Cardiotrophin-1: Expression in Post-MI Heart and Effect on Primary Adult Cardiac Myofibroblasts *in vitro*

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In partial fulfillment of the Requirements

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by

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Cardiotrophin-1: Expression in Post-MI Heart and Effect on Primary Adult Cardiac Myofibroblasts *in vitro*

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Abstract

Ischemic heart disease is the most common cause of mortality worldwide. This is due to primary left ventricular failure, arrhythmias or congestive heart failure. Cardiac fibroblasts play an important role in myocardial healing and are responsible for accumulation of collagen in the infarct scar as well as viable myocardium, thereby directly contributing to the onset of systolic and diastolic heart failure. Cardiotrophin-1 (CT-1), a member of the IL-6 family of cytokines, has been shown to be elevated in the serum of patients with ischemic heart disease and valvular heart disease, and induces cardiomyocyte hypertrophy *in vitro*.

We investigated expression of CT-1 in post-MI rat heart and the effect of CT-1 on cultured primary adult rat cardiac fibroblasts with respect to proliferation, protein synthesis and cell migration. Elevated CT-1 expression was observed in the infarct zone at 24 hours and continued through 2, 4 and 8 weeks post-MI, compared to sham-operated animals. CT-1 induced activation of the Jak/STAT, p38 and p42/44 MAPK, PI3K/Akt and Src pathways in cultured adult cardiac fibroblasts. CT-1 induced cardiac fibroblast protein synthesis as indicated by incorporation of ^{3}H-leucine. Protein synthesis was dependent on activation of Jak/STAT, MEK1/2, PI3K and Src pathways as evidenced by decreased ^{3}H-leucine and ^{3}H-thymidine incorporation after pretreatment with AG490, PD98059, LY294002 and genistein respectively. CT-1 treatment increased procollagen-1-carboxypropeptide (P1CP) synthesis, a marker of mature collagen synthesis. CT-1 induced proliferation of rat cardiac fibroblasts as indicated by increased incorporation of ^{3}H-thymidine and total cell number, expression of cyclins A and E, hyperphosphorylation of retinoblastoma

protein and nuclear accumulation of PCNA. DNA synthesis was dependent on activation of Jak/STAT, MEK1/2, PI3K and Src pathways as evidenced by decreased ^{3}H-thymidine incorporation after pretreatment with AG490, PD98059, LY294002 and genistein respectively. CT-1 induced cell migration of rat cardiac fibroblasts in a dose-dependent manner. This was dependent on the Jak/STAT, PI3K and p42/44 MAPK pathways, as well as intact integrin function. Migration was also dependent on potassium channel function and myosin light chain kinase activity, as evidenced by decreased migration when co-incubated with 4-AP or TEA or ML-7. CT-1 treatment was associated with myosin light chain (MLC) phosphorylation and hyperpolarization of the myofibroblast cell membrane. Phosphorylation of MLC was dependent on intact MLCK and calmodulin function, indicating that CT-1 signaling is associated with a rise in intracellular calcium.

Our results suggest that CT-1, as expressed in post-MI heart, may play an important role in infarct scar formation and ongoing remodeling of the scar. CT-1 was able to initiate each of the processes considered important in the formation of infarct scar including cardiac myofibroblast migration as well as fibroblast proliferation and collagen synthesis. Taken together, these results suggest that CT-1 plays an important, heretofore unrecognized role in infarct scar formation and angiogenesis in this model of experimental MI. Further work is required to determine factors that induce CT-1 expression, its interplay with other mediators of cardiac infarct wound healing in the setting of acute cardiac ischemia and chronic post-MI heart failure, and whether it confers a beneficial effect or contributes to maladaptive cardiac fibrosis.

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List of Abbreviations

4-AP	4-aminopyridine
ACE	angiotensin converting enzyme
Ang II	angiotensin II
Arp	actin related protein
αSMA	α-smooth muscle actin
AT_1	angiotensin type I receptor
AT_2	angiotensin type II receptor
CAD	coronary artery disease
BMP	bone morphogenic protein
CDK	cyclin dependent kinase
CHF	congestive heart failure
CNTF	ciliary neurotrophic factor
CT-1	cardiotrophin-1
DMEM	Dulbecco's modified Eagle medium
ECM	extracellular matrix
EGF	epidermal growth factor
eIF	eukaryotic initiation factor
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FA	focal adhesion
FAK	focal adhesion kinase
FGF	fibroblast growth factor

FRAP	FKBP 12-rapamycin-associated protein
HSP	heat shock protein
IL	interleukin
iNOS	inducible nitric oxide synthase
JAB	Jak-binding protein
Jak	janus-actived kinase
JNK	c-jun N-terminal kinase
LIF	leukemia inhibitory factor
LIFR-β	leukemia inhibitory factor receptor-β
LV	left ventricle
MAPK	mitogen activated protein kinase
MEK	mitogen activated protein kinase kinase
MI	myocardial infarction
MLC	myosin light chain
MLCK	myosin light chain kinase
MMP	matrix metalloproteinase
mTOR	mammalian target of rapamycin
OSM	Oncostatin M
P4H	prolyl-4-hydroxylase
P1CP	procollagen-1-carboxypropeptide
PAGE	polyacrylimide gel electrophoresis
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen

PDGF	platelet derived growth factor
PI3K	phosphoinositol-3-kinase
PLC	phospholipase C
Pmn	progressive motor neuropathy
RAAS	renin angiotensin aldosterone system
Rb	retinoblastoma protein
RIPA	radioimmunoprecipitation assay
rMLC	regulatory myosin light chain
SOCS	suppressor of cytokine signaling
SCF	stem cell factor
SDS	sodium dodecyl sulfate
SSI	STAT induced STAT inhibitor
STAT	signal transducer activator of transcription
TGF-β	transforming growth factor-β
TNF	tumor necrosis factor
TEA	tetraethylammonium
VEGF	vascular endothelial growth factor
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP-family verprolin-homologous protein

I. Introduction and Statement of the Problem

Myocardial infarction (MI) is a major contributor to mortality and morbidity in all developed nations [1]. If the initial infarct is large the prognosis of post-MI patients is grim; this is due to the relatively rapid development of congestive heart failure. Despite a concerted effort by many groups, a clear understanding of the precise mechanisms that contribute to the pathogenesis of post-MI heart failure remains elusive. Myocardial healing after infarction is characterized by complex time-dependent alterations of ventricular architecture of the remnant (non-infarcted) myocardium as well as the infarct zone itself [2-4]. The sum of these gross ventricular changes, including cardiac hypertrophy and altered gross ventricular geometry, are referred to as ventricular remodeling. A component of this process is interstitial (or extracellular matrix) remodeling of the remnant heart and wound healing of the infarct scar [3]. Thus matrix remodeling is manifest as interstitial fibrosis of the remnant heart and the progressive evolution of the structure of the infarct scar [5]. In normal heart tissue, matrix protein secretion and deposition is carried out exclusively by cardiac fibroblasts with relatively low turnover of proteins. In the post-MI heart, however, turnover of the extracellular matrix is accelerated [4,6,7], a process that is modulated by interstitial fibroblasts and myofibroblasts, which are phenotypic derivatives of interstitial fibroblasts [8].

The primary function of adult cardiac myofibroblasts is to synthesize fibrillar collagens to maintain the integrity of the cardiac matrix. While fibrosis contributes to the development of heart failure, the mechanisms by which these cells come to populate the scar are not well understood. In the early period after infarction, the

scar must become repopulated with cells. This happens via migration and proliferation of interstitial fibroblasts from the adjacent non-injured myocardium. Much of the current literature that addresses cardiac fibroblast function deals with the effects of a limited number of profibrotic factors. Little information to specifically address fibroblast migration and proliferation is available in the literature. Thus the mechanisms governing fibroblast and myofibroblast movement to the infarct site is largely unknown.

Cardiotrophin-1 (CT-1) is a member of the IL-6 superfamily of cytokines, and is known to induce hypertrophy of cardiac myocytes via induction of sarcomeric proteins in series [9]. While the hypertrophic effect of CT-1 on cardiac myocytes is fairly well characterized [9-13], its expression in the post-MI heart and its influence on nonmyocyte behavior remains to be defined. Although other members of the IL-6 family of cytokines have been postulated to play a role in the acute inflammatory response of post-MI heart, they are overexpressed in a highly transient manner, wherein expression rapidly increases and declines to normal levels within days after infarction [14]. Thus, it would appear that other factors are involved in repopulation of the infarct scar with myofibroblasts after MI. As there is very little information concerning this phenomena is available in the literature, we have undertaken a study to address the effects of CT-1 on myofibroblasts. Specifically, this thesis addresses the expression of CT-1 in post-MI rat heart and its effects of CT-1 on cardiac myofibroblast migration, proliferation and protein synthetic function with a view to furthering our understanding of how the heart is repaired after myocardial infarction. The general premise to be investigated is that CT-1 influences cardiac myofibroblast

function, migration and proliferation in a manner which is essentially opposed to profibrotic hormone effects such as those associated with angiotensin II (angiotensin) and TGF-β1. In other words, we expect that while CT-1 exerts a weak influence over the synthesis and secretion of collagen by myofibroblasts (relative to known profibrotic factors), it strongly induces migration and proliferation in these cells. As such, CT-1 may serve as a major stimulus for repopulation of the infarct scar, and may serve to antagonize the detrimental effects of over-driven angiotensin and TGF-β1 signaling.

The first aim within the framework of our working hypothesis is to assess the time-course of CT-1 expression in post-MI heart, including the infarct zone and the remnant, viable myocardium. We will show that CT-1 is rapidly expressed after permanent coronary artery ligation and stays elevated in the infarct scar out to 8 weeks. We also suggest that CT-1 expression in the remnant heart is not elevated until the onset of overt hypertrophy and fibrosis (~ 8 wks). Thus we expect that CT-1 expression is very different from either that of other IL-6 family members as well as that of the established profibrotic factors. *The second aim* within the working hypothesis is to determine signaling pathways utilized by CT-1 in transducing its effects on cardiac fibroblasts. We will show that the effects of CT-1 require activation of multiple signaling pathways in these cells. *The third aim* is to assess whether CT-1 augments DNA and protein synthesis as well as myofibroblast proliferation, and we aim to provide linkage between activation of salient signaling pathways and cell proliferation. We will also show that the effect of CT-1 is much greater on cell proliferation than on mature collagen synthesis. *Fourth*, we will

examine whether CT-1 is a chemokine for cardiac myofibroblasts cultured. We will show that CT-1 is a chemoattractant for cardiac fibroblasts, and this phenomenon is dependent on specific intracellular signaling pathways, integrin function and potassium channel function. We will also show that CT-1 induces hyperpolarization of the cell membrane, consistent with the requirement for intact potassium channel function for effective cell migration. In short, our data will show that CT-1 may subserve acute infarct scar healing and maturation through activation of cardiac myofibroblasts. By demonstrating elevated CT-1 in the remnant heart in the chronic state, we suggest that CT-1 contributes to the ongoing wound healing and the pathogenesis of post-MI heart failure with particular reference to myocyte hypertrophy.

II. Review of the Literature

1. Introduction: Epidemiology, clinical correlation, and the molecular consequences of acute MI. Coronary artery disease (CAD) is the leading cause of death in the world [1]. The sequelae of CAD include angina pectoris, myocardial infarction (MI) and congestive heart failure (CHF). Myocardial infarction occurs when there is an abrupt cessation of coronary blood flow, usual secondary to rupture of an atherosclerotic plaque with thrombus formation [15]. The mortality associated with MI has been reduced over the past 15 years with widespread clinical use of thrombolytic agents, resulting in an increased number of survivors who are subject to the sequelae of MI such as CHF and arrhythmias [16]. Myocardial healing after MI includes proliferation of fibroblasts and deposition of new extracellular matrix proteins, not only in the infarct zone, but also in the remnant, viable myocardium [3]. This process is required to maintain the integrity of the ventricular wall, but can lead to CHF from both systolic and diastolic failure [7]. Congestive heart failure is the leading hospital discharge diagnosis in the United States, and consumes a significant amount of health care resources [17]. As such it is the focus of a significant amount of clinical and basic science research aimed at prevention and altering the course of the disease.

Upon cessation of coronary blood flow, resident mast cells degranulate and initiate an inflammatory response. This response is aggravated by trapping of neutrophils and monocytes within the myocardial capillary network. These cells secrete cytokines that activate monocytes to begin the process of clearing necrotic myocytes and other debris [18]. It has been shown that outcomes are improved if

myocardial blood flow is re-established, even after the infarct has been completed (the open-artery hypothesis) [19]. This may indicate that the leukocyte mediated inflammatory response is necessary for appropriate wound healing.

Remaining cells also secrete chemokines that induce migration of interstitial fibroblasts from the adjacent viable myocardium to the infarct zone. As they migrate into the infarct zone, these cells activate matrix metalloproteinases (MMPs) that degrade the matrix [20]. Once taking up residence in the infarct zone, these fibroblasts proliferate, transform into actin expressing myofibroblasts, and synthesize new extracellular matrix proteins [21]. These cells persist for many years after infarction [22] and in the late stages of wound healing, mediate contraction of the scar, which results in scar thinning and dyskinesis with ventricular systole [23]. Thus cardiac myofibroblasts constitute a key component of the abnormal cellular complement attending the chronic phase of wound healing after myocardial infarction. Consideration of global cardiac remodeling, which includes the noninfarcted or remnant myocardium, in addition to the specific events that occur within the site of infarct facilitates an understanding of the pathophysiology of post-MI heart failure.

2. Chronic phase of wound healing after myocardial infarction, cardiac hypertrophy and failure. The consequences of large myocardial infarction in the chronic phase are well studied. The overloaded heart adapts with increased muscle mass (cardiac hypertrophy), usually preceding the occurrence of congestive heart failure (CHF), a major cause of death in the North American population [24,25].

Cardiac hypertrophy occurs in compensation for loss of heart tissue due to MI [26], and its magnitude is variable depending on the size of infarction [27,28]. In the event of large MI, the ventricular chamber may remodel by an increase in volume [29] and severe hypertrophy which is associated with increased myocyte size and decreased intrinsic cardiac performance [24]. Using an experimental model of post-MI heart failure, the consequences of post-MI remodeling has been observed [30-39] including progressive cardiac dysfunction, to which has been assigned arbitrary designations of prefailure, moderate, and severe failure stages. This model is relatively well characterized and overt cardiac fibrosis in remnant heart and infarct scar from animals with moderate heart failure has been observed [34].

Chronic cardiac wound healing and the development of fibrosis in congestive heart failure is a complex process and may involve input from multiple factors [14,34,40,41]. It is becoming clear that myofibroblast behavior may also potentiate wound healing and eventual cardiac fibrosis. TGF-β_1 is widely studied as a stimulus for fibroblast and myofibroblast function in the setting of myocardial infarction [42]. TGF-β_1 is known to stimulate focal adhesion (FA) supermaturation in myofibroblasts [43] which is associated with reduced turnover and decreased cell motility [44]. Enhanced focal adhesion turnover is regulated by increased phosphorylation of focal adhesion kinase (FAK) and c-Src activity [44].

The myocardial ECM (comprised mainly of fibrillar collagens) is an organized network intimately associated with cardiac function, serving to direct, transmit, and distribute myocyte-generated contractile force [45]. It participates in active restoration of sarcomeric length, via release of stored potential energy in

matrix proteins [46-49]. Collagen types I and III (fibrillar collagens) form struts between myocytes and among muscle fibres [46,47,50,51]. Other functions of the matrix include regulation of cell death, gene expression and parenchymal cell differentiation [48,49]. Nonetheless, elevated fibrillar collagen expression may be responsible for changing heart function in heart disease based on its adverse influence on myocardial stiffness [37,52,53]. The majority of DNA synthesizing cells in the surviving myocardium and infarct scar of experimental animals are fibroblasts and myofibroblasts [21,39,54]. Pathological cardiac hypertrophy is associated with interstitial and perivascular fibrosis in remnant heart or as replacement fibrosis for necrosed muscle [26,33,55]. In the latter, ongoing collagen remodeling may contribute to decompensated cardiac function in severe heart failure stage [35,56]. Limited fibrosis in the healing infarct scar may help to preserve ventricular function, as the new scar tissue selectively resists circumferential deformation [57]. Thus the scar is a distinctly anisotropic tissue with large collagen fibers oriented within 30 degrees of the local circumferential axis [57], and elevated crosslinking of collagens [38,56]. Angiotensin is implicated in the stimulation of collagen biosynthesis in the heart [34,40,58-60]. Suppression of angiotensin is associated with prevention of ventricular dilatation, improved exercise capacity, attenuation of scar remodeling, and survival in patients and experimental animal models of heart post-MI heart failure [61-66]. While the stimulatory effect of angiotensin on collagen synthesis in cultured rat adult cardiac fibroblasts is well known [67,68], its role as a proliferative agent is questionable [69] and in adult myofibroblasts, antiproliferative effects have been noted [70 and unpublished

observations]. The *in vitro* and *in vivo* models to be used in these studies are suitable for the examination of fibroblast function as well as the functional and morphological characteristics of hypertrophied and failing hearts. Further, the experimental rat model of myocardial infarction is suited for the examination of responsiveness of gene expression in the remnant myocardium and infarct scar [33-35,39,65,71-74].

3. Experimental rat model of myocardial infarction.

There are few reliable models of MI suitable for the study of transmural scar tissue and development of congestive heart failure [75]. The rat model of MI was developed by Selye *et al* in Canada [76]. Vascular proliferation and fibrosis of the infarct zone and resorption of necrotic tissue occurs from the onset of infarction, and discrete scar formation is apparent at 21 days [46]. Morphometric techniques in rat heart have revealed that scar shrinkage and transmural thinning occurs over the first 40 days [77]. Clinical investigation has linked infarction size to the severity of ensuing cardiac hypertrophy and subsequent heart failure [27,29,78]. To verify that the rat model of chronic infarction is an acceptable approximation of the clinical condition, comparisons of healed infarct size and occurrence of heart failure have been carried out [64,71-73]. Large (> 30%) infarct size is associated with significant myocyte hypertrophy [64], and rats with very large infarcts (> 45%) had overt heart failure characterised by elevated filling pressures, reduced cardiac output and low capacity to respond to preload and afterload stress [79]. Thus this is an experimental model of graded left ventricular dysfunction, whose magnitude is closely related to the extent of the healed MI [80]. As occurs in human MI patients [81], left

ventricular distension and dilatation in this animal model is characterised by upward and rightward movement on the pressure-volume relationship *vs.* control [72,80]. Decreased blood flow to systemic organs suggests the presence of progressive cardiac decompensation in these animals and that this sequelae of events mimics the human condition [73].

As the rat model of congestive heart failure became established, it was generally believed that the function of the scarred ventricle would depend not only on size and location of the infarct but also on evolving changes in the neurohumoral state. A pathogenic role for elevated levels of angiotensin in this model has been well established [33,34,61,64,82-84].

