotyping of VN -/- mice

4.2.1.1 Isolation of genomic DNA

Cryo-derived CD57BL/6J VN-/– mice were obtained from Jackson Laboratories (Bar Harbor, Maine) and were cross-bred with mice on a CD57B6/6J background. In order to determine whether mice expressed the VN gene, mouse tails were clipped, genomic DNA isolated and subjected to PCR. Tail biopsies (~0.5 cm) were obtained from 3 week old mice. Tail clips were placed in TNES buffer (10mM Tris, pH = 7.5, 400mM NaCl, 100mM EDTA, 0.6% SDS) containing 35 µL Proteinase K (10 mg/mL) and incubated overnight at 55°C. Following the incubation period 167 µL of 6M NaCl was added to the digested tail clips, shaken vigorously for 15 seconds, then centrifuged at 14000 x G for 5 minutes at room temperature. Supernatants were then removed and 1 volume of 95% cold ethanol added. DNA was precipitated by centrifugating for 10 minutes at 14000 x G. Following discarding of the supernatants the DNA was washed with 70% ethanol, then allowed to air dry. DNA pellet was then resuspended in 100 µL of TE buffer (10 mM Tris, 1mM EDTA pH = 8.0) and placed at 65°C to assist in dissolution. DNA yield was determined using spectrophotometry and stored at 4°C.

4.2.1.2 Determining mouse strain using PCR

Five microliters (5-20 ng) of DNA was used per reaction to a final reaction volume of 30 µL. DNA was amplified for 35 cycles at the following primer conditions used 94°C for 3 minutes, 94°C for 30 seconds, 67°C for 1 minutes, 72°C for 1 minutes, 72°C for 2 minutes, then 4°C until further use. DNA was amplified in
four parallel PCR reactions using specific primers (Table 4-1) and Taq polymerase. RT-PCR products were separated on 1.5 % agarose gels containing ethidium bromide. Results were evaluated as the reaction product appeared as either 442 bp band (+/+, VN homozygous), 442 bp & 280 bp bands (+/-, heterozygous), or 280 bp band (-/-, Neomycin homozygous) (Figure 4-1). After establishing the phenotypes, 5 sets of breeding pairs from both WT and VN-/- mice were selected for propagation of the colonies.

<table>
<thead>
<tr>
<th>Designation</th>
<th>Sense Strand Primer (5' - 3')</th>
<th>Anti-Sense Strand Primer (3' - 5')</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neomycin</td>
<td>CTTGGGTGGAGAGGCTATTC</td>
<td>CTAGAGGACAGTAGAGGTGGA</td>
<td>280</td>
</tr>
<tr>
<td>VN</td>
<td>GTGCCAGTGCTTGGGAATTGGG</td>
<td>CTTCTTATCTGAGACTGGTGATG</td>
<td>442</td>
</tr>
</tbody>
</table>

Table 4-1. Primer sequences used for genotyping of VN+/+ and VN-/- mice. Sequences were obtained from http://jaxmice.jax.org/

**4.2.2 Mouse model of left anterior descending coronary artery ligation**

The mouse model of wound healing following acute MI has been described previously [241] [242]. All animal experiments were approved by the University of British Columbia Committee on Animal Care. Briefly, 12-week-old C57B6/6J WT and VN-/- mice (n = 201, weight 20–25-g) underwent surgical ligation of LAD. Infarction was sustained for 3, 7, 14, 28, 60 and 90 days. Prior to sacrifice mice were weighed, then a thoracotomy was performed and hearts rapidly excised into ice-cold saline, then manually compressed to remove residual blood. For hearts designated for protein and RNA studies, infarcted LV, the right ventricle (RV) and atria were dissected out under a surgical microscope and weighed. The tissues were flash-frozen in liquid nitrogen or placed in RNAlater® solution (Ambion, Austin, TX) and stored at −80°C. For histological studies hearts were sectioned mid-ventricle into two pieces and designated as either apex or base. These were
perfused-fixed under constant pressure then placed in 4% paraformaldehyde. Specific experiments and sample size for each time-point is described in Figure 4-2.

4.2.2.1 Anesthetic technique

Mice were weighed before administering the appropriate amount of anesthetic subcutaneously; 50 µL/10 g of weight of a mixture of xylazine (120 µL) and ketamine (900 µL) in 0.9% saline (6980 µL), equivalent to 28 µg ketamine and 3.8µg xylazine per gram bodyweight. Ophthalmologic ointment was applied to the eyes to prevent corneal drying. Once anesthetized, chest hair was removed using Nair™ hair removal lotion. Following a short period of isoflurane inhalation in an anesthetic box, intubation was performed using an intravenous cannula into the trachea (a 22-gauge cannula for animals up to 30 g of weight and a 20-gauge cannula for animals heavier than 30 g). The cannula was connected to the ventilator outlet delivering a mixture of oxygen (4L/minute) and isoflurane 1-2% to keep the animal under deep anesthesia (tidal volume 0.5 mL, rate 120 strokes/minute).
Figure 4-1. **Mouse genotyping performed by PCR.** Genomic DNA was isolated from mouse tails at 3-5 weeks of age. Presence of a 280 bp band signified the presence of a neomycin cassette (Neo) (lanes 1) while the presence of a 442 bp band signified the presence of VN (lanes 2) which replaced the entire VN locus. Mice with a single band at 442 bp represented VN+/+ progeny. Mice with bands at 442 bp and 280 bp represented the heterozygous (VN+/-) progeny. Mice with a single band at 280bp represented the VN-/- progeny.
Figure 4-2. Experimental methods utilized in mouse model of LAD ligation. Detailed experimental protocol describing the WT and VN-/- mice sample size and experiments conducted at different times of infarction.
4.2.2.2 Surgical ligation of left anterior descending (LAD) artery

The animal was placed in a supine position and tilted to its right side on a heated pad and secured by tape. A skin incision was made parallel with the midline and halfway between the midline and left leg. Skin and subcutaneous muscles were separated from the layers of muscle underneath using serrated forceps and the transverse pectoral muscle was retracted using a suture to expose the rib cage. Sharp pointed scissors were used to separate rib muscles longitudinally between the second and third ribs just above the left lung contour. Blunt-tipped bone scissors were then inserted into the opening and underneath the chest wall being careful to avoid cutting any major blood vessels whilst cutting three ribs. The chest was then opened with a retractor.

The LAD was identified as it appeared from under the left atrial appendage. At time of ligation the initial strategy was to ligate mid-LAD after the origin of the first diagonal branch. Occasionally, a suture placed at this point did not result in LAD occlusion and a second suture had to be placed. The second suture was usually positioned at the level of the left main stem, just below the left atrial appendage. In some animals the LAD could not be visualised and occasionally only a large left diagonal branch to the apex could be seen in which case it was tied; otherwise the main vessel was tied using 8-0 polypropylene monofilament it was ligated. If blanching of the myocardium was not observed, ligation was repeated. The chest was closed using a two-layer closure with 5/0 Vicryl for ribcage and skin. Isofluorane was discontinued and ventilation maintained until the return of spontaneous respiration. Mice were allowed to recover in a heated chamber under observation for a few hours. Thereafter, they were monitored daily until time of sacrifice.
4.2.3 Assessment of cardiac function

To evaluate the effects of VN on myocardial healing and cardiac function, hemodynamic measurements were obtained from WT and VN-/- at baseline and mice at 28 days post-MI using transthoracic echocardiography and pressure-volume conductance Millar catheterization.

4.2.3.1 Echocardiography

Echocardiographic evaluation was performed at 28 days using a Visual Sonics 700 VEVO system, a platform, a 30 MHz probe, probe holder and data analysis unit. Animals were lightly sedated with 0.1 mL/30 g of a mixture of ketamine (300 µL) and xylazine (40 µL) solution (1/3 of the dose used for ligation purposes), using the lightest sedation compatible with keeping animals sedated whilst in the supine position. Chest wall hair was removed and the animal was taped in the left lateral position on the examination table. Liberal quantities of Depo™ Cardiac Contrast agent was applied to the chest wall. By using different size probes and various table positions multiple echocardiographic images were obtained, including a parasternal long axis view and parasternal short axis view at the levels of (a) immediately below the mitral valve (b) papillary muscle (c) sub-papillary muscle and (d) apex.

Ejection fraction (EF) was calculated using Simpson’s method in all animals where adequate images could be obtained as described in Figure 4-24. Off-line analysis was performed in a blinded manner using only numeric identifiers. The largest circumference in diastole and the smallest diameter in systole were measured for three different beats at all four parasternal short axis levels. Finally, using the parasternal long axis views, the longest distance between the apex and
the base of the mitral valve leaflets in diastole and the shortest axis between the same points were measured on three separate beats. Measurements resulted in three separate EF calculations with mean average determined for each animal. Averaged EF was compiled and the group means were compared using the Student's t-test. M-mode measurements were obtained at papillary muscle level; where possible measurements were made while directing the beam between the papillary muscles at the maximal diameter. M-mode measurements were used to calculate heart rate and estimate EF (Teicholz method). EF was calculated using the following formula:

\[
\text{Ejection Fraction} = \left( \frac{\text{Stroke Volume}}{\text{End-Diastolic Volume}} \right) \times 100
\]

4.2.3.2 Pressure-volume conductance

Following echocardiographic evaluation, animals were again sedated using Ketamine and Xylazine to a total of 0.1 mL per 10 g. Intubation was performed under direct visualisation and animals were ventilated using a small animal ventilator (MiniVent Ventilator for Mice, 230 VAC, Harvard Apparatus, Holliston, Massachusetts). Under a dissecting microscope (Carl Zeiss Canada Ltd., Toronto, Canada), the chest wall was opened using a mid-sternal incision, which was extended caudally into the abdomen. The pericardium was opened and dissected away from the infarct to allow access to the apex. A visual estimation of infarct size was made and recorded (none, small, medium, large). The apex was grasped with a sharp forceps and punctured using a 21 gage needle. A 1.2 French fine-tipped pressure transducer (Millar Pressure-Volume System, Millar Instruments, Houston,
TX) was introduced into the apex of the LV and manipulated as required to obtain the best possible wave-form.

Once adequate steady-state loops were obtained aortic occlusion was performed. This was done using a cotton-tipped swab placed just below the diaphragm after re-locating the intestines and mesentery to the left. This occlusion was performed for approximately 10 beats, allowing the wave-form to return to baseline. Occlusion was repeated 2 to 4 times as required to obtain a satisfactory increase in aortic pressure. A similar process was repeated by performing occlusion of inferior vena cava (IVC). Pressure was applied to the IVC until LV pressure dropped satisfactorily. The swab was then removed to allow recovery to baseline. This process was repeated 2 to 4 times.

After obtaining measurements the catheter was removed and animals were sacrificed under anaesthesia and the hearts excised. The heart was rinsed in cold saline to remove residual blood. Hearts were arrested by immersion in a 0.1mM solution of potassium chloride.

4.2.4 Histology and morphometry

4.2.4.1 Infarct size and infarct expansion

Following sacrifice the hearts were excised and weighed. Hearts were cannulated by the aorta and perfused-fixed with 4% paraformaldehyde at a constant pressure. Hearts were kept in 10% formalin overnight then embedded in paraffin. Paraffin embedded sections were cut into 5-μm slices, which were stained with H&E and picrosirius red (1mg Sirius red per 1mL picoric acid). Slides were scanned under high resolution (300 dpi) then analyzed using a computerized morphometric system (Image Pro Plus, Media Cybernetics). The area occupied by
the infarct was measured and expressed as ratio of picrosirius red positive scar area to total LV area. Measurements were performed in serial sections. Successive heart slices were taken beginning at mid-ventricle and spaced every 500 μm until the apex (Figure 4-3A). Final infarct size was expressed as the mean area of each heart. Additional methods used to determine collagen content was also performed in which the internal circumference of the picrosirius red positive scar was measured and expressed as a ratio to total LV chamber circumference.

4.2.4.2 LV, RV and septum geometry

As outlined by Hochman [19] et al and Weisman et al [35], LV diameter was measured as the maximum distance from the endocardial surface of the septum to the endocardial surface of the LV anterior free wall along a perpendicular line. LV anterior free wall thickness in infarcted rat hearts was measured at three distinct points as described in Figure 4-3B, and the mean of the three points determined. Septum and RV thickness were measured at three distinct points, two located at the origin of attachment and one through the centre. Mean of the three points was then determined (Figure 4-3B).

4.2.4.3 Inflammatory infiltration and angiogenesis

Paraffin-embedded mouse heart tissue sections were subjected to routine immunohistochemical staining for myeloperoxidase (MPO), platelet-endothelial cell adhesion molecule 1 (CD31) and von Willebrand factor (vWF). Staining was visualized with the chromagen Vector Red (Vector Laboratories, Burlingame, CA), which possesses both, colorimetric and fluorescent properties. Slides were examined by light microscopy under a 40 x objective across 5 fields of view in the anterior free wall (infarct and peri-infarct zones). Same methodology was followed
Figure 4-3. Schematic representation used to determine tissue morphology analysis. A) Mouse hearts (lateral view) were sectioned in 500 μm intervals beginning mid-ventricularly and progressing toward the apex. B) Representative tissue slice in cross section. LV diameter was determined by taking the single most-widest measurement from the endocardial aspect of the septum to the endocardial aspects of the anterior LV free wall. LV thickness, RV thickness and septum thickness were each determined by averaging three measurements obtained from various myocardial areas.
for sections stained with CD31 and vWF. Number of MPO positive inflammatory cells, and CD31 and vWF positive vessels were identified using a computerized morphometry system (Image Pro Plus, Media Cybernetics) and counted manually.

4.2.5 Immunohistochemistry

Paraffin-embedded mouse heart tissue sections were subjected to routine immunohistochemistry (as described in Chapter IV) using purified Abs to VN, t-PA, u-PA, PAI-1 or pre-immune IgG obtained from Molecular Innovations. Staining was visualized with the chromagen Vector Red, or peroxidase diaminobenzidine tetrahydrochloride (DAB). Nuclei were counterstained with hematoxylin.

4.2.6 Statistics

Statistical significance of differences was assessed using Student's t-tests and comparison among multiple groups was performed one-way analysis of variance (ANOVA); p-value of 0.05 or less was considered significant. All values are expressed as means ± SEM or means ± SD.
4.3 Results

4.3.1 Genotyping

Detection of the VN gene by PCR demonstrated unexpected ratios of WT : VN+/− : VN−/− mice (10:85:5). Homozygous WT and VN−/− mice were then used to propagate each phenotype. Heterozygous mice were excluded from the study.

4.3.2 Coronary artery anatomy

Ligation of the mid-LAD resulted in infarction of the LV free wall with blanching usually evident within moments of ligation. LAD ligation did not result in septal or papillary muscle infarction, and the apex was generally spared. Two major variants were recognized in mouse coronary anatomy. In the first variant a large single artery coursed from the left atrial appendage down to the apex with no major branches (Figure 4-4A). In the second variant there was a single large branch of this artery, analogous to a large first diagonal branch (or the circumflex artery) in the human. This branch usually was located just below the tip of the left atrium passing laterally to the left (Figure 4-4B). Occasionally only the diagonal branch was visualized passing laterally with no obvious LAD.

MI was induced in a combined total of 167 mice, with an additional 34 sacrificed as sham-operated controls. Infarcted WT mice experienced a mortality rate of 20.5%, of those, 3 mice suffered peri-operative mortality and 12 expired post-operatively. VN−/− mice suffered 17.6% mortality, of which 4 died peri-operatively and 11 post-operatively. Seven mice from both, WT and VN−/− MI groups died as a consequence of cardiac rupture within first 7 days of infarction. Sample sizes and allocation of mice at each time-point are provided in Figure 4-2.
4.3.3 Establishment of murine model of left anterior descending coronary artery ligation

Prior to performing the procedure on mice used in our study, practice C57B6/6J mice (n = 100) were subjected to ligation of the LAD to ensure accuracy and consistency of infarct size. Once the mouse model of MI was established and the surgeons were confident in obtaining reproducible results WT and VN-/- mice were randomly selected into two groups; sham-operated and MI. Ligation of the mid-LAD resulted in infarction of the LV free wall with blanching usually evident within moments of ligation. Some infarcts were located antero-apically rather than in LV free wall. This was likely caused by differences in mouse coronary artery anatomy. Mid-LAD ligation did not result in septal or papillary muscle infarction and the apex was generally spared. Generally a mid-LAD ligation resulted in a small-to-medium sized infarct, whereas a main trunk ligation resulted in a large infarct. Mid-LAD ligation resulted in 13% mouse mortality, whereas mortality after left main trunk ligation was 30%. Mortality post-operatively occurred almost exclusively between days three and nine. In all mice that were analysed by post-mortem cardiac rupture appeared to be the cause resulting in either massive haemopericardium or haemothorax. Surviving mice lost approximately 10% total body weight by day 2 with recovery back to baseline by the end of week one. By four weeks post-MI body weight had increased approximately 15% above baseline (Figure 4-5).
Figure 4-4. Differences encountered in mouse coronary artery anatomy. In our model of LAD ligation two major variants in mouse coronary anatomy were predominant. In the first, a major single artery courses from the left atrial appendage and concluding at the apex with no major branches (A). In the second variant there is a single large (main) branch of this artery, analogous to a large, first diagonal branch (circumflex artery in the human). This branch is usually given off just below the tip of the left atrium passing laterally to the left (B). In rare instances only the diagonal branch was seen passing laterally without a noticeable LAD (not shown).
4.3.4 **Comparison of post-MI mouse body weights**

Both WT (n = 57) and VN/-/- (n = 73) mice demonstrated an 5-7% decline in weight during the first 3 days post-MI. Weight returned back to baseline by 14 days post-MI and increased an additional 2% in WT and 5% in VN/-/- mice above baseline by day 28 (Figure 4-6). No statistically significant differences were noted at any time-point between the WT and VN/-/- groups.

4.3.5 **Assessment of infarct induction in WT and VN/-/- mice 28 days post-MI**

There were no significant differences in age or weight recorded between the WT (n = 27) and VN/-/- (n = 31) groups at baseline. In majority of the mice mid-LAD ligation was performed (84%). At time of sacrifice at 28 days post-MI, no significant differences in age, weight or time of infarction were noted between WT (n = 24) and VN/-/- (n = 27) mice. Similarly, no significant differences in infarct size were noted at time of initial ligation or at time of sacrifice, although smaller infarcts were more commonly observed in VN/-/- mice. (p = 0.07, Chi-squared test for trend, Table 4-3)
Table 4-2. Measurements of age, body weights, and the site of ligation in WT and VN-/− mice at baseline and 28 days post-LAD ligation. Mid-LAD ligation was performed in most cases (23 of 26 WT and 26 of 31 VN-/−), with no significant differences in site of ligation or visual estimate of infarct size observed between the groups peri-operatively. No significant differences in age or weight were noted at baseline or at 28 days post-MI between the WT and VN-/− mice. P value as determined by a paired Student’s t-test.

### 4.3.6 Accumulation of VN in the infarcted myocardium over time

We have examined the accumulation of VN following MI in WT and VN-/− mice by Western blot and immunohistochemistry. VN was accumulated in the Triton-X insoluble cytoskeletal fraction from isolated LV. VN was present in WT mice at 3, 7, 14, 28 and 60 days post-MI. Peak amounts of VN were noted 7 days post-MI as determined by densitometry, following which a gradual decline was observed over time (Figure 4-7 A and B). VN was absent from the Triton-X soluble cytoplasmic fraction of the infarcted LV of WT. No VN was found in the infarcted VN-/− mice at any timepoint. Similarly, immunostaining for VN in WT mice showed its accumulation throughout the infarcted myocardium primarily localized to the LV. Corresponding to the Western blots, VN staining appeared progressively weaker in intensity although thinning of the LV anterior wall was also evident. (Figure 4-7C).
Figure 4-5. Distribution of body weight of practice mice over time following LAD ligation. Mouse weight declined 10% by day 2 post-MI with a return to baseline by day 7. At four weeks post-MI, mouse weight had increased 15% above baseline.
Figure 4-6. WT and VN-/- mice exhibit no changes in body weights post-MI. Both, WT and VN-/- mice experienced approximately a 10% decline in body weight in the first 3 days post-MI. Mouse weight returned back to baseline by 14 days post-MI and increased an additional 3 - 5% above baseline weight by day 28. Similar trends were observed in WT and VN-/- mice with no significant differences noted between the groups at any timepoint.
**Figure 4-7. VN accumulates in the infarcted myocardium.** A) Western blotting showed increased levels of VN in the Triton-X insoluble cytoskeletal fraction of infarcted LV. No VN was present in the cytoskeletal heart extracts of VN−/− mice. B) VN accumulated progressively reaching peak levels at 7 days post-MI, followed by a gradual decline up to 60 days post-MI. C) Staining for VN (red) in infarcted hearts (10x objective) of WT mice demonstrated an accumulation of VN in the anterior LV free wall which gradually decreased in size (top panel: brightfield and bottom panel: darkfield). Scale bar = 1 mm.
4.3.7 Examination of post-MI fibrous scar formation

To determine if VN affected myocardial healing, we quantified the area of picrosirius red positive collagen staining as percent of LV at 28 days post-MI. Collagen staining was absent from both, WT (n = 8) and VN-/-(n = 8) non-infarcted control groups (WT controls 0.58 ± 0.2 vs. VN-/ controls 0.68 ± 0.1). (Figure 4-8, A and C). It appeared that at 28 days post-MI, WT mice exhibited prominent and altered remodeling of the infarcted LV when compared to VN-/-(Figure 4-8, arrows). Morphometric analysis showed a significant reduction in total collagen accumulation in the VN-/ MI group (n = 8) when compared to WT MI mice (n = 8) at 28 days (WT MI 19.2 ± 3.6 vs. VN-/ MI 8.1 ± 1.9, p < 0.05) (Figure 4-8C). Staining for interstitial collagen was included in the measurement of the LV scar. To further evaluate the effects of VN on post-MI heart remodeling infarct expansion was measured and expressed as percent of internal circumference. Surprisingly, only a slight difference was noted between the WT and VN-/ MI groups (WT MI; 34.6 ± 3.01 vs. VN-/ MI; 31.3 ± 2.3, p = 0.43) (Figure 4-9).

