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R-SMAD 2 ACTIVATION IN INFARCTED RAT HEART: CROSSTALK BETWEEN ANGIOTENSIN II AND TGF-β_1

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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LIST OF ABBREVIATIONS

ACE	angiotensin converting enzyme
Ad DN-TβRII	adenoviral dominant-negative TGF-β type II receptor
Ad β-Gal	adenoviral β-Galactosidase expression control
Angiotensin	angiotensin II
AT_1 receptor	angiotensin II type I receptor
AT_2 receptor	angiotensin II type II receptor
BCA	bicinchoninic acid
BMP	bone morphogenic protein
CAD	coronary heart disease
cAMP	cyclic AMP
CHF	congestive heart failure
CMP	cardiomyopathy
Co-Smad	common Smad
CREB	cAMP response element binding protein
±dP/dtmax	the maximum rate of isovolumic pressure development or decay
ECM	extracellular matrix
ELISA	enzyme-linked immunoabsorbant assay
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ET	endothelin
FAST	fork head activin signal transducer

GAG	glycosaminoglycan
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
HRP	horseradish peroxidase
IL-1α	interleukin-1α
INF-γ	interferon-γ
JNK	c-jun NH2-terminal kinase
LAP	latency associated peptide
Los	Losartan
LTBP	latent TGF-β-binding protein
LV	left ventricular/left ventricle
LVEDP	left ventricular end-diastolic pressure
LVSP	left ventricular systolic pressure
β-MHC	β-myosin heavy chain
MAPK	mitogen activated protein kinase
MI	myocardial infarction
MMP	matrix metalloproteinase
MOPS	3-[N-morpholino] propanesulfonic acid
MOI	multiplicity of infection
PBS	phosphate-buffered saline
PVDF	polyvinylidene difluoride
PKC	protein kinase C
RAAS	renin angiotensin aldosterone system
R-Smad	regulatory Smad

TBS-T	tris-buffered saline with 0.2% Tween-20
SARA	smad anchor for receptor activation
α-SMA	α-smooth muscle actin
SMM	smooth muscle myosin
TβR-I	transforming growth factor-β receptor type I
TβR-II	transforming growth factor-β receptor type II
TGF-β	transforming growth factor-β
TGIF	TG-interacting factor
TIMP	tissue inhibitor of matrix metalloproteinase
t-PA	tissue plasminogen activator
VSMC	vascular smooth muscle cell

ABSTRACT

After a large myocardial infarction (MI), ventricular chamber dilatation and sphericalization are attended by cardiac hypertrophy and interstitial fibrosis, leading to the loss of normal cardiac function. We have previously shown that nonmyocytes present in the healed 8-week infarct scar overexpress transduction proteins required for initiating the elevated deposition of structural matrix proteins in this tissue. Other studies indicate that TGF-β_1 may be involved in cardiac fibrosis and myocyte hypertrophy. Despite the existence of this evidence, the significance of altered TGF-β signaling in heart failure in the chronic phase of post-myocardial infarction (MI), particularly in the ongoing remodeling of the infarct scar, is unclear. Patterns of cardiac TGF-β_1 and Smad 2, 3, and 4 protein expression were investigated 8 weeks after MI and were correlated to relative collagen deposition in border tissues (containing remnant myocytes) and the infarct scar (nonmyocytes). Both TGF-β_1 mRNA abundance and protein levels were significantly increased in the infarct scar *vs.* control values and this trend was positively correlated to increased collagen type I expression. Cardiac Smad 2, 3 and 4 proteins were significantly increased in border and scar tissues *vs.* control values. Immunofluorescence studies indicated that Smad proteins localized proximal to the cellular nuclei present in the infarct scar. Decorin mRNA abundance was elevated in border and infarct scar and the pattern of decorin immunostaining was markedly altered in remote remnant heart and scar vs. staining patterns of control sections. TβRI (53 kDa) protein expression was significantly reduced in the scar, while the 75 kDa and 110 kDa isoforms of TβRII were

unchanged and significantly increased in scar, respectively. Using double immunofluorescent analyses, we identified that the major cell type populating the 8 week infarct scar was myofibroblasts. These results indicate that TGF-β_1/Smad signaling may be involved in the remodeling of the infarct scar after the completion of wound healing *per se*, via ongoing stimulation of matrix deposition. Cardiac myofibroblasts may play important roles in scar remodeling as well as cardiac hypertrophy and fibrosis of the surviving tissue in post-MI rat heart. As it is known that angiotensin II (angiotensin) and transforming growth factor -β_1 (TGF-β_1) play an important role in cardiac fibrosis and infarct scar remodeling after myocardial infarction (MI), we further characterized 8 week post-MI rat hearts for altered expression of Smad proteins with and without losartan treatment. AT_1 blockade was associated with attenuated activation of the latent form of TGF-β_1 in remnant (viable) myocardium and infarct scar. Immunofluorescence (IF) studies revealed Smad 2 localization to myofibroblasts in target tissues (many intensely stained cells in the infarct scar) with less intense staining in cardiac myocytes. The pattern of relatively intense staining in the remnant myocardium was limited to the interstitial space. Cardiac myocytes stained weakly in the remnant heart, and these cells are the predominant feature of this tissue. Losartan administration (15 mg/kg/day) for 8 weeks was associated with normalization of total cellular Smad 2 and Smad 4 overexpression in the infarct scar as well as Smad 2 overexpression in remnant heart tissue. On the other hand, phosphorylated Smad 2 (P-Smad 2) staining was reduced in cytosolic fractions from failing experimental heart tissues *vs.* controls and these trends were normalized in the presence of losartan, suggesting augmented P-Smad 2 movement into (myo)fibroblasts nuclei in untreated hearts. Using cultured adult primary rat

fibroblasts treated with 10^{-6} M angiotensin, we noted rapid translocation (15 min) of P-Smad 2 into the cellular nuclei from the cytosol. Nuclear P-Smad 2 protein levels were increased in cultured fibroblasts following 15 min angiotensin treatment, and this response was blocked by losartan treatment. We conclude that angiotensin may influence total Smad 2 and 4 expression in post-MI heart failure, and that angiotensin treatment is associated with rapid P-Smad 2 nuclear translocation in isolated fibroblasts. The results of this study indicate that crosstalk between angiotensin and Smad signaling is associated with fibrotic events in post-MI hearts.

To determine whether angiotensin stimulation alone is sufficient to stimulate Smad 2 phosphorylation/transfection, we blocked TGF-β_1 signaling in cultured cardiac fibroblasts using TGF-β neutralizing antibody (1.5 μg/ml) in cultured cardiac fibroblasts. We found that both angiotensin and TGF-β_1 (10 ng/ml) increased the accumulation of phosphorylated Smad 2 in the nuclei in fibroblasts. This effect of TGF-β_1 was abrogated in the presence of TGF-β neutralizing peptide. However, the effect of angiotensin on Smad 2 activation was not blocked in the presence of TGF-β neutralizing peptide.

Adenoviral dominant-negative TGF-β type II receptor (ADvDNTβRII) was administered to cultured cardiac fibroblasts [Multiplicity Of Infection (MOI) = 25]. Immunoreactive P-Smad 2 localization was examined using immunofluorescent staining. ADvDNTβRII–infected fibroblasts showed similar Smad 2 phosphorylation/translocation responses to angiotensin stimulation (10^{-6} M for 15 min) as controls treated with Adenovirus expressing β-gal receptor gene (MOI=25), suggesting a direct effect of angiotensin on Smad 2 activation. Thus angiotensin activates Smad 2 in cardiac fibroblasts through a TGF-β_1 ligand-independent pathway.

Our studies indicate that R-Smad 2 is activated in post-MI rat hearts and may specifically mediate both angiotensin and TGF-β_1 signaling, which may be involved in the pathogenesis of cardiac fibrosis and subsequent heart failure. Modulation of Smad 2 mediated signaling may provide a therapeutic target for the prevention of cardiac fibrosis and heart failure and further investigation of this possibility is warranted in this regard.