4. Fibroblast Migration

Migration of interstitial fibroblasts from the adjacent viable myocardium contributes to repopulation of the infarct scar [85]. Although there are differences between cell types, in general there are 5 physical processes which must occur for cells to migrate: 1) front *vs.* rear asymmetry, 2) membrane extension, 3) attachment formation, 4) contractile force generation, and 5) cell rear detachment [86]. This occurs in response to activation of ligand dependent signaling pathways and extracellular matrix (ECM) interactions through focal adhesions. Although it is unclear how a receptor mediated, extracellular ligand initiated migration response occurs, a great deal of information has accumulated on the role of cell-matrix interactions. Focal adhesions (FA) are areas of the cell membrane where there is a concentration of proteins that interact with the ECM [87]. These complexes form the

adhesive connection between the cell and substratum and assembly or disassembly of focal adhesions are involved in attachment and detachment of the cell during migration [88]. Focal adhesions serve as anchor points for actin stress fibres [89] and they assist in migration by forming along the leading edge of the cell, remaining fixed as the cell migrates over them, and then detaching at the rear (FA turnover)[90]. It is this dynamic assembly and disassembly of FAs that plays a critical role in regulating the speed of migration [87]. The ECM receptors, integrins, are the hallmark component proteins of focal adhesions, although the complex also contains syndecan and other structural/adapter proteins and kinases, such as vinculin, talin, paxillin, p130CAS, PI3-K, src, integrin linked kinase, and focal adhesion kinase (FAK). FAK is a tyrosine kinase the activity of which is modulated by multiple tyrosine and serine phosphorylation sites. Tyrosine 397 of FAK becomes autophosphorylated upon engagement of integrins and regulates formation of a conglomerate of proteins and kinases including Src, the 85 kDa subunit of PI3K, PLCγ, and the adapter protein Grb7 [91]. Tyrosines 576/577, which lie in the catalytic domain, are regulated by Src kinases and phosphorylation of these residues is required for maximal catalytic activity [92,93]. Rho also plays a central role in control of focal adhesion assembly and disassembly: activated Rho is required for the assembly of focal adhesions, and inactivation is implicated in the disassembly of focal adhesions [88]. The calcium sensitive protease calpain also plays a role in decreasing adhesiveness at the rear of the cell through cleavage of focal adhesion constituents [94].

Two types of force must be generated by the cell for movement to occur: protrusive force, resulting in the formation of membrane protrusions (pseudopodia, lamellipodia, filopodia) and contractile force to move the cell body over the substratum [86,95]. The first likely occurs through actin polymerization and cytoskeletal reorganization independent of myosin, whereas the later is accomplished by active myosin motors [86,95]. Filopodia are spikes of cell membrane supported by a tight cylinder of actin whereas lamellipodia are thin protrusive sheets of cell membrane supported by a web of actin filaments [95]. Actin stabilizing proteins such as cortactin, ARP 2/3, WASP and WAVE play an central role in formation and stabilization of these filaments [96-98].

In smooth muscle, the activity of myosin motors is regulated by the phosphorylation of regulatory myosin light chains (rMLC), in turn regulated by myosin light chain kinase (MLCK) [99] and phosphatase [100]. Phosphorylation of rMLC allows activation of the myosin ATPase by actin, with subsequent cross-bridge cycling and contractile activity. Although there is new evidence suggesting that MLCK can be activated by Src kinases [101,102], MLCK is classically activated by elevated intracellular calcium which binds to calmodulin, in turn binding to and activating MLCK [100]. Intracellular calcium is an important second messenger that regulates a large number of physiologic functions [103,104]. Its level is controlled by influx of extracellular calcium and mobilization of intracellular stores, mainly from the sarcoplasmic and endoplasmic reticulum [105]. In non-excitable cells, such as fibroblasts, which do not express significant levels of voltage sensitive calcium channels, an important regulator of calcium entry is membrane potential [106].

Membrane potential is primarily regulated by potassium channels, which when open induce hyperpolarization of the cell membrane. This regulates the cytosolic calcium content by altering the driving force for calcium entry through capacitive or non-selective cation channels [106-108]. Potassium channels can be regulated by phosphorylation [109,110], and have been shown to be associated with cytoskeletal organizing proteins such as cortactin [111]. MLCK has also been shown to be associated with cortactin [101,112]. These interactions likely play a role in actin based cytoskeletal reorganization leading to membrane protrusion, since cortactin is known to localize with lamellipodia and on endosomal vesicles [113] and plays an important role in cortical actin assembly [96]. This may also be the mechanism that produces front-rear asymmetry of the cell and guides the cell towards a specific stimulus.

5. The Cardiac Myofibroblast

Fibroblasts and myofibroblasts are abundant in the heart [55], and wound healing/interstitial cardiac fibrosis is mediated by primarily the latter type [39,114-116]. During wound healing, circulating myofibroblast progenitors and normal interstitial fibroblasts transform into myofibroblasts that are hypersynthetic, less migratory and possess contractile properties [8,18,115]. This phenotype is marked by expression of α-smooth muscle actin, vimentin, AT_1 receptors, TGF-β receptors, LIFR/gp-130, ACE, and fibrillar collagens [5,59,60,116-120]. Additional features of myofibroblasts are a well developed rough endoplasmic reticulum, myofilaments (stress fibres) with focal densities, collagen secretion granules [121] and gap

junctions [115]. A marker of myofibroblasts that may be more specific than α-smooth muscle actin expression is expression of the embryonic isoform of myosin, Smemb [122]. This protein has been found to be expressed in infarct scar [122], and in hibernating myocardium [123]. Unlike dermal myofibroblasts which disappear during the transition from granulation tissue to scar [124], cardiac myofibroblasts can be found in the infarct scar many years after injury [22]. The main stimuli for fibroblast - myofibroblast transformation is PDGF, SCF and most importantly, TGF-β [8,115], whereas LIF may antagonize this phenotypic change [125]. In cultured cells, this phenotype is induced by TGF-β_1 and *in vitro* culture seeding at low density [43,126,127]. We have demonstrated the predominance of myofibroblasts in the infarct scar [39] in post-MI rats and in adult cells when plated at low initial density (unpublished observations). Cultured adult myofibroblasts are phenotypically stable and hypersynthetic [126].

In clinical and experimental models of post-MI heart, it is generally believed that the function of the scarred ventricle depends upon the size and location of the infarct and also on alterations in neurohumoral state. In the context of cardiac fibrosis, only a small number of growth factors or cytokines have been well investigated. Among these, perhaps angiotensin II (angiotensin) and TGF-β_1 been the most extensively characterized. Suppression of angiotensin is associated with prevention of ventricular dilatation, improved exercise capacity, attenuation of scar remodeling, and survival in patients and experimental animal models of heart post-MI heart failure [33,34,61,63-66,82-84,128,129]. Angiotensin stimulation of cultured cardiac fibroblasts is marked by increased collagen synthesis [67,68,130].

Most of the known effects of angiotensin on cardiac fibroblasts are mediated by the AT_1 receptor [131], whereas the function of the AT_2 receptor in the heart is less well-defined [132]. ACE inhibitor therapy is associated with attenuation of left ventricular remodeling in patients with myocardial infarction [41,62,133,134]. In post-MI rat heart, fibrosis is marked by an increase in local angiotensin via increased ACE activity and AT_1 receptor density [59,60,135] in fibroblasts and myofibroblasts which appear at the infarct 3 days post–MI [59]. Myofibroblasts of the borderzone and infarct scar overexpress signal proteins associated with angiotensin signaling [35,39].

With respect to myofibroblast function, TGF-β_1 mediates cell growth and differentiation, tissue wound repair, and extracellular matrix production [136-138], including regulation of fibrillar collagens [139] and is expressed in the normal and hypertrophied myocardium [130,137,140,141]. TGF-β_1 ligand signaling from cell-surface receptors to the nucleus is transduced by Smads and their DNA-binding partners [142-149]. TGF-β_1 receptor type I and II are Ser/Thr kinase class proteins, and signal through receptor-regulated Smads (R-Smad 2 or 3) by specific recognition and phosphorylation steps [144,150,151]. Evidence for ligand-level crosstalk between cardiac angiotensin and downstream TGF-β_1 release exists [140,144,152-157]. TGF-β_1 is also associated with enhanced collagen synthesis [5,115,158]. Finally, R-Smads may subserve a nexus for post-receptor crosstalk between angiotensin and TGF-β, as they serve as a common downstream effectors [33]. While TGF-β_1 is likely to exert effects on fibroblasts that impair their motility [8,43] and reduce their overall proliferation [159 and unpublished observations], and as

angiotensin's role as a proliferative agent has been recently called into question [69 and unpublished observations], investigation of alternative signaling mediators with clear-cut proliferative effects is of considerable interest.

Myofibroblast motility and proliferation contribute to net matrix deposition in the pathogenesis of cardiac fibrosis [56]. Myofibroblasts produce isometric tension within granulation tissue *in vivo* and in cultures [8]. Tension is exerted at the level of focal adhesions (FAs), which connect cells to matrix [43]. TGF-β_1 stimulation leads to FA maturation facilitating tension transmission from the cell to the matrix [43]. Myofibroblast contraction likely mediates infarct scar thinning that occurs in the late stages of post-MI wound healing [23]. Stimulation of focal adhesion kinase (FAK) facilitates FA turnover, motility [44] and cell cycle progression [160]. Mature and supermature FAs favour enhanced cellular anchoring, which tends to retard myofibroblast motility and inhibit cell proliferation [43]. It is possible that this "anchoring" phenomenon is an adaptation for efficient collagen synthesis and scar contraction.

6. Cell Proliferation

Upon taking up residence in the infarct zone, these fibroblasts proliferate. The cell cycle is divided into four distinct phases: G1, S, G2 and M. The two G (gap) phases are referred to as growth phases, DNA synthesis occurs during S phase while cell division (mitosis) occurs during M phase [161-164]. Proper progression through the cycle is assured by "checkpoints." These are points in the cell cycle where certain conditions have to be met before the cell can proceed into the next

phase. These transitions are governed by the cyclin dependent kinases (CDKs) and by inhibitory and activating phosphorylation events [161,165]. The activity of these enzymes is dependent on their molecular partners, cyclins and CDK inhibitors, the levels of which fluctuate depending on the specific phase of the cell cycle, whereas the CDKs are more stably expressed [165]. To date over 12 cyclins and 9 CDK subunits have been described; however, the cyclins are categorized into 5 groups, A through E [165]. Progression through G1 is mediated by sequential activation of D type CDKs (cyclinD with CDK4 or CDK6), and cyclinE complexed with CDK2 [166]. CyclinD is upregulated by growth factors, and is expressed in any cell that is in the cell cycle. A target of the G1 CDKs is Rb, which is increasingly phosphorylated during mid-late G1 phase, first CyclinD-CDK4 and CDK6, then later CyclinE-CDK2 as cells enter S phase [161,166]. This is a principle endpoint of growth factor induced ERK1/2 activation: phosphorylation and activation of transcription factors, with resultant increased expression of cyclinD and phosphorylation of Rb which leads to disinhibition of E2F, leading to transcription of target genes and cell cycle progression [162,167]. CyclinA and cylinB are expressed in a phasic manner, with low expression throughout the cell cycle except during the transition from G2 to M phase [165]. After entry into S phase, Rb is held in its hyperphosphorylated inactive state by CyclinA-CDK2, and by CyclinB-CDK1/CDC2 during M phase. The early events in the cell cycle are dependent on the presence of growth factors, whereas once the cell has entered S phase, the remainder of the cell cycle can be completed even if growth factors are removed. This time point has been designated the restriction (R) point [168]. Beyond this point

the cells can not only complete S phase, but also traverse G2 and M phases in the absence of growth factor stimuli [161,163,164,166].

Cell cycle progression is not only dependent on CDKs, cyclins and CDK inhibitors. Normal, non-transformed cells have to be adherent to the substratum in order for effective proliferation to occur [169]. FAK has been shown to independently induce activation of the cyclinD promoter, resulting in enhanced cell cycle progression [170-173]. Additionally, non-adherent cells fail to phosphorylate Rb and fail to activate cyclinE-CDK2 [173]. Therefore ECM receptors play a critical role in regulating cell cycle progression by facilitating optimal activation of intracellular signaling pathways and induction of cyclins, in particular, cyclinD [169]. In addition, adhesion may also play a role by suppressing the total levels of the CDK inhibitors, p21 and p27 [174]. Cell adhesion exerts its regulatory role primarily in the G1 restriction point, since the events that are dependent on adhesion also regulate the G1-S transition. For a cell to complete M phase and cytokinesis, it must be able to detach momentarily from the substratum. Therefore focal adhesions must be disassembled and reassembled, a phenomenon mediated by focal adhesion kinase (FAK) [175-177].

7. Extracellular Matrix Protein Synthesis

Synthesis of new extracellular matrix proteins is a tightly regulated process involving gene transcription and translation. Initiation of protein synthesis is a tightly regulated process that culminates in the positioning of a charged ribosome at an initiation codon [178]. The ribosome does not bind directly to the mRNA 5' cap,

but is directed there by the concerted action of a large number of eukaryotic translation initiation factors (eIFs). In this regard, eIF4E and eIF4G are important components of a trimeric complex eIF4F, and it is known that the levels of eIF4F fluctuate with varying translational rates. Growing or stimulated cells contain high levels of eIF4F, whereas starved or quiescent cells contain low levels of eIF4F. The phosphorylation status of eIF4E also correlates with the degree of translational activity, since the phosphorylated form has a greater affinity for cap structures [179]. Formation of eIF4F is regulated at least in part by a family of translation repressors, the eIF4E binding proteins (4E-BPs). Binding of 4E-BP1 to eIF4E prevents association of eIF4E and eIF4G, thereby preventing formation of the initiation complex. Regulation of 4E-BP1 is accomplished through phosphorylation: hypophosphorylated 4E-BP1 binds tightly to eIF4E, whereas phosphorylation of 4E-BP1 releases eIF4E and allows formation of the initiation complex [180]. The primary target of FRAP/mTOR kinase activity is 4E-BP1 [180], while the MAPK-related kinase Mnk1 is the most likely candidate kinase in regulation of eIF4E phosphorylation [181]. Thus integration of the PI3K and MAPK pathways is implicit at the level of initiation of translation.

Control of the collagen synthesis is a complex process involving many different signaling pathways and post-translational modification check-points. It is becoming clear that R-Smads (receptor activated Smads) are activators of procollagen gene transcription, whereas STATs and MAP kinases have been viewed as negative regulators (reviewed in [182]). Following translation, there is a great deal of modification of the collagen gene product. After synthesis of the initial

protein, prolyl residues are hydroxylated by prolyl-4-hydroxylase (P4H) within the endoplasmic reticulum [183]. P4H is a multisubunit protein that possesses not only hydroxylase activity, but also functions as a molecular chaperone (with HSP47) to aid in procollagen folding and assembly of trimeric fibrillar collagens [184,185], and is considered a rate limiting step in the synthesis of mature collagen [186]. There are several cofactors required for optimal P4H activity: ascorbic acid, molecular oxygen, ferrous iron and 2-oxoglutarate [183]. When one or more of these factors is deficient, there is retention of procollagen within the endoplasmic reticulum [187]; these immature proteins then undergo degradation [188] and there is an overall deficiency of mature collagen. After prolyl hydroxylation, the procollagen protein is expelled from the cell and undergoes proteolytic cleavage of the carboxy and amino terminal propeptides by the extracellular metalloproteinases, procollagen C- and N-proteinases [189]. The mature collagen molecule is then further cross-linked by lysyl oxidase [190].

8. Cytokine Expression in Post-MI Heart

Upon cessation of blood flow, there is rapid degranulation of preformed mast cells in the heart. These cells release histamine and TNF-α, which is considered to be important in initiating the inflammatory cascade [191]. This resulted in an increase in IL-6 expression in mononuclear cells as well [18] with subsequent activation of adhesion molecules, leukocyte trapping and neutrophil-induced injury [191-193]. In addition to its effects on adhesion, IL-6 may also act as a nitric oxide-dependent cardiac depressant, and may therefore may be associated with stunned

myocardium [18,191]. Furthermore, IL-6 plays a role in tissue repair, since knock-out mice demonstrate significantly delayed cutaneous wound healing [194]. There are several cytokines that are expressed during the various stages of post-MI wound healing, including members of the IL-6 family, IL-1β [14], TGF-β [42,141], EGF and FGF [195]. In the subacute and chronic phase, these are elevated, at least in part, by activation of the renin-angiotensin-aldosterone system (RAAS) [196-198]. It is well known that inhibition of the RAAS results in improved outcomes in animal models and patients suffering MI and CHF [34,61,64,66,82-84,128,129,141], and this is in part related to a reduction of cytokine expression. Angiotensin has been shown to increase TGF- β expression in post-MI heart, and its antagonism results in decreased cardiac fibrosis and improved ventricular function [120,141,199]. Cardiotrophin-1 has also been shown to be elevated in the myocardium after myocardial infarction [200], but the relationship of its expression to the RAAS is not clear.

9. Cardiotrophin-1

Cardiotrophin-1 (CT-1), a member of the IL-6 family of cytokines, was isolated in 1995 based in its ability to induce hypertrophy of neonatal cardiac myocytes *in vitro* [201]. The IL-6 family of cytokines includes IL-6, IL-11, LIF, OSM, CNTF and CT-1 [202]. To identify factors that induce cardiac hypertrophy, an expression cloning system was coupled to an in vitro hypertrophy assay. From this, a 1.4 kb cDNA was identified that produced a 214 amino acid, 21.5 kDa protein which was named Cardiotrophin-1 [201]. This protein was found to induce hypertrophy more potently than other members of the IL-6 family of cytokines or other known

mediators of cardiomyocyte hypertrophy [201]. The 1.4 kb band was observed in adult mouse mRNA from heart, skeletal muscle, liver, lung and kidney [201] and in adult rat heart, lung, liver, kidney, skeletal muscle, stomach and urinary bladder [203]. In humans, a 1.7 kb mRNA encoding CT-1 was found in heart, skeletal muscle, prostate and ovary [204]. CT-1 was observed in the early mouse embryonic heart tube, primarily in myocardial cells, with less expression in the endocardial cushion or outflow tract tissues. After E12.5, CT-1 expression was also observed in liver, dorsal root ganglia and skeletal tissues [205]. The expression of CT-1 in the spinal cord appears required for survival of developing motoneurons, since CT-1 deficiency (knock-out) causes increased motoneuron cell death in spinal cord and brainstem of mice during a period between embryonic day 14 and the first postnatal week [206]. CT-1 also appears to induce astrocyte differentiation from neuroepithelial cells in a synergistic fashion with BMP-2 [207].

Expression of CT-1 has been observed in various disease states. Increased CT-1 mRNA was observed in the ventricles of genetically hypertensive rats [203,208] and in the ventricles of rats subjected to pressure overload [209]. Elevated CT-1 and gp130 mRNA and protein were observed in the ventricles from rats with myocardial infarction, and increased expression persisted into the chronic phase of wound healing [200]. Elevated CT-1 was observed in the ventricles from dogs with pacing induced heart failure, and the degree of CT-1 mRNA expression correlated with left ventricular mass index [210]. Mechanical stretch of cardiomyocytes has been shown to increase CT-1 mRNA expression [211], as has norepinephrine stimulation [212], isoproterenol stimulation [213] and hypoxic stress [214].