4.3.8 Assessment of viable LV and RV myocardium in WT and VN-/ mice

No significant differences in viable myocardial areas were noted in either the RV or LV of sham-operated WT and VN-/ mice as determined by area of viable myocardial tissue (Figure 4-10). At 28 days post-MI there was evident increase in the viable RV and LV area in WT and VN-/ mice which may be attributed to compensatory hypertrophy. Differences found were not significant in nature, either in the RV (WT MI mice; 3590.34 ± 463.22 pixels/area vs. VN-/ MI mice 4139.97 ± 516.96, p = 0.44) or LV (WT MI mice; 22097.29 ± 1767.77 VN-/ MI mice; vs.
23238.65 ± 1483.13, p = 0.63) were noted between the WT and VN-/- MI groups (Figure 4-10). Thus, there was an increase in total myocardial area (RV + LV) in MI WT mice when compared to sham-operated mice. Interestingly, VN-/- MI mice exhibited only a nominal change in the total myocardial area when compared to VN-/- sham-operated mice. Right and left atria were excluded when quantitating viable myocardial area.
Figure 4-8. VN-/- mice exhibit a decrease in collagen accumulation 28 days post-MI. A) Heart serial sections from non-infarcted (WT; n = 8, VN-/-; n = 8) and MI mice (WT; n = 8, VN-/-; n = 8) were stained with picrosirius red stain (red) for collagen. Picrosirius red stain demonstrated an accumulation of collagen in the thinning anterior LV free wall of both WT and VN-/- MI mice. B) Higher magnification images taken at mid-ventricularly designated localization of collagen (arrows) in the fibrosed LV. C) WT mice exhibited a significant increase in collagen staining when compared to VN-/- (WT MI 19.2 ± 3.6 % LV area vs. VN-/- MI 8.1 ± 1.9, p < 0.05). Only trivial amount of collagen staining was evident in LV of sham-operated mice.
Figure 4-9. VN/- mice exhibit no significant changes in amount of subendocardial fibrosis at 28 days post-MI. Morphometric analysis of subendocardial fibrosis quantitated as a percentage of internal LV circumference demonstrated only trivial differences between infarcted WT (n = 8) and VN/- (n = 8) mice (WT MI; 34.6 ± 3.01 vs. VN/- MI; 31.3 ± 2.3, p = 0.43).
Figure 4-10. VN-/- and WT mice exhibit no differences in remote areas of myocardium. Area of normal myocardium was quantitated in both sham-operated (WT; n = 8, VN-/-; n = 8) and MI (WT; n = 8, VN-/-; n = 8) mice at 28 days post-MI. Sham-operated VN-/- mice exhibited slightly larger LV area when compared to sham-operated WT mice, however no differences in RV areas were noted. At 28 days post-MI VN-/- mice exhibited a similar LV and RV sizes when compared to infarcted WT mice. Interestingly, there was no change in the total myocardial area (LV + RV) between the sham-operated and MI VN-/- mice.
4.3.9 Examination of post-MI wall structure and chamber geometry

Morphometric analysis of infarcted mice (n = 24) at 28 days showed profound differences between WT and VN-/- mice. Analysis of cardiac architecture determined as measures of LV diameter, LV anterior free wall thickness, RV thickness and septum thickness are presented in Table 4-4. There were no differences in parameters between sham-operated WT and VN-/- mice. As anticipated, LV free wall thickness of infarcted mice was diminished when compared to sham-operated VN-/- and WT mice. However we also found that infarcted LV free wall of VN-/- mice was increased in thickness when compared to WT (WT MI; 0.72 ± 0.05mm VN-/- MI; vs. 0.82 ± 0.04mm, p = 0.1) (Figure 4-11). Despite the apparent changes to infarcted mouse hearts no significant differences were noted in the RV and septum thickness between infarcted WT and VN-/- mouse groups (WT MI; 0.31 ± 0.01mm vs. VN-/- MI; 0.28 ± 0.01mm, p = NS) and (WT mice; 0.87 ± 0.01mm vs. VN-/- mice; 0.9 ± 0.01mm, p = NS) respectively (Figure 4-11). However, both the RV and septum thickness were notably increased in infarcted WT or VN-/- mice compared to their representative sham-operated mice (RV thickness, WT mice; 0.25 ± 0.02 mm vs. 0.31 ± 0.02, p = 0.06) and (RV thickness, VN-/- mice; 0.28 ± 0.01 vs. 0.33 ± 0.02, p = 0.1) and (Septum thickness, WT mice; 0.7 ± 0.1 vs. 0.87 ± 0.05, p = 0.12) and (Septum thickness, VN-/- mice; 0.74 ± 0.01 vs. 0.9 ± 0.01, p = 0.09) (Figure 4-11). Alterations in cardiac architecture occur likely as a compensatory hypertrophic response to excessive LV thinning to maintain sufficient function. LV diameter was increased in the MI groups of both, WT (WT controls; 2.99 ± 0.33 vs. WT MI 3.73 ± 0.23 mm, p = 0.09) and VN-/- (VN-/- controls; 2.5 ± 0.4 mm vs. VN-/- MI 3.77 ± 0.17 mm, p = 0.006) mice compared to their respected sham-operated controls (Figure 4-12). Surprisingly,
sham-operated WT mice appeared to exhibit an increase in LV diameter compared to VN-/-, although the difference was not significant (p = 0.38).

4.3.10 Examination of leukocyte infiltration 3 days post-MI

Three days post-MI VN-/- mice experienced reduced myocardial necrosis and showed a reduction in inflammatory cell infiltration determined by accumulation of MPO positive cells in the infarcted LV (WT MI 167 ± 33 vs. VN-/- MI 118 ± 34 cells/500μm²; p = 0.34). The majority of MPO positive cells were localized to the infarct area abundant in enucleated (dead) cardiomyocytes in both WT and VN-/- mice. MPO positive cells were mainly absent from the RV and posterior non-infarcted LV in both WT and VN-/- MI mice (Figure 4-13).
Figure 4-11. VN-/− mice exhibit altered wall structure 28 days post-MI. VN-/− mice experienced a marked decrease in the infarcted LV free wall thickness when compared to WT mice at 28 days post-MI (WT MI; 0.57 ± 0.02mm VN-/− MI; vs. 0.74 ± 0.04 mm 05, p = 0.1). No significant differences were noted in the RV and septum thickness between infarcted WT and VN-/− mice (WT controls; 0.31 ± 0.01mm vs. VN-/− controls; 0.28 ± 0.01mm, p = NS) and (WT controls; 0.87 ± 0.01mm vs. VN-/− controls; 0.9 ± 0.01mm, p = NS) respectively. Slight increase in the thickness of anterior LV free wall was noted between the WT and VN-/− sham-operated controls, however no differences were noted in either the RV and septum thickness.
Figure 4-12. VN-/- mice exhibit no change in LV diameter 28 days post-MI. Morphometric analysis demonstrated no significant changes in LV diameter between WT and VN-/- sham-operated controls (WT controls; 2.99 ± 0.33. VN-/- controls; vs. 2.5 ± 0.4 mm, p = 0.38) and between WT and VN-/- MI groups (WT MI; 3.73 ± 0.23 vs. VN-/- MI; 3.77 ± 0.17 mm, p = 0.89). 28 days post-MI infarcted LVs of both WT and VN-/- were significantly increased in size compared to their respective sham-operated control groups.
Figure 4-13. VN-/– mice exhibit reduced MPO staining in the infarcted LV at 3 days post-MI. A) Myeloperoxidase (MPO) positive inflammatory cells (red) in the infarcted LV of WT and VN-/– mice at 3 days. Localization of MPO positive cells was particularly observed in area of myocardial necrosis (insets, lower right). Staining was absent from WT and VN-/– sham-operated control mice. B) Quantitative morphometry (40x objective, 5 fields of view) demonstrated a reduction in the MPO positive cells in the infarcted VN-/– mice when compared with WT (WT MI; 167 ± 33 vs. VN-/– MI; 118 ± 34 cells/100μm²). Scale bar = 100 μm.
4.3.11 Examination of vessel formation 7 and 28 days post-MI

Angiogenesis is an essential part of post-MI remodeling. In order to investigate the effects of VN on development of new microvessel, slides were stained with CD31 and examined by brightfield microscopy (Figure 4-14). Quantitative morphometry of CD31 staining 3 days post-MI demonstrated a decline in the number of newly formed capillaries in the infarct (WT MI 9.75 ± 1.89 vs. VN-/− MI 4.67 ± 2.09 vessels/500μm², p = 0.13) and peri-infarct regions (WT MI 10.38 ± 1.82 vs. VN-/− MI 5.42 ± 1.72 vessels/500μm², p = 0.09) of VN-/− mice. Quantification of total CD31 positive vessels throughout the entire anterior LV further showed a significant reduction in VN-/− MI mice when compared to WT MI (WT MI 13.5 ± 0.71 vs. VN-/− MI 6.08 ± 2.5 vessels/500μm², p = 0.04). Furthermore, although significant differences in the number of CD31 positive vessels was noted between WT sham-operated and WT MI groups (WT Controls 2.15 ± 1.83 vs. WT MI 13.5 ± 0.71 vessels/500μm², p < 0.001), sham-operated VN-/− mice exhibited no differences when compared to the VN-/− MI mice (VN-/− Controls 5 ± 3.89 vs. VN-/− MI 6.08 ± 2.5, p = 0.81) (Figure 4-15).

vWF staining was used to identify vascular endothelial cells in mature vessels. Examination of vWF positive vessels demonstrated a reduction in the number of vessels found in the infarct and peri-infarct regions of VN-/− mice at 28 days. There was an overall increase in vessel number found in LV of WT mice when compared to VN-/− (WT MI 32.06 ± 6.83 vs. VN-/− MI 23.9 ± 3.01 vessels/500μm², p = 0.27). Distribution of vWF positive vessels by region showed an increase in the peri-infarct zone of WT mice when compared to VN-/− (WT MI 37.14 ± 9.53 vs. VN-/− MI 19.94 ± 2.77 vessels/500μm², p = 0.09), with no
significant changes noted in the infarct zone between the two groups (WT MI 30.86 ± 7.09 vs. VN/- Ml 21.75 ± 4.28 vessels/500μm², p = 0.76) (Figure 4-16).

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<th>WT Sham</th>
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**Table 4-3.** Summary of morphological analysis associated with sham-operated and infarcted WT and VN/- mice. P value as determined by a paired Student’s t-test.
Figure 4-14. Accumulation of new vessels in hearts of WT and VN-/- mice 3 days post-MI. Brightfield images stained for CD31 showed immature vessels (red) localized in the peri-infarcted region at 3 days post-MI. Marginal staining was also observed in sham-operated control mice. Scale bar = 100 μm.
Figure 4-15. VN-/— mice exhibit a reduction in CD31 positive microvessels 3 days post-MI. Morphometric analysis of CD31 positive vessels in WT and VN-/— hearts demonstrated a marginal increase in the infarct and peri-infarct regions of WT mice when compared to VN-/—. Overall, no significant differences were observed between the WT and VN-/— sham-operated control mice groups (WT controls 2.15 ± 1.83 vs. VN-/— controls 5.0 ± 3.89, p = 0.53). In contrast, significant increases were noted in WT MI mice when compared to VN-/— MI mice (WT MI 13.5 ± 0.71 vs. VN-/— MI 6.08 ± 2.5, p < 0.05). Furthermore, there was a significant increase in vessel formation between WT MI mice 3 days post-MI when compared to WT controls (p < 0.001). No changes were noted between VN-/— sham-operated controls and VN-/— MI mice (p = 0.81).
Peri-infarct Region

Infarct Region

Overall

Figure 4-16. VN/- mice exhibit a reduction in vWF positive vessels 28 days post-MI. Morphometric analysis of von Willebrand factor (vWF) staining for mature endothelial cells in WT and VN/- hearts demonstrated a notable increase in peri-infarct region (WT MI 37.14 ± 9.53 vs. VN/- MI 19.94 ± 2.77, p = 0.09), with only trivial increase noted in the infarct region (WT MI 30.86 ± 7.09 vs. VN/- MI 21.75 ± 4.28, p = 0.76) of WT mice when compared to VN/-. Overall, there was an increase in vWF positive vessels throughout the LV of WT MI mice when compared to VN/- MI mice (WT MI 34 ± 5.77 vs. VN/- MI 24.43 ± 3.43, p = 0.15).
4.3.12 Assessment of cardiac function post-MI

4.3.12.1 Evaluation of hemodynamic parameters by echocardiography

Details regarding body weight, age and site of ligation of mice are detailed in Table 4-3. Baseline evaluation of hemodynamic parameters was performed on 21 mice, 11 WT and 10 VN-/- . There was no difference in age or weight at time of assessment. Dimensions and function were assessed by both M-mode and by Simpson's method. No significant differences were noted in any of the parameters measured between baseline control WT and VN-/- mice. Initial reduction in body weight was noted in the post-MI WT or VN-/- groups however the weight returned back to baseline by day 28. Cardiac function was evaluated by M-mode and 2D transthoracic echocardiography at baseline and 28 days post-MI (Figure 4-17 and 4-18). No differences in hemodynamic parameters were evident between WT and VN-/- baseline controls (Table 4-5). Significant differences in hemodynamic parameters were observed between WT and VN-/- mice 28 days post-MI (Table 4-5). Both systolic and diastolic volumes were greatly diminished in post-MI VN-/- mice when compared to WT. VN-/- mice showed significant improvement over WT mice in EF (41.8 ± 7% vs. 48.5 ± 7.5%, p < 0.001) (Figure 4-19). Furthermore, significant changes in EF were observed in both, WT and VN-/- baseline control mice when compared to the corresponding MI groups. Major differences were also noted in fractional area of contractility between the two MI groups (WT MI 35.9 ± 9.3% vs. VN-/- MI 43.7 ± 7.4%, p < 0.01) (Figure 4-19B). Significant changes in fractional area of contractility were also noted, in both WT and VN-/- baseline control mice when compared to the corresponding MI groups. No significant differences in stroke volume, cardiac output, and heart rate were evident between WT and VN-/- mice at baseline or 28 days post-MI (Figure 4-19B).
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<tr>
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</tr>
<tr>
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<td>66.1±22.5</td>
<td>62.1±15.4</td>
</tr>
<tr>
<td>Diastolic Volume (µL/sec)</td>
<td>37.3±13.2</td>
<td>34.6±8.4</td>
</tr>
<tr>
<td>Ejection Fraction (%)</td>
<td>56.3±7.0</td>
<td>55.9±4.9</td>
</tr>
<tr>
<td>Cardiac Output (mL/min)</td>
<td>9.0±6.5</td>
<td>8.1±3.2</td>
</tr>
<tr>
<td>Fractional Area of Contractility (%)</td>
<td>51.2±6.8</td>
<td>51.9±8.4</td>
</tr>
<tr>
<td>Heart Rate (beats/min)</td>
<td>28.8±10.7</td>
<td>27.5±8.2</td>
</tr>
</tbody>
</table>

Table 4-4. Summary of measurements for hemodynamic parameter acquired by echocardiography in baseline and infarcted WT and VN-/− mice.

4.3.12.2 Evaluation of hemodynamic parameters by Millar catheter analysis

Pressure-volume conductance data adequate for analysis were obtained from 18 of 22 non-infarcted mice (WT, n = 10 and VN-/−, n = 8). Millar catheter insertion was attempted on infarcted mice following echocardiography with adequate data obtained from 16 of 20 WT and 16 of 19 VN-/− mice (other seven animals either died before or shortly after catheter insertion), although standard deviation were substantial accounting for approximately one-third of the mean. No differences in any hemodynamic parameter were noted between WT and VN-/− mice at baseline or at 28 days post-MI. Data detailing all pressure-volume assessment using Millar catheters are detailed in Tables 4-6, 4-7, 4-8, 4-9, 4-10 and 4-11.
### Steady State Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT Mice (n = 10)</th>
<th>VN-/ Mice (n = 8)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Rate (bpm)</td>
<td>203.9 ± 49.9</td>
<td>214.7 ± 66.1</td>
<td>NS</td>
</tr>
<tr>
<td>Maximum Volume (µL)</td>
<td>53.5 ± 11.4</td>
<td>45.8 ± 5.2</td>
<td>NS</td>
</tr>
<tr>
<td>Minimum Volume (µL)</td>
<td>35.3 ± 5.4</td>
<td>33.5 ± 5.6</td>
<td>NS</td>
</tr>
<tr>
<td>End-systolic Volume (µL)</td>
<td>36.5 ± 5.9</td>
<td>34.9 ± 6</td>
<td>NS</td>
</tr>
<tr>
<td>End-diastolic Volume (µL)</td>
<td>52.9 ± 11.3</td>
<td>45.3 ± 5.3</td>
<td>NS</td>
</tr>
<tr>
<td>Maximum Pressure (mmHg)</td>
<td>97.9 ± 37.1</td>
<td>92.9 ± 35.6</td>
<td>NS</td>
</tr>
<tr>
<td>Minimum Pressure (mmHg)</td>
<td>4.6 ± 4.9</td>
<td>4.5 ± 4.3</td>
<td>NS</td>
</tr>
<tr>
<td>End-systolic Pressure (mmHg)</td>
<td>96.2 ± 37.9</td>
<td>92.9 ± 35.6</td>
<td>NS</td>
</tr>
<tr>
<td>End-diastolic Pressure (mmHg)</td>
<td>8.2 ± 4.4</td>
<td>7.2 ± 4.3</td>
<td>NS</td>
</tr>
<tr>
<td>Stroke Volume (µL)</td>
<td>18.2 ± 8.5</td>
<td>12.3 ± 3.4</td>
<td>NS</td>
</tr>
<tr>
<td>Ejection Fraction (%)</td>
<td>32.5 ± 9.9</td>
<td>27 ± 8</td>
<td>NS</td>
</tr>
<tr>
<td>Cardiac Output (µL/min)</td>
<td>3423.2 ± 1172.6</td>
<td>2604 ± 1050.1</td>
<td>NS</td>
</tr>
<tr>
<td>Stroke Work (mmHg x µL)</td>
<td>1125.2 ± 489.7</td>
<td>760.9 ± 226.8</td>
<td>NS</td>
</tr>
<tr>
<td>dP/dt max (mmHg/sec)</td>
<td>4530.6 ± 1143.9</td>
<td>5055.9 ± 1239</td>
<td>NS</td>
</tr>
<tr>
<td>dP/dt min (mmHg/sec)</td>
<td>-2253.2 ± 761.8</td>
<td>-2554.9 ± 692.1</td>
<td>NS</td>
</tr>
<tr>
<td>dV/dt max (µL/sec)</td>
<td>321.3 ± 123</td>
<td>232.3 ± 55.5</td>
<td>NS</td>
</tr>
<tr>
<td>dV/dt min (µL/sec)</td>
<td>-425.2 ± 179.3</td>
<td>-325 ± 132.8</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 4-5. Hemodynamic parameter evaluation in baseline control WT and VN-/ mice using Millar catheter. P value as determined by a paired Student’s t-test. All t-tests assume Gaussian distribution and unequal variance between groups.
<table>
<thead>
<tr>
<th>Steady State Parameters</th>
<th>WT Mice (n = 10)</th>
<th>VN/- Mice (n = 8)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Rate (bpm)</td>
<td>169.3 ± 31.4</td>
<td>165.8 ± 56.3</td>
<td>NS</td>
</tr>
<tr>
<td>Maximum Volume (µL)</td>
<td>47.2 ± 20</td>
<td>49.6 ± 16.6</td>
<td>NS</td>
</tr>
<tr>
<td>Minimum Volume (µL)</td>
<td>38.3 ± 17</td>
<td>39.1 ± 12.4</td>
<td>NS</td>
</tr>
<tr>
<td>End-systolic Volume (µL)</td>
<td>39.8 ± 17.4</td>
<td>40.1 ± 12.7</td>
<td>NS</td>
</tr>
<tr>
<td>End-diastolic Volume (µL)</td>
<td>46.1 ± 20.4</td>
<td>48.8 ± 16.1</td>
<td>NS</td>
</tr>
<tr>
<td>Maximum Pressure (mmHg)</td>
<td>67.3 ± 19.3</td>
<td>76.9 ± 21.1</td>
<td>NS</td>
</tr>
<tr>
<td>Minimum Pressure (mmHg)</td>
<td>3.4 ± 4.6</td>
<td>1.6 ± 3.1</td>
<td>NS</td>
</tr>
<tr>
<td>End-systolic Pressure (mmHg)</td>
<td>66.3 ± 19.6</td>
<td>76.2 ± 21</td>
<td>NS</td>
</tr>
<tr>
<td>End-diastolic Pressure (mmHg)</td>
<td>5.8 ± 4.8</td>
<td>4.6 ± 3.2</td>
<td>NS</td>
</tr>
<tr>
<td>Stroke Volume (µL)</td>
<td>8.9 ± 6.2</td>
<td>10.6 ± 6.2</td>
<td>NS</td>
</tr>
<tr>
<td>Ejection Fraction (%)</td>
<td>21.1 ± 15.6</td>
<td>20.5 ± 7.8</td>
<td>NS</td>
</tr>
<tr>
<td>Cardiac Output (µL/min)</td>
<td>1449.6 ± 993</td>
<td>1629.4 ± 938.7</td>
<td>NS</td>
</tr>
<tr>
<td>Stroke Work (mmHg x µL)</td>
<td>467.5 ± 501.6</td>
<td>597.8 ± 441.6</td>
<td>NS</td>
</tr>
<tr>
<td>dP/dt max (mmHg/sec)</td>
<td>3314.4 ± 1471.9</td>
<td>3480.1 ± 1234.9</td>
<td>NS</td>
</tr>
<tr>
<td>dP/dt min (mmHg/sec)</td>
<td>-1655.2 ± 773.9</td>
<td>-1825.3 ± 875</td>
<td>NS</td>
</tr>
<tr>
<td>dV/dt max (µL/sec)</td>
<td>208.5 ± 127.7</td>
<td>239.3 ± 165.5</td>
<td>NS</td>
</tr>
<tr>
<td>dV/dt min (µL/sec)</td>
<td>-225.6 ± 154.3</td>
<td>-304.1 ± 242.3</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 4-6. Hemodynamic parameter evaluation in WT and VN/- mice 28 days post-MI using Millar catheter. P value as determined by a paired Student's t-test. All t-tests assume Gaussian distribution and unequal variance between groups.
### Hemodynamic Parameters

<table>
<thead>
<tr>
<th></th>
<th>WT Mice (n = 10)</th>
<th>VN-/- Mice (n = 7)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ees</td>
<td>10.58 ± 9.59</td>
<td>12.86 ± 9.24</td>
<td>NS</td>
</tr>
<tr>
<td>Emax</td>
<td>17.08 ± 11.42</td>
<td>22.74 ± 14.25</td>
<td>NS</td>
</tr>
<tr>
<td>dP/dt - EDV</td>
<td>257.55 ± 372.03</td>
<td>573.74 ± 505.96</td>
<td>NS</td>
</tr>
<tr>
<td>dP/dt – EDV intercept</td>
<td>48.01 ± 12.26</td>
<td>27.88 ± 23.34</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 4-7. Baseline hemodynamic parameter following aortic occlusion evaluated by Millar catheterization in WT and VN-/- mice. P value as determined by a paired Student’s t-test. All t-tests assume Gaussian distribution and unequal variance between groups.

<table>
<thead>
<tr>
<th></th>
<th>WT Mice (n = 13)</th>
<th>VN-/- Mice (n = 13)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ees</td>
<td>10.88 ± 8.81</td>
<td>9.77 ± 6.03</td>
<td>NS</td>
</tr>
<tr>
<td>Emax</td>
<td>17.38 ± 10.16</td>
<td>11.86 ± 6.46</td>
<td>NS</td>
</tr>
<tr>
<td>dP/dt - EDV</td>
<td>303.61 ± 561.62</td>
<td>151.36 ± 214.13</td>
<td>NS</td>
</tr>
<tr>
<td>dP/dt – EDV intercept</td>
<td>52.26 ± 59.62</td>
<td>50.79 ± 52.12</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 4-8. Hemodynamic parameter following aortic occlusion evaluated by Millar catheterization in WT and VN-/- mice at 28 days post-MI. P value as determined by a paired Student’s t-test. All t-tests assume Gaussian distribution and unequal variance between groups.