Chapter 1. INTRODUCTION AND STATEMENT OF THE PROBLEM

After left ventricular (LV) myocardial infarction (MI), the infarcted myocardium undergoes a repair process, referred to as cardiac fibrosis. This general phenomenon includes the occurrence of fibroblast proliferation and concomitant deposition of extracellular matrix (1-3). Fibrosis occurs not only in the necrotic tissues (i.e., zone of infarction), but also in remnant cardiac tissues (non-infarcted LV region) and in right ventricle (RV) of post-MI hearts. It has been well documented that cardiac collagen is an important determining factor for passive cardiac stiffness and that excessive collagen accumulation in the otherwise normal cardiac interstitium may contribute to increased myocardial stiffness (4-7). The accumulation of interstitial collagen also leads to disruption of electrical coupling among myocytes, reduced capillary density and increased diffusion distance for oxygen and carbon dioxide (CO_2), which in turn may lead to an increase of metabolic stress or even overt ischemia with increased incidence of myocyte apoptosis (8-10). Thus, the occurrence of cardiac fibrosis in remnant myocardium after MI may result in an impairment of cardiac function leading to the development of congestive heart failure. However, the underlying mechanisms for the pathogenesis of cardiac fibrosis remain unclear to date. Increasing evidence has suggested that the pleiotropic angiotensin II (angiotensin) and transforming growth factor (TGF)-β_1 play crucial roles in the progression of cardiac fibrosis during developing cardiac hypertrophy and heart failure (11-15). TGF-β_1 is a powerful initiator for the production of collagens and other major extracellular matrix (ECM) components in a variety of cell types (16). Increased expression of TGF-β_1 has been noted in the myocardium during pressure overload-induced hypertrophy (17) and early after

myocardial infarction (18). Conversely, other evidence suggesting that angiotensin is associated with cardiac fibrosis in different models of heart failure including myocardial infarction exists (15,19-21). Chronic administration of an ACE inhibitor or an AT_1 receptor antagonist, significantly attenuates fibrosis in both infarcted and non-infarcted rat myocardium (12,14,15,22). Nevertheless, the precise signaling pathways and in particular the post-receptor mechanism of TGF-β and angiotensin in post-MI heart as well as the relationship between these two cytokines is far from clear. Very recently, Smad 2 has been identified as the downstream effectors of TGF-β_1 (23,24). Receptor-activated Smad 2 dimerizes with Smad 4 upon phosphorylation of tyrosine residues on the Smad 2 C-terminal region (24,25). The phosphorylated Smad 2-Smad 4 dimer then translocates to the nucleus and initiates gene transcription (24,26) by association with eukaryotic nuclear transcription factors via their specific binding to Smad 2 (24,26). Thus the phosphorylation of Smad 2 and its subsequent translocation to the nucleus may be the critical steps in modulation of signaling by this pathway in cardiac (myo)fibroblasts. However, little information is available regarding the TGF-β_1 signaling during development of post-MI heart failure. There is little information dealing with reports on the expression of Smad proteins in normal or failing heart of any etiologies. Our working hypotheses are that: 1). Smad 2 mediates TGF-β_1 and angiotensin-induced cardiac fibrosis, 2). blockade or inhibition of the phosphorylation or translocation of Smad 2 can attenuate the post-MI fibrosis syndrome and alter the development of heart failure, and 3). angiotensin may stimulate Smad 2 independently of TGF-β_1 receptors activation.

Chapter 2. LITERATURE REVIEW

2.1 Cardiac Fibrosis Post-Myocardial Infarction

After myocardial infarction (MI), the myocardium undergoes a repair process involving scar formation at the site of infarction that includes fibroblast and myofibroblast proliferation and concomitant deposition of extracellular matrix proteins (3). During the early phase of MI, activation of these processes is critical for normal wound healing in the infarcted region. However, eventual interstitial fibrosis also occurs in remnant tissue and acts to increase myocardial stiffness. Further expansion of the extracellular matrix impairs diastolic stiffness and compromises systolic mechanics contributing to subsequent cardiac hypertrophy and heart failure (27,28). Thus, the investigation of mechanism(s) underlying post-MI cardiac fibrosis has attracted considerable attention in recent years.

2.2 Collagen Remodeling and Heart Failure after Myocardial Infarction

The remodeling of non-infarcted cardiac tissue includes the appearance of ventricular hypertrophy, interstitial fibrosis and loss of normal ventricular geometry followed by the development of heart failure. The role of collagen in the remodeling of both scar and remnant cardiac tissues after MI has been increasingly recognized as an important factor in heart failure during recent years (3,29).

2.3 Cardiac Myofibroblast Activation

After the initial inflammatory cell response, fibroblasts arrive at the site of repair where they undergo phenotypic transformation to myofibroblasts (30). Myofibroblasts have extensive rough endoplasmic reticule and Gulgi apparatus characteristic of fibroblasts. In contrast to fibroblast, these cells express α-smooth muscle actin (α-SMA), which resemble the myofibrils of smooth muscle cells. Fibroblasts have an extensive clonal heterogeneity and phenotypically transformed fibroblast-like cell populations, having broad-ranged functional diversity, including their ability to promote fibrous tissue contraction. Myofibroblasts proliferate and express cytokine and other peptide receptors essential to their subsequent behavior (30).

The presence of myofibroblasts in actively contracting granulation tissue and hypertrophic scars and their morphologic characteristics have led to the proposal that the myofibroblast is the cellular agent responsible for tissue contraction (31,32). Myofibroblast contraction governs matrix remodeling, including scar thinning(33). The presence of myofibroblasts in myocardial granulation tissue following an infarct has been shown to persist for a long time (34,35). Cardiac myofibroblasts are abundant at the site of cardiac tissue repair, are known to synthesize fibrillar collagens and are major players in the formation of infarct scar structure in the post-MI heart (36). Although signals that determine the appearance of the myofibroblast phenotype are not entirely certain, it has been demonstrated that TGF-β_1 is able to induce, *in vitro,* the differentiation of adult rat cardiac fibroblasts to an angiotensin-converting enzyme containing phenotype of myofibroblasts (37). A recent study has shown that the TGF-β_1 stimulated collagen production in cultured second passage of adult rat cardiac fibroblasts is positively correlated with the appearance of α -SMA (38). TGF-β_1 increases the collagen

production and stimulates the differentiation of fibroblasts to myofibroblasts. The maximal stimulation of collagen production with TGF-β_1 for 48 hours is accompanied by a maximal stimulation of α -SMA expression when cultures consist mainly of myofibroblasts, which have a higher activity for collagen production than fibroblasts (38). Myofibroblast also elaborate and metabolize various substances that regulate their turnover of collagen and govern fibrous contraction in an autocrine manner. These include angiotensin, endothelin-1 (ET_1), catecholamines, bradykinin, and serotonin (39). Myofibroblasts are eliminated by apoptosis when the fibrotic process is complete (32). However, at the infarct site, a population of myofibroblasts persists long after healing is complete and might contribute to an ongoing process of collagen turnover at the MI site (34,40).

2.4 Early Remodeling of Collagen After Myocardial Infarction

In the rat model of MI, total collagen content in the infarct zone is known to decrease by 25% - 50% one to three hours after the induction of MI when compared with non-infarcted myocardium (41). Results from an electron microscopic study indicate that collagen fibrils and elastic fibers are rapidly broken down in acute ischemic conditions (42). Cardiac interstitial collagenase, elastase, and cathepsin G activities are significantly increased in infarcted tissue compared with noninfarcted control values, and these findings suggest that the increased activities of collagenase and other neutral proteases may be responsible for these changes (41,43,44). The acute loss of cardiac matrix may lead to myocyte slippage in the ischemic zone causing thinning and dilatation of the necrotic region (infarct expansion) and even rupture of the myocardium (4,45). Infarct

expansion may impair heart function via an increase of ventricular volume beyond optimal geometry in the early post-MI phase. This rapid dilatation thereby reduces the contractile efficiency of the remnant myocardium by invoking time-dependent secondary changes in the noninfarcted tissue (2,27).