Angiotensin II was shown to induce CT-1 expression in cardiac fibroblasts [215], and cardiac non-myocytes (mainly fibroblasts) were observed to have 3.5 times higher CT-1 mRNA expression than cardiac myocytes [216]. CT-1 expression has been observed in rats with Chagasic cardiomyopathy (*Trypanosoma cruzi* infection) [217] and in mice with acute Coxsackievirus B3 myocarditis [218].

In humans, elevated serum levels of CT-1 have been observed in patients with unstable angina [219], acute myocardial infarction [220] and heart failure [221]. The level of CT-1 expression was correlated with the degree of left ventricular systolic dysfunction. Elevated CT-1 was also observed in patients with aortic stenosis [222] and mitral regurgitation; the degree of regurgitation correlated with the level of CT-1 in serum [223]. There has been a report of a mutation/polymorphism in the promoter region and coding region of the human CT-1 gene in some patients with idiopathic dilated cardiomyopathy [224]. The significance of this is unknown, although elevated CT-1 was observed in the serum of patients with dilated cardiomyopathy and its expression was positively correlated with left ventricular mass index [225]. Elevated mRNA and protein levels of CT-1 were observed in the hearts from patients with end stage cardiomyopathy undergoing heart transplantation. This increased expression was accompanied by a decrease in gp130, suggesting that receptor downregulation plays a role in balancing enhanced CT-1 expression [226].

When administered intravenously, CT-1 induces a drop in mean arterial pressure and reflex increase in heart rate without affecting cardiac output, an effect that is mediated by iNOS [227]. This effect was also seen in spontaneously hypertensive rats [228]. Male Wistar rats became resistant to repeated injections of

CT-1, by induction of endogenous suppressors of IL-6 family cytokine signaling, namely Jak-binding protein (JAB)/Suppressor of Cytokine Signaling-1/STAT induced STAT Inhibitor-1 and CIS3/SOCS-3/SSI-3 [229]. Chronic administration of CT-1 to mice by intraperitoneal injection lead to increased heart to body weight ratio, as well as increased liver, spleen and kidney weight, but induced a reduction of thymus weight [230].

Cardiomyocyte hypertrophy produced by CT-1 was observed to be different than the other hypertrophic stimuli. Whereas hypertrophy typically results in assembly of sarcomeres in parallel, CT-1 induced hypertrophy resulted in sarcomere assembly in series with subsequent increase in cardiomyocyte cell length [9,201]. This hypertrophic response was transduced by a receptor complex composed of the leukemia inhibitory factor receptor subunit β (LIFRβ) and gp130. Upon CT-1 stimulation, both LIFRβ and gp130 become tyrosine phosphorylated by Janus kinases (Jaks), since neither gp130 nor LIFRβ contain any inherent kinase activity [9,202,231]. Because Janus kinases are activators of multiple signaling pathways, there is activation of STATs, the mitogen activated protein kinase pathway, the phosphoinositol-3-kinase pathway and the Src pathway [232,233]. Activation of these pathways results in hypertrophy and protection from ischemia/reperfusion injury [234].

In keeping with its role as a growth factor, CT-1 also has cyoprotective effects. CT-1 prevented apoptosis of serum starved neonatal ventricular myocytes in a PI3K-dependent manner [234] and a MAPK dependent manner [235,236]. NF-κB also plays a central role in the cytoprotective effect of CT-1 [237]. CT-1 was also

able to protect isolated rat cardiac myocytes from apoptosis when added before simulated ischemia or when added at reoxygenation [238]. The same effect was observed in whole rat heart *ex vivo* [239]. The protective effect of CT-1 on human right atrium was found to be as effective as ischemic preconditioning when cells were treated for up to 24 hours before ischemia [240].

CT-1 also appears to be cytoprotective in the brain: In a mouse model of amyotrophic lateral sclerosis, adenoviral administration of CT-1 was found to delay the onset of motor impairment and axonal degeneration [241]. Prolonged survival and improved muscle function was also noted in neonatal progressive motor neuropathy (pmn) mice treated with adenoviral CT-1 [242]. In the Wobbler mouse motor neuron disease model, subcutaneous administration of CT-1 after disease diagnosis reversed the progressive decline in neurological and muscle function [243].

To date there is only one paper on the effect of CT-1 on cardiac fibroblast function. Tsuruda et al. showed that canine cardiac fibroblasts express CT-1, gp130 and the LIF receptor. CT-1 stimulation of canine cardiac fibroblasts increased proliferation and incorporation of ^{3}H-proline, an effect that could be inhibited by coincubation with a gp130 blocking antibody or the endothelin-1 receptor antagonist BQ123 [118]. It was in this setting that we sought to determine the time course of CT-1 expression in post-MI rat heart, and its effect on post-MI wound healing, with particular respect to cardiac fibroblast proliferation, migration, and protein (collagen) synthesis. To that effect we performed coronary artery ligation in rats, and performed experiments on cultured primary adult rat cardiac fibroblasts to more closely examine the effects of CT-1 on this cell type.

III. MATERIALS AND METHODS

1.0 Myocardial Infarction in Rats

All experimental protocols for animal studies were approved by the Animal Care Committee of the University of Manitoba, following guidelines set forth by the Canadian Council on Animal Care. MI was produced in 200-250 gm anesthetized (0.01-0.05 mg/kg buprenorphine subcutaneous premedication, 2-2.5% isofluorane inhalation anesthetic) male Sprague-Dawley rats by ligation of the left coronary artery as previously described [30]. The mortality rate associated with this procedure was ~40% within 48 hours and the resultant scar occupied ~40% of the left ventricle. Sham operated animals received the same procedure, lacking only the coronary artery ligature, and were used for comparative purposes. Animals were sacrificed at specified time points and tissue was isolated from two regions of the left ventricle: remnant/viable and infarct zone/scar. These specimens were pulverized under liquid nitrogen, homogenized, and lysed in 2x sodium dodecyl sulfate (SDS) buffer (0.125 M Tris, 2% SDS, 20% glycerol) at 4°C for 60 minutes then sonicated for 15 seconds. Insoluble material was removed by centrifugation and the clear supernatant was used for Western analysis of CT-1 expression.

2.0 Isolation and Characterization of Cardiac (myo)fibroblasts

Fibroblasts were isolated from the hearts of 200-250 g adult male Sprague-Dawley rats as previously described [33]. Briefly, hearts were subjected to Langendorff perfusion at 37°C with Joklik's medium containing 0.1% collagenase (Worthington Biochemical Corporation, Lakewood, NJ) for 20-25 minutes.

Collagenase was neutralized by addition of an equal volume of DMEM/F12 medium containing 10% FBS and liberated cells were collected by centrifugation at 500 x g for 5 minutes. Cells were resuspended in fresh DMEM/F12 containing 10% FBS and plated on 75 cm^2 culture flasks at 37°C with 5% CO_2 for 3 hours. Non adherent cells (myocytes) were removed by changing the culture media and adherent cells (mainly fibroblasts) were incubated in DMEM/F12 containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 100 μM ascorbate. Fibroblasts were used for experiments after the second passage and the purity of these cells was $\geq$ 95%, using routine phenotyping methods as previously described [39]. Figure 2 demonstrates that these cells begin to express α-smooth muscle actin by the first passage, indicating that these cells are myofibroblasts. Cells were rendered quiescent by incubation in serum-free medium for 24 hours then stimulated with CT-1 for specified times and lysed in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris) with 1x protease inhibitor cocktail (Sigma-Aldrich, Oakville, Ontario) and 10 mM NaF, 1mM Na_3VO_4 and 1 mM EGTA.

3.0 Western Analysis

Protein concentrations of tissue and cell lysates were determined by the BCA method [244]. Proteins were separated by 8-12% SDS-PAGE and transferred to PVDF membrane (Roche, Indianapolis, IN) for 1 hour at 300 mA for small proteins or for 2 hours at 500 mA for proteins greater than 100 kDa. Membranes were blocked with 5% non-fat skim milk in tris-buffered saline containing 0.2% Tween 20 (TBST). Proteins were visualized with ECL Plus (Amersham) after probing with

primary and secondary antibodies. Band intensity was quantified using a CCD camera imaging densitometer (GS670, Bio-Rad Laboratories (Canada) Ltd. Mississauga, Ontario).

4.0 Immunofluorescence

Cardiac fibroblasts were seeded onto coverslips in 6 well dishes and allowed to attach overnight in media containing 10% serum. The cells were rendered quiescent in serum free media for 24 hours before being stimulated with CT-1 for specified times. The media was removed, cells were rinsed with PBS and fixed with 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 and incubated with primary antibodies, biotinylated secondary antibodies, and streptavidin FITC. Nuclei were stained with Hoechst 33342. The cells were visualized with epifluorescent microscopy with appropriate filters (Nikon Canada).

5.0 Proliferation Assay

5.1 ^{3}H Thymidine Incorporation

DNA synthesis was measured by uptake of ^{3}H-thymidine as previously described [245] with modification. Briefly, 2.5 x 10^{4} cells (counted with a hemacytometer) in DMEM/F12 with 10% FBS were loaded into each well of 24-well culture plates, allowed to attach overnight and rendered quiescent in serum-free DMEM with 100 μM ascorbate for 24 hours. S-phase re-entry in response to CT-1 was measured in two ways: pulse labeling with ^{3}H-thymidine for 30 minutes every 6 hours, or labeling for the last 30 minutes of 6 or 12 hour incubation. DNA in cell

lysate was precipitated with cold 20% trichloroacetic acid (TCA) and filtered through GF/A filters (Fisher). Beta emission from the dried filers was measured with Cytoscint scintillation fluid (ICN Pharmaceuticals, Costa Mesa, CA) and a scintillation counter (LS6500, Beckman Coulter, Fullerton, CA).

5.2 Cell Counting

To determine absolute cell numbers after stimulation with CT-1, the total number of cells were counted. Equal numbers of cells were loaded into each well of 24 well plates and incubated with CT-1 for 24 hours. The cells were detached with trypsin, diluted in filtered phosphate buffered saline, and counted with Model ZM Coulter cell counter (Beckman Coulter, Fullerton, CA).

5.3 Nuclear Accumulation of PCNA

Cardiac fibroblasts were seeded onto cover slips and allowed to attach overnight. The cells were incubated with serum free media for 24 hours prior to stimulation with CT-1 for an additional 24 hours. The cells were then fixed with cold acetone, permeabilized and immunostained for PCNA and counter stained with Hoechst 33342. Images from 5 high power fields per slide were captured and cells were counted. Nuclei that were positive for PCNA were counted and expressed as a ratio to total nuclei.

6.0 Protein Synthesis Assay

Protein synthesis was determined using the methods of Wolf and Neilson [246]. 2.5 $x10^4$ cells (counted with a hemacytometer) were loaded into each well of 24-well plates, allowed to attach overnight, then rendered quiescent in serum free

DMEM/F12 for 24 hours. Cells were stimulated with CT-1 for an additional 24 hours in the presence of 2 μCi/mL ^{3}H-leucine with or without inhibitors of signaling pathways. The culture media was aspirated, cells rinsed twice with phosphate buffered saline and proteins precipitated by two incubations with 10% TCA at room temperature. The precipitated protein was solubilized in 300 μl lysis buffer containing 0.5 M NaOH and 1% Triton X-100 at room temperature for 15 minutes. The lysate was transferred to scintillation vials, and beta emission was determined with 3 ml Ecolume scintillation fluid (ICN Pharmaceuticals) and a scintillation counter (LS6500, Beckman Coulter, Fullerton, CA).

7.0 Collagen Synthesis

Synthesis of mature type I collagen was determined by measuring the concentration of the carboxyterminal propeptide of type I collagen (P1CP) in conditioned media from CT-1 treated or untreated cardiac fibroblasts. 5 x 10^4 cells were loaded into each well of a 6 well plate, allowed to attach overnight, and serum starved for 24 hours. Cells were treated with CT-1 at specified concentrations for an additional 24 or 48 hours. P1CP content in 0.1 mL conditioned media was determined by radioimmunoassay according to the manufacturer's specification (Diasorin Inc, Stillwater, MN). Radioactivity was quantified with a gamma counter (Gamma 5500, Beckman Coulter, Fullerton, CA) and the concentration of P1CP was determined by the % B/B_0 versus log concentration. This method has been shown to correlate well with ^{3}H-proline incorporation into collagenase sensitive proteins [247].

8.0 Migration Assay

8.1 Boyden Chamber

Migration of rat cardiac fibroblasts was measured using Boyden chamber (Neuro Probe Inc. Gaithersburg, MD) assay [248]. Chemoattractants were diluted in DMEM/F12 and loaded into the lower wells at specified concentrations. The polycarbonate membrane (5 μm pore) was placed over the wells, and 55 μL cell suspension in DMEM/F12 supplemented with 100 μM ascorbate was loaded into each well (1000 cells/mm^2). The chamber was incubated overnight in 5% CO_2 and 100% humidity at 37°C. Cells that had migrated through the membrane and become adherent to the lower surface were fixed with methanol and stained with Diff-Quick stain (Dade Behring AG, Düdingen, Switzerland). Cell migration was determined by counting the number of cells per high power field.

8.2 Wounded Monolayer Model

Cardiac fibroblasts were seeded onto coverslips in 6-well dishes and allowed to proliferate. Once the cells were near confluency, they were serum deprived for 24 hours. A scrape was made down the middle of the coverslip with the blunt end of a 1 ml syringe and the media was changed again. Cells were further incubated in the presence or absence of CT-1 and inhibitors at specified concentrations for specified times. The cells were then subjected to immunofluorescence staining as per section 4.0.

9.0 Estimation of cardiac myofibroblast membrane potential

Myofibroblast membrane potential was estimated using voltage sensitive $DiBAC_4(3)$ dye (Molecular Probes, Eugene, OR). $DiBAC_4(3)$ is a potentiometric bisoxonol dye that partitions into the cell membrane and exhibits increased fluorescence upon depolarization and decreased fluorescence upon hyperpolarization [249]. First passage myofibroblasts were plated on glass coverslips, allowed to attach, then incubated with 1 μM $DiBAC_4(3)$ in control solution containing: NaCl (140 mM), KCl (5 mM), HEPES (10 mM), $CaCl_2$ (2.0 mM), $MgCl_2$ (1.4 mM) and glucose (10 mM) for 20 minutes. The cells were visualized at 400X on an inverted microscope (Nikon Canada). $DiBAC_4(3)$ was excited at 470 nm wavelength and emitted light at 525 nm was digitized and stored (Photon Technology International, Lawrenceville, NJ). Fluorescence intensity was normalized to maximum fluorescence observed throughout the experiment so that data are expressed as percent change relative to baseline. According to the manufacturer's specifications, 1% change in fluorescence intensity is equivalent to 1 mV change in membrane potential. To ensure fluorescence intensity changes in response to altered membrane potential were occurring as expected, the cells were superfused with Tyrode's solution containing 1 μM $DiBAC_4(3)$ and 1.5 mM KCl or 15 mM KCl. Under these control conditions, $DiBAC_4(3)$ fluorescence decreased or increased indicating hyperpolarization or depolarization, respectively. For CT-1 experiments, CT-1 was diluted in control solution containing 1 μM $DiBAC_4(3)$. After ensuring a stable fluorescence signal, the cells were superfused with the CT-1 containing solution for 4 minutes, followed by washout. Fluorescence was monitored for a total of 10 minutes.

10.0 Use of pharmacologic inhibitors

The use of pharmacologic inhibitors of signaling pathways has been widely practiced in determining signaling by a ligand. Tyrphostin B42 (AG490) was identified as an inhibitor of Jak2 in 1996 [250], and has been shown to have beneficial effects on various hematologic malignancies [250,251]. AG490 was shown to attenuate angiotensinogen mRNA production in response to CT-1 stimulation [11]. Genistein is a phytoestrogen that possesses tyrosine kinase inhibiting activity [252]. PD98059 is a highly specific inhibitor of MEK1/2, and therefore an inhibitor of p42/44 MAPK activation. It maintains its selectivity at concentrations as high as 50 μM [253]. SB203580 is a highly specific inhibitor of p38 MAPK with an IC_{50} of 50 to 500 nM, depending on the isoform, and maintains its selectivity when used at 10 μM [253]. Rapamycin is a highly specific inhibitor of mTOR and does not inhibit other kinases when used at 1 μM [253]. LY294002 is a specific inhibitor of PI3K, but also inhibits casein kinase 2 with equal efficacy. The IC_{50} for LY294002 inhibition of PI3K is 10 μM [253]. These inhibitors are used at published, accepted concentrations.

11.0 Reagents

Cell culture reagents were purchased from Gibco BRL unless otherwise specified. Recombinant human CT-1, and mouse monoclonal CT-1 antibody were purchased from R&D Systems, Inc. (Minneapolis, MN). Polyclonal antibodies against STAT3, STAT3 pY^{705} and pS^{727}, STAT1 pY^{701}, Akt pS^{473}, Rb pS^{795} and $pS^{807/811}$, myosin light chain 2 pT^{18}/S^{19} and mouse monoclonal phosphotyrosine

antibody and goat anti-rabbit HRP-linked secondary antibody were purchased from Cell Signaling (New England Biolabs Ltd., Mississauga, Ontario). Polyclonal Jak1 $pYpY^{1022/1023}$ and Jak2 $pYpY^{1007/1008}$ antibodies were from Biosource International (Camarillo, CA). Rabbit polyclonal gp130 and Jak1 antibodies and Jak2 antisera were purchased from Upstate (Lake Placid, NY). Antibodies to actin, cyclin A (BF683), cyclin E2 (C-19), Erk1/2 pT^{204}, JNK1/2 pT^{183}/pY^{185} and p38 MAPK pY^{182} were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). PCNA antibody was from DakoCytomation (Carpinteria, CA). α-smooth muscle actin antibody was from Sigma-Aldrich Canada Ltd (Oakville Ontario). Mouse monoclonal antibody against procollagen (SP1.D8) was from Developmental Studies Hybridoma Bank (Iowa City, IA). Biotinylated secondary antibodies and streptavidin FITC were from Amersham-Pharmacia (Baie d'Urfe QC). P1CP radioimmunoassay kit was from Diasorin Inc. (Stillwater, MN). Inhibitors of cell signaling pathways were from Calbiochem (San Diego, CA). [methyl-^{3}H]-thymidine and L-[3,4,5-^{3}H(N)]-leucine were from Perkin Elmer Life Sciences, Inc. (Boston, MA). Other laboratory grade reagents were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario).

12.0 Statistics

Data are expressed as mean +/- standard error and are compared to control conditions using student's t-test. A p value less than 0.05 is considered significant.

IV. RESULTS

Expression of CT-1 in post-MI heart.