<table>
<thead>
<tr>
<th></th>
<th>WT Mice (n = 7)</th>
<th>VN-/- Mice (n = 4)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ees</td>
<td>8.66 ± 11.53</td>
<td>16.14 ± 20.96</td>
<td>NS</td>
</tr>
<tr>
<td>Emax</td>
<td>11.55 ± 12.69</td>
<td>19.66 ± 21.11</td>
<td>NS</td>
</tr>
<tr>
<td>dP/dt - EDV</td>
<td>106.64 ± 65.52</td>
<td>234.59 ± 87.78</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>dP/dt – EDV intercept</td>
<td>6.45 ± 19.28</td>
<td>24.55 ± 10.7</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 4-9. Baseline hemodynamic parameter following occlusion of the inferior vena cava as evaluated by Millar catheterization in WT and VN-/- mice. P value as determined by a paired Student’s t-test. All t-tests assume Gaussian distribution and unequal variance between groups.

<table>
<thead>
<tr>
<th></th>
<th>WT Mice (n = 9)</th>
<th>VN-/- Mice (n = 5)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ees</td>
<td>16.85 ± 16.98</td>
<td>15.66 ± 10.69</td>
<td>NS</td>
</tr>
<tr>
<td>Emax</td>
<td>22.98 ± 17.59</td>
<td>19.95 ± 12.56</td>
<td>NS</td>
</tr>
<tr>
<td>dP/dt - EDV</td>
<td>389.39 ± 314.58</td>
<td>178.53 ± 259.73</td>
<td>NS</td>
</tr>
<tr>
<td>dP/dt – EDV intercept</td>
<td>33.15 ± 13.97</td>
<td>44.49 ± 23.26</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 4-10. Hemodynamic parameter following occlusion of the inferior vena cava evaluated by Millar catheterization in WT and VN-/- mice at 28 days post-MI. P value as determined by a paired Student’s t-test. All t-tests assume Gaussian distribution and unequal variance between groups.
Intraventricular length (L) and ventricular cross sections at the sub-mitral valve (A1), papillary (A2), sub-papillary (A3) and apical (A4) levels in diastole and systole were obtained using echocardiography. The images were used to determine the systolic and diastolic volume of the hearts, which were then used to calculate ejection fraction and other hemodynamic parameters in sham-operated and infarcted hearts by using Simpson’s method.
Figure 4-18. Representative echocardiographic M-mode tracing. M-mode tracing across the LV at the level of the papillary muscles was used to measure interventricular septal (IVS) and posterior wall thickness (PWT) in systole and diastole. M-mode measurements were also used to obtain LV internal diameter (LVID) in systole and diastole at baseline controls which allowed for estimation of the ejection fraction using the Teicholz method.
Figure 4-19. VN-/- mice exhibit significant improvement in cardiac function 28 days post-MI. Echocardiographic evaluation of ejection fraction and fractional area of contractility displayed no differences between WT and VN-/- mice at baseline. In contrast, infarcted VN-/- mice exhibited significant improvements in ejection fraction (WT MI 41.4 ± 7.6% vs. VN-/- MI 48.4 ± 7.0%, p < 0.005) and fractional area of shortening (WT MI 35.9 ± 9.3% vs. VN-/- MI 43.7 ± 7.4%, p < 0.01) when compared to WT mice at 28 days post-MI.
4.4 Discussion

Post-MI wound healing is a complex process that involves proteolysis, cell migration and proliferation, and fibrosis. Many of the functions attributed to VN overlap with the responses to wound healing following ischemic injury [243]. Multiple mechanisms can be implicated in regulation of myocardial wound healing and here we investigated the potential role of VN in regulation of wound repair following MI. Following MI, VN may regulate post-MI healing by providing abundant attachment sites for various cell types through interactions with integrins and is therefore crucial in the adhesion, migration and proliferation processes particularly in area of damage. Additionally, following infarction VN may have a role in regulating cardiac function by potentially modulating/interfering with sarcomeric and/or cytoskeletal proteins, or another unidentified mechanism.

We have examined the effects of VN on cardiac structure and function following MI. The major novel finding from our study demonstrated that mice lacking VN exhibit an improvement in healing and cardiac function following infarction. Overall, deficiency in VN proved to be beneficial to post-MI wound healing which was further established by morphometric analysis of infarcted mouse hearts at 3, 7 and 28 days. Improvements could be mainly attributed to a reduction in inflammatory infiltrate and attenuation in tissue fibrosis, despite a diminished angiogenic response. A dramatic reduction in collagen scaring of LV anterior free wall was noted in VN-/- mice when compared to WT. Furthermore, both the anterior LV free wall thickness and total non-infarcted myocardial regions were increased at 28 days post-MI in VN-/- mice, suggesting of diminished remodeling taking place. The differences in wall structure of the infarcted LV observed between WT and VN-/- mice at 28 days were probably instigated by an apparent arrest in LV remodeling
due to a reduction in leukocyte infiltration in the absence of VN. LV remodeling in infarcted mice was accompanied by changes to the unaffected myocardium. However, no changes in LV diameter, RV or septum thickness were noted between WT and VN-/- at 28 days post-MI. Moreover, no significant changes in wall structure and chamber geometry were noted between the sham-operated groups. Alterations in post-MI remodeling between WT and VN-/- mice were perhaps best exemplified by the apparent differences in cardiac function. Hemodynamic measurements acquired by echocardiography demonstrated that post-MI VN-/- mice showed a significantly improved cardiac function compared to WT mice at 28 days.

The dramatic differences associated with the manifestation of VN at sites of MI are likely attributed to either plasmin dependent and/or independent mechanism(s). VN has previously been shown to have a dominant role in cell adhesion, spreading and migration. VN contains attachment sites for a number of cells including inflammatory cells, SMC, myofibroblasts and endothelial cells [244-246]. Hours to days following ischemic injury the accumulation of VN in the myocardium can promote migration and adhesion of inflammatory cells which are responsible for removal of necrotic tissue and clearing debris for the formation of new collagen network. VN which enters from plasma is localized in the ECM via the collagen binding domain and glycosaminoglycan domain [73, 74]. In the ECM, migrating cells bind via RGD sequence of VN by forming interactions with integrins (αvβ3, αvβ5) and uPAR expressed on the surface of the cell [127, 247, 248]. Days following infarction, fibroblasts begin the remodeling process by producing collagen and other structural proteins. Additionally, endothelial progenitor cells migrate to areas of damage to stimulate angiogenesis [248]. Thus, ECM VN can significantly
influence post-MI remodeling by modulating the localization of various cell types to the area of injury (Figure 4-20).

The function of VN in regulation of vascularization following injury was previously demonstrated in a number of models [109, 249-251]. These studies suggest the absence of VN considerably reduces angiogenesis, while the reverse was noted in its presence. Our findings are consistent with the above allegation as VN-/− mice experienced a reduction of CD31 and vWF positive vessels post-MI. These observations can be rationalized two-fold. First, VN is a ligand for integrin αvβ3 and αvβ5 on the surface of endothelial cells and is therefore important for vascular development [247, 252]. The absence of VN can not only limit the migration, and proliferation of endothelial cells through integrin-cytoskeletal interactions, but also lead to changes in intracellular signaling pathways which enlists an apoptotic response [109, 249, 250, 253]. Secondly, PAI-1 is a substantial factor in the angiogenic response. While a lack of PAI-1 completely abolished angiogenesis in mice, at physiological concentrations PAI-1 promotes angiogenesis [254, 255]. Although such effects are accomplished through PAI-1's antiproteolytic activity rather than by its interactions with VN, in the absence of VN levels of PAI-1 are considerably reduced [254, 256]. However, despite the unfavorable antiangiogenic effects, VN-/− mice sustained better cardiac healing and function post-MI.

The importance of integrins in post-MI cardiac remodeling has been recognized for some time. In addition to controlling growth factors and hemodynamic effects, regulation of integrin activity through ECM protein interactions also affect cardiac fibrosis and remodeling process. ECM proteins can
Figure 4-20. VN modulates cell migration at sites of post-MI injury. In the ECM, migrating cells bind VN and fibronectin (FN) via RGD sequence by forming interactions with integrins (αvβ1, αvβ3, αvβ5) and u-PA receptor (uPAR) expressed on the surface of the cell. In days post-infarction the accumulation of VN in the myocardium promotes migration and adhesion of inflammatory cells such as monocytes (Mono), eosinophils (Eos) and polynuclear monocytes (PMN) which are responsible for clearing of necrotic debris. The binding of fibroblasts (FB) and endothelial cells (EC) also assist in the remodeling process by producing collagen and other structural proteins, and stimulate vascular genesis.
influence integrin activity and vice versa. For example, both collagen and FN induces MMPs, collagenases and gelatinases through their interactions with integrins [257, 258]. Additionally, shape, migration, proliferation and apoptosis are all regulated by ECM molecules through integrin-cytoskeleton and intracellular pathways in SMC and endothelial cells [252, 258]. Integrins which bind to the RGD motif are also imperative in the angiogenic process [249, 257, 259]. VN binds to various integrins and interacts preferentially with αvβ1, αvβ3, αvβ5 which may be responsible for attachment of cells, prevention of microvascular cell apoptosis, as well as neo-vessel synthesis and regeneration in the infarcted myocardium [260, 261]. Specifically, integrin αvβ3, also known as the VN receptor has a significant role in vascular remodeling and cardiac fibroblast action [247, 262]. Thus, as anticipated VN-/- mice exhibited some attenuation of angiogenesis and fibrosis post-MI. However, it is highly improbable that VN is the sole ECM RGD protein involved in the post-MI ECM remodeling process [247, 263, 264]. Undoubtedly, other RGD proteins such as FN are involved in leukocyte recruitment and migration, angiogenesis, and tissue fibrosis. Therefore, although the wound healing response following MI is notably altered in the VN-/- mice compared with WT it is not drastic in nature. Work from our laboratory also demonstrates that bone marrow cells localize particularly in the VN-rich infarct region (Figure 7-3). Such cells have the potential to differentiate into functional cardiomyocytes or other cardiogenic cell types [265-267] [268, 269]. Thus, in absence of VN, there are profound changes to integrin-dependent attachment of cell to ECM scaffold, as well as VN-stimulated intracellular signaling pathways.

uPAR is another important regulator of cellular communication which is in-part controlled by interacting with VN. This molecule is expressed on a number of
cell types including leukocytes, endothelial, SMC, fibroblasts and bone marrow cells [248, 270]. uPAR is a receptor for VN, and by binding u-PA it increases its affinity for VN and enhances the adhesiveness of uPAR-expressing cells [271-273]. Furthermore, the association of uPAR with integrins and/or VN allows for regulation and modulation of ligand binding, cellular signaling, as well as control cell uptake/invasion [259, 270]. Thus, uPAR may also play an integral role in post-MI wound healing through its participation in cell adhesion and signaling. However, through differential interactions uPAR and integrin αv mediated cell adhesion is competed for by PAI-1 [270, 274, 275]. Binding sites for uPAR and PAI-1 lie within close proximity along the amino-terminal domain of VN, thus there is direct competition between uPAR and PAI-1 VN binding. Previous studies demonstrated that in mice deficient in PAI-1, neovascularization is significantly impaired suggesting an anti-adhesive function of PAI-1 in cellular interactions [276]. Although the role of uPAR and its interactions with VN and integrins was not explored in this study, uPAR expression is observed in organs undergoing extensive tissue remodeling [277]. Thus, it is probable that uPAR is also involved in the regulative processes associated with post-MI wound healing by interacting with VN although the exact role remains to be investigated.

Integrin-dependent migration of cells to sites of injury is in-part contingent on the accumulation of VN in the ECM [67, 274]. However, binding of PAI-1 to the somatomedin B domain of VN sterically inhibits the integrin recognition site, thus preventing cell migration, adhesion and spreading [251, 278]. Recent studies with mouse models of pulmonary and dermal injury suggest that through interactions with PAI-1, VN is the driving force in preventing repair and commencing fibrosis [279, 280]. Similar to human studies, post-MI, WT mice experience elevated PAI-1
levels which block integrin binding sites on VN partially limiting the attachment of cells, however in absence of VN the binding of cells is severely limited. Therefore, a deficiency in VN reduces PAI-1 levels and activity in the wound matrix, concurrently reducing cell adhesion and migration. Despite the fact that VN is involved in various physiological and pathological functions, the VN-/- phenotype is not embryonic lethal [115]. Thus, it is probable that other protein(s) facilitate similar function in the absence of VN. FN is one such potential candidate, since like VN it is a major adhesion protein with common structural and functional characteristics [281]. Like VN, FN mediates a wide variety of cellular interactions with the ECM and plays important roles in cell adhesion, migration, growth and differentiation [282, 283]. Moreover, besides binding to cell surfaces via integrins, FN binds to a number of biologically important molecules including heparin, collagen/gelatin, and fibrin [282].

It is also probable that VN may influence post-MI cardiac function directly by modulating cardiomyocyte contractility. The regulation of cardiomyocyte contractility may potentially involve the binding of VN to sarcomeric and/or cytoskeletal proteins. One such candidate protein includes desmin IF, as discussed in detail in Chapter 6. Additionally, VN may also regulate cell contractility via integrin specific interactions. Previous studies reported that by binding to αv and β1 integrins on SMC, VN inhibits contractility by stimulating phenotypic cell transformation [245]. Furthermore, VN reduced the contractile ability and altered the phenotype of human lung fibroblasts down-regulating α-smooth muscle actin expression via integrin- dependent interactions [284]. Adult cardiomyocytes express numerous integrins including alpha 1, 3, 5, 6, 7, 9, and 10, as well as beta 1, 3 and 5 [76, 285]. Thus, it is plausible that through specific interactions with
integrins, VN is involved in signaling pathways which may depress contractility by regulating sarcomeric protein(s) expression. Alternatively, improvement in cardiac function can be attributed to increased numbers of surviving myocytes and/or a reduction in fibrosis. VN-/- mice sustained a substantial decrease in post-MI collagen deposition in the infarcted LV as demonstrated by picrosirius red staining at 28 days. Moreover, when compared to WT mice, VN-/- exhibited an increase in area of viable muscle 28 days post-MI. Thus, the VN-/- phenotype may be inclined to protect the myocardium from ischemic damage and/or prevent excessive remodeling, thus improving cardiac function.

4.5 Conclusions

Here we have shown the detrimental effects of VN in wound healing following MI. Ligation of the LAD in mice deficient in VN demonstrated a reduction in the inflammatory response, decreased collagen scar formation, altered angiogenesis and improved cardiac function. Together, our findings suggest that VN plays a detrimental role in post-MI wound healing. Thus, potential strategies aimed at preventing VN deposition at sites of ischemic myocardium may be a novel means of preserving function and improving healing post-MI. VN may modulate cardiac fibrosis through integrin-dependent attachment of inflammatory cells at sites of infarction although PAI-1-dependent anti-proteolytic properties may also play a significant role. The precise role of VN in post-MI remodeling remains to be further consideration, however our work suggests that absence of VN exhibits substantial cardioprotective effects by preventing damage to the myocardium during ischemia and/or promoting post-MI healing. Exploring and understanding the means of VN regulation of processes involved in post-MI remodeling and analysis of
mechanism(s) of action can advance our knowledge with respect to post-MI cardiac healing. Consequently, this may stimulate exciting opportunities to develop new therapeutic strategies to prevent or treat MI.

4.6 Additional Considerations

Although VN is also thought to participate in various other pathological processes such as the regulation of the immune and complement systems, the work presented here did not investigate the role of VN in that respect. At sites of myocardial ischemic injury VN is co-deposited with membrane attack complex (MAC) and may encourage the clearance of injured cells [286]. Furthermore, VN may also play a role in post-MI apoptosis. Previous reports demonstrated that VN is involved in ECM remodeling via granzyme B dependent activity. Granzyme B which is released from cytotoxic lymphocytes has a role in the induction of target cell apoptosis and anoikis, and is stimulated in part by cleaving the RGD motif of VN affecting the integrin-binding site [287]. Preliminary immunohistochemical studies from our laboratory demonstrated that VN-/- mice exhibited an increase in terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) stained cells at 3 and 7 days post-MI, contrary to expectations. However, further studies are necessary in order to examine the role of VN in post-MI apoptosis.

Furthermore, despite only minor complications experienced in obtaining functional measurements using echocardiography, it should be stated that these experiments were performed on mildly sedated animals. Though previous studies have successfully performed echocardiography in non-sedated animals, we were unable to use this technique in our facility. The probe used contained a mechanical transducer in a liquid medium which could only be mounted in a vertically and
required the animal to be oriented in a supine position. Attempts to orient the probe in a different fashion resulted in formation of air bubbles which prevented obtaining usable images.

Additionally, we experienced difficulties obtaining open-chest hemodynamic assessment using pressure-volume conductance systems. By using a 1.2 French probe inserted trans-apically we were able to obtain data only 70% of time and were not always able to obtain adequate wave-forms for aortic and IVC occlusions. Aortic occlusion seemed to be more reliably reproducible and IVC occlusion could only be repeated a few times before the animal succumbed. Although smaller probes are currently available, we did not wish to substitute the probe used during the course of the experiment and managed to conduct all the tracings for the whole experiment using a single catheter.

Finally, functional evaluation of infarcted mice was performed at 28 days only, with no subsequent testing taking place. Development of heart failure traditionally begins to manifest six to eight weeks in murine models post-MI which generally results in rapid increase in weight caused by excessive fluid retention and development of dyspnoea caused by LV dysfunction. These outcomes were not observed in the infarcted animals at the time of the experiment as the duration of the experiment was relatively short. Although we were able to study the effects of VN on post-MI remodeling, we were unable to investigate the function of VN in the development of heart failure.
5.1 Rationale

Although in the last decade our understanding of post-MI remodeling has considerably advanced, to date the precise mechanism(s) of wound healing requires additional clarification. Studies described in Chapter 4 demonstrated that VN plays a significant role in post-MI remodeling by influencing cardiac structure and function. The PA/plasmin and MMP systems play a major role in wound healing post-MI. VN is an acute phase plasma protein which closely interacts with components of PA/plasmin system, regulating the deposition of PAI-1 in damaged tissues. Many unique VN-dependent binding interactions which regulate the deposition of PAI-1 in thrombi and damaged tissues have been studied. These include the biochemical, morphologic and functional characterization of the multimeric form of the VN:PAI-1 complexes, and the VN-dependent binding of these multivalent VN:PAI-1 complexes to the surface of fibrin fibrils expressed on the surface of activated platelets and platelet microparticles. Additionally, studies demonstrated that VN prevents proper remodeling at sites of skin and lung injury directly by interacting with PAI-1.

Hence, it is plausible that VN by mediating the binding of VN:PAI-1 complexes to polymeric structures in damaged myocardium, may also be an important determinant of post-MI ventricular revascularization and remodeling. Since we have established that VN has a significant role in post-MI remodeling, we wanted to determine whether it does so through the regulation of the PA/plasmin system.
5.2 Experimental Methodology

5.2.1 mRNA expression using RT-PCR

Tissue samples stored in RNAlater® solution at −80°C were lysed by mechanical homogenization at medium speed to avoid RNA degradation. Total RNA was extracted using RNeasy Protect Mini Kit (Qiagen) RNA expression within the infarcted LV and non-infarcted RV were measured using spectrophotometry following which 6 μg of total RNA was reverse transcribed in 60 μL volume using reverse transcriptase with 1 μg random primers. For each RT product, aliquots (2–10 μl) of the final reaction volume were amplified in four parallel PCR reactions using VN, PAI-1, MMP-2 and 9, t-PA, u-PA, desmin and GAPDH-specific primers (NAPS, University of British Columbia, Vancouver) (Table 5-1) and Taq polymerase. RT-PCR products were separated on agarose gels containing ethidium bromide and quantification performed by densitometry using Image J v1.38. All measurements were expressed as the ratio between the target genes and GAPDH, or as a comparison of target genes from control mice and MI mice. mRNA from three animals from each group at each time-point were used and the mean value calculated and compared by ANOVA.

<table>
<thead>
<tr>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td><strong>Designation</strong></td>
</tr>
<tr>
<td>VN</td>
</tr>
<tr>
<td>u-PA</td>
</tr>
<tr>
<td>t-PA</td>
</tr>
<tr>
<td>PAI-1</td>
</tr>
<tr>
<td>MMP-2</td>
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<tr>
<td>MMP-9</td>
</tr>
<tr>
<td>GAPDH</td>
</tr>
</tbody>
</table>

Table 5-1. Sequences and sizes of primers used for RT-PCR.
5.2.2 **Protein distribution in the infarcted LV**

Modified Triton-X soluble and insoluble LV heart extracts (50 µg/lane) were treated with sample buffer (100 mM Tris buffer, pH 8.3 containing 2% SDS, 5 mM EDTA, 10% glycerol, 15 mM dithiothreitol) and fractionated by SDS-PAGE. The gel was washed in distilled water and incubated with EZBlue™ gel staining reagent (Sigma Aldrich) overnight at room temperature. The EZBlue™ gel staining reagent was then removed and gel washed in distilled water for 1 hour. The gel was scanned in and visualized (Adobe Photoshop Professional 7.0).

5.2.3 **Western blot protein analysis**

Western blot analysis was conducted as previously described [287]. Briefly, Triton-X soluble and insoluble heart extracts (20 µg/lane) were treated with lysis buffer (100 mM Tris buffer, pH 8.3 containing 2% SDS, 5 mM EDTA, 10% glycerol, 15 mM dithiothreitol) and fractionated by SDS-PAGE. After fractionation, the gels were electrophoretically transferred onto nitrocellulose membranes. Subsequently, the membranes were blocked with PBS containing 10% casein (PBS/casein), then incubated with either anti-mouse t-PA IgG, anti-mouse u-PA IgG, anti-mouse PAI-1 IgG, anti-mouse desmin and anti-mouse β-actin IgG (Sigma Aldrich) for 1 hour. The membranes were then incubated with alkaline phosphatase-conjugated secondary Ab and processed for colour development with the Fast Red system.

5.2.4 **MMP-2 and 9 activity gelatin zymography**

Gelatin zymography for MMP-2 and MMP-9 activity in extracts reflects the influence of plasmin-mediated activation of MMPs [189]. Homogenized tissue was
separated on SDS-PAGE gels containing gelatin substrate to analyze MMP-2 and 9 activity. Following electrophoresis, gels were washed in distilled water containing 5% Triton-X-100 for 1 hour, then incubated in incubation buffer and stained with Coomassie blue staining reagent. Gels were then de-stained and bands indicating areas of substrate proteolysis were visualized. Clear bands seen on gelatin gels were confirmed as MMPs by addition of 10mM EDTA to the incubation buffer to inhibit enzyme activity. Digestion bands were quantified by densitometry using Image J v1.38.