2.5 The Chronic Phase of Collagen Matrix Remodeling After Myocardial Infarction

The heart has a three-dimensional extracellular fibrillar collagen scaffolding that normally serves a variety of functions important to tissue integrity and efficiency of muscular systolic pump and diastolic suction pump function (46,47). An adverse accumulation of extracellular matrix structural protein compromises tissue stiffness and adversely affects myocardial viscoelasticity leading to ventricular diastolic and systolic dysfunction. Hormonal factors, such as chronic inappropriate (relative to dietary salt intake and intravascular volume) elevations in circulating angiotensin II and aldosterone, are accompanied by fibrosis of right and left sides of the heart. Hemodynamic factors regulate cardiac myocyte work and their adaptive hypertrophic growth. The relative contributions of hormonal and hemodynamic factors in regulating growth of muscular and nonmuscular compartments must form the basis for the selection of pharmacologic intervention that will optimize the management of symptomatic heart failure that accompanies hypertensive heart disease and ischemic cardiomyopathy (CMP) . Cardioprotective strategies that prevent alteration of normal cardiac tissue structure by fibrosis and appearance of abnormal ventricular stiffness (viscoelasticity) are based on

negating the generation of these hormones or interfering with their receptor-ligand binding. A regression of established cardiac fibrosis and an improvement in abnormal ventricular stiffness is feasible. Experimental and clinical findings with lisinopril in hypertensive heart disease, where cardiac fibrosis and abnormal ventricular stiffness are present, indicate that such cardioreparation should be a targeted objective of pharmacologic intervention. Systematic analysis of this approach using a controlled clinical trial format is warranted. In recognizing the importance of viscoelastic elements in regulating the mechanical behavior of cardiac tissue and in turn systolic and diastolic ventricular function, a broader tissue compartment based paradigm (ECM versus myocyte) for the management of heart failure emerges (48).

Cardiac interstitial fibrosis, present in the chronic phase of MI, is recognized as a marker of irreversible cardiac hypertrophy and heart failure in post-MI hearts (49). Collagen deposition in the infarct zone is progressively increased from one to six weeks after the induction of MI (50). Cardiac collagen is also found to be increased in the myocardium from patients with coronary artery disease (51,52). Results from our laboratory have indicated that the total collagen concentration in remnant LV and RV, as determined by 4-hydroxyproline measurement, was increased at 2, 4, and 8 weeks after MI (53). Moreover, others have shown that the mRNA abundances of collagen types I, III and IV, as well as fibronectin are all increased in remnant myocardium after MI (43,54). It is known that the stability and functionality of collagen are dependent not only on the total amount of collagen but also on the degree of covalent cross-linkage and organization among fibrils. A recent description of the degree of cross-linking of collagen fibrils as assessed by hydroxylysylpyridinoline (HP) assay, indicates that cross-

linkage was increased in remnant free wall, but was unchanged in cardiac septum after infarction (55). HP is the major lysine aldehyde-derived, non-reducible collagen cross-link in myocardium and the concentration of trivalent HP is directly proportional to the tensile strength of constitutive collagen (56). It has been well documented that cardiac collagen is an important determining factor influencing cardiac muscle passive stiffness and that excessive collagen accumulation may contribute to abnormal (increased) myocardial stiffness (4-7). Further work has revealed that increased myocardial stiffness may also be a consequence of an enhanced collagen cross-linking (57). Increased stiffness was found in papillary muscle from post-MI hearts and depression of normal cardiac contractility was associated with an increase in myocardial collagen content (58). The accumulation of collagen proteins may also lead to morphologic and functional separation of myocytes (8). Enlarged or expanded interstitium due to excessive deposition of collagen results in the inhibition of electrical coupling of these myocytes, as well as, increased diffusion distance for oxygen and all metabolic substrates (8,9). Apoptotic cardiocytes have been noted to be localized in collagen-encased myocytes bordering the infarct scar (10). This suggests that cardiac fibrosis may contribute to the development of heart failure by the loss of myocytes via the creation of conditions that favour myocyte apoptosis (10). Thus, cardiac fibrosis *per se* in post-MI hearts may contribute to the development of CHF in this primary, as well as several secondary (aforementioned) mechanisms.

Recent studies have shown that MMP-1, MMP-2 and TIMP-1 are co-expressed in heart tissue (43). The expression of these proteins in myocardium has been localized to both cardiac fibroblasts and endothelial cells (59). The activation of a number of MMPs

has been hypothesized to play a role in matrix remodeling in both coronary artery disease and idiopathic dilated cardiomyopathy in humans (60-62). Increased MMP activities and a steady-state shift in the rate of removal of fibrillar collagens for the supportive matrix structures has been suggested to cause myocyte slippage and misalignment within the myocardium, thus, resulting in abnormalities in contractile force production (63). This data support the hypothesis that abnormal turnover of collagen mediated via increased MMP activities leads to the development of heart failure.

2.6 TGF-β IN THE MYOCARDIUM

TGF-β_1 has been shown to control diverse cellular events such as development, differentiation, tissue repair, tumorigenesis, and many immune and endocrine functions (64). TGF-β proteins (i.e., TGF-$\beta_{1, 2 \text{ and } 3}$) are secreted by most mammalian cells and their actions are locally mediated through autocrine/paracrine release (65,66). It has been demonstrated that cardiac myocytes, endothelial cells, fibroblasts and myofibroblasts may generate TGF-β_1 (65). However, the expression of cardiac TGF-β_1 is mainly localized to cardiac fibroblasts (65,67). Typically, any given cell secretes TGF-β in biologically inactive or latent form, as it is normally bound to latency- associated peptide (LAP). Latent TGF-β can be activated through dissociation from LAP by heating, cell-cell interactions(68) and proteolytic treatments including extreme pH, urea, sodium dodecyl sulfate (SDS), or exposure to plasmin, cathepsin D, and glycosidases (69). Despite this information, the mode of physiological activation is as yet unclear. It is known that all active TGF-β isoforms are covalently linked homodimers of ~12.5 kDa subunit proteins. The 12.5 kDa band can be visualized in the presence of strong reducing

conditions with specific antibodies (70). Once released from the latent complex, active TGF-β binds to components of the extracellular matrix (ECM or matrix) and accumulates in the cardiac interstitium. This action may serve to protect TGF-β from degradation and may function as a long-term source of this cytokine, also subserving normal turnover and as a sink, ready for release in the occurrence of an exceptional event i.e., tissue damage and wound healing. Decorin is yet another protein that binds TGF-β_1 and in doing so may neutralize its activity. Some current investigation is ongoing to address potential therapeutic avenues in this regard (71,72). For example, it has been hypothesized that overexpression of decorin may lead to a marked inhibition of TGF-β_1- induced fibrosis (71,72).

2.7 TGF-β Receptors in the Myocardium

Affinity labeling technology has been applied to identify three major TGF-β receptors present in most mammalian cell types and they includ type I (TβRI, mass 53 kDa), type II (TβRII, mass 70-100 kDa) and type III (a relatively large betaglycan of 200-400 kDa) (16). These receptors have been identified in both cardiac myocytes and cardiac (myo)fibroblasts (73,74). Cardiac myocytes may have ~ 2000 TβRI binding sites and ~5000 TβRII binding sites per cell (75,76). The dogma surrounding our current understanding of TGF-β_1 receptor function is that this is mediated through transmembrane TβRI and TβRII receptors, which normally display serine/threonine kinase activity. TGF-β receptor activation is initiated upon the binding of TGF-β to TβRII, which then recruits and phosphorylates TβRI (26). Although these early receptor events were initially characterized using epithelial cells, it has been shown that receptor

dimerization and subsequent phosphorylation of TβRI in TGF-β signaling also exists in cardiac myocytes and fibroblasts (77,78).

It has been shown that overexpression of TβRIII results in increased responsiveness of vascular endothelial cells and this was interpreted to suggest that TβRIII may facilitate TGF- β_1 binding (79). Despite this report, a complete understanding of the function of TβRIII is far from clear and requires further investigation.