To examine the tissue expression of CT-1 in the setting of myocardial ischemic injury, we used the rat model of myocardial infarction. Ligation of the left coronary artery was performed in rats, and only those animals which developed an infarct occupying greater than 40% of the left ventricle were used for analysis. Tissue lysates from the infarct zone/scar, and remnant or viable left ventricle and the left ventricles of sham operated animals were prepared at 24 and 48 hours, and 2, 4 and 8 weeks after the ligation. These lysates were subjected to SDS-PAGE and Western blotting with specific CT-1 monoclonal antibodies. Elevated CT-1 expression (relative to sham and viable LV) was observed in tissue lysates from the infarct zone at the 24 hour time point, and elevated expression was observed up to 8 weeks after MI. At 8 weeks, elevated CT-1 was also observed in the viable LV, consistent with the development of left ventricular hypertrophy, as has previously been described [30,37] (Figure 1). A graphic representation of relative CT-1 expression is shown in Figure 2. Knowing that CT-1 was expressed in the infarct zone, we sought to determine the effects of CT-1 on isolated cardiac fibroblasts, the sole function of which is to maintain the extracellular matrix.

Phenotypic modulation of rat cardiac fibroblasts when cultured.

During myocardial wound healing, α-smooth muscle actin expressing myofibroblasts appear in the infarct zone. To determine if cultured cardiac fibroblasts undergo a phenotypic change to myofibroblasts when cultured, we

isolated fibroblasts from the hearts of Sprague-Dawley rats and plated them on glass coverslips. After 1 day in culture, these cells are negative for α-smooth muscle actin, but stain positively for procollagen, indicating that they are fibroblasts. After the first passage (5-7 days after isolation), these cells stain positively for α-smooth muscle actin and procollagen, indicating that they are myofibroblastic cells (Figure 3).

Signaling pathways activated by CT-1.

Cytokines of the IL-6 family typically activate the Jak pathway, which can subsequently activate multiple down-stream signaling pathways [202,232]. To screen for the signaling pathways activated by CT-1, cell lysates of cardiac fibroblasts stimulated with CT-1 were subjected to SDS-PAGE and Western blotting with phospho-specific antibodies to common intracellular signaling mediators. To examine phosphorylation of gp130, immunoprecipitation of gp130 from the cell lysates was first performed, followed by SDS-PAGE and Western blotting with anti-phosphotyrosine antibody. As expected, incubation of cardiac fibroblasts with CT-1 resulted in phosphorylation of gp130, Jak1, Jak2 (Figure 4), STAT3, STAT1 and nuclear accumulation of STAT3 (Figure 5a and Figure 6). Phosphorylation of STAT5 or STAT6 was not observed (data not shown). Consistent with their enzymatic activation, we observed phosphorylation of p42/44 MAPK, p38 MAPK, JNK and Src (Figure 5b).

Carditrophin-1 induces cardiac fibroblast proliferation.

To determine if CT-1 possesses mitogenic properties for cardiac fibroblasts, we used incorporation of ^{3}H-labeled thymidine as a measure of DNA synthesis. Cardiac fibroblasts were isolated from the ventricles of rats and incubated with CT-1 for specified times and at specified concentrations. Figure 7 demonstrates that upon removal of serum, cardiac fibroblasts complete S-phase and become quiescent. Addition of CT-1 results in S-phase re-entry within 6 hours of stimulation (Figure 7). Elevated incorporation of ^{3}H thymidine was observed with as little as 1 ng/mL CT-1, and peak incorporation was observed at a dose of 10-20 ng/mL CT-1. (Figure 8). CT-1 induced cell proliferation was dependent on Jak, PI3-K, MAPK, and Src kinases (Figure 9). To determine the effect of CT-1 on cell cycle regulatory proteins, we incubated cardiac fibroblasts with CT-1 and lysed them at specified times. CT-1 treatment resulted in an increase in expression of cyclins and hyperphosphorylation of the retinoblastoma protein (Figure 10), which relieves its inhibition of E2F and allows activation of target genes and progression through the cell cycle [161]. CT-1 stimulation resulted in increased nuclear expression of PCNA, a DNA polymerase co-factor (Figure 11). To determine if increased DNA synthesis was accompanied by an increase in cell number, we repeated the experiments in the absence of ^{3}H-thymidine, detached the cells from the plates and counted the total number of cells. Increased incorporation of ^{3}H-thymidine was accompanied by an increase in total cell number (Figure 12). These results strongly support the hypothesis that CT-1 is a mitogen for primary adult rat cardiac fibroblasts.

Cardiotrophin-1 induces protein synthesis in cardiac fibroblasts.

To determine the effect of CT-1 on protein synthesis in cardiac fibroblasts we measured incorporation of ^{3}H-labeled leucine. Stimulation of cultured cardiac fibroblasts with CT-1 induced a dose-dependent increase in protein synthesis (Figure 13). Since the activity of translational regulatory proteins is modified by phosphorylation, we used phospho-specific antibodies to determine if CT-1 directly influenced the activity of these regulatory proteins. Stimulation of cardiac fibroblasts with CT-1 induced an increase in phosphorylation of Akt at threonine 308 and serine 473, and increased phosphorylation of p70 S6 kinase at threonine 389 and serine 421/threonine 424, as well as phosphorylation of Mnk1, eIF4E, 4E-BP1 and S6 ribosomal protein (Figure 14).

We then determined the effect of pharmacologic inhibitors on the activation of this signaling cascade. AG490, an inhibitor of Jak2 [250], suppressed CT-1 induced phosphorylation of Akt at both sites, p70 S6 kinase T^{389}, Mnk1, eIF4E and 4E-BP1, but did not significantly affect CT-1 induced activation of ERK 1/2, p38 MAPK, mTOR, p70 S6 kinase S^{421}/T^{424}, or S6 ribosomal protein. PD98059, an inhibitor of MEK 1/2 [253], had the greatest inhibitory effect on basal and CT-1 induced phosphorylation of ERK 1/2, Mnk1, and eIF4E, but had a lesser effect on CT-1 induced phosphorylation of Akt, mTOR, p70 S6 kinase, 4EB-P1 and S6 ribosomal protein. SB203580, an inhibitor of p38 MAPK [254], suppressed CT-1 induced phosphorylation of Akt, p70 S6 kinase and Mnk1, a finding that supports previous work demonstrating that p38 MAPK can activate Akt [255], p70 S6 kinase [256] and Mnk1 [257]. As expected, LY294002, an inhibitor of PI3K [253],

suppressed basal and CT-1 induced phosphorylation of Akt, mTOR, p70 S6 kinase, Mnk1, eIF4E, 4E-BP1 and S6 ribosomal protein. LY294002 also attenuated activation of ERK 1/2 and p38 MAPK suggesting that PI3K participates in CT-1 induced activation of these signaling mediators. Rapamycin, an inhibitor of FRAP/mTOR [253], decreased basal and CT-1 induced phosphorylation of p70 S6 kinase and S6 ribosomal protein phosphorylation, and had a lesser effect on 4E-BP1 phosphorylation (Figure 15).

To determine if the inhibitory effect of these pharmacologic inhibitors impacted CT-1 induced protein synthesis, we again measured CT-1 induced ^{3}H-leucine uptake in the presence of inhibitors of the Jak/STAT, PI3K, p42/44 and p38 MAPK and mTOR pathways. CT-1 induced protein synthesis could be suppressed by co-incubation with AG490, PD98059, SB203580, LY294002 or Rapamycin (Figure 16). Our interpretation of how these pathways are integrated is shown in Figure 17. These results demonstrate that CT-1 induced protein synthesis occurs through activation of typical translation regulatory proteins.

To examine synthesis of mature collagen, we utilized a radioimmunoassay to measure the expression of procollagen-1-carboxypropeptide (P1CP) in conditioned media from cardiac fibroblasts stimulated with CT-1. This method has been shown to correlate well with incorporation of ^{3}H-proline into collagenase sensitive proteins [247], but is much easier to perform. CT-1 stimulation of cardiac fibroblasts increased expression of P1CP in the culture media, indicating an overall increase in mature collagen synthesis. Since CT-1 also induces cardiac fibroblast proliferation, we normalized P1CP expression to the number of cells present at the end of the

experiment. Although equal numbers of cells were loaded at the beginning of the experiment, the proliferative effect of CT-1 was much greater than its collagen synthetic effect resulting in an apparent decrease in overall mature collagen synthesis (Figure 18). This has observation has important implications with reference to the effect of CT-1 on cardiac fibrosis *in vivo*, and suggests that CT-1 does not contribute to the overt cardiac fibrosis that follows MI, and may in fact dampen the effects other putative profibrotic agents.

Cardiotrophin-1 induces cardiac fibroblast migration.

The infarct zone is repopulated by interstitial fibroblasts that infiltrate the scar after MI. To determine if CT-1 possesses chemoattractive properties, we used a Boyden chamber. Stimulation of cardiac fibroblasts with a CT-1 gradient induced cardiac fibroblast migration in a dose dependent manner (Figure 19). Similar results were obtained when using the Costar Transwell® system (data not shown). To determine how the chemotactic response is transduced, we used specific inhibitors of intracellular signaling pathways. CT-1 chemotaxis is dependent on intracellular signaling pathways (Figure 20) and integrin function (Figure 21). Stimulation of migrating cardiac fibroblasts with CT-1 resulted in localization of the actin stabilizing protein, cortactin, at the leading edge of lamellipodia. This effect could be blunted by co-incubation with AG490 (Figure 22). CT-1 induced migration is dependent on functioning potassium channels and myosin light chain kinase (Figure 23). CT-1 induced a pronounced hyperpolarization of the cell, as indicated by decreased fluorescence of the voltage sensitive dye $DiBAC_4(3)$ (Figure 24). Since

activation of myosin motors is required for effective cell migration, and CT-1 induced migration could be inhibited by co-incubation with a myosin light chain kinase inhibitor, we sought to determine the effect of CT-1 on the phosphorylation status of myosin light chain. Myosin light chain regulates the ATPase activity of myosin heavy chain, and thus overall contractile activity. As seen in Figure 25a, CT-1 induced phosphorylation of myosin light chain at threonine 18/serine 19. Myosin light chain phosphorylation could be prevented by co-incubation with ML-7 or W7 but not AG490 or PP2 (Figure 25b). This suggests that CT-1 induced phosphorylation of myosin light chain in cardiac myofibroblasts occurs through activation of calmodulin and not through activation of Src kinases. This also suggests that CT-1 induced cell membrane hyperpolarization is attended by an increase in intracellular calcium.

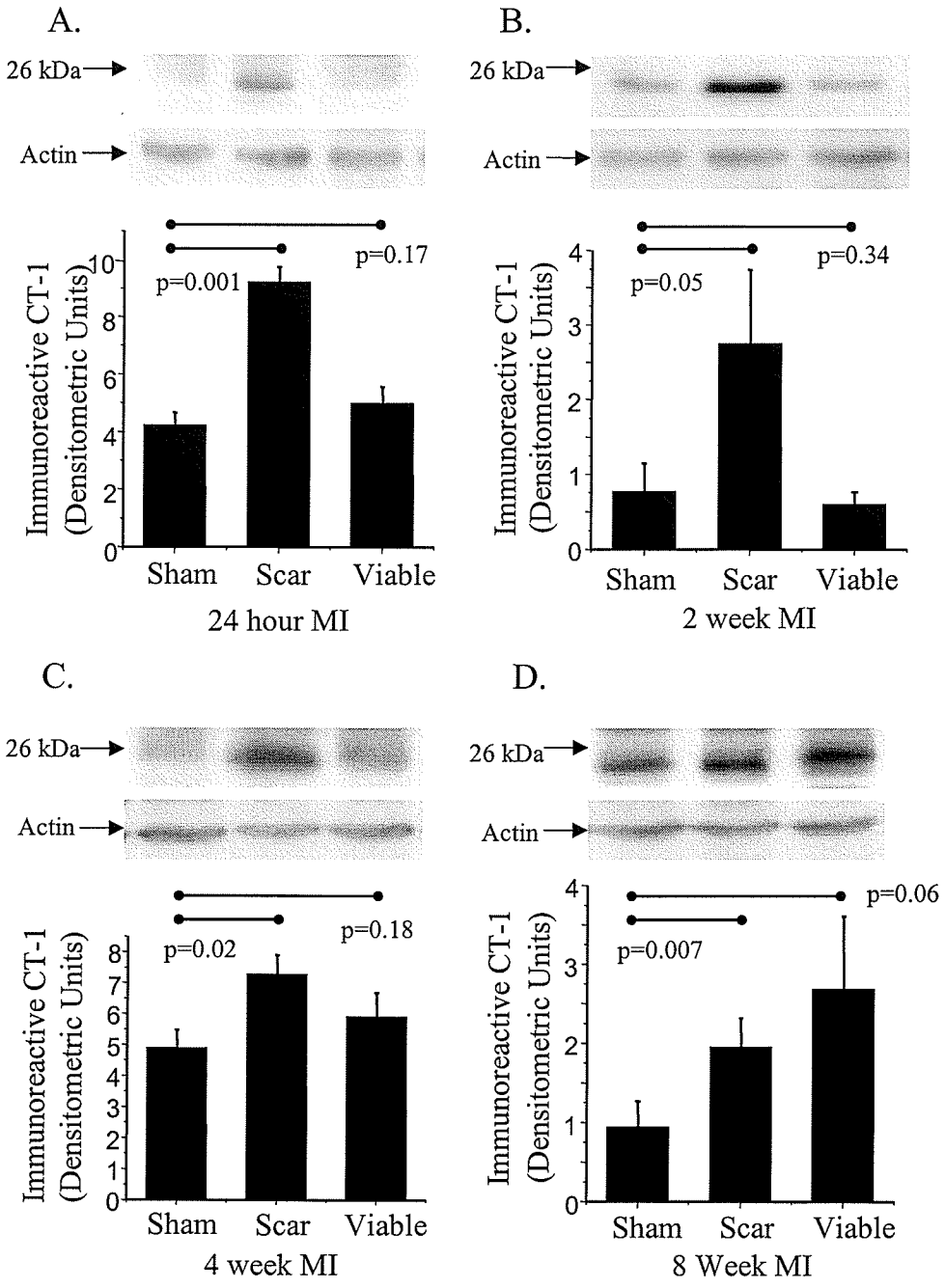


Figure 1: Expression of CT-1 at 24 hr, 2, 4 and 8 weeks after MI. Cardiotrophin-1 expression was determined in 50μg cardiac tissue lysate by Western analysis with anti-CT-1 monoclonal antibody. Samples from 3 separate animals were quantified and relative intensity is expressed in arbitrary densitometric units (mean ± SEM).

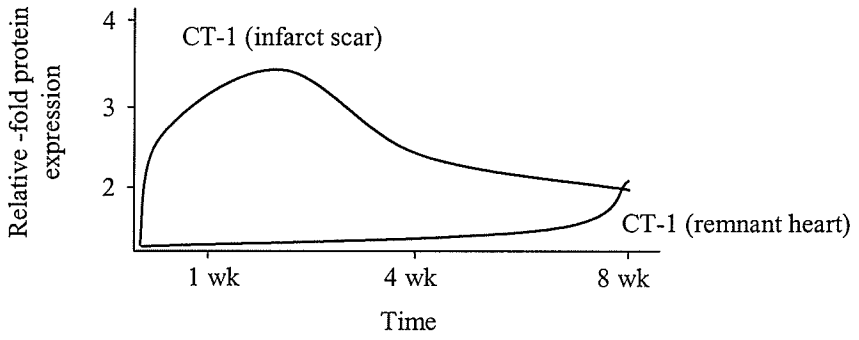


Figure 2. Graphic representation of CT-1 expression after permanent coronary artery ligation in the rat in the infarct zone and in viable/remnant myocardium.

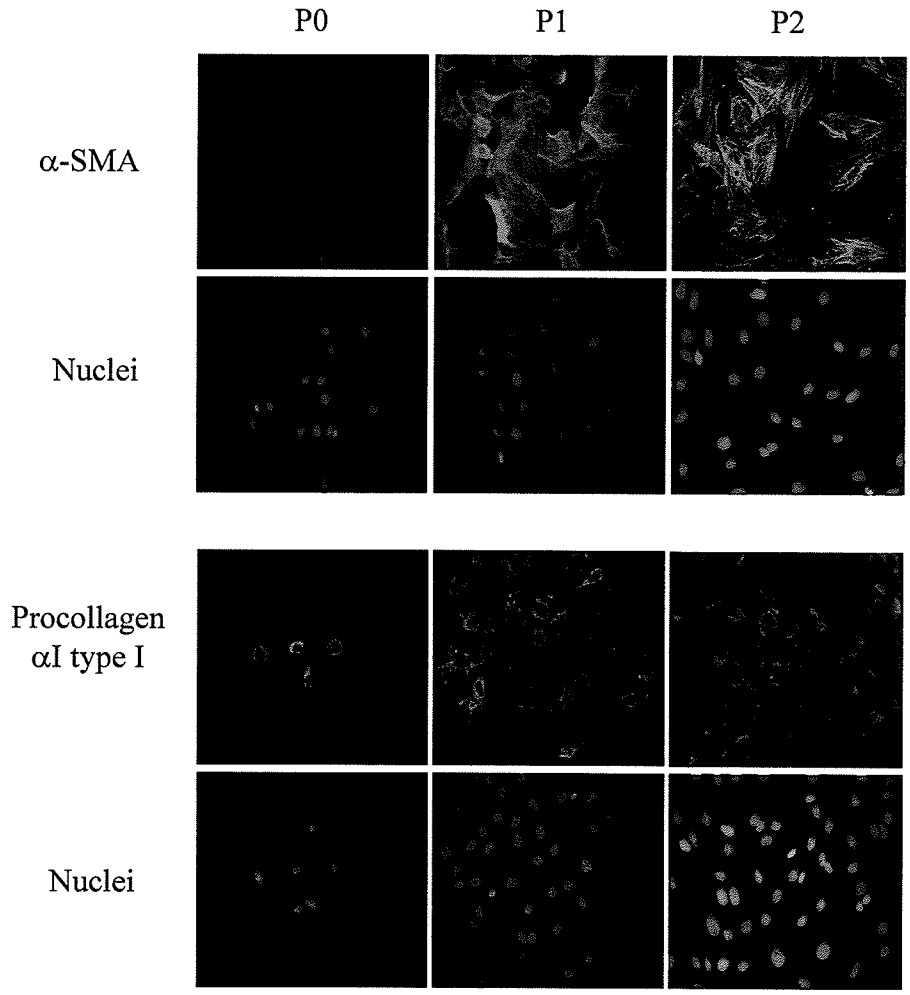
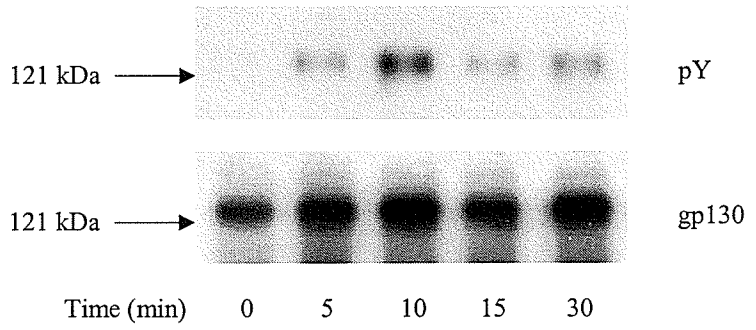


Figure 3: Expression of α smooth muscle actin after isolation of primary adult rat cardiac fibroblasts. (400x)

A.