5.2.5 Statistics

Statistical significance of differences was assessed using Student's *t*-tests and comparison among multiple groups was performed one-way analysis of variance (ANOVA); *p*-value of 0.05 or less was considered significant. All values are expressed as means ± SEM or means ± SD.
5.3 Results

5.3.1 Differential gene expression of PA/plasmin system components post-MI

We have assessed mRNA expression levels of genes associated with the PA/plasmin and MMP systems (u-PA, t-PA, PAI-1, MMP-2 and 9), involved in the regulation of ECM remodeling at 3, 7, 14 and 28 days post-MI in WT and VN-/− mice. Representative gels demonstrated differences in mRNA expression levels between WT and VN-/− mice (Figure 5-1a). VN-/− MI mice exhibited more robust band in t-PA at 3 days post-MI. At 7 days post-MI VN-/− exhibited increased u-PA mRNA, while PAI-1 was decreased when compared to WT. MMP-2 levels were slightly higher in WT mice particularly at 3 and 7 days post-MI, with only trivial differences noted in MMP-9 mRNA levels. Desmin mRNA was expressed at all time-points in WT and VN-/− mice with slightly more robust bands noted in sham-operated and infarcted WT mice at 3 days post-MI.

Pooling and averaging of data gathered from individual WT (n = 3) and VN-/− (n = 3) mice demonstrated common trends in mRNA expression (Figure 5-1b). VN-/− exhibited a decrease in RNA levels of all genes at 3 and 7 days when compared to WT MI mice. At 3 days post-MI all genes in WT mice were elevated with at least a 2-fold difference compared to VN-/− mice. Interestingly, mRNA levels for all genes investigated were elevated above baseline at 3 and 7 days post-MI in the WT MI group, then returning near baseline levels by day 28. No distinct pattern in mRNA expression was noted in the VN-/− MI group at 3, 7 and 28 post-MI. Results of mRNA expression levels are detailed in Table 5-1.
<table>
<thead>
<tr>
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<th>Fold Change vs. Control (n = 12)</th>
<th>% of GAPDH (n = 12)</th>
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<tr>
<td></td>
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<td>VN-/- MI</td>
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<tr>
<td>GAPDH</td>
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<td>1.02</td>
</tr>
</tbody>
</table>

Table 5-1. Numerical data corresponding to quantitative RT-PCR from histograms in Figure 5-1. All mRNA level measurements are expressed as a ratio of MI group/control group or as % GAPDH.

5.3.2 Protein distribution of infarcted myocardium of WT and VN-/- mice

Proteins from infarcted LV were fractionated using Triton-X 100 soluble and insoluble lysis buffer to depict the relationship between the morphological fractionation and the protein populations obtained after each fractionation step. Proteins obtained from each fraction step were visualized using EZblue stain.
The Triton-X-100-soluble “cytoplasmic” proteins constituted ~75% of the total cell protein. The Triton-X-insoluble “cytoskeletal” fraction was ~20% with the remaining 5% of the total cell protein regarded as the “chromatin” fraction. The soluble fraction represents the majority of cellular proteins while the remaining proteins were primarily cytoskeletal in composition.

5.3.3 Post-MI t-PA, u-PA, PAI-1 and desmin expression

We have investigated the effects of VN in regulation of components of PA/plasmin system following MI. Western blotting analysis demonstrated presence of t-PA in the infarcted LV of WT and VN-/- mice. Increased levels of t-PA were noted in VN-/- primarily at 3 and 7 days post-MI compared to WT mice, with peak accumulation seen at day 7 (Figure 5-3). u-PA was noted from 3 to 28 days post-MI, with slight differences in levels noted between WT and VN-/- mice at 3, 7 and 14 days (Figure 5-3). Additional strong bands ~ 82 kDa were noted in VN-/- mice at 3 and 7 days post-MI (arrowheads). Presence of bands ~ 100 kDa likely represent u-PA : PAI-1 complex formation and were more pronounced at 3 and 7 days post-MI in WT and VN-/- mice. Unidentified high molecular weight bands that appeared ~170 kDa may represent additional complex formation between u-PA, PAI-1 and uPAR (arrowhead). Reduced levels of PAI-1 were primarily noted in control and infarcted mice from 3 to 28 days post-MI. In contrast to both PAs, PAI-1 bands were markedly reduced in VN-/- at all time-points (Figure 5-3). Furthermore, robust desmin bands were observed at 7, 14 and 28 post-MI in WT and VN-/- mice. Interestingly, WT mice exhibited an increase in desmin levels when compared to VN-/-, which was particularly evident at 7 and 14 days post-MI (Figure 5-3).
Figure 5-1a. Representative gels of PAI-1, t-PA, u-PA, MMP-2 and 9, and desmin mRNA expression levels at 3, 7 and 28 post-MI. Infarcted VN-/− mice exhibited notable differences between of t-PA, u-PA, PAI-1, MMP-2, MMP-9 and desmin mRNA expression levels when compared to WT mice.
Figure 5-1b. VN-/– mice exhibit different mRNA expression of PAs, PAI-1 and gelatinases in the infarcted LV over time. Assessment of mRNA expression for genes involved in regulation of post-MI ECM remodeling indicated substantial variability between WT and VN-/– mice at 3, 7 and 28 days post-MI. Generally, mRNA expression levels were markedly elevated in WT mice with a minimum 2-fold difference above baseline. WT mice followed a common pattern in which mRNA levels were increased between day 3 and 7 post-MI, returning back to baseline levels by day 28. No steady patterns of mRNA expression were evident in VN-/– mice, although levels for all evaluated genes were lower when compared to WT mice at days 3, 7 and 28 post-MI respectively.
Figure 5-2. Protein distribution in Triton-X soluble and insoluble LV fractions of WT and VN/- mice. EZblue stain shows an accumulation of proteins in the Triton-X 100 soluble “cytoplasmic” and insoluble “cytoskeletal” fractions derived from normal and infarcted LVs.
Figure 5-3. VN affects the regulation of the PAs and PAI-1 post-MI. Western blotting of Triton-X soluble extracts of sham-operated and infarcted adult rat hearts (20 µg/lane) reveals the accumulation of the PAs, PAI-1 and desmin. Infarcted VN-/- mice exhibited notable differences between of t-PA, u-PA, PAI-1, compared to WT mice. Levels of t-PA and u-PA appeared increased in VN-/− mice mainly in the first 7 days post-MI. Conversely, PAI-1 was found predominantly in WT mice.
5.3.4 Immunostaining for t-PA, u-PA and PAI-1 at 3, 7 and 28 days post-MI

Staining for both t-PA and u-PA was absent from the hearts of sham-operated controls. PAI-1 staining was present in hearts of both WT and VN-/- sham-operated mice (Figure 5-4). Staining for t-PA was localized in WT mouse hearts primarily at 3 days post-MI, with reduced staining observed at 7 and 28 days (Figure 5-4). Similar pattern in t-PA staining was noted in hearts of VN-/- mice at 3, 7 and 28 post-MI. Positive staining for u-PA was observed at 3 days post-MI in heart of WT mice, while staining was primarily absent at 7 and 28 days (Figure 5-4). VN-/- mice experienced faint staining for u-PA at 3 days post-MI, and more prominent staining noted at 7 days. Strong staining for PAI-1 was observed in both WT and VN-/- mouse hearts and distributed in throughout the viable myocardium at 3 and 7 days post-MI, with faint staining observed in WT mice at day 28 (Figure 5-4).

5.3.5 Evaluation of MMP 2 and 9 activity in infarcted myocardium

Gelatin zymography was performed in order to investigate the effects of post-MI MMP 2 and 9 activity. MMP-2 and 9 activity was absent from control mice. We observed a modest increase in MMP-9 activity in the cytoplasmic fraction of the infarcted LV of VN-/- mice 3 and 28 days post-MI. Levels of both pro- and active MMP-2 levels were also significantly elevated 14 days post-MI in VN-/- mice when compared to WT (Figure 5-5). The increase in MMP activity noted in infarcted VN-/- mice were likely attributed to the elevated PA levels. Moreover, cytoskeletal fraction of the infarcted LV from both, WT and VN-/- mice showed comparable
results to those observed in the cytoplasmic fraction with negligible reduction in MMP 2 and 9 activity (Figure 5-6).

5.3.6 Post-MI distribution of alpha v integrin

We have investigated the localization and distribution of αv integrin present on the surface of FB, SMC and endothelial cells by confocal microscopy. In WT mice, intense staining for αv integrin was observed in the infarcted LV at 14 days post-MI where it was colocalized with VN (Figure 5-7, arrow). Faint αv staining was also noted in the healthy myocardium of the peri-infarct region in which staining for VN was absent (Figure 5-7,*). Higher magnification images of the infarct region showed accumulation of VN distributed in between cells positive for αv. Normal myocardium from sham-operated control mice exhibited uniform staining for αv throughout the myocardium (Figure 5-7, inset).
Figure 5-4. Localization of t-PA, u-PA and PAI-1 in infarcted LV of WT and VN-/- mice. Brightfield images (4x) depict the accumulation of t-PA, u-PA and PAI-1 in the infarcted LV at 3, 7 and 28 days post-MI. Scale bar = 1000 µm.
A Days Post-MI

<table>
<thead>
<tr>
<th>Control</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>28</th>
<th>60</th>
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<tbody>
<tr>
<td>+/+</td>
<td>-/-</td>
<td>+/+</td>
<td>-/-</td>
<td>+/+</td>
<td>-/-</td>
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<tr>
<td>-/-</td>
<td>+/+</td>
<td>-/-</td>
<td>+/+</td>
<td>-/-</td>
<td>+/+</td>
</tr>
<tr>
<td>proMMP-2</td>
<td>MMP-9</td>
<td></td>
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<td>MMP-2</td>
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</table>

B

Figure 5-5. MMP-2 and MMP-9 activity in Triton-X soluble LV fraction of WT and VN-/- mice. A) Representative zymograms for MMP-2 and MMP-9 in infarcted WT and VN-/- mice. B) Relative levels of pro and active MMP-2 were significantly increased in both WT and VN-/- mice post-MI. Compared to WT, VN-/- mice had increased MMP-2 activity at 14 days post-MI. MMP-9 activity was elevated in WT and VN-/- mice 3 days post-MI, following which only trivial activity was noted until day 28.
Figure 5-6. MMP-2 and MMP-9 activity in Triton-X insoluble LV fraction of WT and VN-/- mice. Representative zymograms demonstrated increased pro and active MMP-2 in both WT and VN-/- mice beginning at 7 days post-MI. Compared to WT, VN-/- mice exhibited increased pro and active MMP-2 activity at 14 days post-MI, however activity was minimally increased in WT at days 28 and 60. Weak signal for MMP-9 was only observed at 3 days post-MI in WT and VN-/- mice.
Figure 5-7. VN localizes to integrin alpha v in the infarcted LV 14 days post-MI. Confocal images from infarcted WT mice demonstrated the accumulation of VN (red) in the infarct region (arrow) which localized to areas of alpha v integrin (blue). Accumulation of VN and alpha v staining were more pronounced in areas of the infarcted LV. Reduced staining for both alpha v integrin and VN were localized in the peri-infarct region (*). Alpha v staining was prominent throughout the myocardium of sham-operated control mouse heart (inset). Scale bar = 200 μm (10x) and 40 μm (63x).
5.4 Discussion

The PA/plasmin and MMP systems have been implicated in regulating post-MI wound repair process [117, 216]. Although excessive PA and/or MMP levels, or the absence of their inhibitors are associated with a detrimental outcome post-MI [156, 181, 185], modest expression/activity are essential to normal wound healing. PAI-1, the major inhibitor of PAs, has been implicated in adverse post-MI remodeling as its increased expression prevents matrix degradation and promotes cardiac fibrosis [131, 216]. By interacting with PAI-1, VN stabilizes PAI-1’s conformation and promotes its inhibitory activity in the blood and tissue matrix [82, 288, 289]. Thus, the binding of VN to PAI-1 inhibits fibrinolysis, interferes with cell adhesion and migration, and therefore alters tissue remodeling.

Investigation at the molecular level demonstrated that VN manipulated the remodeling process by regulating the PA/plasmin system as observed when comparing WT and VN-/- mice post-MI. VN-/- mice demonstrated reduced mRNA expression of PAI-1, u-PA, t-PA and MMPs 2 and 9 when compared to WT mice, particularly in the first week post-MI. At 28 days post-MI mRNA expression levels returned back to baseline or were only slightly elevated in WT and VN-/- mice. Protein levels were not consistent with that of mRNA expression levels at the specified time-points as VN-/- mice exhibited increased t-PA at seven days post-MI and differences in u-PA levels were negligible between WT and VN-/- mice. Interestingly, unknown bands noted at ~ 82 kDa were found only in the infarcted VN-/- hearts when detected with anti-u-PA Ab. This may suggest that in the absence of VN, u-PA is better able to interact with a specific protein(s) of unknown origin. Binding interactions of u-PA with other proteins in the absence of VN should be explored in more detail as they may be relevant to post-MI wound healing.
Interestingly, despite the elevation of t-PA and u-PA, and decrease in PAI-1, we did not observe delayed clotting or excessive bleeding in VN/-/- mice at time of surgery. Conversely, infarcted WT mice exhibited increased levels of PAI-1, with a reduction of t-PA and u-PA. Additionally, bands indicative of complexes formed between PAI-1 with either t-PA and u-PA were more profound in WT mice, suggesting an increase in PAI-1 levels and/or activity.

Desmin levels were also increased in both, WT and VN/-/- particularly at 7 and 14 days post-MI. These findings were likely associated with post-MI cardiomyocyte hypertrophy which initiates changes in sarcomeric proteins commonly noted in the progression to heart failure [290, 291]. Additionally, the increase in desmin noted in infarcted WT mice was consistent with increases in viable myocardial areas, not observed in infarcted VN/-/- mice. In comparison to VN/-/- mice, increased desmin levels in infarcted hearts of WT mice was potentially attributed to an elevated hypertrophic response resulting from increased myocardial loss which was reflected by augmented fibrosis.

Even though discrepancies in expression levels of mRNA and protein were observed, such results are relatively common and have been previously described in the literature [292-294]. Poor correlations between protein and mRNA can be attributed to a number of reasons. First, there are many complicated and varied post-transcriptional mechanisms involved in turning mRNA into protein which lack sufficient understanding to be able to compute protein concentrations from mRNA; furthermore, proteins may express substantial differences in half-life; and/or significant amount of errors and noise in both protein and mRNA experiments are present [292, 293]. Interestingly, gelatin zymograms were in accordance with protein expression levels as determined by Western blotting, as post-MI VN/-/- mice
exhibited increased MMP-2 and -9 activity compared with that of WT. These findings suggest that activation of the MMPs in the post-MI myocardium is primarily a plasmin-dependent event which is, at least in part, manipulated by VN [181, 295].

The PA/plasmin and MMP systems are involved in cell migration, activation of proteases and inflammatory cytokines, as well as ECM synthesis and degradation [181]. Remodeling following MI requires modifications to the ECM which is achieved in-part by a tight regulation of proteases and their inhibitors for the maintenance of proper function. PAI-1 plays a major role in post-MI myocardial fibrosis by preventing the activation of PA (and hence MMPs) which may contribute to progression of LV dysfunction [216, 296]. Significant increase in PAI-1 levels have also been observed in aging hearts and scars from failing hearts [296-298]. Moreover, deficiency in PAI-1 is not only protective from myocardial, pulmonary and cutaneous fibrosis but also accelerates wound healing [129, 297-299]. Deposition of VN conceivably regulates the fibrinolytic response in the provisional wound matrix by binding and stabilizing PAI-1 [300]. By forming complexes with PAI-1, VN extends the half-life of PAI-1 ultimately reducing plasmin activity. Additionally, binding of VN to PAI-1 causes a conformational change which promotes VN multimerization [288] and stimulates additional PAI-1 binding [82]. Prolonged accumulation of VN at sites of damage of various tissues reflects chronic injury and is associated with fibrosis [301-303]. We also found that VN localized at sites of chronically infarcted and scarred myocardium in both human and mouse. In our study the absence of VN in infarcted mice was correlated with reduced leukocyte infiltration and angiogenesis, and importantly a dramatic reduction in myocardial fibrosis. Thus, it is possible that VN may participate in regulating fibrosis and progression to heart failure by modulating PAI-1 activity. One prospective
mechanism for induction of post-MI fibrosis is based on increased activation of TGF-β which is caused by increased PAI-1 and decreased plasmin activity [304]. Based on our study it is apparent that the results from infarcted VN-/− mice are at least partially attributed to a decline in PAI-1 expression and activity levels, which drastically affected PAs and MMPs. Thus, VN appears to be a modulator of proteolytic cascades responsible for plasmin-dependent ECM proteolysis by interacting with PAI-1.

5.5 Conclusions

VN plays an important role in wound healing following MI. Here, we have established that VN mediates post-MI remodeling in a plasmin-dependent manner likely by regulating PAI-1 levels. Mice deficient in plasminogen lack proper wound healing post-MI which suggests that u-PA, possibly in conjunction with t-PA, is important in the pathogenesis of post-MI myocardial fibrosis. Thus, the increased levels of the PAs and MMP-2 and 9 promote tissue healing, and hence cardiac function as noted in the post-MI VN-/− mice. Although further studies are necessary to establish the precise involvement of VN in regulating the PA/plasmin and MMP systems, it can be concluded that VN is involved in modulation of these systems post-MI.

5.6 Additional Considerations

We have suffered unfortunate complications identifying the expression of various proteins using Western blotting. Difficulty arose mainly from the inability to visualize proteins on our membrane, even though experiments were excessively
replicated. Due to a limited number of mouse heart tissue samples designated for protein studies we could only perform a finite number of experiments for each individual protein of interest. We have failed to obtain suitable Western blots for MMPs 2 and 9, uPAR, or TIMPs 1 and 4. Additionally, it would be both interesting and beneficial to investigate the function of various other proteins which are important in post-MI remodeling. These proteins can come from diverse families including inflammatory cytokines; TGF-β, TNF-α, IL-6, structural and motility proteins; TIMPs, myosin, actin, collagen and vimentin, cell defense; complement protein and MHC class I, to list a few. Regrettably lack of sufficient protein and mRNA tissue samples significantly hindered our efforts in looking at expression and activity levels of several other proteins of interest. We have performed a thorough assessment with regard to mRNA and protein expression and activity in the infarcted myocardium of WT and VN-/− mice. However, further exploration on the molecular events occurring in the viable, non-infarcted myocardium would be useful in developing a better understanding in the role VN plays in post-MI healing. Although we did not commence such experiments at the present time, we are planning on pursuing them in the near future.
CHAPTER VI EFFECTS OF VITRONECTIN AT SITES OF HYPOXIA AND MYOCARDIAL ISCHEMIA

6.1 Rationale

Results from Chapter 4 and 5 demonstrate that the presence of VN in the infarcted myocardium significantly reduces cardiac function. Upon closer examination of infarcted myocardium we noted that VN accumulated in ischemic cardiomyocytes and localizes to sarcomeric proteins. Although the accumulation of VN at sites of MI has previously been observed the exact mechanism(s) involved in its regulation of cardiac function have not been elucidated. Previously we have shown that VN-dependent binding of multivalent VN:PAI-1 complexes is mediated by vimentin. Vimentin expresses high affinity binding of VN on surface of activated platelets.

Desmin, an IF structurally similar to vimentin is integral to maintaining the structural and functional integrity of cardiomyocytes by linking myofibrils in bundles and the entire contractile apparatus to the sarcolemmal cytoskeleton, cytoplasmic organelles and nucleus. Additionally, through the inter-myofibrillar connections desmin filaments align Z-lines in neighboring cells stabilizing sarcomeres in contracting muscle and protecting cells from repeated mechanical stress. Numerous types of cardiomyopathies are associated with acquired desmin mutations in humans or transgenic mice which underscores the importance of desmin in cardiac function.

Furthermore, it was previous established that contractility was significantly reduced in myofibroblasts and smooth muscle cells in the presence of VN. Thus, in this study we explored the binding interactions of VN with desmin IF at sites of
myocardial ischemia and infarction and investigated whether the accumulation of VN within ischemic rat cardiomyocytes was associated with a reduction of contractility.
6.2 Experimental Methodology

6.2.1 *Ex vivo* Langendorff perfusion of rat heart with rat serum and fibrinogen

Adult male Sprague Dawley rats were sedated with isofluorane and the hearts excised and collected for *ex-vivo* Langendorff perfusion. The perfusate was a modified Tyrode solution ($128 \text{ mM } \text{NaCl}, 4.7 \text{ mM } \text{KCl}, 1 \text{ mM } \text{MgCl}_2, 0.4 \text{ mM } \text{NaH}_2\text{PO}_4, 1.2 \text{ mM } \text{Na}_2\text{SO}_4, 20.2 \text{ mM } \text{NaHCO}_3, \text{ and } 1.3 \text{ mM } \text{CaCl}_2$) containing 11.0 mM glucose. Perfusate temperature was kept at 37°C and equilibrated with either 95% O$_2$-5% CO$_2$ at pH 7.35 for up to 1 hour. In some experiments rat serum or Alexa 594 conjugated fibrinogen was added to the perfusate. Following the experiment the hearts were perfused-fixed with 4% paraformaldehyde and sectioned serially for immunofluorescence studies.

6.2.2 Mouse model of left anterior descending coronary artery ligation

Detailed mouse model of MI by LAD ligation has been described previously [304]. All animal experiments were approved by the University of British Columbia Committee on Animal Care. Briefly, 12-week-old C57B6/6J mice ($n = 18$, weight 20–25-g) underwent surgical ligation of the LAD, resulting in anterior wall MI. Animals were intubated and ventilated with room air at 100 breaths per minute using a rodent ventilator (Inspira, Harvard Apparatus, Holliston, MA). Under microscopy (Zeiss Contraves, Carl Zeiss Inc, Thornwood, NY), sternotomy was performed and the proximal LAD was identified as it appeared below the left atrium and ligated with 8/0 Prolene (Surgical Specialities Corp, Reading, PA). Blanching and dysfunction of the anterior wall verified LAD ligation. Ligation was sustained for 45 minutes, 90 minutes, 3 hours, 6 hours and 1 day, following which a thoracotomy
was performed and hearts rapidly excised into ice-cold saline, then manually compressed to remove residual blood. Hearts for histology were cannulated by the aorta and perfused-fixed with 4% paraformaldehyde at a constant pressure. Hearts were kept in 10% formalin overnight and then embedded in paraffin. In hearts for protein analysis, infarct regions were dissected out under a surgical microscope and both infarcted (LV) and non-infarcted (RV and interventricular septum) myocardium was flash-frozen in liquid nitrogen and stored at −80°C.