2.8 SMAD PROTEINS

Recent results have provided an indication that Smad proteins serve as important post-receptor TGF-β signal transducing components (80,81). The phosphorylated (activated) TβRI then phosphorylates in turn, the downstream target R-Smad 2 (or R-Smad 3) carboxy-terminal serine residues. Specifically, this has been identified as the SSXS motif (24,24,80). Phosphorylated Smad 2 (and/or Smad 3) then form(s) a heteromeric complex with Co-Smad 4 and this protein bundle rapidly translocates (within minutes) and accumulates in the cellular nucleus (24,25,82,83). In the nucleus, R-Smad 2/Co-Smad4 complexes then regulate cellular transcriptional responses by specifically interacting with DNA-binding proteins. Each cell type expresses a specific complement of these DNA-binding proteins known as Smad coactivators and corepressors (84).

In addition, Smad complexes may directly bind promotor regions in specific Smad binding elements (SBE's) of many different genes, and some work has indicated a direct activation of specific genes in this mode (85,86). The role that direct binding Smad DNA plays in cellular physiology is controversial, but much new research points to

the reliance of the Smad signal on coactivator and corepressor involvement. Recent studies indicate that TGF-β_1 signaling may be inhibited by other Smad proteins such as I-Smad 6 and I-Smad 7 (70,87-89).

2.9 The TGF-β Signaling Pathway

TGF-β_1 signaling is involved in two major membrane receptor kinases, type I TGF-β receptor (TβRI) and type II TGF-β receptor (TβRII), and a family of receptor substrates the Smad proteins (64). To initiate the TGF-β signaling cascade, two different transmembrane protein serine / threonine kinases, known as TGF-β_1 receptor types I and II are brought together by the ligand, which effectively acts as a receptor assembly factor. In the ligand-induced complex, the type I receptor kinase is activated by the phosphorylation of its GS region by receptor type II. The type I receptors specifically recognize the Smad subgroup known as receptor-activated Smads (R-Smads, Smad 2 and Smad 3) (64), which are recognized by TGF-β receptors. The R-Smads consist of two conserved domains that form globular structures separated by a linker region (90,91). The N-terminal MH1 domain has DNA-binding activity whereas the C-terminal MH2 domain drives translocation (upon phosphorylation, which equates to the activation step) into the nucleus and possesses transcriptional regulatory activity. Receptor-mediated phosphorylation of the C-terminal SSXS motif appears to relieve these two domains from a mutually inhibitory interaction and leads to R-Smad activation. Once activated, the R-Smad then binds to a common-mediator Smad (Co-Smad, Smad 4 in TGF-β signaling) and this complex translocates to the nucleus (92). In the nucleus, it appears that Smad proteins can interact with a variety of nuclear factors that may act as DNA-binding

proteins, transcriptional co-activators or co-repressors. In fact, a general underlying hypothesis to explain the pleuripotentiality of TGF-β_1 is embodied in the suggestion that the type of responsiveness of a given cell is determined by its precise complement of cofactors and corepressors. Fast 2 (i.e., FOXH1) is one such DNA-binding partner that associates with the Smad proteins in the nucleus, allowing the entire complex to assemble on a sequence-specific DNA element to strongly activate transcription (93,94). Recent work from our laboratory indicates the presence and modulated expression of FOXH1 in cardiac fibroblasts and that it may help to bind Smad proteins to the promoter region of the collagen type I gene (Roth and Dixon, personal communication, unpublished data). Further enhancement of Smad-mediated transcription occurs through the direct interaction of CBP/P300 with the activated R-Smad, an interaction that is enhanced by TGF-β-induced phosphorylation (95). Smads can also bind transcription repressors such as TGIF, c-Ski or SnoN to inhibit or down-regulate the transcription of target genes (96,97). Thus, the R-Smad-Co-Smad complex may be able to regulate transcription in a positive or negative manner, depending on the interacting partners. In this sense, the mix of Smad partners and regulators present in a given cell at the time of TGF-β stimulation may decide the ultimate cellular response.

In addition to R-Smads and Co-Smads, which carry signals from receptors to the nucleus, a third group of Smads called inhibitory Smads (I-Smads), including Smad 6 and Smad 7 may abrogate TGF-β signal transduction. Smad 7 inhibits Smad phosphorylation by occupying TGF-β type I receptors (98). The expression of both I-Smad 6 and I-Smad 7 are increased in response to TGF-β, supporting the existence of roles in negative feedback of these pathways (99). The expression of Smad 7 can also be regulated by

other cytokines such as interferon-γ (100). Interferon-γ stimulates the expression of Smad 7 through Jak 1 tyrosine and the Stat 1 transcription factor and thus exerts an inhibitory effect on TGF-β signaling (100).

2.10 TGF-β IN CARDIAC FIBROSIS, MYOCYTE HYPERTROPHY, AND HEART FAILURE

TGF-β_1 is a powerful initiator for the synthesis of collagen and other major extracellular matrix (ECM) components in a variety of cell types (101). The expression of TGF-β_1 is increased in the myocardium during pressure overload-induced hypertrophy (102) and early after myocardial infarction (103). We have observed activation of TGF-β_1 and the increased expression of novel downstream Smad 2 and Smad 4 proteins in infarct scar and remnant myocardium during the chronic phase of MI (104). These events were positively correlated to ongoing cardiac fibrosis in remnant tissues as well as scar remodeling in post-MI heart, which is modulated exclusively by cardiac fibroblasts and myofibroblasts (104,105). Eghbali *et al.,* found that the mRNA for TGF- β_1 could be detected only in the nonmyocyte fraction of heart cells (106).

In a variety of cell types TGF-β_1 is known to stimulate matrix component protein synthesis, including the synthesis of fibrillar collagen species. It has been demonstrated that TGF-β1 is involved in many fibrotic disorders including glomerulonephritis, cirrhosis, lung fibrosis and vascular restenosis (107). As a potent stimulus for collagen synthesis, this cytokine may be important for the induction and development of cardiac fibrosis (108). In adult rat cardiac fibroblasts, TGF-β_1 induces a dose-dependent increase in collagen production and secretion from 0.3 to 15 ng/ml (109).

The binding density of TGF-β_1 receptors are markedly increased in both infarcted and noninfarcted myocardium and this increase localizes within microdomains that are characterized by the appearance of abnormal matrix deposition (110). Furthermore, it has been suggested that TGF-β regulates not only cardiac collagen synthesis, but also collagen degradation by MMP (111). TGF-β_1 receptor activation in cultured human cardiac fibroblasts has been shown to elevate the matrix protein deposition by decreasing via inhibition of MMP-1 activity and/or by stimulating TIMP expression in cultured cells (111-113). In addition to the direct effects of TGF-β superfamily ligands on MMPs and TIMP expression and function, TGF-β_1 may regulate the activation of MMPs through the inhibition of t-PA and stimulation of PA inhibitor (PAI-1) gene expression (114). In summary, abnormal elevation of TGF-β is associated with elevated synthesis of matrix proteins by fibroblasts and at the same time, it may inhibit its degradation via MMP-1 modulation thereby increasing the matrix protein production.