B.

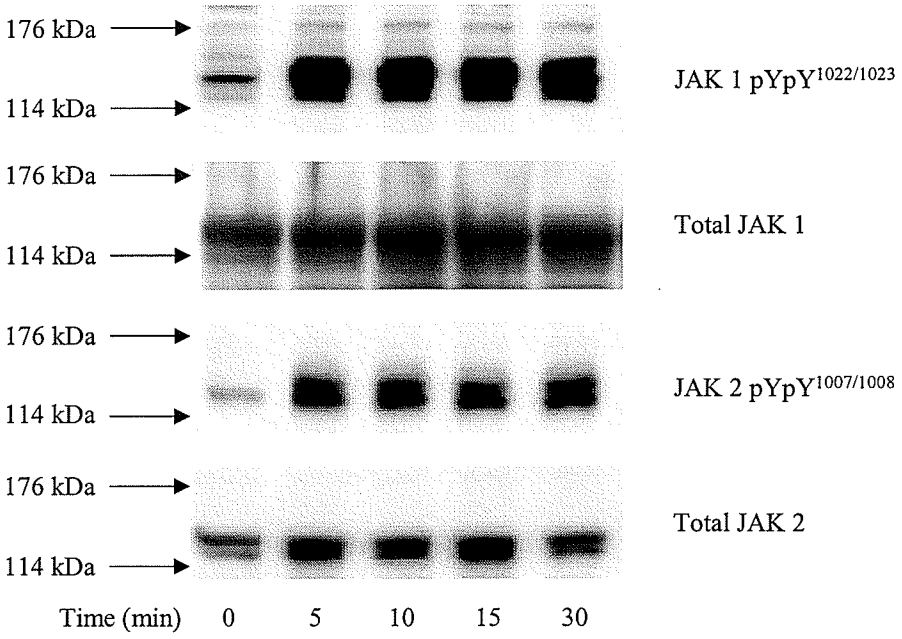
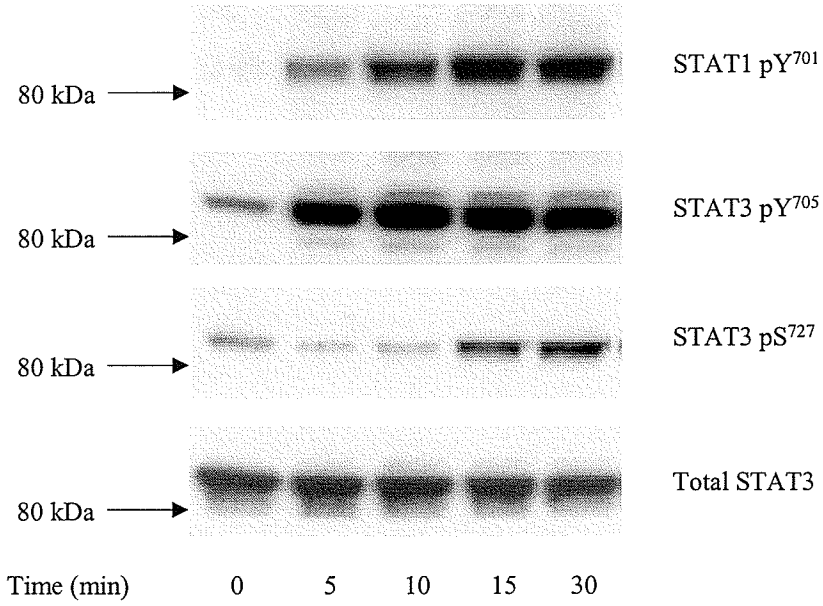


Figure 4. CT-1 induced phosphorylation of gp130 and Jak1/2. Panel A. Phosphorylation of gp130 was investigated by immunoprecipitating gp130 from cell lysates of CT-1 treated or non-treated cells and probing with anti-phosphotyrosine antibody. To verify equal protein loading between different lanes, the membrane was stripped and probed for gp130. Panel B. Jak1 and Jak2 phosphorylation was analyzed by western analysis. Equal protein loading was verified by stripping the membranes and probing for total Jak1 and total Jak2. Representative western blots from 3 separate experiments are shown.

A.



B.

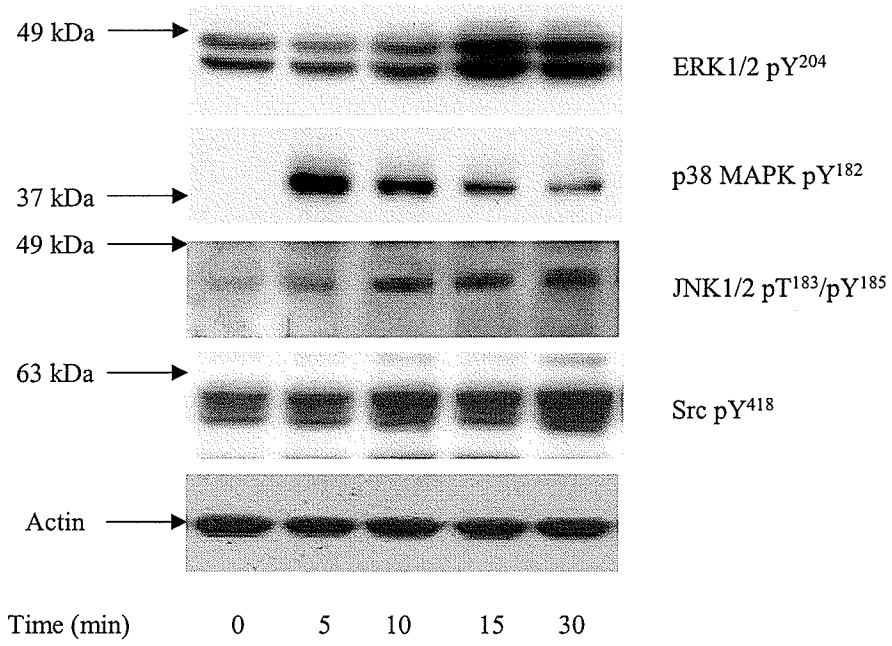
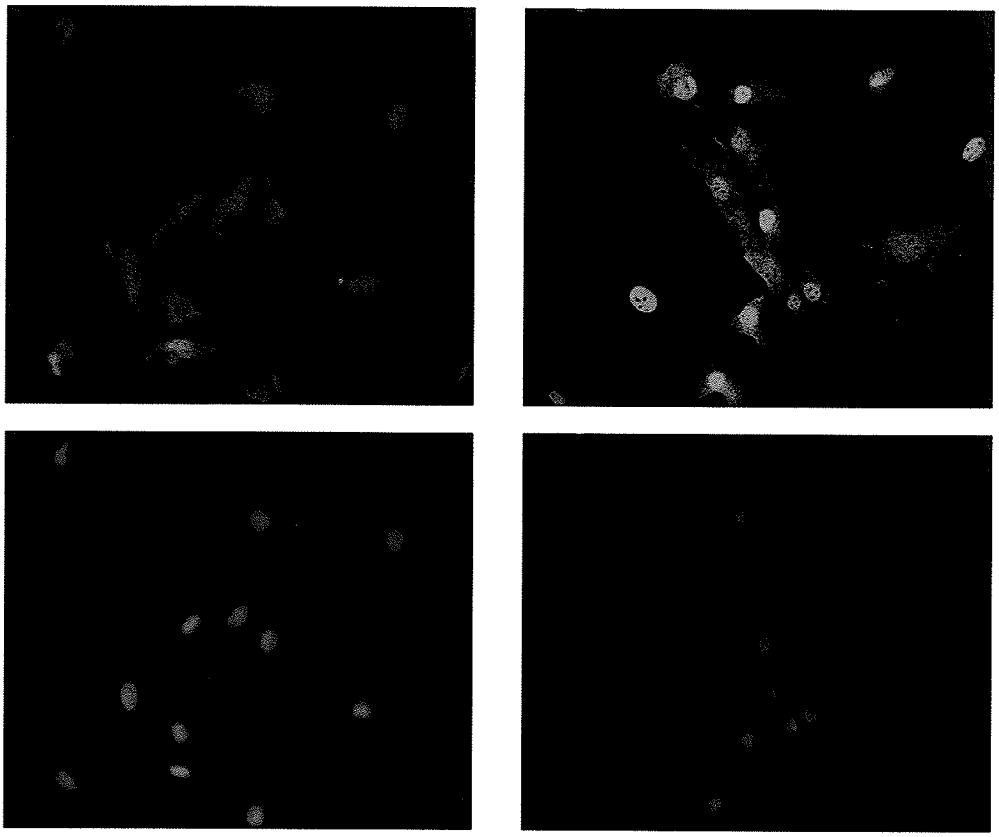


Figure 5. CT-1 Induced Phosphorylation of Signaling Pathways. Panel A. CT-1 induces phosphorylation of STAT1 and STAT3. To verify equal protein loading, the membranes were stripped and probed for total STAT3. Panel B. CT-1 induces phosphorylation of ERK1/2, p38 MAPK, JNK and Src. To verify equal protein loading, the membranes were stripped and probed for actin. Representative Western blots from 3 separate experiments are shown.



Non-stimulated cells CT-1, 10 ng/mL 5 minutes

Figure 6. CT-1 induces nuclear accumulation of STAT3. Cells were stimulated with CT-1 for 5 minutes and immunostained with anti-STAT3 antibody. Nuclei were identified by staining with Hoechst 33342 (lower panels). Representative images are shown from 3 separate experiments.

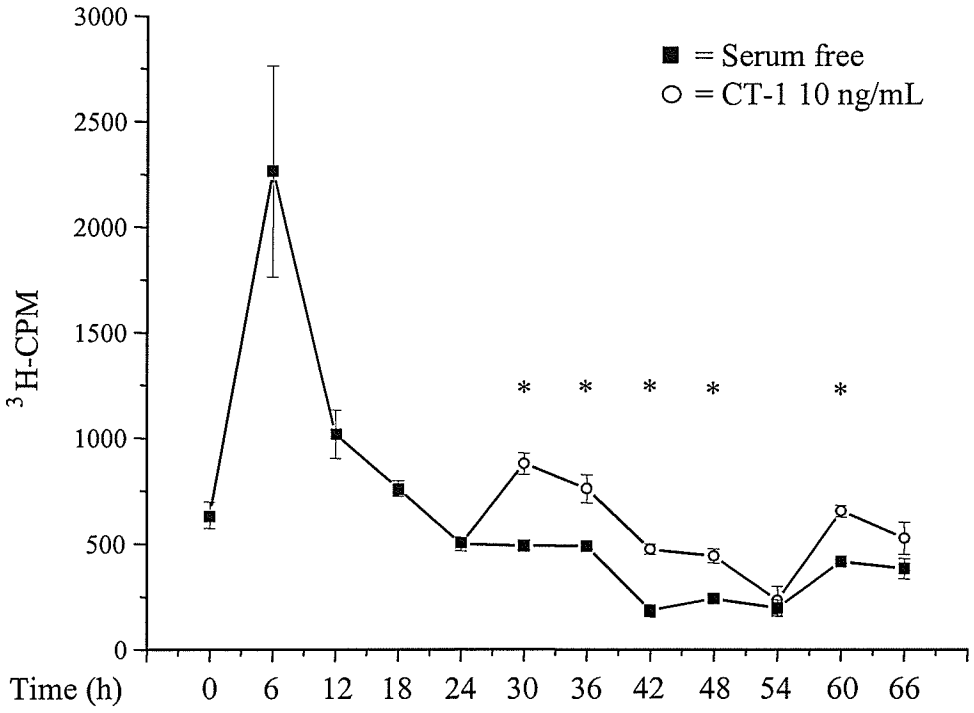
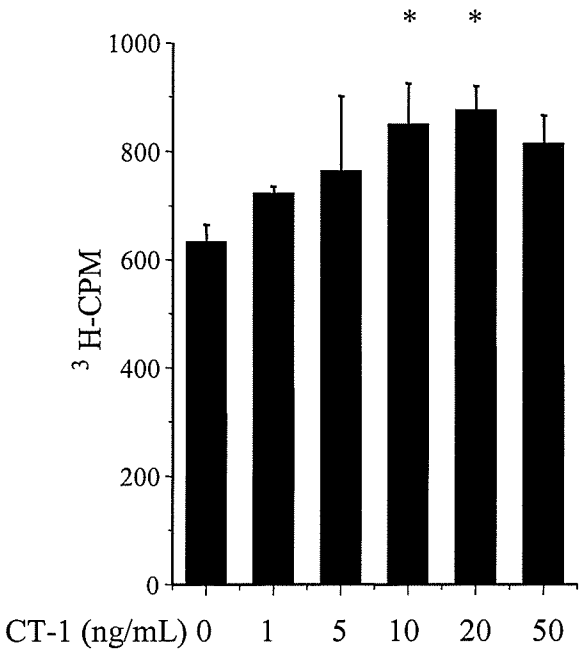


Figure 7: CT-1 induces cardiac fibroblast DNA synthesis. DNA synthesis was measured by incorporation of 3H-thymidine. Cardiac fibroblasts were incubated in serum-free media from time zero, then pulse labeled with ^{3}H-thymidine for 30 minutes. At 24 hours, cells were stimulated with 10 ng/mL CT-1, or further incubated in serum-free media. Samples from 7 separate experiments were analyzed in triplicate. Results are displayed as mean ± SEM. *$p < 0.05$ vs serum-free at the same time point.

A.



B.

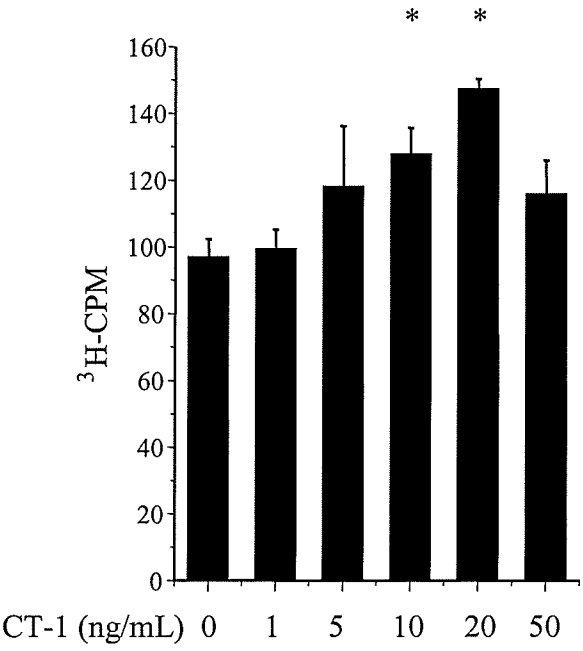
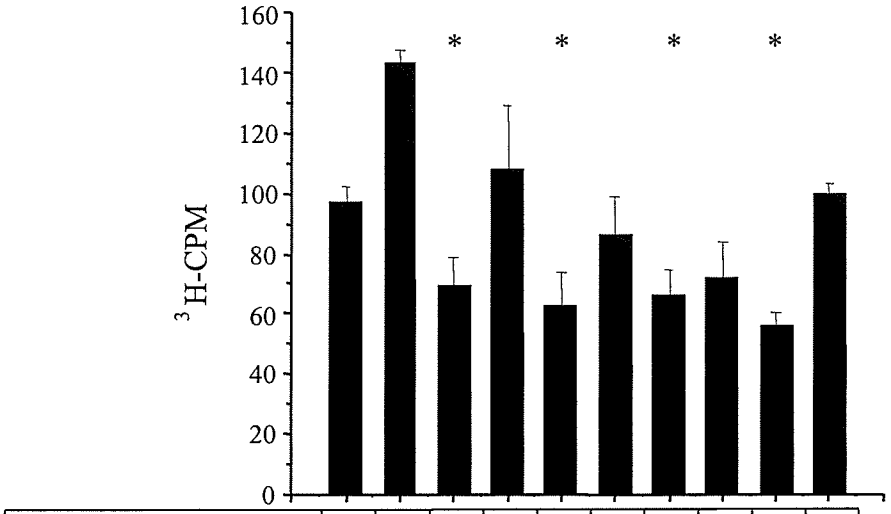


Figure 8: Dose-response effect of CT-1 on DNA synthesis. Quiescent cardiac fibroblasts were incubated with CT-1 at specified concentrations for 6 hours (A) or 12 hours (B) and labeled with ^{3}H-thymidine for 30 minutes. Samples from 7 separate experiments were analyzed in triplicate. Results are displayed as mean ± SEM. *$p < 0.05$ vs non-stimulated control.



CT-1 (10 ng/mL)	-	+	+		+		+		+	
AG490 (25 μM)			+	+						
PD98059 (50 μM)					+	+				
LY294002 (10 μM)							+	+		
Genistien (10 μM)									+	+

Figure 9: CT-1 induced DNA synthesis is dependent on the Jak/STAT, MAPK, PI3K and Src pathways. Quiescent cardiac fibroblasts were pretreated with inhibitors at specified concentrations, incubated with CT-1 for 12 hours and labeled with ^{3}H-thymidine for 30 minutes. Samples from 7 separate experiments were analyzed in triplicate. *$p < 0.05$ vs CT-1 10 ng/mL.

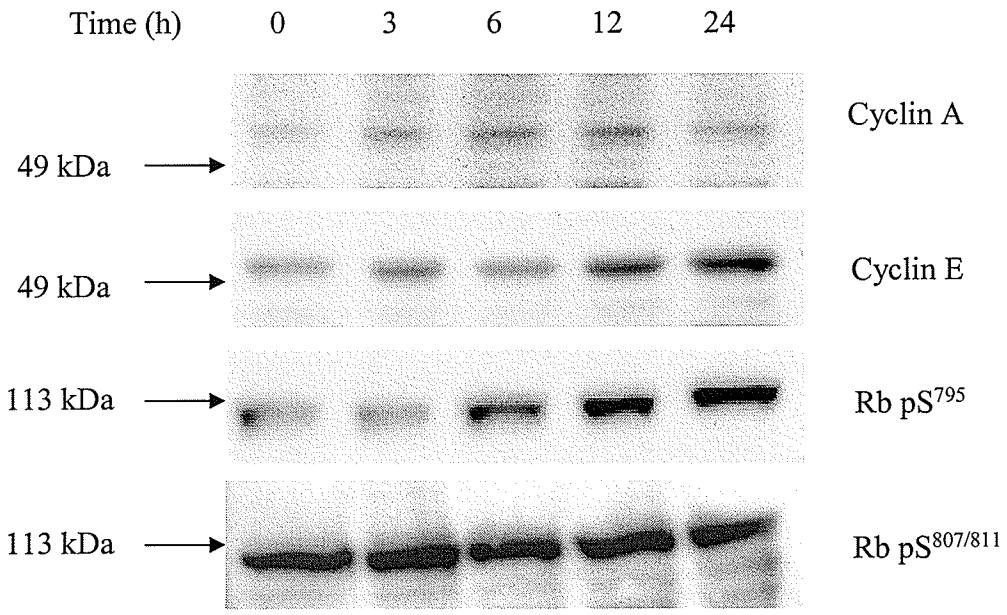


Figure 10: CT-1 induces expression of cyclins and Rb phosphorylation. Quiescent cardiac fibroblasts were incubated with CT-1 (10 ng/mL) for specified times. Cell lysates were subjected to Western analysis with antibodies against cyclins A, E, Rb pS795, and pS807/811. Representative blots from 3 separate experiments are shown.