6.2.3 Immunohisto- and immunocytochemistry

Paraffin-embedded human heart tissues were obtained from the Cardiac Registry at St. Paul’s Hospital in Vancouver, British Columbia. For brightfield/polarized light microscopy mouse hearts were serially sectioned and subjected to routine immunohistochemistry using purified polyclonal rabbit anti-VN antibody (Ab) (Molecular Innovations) and polyclonal anti-human albumin Ab (Sigma) or pre-immune IgG. Anti-albumin Ab was used as a control to determine whether accumulation in the damaged myocardium was specific to VN and not artifactual. Slides were baked at 60°C for 1 hour, then dewaxed in xylene then rehydrated in graded alcohols (100% Ethanol 2 x 5 minutes, 90% Ethanol 1 x 5 minutes, 70% Ethanol 1 x 5 minutes and distilled water 1 x 5 minutes). Slides were washed TBS-T pH 7.6 and carefully dried and serum block applied in 10% normal serum (NS) solution then incubated for 1 hour. Following the serum block primary Ab was prepared in NS (10 - 20 µg/mL) and applied to the tissues for a minimum of 1 hour in a humidity chamber. Slides were then washed 3 x 5 minutes in TBS-T pH 7.6 and secondary Ab solutions (1-2 µg/mL) applied for 1 hour. Slides were washed 3 x 5 minutes in TBS-T pH 7.6, dried and coverslipped. Staining was
visualized with Vector Red, which possesses both, colorimetric and fluorescent properties (Vector Laboratories, Burlingame, CA) or peroxidase diaminobenzidine tetrahydrochloride (DAB). Nuclei were counterstained with Mayers' hematoxylin.

Cardiomyocytes were isolated from Langendorff-perfused adult male Sprague-Dawley rat hearts [304] were fixed in 4% paraformaldehyde in TBS, washed, then neutralized for 10 minutes in 0.2 M glycine in TBS. Cells were permeabilized with 0.01% Triton-X-100 in TBS for 5 minutes. For confocal microscopy immunolabeling for cardiomyocytes and cardiac tissues was performed with polyclonal murine anti-VN (Molecular Innovations), polyclonal rabbit anti-desmin (Abcam) or goat anti-murine albumin (Sigma Aldrich) and specific mouse or human and monoclonal IgG Ab. Tissue sections were then incubated with AlexaFluor® 594 donkey anti-sheep and AlexaFluor® 488-conjugated goat-anti-rabbit IgG diluted to 2 μg/mL, then incubated with Hoechst 33342 nuclear dye diluted to 1 μg/mL. Rat cardiomyocytes were incubated for 1 hour with either 488 AlexaFluor® phalloidin, AlexaFluor® 594-conjugated goat-anti-rabbit IgG and Hoechst 33342 nuclear dye. The slides were washed, cover slipped and then examined by Leica SP2 AOBS confocal laser scanners on a Leica DM IRE inverted microscope. 3-D volume rendering was achieved using MetaMorph (Universal Imaging Corp, Downingtown, PA) and Volocity software (Improvision, Lexington, MA). Line-scanning was performed using LiCore® confocal software (Leica Microsystems).
6.2.4 Rat heart and neonatal cardiomyocyte cytoskeleton preparation

Neonatal cardiomyocytes were isolated from the ventricles of a litter of 1-day-old Sprague Dawley rat pups (n = 16) using a modified method described by Simpson et al. [305] and grown in 10% fetal calf serum (FCS) and 90% of media containing insulin, transferin, lithium, ascorbic acid and vitamin E. The cells were then incubated at 37°C for 72 h, collected, centrifuged and the cell pellets stored at -4°C until processed.

Sections of frozen myocardium or neonatal cardiomyocytes cell pellets were thawed out and washed with PBS, then soluble proteins were extracted with 1 mL of cytoskeletal extraction buffer (100 mM NaCl, 300 mM sucrose, 10 mM PIPES [pH 6.8], 3 mM MgCl₂, 0.5% Triton-X-100 and 1.2 mM PMSF) by manual homogenization for 10 minutes at 0°C then centrifuged 2000 x g for 2 minutes. The supernatant (Triton-X soluble fraction) was removed and insoluble structures (Triton-X insoluble fraction) further extracted by addition of modified cytoskeletal extraction buffer (250 mM ammonium sulfate, 300 mM sucrose, 10 mM PIPES [pH 6.8], 3 mM MgCl₂, 1.2 mM PMSF and 2% SDS) for 10 minutes at 0°C, then centrifuged 2000 x g for 2 minutes. The supernatant (Triton-X insoluble fraction) containing cardiomyocyte cytoskeleton was then removed. The samples were stored at -80°C until further use.

6.2.5 Western and ligand blot analysis

Western and ligand blot analysis for VN and VN-binding proteins was optimized and described previously [306]. Cytoskeletal heart extracts (10 µg/lane) or neonatal cardiomyocytes (10 µg/lane) were treated with sample buffer (100 mM Tris buffer, pH 8.3 containing 2% SDS, 5 mM EDTA, 10% glycerol, 15 mM
dithiothreitol) and fractionated by SDS-PAGE. Purified standards of human endothelial cell vimentin (0.25 μg/lane) and recombinant human desmin (0.25 μg/lane) were used as controls. After fractionation, the gels were electrophoretically transferred onto nitrocellulose membranes. Subsequently, the membranes were blocked with PBS containing 10% casein (PBS/casein), then incubated with PBS/casein containing either biotinylated VN, anti-vimentin IgG, mouse anti-desmin IgG or goat anti-albumin for 1 hour. The membranes were then incubated with alkaline phosphatase-conjugated streptavidin and processed for colour development with the Fast Red chromagen system (Axxora). Relative intensity of bands was compared by densitometry using Image J v1.38 (http://rsb.info.nih.gov/ij/download.html) and tabulated using SigmaPlot 8.0 (Systat Software, Point Richmond, CA).

6.2.6 Vitronectin binding activity

To examine the binding properties of VN (Molecular Innovations) to desmin, 96-well microtitre plates (Costar, Dallas, TX) were coated with purified recombinant human desmin (Research Diagnostics) in PBS [pH 7.7] (3.3 μg/mL) overnight at 4°C, and the excess protein binding sites were blocked with 3% BSA in PBS. The cells were washed with SPIRA buffer, then were incubated with various concentrations of biotinylated-VN diluted in dilution buffer (PBS containing 3% BSA, 0.1% Tween 80, 5mM EDTA and 20 U/ml aprotinin) at 37°C for 1 hour. Specific binding of biotinylated-VN was determined by measuring the change in absorbance at 405 nm after the addition of streptavidin-conjugated alkaline phosphatase/pNPP substrate and subtracting the background of biotinylated-VN binding to BSA-coated wells.
6.2.7 Isolation of adult rat ventricular myocytes

Male Sprague-Dawley rats (250–300g) were anesthetized using 3% halothane, and the heart was excised and mounted on a modified Langendorff apparatus and perfused with oxygenated (95% O₂-5% CO₂) HEPES-Joklik-modified MEM buffer (GIBCO-BRL, Grand Island, NY) at 37°C for 2 minutes. The perfusate was changed to 30 mL of recirculating Ca²⁺-free MEM containing 236 U/mL collagenase (Worthington Biochemical, Freehold, NJ). At 15 and 20 minutes, the Ca²⁺ concentration was increased stepwise to 0.025 and 0.075 mmol/L. After 30–40 minutes, the ventricles were removed from the perfusion system, gently teased apart, and agitated for 2–3 minutes at 37°C. The tissue and dispersed cells in solution were filtered through a 200-μm nylon mesh. The cells were washed three times at 37°C in MEM containing increasing Ca²⁺ concentrations (200 μM, 500 μM, and 1 mM), with the cells allowed to settle by gravity for 10 minutes between each wash. Cells were resuspended in 37°C HEPES-modified M199 buffer (GIBCO-BRL) with 1% BSA. The cells were diluted to a final concentration of 5 x 10⁴ cells/mL, 100 μl were loaded into each well of 96-well plates, and the plates were incubated at 37°C in 95% O₂-5% CO₂. At 90 minutes, the medium was changed to fresh M199 with BSA, and the cardiomyocytes were incubated for 24 hour to allow them to become relatively quiescent. After 24 hour, the cells were considered viable if they demonstrated a characteristic rod shape without cytoplasmic blebbing. This morphometric assessment of viability was confirmed in a subset of experiments with trypan blue exclusion. We have found that the fraction of viable cardiomyocytes is always >85%.
6.2.8 Measurement of cardiomyocyte fractional shortening and shortening velocity

Cultured adult rat cardiomyocytes were incubated at normal conditions (95% O₂, 5% CO₂ at 37°C) or subjected to hypoxic conditions (90min, PO₂ <15mmHg at 37°C) in the presence or absence of 10% fetal bovine serum (FBS) containing VN or purified monomeric VN at 1, 2.5, 10 and 20 µg/mL, Molecular Innovations).

For the measurement of fractional shortening and shortening velocity a platinum electrode was lowered into each well of a 96-well plate and the cardiomyocytes were electrically stimulated (Grass S48 stimulator, West Warwick, RI; 45 mV, 2.2-ms duration, 25-Ω resistance) while being recorded by video-microscopy (Sony SLV-760HF). The electrical stimulus was chosen from preliminary threshold experiments as two times the minimum electrical stimulus required to maximally contract the cardiomyocytes. Still frames of cardiomyocytes in diastole (unstimulated) and systole (stimulated) were captured and the time of contraction recorded (Figure 6-1). Based on the video recording, myocyte fractional shortening and shortening velocity were then determined using Scion ImagePC (Scion, Frederick, MD). Experiments were performed in quadruplicate each time with cardiomyocytes isolated from individual rat hearts. To determine effects on contractility, cardiomyocytes under either normal or hypoxic conditions were treated with either FBS and/or recombinant VN (20 µg/mL), following which fractional shortening was measured as described above. Cardiomyocyte fractional shortening and shortening velocity were calculated using the following formulas:

Fractional Shortening = \( \frac{(\text{Diastolic length} - \text{systolic length}) \times 100}{\text{Diastolic length}} \)

Shortening Velocity = \( \frac{\Delta \text{length}}{\Delta \text{time}} \)
6.2.9 Desmin and vimentin alignment

In order to determine sequence homology, protein sequences of human, rat and mouse desmin [307] and vimentin [308] were obtained from the NCBI website www.ncbi.nlm.nih.gov. The sequences were aligned using “BLAST 2 Sequences” engine for local alignment found on the NCBI website http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi.

6.2.10 Statistics

Statistical significance of differences was assessed using ANOVA; p-value of 0.05 or less was considered significant.
Figure 6-1. Measuring rat cardiomyocyte contractility using soft edge detection and IonWizard®. Isolated cells were treated with VN under normal or hypoxic conditions for 45 minutes. Cells were then electrically stimulated with the length, as well as the duration of contraction recorded. A) Graphical and visual representation of an unstimulated, cardiomyocyte in diastole. In a resting state there is no change in cardiomyocyte size (blue circle). B) Graphical and visual representation of a stimulated cardiomyocyte in systole. During stimulation the cell contracts maximally following which normal size is gradually re-established (blue circle).
6.3 Results

6.3.1 Exudation and localization of plasma proteins in ischemic and ischemia-reperfused rat myocardium

We examined the distribution of plasma proteins VN and fibrinogen in rat hearts following varied periods of acute ischemia and ischemia/reperfusion by coronary artery occlusion in situ, or in isolated hearts subjected to Langendorff perfusion with plasma or saline ex vivo. Hearts were perfused with Alexa 594-conjugated-fibrinogen in order to fluorescently define areas of vascular damage and permeability. Fibrin(ogen) deposits were evident in the coronary vessel wall and peri-vascular area (Figure 6-2A). In hearts infused with rat serum, fibrin(ogen) colocalized with VN in ischemic blood vessel walls and the perivascular myocardium (Figure 6-2B). Examination of the VN within the ischemic cardiomyocytes revealed the characteristic banding pattern of sarcomere-associated proteins. These studies provide evidence that the accumulation of plasma VN in the ischemic myocardium characteristically defines the area of risk as it was noted within minutes following the onset of ischemia and reflects the binding of VN to sarcomeres-associated proteins of reversibly and irreversibly injured cells (Figure 6-2C). Both fibrin(ogen) and VN staining was absent from normal rat hearts.

6.3.2 Influx of VN into ischemic and infarcted myocardium

Following LAD ligation, VN immunostaining of the injured mouse myocardium was first apparent within 45 minutes, with progressively more positive staining evident up to 1 day (Figure 6-3A). Extravascular VN was absent from non-
Figure 6-2. \textit{In situ} and \textit{ex vivo} acute ischemia-reperfusion injury results in increased vascular permeability and accumulation of circulating VN and fibrin(ogen) within the myocardium and walls of coronary blood vessels. Brightfield/fluorescence micrograph of frozen sections from isolated rat hearts subjected to 20 minutes global ischemia (A and B), or LAD ligation followed by 15 minutes reperfusion (C). Prior to reperfusion, trace quantities of fluorescently-labeled Alexa 594-fibrinogen (red) was infused into the circulation (A and B). A) Fibrin(ogen) was localized to ischemic vessel wall with trace amounts found in the myocardium (arrows). B) Immunolocalization of VN (green) accumulated in the blood vessel wall (arrows) and peri-vascular space, and colocalized with fibrin(ogen) deposits. C) VN staining is localized to sarcomeres of ischemic cardiomyocytes.
ischemic myocardium. VN progressively accumulated in the myocardium with prolonged exposure to ischemia, and was localized particularly to myofibers. To confirm that the influx into the ischemic myocardium was a VN-specific event, we also stained for albumin. In contrast to VN staining, faint albumin staining was observed throughout the acute ischemic myocardium between 45 minutes and 6 hours with majority localized to the extracellular space. Slightly increased staining was visualized 1 day post-MI (Figure 6-3A). Furthermore, magnified images taken 1 day following LAD ligation displayed an accumulation of VN staining that was localized primarily to ischemic cardiomyocytes while staining for albumin was primarily absent (Figure 6-3B). Triton-X soluble and insoluble protein fractions isolated from mouse hearts were fractionated using gel electrophoresis and their distribution visualized by EZBlue™ gel staining reagent. A majority of the bands appeared in the Triton-X soluble fraction, fewer bands in the cytoskeletal Triton-X insoluble fraction.

Western blotting of tissue extracts isolated from the ischemic LV showed a lack of VN in the Triton-X soluble cytoplasmic fraction (Figure 6-4). Conversely, prominent albumin levels were evident in the cytoplasmic fractions which increased over time from 45 minutes up to 1 day post-MI. VN was present in the Triton-X insoluble cytoskeletal fraction of ischemic myocardium commencing at 45 minutes. Between 45 minutes and 6 hours VN levels appeared constant, with increased accumulation observed at 1 day post-MI (Figure 6-4). In contrast albumin was relatively absent from the Triton-X insoluble cytoskeletal fraction extracts from the ischemic myocardium. Accumulation of VN within cardiomyocytes occurred at sites of ischemia. Unlike VN, low levels of albumin were present in the Triton-X soluble
Figure 6-3. VN progressively accumulates in the ischemic myocardium of LAD ligated mice. A) Immunostaining in ischemic mouse hearts showed progressive localization of VN in the LV from 45 minutes to 1 day post-ischemia. No VN was observed in non-ischemic control hearts. Albumin staining was primarily absent from the normal or ischemic myocardium for up to 1 day post-ischemia. (Insets, lower right). Higher magnification images of VN accumulation in the ischemic myocardium over time. B) Magnified image taken from LV of ischemic mice at 90 minutes demonstrated VN staining localized to sarcomeres (arrows) of viable cardiomyocytes (intact nuclei). Scale bars equals 500 μm (A, 100x magnified) and 10 μm (B, 800x magnified).
Figure 6-4. VN accumulates in the Triton-X insoluble cytoskeletal fraction of ischemic myocardium. Western blotting showed absence of VN, but increasing levels of albumin in the Triton-X soluble cytoplasmic fraction between 45 minutes and 1 day post-LAD ligation. In contrast, VN accumulated in a time-dependent manner in Triton-X insoluble cytoskeletal fraction, while albumin was primarily absent. VN was detected as early as 45 minutes post-LAD ligation with notable increase observed at 1 day. Both VN and albumin were absent from non-ischemic Triton-X insoluble controls.
Figure 6-5. VN mRNA is absent from differentiated rat embryonic ventricular myocytes. Semi-quantitative RNA analysis using real-time PCR demonstrated a lack of VN expression in differentiated H9C2 cells as tested at different primer concentrations (coloured lines).
fraction of control hearts. Albumin and VN were absent from the Triton-X insoluble fractions of sham-operated control hearts (Figure 6-4). Furthermore, embryonic ventricular cardiomyocytes were not an active source of VN as semi-quantitative RT-PCR demonstrated an absence of VN mRNA expression in these cells (Figure 6-5).

6.3.3 Localization of VN at Z-lines of cardiomyocyte sarcomeres coincides with desmin at sites of acute MI

Immunohistochemical staining of acutely infarcted human myocardium obtained from St. Paul's Hospital Cardiovascular Registry showed the accumulation of VN in both necrotic and viable cardiomyocytes with no VN visible in the non-ischemic cells in the peri-infarct region (Figure 6-6A). Immunofluorescent staining showed localization of VN to sarcomeric bands within ischemic cardiomyocytes. Moreover, staining for sarcomeric desmin revealed co-localization of VN with desmin IF at the Z-lines of ischemic cardiomyocytes (Figure 6-6 B-F). VN staining was absent from adjacent healthy cardiomyocytes.

6.3.4 Association of VN with desmin and vimentin cytoskeleton of neonatal and adult cardiomyocytes

To demonstrate the presence of polymeric IF proteins in the myocardium, equal quantities of protein extracts from the Triton-X soluble and insoluble cytoskeleton fractions of adult rat hearts, neonatal rat cardiomyocytes, as well as purified recombinant human desmin and endothelial cell vimentin were electrophoretically fractionated by SDS-PAGE, and then subjected to Western blot analysis with Abs directed against desmin and vimentin. Figure 6-7 illustrates that both the heart and cardiomyocyte samples contained a single band of desmin (MW
that was also concentrated in the Triton-X insoluble fraction. Similarly, both heart and cardiomyocyte samples contained two major vimentin bands that migrated at 58 and 56 kDa molecular weight, and was associated exclusively with the Triton-X insoluble fraction (Figure 6-8). Both desmin and vimentin IFs were absent from the Triton-X soluble heart and cardiomyocyte extracts. Ligand blot analysis on equal quantities of SDS-PAGE fractionated neonatal cardiomyocytes-derived Triton-X insoluble protein fractions and purified protein controls was performed to determine whether myocardial IF cytoskeletal proteins interact with VN. VN bound specifically to the bands corresponding to the native vimentin and desmin, as well as the purified desmin control (Figure 6-9). Additional unidentified higher molecular weight bands displayed VN binding activity, suggesting that other cytoskeleton-associated protein, or possibly IF multimers, may also bind VN.

6.3.5 VN binds directly to cardiomyocyte desmin IFs with high affinity

Analysis of the binding affinity of VN for desmin showed protein interactions in a dose-dependent, saturable manner, with an apparent dissociation constant (Kd) of 25 nM (Figure 6-9). High affinity binding interaction between VN and vimentin were also noted and involved the amino terminal head domain of vimentin (kD = 80 nM) as previous demonstrated in our laboratory [307].
Figure 6-6. VN localizes to desmin in Z-lines of sarcomeres in ischemic human cardiomyocytes. A-B) Polarized light and immunofluorescent imaging demonstrated VN localization in sarcomeres of ischemic and necrotic cardiomyocytes, however no staining was observed in non-ischemic healthy cells in the peri-infract zone. C-F) Magnified image from region of acutely infarcted human myocardium stained for desmin (green), VN (red) and nuclei (blue) revealed co-localization of VN with desmin on the Z-line of sarcomeres in the damaged cells. Scale bars = 40 μm.
Figure 6-7. Desmin IF are associated with Triton-X insoluble extracts from rat hearts and cultured cardiomyocytes. Triton-X soluble extracts from adult rat hearts (lane 1) and neonatal rat ventricular cardiomyocytes (lane 2) were both deficient in desmin. In contrast, desmin was present in Triton-X insoluble extracts from adult rat hearts and neonatal rat ventricular cardiomyocytes. Purified recombinant human desmin used as a positive control (lane 3).
Figure 6-8. Vimentin IF are associated with the Triton-X insoluble extracts from rat hearts and cultured cardiomyocytes. Triton-X soluble extracts of adult rat hearts (lane 1) and neonatal rat ventricular cardiomyocytes (lane 2) were both deficient in vimentin. In contrast, vimentin (double band 56 and 58 kDa) was present in Triton-X insoluble extracts from adult rat hearts and neonatal rat ventricular cardiomyocytes. Endothelial cell vimentin was used as a positive control (lane 3).
Figure 6-9. VN binds to desmin IF with high specificity. A) Western (left) and ligand (right) blot analysis of biotinylated VN binding to purified desmin from extracts of neonatal rat cardiomyocytes. Triton-X insoluble extracts (10µg/lane) of neonatal rat ventricular cardiomyocytes (Cardiomyocytes) and purified recombinant human desmin (Desmin Std) were fractionated and subjected to Western blotting ligand binding using biotinylated-VN. In neonatal cardiomyocyte extracts VN was bound to both vimentin and desmin at 56 and 53 kDa respectively. B) Affinity binding assay in which increasing concentrations of biotinylated VN was added to purified desmin. VN formed specific, saturable, high affinity binding interaction with desmin. (kD apparent = 25 nM).
6.3.6 Influx of VN into hypoxic cardiomyocytes localizes to desmin IF in an energy-dependent manner

In order to test whether VN accumulation into ischemic cardiomyocyte was energy-dependent, isolated adult rat cardiomyocytes were subjected to hypoxic conditions for 45 minutes at either 4°C or 37°C. Cells were then fixed, permeabilized, fluorescently stained and imaged by confocal microscopy. Staining for VN and desmin was evident in viable cultured cardiomyocytes subjected to hypoxic conditions, both at 4°C or 37°C in the presence of purified VN (20 μg/mL) (Figure 6-10A), however increased VN staining was evident in cells at 37°C as determined using color segmentation (ImagePro). Furthermore, staining was localized to sarcomeres (insets, upper right, arrows) of ischemic cardiomyocytes and appeared co-localized with desmin IF. In contrast, staining for VN was noted in normoxic cells at 4°C or 37°C localized primarily only to the sarcolemma. Staining for albumin was mostly uniformly distributed throughout the cytoplasm with increased accumulation noted in the cell periphery of both normoxic and hypoxic cells at both 4°C and 37°C (Figure 6-10A, insets, lower right). Line scanning for the relative signal intensity of VN with desmin present at sarcomeric Z-lines (Figure 6-10A, insets, arrowheads) showed an overlap of VN and desmin (at ~ 0.3 and 2.9 μm) at areas indicating colocalization at sarcomeric Z-lines (Figure 6-10B). This distance was consistent with that of a single sarcomere.

6.3.7 FBS reduces cardiomyocyte contractility in normoxic and hypoxic cardiomyocytes

To test whether serum proteins have an effect on cardiomyocyte contractility, isolated rat cardiomyocytes have been subjected to electrical stimulation in the presence of 10% FBS which contains micromolar quantities of VN under either
normal or hypoxic conditions. Fractional shortening of both normoxic and hypoxic cardiomyocytes decreased in the presence of FBS (24.14% ± 1.18 vs. 19.25% ± 1.11, p = 0.003; 16.95% ± 1.33 vs. 13.7% ± 1.19, p = 0.06) (Figure 6-11A). Similarly, shortening velocity of both normoxic and hypoxic cardiomyocytes decreased in the presence of FBS (normoxic conditions; 52.56% ± 3.72 vs. 41.46% ± 3.97, p = 0.04; hypoxic conditions 41.43 μm/second ± 4.04 and 34.85 μm/second ± 3.07, p = 0.18) (Figure 6-11B).