In addition to effects on fibroblasts and matrix protein deposition in the heart, TGF-β_1 is known to alter myocardial gene expression in cardiac myocytes. For example, in cultured neonatal cardiac myocytes this ligand is associated with the induction of β-myosin heavy chain (β-MHC), skeletal α-actin genes, smooth muscle α-actin, atrial natriuretic peptide (ANP) as well as downregulation of α-MHC and sarcoplasmic reticular Ca^{2+} ATPase (SERCA2) mRNAs (65). These alterations mimic the changes that characterize usual events in the development of cardiac hypertrophy and failure (65) and these findings underscore the importance of this cytokine in the development of cardiac hypertrophy and failure. Work by Long *et al.*, have demonstrated that TGF-β_1, TGF-β_2 and TGF-β_3 may all stimulate protein synthesis in cultured neonatal cardiac myocytes

during the absence of other mitogens and under serum-free conditions (115,116). Despite this finding, some controversy exists with respect to myocyte responsiveness. For example, Parker *et al.*, have reported that TGF-β_1 had no effect on the growth of neonatal cultured cardiac myocytes (117). It has been pointed out that the developmental age of cells is critical to TGF-β responsiveness and that adult cells may respond differently than the characteristic response observed in neonatal cells (118). Despite difficulties in comparing different data sets using different cell types, the burden of evidence seems to indicate a major role for TGF-β in the pathogenesis of heart failure. Furthermore, this cytokine may act in concert with other known cardiac trophic factors. For example, increased total protein synthesis in adult myocytes induced by isoproterenol was abolished by the application of TGF-β_1 neutralizing antibody, while the administration of TGF-β_1 was associated with restoration of this hypertrophic response (119). Although TGF-β does not induce c-fos expression in myocytes, it is known to potentiate norepinephrine and stretch-induced c-fos expression and protein synthesis in this cell (120). On the other hand, the *in vivo* effects of TGF-β_1 on cardiac myocytes are less clearly defined in the literature (121-123). Increased cardiac expression of TGF-β mRNA is associated with the development of cardiac hypertrophy in a variety of experimental models (122-124). As alluded to in the preceding discussion, TGF-β_1 is implicated in myocardial remodeling *via* autocrine and/or paracrine mechanisms and that both cardiac myocytes and fibroblasts participate in this remodeling. Recent studies have indicated that TGF-β_1 protein is increased in the myocardium from chronically pressure-overloaded experimental rat hearts and human idiopathic cardiomyopathy (17,125). These data support the hypothesis that TGF-β may be involved in the development of

cardiac hypertrophy. A recent study has shown that TGF-β_1 modulates enhanced iNOS expression and phosphorylation of Akt/protein kinase B in rat myocytes exposed to hypoxia-reoxygenation (126). The latter finding is of some significance as Akt/protein kinase B are downstream of phosphatidyl inositol (PI) 3-kinase and are involved in many cellular processes, including proliferation and apoptosis (127). In this regard, it is known that supplementation with exogenous TGF-β_1 can protect the heart from hypoxia-reoxgygenation injury under controlled conditions (128,129). *In vivo* genetic induction of TGF-β in the lungs resulted in prolonged and severe interstitial and pleural fibrosis characterized by extensive deposition of the extracellular matrix (ECM) proteins collagen, fibronectin, and elastin and by the emergence of cells with the myofibroblast phenotype (130). Expression of TGF-β_1 in normal arteries resulted in substantial extracellular matrix production accompanied by intimal and medial hyperplasia. Increased procollagen, collagen and proteoglycan synthesis in the neointima was also noted (131). A major difficulty in the propagation of TGF-β_1 knockout mice is the occurrence of early inflammatory events in most parenchymal tissues (132,133) including the heart, this in turn culminates in death at approximately 3 weeks post-partum (132,133). On the other hand, effective "knock-in" approaches have been made available through the development of some elegant molecular strategies. For example, targeted expression of a human TGF-β_1 cDNA that harbors a cysteine-to-serine substitution at amino acid residue 33 of the LAP markedly increased active TGF-β_1 levels in adult transgenic hearts (134).

2.11 The Renin-Angiotensin System (RAS) in the Cardiovascular System

The cardiac RAS components include renin, angiotensinogen, angiotensin converting enzyme (ACE) as well as angiotensin I (Ang I) and angiotensin II (angiotensin). Classically, research led to a relatively early understanding that the liver is a major source of angiotensinogen which is a large plasma protein and the only known natural substrate of renin (produced by the juxtaglomerular apparatus of the kidney). Renin cleaves angiotensinogen to release the Ang I decapeptide and this inactive precursor is immediately converted to active angiotensin (an octapeptide) by ACE. ACE is a well-characterized protein and is widely acknowledged to be synthesized by endothelial cells lining the vascular system. Angiotensin also may be produced by an ACE-independent pathway in cardiovascular system. For example, angiotensin can be released by direct cleavage of angiotensinogen and AI by tissue plasminogen activator (t-PA), cathepsin G, tonin and elastase in the vessel wall or from AI by a cardiac chymase (135). During the past several years, the existence of a local or tissue RAS system in the cardiovascular system has been acknowledged and has gained considerable attention. This finding is supported by evidence indicating that most of the components of the RAS are synthesized in cardiovascular tissues. However, the question as to whether renin is synthesized by cardiac and extra-renal vascular tissues remains controversial (136). Local cardiac RAS is defined by the ability of the heart to express most RAS components leading to generation of angiotensin (137). It has since been demonstrated that angiotensin is generated and released by cardiac myocytes and cardiac fibroblasts (138,139). As the heart may use circulating renin for *in situ* synthesis of angiotensin, cardiac synthesis of this component may not be crucial for local generation of angiotensin (140). The observation that Ang I and angiotensin levels are greater than 100-fold higher

in interstitial fluid than in plasma strongly supports the hypothesis emphasizing the role of local production in cardiac myocytes and fibroblasts (141). Unlike the half-life of ^{125}I-Angiotensin in the myocardium *in vivo*, which is approximately 15 minutes, the $t_{1/2}$ of angiotensin in the circulation is only 30 seconds (142). Thus, the accumulation and compartmentalization of angiotensin in the myocardium can be seen to prevent its rapid degradation. Whether generated locally or not, angiotensin may influence the myocardium in both a direct and indirect manner. The direct actions of angiotensin on the cardiovascular system include potent vasoconstriction, positive cardiac inotropism and positive cardiac chronotropism (143). Indirect actions of angiotensin on the heart include increasing cardiac load due to activation of the sympathetic system and stimulation of aldosterone synthesis (143).

2.12 Angiotensin Receptors in Myocardium

Cellular responses induced by angiotensin are dependent upon the balanced activation of different angiotensin receptors. Biochemical, pharmacological and functional studies have revealed the presence of two main subgroups which are further divided into multiple receptor subtypes (144). Studies on binding affinities for plasma membrane receptors to nonpeptide antagonists such as losartan and PD123177 have defined the existence of AT_1 and AT_2 receptors, respectively (145-147). To date, the majority of known physiological functions mediated by angiotensin within the cardiovascular system are carried out by angiotensin binding to the AT_1 receptor (148), a "typical" seven transmembrane domain membrane receptor protein. The AT_1 group of angiotensin receptors is further subdivided into AT_{1A} and AT_{1B} classes (144). Among

them the AT_{1A} and AT_{1B} isoforms contain 18-22 different amino acids and yet maintain similar binding profiles for angiotensin and nonpeptide antagonists (including losartan) as well as peptide AT_1 receptor antagonists (144,148). The AT_{1A} subtype is localized mainly in vascular smooth muscle cells, hypothalamic tissue, lung, kidney, and adrenal tissues (144,149). In the cardiovascular system, the AT_{1A} receptor is constitutively expressed in all developmental stages (150). The AT_{1B} receptor has been noted to localize in the zona glomerulosa of the adrenal medulla, uterine, anterior pituitary and renal tissues (144,149). Recent work has shown that the AT_{1B} receptor contributes to the regulation of blood pressure in AT_{1A} receptor-deficient mice (151). In a rat heart, both AT_1 and AT_2 receptors density are roughly equal based on binding assay (152). On the other hand, the ratio of AT_1 to AT_2 receptors in human heart (including myocytes and nonmyocytes) remains controversial (153). In general, both neonatal and adult cardiac fibroblasts are characterized by the predominant presence of AT_1 receptor with very low levels of the AT_2 receptor (154,155).