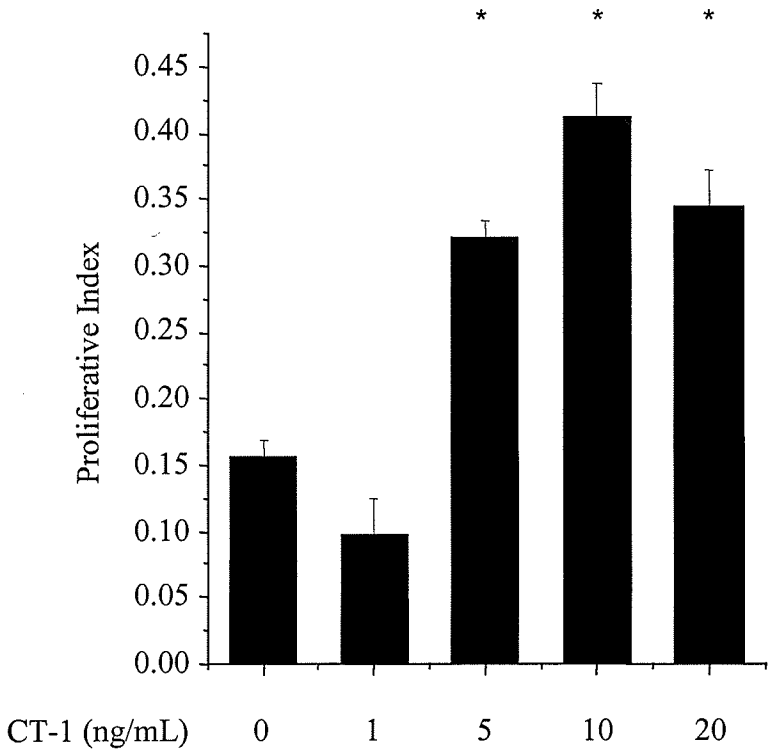


Figure 11. CT-1 induces nuclear accumulation of PCNA. Quiescent cardiac fibroblasts were incubated with CT-1 at specified concentrations for 24 hours, fixed and immunostained with anti-PCNA antibody. The number of nuclei positive for PCNA are expressed as a ratio with total nuclei (proliferative index). *$p < 0.05$ vs non-stimulated control.

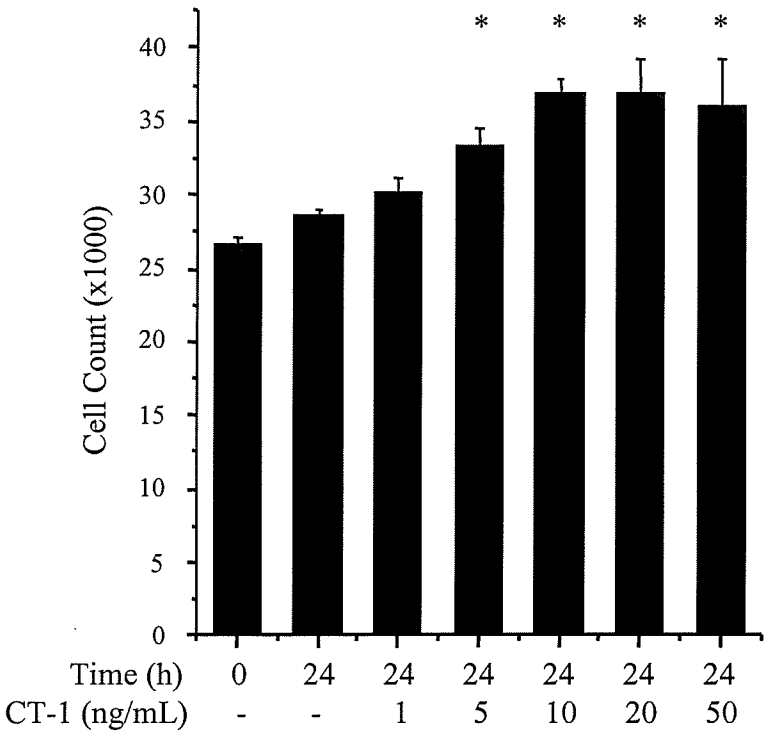


Figure 12: CT-1 increases total cell number. Equal numbers of cardiac fibroblasts were loaded into 24 well plates, rendered quiescent by incubation in serum free media for 24 hours, then stimulated with CT-1 at indicated concentrations for 24 hours. The cells were trypsinized, diluted in filtered phosphate buffered saline and counted with a Coulter counter. *$p < 0.05$ vs non-stimulated control.

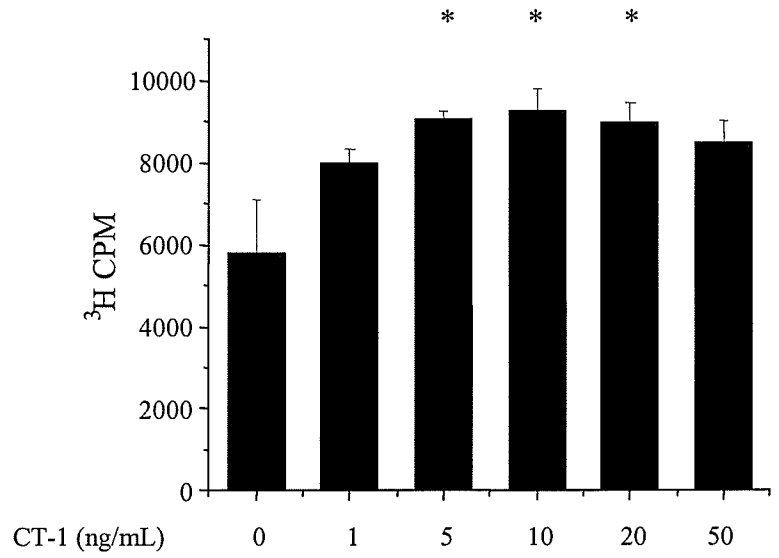


Figure 13. CT-1 increases protein synthesis. Protein synthesis was measured by incorporation of ^{3}H-leucine. Quiescent cardiac fibroblasts were stimulated with indicated concentrations of CT-1 for 24 hours. Results are displayed as mean ± SEM. Samples from 5 separate experiments were analyzed in triplicate. *$p < 0.05$ vs control.

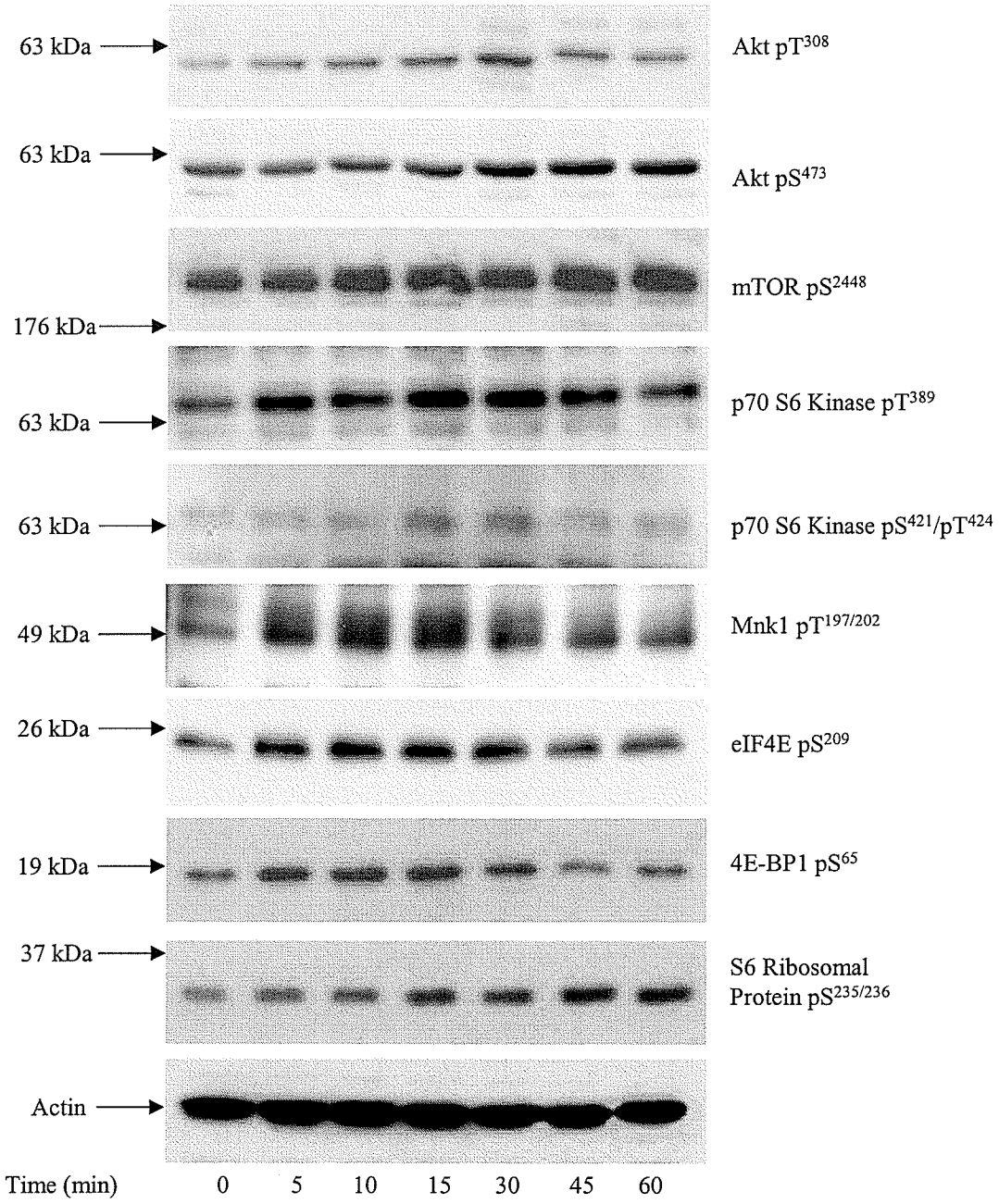
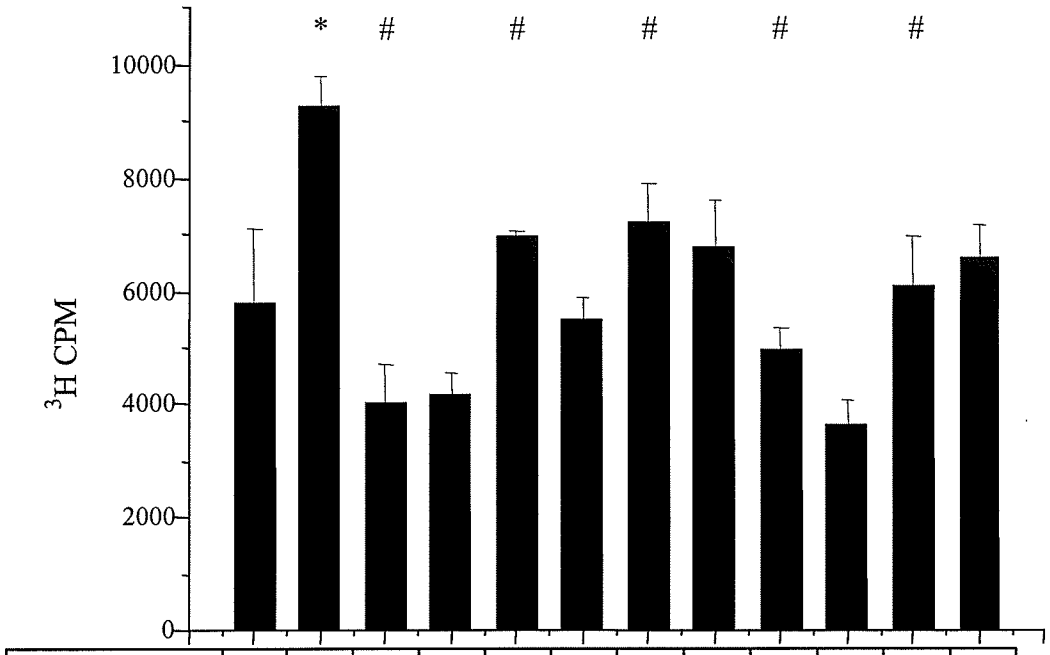
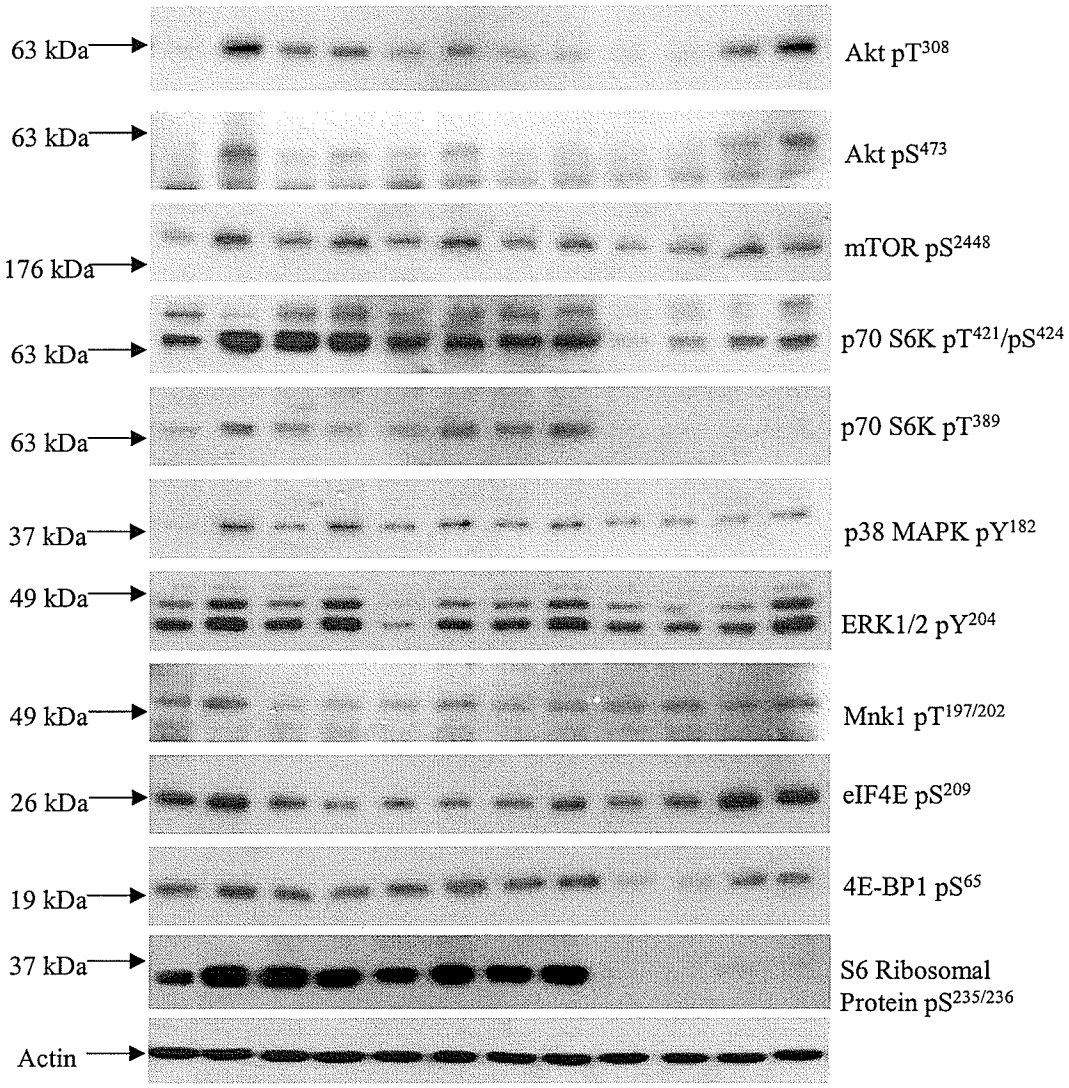


Figure 14. CT-1 increases phosphorylation of protein synthesis regulatory proteins. Quiescent cardiac myofibroblasts were treated with CT-1 (10 ng/mL) for indicated times. Protein lysates were prepared using RIPA buffer containing phosphatase and protease inhibitors, and subjected to Western blot analysis. Membranes were probed with phospho-specific antibodies. To verify equal protein loading, the membranes were stripped and probed for actin. Representative blots from 3 separate experiments are shown.



CT-1 (10ng/mL)		+	+		+		+		+		+	
AG490 25 μ M			+	+								
PD98059 10 μ M					+	+						
SB203580 10 μ M							+	+				
LY294002 10 μ M									+	+		
Rapamycin 10 μM											+	+

Figure 15. Incubation with inhibitors of signaling pathways depresses protein synthesis. Protein synthesis was measured by incorporation of ^{3}H-leucine. Quiescent cardiac fibroblasts were stimulated with CT-1 with or without inhibitors for 24 hours. Results are displayed as mean ± SEM. Samples from 3 separate experiments were analyzed in triplicate. *$p < 0.05$ vs control. #$p < 0.05$ vs CT-1 10 ng/mL.



CT-1 (ng/mL)		+		+		+		+		+		+
AG490 25 μM			+	+								
PD98059 10 μM					+	+						
SB203580 10 μM							+	+				
LY294002 10 μM									+	+		
Rapamycin 10 μM											+	+

Figure 16. Incubation with inhibitors of signaling pathways depresses phosphorylation of translational regulatory proteins. Quiescent cardiac fibroblasts were stimulated with CT-1 with or without inhibitors for 15 minutes. Membranes were probed with phospho-specific antibodies. To verify equal protein loading, the membranes were stripped and probed for actin.