6.3.8 Dose dependent effects of VN on hypoxic cardiomyocytes

To investigate whether VN accumulation exhibited effects on cell function, isolated adult rat cardiomyocytes were incubated under either normal or hypoxic conditions in the presence or absence of purified VN, then electrically stimulated to measure cell contractility. Treatment with VN had a profound effect on hypoxic isolated rat cardiomyocytes by significantly reducing contractility. Exposure to increasing concentrations of VN (1, 2.5, 10, 20 μg/mL) showed a significant, dose-dependent decrease in fractional shortening of hypoxic cardiomyocytes (Figure 6-12). VN has minimal effects on fractional shortening of the normoxic groups. As expected, compared to normoxic cells, hypoxia alone had a significant effect on the isolated cardiomyocytes, reducing fractional shortening (24.1% ± 1.2 vs. 17.2% ± 1.3, p < 0.0001). However, addition of VN to hypoxic cardiomyocytes led to further reduction in fractional shortening with the greatest reduction observed at 20 μg/mL (12.3 μm/second ± 0.9 vs. 24.0 μm/second ± 1.3, p < 0.000001) (Figure 6-12). Reduction in the shortening velocity was also noted in hypoxic cells, with a significant difference noted only at 20 μg/mL of VN treatment. All results are summarized in Table 6-1.
### Table 6-1. Summary of results corresponding to fractional shortening and shortening velocity of VN treated adult rat cardiomyocytes.

<table>
<thead>
<tr>
<th>VN (µg/mL)</th>
<th>Fractional Shortening (%)</th>
<th>Shortening Velocity (µm/sec)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Normoxic</td>
<td>Hypoxic</td>
</tr>
<tr>
<td>0</td>
<td>24.14 ± 1.18</td>
<td>17.16 ± 1.33</td>
</tr>
<tr>
<td>1</td>
<td>22.9 ± 1.08</td>
<td>17.23 ± 1.21</td>
</tr>
<tr>
<td>5</td>
<td>20.46 ± 1.03</td>
<td>16.1 ± 0.97</td>
</tr>
<tr>
<td>10</td>
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<td>14.18 ± 1.15</td>
</tr>
<tr>
<td>20</td>
<td>24.05 ± 1.27</td>
<td>12.33 ± 0.86</td>
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### 6.3.9 Vimentin and desmin amino acid sequence alignment

Amino acid sequence comparison of the protein structures for desmin and other IF subunits revealed a highly conserved region (80%) among different members of the IF protein gene superfamily [307]. The global alignment of the desmin and vimentin sequences showed 61.1% homology between the IF proteins. Significant sequence homology was observed within the first 120 residues of the amino terminal head domains of both IF proteins, which share two specific regions; a nonapeptide (SSYRRTFGG) and an arginine and proline-containing RP-box (RLGSFRAPRAGALRLP). These two regions have been previously recognized to contain the high affinity VN binding site for vimentin [309-311]. Both sequences contain phosphorylation sites that are critical for the assembly and disassembly of type III IF proteins. Both regions are possible binding sites and may exhibit high affinity for VN.
Figure 6-10. Localization of VN to sarcomeres of hypoxic cardiomyocytes. A) Confocal images of VN localization in cultured adult rat cardiomyocytes in the presence or absence of VN (20μg/ml) under normal and hypoxic conditions (45 minutes) at 4°C and 37°C. Cardiomyocytes were stained for VN (red) and desmin (green). Normoxic cells at 4°C and 37°C showed VN staining in cellular periphery (arrow), but was absent intracellularly. Staining for VN was localized to the sarcomeres of hypoxic cardiomyocytes treated with purified recombinant VN both at 4°C and 37°C (inset, upper right). Insets, lower right: Staining of albumin (pink) and desmin (green). Staining for albumin was present throughout on cellular periphery and diffusely throughout cytoplasm under normal and hypoxic conditions both at 4°C and 37°C. No albumin staining was localized to cardiomyocyte sarcomeres. B) Representative line scan of a single sarcomere (A, insert, arrowsheads) demonstrates the colocalization of VN (red line) with desmin (green line) at the Z-bands. At peak signal intensities ~ 0.3 and 2.9 μm (arrows) there was an overlap of VN and desmin corresponding to areas of colocalization.
**Figure 6-11.** FBS reduces contractility of isolated rat cardiomyocytes. Isolated rat cardiomyocytes have been incubated with or without 10% FBS added to the culture medium under either normal or hypoxic conditions (30 minutes). Cardiomyocytes have been electrically stimulated (45 mV) and the distance and duration of the contraction measured. A) In the presence of FBS a noticeable decrease in fractional shortening of both normoxic and hypoxic cardiomyocytes was observed (Control 24.14 ± 1.18 vs. 19.25 ± 1.11, p = 0.003; FBS 16.95 ± 1.33 versus 13.70 ± 1.19, p = 0.06). B) Similarly, shortening velocity of both normoxic and hypoxic cardiomyocytes decreased in the presence of FBS (Control 52.56 ± 3.7158 vs. 41.46 ± 3.97, p = 0.04; FBS 41.43 ± 4.04 and 34.85 ± 3.07, p = 0.18).
Figure 6-12. VN exhibits dose dependent effects on fractional shortening and shortening velocity of cultured rat cardiomyocytes. Isolated adult rat cardiomyocytes were incubated with increasing concentrations of purified VN (1, 2.5, 10 and 20 µg/mL) under either normal or hypoxic conditions (45 minutes). Following the incubation period cells were electrically stimulated with the distance and duration of contractions measured. Fractional shortening (top panel) and shortening velocity (bottom panel) progressively decreased in hypoxic cardiomyocytes with increasing doses of VN. Untreated hypoxic cardiomyocytes exhibited a marked reduction of both fractional shortening and shortening velocity when compared to normoxic cells. Significant reduction in fractional shortening was noted between normoxic and hypoxic in all VN treated groups independent of the dose (top panel). Significant differences in shortening velocity were observed only in cardiomyocytes treated with 20 µg/mL purified VN (bottom panel).
6.4 Discussion

VN accumulates in the ECM of the heart [286] and localizes at sites of MI [309-311]. We have further investigated the localization, distribution and function of VN following hypoxic and ischemic injury by using in vitro and in vivo models. The work described here demonstrated that VN is found at sites of hypoxic (in vitro) and ischemic (in vivo) damage and localizes not only in the ECM but also within compromised cardiomyocytes wherein it is involved in regulation of cell function. Initial observations gathered from infarcted human myocardial tissue revealed VN accumulation at sites of ischemia, acute and chronic MI, as well as cardiac rupture (Figure 7-8). Thus, VN is deposited at the site of injury within hours of myocardial ischemia, and persists for a prolonged period of weeks to months post-MI. Furthermore, accumulation of VN was associated with staining of other proteins important in ischemic tissue remodeling such as PAI-1, t-PA and u-PA which may be influenced by its versatile nature. Thus, VN likely plays an important role at sites of ischemic myocardial injury, and it is essential to investigate the function(s) and dissect its mechanism(s) of action in more detail.

Following LAD ligation in mice, VN begins to accumulate in ischemic myocardium within 45 minutes and progressively increases over time localizing in both ischemic and necrotic cells. VN was primarily observed in the LV free wall and partially in the lateral wall supplied by the LAD. Closer examination of VN localization in the infarcted human myocardium revealed that it was found intracellularly within ischemic and necrotic cardiomyocytes. Furthermore, diminutive amounts of VN were found in the cell cytoplasm while the majority was associated with the sacromeric cytoskeleton, and particularly noted at the Z-lines. The clear VN striation pattern co-localized with desmin in the Z-line, thus we
investigated the potential binding interactions that occur between these two proteins. Desmin which exists in adult cardiomyocytes, is a type III IF protein homologous to vimentin, another structural IF protein found in neonatal and regenerating myocardium. In cardiac muscle, desmin has a function in maintaining structural and functional integrity, as well as differentiation [312-314] and both desmin and vimentin play a role in muscle regeneration [314, 315]. However, unlike vimentin, desmin persists between the Z-lines after sarcomeres are properly aligned and can also be found at the intercalated disk region of adult cardiomyocytes [316, 317]. Thus, we perceived that the accumulation of VN within viable cardiomyocytes within minutes to hours of ischemia encompasses a specific function, rather than simply general exudation of plasma proteins caused by augmented vessels permeability.

In vivo studies using a mouse model of myocardial ischemia confirmed our human-based observations in which VN accumulates in cardiomyocytes and is bound to sarcomeric Z-lines. Confocal line scanning verified that peak signal intensities were noted overlapping in areas of VN-desmin co-localization notably at sarcomeric Z-lines. Protein fraction extraction from ischemic hearts demonstrated that majority of VN was observed in the IF cytoskeletal fraction and is potentially associated with sarcomeric or IF proteins as previously reported [307]. The deposition of other plasma antigens such as fibrinogen, FN and the terminal complement complex C5b-9 was previously noted within the ischemic heart [286, 307, 318]. Serum albumin is another abundant plasma protein with similar molecular weight to VN and was therefore used as a control. Interestingly, unlike VN, absence of albumin from the cytoskeletal fraction of ischemic hearts suggests that this phenomenon was not simply a passive effect of ischemia causing an
increase in membrane permeability. This offers additional support for a specific function of VN in binding to cytoskeletal proteins of compromised cardiomyocytes.

Although incidence of VN in the ischemic myocardium has also been described, the focus was mainly on its immunolocalization within the ECM rather than cardiomyocytes [319, 320]. We did not re-examine the function of VN deposition within the ECM, but rather concentrated on the novel observation of VN accumulation in ischemic cardiomyocytes. *In vitro* studies with cultured adult rat ventricular cardiomyocytes indicated that VN is not expressed by the muscle cells. We found that the route of VN influx into ischemic/hypoxic cardiomyocytes occurs both dependent and independent of energy as VN accumulated in hypoxic cells at 4°C or 37°C consistent with passive and active transport respectively. However, further studies are required in order to clearly determine whether VN transport through the cell sarcolemma is receptor-mediated and potentially associated with the transverse tubular system (T-system). Cardiac muscle fibers possess a well developed T-system that traverses every fiber at the level of the Z band [321, 322] [321-324]. The lumen of the T tubules connects directly to the extracellular space permitting entrance of large, serum derived proteins as elegantly shown in previous studies [321-324]. Thus it is possible that VN may also be internalized via the T-system.

We have previously described the specific binding interactions of vimentin IF with VN and PAI-1 on activated platelets and platelet microparticles promoting the regulation of fibrinolysis in plasma and thrombi [307]. Studies with neonatal cardiomyocytes demonstrated that VN binds vimentin, as well as to desmin IF. Biochemical studies indicated that VN binds desmin specifically and with high affinity forming Triton-X soluble complexes. Desmin plays essential roles in cell
survival [325], maintenance of functional and mechanical integrity of the cells [314], sarcomerogenesis [316], as well as counteracting external stresses [326]. Desmin, along with other cytoskeletal proteins is more resistant to ischemia than contractile filaments, although significant losses of cross striations occur as early as within ninety minutes of onset [318, 323]. One potential theory is that rapid influx of VN into ischemic cardiomyocytes may inhibit depolymerization of desmin by binding the IF, stabilizing its conformation and cross linking the desmin tetramers. Alternatively, binding of VN to desmin may serve as a mediating step that promotes association of VN-desmin complexes with other protein(s) which together maintain and regulate cellular function during ischemia. Similarly, interactions between platelet-derived VN-PAI-1 complexes and vimentin on activated platelets and endothelial cells have previously been observed [307].

We observed a significant compromise of contractile function following in vitro hypoxic injury, which was further exacerbated by VN. Loss of contractile function was also noted in cardiomyocytes subjected to FBS treatment although we could not attribute these effects solely to the presence of VN in the serum. Unexpectedly, along with a reduction of function in hypoxic cells, normoxic cells treated with FBS also exhibited a significant decline in contractility, unlike those treated with purified VN. Thus, VN potentially reduces contractility in cardiomyocytes compromised by hypoxia, but exhibits trivial effects under normal conditions. This compromise to cardiomyocyte function may possibly be attributed to the binding of VN to desmin IFs potentially reducing metabolic demands. Our findings suggest that VN binds to desmin within ischemic cardiomyocytes, potentially disrupting IF-dependent regulation of sarcomere contractility, reducing cardiomyocyte shortening and decreasing the duration of contraction. Desmin is
connected with the sarcomeres at the Z disks and, along with other IF proteins, plays a regulatory role on the normal contractile function of myofibrils [234]. Thus, there likely exists a myofibril-desmin relationship in the sarcomere and its integrity is essential for normal contraction of the myocyte. Our work demonstrated that VN binds to desmin IF with high affinity, an interaction which was noted in ischemic/infarcted cardiomyocytes. Reduction of cardiomyocyte contractility may represent a protective effect during ischemia by reducing metabolic requirements in favor of sustaining critical function for survival of the cell. Thus, VN may potentially exhibit an additional roles in regulation of stunning/hibernating of the myocardium through its interactions with desmin IF. Structural proteins such as desmoplakin, cardiotin, actin, titin and desmin commonly undergo changes in stunned myocardium [240]. Of these, cytoskeletal desmin levels are reduced throughout the dysfunctional myocardial regions while a global decrease is noted during hibernation [327]. Disorganized expression and loss of cytoskeletal, as well as intercalated disk desmin have been commonly noted in stunned myocardium. Desmin is indispensable for force generation and maintenance of passive tension. The absence of desmin significantly hinders the mechanical properties of muscle tissue. In visceral smooth muscle the absence of desmin caused contractile dysfunction allowing for only 40% of the normal contractile force and a 25–40% reduction of maximal shortening velocity [328]. Disruption of cytoskeletal proteins including desmin is a possible mechanism for stunning, although it may be a secondary effect of protease activation. Furthermore, in end-stage heart failure total absence of desmin from some myocytes is associated with cellular loss of the IF protein and exhaustion of its compensatory reserve [329]. Such desmin alterations are responsible for LV dysfunction causing a reduction in EF [330, 331].
Thus binding of VN to desmin, the major stress-bearing element of the sarcomere, may promote desmin stability, in an effort to maintain sarcomeric integrity and cellular function. Although the role for VN in regulation of ischemic cardiomyocyte contractility and maintenance of cellular integrity is sensible, additional functions should also be considered. For example, by regulating cardiomyocyte contractility VN may partake in lowering cellular energy demands during a period of reduced oxygen concentration [331].

Furthermore, VN-desmin interactions may also play a role in progression to heart failure. Systolic heart failure results from an inadequate number of cardiomyocytes, or reduced contractile function of the muscle cells. In the latter situation myocardial function is impaired although the pathophysiological mechanism behind this cellular defect is unknown. It has been suggested that during extensive oxygen deprivation, cell viability is severely compromised and results from lysis of lateral Z-, M-, and A-band IF attachments, vesiculation of sarcoplasmic reticulum, and rupture of sarcolemmal membranes. Furthermore, IF attachments with nuclear membranes are disrupted and microtubules disappear from the perinuclear space. Sarcomere changes include distortion of Z bands, loss of lateral Z band-sarcolemmal attachments, and separations of myofibrils from internal faces in intercalated disks [331-333]. These alterations could help explain the reduced contractility observed during hypoxia, ischemia, and in the failing hearts [229, 333]. Although the role of desmin on cardiomyocyte function has been well described [314, 334] the importance of VN-desmin interactions in ischemic myocardium are unclear and require closer investigation.

Our findings determined that VN binds to desmin with a high affinity, however the details of this interaction are yet to be determined. Previous findings from our
laboratory have shown that VN binds to the amino-terminal end of vimentin [307]. Amino acid sequence comparison of desmin to other IF subunits revealed a region that is highly conserved (80%) among different members of the IF protein gene superfamily [307]. Global alignment of human desmin and vimentin sequences showed 61.1% homology between the two IF proteins. Significant sequence homology exists within the first 120 residues of the amino-terminal head domains, including the highly conserved nonapeptide motif SSYRRTFGG (residues 12-21 of desmin) and an arginine and proline containing domain known as the RP-box found in type III IF such as vimentin, desmin, peripherin and glial fibrillary acidic protein [224, 335]. Both of these sequences contain phosphorylation sites that are critical for the assembly and disassembly of type III IF proteins [336]. It is probable that VN also binds to the amino-terminal end of desmin, similar to vimentin which contains a high affinity VN binding site [307]. These are possible binding regions that exhibit high affinity for VN, thus promoting its incorporation into the cardiomyocytes. Further experiments using peptides generated from the amino-terminus of human desmin are essential to identify the specific region involved in binding of VN. Three peptides have been designed to illuminate the specific region responsible for the binding to VN; peptide 1 a 25-mer which includes a nonapeptide (SSSQRVSSYRRTFGGAPGFPLGSPL), peptide 2 is a 27-mer which spans the middle of the amino-terminus (SPVFPRAGFGSKGSSSSVTSRVVYQVS) and peptide 3 a 26-mer containing the RP-box (RTSGGAGGLGSLRASRLGTTTPSSY). Future experiments using these peptides will provide us with a clearer idea about the specific region(s) responsible for the interaction between these two proteins.

Inhibition of VN-desmin interaction may potentially exhibit cardioprotective effects by preventing VN multimerization and binding to sarcomeric desmin IF, thus
restoring cardiomyocyte contractility. Therefore, plausible novel therapeutics could target methods and compositions for inhibiting the multimerization of VN in cardiovascular tissue. Development of specific compound(s) that specifically inhibits VN polymerization and the binding of polymerized VN to desmin IF are necessary for further investigation of interactions between these proteins, particularly in ischemic cardiomyocytes.

6.5 Conclusion

The roles of VN in growth, motility and matrix remodeling strongly indicate its importance as a mediator in response to injury, both in the extra- and intracellular environment. In this study we have shown that during acute ischemia VN influx into damaged myocardium progressively accumulates over time. Furthermore, VN enters ischemic cardiomyocytes specifically binding to desmin with high affinity wherein it may play a role in regulating cardiac function by significantly inhibiting cardiomyocyte contractility as proposed in Figure 6-14. The binding of VN to desmin and inhibition of contractility during the early ischemia period in injured cells suggests a novel role of VN that may be independent of its role as a PAI-1 binding cofactor. Based on our findings we suggest that through direct binding to cardiomyocyte desmin IF, VN compromises cellular function in response to ischemic injury. In an ongoing and exciting avenue of this investigation we are working towards identifying and assessing the functional roles of VN through comprehensive in vitro and in vivo methodologies using transgenic models in order to determine: 1) mechanism of entry for VN into ischemic cardiomyocytes, 2) whether the accumulation of VN in cardiomyocytes has an impact on long-term cardiac function, and 3) whether VN affects post-MI wound healing. By
Figure 6-14. Proposed model of VN-desmin interactions in regulation of cardiomyocyte sarcomere contractility. A) Under physiologic conditions actin thin filaments slide past myosin thick filaments allowing the sarcomeres to maximally contract. B) During myocardial ischemia there is an influx of plasma VN into compromised cardiomyocytes reducing sarcomeric contraction potentially by binding to desmin IFs.
understanding specific roles of VN in acute and chronic phases of cardiomyocyte response to injury and repair, approaches to alter pathological aberrations could be developed. These developments will provide vital groundwork to the discovery of future molecular therapy targets using endogenous elements providing potential therapies for various diseases.

6.6 Additional Considerations

Although we have established that VN influxes into ischemic myocardium, and more specifically into cardiomyocytes in which it exhibits a role in mediating cardiomyocyte contractility, effects of VN on myocardial wound healing and regeneration remain unknown. VN has multiple ligands, the major one of which appears to be PAI-1 [280, 337, 338]. Binding to VN stabilizes the conformation of PAI-1, in turn promoting its inhibition of t-PA and u-PA preventing plasmin generation, hence limiting ECM degradation [339]. VN also mediates cell adhesion and migration by binding to integrins and the uPAR. When bound to VN, PAI-1 can inhibit both uPAR activity and integrin association [136, 274, 275], impeding inflammatory cell recruitment to the area of injury. Thus, following acute ischemia and infarction, VN rapidly influxes into the injured myocardium where, by binding to PAI-1, it may modulate ECM remodeling and fibrosis. Moreover, following infarction, hypertrophic and failing hearts exhibit disorientation and increase in desmin filaments [340, 341] which likely occurs to counteract abnormal stresses experienced by cardiomyocytes. Thus, binding of VN to desmin may also be involved in mediating and reducing myocardial stresses through inhibition of contractility in response to post-MI remodeling. Further studies need to be undertaken to determine the immediate and long-effects of VN-desmin interactions.
post-MI. These would best be investigated using a double knockout (VN and desmin) mouse model of LAD ligation. Alternatively, to test the specific effects of VN-desmin interaction on cell contractility an *in vitro* study can be conducted in which cardiomyocytes from desmin knockout mice treated with VN under hypoxic conditions. Additionally, WT cardiomyocytes can be blocked with various anti-VN Abs. Although at this time we have preliminary data supportive of VN binding to the desmin N-terminus, further experiments are essential to determine the exact location and characterization of the binding site on VN for desmin.
CHAPTER VII EXPRESSION OF CARDIOMYOCYTE TISSUE PLASMINOGEN ACTIVATOR FOLLOWING MYOCARDIAL INFARCTION

7.1 Rationale

Impaired cardiovascular fibrinolytic activity is implicated in the pathogenesis of acute MI. Subsequently, impaired fibrinolytic activity in the infarct region promotes fibrosis and a loss of myocardial function. Tissue remodelling is dependent on the activity of proteinases, such as the PA, that convert plasminogen to plasmin, the biologically active form. Although physiologically the function of plasmin is primarily related to fibrinolysis, it also activates MMPs capable of degrading the fibrillar collagen that accumulates at sites of myocardial injury.

Activation of PAs is impaired by PAI-1. Elevated PAI-1 post-MI impaires angiogenesis and promotes fibrosis in the infarct region which may eventually result in heart failure. PAI-1 activity is maintained by VN, which stabilizes its active inhibitory conformation and mediates its binding to structural proteins. Although u-PA is a major PA associated with post-MI tissue remodeling we observed increased cardiac t-PA levels in infarcted VN/-/- mice. Thus, we investigated the previously unrecognized effects of t-PA in contribution to post-MI remodelling.