The function of angiotensin type II (AT_2) receptors is unclear but is an area of intense investigation in the pathogenesis of cardiovascular disease (156). It has been noted that transgenic mice overexpressing AT_2 in a heart subjected to angiotensin infusion for a period of 28 days failed to induce cardiomyocyte apoptosis (157). Thus, it would appear that AT_2 receptors have little influence over the initiation of cardiomyocyte apoptosis *in vivo* (157). In contrast, another study used transgenic mice overexpressing angiotensinogen in cardiomyocytes, the transgenic mice were characterized by cardiac hypertrophy without fibrosis and normal blood pressure. Angiotensin-converting enzyme inhibition and angiotensin II type 1 receptor blockade can prevent or normalize

ventricular hypertrophy. Surprisingly, in control mice, receptor blockade decreases tissue angiotensin II despite increased plasma levels. This suggests that angiotensin II may be protected from metabolization by binding to its receptor. Blocking of the angiotensin II type 1 receptor rather than enhanced stimulation of the angiotensin II type 2 receptor may prevent remodeling and may account for the beneficial effects of angiotensin antagonists (158). A great deal of research indicates that the clinical or experimental use of ACE inhibitors or AT_1 receptor blockers may prevent or normalize ventricular hypertrophy (159). Recent experimental evidence indicates that in normal mice subjected to AT_1 receptor blockade therapy, a decrease in tissue angiotensin levels is observed despite an increase in plasma angiotensin levels (158). This suggests that angiotensin may be protected from becoming rapidly metabolized in the plasma by binding to its receptor. Recent evidence suggests that AT_2 receptor actions oppose those of AT_1 activation (160), and overexpression of angiotensin type 2 receptor preserves left ventricular function after myocardial infarction (161), thus either blocking of the angiotensin II type 1 receptor or stimulating the angiotensin II type 2 receptor may prevent remodeling (158).

Unlike the AT_1 receptors, AT_2 receptors do not undergo ligand-mediated endocytosis upon ligand binding (156). Despite the recent intense investigation of the function of AT_2 receptor in the pathogenesis of cardiovascular disease (162,163), the precise role of this receptor in cardiac hypertrophy and heart failure is unclear. Nonetheless, some lines of evidence point to multiple putative functions in various tissues. These functions include i) mediation of apoptosis in PC12W cells (rat pheochromocytoma cell line) and R3T3 cells (mouse fibroblast cell line) (164), ii) the inhibition of cellular proliferation in coronary endothelial cells (165), iii) an

antiproliferative effect on VSMCs in neointima after vascular injury (165) and iv) maintenance of normal cardiovascular and central nervous system function (160,166,167). Thus, the observed response to angiotensin in the whole organism may represent a balanced activation of AT_1 and AT_2 receptors, and that the response of a given organ may depend on the relative level of expression of each receptor subtype.

2.13 Regulation of Angiotensin and Angiotensin Receptors

The mechanisms for release of angiotensin into the heart tissue and for the regulation of AT_1 and AT_2 receptors have been well examined during the past several years. Sadoshima *et al.,* have clearly demonstrated that mechanical stretching of cardiac myocytes induces angiotensin secretion from these cells *in vitro* and have suggested that stretch-induced release of angiotensin is an important mechanism for stimulating myocyte hypertrophy (168). In cultured neonatal cardiac myocytes, elevation of angiotensin receptor expression at transcriptional and posttranscriptional levels may also occur via mechanical stretching (169). Mechanical stretching of cultured myocytes and pressure-overload in vivo results in the upregulation of AT_1 and AT_2 mRNA as well as receptor densities (169,170). These studies strongly support the role of mechanical stretch i.e., abnormal hemodynamic cardiac loading is pivotal in the development of cardiac hypertrophy. Furthermore, in a canine model of RV chronic hypertrophy a study has revealed that in failure induced by tricuspid valve avulsion and pulmonary artery constriction, cardiac ACE, chymase, as well as AT_1 and AT_2 receptor mRNAs were subject to regulation by local mechanical stimuli (171). Several *in vitro* studies have shown that binding of angiotensin to AT_1 receptor initiates internalization of receptor-

ligand complex leading to receptor desensitization in different cell type including VSMC (156,172-174). As only ~25% of the internalized receptors are recycled to the plasma membrane (175), the degradation of an internalized receptor may be the main mechanism by which angiotensin may regulate its receptor numbers. In addition, the AT_1 receptor is regulated through inhibition of transcription, whereas the AT_2 receptor synthesis/expression is regulated mainly by decreasing mRNA stability (176).

2.14 Angiotensin in Myocyte Hypertrophy, Cardiac Fibrosis, and Heart Failure

2.14.1 Angiotensin and Myocyte Hypertrophy

Studies using neonatal cardiac myocytes have shown that angiotensin causes an increase in protein synthesis via AT_1 receptor activation in these cells and along with the induction of "early" and "late" markers for myocyte hypertrophy (168). This trophic effect was enhanced by the administration of AT_2 receptor antagonist suggesting AT_2 receptors may mediate an anti-growth effect in cardiac myocytes (177,178). Angiotensin also increases protein synthesis in isolated adult heart via AT_1 receptor activation (179). However, angiotensin-mediated enhancement of protein synthesis in unloaded perfused adult rat heart may occur without preceding protooncogene expression (179). Recent studies suggested that cardiac fibroblasts play an important role in angiotensin-induced myocyte hypertrophy (180-182). Specifically, angiotensin failed to increase protein synthesis in cultured pure neonatal myocytes. However, the addition of nonmyocytes (mainly cardiac fibroblasts) to the myocyte culture is observed to restore angiotensin-induced protein synthesis (181). Similarly, although angiotensin may stimulate protein

synthesis in adult myocytes, this stimulation was significantly inhibited when bromodeoxyuridine was added to the culture to inhibit proliferation of fibroblasts (182). Furthermore, the conditioned medium from untreated fibroblasts is seen to increase protein synthesis in both angiotensin-treated and untreated myocytes (180,182). These findings support the suggestion that the interaction between myocytes and (myo)fibroblasts for the development of myocyte hypertrophy may be triggered by angiotensin. In addition to the in vitro trophic effect of angiotensin on myocytes, some lines of evidence also support the role of angiotensin in cardiac hypertrophy in vivo. It has been shown that chronic infusion of suppressor doses of angiotensin caused ventricular hypertrophy in rats without alteration of blood pressure. This supports the hypothesis that angiotensin has a direct trophic effect on the heart (183). It is well documented that suppression of angiotensin by administration of ACE inhibition is associated with the attenuation of cardiac hypertrophy in post-MI rat heart (159,184,185). It has been postulated that the beneficial effect of ACE inhibitor in this regard may be caused in part by elevation of bradykinin level (185). As it has been shown that kinins may inhibit the interstitial accumulation of collagen but do not modulate myocyte hypertrophy after MI, the inhibitory effects of ACE on myocyte hypertrophy has been suggested to be related only to the reduction of angiotensin (185). This suggestion is supported by recent findings in studies using AT_1 receptor antagonists that effectively inhibit cardiac hypertrophy without alteration of bradykinin post-MI rat (13,186,187).

2.14.2 Angiotensin and Cardiac Fibrosis

Cardiac fibrosis occurs not only in the scar tissue but also in the remnant myocardium in post-MI heart. Recently, both *in vitro* and *in vivo* studies have suggested that angiotensin plays a crucial role in the development of cardiac fibrosis. In cardiac fibroblasts, the AT_1 receptor is known to participate in the induction of ECM protein component synthesis and gene expression mediating mitogenic responses (168,188). The results, from *in vivo* administration of angiotensin to experimental animals, have demonstrated an association with increased cardiac collagen and fibronectin via AT_1 receptor activation (54,180,189,190). Evidence from experimental studies undertaking the investigation of ACE inhibition and AT_1 blockade indicates that their use is associated with attenuation of cardiac fibroblast proliferation and deposition of cardiac collagen (186,191,192). In the post-MI rat model of heart failure, myocardial collagen concentration was significantly increased in remnant tissue at 7, 14, 21 and 35 days post-MI (191). These investigators found that this increase of cardiac collagen concentration was inhibited by captopril treatment. In another study, ramipril (ACE inhibitor) is found to inhibit cardiac fibrosis without lowering blood pressure in spontaneous hypertensive rats compared to Wistar-Kyoto control (193). These studies suggested that ACE inhibitor therapy may attenuate cardiac collagen deposition, and that this inhibitory effect is independent of afterload reduction. The molecular mechanisms responsible for the attenuation of interstitial fibrosis by ACE inhibition remain obscure. It may be argued that ACE inhibitor treatment may potentiate an increase in the concentration of bradykinin in heart by inhibition of the kininase II enzyme (i.e. ACE) (185). In this respect, chronic treatment of infarcted hearts with AT_1 receptor antagonist is effective in partial attenuation of collagen protein deposition to a level comparable to that by ACE

inhibition (194). Thus, the efficacy of ACE inhibition may lie in the ability of these agents to suppress angiotensin in the stimulation of cardiac fibroblasts and potentiate the inhibitory effect of bradykinin on collagen in post-MI hearts. Therefore, these results support the hypothesis that angiotensin may be a causal factor for the stimulation of cardiac fibrosis after MI in rat heart.