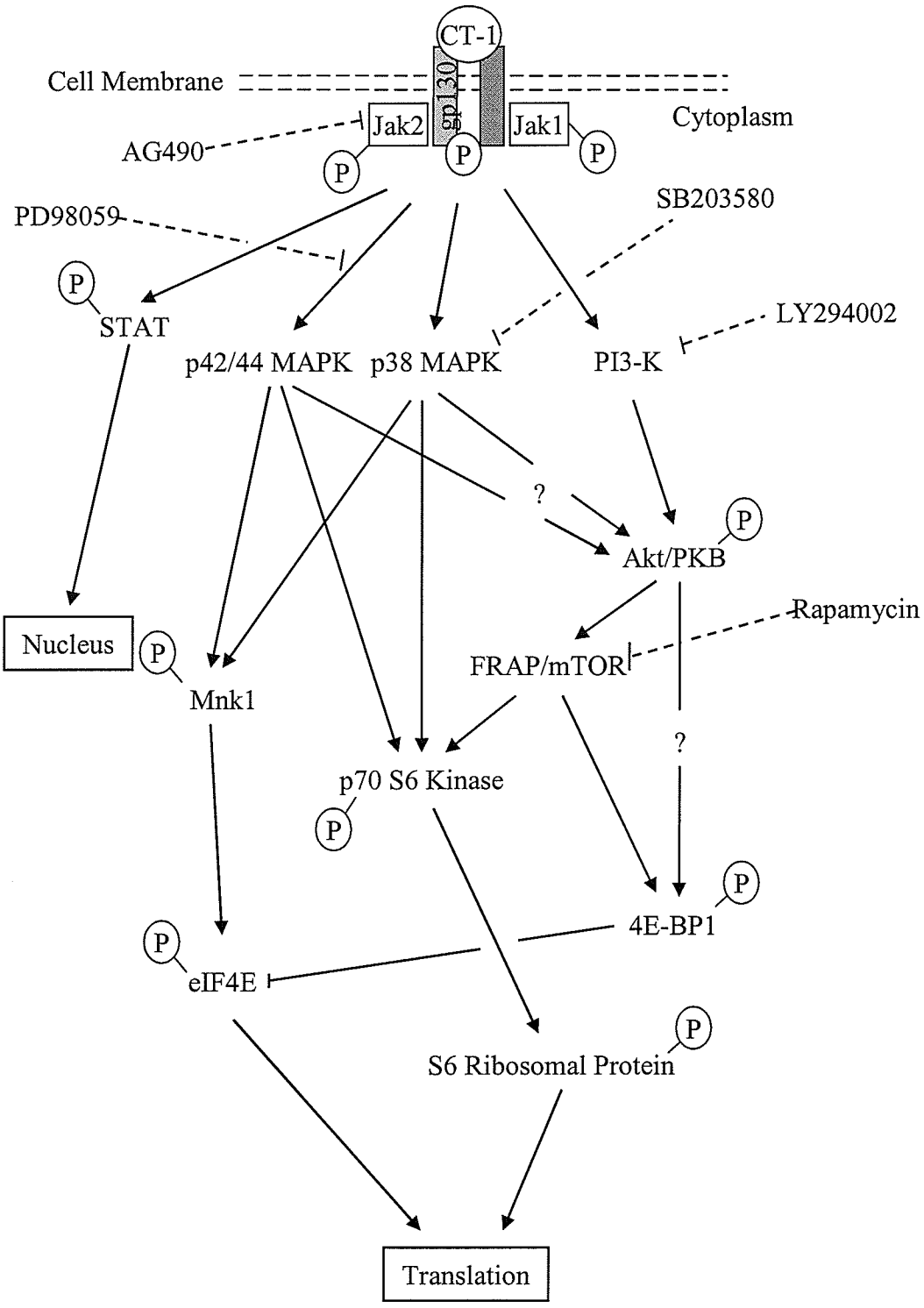


Figure 17. Proposed scheme of the CT-1 signaling cascade in cardiac fibroblasts leading to induction of protein synthesis.

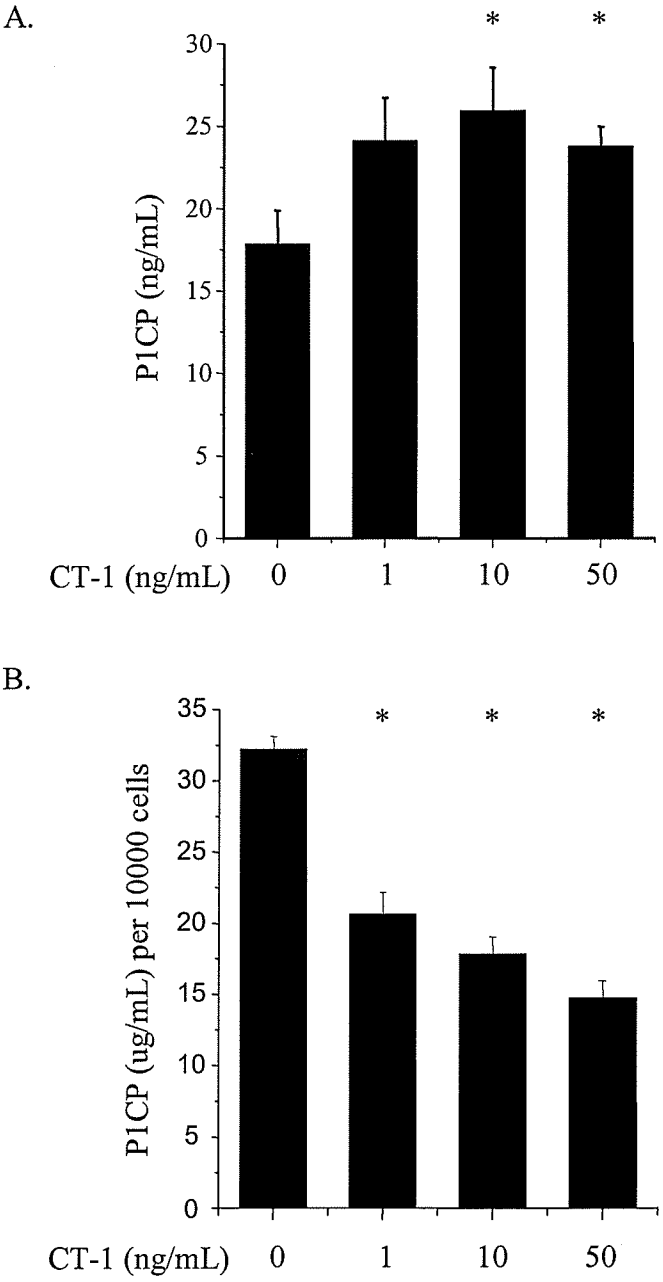


Figure 18: Effect of CT-1 on mature collagen synthesis. Collagen synthesis was determined by radioimmunoassay for procollagen-1 carboxy-propeptide (P1CP) in conditioned media from quiescent cardiac fibroblasts stimulated with specified concentrations of CT-1 for 24 hours. Conditioned media from 3 separate experiments was analyzed in triplicate. Results are displayed as mean ± SEM. Panel A shows the P1CP content of media collected from CT-1 stimulated cells. Panel B shows the P1CP concentration corrected for the number of cells at the end of the experiment. *$p < 0.05$ vs non-stimulated control.

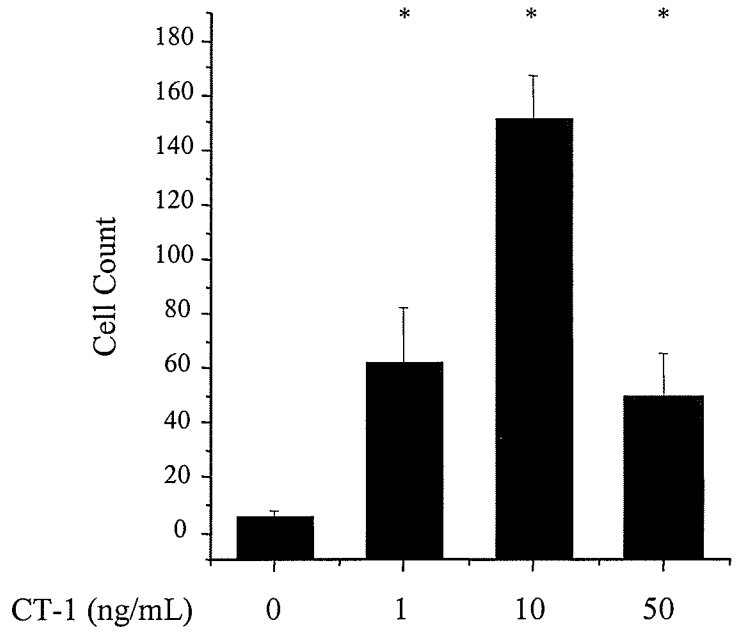


Figure 19: CT-1 is a chemoattractant for rat cardiac fibroblasts. The ability of CT-1 to induce cell migration was analyzed with a Boyden chamber. Indicated concentrations of CT-1 in serum free media were loaded into the lower wells and cardiac fibroblasts (1000 cells/mm^2) were loaded into the upper wells. Results displayed represent the number of cells that migrated through the membrane separating the cells and media containing CT-1. *$p < 0.05$ vs non-stimulated control.

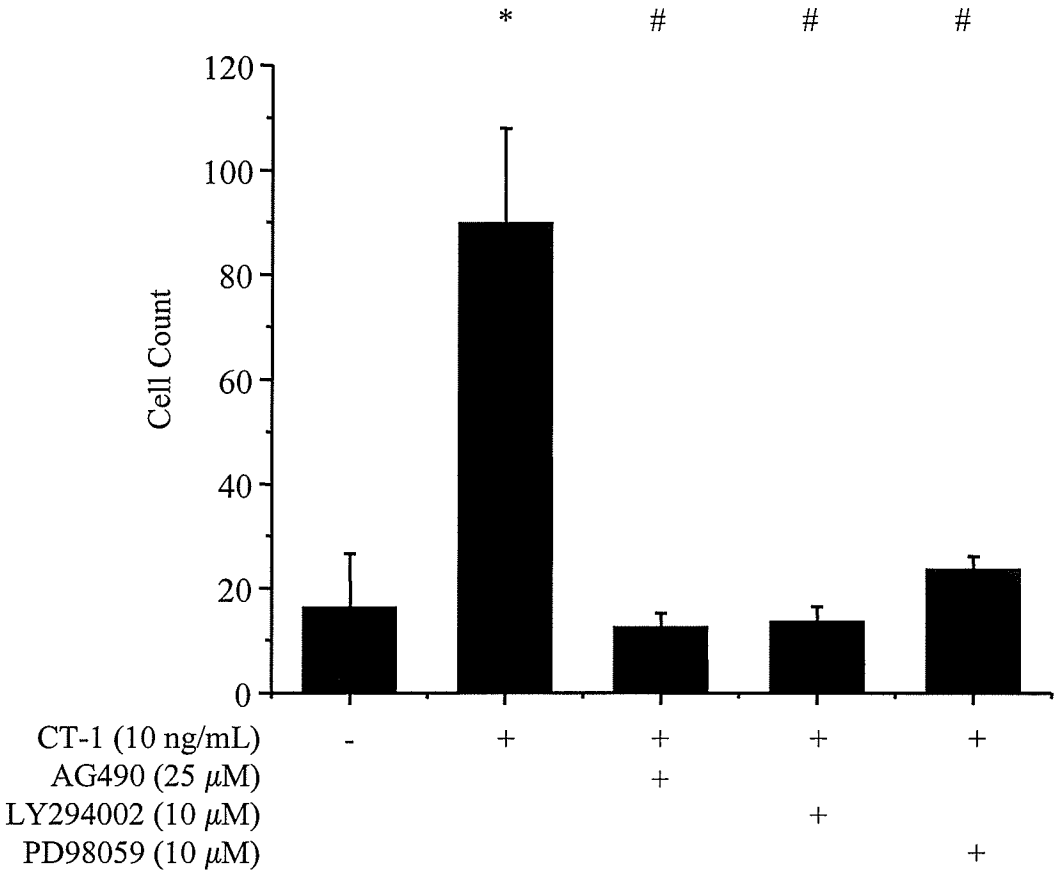


Figure 20: CT-1 induced chemotaxis is dependent on signaling pathways. The ability of CT-1 to induce cell migration was analyzed with a Boyden chamber. Indicated concentrations of CT-1 and inhibitors in serum free media were loaded into the lower wells and cardiac fibroblasts and inhibitors (1000 cells/mm2) were loaded into the upper wells. Cells that had migrated through the membrane were stained and counted. *$p < 0.05$ vs non-stimulated control. # $p < 0.05$ vs CT-1.

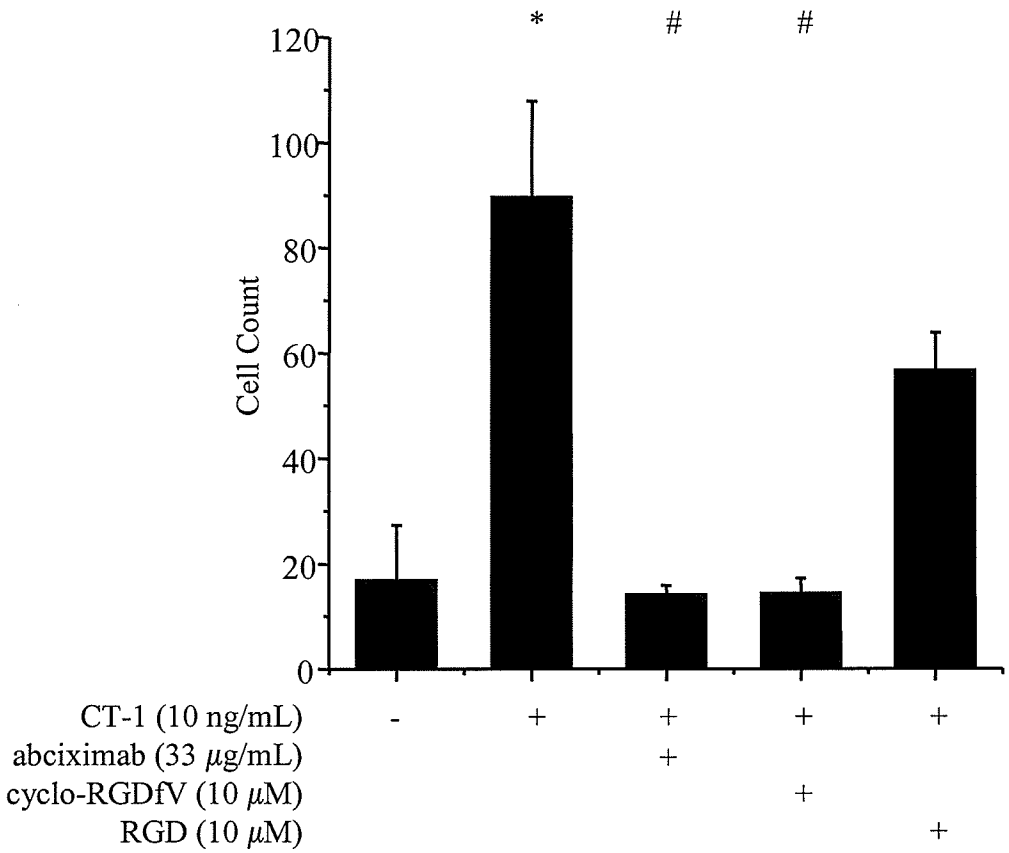


Figure 21: CT-1 induced chemotaxis is dependent on integrins. The role of extracellular matrix interactions utilized in CT-1 induced chemotaxis were examined using a Boyden chamber and integrin inhibitors. Indicated concentrations of CT-1 and inhibitors in serum free media were loaded into the lower wells and cardiac fibroblasts and inhibitors (1000 cells/mm2) were loaded into the upper wells. Cells that had migrated through the membrane were stained and counted. *$p < 0.05$ vs non-stimulated control. # $p < 0.05$ vs CT-1.

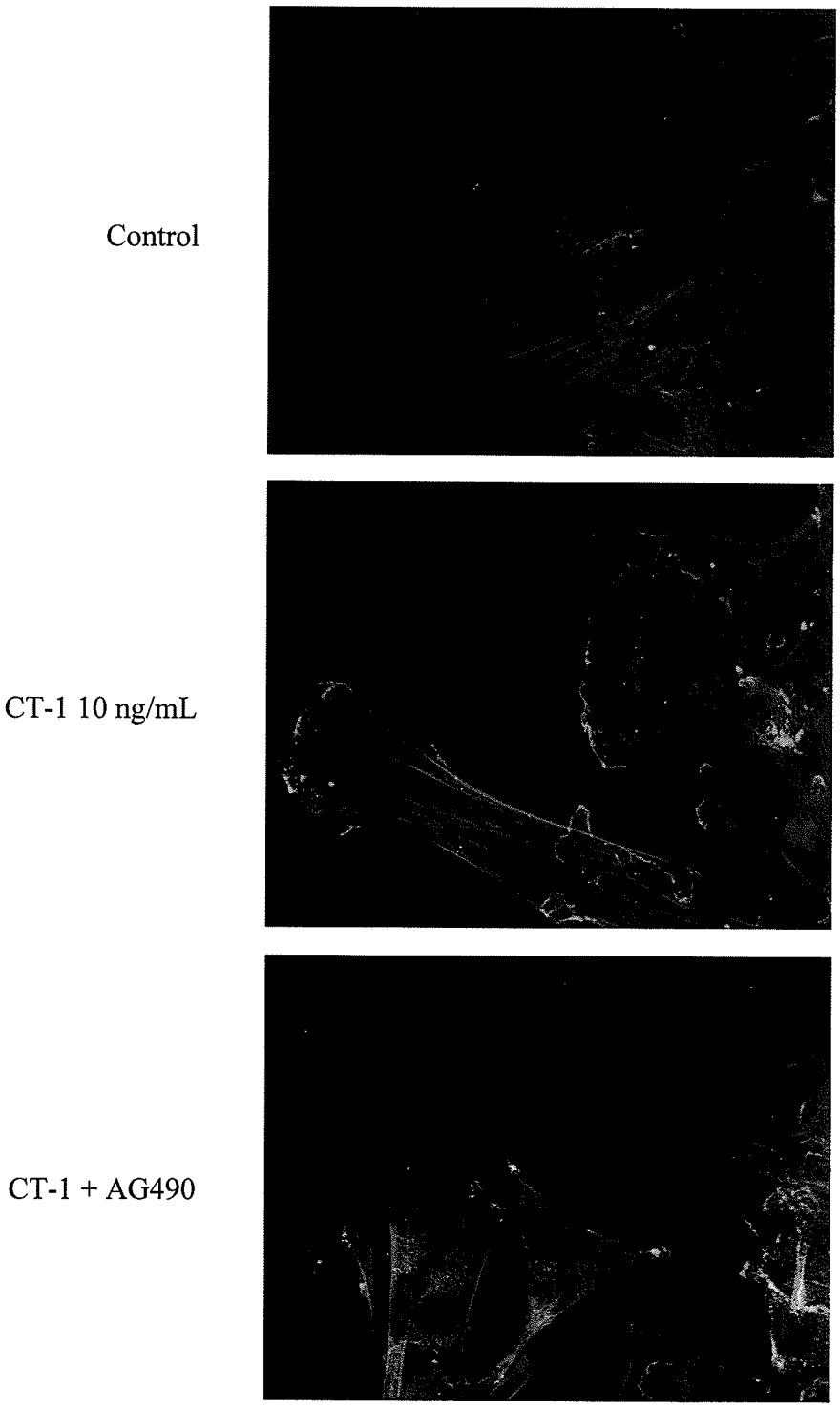


Figure 22: Cardiotrophin-1 induces accumulation of cortactin at the leading edge of migrating cells. Localization of the actin stabilizing protein, cortactin, was examined using the "wounded monolayer" model. Cells were immunostained for cortactin 4 hours after the creation of the wound. Green: cortactin. Red: Actin (rhodamine phalloidin). Blue: Nuclei.