Additionally, t-PA levels increase with various hypertrophic stimuli. Exercise training is known to induce an increase in systemic fibrinolytic activity, suggestive of an enhanced fibrinolytic reserve including increased levels of plasma t-PA. Therefore, following MI the beneficial effect of exercise training may be related to increases in t-PA activity. Here we investigated whether hypertrophy instigated by exercise training or ISO stimulation induced cardiac t-PA synthesis and whether cardiomyocytes are a contributing source of t-PA in the heart.
7.2 Experimental Methodology

7.2.1 Rat MI model

A rat model of MI was performed as described by Orenstein et al [198]. Male 12-14 week-old Sprague Dawley rats were randomized prior to proximal LAD occlusion or sham-operated groups. To create the model, animals were anesthetized with ketamine hydrochloride (90 mg/kg) and xylazine (10 mg/kg) intraperitoneally. After adequate anaesthesia they were intubated with a 14-gauge polyethylene catheter and ventilated with room air using a small animal ventilator (model 683; Harvard Apparatus, Boston, MA). A left thoracotomy was performed in the fifth intercostal space and the pericardium was opened. The proximal left coronary artery under the tip of the left atrial appendage was encircled and ligated using a 6-0 silk suture. Proximal LAD ligation in a rat model created a reproducibly large lateral wall infarction. The muscle and skin were closed in layers. In sham-operated animals, the LAD was encircled but not ligated and the muscle layers and skin were closed similarly. Five weeks post-surgery, rats from both groups were randomized to exercise training or sedentary lifestyles. Rats in the exercise treated group swam daily for 6 weeks with increasing time periods from 5-40 minutes. This resulted in four final groups: MI sedentary (n = 28), MI exercise (n = 30), sham-operated sedentary (n = 11), and sham-operated exercise (n = 11). During the 6 week of intervention, five rats died in the infarction sedentary group, and two rats died in the infarction exercise group. To determine the natural progression of molecular changes within the myocardium in the infarct and non-infarction regions of the heart following coronary occlusion an additional 50 rats of similar weight and age were subjected to sedentary conditions. The rats were randomized to be
sacrificed on days 1, 2, 7, 14, 21, 28, and 35 after ligation, and some hearts were processed for histology. Of these, five animals were also randomized to receive sham ligation and were sacrificed on day 0 to act as controls. All animal experiments were approved by the University of Toronto Committee on Animal Care.

7.2.2 Exercise protocol

The swimming protocol began on day 35 for 5 minutes/day initially, and increased by an additional 5 minutes/day until the rats were swimming continuously for 40 minutes/day. Swim frequency was 5 days/week for a total duration of 6 weeks. The rats swam in a 60-cm-deep tub with water temperature maintained at 35°C in groups of four animals, and were towel-dried following each session. The rats randomized to sedentary conditions were dipped in the water for 30 seconds and towel-dried 5 days/week. All rats were then sacrificed at 11 weeks and randomized to hemodynamic/morphometry analysis or molecular studies. To ensure that we investigated a model of severe LV dysfunction, all hearts with infarction involving less than 45% of LV perimeter were excluded after pathologic and morphometric analysis. Thus, seven sedentary and seven exercise infarcted hearts were excluded from their respective groups. In total, 51 infarcted rats were used in the study protocol.
7.2.3 Isolation and culture of adult and neonatal rat cardiomyocytes

Adult cardiomyocytes were isolated from Langendorff perfused adult male Sprague-Dawley hearts as previously described [198]. Neonatal cardiomyocytes were isolated from the ventricles of a litter of 1-day-old Sprague Dawley rat pups (n = 14) using a modified method described by Simpson et al. [198]. Rat endothelial cells were isolated from epididymal fat pad and maintained in M199 media with 20% fetal calf serum and supplemented with 10 ng/mL endothelial cell growth factor [305] (ICN, Montreal, P.Q.). Neonatal cells were treated with 10nM isoproterenol for 72 hours. Adult rat cardiomyocytes were 10nM isoproterenol for 30, 60 and 90 min.

7.2.4 Immunohistochemistry and immunocytochemistry

Rats heart tissues were fixed in 4:1 formaldehyde/glutaraldehyde. The tissues were paraffin embedded and serially sectioned, then stained for H&E and Massons' trichrome or for immunohistochemical staining using polyclonal Abs to t-PA, PAI-1 and VN (Affinity Biologicals, Hamilton, ON, Canada).

Isolated neonatal and adult rat cardiomyocytes were grown on 2-well chamber slides (Lab-Tek®, Campbell, California), fixed in 4% paraformaldehyde in TBS, washed, neutralized for 10 minutes in 2 µl/mL glycine in TBS then permeabilized with 0.01% Triton-X-100 in TBS for 5 minutes. Immunolabeling for neonatal cardiomyocytes was performed with polyclonal rat anti-GLUT 4 (Santa Cruz Biotechnology, Inc., Santa Cruz, California), polyclonal anti-murine t-PA (Molecular Innovations, Southfield, MI) and polyclonal anti-murine actin (Sigma Aldrich) then incubated for 1 hour with either AlexaFluor® 488 goat anti-murine and AlexaFluor® 594 goat-anti-rabbit IgG (Molecular Probes). Adult cardiomyocytes
were stained with polyclonal murine anti-t-PA (Molecular Innovations), anti-desmin (Abcam) and specific mouse and rabbit monoclonal IgG Ab, then incubated for 1 hour with either AlexaFluor® 594 goat-anti-murine, AlexaFluor® 488-conjugated goat-anti-rabbit IgG and Hoechst 33342 nuclear dye (Molecular Probes). The slides were washed, mounted with cover slips using SlowFade® (Molecular Probes) mounting medium.

7.2.5 Cell and tissue RNA extraction and RT-PCR

Neonatal rat heart cell or whole block RNA extraction on paraffin embedded rat heart tissue was performed. Previous studies describing efficacy of extracting RNA from fixed tissues yielded good quality results despite the high probability of degraded RNA signal [198, 306, 342]. Since the majority of RNase activity was localized to the 5' and 3' ends of mRNA transcripts, primers were designed to amplify internal regions of the rat t-PA mRNA sequence (GI: M23697). For RNA extracted from paraffin-embedded tissues two short PCR amplicons of 130 bp and 79 bp were chosen, representing regions of the internal sequences of rat t-PA mRNA with low likelihood of mRNA degradation (Table 7-1). The short length of these primers further increased the likelihood of PCR amplification from interrupted and degraded mRNA. Both primers (NAPS, University of British Columbia, Vancouver) were used to determine t-PA mRNA expression in each group. RNA isolated from cultured Bowes melanoma cells [343] and 18S primers were used as positive controls.

For RNA extracted from neonatal rat hearts a different set of primers was used specific for rat t-PA mRNA expression (800 bp) from both cardiomyocyte and non-cardiomyocyte populations of cells. Atrial natriuretic factor (ANF) mRNA
primers (246 bp) were also designed for the detection (Table 7-2). Reverse transcription reactions were carried out with ~ 0.1µg/µL of total RNA using random decamers supplied with the RetroscriptRT reaction kit (Ambion Inc., Austin, TX). Briefly, RNA and random decamers were heated to 70°C for 2 minutes and then placed on ice. 5X first strand buffer, 25 mmol/L MgCl₂, 10 mmol/L dNTPs, 50 U/µL RNase inhibitor, 100 mmol/L DTT and M-MLV reverse transcriptase (Invitrogen, Burlington, ON) were added to the reaction which was performed at 42°C for 1 hour and stopped by heating at 70°C for 10 minutes. PCR reactions were carried out in a Thermohybrid PCR express machine in a final volume of 20 µL using HotStarTaq Polymerase (Qiagen, Mississauga, Ontario). Cycling conditions were at 94°C for 15 minutes; then 94°C for 30 seconds, followed by 48°C for 30 seconds and 72°C for 30 seconds for 35 cycles; followed by a final extension at 72°C for 3 minutes. All PCR products were visualized by gel electrophoresis, additionally the products obtained from paraffin embedded hearts were subjected to nucleotide sequencing.

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<tr>
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<th>Sense Strand (5' - 3')</th>
<th>Anti-Sense Strand (5' - 3')</th>
<th>Size (bp)</th>
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<tr>
<td>t-PA 1</td>
<td>TAACAAAACCATCACGAGCA</td>
<td>AGTCATCGCTTATCGATCA</td>
<td>130</td>
</tr>
<tr>
<td>t-PA 2</td>
<td>AATATTGCGACATGTCCCCA</td>
<td>GAGTCCTCCTTTAATTCGAA</td>
<td>79</td>
</tr>
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**Table 7-1.** Sequences and sizes of primers used for amplification of rat t-PA.

<table>
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<tr>
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<th>Sense Strand (5' - 3')</th>
<th>Anti-Sense Strand (5' - 3')</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-PA</td>
<td>TGGCACCCACAGCTTTACC-ACATCCAAGG</td>
<td>CTCCAGGTACCTGGCA-CGCGTCATGG</td>
<td>800</td>
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<tr>
<td>ANF</td>
<td>ATCTGCCCTCTTTGAAAGCA</td>
<td>CTCCAGGAGGGTATTCCACCA</td>
<td>246</td>
</tr>
</tbody>
</table>

**Table 7-2.** Sequences and sizes of primers used for amplification of rat t-PA and ANF.
7.2.6 **Microscopy and image analysis**

Stained tissue sections were imaged and captured on Nikon Eclipse 600 using 20x and 40x objective lenses and analyzed. Quantitation of relative tissue t-PA staining intensity was measured over 20 fields of view (40x objective) per stained section using Northern Exposure software (Empix Imaging, Mississauga, ON) \[344\]. Images of cells were captured by either florescence or phase contrast microscopy (neonatal cardiomyocytes) on a Nikon Eclipse 600 using 20x and 40x objective lenses, or a Leica SP2 AOBS confocal laser scanners on Leica DM IRE inverted microscope using 40x objective lenses (adult cardiomyocytes). For some images 3-D volume rendering was performed using MetaMorph (Universal Imaging Corp, Downingtown, PA) and Volocity imaging software (Improvision Inc, Lexington, MA). The point spread functions of the microscope optical system was measured by imaging standardized fluorescent beads (PS$^\text{TM}$-Speck Microscope point source kit; Molecular Probes) and processing these images with those of the z-scan stacks of the cells using Openlab deconvolution software and the 3-D restoration module (Improvision Inc).

7.2.7 **Western immunoblotting**

Culture supernatants or cell fractions were run on 8% SDS-PAGE gel under non-reducing conditions and transferred to Immobulon-P membranes (Millipore). The membranes containing neonatal heart extracts were probed with murine anti-t-PA polyclonal Ab (Molecular Innovations) or mouse anti-human PAI-1 monoclonal Ab (Affinity Biologicals). Membranes containing adult cardiomyocyte culture supernatants were probed with murine anti-t-PA polyclonal Ab. Membranes were visualized using LI-COR Odyssey infrared imaging system (LI-COR Inc. Lincoln,
Relative intensity of bands was compared using Image J v1.38 and tabulated using SigmaPlot 8.0 (Systat Software, Point Richmond, CA).
7.3 Results

7.3.1 Increased expression of t-PA, PAI-1 and VN in the infarcted LV

LVs from sedentary sham-operated controls and RVs from sedentary acute MI rat heart showed normal morphology using H&E staining (Figure 7-1 A-B). In contrast, infarctions were evident in LVs of LAD ligated rats (Figure 7-1 C). Minimal PAI-1 or VN staining was observed in the normal LV of sedentary sham-operated rat hearts, or in the RV of sedentary infarcted rat hearts using immunohistochemistry (Figure 7-1 J-K, M-N). Similarly, only faint t-PA staining was found in the myocardium of sham LV and normal RV of MI sedentary rat hearts (Figure 7-1 G-H). Following MI, t-PA staining was present throughout the LV, both in cardiomyocytes and with marked intensity in small blood vessels within the peri-infarct zone (Figure 7-1 I, arrow, high magnification inset). Intense staining of both PAI-1 and VN co-localized in the infarct with faint staining also noted in the peri-infarct zones (Figure 7-1 L, O). Both PAI-1 and VN were absent from the non-infarcted RV.

7.3.2 Exercise training induces t-PA expression in normal and post-MI ventricles

A marked increase of t-PA immuno-reactivity was seen in cardiomyocytes in hearts of exercise-trained MI rats particularly in the peri-infarct region (Figure 7-2). Moreover t-PA staining was limited primarily to cardiomyocytes, with only relatively trivial expression in endothelial cells within the myocardial vessels (Figure 7-2, arrows). Increased t-PA was also observed in sham-operated exercised rat hearts. Similar to hearts of exercise-trained MI rats, intracellular t-PA was visible throughout the myocardium, primarily in cardiomyocytes. In contrast, only minimal t-PA staining was evident in both, sham-operated or acute MI hearts of sedentary rats.
Figure 7-1. t-PA, PAI-1 and VN accumulate in infarcted rat myocardium. A-C) Haematoxylin and eosin staining displayed the morphology of sham-operated LV, and infarcted RV and LV of sedentary rats. LV of sham-operated or contra-lateral non-infarcted RV of infarcted animals showed occasional, faint staining for t-PA (G-H) and negligible staining for PAI-1 (J-K) and VN (M-N). Marked accumulation of PAI-1 and VN staining was evident within the peri-infarct and infarcted region (L, O). Elevated t-PA staining was evident within the endothelium of small blood vessels of the peri-infarct zone (I, arrow, inset high magnification). Faint staining was also present in ventricular cardiomyocytes. No staining was noted in control normal rabbit IgG (NRG) (D-F). Scale bar = 100 μm.
Figure 7-2. Exercise increases t-PA in rat myocardium. Immunohistochemical staining (40x objective) demonstrated an accumulation of t-PA in hearts of rats subjected to a regular exercise regimen, with increased intensity noted particularly post-MI. t-PA staining was observed localized primarily to ventricular cardiomyocytes and was reduced in vascular endothelial cells (arrows). Scale bar = 100 μm.
Exercise-trained MI rats showed highest levels of t-PA staining which was significantly increased when compared to MI hearts of sedentary animals (13.3 ± 1.5% LV vs. 1.2 ± 0.4% LV t-PA positive area, p < 0.01) (Figure 7-3 A). Sham-operated exercised rat hearts also showed increased t-PA staining which was significantly elevated when compared to hearts of sham-operated sedentary animals (6.9 ± 2.9% vs. 1.8 ± 1.1% p < 0.01) (Figure 7-3 A). Furthermore, t-PA was also considerably increased between the sham-operated and MI exercise groups (P < 0.05) (Figure 7-3 A).

Levels of t-PA mRNA levels extracted from the paraffin embedded rat hearts were examined using RT-PCR. Low levels of the two selected t-PA amplicons (130 bp and 79 bp) were detected in rat hearts from the sham-operated and acute MI sedentary groups (Figure 7-3 B). Conversely, both t-PA amplicons were detected at increased intensity in rat hearts of both sham-operated and MI exercise groups, where band were consistent with those expressed by the Bowes melanoma cell standard. The use of primer set t-PA-1 revealed a strong band in both sham-operated and MI animals subjected to exercise. DNA sequencing of the 130 bp PCR product revealed a 95% sequence identity to rat t-PA mRNA and corresponded to the correct site on the known rat t-PA mRNA sequence. Additionally, an unexpected distinct band (~ 200 bp) was detected above the 130 bp t-PA amplicon when using the t-PA primer (t-PA-1, Table 7-1) and was found exclusively in hearts of rats from both exercise groups (Figure 7-3 B). The upper ~ 200 bp band also revealed homology to sequences corresponding to rat t-PA mRNA. The use of primer set t-PA-2 also revealed a distinct band in animals from all groups consistent with rat t-PA mRNA. This band was noted in both exercise groups, and was particularly robust in the exercised MI mice.
Exercise training increases rat cardiomyocyte t-PA synthesis. A) Quantitation of t-PA positive rat myocardium demonstrated a significant increase in t-PA positive area in rats subjected to an exercise regimen, both sham-operated and infarcted. Bars represent mean ± standard error of mean, (*) represents significant difference in optical densities; p < 0.05. B) Two t-PA amplicons at 130 bp (lane 1) and 79 bp (lane 2), and positive control mRNA isolated from Bowes melanoma cells (lane 3) were present in all four groups of rats. Increased expression of both t-PA amplicons (lanes 1 and 2) was observed in sham-exercised and AMI exercised groups. Sequence analysis of an intense band located above the 139 t-PA amplicon, at ~200 bp, exclusive to both exercised groups was found to encode the rat t-PA gene (lane 1). Amplicon expression of 18S mRNA (488 bp) was present in all four groups.
7.3.3 t-PA synthesis, storage and secretion by neonatal rat cardiomyocytes

Neonatal rat cardiomyocytes were cultured under serum free conditions in the absence or presence of ISO for 72 hours. Total RNA was subjected to reverse transcription and PCR amplification using primers specific for rat t-PA. mRNA was detected as a 800 bp band corresponding to the 5' and 3' untranslated regions of the rat t-PA cDNA. The t-PA message was found in the isolated neonatal cardiomyocytes, as well as in the non-cardiomyocytes cell population (endothelium and fibroblasts) and positive control rat cDNA from cultured endothelial cells (Figure 7-4A, upper bands). Additional PCR for ANF was used to validate the identity of the isolated cells as cardiomyocytes. ANF mRNA expression was identified as a 246 bp cDNA fragment in the cultured cardiomyocytes, but was absent from non-cardiomyocytes cultures (Figure 7-4A, lower bands).

Western immunoblotting of the 72 hour serum free culture supernatants from the neonatal cardiomyocytes treated in the presence or absence of ISO confirmed inducible t-PA antigen secretion. The majority of the t-PA in the cardiomyocyte culture supernatants was complexed with released PAI-1 (~115 kDa), with low levels of free t-PA (60 kDa) (Figure 7-4B). In contrast, the majority of cardiomyocyte PAI-1 was in the free form detected as a heavy band at 45 kDa (Figure 7-4B, top, lane 2-3). Treatment of cardiomyocytes with ISO increased the total secreted t-PA and PAI-1 by an estimated 2-fold as detected by densitometry.
Figure 7-4. ISO-stimulated neonatal rat cardiomyocytes produce and express t-PA. Cells from neonatal rat hearts were cultured and separated into cardiomyocytes (CM) and non-CM populations. A) Primers specific for rat t-PA were used to identify t-PA (800 bp) mRNA expression from both CM and non-CM populations of cells. ANF (246 bp) mRNA was only detected in the CM population. B) Western blot of serum free (SF) culture supernatants from rat endothelial cells (ECs) and CM treated in the presence or absence of isoproterenol (ISO) for 72 hours. Majority of t-PA in both untreated and ISO treated cells was complexed with PAI-1 while majority PAI-1 was in the free, uncomplexed form.
Distribution of t-PA in neonatal cardiomyocytes was also examined by confocal microscopy analysis. In the absence of ISO treatment, neonatal cardiomyocytes were generally small in volume with low t-PA and actin immunoreactivity (Figure 7-5A). ISO stimulation increases cardiomyocytes size, with concurrent increases in the actin cytoskeleton along with the number of t-PA positive vesicles per cell (Figure 7-5B). To test whether t-PA was secreted in a regulated secretory manner dual labelling with Abs against t-PA and GLUT-4 was performed. t-PA-positive granules co-localized with GLUT-4 in the ISO treated neonatal cardiomyocytes (Figure 7-5 C-F).

7.3.4 t-PA synthesis, storage and secretion by adult rat cardiomyocytes

To test whether acute t-PA secretion can be induced in cultured adult rat cardiomyocytes we tested the effects of β-adrenergic agonist ISO on the acute inducible secretion of t-PA. Immunofluorescence staining revealed a time-dependent increase in the accumulation of t-PA evident throughout the cytoplasm of ISO-treated cells (Figure 7-6 E-H and I-L) relative to non-stimulated cardiomyocytes (Figure 7-6 A-D). t-PA staining was absent from IgG treated control cells (Figure 7-6 A-D). Desmin staining demarcated the sarcomeric Z-lines and intercalated discs of the isolated cardiomyocytes (Figure 7-6 B, F, J). Intracellular t-PA staining demonstrated a time-dependent accumulation of t-PA in discrete secretory granules within ISO-treated cardiomyocytes, and became progressively more evident by 30 and 90 minutes after treatment (Figure 7-6 E, I). A deconvolved higher magnification image of a single cardiomyocyte after 90 minutes of treatment with ISO reveals distinct t-PA vesicles dispersed throughout the cell (Figure 7-6 M).
Figure 7-5. ISO-stimulated neonatal rat cardiomyocytes store t-PA in vesicles. A-B) Confocal microscopic images of immunolabelled cultured neonatal cardiomyocytes in the absence (A) or presence (B) of ISO for 72 hours showed an ISO-inducible increase in intracellular t-PA (yellow/green) localized within discrete peri-nuclear vesicles (arrow). C-F) Hypertrophic effects of ISO were also evident on cardiomyocyte growth and actin cytoskeleton development (red). GLUT-4 (C) and t-PA (D) within ISO-treated cells. E-F) Higher magnification images of serial 2-plane composite of GLUT-4 (C), t-PA (D), and phase contrast images revealed colocalization of t-PA with GLUT-4 (arrows, yellow, Panels E-F). Scale bars = 5 μm.
Figure 7-6. ISO-stimulation increases t-PA accumulation in adult rat cardiomyocytes over time. Immunofluorescent staining of cultured adult rat cardiomyocytes at 0 (A, B, C, D), 30 (E, F, G, H) and 90 (I, J, K, L) minutes following treatment with ISO stained for t-PA (green; A, E, I), desmin (red; B, F, J) and nuclei (blue; C, G, K). Intracellular t-PA accumulation increased over time of treatment with ISO. t-PA was present within discrete secretory vesicles and localized to cellular periphery (D, H, L). Scale bar = 20 μm. Higher resolution images demonstrated distinct t-PA granules (arrow) confined to the cytoplasm and sarcolemma following 90 minutes of ISO stimulation (M, N). Scale bar = 10 μm.
Closer examination of a magnified region (box) reveals t-PA positive secretory granules localized within the cytoplasm (Figure 7-6 N, arrow), as well as at the cell periphery.