The AT_1 receptor has been shown to be highly expressed in cardiac fibroblasts and myofibroblasts which appear at the site of infarct in 1 week post-MI hearts (43,195). On the other hand, the expression of the AT_2 receptor is known to be elevated in tissue undergoing wound repair, as well as in vascular injury and cardiac hypertrophy associated with MI (165,196). However, the role of this receptor in cardiac fibrosis is not clear. An AT_1 (but not AT_2) receptor blockade is associated with attenuated cardiac collagen accumulation in post-MI heart suggesting that cardiac fibrosis is mediated by AT_1 receptor activation (187). Furthermore, AT_2 receptor antagonism is seen to abolish the attenuation of cardiac collagen deposition mediated by AT_1 receptor antagonism, indicating that the effects of AT_1 blockade are mediated by activation of the AT_2 receptor (187). Adenovirus-mediated overexpression and stimulation of the human angiotensin II type 2 receptor in porcine cardiac fibroblasts does not modulate proliferation, collagen I mRNA expression and ERK1/ERK2 activity, but inhibits protein tyrosine phosphatases. Stimulation of the overexpressed human AT_2 receptor in porcine cardiac fibroblasts inhibited tyrosine phosphatase activity but had no significant effect on fibroblast functions related to cardiac fibrosis (197). It is conceivable that possible antifibrotic AT_2 r eceptor effects are species specific and/or require the interaction between fibroblasts and cardiomyocytes, probably *via* paracrine factors, or mechanical load (197). In a study

using human cardiac fibroblasts, angiotensin treatment was associated with an increase in p38 MAP kinase activity and DNA synthesis, enhanced PAI-1 expression, upregulated TGF-β_1, as well as inhibition of MMP expression (198).

2.14.3 Angiotensin and Heart Failure

A number of studies support the hypothesis that angiotensin is important in the onset of irreversible cardiac hypertrophy and heart failure post-MI (199). Ventricular remodeling in post-MI hearts is now strongly associated with the activation of RAS. Specifically, there is an increase in angiotensin concentration in scar and a transient increase of angiotensin in the remnant tissue after MI (200,201). Furthermore, the angiotensin receptor density is considerably elevated in both noninfarcted myocardium and scar (35,195,196). For example, a 4.2-fold and 3.2-fold increase in AT_{1A} and AT_2 mRNA levels, respectively, are found in infarcted regions, while a ~2-fold increase in these mRNAs for both AT_{1a} and AT_2 receptors are observed in non-infarcted regions of the myocardium in the 7-day post MI group (196). Similarly, cardiac angiotensin and its receptors have been shown to be upregulated in rapid pacing induced heart failure in dogs and in hearts of hamster with genetic cardiomyopathy (202,203). The contribution of locally generated angiotensin appears to be important for the development of cardiac hypertrophy and failure (204,205). The local RAS may act in concert with circulating RAS, and the efficacy of ACE inhibitor therapy for patients with heart failure wherein the overall effect of treatment is due to inhibition of both systems. Administration of ACE inhibitors is well known to reduce afterload and preload in rat, and to cause an elevation of bradykinin, as well as to be associated with prolongation of life after MI in rat

(206,207). In two large clinical studies, long-term ACE inhibitor therapy for patients with heart failure after MI has been shown to reduce mortality regardless of the degree of LV dysfunction (208,209). Nevertheless, ACE inhibition is unable to prevent the formation of angiotensin by non-ACE pathways such as by the activation of chymase (210). For this reason, treatment with an AT_1 receptor antagonist such as losartan is an alternative for more efficacious suppression of angiotensin in the cardiovascular system (210). Milavetz *et al.,* have shown that the long-term use of losartan is effective in reducing mortality and in improving heart function in post-MI rats. This evidence supports the role of angiotensin in the development of heart failure (211). Furthermore, in heart failure patients, treatment with losartan was associated with lower mortality than in those patients receiving captopril (212). Taken together, these results strongly support the idea that angiotensin plays a key role in the development of heart failure.

2.15 Crosstalk Between Angiotensin and TGF-β Signaling in the Heart

A significant body of literature indicates that other trophic ligands may act in concert with TGF-β in the heart. For example, elevated angiotensin signaling is associated with the onset of cardiac fibrosis in different models of heart failure, including myocardial infarction (213,214). In the infarcted rat heart, local angiotensin generation is activated in the remnant myocardium and scar (213). The predominant collagen-synthesizing cells in post-MI hearts have been identified as myofibroblasts (215) and AT_1 receptor antagonism significantly attenuates fibrosis in both infarcted and non-infarcted rat myocardium (214,216). Angiotensin-mediated modulation of the expression of TGF-

β_1 ligand occurs *in vitro* (217,218) and *in vivo* (219) in various cell types including cardiac fibroblasts. However, information about crosstalk between angiotensin and TGF-β_1 in post-MI heart at the post-receptor level (Smad proteins) is lacking. Furthermore, the role of putative angiotensin / TGF-β_1 crosstalk in the development of cardiac fibrosis and heart failure is unclear. This thesis addresses whether a known anti-fibrotic strategy (chronic AT_1 receptor blockade) is associated with modulation of cardiac Smad expression and activation in failing rat heart post-MI.

A number of *in vitro* studies have shown that angiotensin stimulates the autocrine production and release of TGF-β1 in rat neonatal and adult rat cardiac fibroblasts in culture by increasing the mRNA levels, biological activity, and total protein levels of TGF-β_1 (220,221). Angiotensin–induced matrix protein synthesis occurs also through the increased expression of TGF-β_1 in cardiac fibroblasts (168,222,223) and myofibroblasts (221). Other studies indicate that norepinephrine and angiotensin may elevate steady-state levels of TGF-β1 mRNAs in VSMCs (224). Early investigations in this field provide evidence that the production of TGF-β linked to induction by angiotensin is a PKC-dependent phenomenon (224,225). Furthermore, angiotensin stimulation is associated with an accelerated rate of conversion of latent TGF-β1 to the active form (224). TGF-β_1 may also play a role in the regulation of RAS, and this hypothesis is supported by results that TGF-β_1 stimulates the release of renin from cultured juxtaglomerular cells (226). It is pointed out that these factors may exert positive feedback on each other in the failing myocardium and may take on an important causal role for the development of pathological hypertrophy involving multiple cell types. Losartan is a widely employed early-generation AT_1 receptor blocker used in the clinical

applications and in basic benchtop research. Chronic losartan administration to renal replacement patients (25 mg/d for the first week and 50 mg/d for the rest of the period total of 6 weeks was associated with a marked reduction of plasma TGF-β_1 by approximately 50% (227). The cTnT-Q (92) troponin T knock-in mouse transgene is associated with myocyte disarray and interstitial fibrosis. Losartan treatment (14.2 ± 5.3 mg/kg/day and for 42 ± 9.6 days) of adult cardiac troponin T (cTnT-Q (92)) transgenic mice was associated with significantly reduced cardiac collagen volume fraction and the mRNA expression of both collagen α (I) and cardiac TGF-β_1 *vs.* nontreated transgenic animals (228). Despite some controversy in this particular area of investigation (i.e., TGF-β was noted to be unchanged in a RAS component transgene line of rats (229)) and that the use of transgenes is now recognized to be somewhat limited by problems in data interpretation, particularly when pleuripotent genes are tested (229). However, the basic hypothesis that TGF-β_1 and angiotensin may be co-participants in the generation of cardiac fibrosis via chronic stimulation of cardiac fibroblasts and myofibroblasts is upheld by the bulk of the experimental evidence in the literature. Further studies dealing with the nature of existence and mechanism of the crosstalk between these factors are warranted and are taken up within the experiments outlined in this thesis.