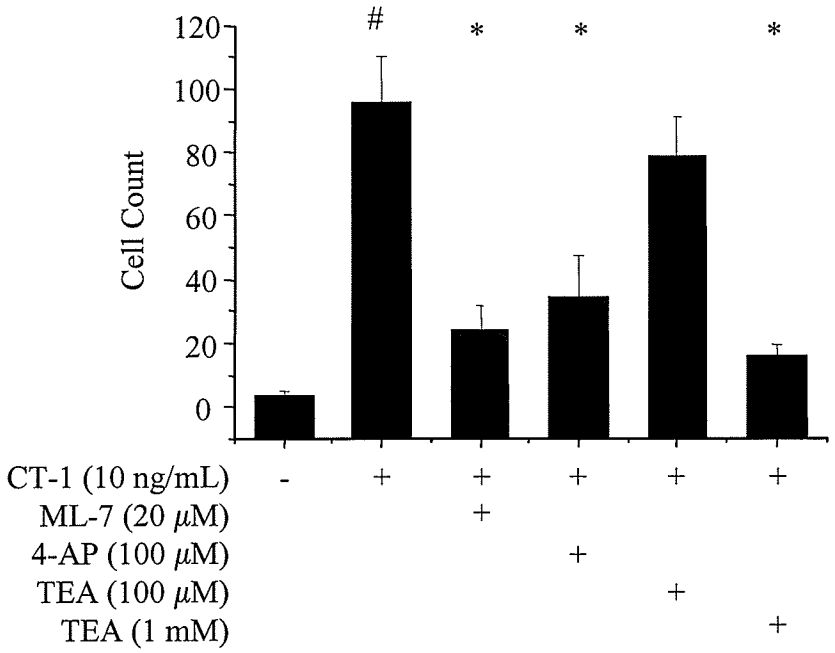


Figure 23: CT-1 induced migration is dependent on ion channel and myosin light chain kinase function. Cells that had migrated through the membrane were stained and counted. *$p < 0.05$ vs non-stimulated control. # $p < 0.05$ vs CT-1.

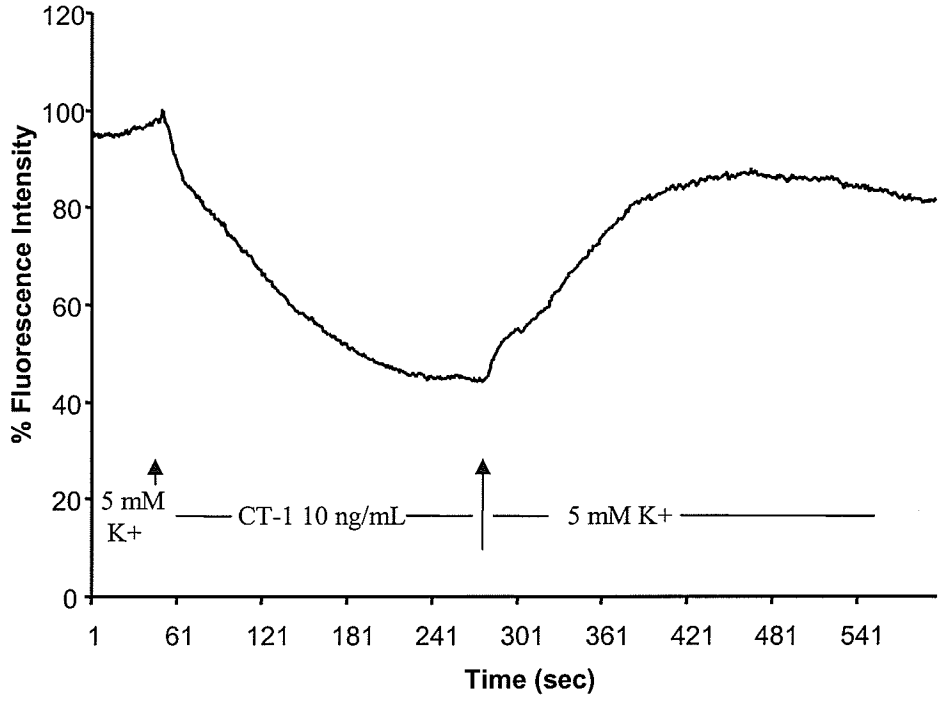
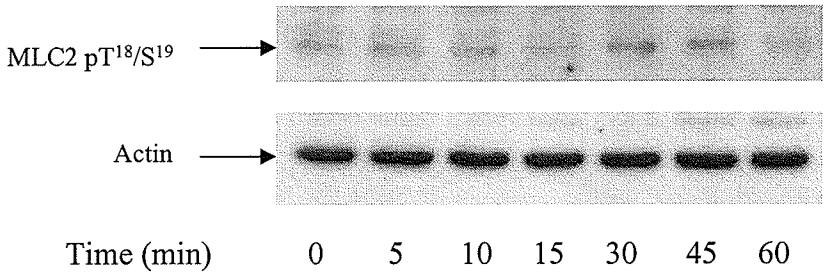


Figure 24. Cardiotrophin-1 induces hyperpolarization of cardiac fibroblasts. Membrane potential was estimated in quiescent cardiac fibroblasts with the use of $DiBac_4(3)$ voltage sensitive dye. The change in fluorescence relative to baseline was recorded, where 1% change ~ 1 mV. Results displayed are representative of 3 separate experiments.

A.



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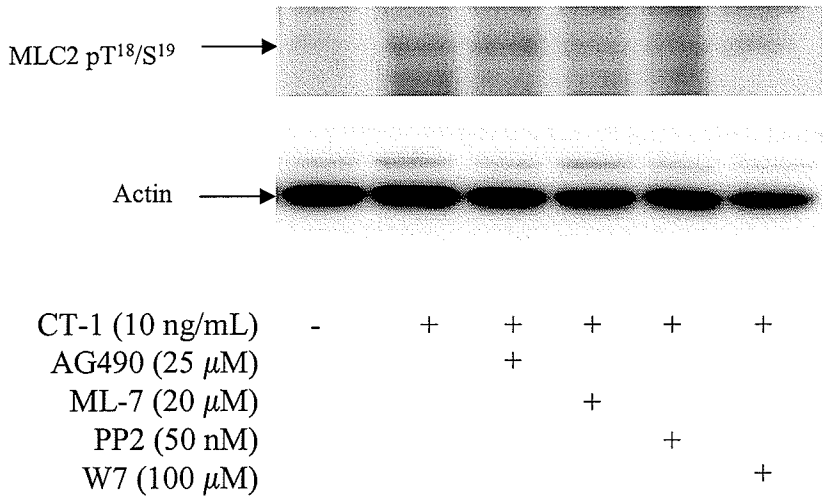

Figure 25: CT-1 induces phosphorylation of myosin light chain. Panel A: Quiescent cardiac fibroblasts were stimulated with CT-1 (10 ng/mL) for specified times. Panel B: Quiescent cardiac fibroblasts were treated for 30 minutes and lysates were analyzed for myosin light chain phosphorylation. The blots were stripped and reprobed for actin to verify equal protein loading. Representative blots from three separate experiments are shown.

V. DISCUSSION

1. Cardiotrophin-1 expression in post-MI heart.

In this study we have examined the expression of CT-1 in the post-MI heart, and have observed elevated expression in the infarct zone at all time points examined. Additionally, we have observed elevated expression in the viable myocardium in the chronic phase of wound healing (8 weeks). The remainder of the experiments performed, coupled with previous published work, allows us to speculate that CT-1 is beneficial during the early period of post-MI wound healing, but in the long term may contribute to ventricular dilation and deterioration of ventricular function. It is known that CT-1 is cardioprotective from ischemia-reperfusion injury, even when added at the point of reoxygenation [236,238,239]. Therefore early expression of CT-1 in the ischemic myocardium may represent an adaptive, protective phenomenon that is beneficial in reducing myocyte loss and inducing hypertrophy of remaining myocytes so that overall ventricular function is maintained. However, it is suggested that CT-1 expression in the late phase of wound healing and the onset of heart failure may contribute to ventricular dilation by inducing hypertrophy of myocytes where sarcomeres are arranged in series (eccentric hypertrophy), rather than in parallel as is seen in concentric cardiac hypertrophy [9], although this hypothesis remains to be proven.

In addition to possibly protecting myocytes, the observation that CT-1 induces migration and proliferation of myofibroblasts suggests that localized expression in the infarct zone may act to stimulate migration of cardiac fibroblasts and other cells from the surrounding viable myocardium into the infarct zone,

thereby initiating repopulation of the scar with cells. It is known that bone marrow mesenchymal stem cells migrate to the scar after myocardial infarction [258]. We speculate that the expression of CT-1 in the infarct zone may provide a critical migratory stimulus for these cells (preliminary data not shown), and may even induce (with other mediators) their differentiation to a myofibroblastic phenotype. The bulk of evidence gathered in the past 5 years which is aimed at muscle cell regrowth in the healed infarct scar indicates that a cellular scar confers improved ventricular performance compared to a "hypocellular" or native infarct scar, regardless of the cell type placed in the scar [259-262]. Infarct scar-specific CT-1 expression may act to induce a more cellular phenotype through induction of cell proliferation and may antagonize the effects of angiotensin and TGF-β, both of which are expressed in the scar and act to induce cell quiescence [69,159,263 and unpublished observations]. Indeed, a common theme in explanation of the beneficial effects of popular ACE inhibiting agents (angiotensin-suppressing drugs) is that while angiotensin is suppressed, other systems are derepressed i.e., ACE inhibition prevents the destruction of bradykinin [264]. Thus the upregulation of a parallel system is invoked and yields a net benefit to the post-MI heart. We suggest that this mechanism may be an additional beneficial effect of angiotensin antagonism insofar as the CT-1 stimulus is allowed to act unopposed. In addition to opposing the action of these mediators, CT-1 expression may antagonize TNF-α induced myocyte apoptosis [265,266], thereby preserving overall ventricular performance.

Although the heart is the major source of circulating CT-1 in humans [267], the cellular source of CT-1 in the post-MI heart is not clear. While induction of CT-1

mRNA has been shown in cardiac fibroblasts [215] and myocytes [211,212,214] *in vitro* in response to a variety of stimuli, it is not clear which cell type is primarily responsible for *in vivo* myocardial CT-1 expression. It is possible that inflammatory cells contribute to CT-1 expression, particularly early on in the course of post-MI wound healing. Irrespective of the cellular source, activation of the gp130 signaling cascade is required for an appropriate myocardial response to injury that allows survival of the animal [268]. It was suggested that cardiac myocyte hypertrophy produced by angiotensin is mediated through CT-1 [269], however, recent studies in humans do not support an upstream connection to angiotensin [220]. Conversely norepinephrine, which is locally and systemically activated after MI [270], is known to elevate CT-1 expression in cardiac myocytes *in vitro* and *in vivo* via a cAMP response element in the 5' flanking region of the CT-1 gene [212]. This finding suggests the involvement of β-adrenergic stimulation in post-MI CT-1 expression.

The infarct scar has been shown to be a vascular structure and myocardial wound healing after infarction invokes a significant upregulation of angiogenesis in the infarct zone [23]. It is suggested that CT-1 induced STAT3 activation as observed in the post-MI heart may act to initiate angiogenesis as STAT3 activation is known to induce VEGF-dependent endothelial tubule formation and vasculogenesis *in vivo* [271].

In this study we have examined the effects of CT-1 on cardiac myofibroblasts to investigate the possible involvement of this IL-6 family member cytokine in remodelling post-MI heart. We are able to confirm that CT-1 activates JAK 1 and 2 (but not JAK3 or Tyk2), leading to the phosphorylation of STAT3 and STAT1 which

then translocate to the nucleus of myofibroblasts. Others have shown that upon gp130 activation, rapid negative regulation of JAKs occurs via induction of suppressor of cytokine signaling 3 (SOCS3) in heart [272], and thus a balance between positive and negative regulatory loops is attained. While CT-1 exerts cardioprotective effects [9,268], myocardial expression of CT-1 is increased in heart failure models [200,210], and in plasma [273] and cardiac tissues of patients with heart failure [226]. It is becoming clear that CT-1 expression generally precedes the development of pathological hypertrophy [208]. As mentioned previously, this may in fact be a beneficial event and required for an appropriate myocardial response to injury by activating the gp130 signaling cascade and inhibiting myocyte apoptosis [268].

2. Significance of the mitogenic effect of Cardiotrophin-1.

Accumulating evidence suggests that a cellular scar is better than a hypocellular scar. The "cellular cardiomyoplasty" literature has shown that irregardless of the cell type that is placed in the scar, ventricular performance is improved, even though these cells may not contribute to synchronous ventricular contraction [259]. Expression of CT-1 in the infarct zone is likely beneficial given the potent induction of cell proliferation and migration. In so doing, CT-1 acts to induce proliferation of and migration of fibroblasts from adjacent viable myocardium thereby repopulating and maintaining the cellularity of the scar. Additionally, CT-1 may act to maintain a proliferative, migratory phenotype and oppose the actions of angiotensin and TGF-β which act to induce a quiescent, contractile, hypersynthetic

myofibroblast phenotype [8,43,69]. LIF, another member of the IL-6 family of cytokines which induces a similar signaling pattern as CT-1, has been shown to reduce collagen expression and antagonized the switch to a myofibroblast phenotype [125]. Although we did not specifically address the effect of CT-1 on cardiac fibroblast phenotype, it is likely that CT-1 has a similar effect, which again supports the notion that CT-1 has beneficial effects on cardiac fibroblast function in the post-MI heart.

The transcription factor STAT3 has been shown to induce VEGF dependent myotube formation when overexpressed [271]. Although this may represent another effect of CT-1 signaling in post MI heart, namely angiogensis, to date there is no information regarding the effect of CT-1 on endothelial cell or smooth muscle cell proliferation.

3. Role of Cardiotrophin-1 in cardiac fibroblast protein synthesis.

Our results show that CT-1 induces protein synthesis in cardiac fibroblasts through typical signaling pathways. Our interpretation of how these pathways may be integrated is shown in Figure 17. Proteins that could be induced by CT-1 stimulation include extracellular matrix proteins (collagens, fibronectin, tenascin, etc), proteins involved in regulation of cell cycle (cyclins, proliferating nuclear antigen, etc) and proteins required for cell adhesion and migration (primarily integrins) [164,169,274]. Our results have shown that the collagen synthetic stimulus of CT-1 is modest without cell number normalization and actually is associated with a reduction of collagen secretion if the normalized data are considered (Figure 18).

These findings indicate that CT-1 may not possess a significant profibrotic stimulus insofar as there is not a significant induction of fibrillar collagen synthesis. While this finding appears to be contradictory to other published data [118] the differences may be due to one of the following extenuating experimental parameters: i) the current dataset is presented in both raw and normalized forms, and it is the former subset that agrees with other published data, which also is not normalized to cell number; ii) we measured collagen synthesis by procollagen-1-carboxy propeptide (P1CP) expression in culture media, as opposed to simple incorporation of ^{3}H-proline; iii) the current data were sourced from samples of culture medium as opposed to cell lysates. It is well known that collagen synthesis is a discoordinate process in cardiac myofibroblasts and therefore changes in collagen type I mRNA abundance may not accurately reflect changes in protein secretion [275], and most importantly, collagen secretion is mediated through a complex series of steps involving extensive post-secondary modification of procollagen monomers, self-winding of the triple helices, and finally cleavage of both N- and C-terminal polypeptides. In cases whereby myofibroblasts are unstimulated or there is a deficiency of cofactors, a high proportion of collagen monomers are cycled within the cell, degraded shortly after synthesis and therefore never reach the point of secretion [188]. Thus the determination of secreted collagen is the preferred method for ascertaining net collagen synthesis in cardiac myofibroblasts, and we feel that the advantages conferred by use of the P1CP method outweigh those that may be realized by addressing collagen type I mRNA expression or incorporation of ^{3}H-proline alone. On balance, we suggest that the proteins that are generated in response

to the CT-1 stimulus are involved in induction of cell functions (such as proliferation or migration) other than synthesis of secreted ECM proteins.

4. Significance of Cardiotrophin-1 induced cardiac fibroblast cell migration.

The observation that CT-1 induces cardiac fibroblast migration coupled with infarct scar-specific expression of CT-1 suggests that it contributes to repopulation of the scar as mentioned previously. Although CT-1 does appear to have a chemotatic effect on porcine coronary artery vascular smooth muscle cells (data not shown), there is conflicting evidence for the role of IL-6 family cytokines on angiogensis. Whereas LIF may act to reduce angiogenesis *in vitro* [276], OSM induces angiogenesis *in vivo* and *in vitro* and is chemoattractant for human microvascular endothelial cells [277]. Clearly the individual effect of CT-1 on angiogenesis needs to be determined.

Induction of cell migration by CT-1 requires cytoskeletal reorganization, increased focal adhesion dynamics and increased contractile activity. We have shown that CT-1 induces myosin light chain phosphorylation, which leads to increased actin-mysoin cross-bridge cycling and contractile activity. The observation that migration was dependent on intact potassium channel function, coupled with the observation that CT-1 induces myofibroblast cell membrane hyperpolarization, indicates that the mechanism of CT-1 induced migration is similar to that observed with other non-excitable cells [106-108,278]. Hyperpolarization of the cell membrane would increase the driving force for calcium entry through non-selective

cation channels. Although our studies have not specifically addressed calcium flux, there is functional evidence that these cells express these channels [279].

CT-1 induced hyperpolarization of the cell membrane, through activation of a potassium channel, is integral to cell functions such as migration, as indicated by our data. Although we haven't specifically demonstrated an increase in intracellular calcium content, it is implied through the observation of increased calmodulin-dependent myosin light chain phosphorylation (Figure 25). Cell membrane hyperpolarization can increase intracellular calcium through an increase in the driving force for calcium flux through capacitive cation channels. This increased intracellular calcium content acts to activate contractile machinery through activation of myosin light chain kinase. This effect is required for cell function such as migration and contraction and for vesicular transport and secretion of proteins from the cell [105].

VI. CONCLUSIONS AND FUTURE DIRECTIONS.

In conclusion, we have shown that cardiotrophin-1 is expressed in the infarct zone after permanent coronary artery ligation, and that it induces migration, proliferation and protein synthesis in cardiac fibroblasts. Taken together, these findings suggest that CT-1 plays a role and is beneficial in post-MI wound healing. To confirm this, gain of function and loss of function experiments need to be done. We plan to do an experiment involving administration of recombinant CT-1 to rats after coronary artery ligation, looking for changes in mortality, infarct size, ventricular performance, cellularity and collagen deposition. A CT-1 knock-out

mouse exists [206] which would be an ideal tool for studying loss of function experiments again looking at the same endpoints. The knock-out model could also be used to determine the role of CT-1 in the accumulation of mesenchymal stem cells in the infarct zone. The hyperpolarizing effect of CT-1 on cell membrane is an intriguing observation that requires further investigation. As myofibroblasts are not only hypersynthetic, but also contractile cells that mediate contraction of the scar (scar thinning), the effect of CT-1 on cell shortening needs to be examined and compared with known mediators of myofibroblast function. It is likely that CT-1 acts to inhibit cell contraction (which is likely stimulated by mediators such as angiotensin and TGF-β).

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