The time-dependent effect of ISO on adult cardiomyocytes t-PA biosynthesis was also confirmed by Western blotting. The constitutive release of t-PA antigen into the supernatants of cultured cells after 24 hours of culture with no ISO-treatment is evident lane 1 (Figure 7-7 A). Furthermore, the acute regulated release of t-PA from the cultured cells was evident following the addition of ISO detected as robust bands at ~ 70 kDa (Figure 7-7 A, arrow). t-PA levels increased by ~ 1.5 fold following 30 minutes and ~ 3.2 fold following 90 minutes of ISO stimulation when compared the untreated controls indicative of release from an apparent t-PA storage pool (Figure 7-7 B).
Figure 7-7. ISO-stimulation increases adult rat cardiomyocyte t-PA secretion over time. A-B) Isolated rat cardiomyocytes cultured for 24 hours then treated with ISO showed an acute time-dependent secretion of cardiomyocyte t-PA into the culture supernatant. A) Western blot of serum-free culture supernatants demonstrated a time-dependent increase in t-PA secretion following treatment with ISO. Low levels of t-PA were detected in the culture supernatants after 24 hours without treatment (lane 1). t-PA was levels progressively increased over time following ISO treatment as noted at 30 and 90 minutes (lanes 2 and 3 respectively). t-PA standard appeared in lane 4.
7.4 Discussion

The PA/plasmin system plays a major role in post-MI wound healing and LV remodeling [116, 181, 185]. In Chapter V we demonstrated the effects of VN on post-MI tissue healing, by means of regulating the PA/plasmin system. Although t-PA levels are elevated in heart post-MI [216], its importance to the myocardial remodeling process is not commonly recognized [117]. Interestingly, our observations from ischemic and infarcted human tissue suggest that t-PA is localized throughout the myocardium and consequently may be involved in the remodeling process (Figure 7-8). In this chapter we have investigated the effects of hypertrophic stimuli on regulation of the fibrinolytic system in infarcted myocardium and cultured rat cells. We provided several lines of evidence which suggests that both neonatal and adult cardiomyocytes are a significant cellular source of t-PA under certain hypertrophic conditions. First, we have shown that following a six-week exercise regimen, expression of t-PA increased in rat hearts; this was observed throughout the infarcted, peri-infarcted and non-infarcted myocardial regions of LAD ligated and sham-operated animals. t-PA staining was not exclusive to endothelial cells, but occurred predominantly in cardiomyocytes. In comparison no increase was evident in sedentary animals following MI or sham-operation. The increased tissue staining was accompanied by increased t-PA mRNA expression in both exercised groups. Furthermore, we were able to confirm that cardiomyocytes were the source of the t-PA, as evidenced by the ISO-induced synthesis, storage and secretion of t-PA by cultured adult rat cardiomyocytes. Additionally, consistent with our mouse studies we discovered that under sedentary conditions accumulation of PAI-1 and VN was observed in the infarct and peri-infarct regions.
following acute MI. Exercise exhibits favorable effects on ventricular remodeling in rats subjected to LAD ligation [117]. Those results demonstrated that exercise can modulate myosin heavy chain (MHC) gene expression, which is normally expressed during fetal development but also occurs following infarction. Others have demonstrated that α-MHC, but not β-MHC, is significantly up-regulated following exercise training in rats [117]. Exercise is known to alter gene expression in the rat heart [198, 345]. The current study confirms that exercise also increases t-PA mRNA expression throughout both infarcted and non-infarcted hearts. These observations are consistent with previous clinical findings demonstrating that exercise increases t-PA antigen and activity in the circulation [345, 346]. Of note, it appears that it is primarily physiological rather than pathological hypertrophic stimuli that is responsible for significant induction of t-PA expression, synthesis and secretion [347]. This may help explain why rats in the exercise groups demonstrated increased cardiac t-PA levels, while t-PA levels in the sedentary acute MI group were not elevated compared to sham-operated sedentary controls. Furthermore, an additional mRNA band observed in the exercise trained groups was absent from sham-operated and infarcted sedentary rat hearts. Sequence homology analysis determined this band to be homologous with t-PA. Thus, it is possible that exercise induces the expression of an additional t-PA splice variant, as previously noted with expression of other factors such as vascular endothelial growth factor (VEGF) and insulin-like growth factor 1 (IGF-1) [348, 349]. It is plausible that the additional t-PA isoform may be responsible for the increase of total t-PA levels present in the myocardium and circulation. Stimulation of isolated cardiomyocytes with ISO, an agent known to induce cardiomyocyte hypertrophy both in vivo and in vitro [350, 351], also demonstrated an accumulation of inducible
Figure 7-8. Localization of t-PA, PAI-1 and VN at sites of myocardial injury. H&E staining demonstrated tissue morphology and Masson's Trichrome (TRI) areas of collagen deposition. Immunohistochemical staining demonstrated an accumulation of t-PA, PAI-1 and VN in the myocardium with severity of infarction. t-PA staining was present at sites of acute MI and at sites of cardiac rupture. PAI-1 and VN staining localized initially at sites of myocardial ischemia and progressively accumulated at sites of acute and chronic MI. Staining for VN was also evident at sites cardiac rupture. Scale bar = 300 μm.
intracellular t-PA. Confocal microscopy of adult rat cardiomyocytes demonstrated ISO-stimulated, time-dependent secretion of t-PA localized in apparent secretory granules. Although vesicular storage and secretion of t-PA in cardiomyocytes is a possibility, additional experiments confirming this phenomenon are required. Moreover, ISO stimulation of neonatal cardiomyocytes in vitro demonstrated the colocalization of t-PA with GLUT-4 suggesting that t-PA may be secreted in a fashion similar to other storage proteins [349, 351]. The trafficking of GLUT-4 in various cell types is accomplished via a regulated secretory pathways including in cardiomyocytes [352]. Thus, it is probable that cardiomyocyte-derived t-PA traffics via the regulated secretory pathway, similar to that of GLUT-4.

Although t-PA is expressed by various known cell types [352, 353], our data suggest that a certain quantity of t-PA can be attributed to the heart muscle itself, particularly when stimulated by exercise training. Interestingly, exercise increases circulating t-PA levels, while simultaneously reducing PAI-1, hence reducing the risk of repeat coronary thrombosis [147, 354]. The concept that impaired plasma fibrinolytic activity is associated with low t-PA activity due to elevated plasma PAI-1 levels and is a risk factor for IHD has been strongly supported by numerous studies [147, 169]. PAI-1 promotes thrombosis and its persistence is a critical determinant in the fibrotic reorganization of the thrombi by inhibiting plasmin-mediated degradation of fibrin and other ECM proteins through MMPs [169, 189, 355, 356]. Thrombi will ultimately prevent revascularization of ischemic myocardium and may also contribute to the increased incidence of reinfarction in patients with impaired fibrinolysis [357, 358]. Reports suggest that impaired thrombolysis of t-PA deficient or PAI-1 overexpressing mice can be completely restored using adenoviral-mediated gene transfer of recombinant t-PA [359]. Thus, increased expression of t-
PA can promote thrombus dissolution restoring myocardial flow, potentially averting MI. We have demonstrated that exercise influences increase of cardiac t-PA levels due to elevated cardiomyocyte synthesis. Whether cardiomyocyte derived t-PA contributes to the systemic t-PA population promoting thrombolysis at sites of vascular occlusion is unclear. However, it is feasible that exercise training may not just influence ventricular remodeling post-MI but also improve the fibrinolytic potential of the heart [360, 361]. Blood samples were not available from the original experiments so we could not examine the relative contribution of t-PA secreted from cardiomyocytes, vessel wall endothelium and that from the systemic circulation. However, it is likely that t-PA from all sources contributes by attempting to regulate the overwhelming pathological effects of PAI-1 in the myocardium [118, 131, 358].

In addition, it is evident that u-PA has a major function in ventricular remodeling [118, 277], and therefore it is also crucial to investigate the effects of exercise training on u-PA expression in the heart.

A central component of post-MI remodeling is tissue fibrosis which leads to compromised cardiac function and heart failure [131, 216]. Cardiac t-PA expression may play an essential role in the wound healing response to MI by stimulating plasmin-dependent fibrin degradation. In addition to increased t-PA expression in the infarcted LVs, we also observed increased accumulation of PAI-1, and its binding cofactor VN, that co-localized at sites of infarction and fibrosis (Figure 7-8). Increased PAI-1 levels post-MI contribute to the development of cardiac tissue fibrosis probably by preventing ECM turnover by inhibiting PAs [216, 362]. Increased synthesis and secretion of t-PA into the ECM, collectively with u-PA, MMPs and other proteases derived from infiltrating mononuclear and various myocardial cells render an important role in remodeling of the infarcted myocardium.
ECM remodeling post-MI is a complex process that involves activation of cytokines, MMP/plasmin systems and synthesis of fibrin and collagen. Collagen accumulation is more profound in t-PA-deficient animals when compared to WT mice in models of lung and skin fibrosis [180, 205, 364]. Thus, it is also probable that insufficient t-PA reduces fibrinolysis and can contribute to enhanced collagen accumulation in the infarcted myocardium. This process may occur by means of one or multiple mechanisms: 1) increase in fibrin deposition may increase fibroblast proliferation and migration; 2) by serving as a reservoir for growth factors increase in fibrin can promote ECM protein synthesis; 3) by reducing plasmin-mediated activation of MMPs. Consequently, increased exercise-induced cardiomyocyte t-PA expression post-MI can avert excessive accumulation of collagen, thus preventing tissue stiffness and ventricular dysfunction which leads to heart failure [180]. In addition, cardiomyocyte-derived t-PA-mediated proteolysis could facilitate the infiltration of other cells required for wound healing [180] including endothelial cells and fibroblasts [58, 117, 365]. Thus, it is possible that induction of t-PA expression by cardiomyocytes at sites of post-MI injury prevents fibrin accumulation and promotes MMP-dependent proteolysis resulting in favourable remodeling and improved cardiac function. These findings are likely secondary to release of acute inflammatory mediators that increase vascular permeability and plasma VN exudate into the tissues, together with stimulation of PAI-1 biosynthesis by activated endothelial cells or cardiomyocytes [365]. Only negligible PAI-1 and VN were noted in the LV of the sham-sedentary group, as well as in the non-infarcted RV of the sedentary acute MI group. However, VN commonly accumulates at sites of tissue injury where it can be bind PAI-1 promoting its activity. The increased presence of PAI-1 and VN in the infarcted LV of sedentary mice are likely responsible for
increased tissue fibrosis. Therefore, a balance between t-PA, PAI-1 and VN is critical in the acute and chronic healing process following MI. In the acute post-MI period, cardiomyocytes produce t-PA in an acute attempt to remodel the interstitium, while PAI-1 limits tissue proteolysis and prevents cardiac rupture by inhibiting both cardiomyocyte t-PA and u-PA secreted by infiltrating leukocytes [117, 212]. We speculate that VN facilitates PAI-1 deposition in damaged tissues promoting its inhibitory activity against t-PA, thereby augmenting fibrosis. Thus, timely intervention with controlled, sustained regular exercise following MI may increase t-PA levels in the peri-infarct region thereby limiting myocardial fibrosis and improving cardiac function.

7.5 Conclusions

In conclusion, we found that exercise trained rats exhibited increased cardiac t-PA levels compared to sedentary animals, with a more significant increase evident following MI. In accordance with previous studies [68] t-PA mRNA levels were also enhanced in exercised rats, both sham-operated and post-MI. The lack of a major increase of t-PA staining in sedentary-MI rats suggests that t-PA gene expression was predominantly related to exercise and not ischemia, however the greater increase in infarcted, exercised rat hearts suggest a synergistic effect. It is plausible that increases in circulating and tissue t-PA following exercise may be regulated by the PA/plasmin system, and more specifically via VN. Thus, exercise-induced t-PA gene expression leading to increased t-PA production by cardiomyocytes may promote favorable ventricular remodeling by reducing post-MI cardiac fibrosis. Furthermore, we have demonstrated that adult rat cardiomyocytes
are capable of inducible synthesis and storage of t-PA, particularly when subjected to hypertrophic stimuli.

7.6 Additional Considerations

It is probable that exercise attributed not only to increased t-PA levels but also to a reduction of PAI-1. Immunohistochemistry for PAI-1 showed a decrease in PAI-1 staining in exercised MI hearts when compared to sedentary. However, PAI-1 is regulated at multiple levels including stimulation/inhibition by inflammatory cytokines as well as by VN in the plasma and ECM. Examination of circulating t-PA, PAI-1 and VN levels and activity post-MI would lead to understanding the therapeutic benefits of exercise. However, since the original study was completed in 1994, there was a significant lack of experimental tissue available while remaining available tissue was excessively degraded and sufficient data was not obtained. Thus we did not investigate the influence of PAI-1 on t-PA levels or other components of the fibrinolytic system. Additionally, tissue used for the experiments described above was derived from rat hearts which have undergone exercise training for six weeks post-MI. It would be of interest to investigate the effects various durations and frequencies of exercise required for an increase in cardiomyocyte t-PA. Another crucial aspect which remains unanswered at the present time is whether cardiomyocyte-derived t-PA contributes to total plasma t-PA. By determining whether cardiomyocyte t-PA enters into circulation would decipher its involvement in maintaining elevated fibrinolytic activity in addition to the potential role in post-MI wound healing.
Although much remains to be answered about the attribution of cardiomyocytes to t-PA synthesis particularly in regulation of post-MI wound healing, it is recognized that exercise significantly increased t-PA levels. Further work must be undertaken to better understand the means by which t-PA synthesis, storage and secretion is regulated in cardiomyocytes.
CHAPTER VIII CONCLUSIONS AND FUTURE DIRECTIONS

During the course of my graduate studies, I have had the opportunity to investigate the effects of VN on hypoxic and ischemic injury both, in vitro and in vivo. Specifically, I have had the privilege to investigate the role of VN in regulation of intracellular and extracellular protein interactions, exploring mechanisms associated with regulation of cardiomyocyte contractility and pursue its function in myocardial healing post-MI.

Although much has been established about the contributions of VN in various pathological states, prior to the commencement of my studies little was understood regarding the role of VN following ischemic injury and post-MI wound healing. To date, limited work is published on the involvement of VN in cell function and post-MI cardiac remodeling. My study made the first attempt to verify the role of VN in the heart, particularly its interactions with sarcomeric proteins, as well as regulation of the PA/plasmin and MMP systems following infarction. Work completed over the last five years has allowed me to elucidate the role of VN in MI. Particularly I tried to gain novel insight into the function of VN at the molecular, cellular and tissue levels in inhibiting cardiomyocyte function and adaptation during the complex process of tissue remodeling. We have made significant strides towards comprehending how VN is involved in regulating processes modulating myocardium healing following ischemic injury. My studies have contributed to several facets of scientific knowledge and have exhibited potential clinical significance which has resulted in obtaining of a provisional patent (US60/836,932). Furthermore, our additional finding related to the healing of the myocardium following infarction
focused on the expression of t-PA in cardiomyocytes, particularly when challenged by physical stimuli. Our work for the first time demonstrated that t-PA is expressed by cardiomyocytes especially following the induction of exercise. These studies which served as a stepping stone and went hand-in-hand with studies exploring the effects of VN in post-MI wound healing, as we recognized a potentially vital relationship between VN and regulation of PA/plasmin system.

Thus, our studies have provided novel evidence that VN is an important component in regulation of contractility in compromised adult cardiomyocytes which is associated with the binding of IF proteins. Moreover, we recognized that VN exhibits adverse effects in the healing process following MI as demonstrated in VN null mice over a period of 60 days. Additionally, based on our findings we propose mechanisms by which VN commands post-MI remodeling. A proposed model summarizing the effects of VN in regulating cell function (intracellular) and ECM remodeling (extracellular) are presented in Figure 8-1.
Figure 8-1. Schematic model of VN in regulation of cardiomyocyte contractility and ECM remodeling. At sites of myocardial ischemia VN enters damaged cardiomyocyte and binds to desmin IF. By binding to desmin VN disrupts sarcomeric machinery inhibiting cell contractility. Influx of VN from plasma into the ECM causes binding to PAI-1, inhibiting t-PA and u-PA which in-turn prevent the generation of plasmin. Reduction in plasmin prevents activation of pro-MMP which in-turn can reduce intracellular and extracellular degradation. Additionally, by binding to integrins and/or uPAR on the cell surface, VN stimulate various signaling pathways which are involved in adhesion, migration, proliferation and contractility.
8.1 Contributions to Scientific Knowledge

1. VN localizes at sites of ischemic heart damage,
   a. Intracellularly; binding to the desmin IF cytoskeleton
   b. Extracellularly; binding to proteins in the ECM
2. Entry of VN into hypoxic cardiomyocytes occurs by:
   a. energy-independent diffusion of permeabilized sarcolemma and/or
   b. energy-dependent process
3. VN binds desmin IF with a high affinity
4. VN significantly impairs in vitro cardiomyocyte contractility under hypoxic conditions
5. VN-/- mice display reduced collagen deposition in the infarcted LV 28 days post-MI
6. Normal and infarcted VN-/- mice exhibit comparable cardiac geometry to those of WT mice
7. VN-/- mice exhibit a reduction in neo-vessel formation at 7 and 28 days post-MI
8. VN-/- mice exhibit reduced inflammatory cell infiltration 3 days post-MI
9. VN-/- mice exhibit reduced t-PA, u-PA and PAI-1 mRNA expression particularly at 3 and 7 days post-MI
10. VN-/- mice exhibit increased protein levels of t-PA and decreased expression of PAI-1
11. VN-/- exhibit trivial differences in MMP-2 and MMP-9 activity compared to infarcted WT mice
12. Adult cardiomyocytes are capable of t-PA expression, storage and secretion
13. Exercise training allows for increased cardiomyocyte t-PA expression particularly following MI

8.2 Strategies for Future Directions

1. Investigate the specific region on desmin responsible for the binding interactions with VN.

To identify the specific binding region(s) on desmin responsible for binding VN we have designed 3 specific peptide sequences from the N-terminus of desmin (Figure 8-2). These peptides were designed based on the previously published studies identifying the region on vimentin responsible for binding VN, which shares over 80% sequence homology to that of desmin. We will perform binding assays in which high-affinity 96 well plates will be coated with purified human desmin at a constant concentration or with blocking buffer containing BSA overnight at 4°C. Wells will then be incubated with either purified human biotinylated-VN (at constant concentration), a mixture of biotinylated-VN with desmin peptide (variable concentrations), or a scrambler peptide as control. These experiments will allow us to determine whether such engineered specific peptides are able to inhibit VN binding to desmin IF.
Figure 8-2. Construction of human desmin peptides and identification of the VN-binding site on the N-terminus of desmin. Schematic representation of peptides generated from human desmin of N-terminus domain used to investigate the specific binding site for VN. The N-terminal region of desmin, shares over 80% homology with vimentin IF, containing a non-α-helical domain with two motifs crucial for filament assembly. Peptide 1 (yellow) is composed of the first 25 amino acids and contains nonameric sequence SSYRRTFGG. Peptide 2 (green) is 27 amino acid and Peptide 3 (blue) 26 amino acids in length. Peptides 2 and 3 contain an arginine and proline-rich region known as the RP-box.
2. Investigate the interaction of VN with integrin and uPAR at sites of myocardial ischemia and infarction.

Specific integrins are implicated in mediating adhesion and functional activation of many different types of cells by binding to ECM proteins. Interactions of various integrins with different ECM proteins regulate homing, secretion and distribution of these cells in the tissue. We would like to examine the effects of VN on integrin expression and integrin regulated adhesion, migration and differentiation of inflammatory, endothelial progenitor, fibroblasts. We propose investigating the VN-integrin interactions by immunofluorescence staining and evaluate the distribution by confocal laser scanning microscopy. This data will provide a morphological correlate to previously reported functional studies, demonstrating the distribution of selected integrins during ECM adhesion at sites of infarction.

3. Investigate the effects on VN on cell signaling pathways.

FN exerts a strong angiogenic influence on endothelial cells which is mediated through the α5β1 and αvβ3 integrins via mitogen-activated protein (MAP) kinase signaling [366]. We would like to investigate whether VN also plays an active part in cell signaling enlisting an angiogenic response post-MI. To analyze the specific signaling pathways involved in angiogenesis that are stimulated by VN through interactions with specific integrins we propose in vitro experiments in which cells are treated with different pathway inhibitors to evaluate their effects.
4. Investigate the effects of VN on remodeling of the non-infarcted myocardium.

It is well known that following MI functionally distinct cardiac regions exhibit distinct mRNA and protein profile [367]. To have a more complete understanding of VN function on post-MI remodeling we propose further studies using the viable, non-infarcted portion of LV. Experiments should follow a similar protocol to these performed on infarcted LV. Specifically, we would like to explore the PA and PAI-1 levels, as well as levels and activity of MMPs and its inhibitors in the non-infarcted myocardium using protein and activity assays. Additionally, we would also determine mRNA levels of genes associated with ECM remodeling and hypertrophy.

5. Investigate the effects of VN on stem cell migration and homing in the infarcted myocardium.

There is growing evidence that stem cells promote regeneration of myocardium following MI. We have performed pilot experiments in which chimeric mice expressed green fluorescent protein (GFP) positive peripheral blood leukocytes and were then subjected to LAD ligation to examine post-MI stem cell distribution. Preliminary data from infarcted WT mice revealed an accumulation of GFP-positive stem cells in the infarcted regions of LV rich in VN at 14 days post-MI (Figure 8-3). It is well established that VN has a major role in cell adhesion and migration. We have found that VN accumulates in areas of ischemia and infarction. Thus, we propose to analyze the precise role of VN in adhesion, proliferation and homing of stem cells and in repair of the infarcted myocardium. We propose investigating stem cell distribution in both, chimeric WT and VN-/- mice post-MI by visualizing the cells by confocal microscopy. By staining for cardiac-specific
proteins such as troponin I, connexin 43 and actin we can determine whether VN promotes cell proliferation into cardiomyocyte-like phenotype. Furthermore, echocardiography would be used to establish whether VN promotes regeneration of damaged myocardium and improves cardiac function.
Figure 8-3. Bone marrow cells migrate to a VN-rich infarct region at 14 days post-MI. Normal (left panel) and infarcted (right panel) myocardium of chimeric WT mice expressing green fluorescent protein (GFP)-positive bone-marrow cells (green) stained for VN (red) and nuclei (blue). 14 days post-MI GFP-positive bone-marrow cells accumulated in the VN-rich region of infarcted myocardium. Only trivial amounts of GFP positive bone-marrow cells were present in the non-infarcted myocardium. Scale bar = 50 μm.
8.3 Final Remarks

In summary, over the course of my graduate studies I have attempted to explore the role of VN with respect to ventricular remodeling in the event of MI. Specifically, my research focused on investigating the effects of VN and its influence on the PA/plasmin and MMP systems in post-MI wound healing. The findings acquired through my investigations provided new insights into the cellular mechanisms of regulation contractility at sites of ischemia and infarction. Further investigation VN regulation of the PA/plasmin and MMP systems as well as purpose of VN-desmin interactions, will hopefully provide a better understanding of the post-MI wound healing process and provide insights into the pathobiology of heart failure. I hope that this newly acquired knowledge can have an impact on future strategies in advancing therapies for the treatment of ischemic heart disease and prevention of heart failure.


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APPENDIX I LIST OF PUBLICATIONS, ABSTRACTS AND PRESENTATIONS

Publications:


**Intellectual Property**

UBC File 07-046US “Improved ventricular remodeling and cardiac function following acute myocardial ischemia”.


**Highlights of Abstracts and Presentations:**


10. **Walinski H, Wang Y, McManus B.M and Podor T.J.** Vitronectin is localized with intermediate filaments in reversibly damaged cardiomyocytes at sites of acute myocardial ischemia and infarction. United States and Canadian Academy of Pathology, Vancouver, BC, Canada, Mod Pathol. 2004;17, Suppl 1: 60A.


32. Walinski H, Pate GE, Bohunek L, McManus BM, Podor TJ. Vitronectin regulates remodeling post-myocardial infarction by regulating PAI-1, MMPs and cell migration. National Research Forum for Young Investigators in Circulatory and Respiratory Health in Winnipeg, Manitoba, Canada. Clin Cardiol. 2006;11(1); 44.
33. **Walinski H**, Pate GE, Bohunek L, McManus BM, Podor TJ. Vitronectin knockout mice exhibit improved healing and function following myocardial infarction. 10th Annual Scientific Meeting of the Heart Failure Society of America, Seattle, Washington. J Card Fail. 2006; 12(6); Suppl 1;S26-S27.


APPENDIX II     AWARDS

Heart Failure Society of America Travel Grant (2006)
Young Investigator Forum Travel Award (2006)
Young Investigator Forum Travel Award (2005)
Canadian Cardiovascular Academy “Have A Heart” Bursary Winner (2004)
The XIIIth International Vascular Biology Meeting Travel Award (2004)
United States and Canadian Academy of Pathology Oral Presentation (2004)
UBC Graduate Student Travel Award (2003)
University of British Columbia PhD Tuition Award (2003-2007)