On the other hand, the information regarding the effect of TGF-β_1 on the regulation of RAS is limited. A recent study has shown that when rat cardiac ventricular fibroblasts were incubated with TGF-β_1 (10 ng/ml) for seven days, TGF-β_1 is able to induce the appearance of ACE in cultures of adult rat cardiac ventricular fibroblasts (37). The appearance of the enzyme is accompanied by the differentiation of fibroblasts to myofibroblasts (37).

Chapter 3. METHODS

3.1 EXPERIMENTAL MODEL

All experimental protocols for animal studies were approved by an appointed Animal Care Committee of the University of Manitoba, following guidelines established by the Canadian Institute of Health Research of Canada. MI was produced in male Sprague-Dawley rats (weighing 200-250 g) by surgical occlusion of the left coronary artery as described previously by Johns and Selye with minor modification(230-232). In short, after isofluorane anesthesia, the chest was opened cutting the third and fourth ribs, and the heart was extruded through the intercostal space. The left coronary artery was ligated 2-3 mm from the origin with a suture (6-0 silk) and the heart was repositioned in the chest. Closure of the wound was accomplished using a purse-string suture. Throughout the operation, ventilation of the lungs was maintained by positive-pressure inhalation of 95% O_2 and 5% CO_2 mixed with isofluorane. Sham-operated animals were treated similarly, except that the coronary suture was not tied. The mortality of all animals operated upon in this manner was about 45% within 48 h (due to acute heart failure subsequent to infarction).

Post-operated animals were divided into three groups, Group 1: sham-operated animals, Group 2: MI animals, and Group 3: MI rats treated with losartan (15 mg/kg/day) (12). All losartan treatment regimens were initiated one day following coronary ligation by implanting an Alzet osmotic mini-pump (Alza Corporation, La Jolla, CA, model 2002) and continued for 8 weeks. To achieve the 8 weeks treatment, two 2-week duration osmotic mini-pumps were implanted consecutively (the $t_{1/2}$ of losartan is 4.7 hours). For comparative purposes, sham-operated controls (group 1) and MI animals were

administered vehicle (0.9% saline) using the same method. After losartan or vehicle infusion, the animals underwent LV functional assessment and infarct size determination, then the remnant LV tissues were used to assess collagen protein profile, fibrillar collagen steady-state mRNA abundance and immunoreactive prolyl 4-hydroxylase concentrations.

The animals were sacrificed 8 weeks after ligation and the hearts were stored in liquid nitrogen (-196°C) for further analysis. Animals from the losartan treatment study and 8 weeks groups underwent assessment of cardiac function, determination of infarct size, and subsequently the remnant LV (non-infarcted LV free wall remote to infarct) and infarct scar tissues were used for further analysis. Animals with small infarcts (<35% of the LV free wall) were excluded from all studies.

3.2 Hemodynamic Measurements

Mean arterial blood pressure (MAP) and LV function of sham-operated control, MI, and MI treated with losartan groups were measured following induction of MI, as described previously (230,233). Briefly, rats were anesthetized by intraperitoneal injection of a ketamine:xylazine mixture (100 mg/kg : 10 mg/kg). A micromanometer-tipped catheter (2-0) (Millar SPR-249) was inserted into the right carotid artery. The catheter was advanced into the aorta to determine MAP and then further advanced to the LV chamber to record LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), the maximum rate of isovolumic pressure development ($+dP/dt_{max}$) and the maximum rate of isovolumic pressure decay ($-dP/dt_{max}$). Hemodynamic data was computed instantaneously and displayed using a computer data acquisition workstation (Biopac, Harvard Apparatus Canada).

3.3 Infarct Size

Following heart function recordings, the LV from different groups was fixed by immersion in 10% formalin and embedded in paraffin. Six transverse slices were cut from the apex to the base. Serial sections (50 μm each) were made from each slice and mounted and stained with Masson's trichrome. The percentage of infarcted LV was estimated after coronary ligation by planimetric techniques as described previously (234).

3.4 Determination of Cardiac Total Collagen

Samples from different groups were ground into powder in liquid nitrogen. Then 100 mg (wet weight) of cardiac tissue was dried to a constant weight. Tissue samples were digested in 6 M HCl (0.12 ml/mg dry weight) for 16 h at 105°C. Hydroxyproline was measured according to the method of Chiariello *et al.*,(235). A stock solution containing 40 mM of 4-hydroxyproline in 1 mM HCl was used as a standard. Collagen concentration was calculated by multiplying hydroxyproline levels by a factor of 7.46, assuming that interstitial collagen contains an average of 13.4 % hydroxyproline (235). The data was expressed as μg collagen per mg dry tissue.

3.5 Adult Cardiac Fibroblast Isolation and Culture

Adult cardiac fibroblasts were isolated from male Sprague-Dawley rats according to the methods of Brilla *et al.*,(236) with minor modifications (237). The adult rat heart was subjected to Langendorff perfusion at a flow of 5 ml/min at 37°C with recirculatory Joklik's medium containing 0.1% collagenase and 2% bovine serum albumin (BSA) for 25-35 minutes. Liberated cells were collected by centrifugation at 2000 rpm for 10 minutes. Following this, the suspension of DMEM/F12 was plated on a 100 mm

noncoated culture flask at 37°C with 5% CO_2 for 2 h. Cardiac fibroblasts attached to the bottom of the culture flask during a 2 h incubation while non-adherent myocytes were removed by changing the culture medium. The cells were maintained in DMEM/F12 supplemented with 10% fetal bovine serum, 100 unit/ml penicillin and 100 μg/ml streptomycin. The cells used for the study were from the second passage (P2) and the purity of fibroblasts used in these experiments was found to be ≥ 95%, using routine phenotyping methods described previously (105,237). For stimulation with angiotensin, fibroblasts were maintained in serum-free media for 24 h before administration of angiotensin (10^{-6} M) for 15 min. Equimolar losartan was added to cultured cells 1 hour before angiotensin treatment to achieve AT_1 blockade.

3.6 IMMUNOFLUORESCENCE

Myocardium from sham-operated animals, remnant LV remote to the infarct, and RV scar and border tissues from various times after induction of MI, were immersed in OCT compound and stored frozen at -80°C. Serial cryostat sections of 7 μm thickness were mounted on gelatin-coated slides. A minimum of 6 sections from different regions of each group was processed. Sections were fixed in 4% paraformaldehyde for 15 min and then washed 6 x 5 min in 1x PBS to eliminate background caused by paraformaldehyde. Immunofluorescent staining was performed using the indirect immunofluorescent technique (238). In brief, the tissue sections were incubated with primary antibodies overnight at 4°C. Sections were then washed 3 x 5 min in phosphate-buffered saline (PBS) and were incubated with biotinylated secondary antibody for 90 minutes at room temperature. Sections were washed 3 x 5 minutes and then incubated with FITC labeled or Texas Red labeled streptavidin. Finally, the slides were mounted

and coverslipped. The results were recorded by photography on Kodak T-MAX 400 black and white film. Quantification of resultant image data from immunofluorescent staining was performed using digital image analysis software (SigmaScan Pro).

The primary antibodies and secondary antibodies used in this study were listed. Primary antibodies are as follows: 1. Goat polyclonal anti-types I & III collagen antibodies were diluted in 1:100 (Southern Biotechnology Associates Inc, Alabama, USA), 2. Polyclonal antibody against active TGF-β_1 was diluted in 1:50 (Promaga Corporation, Madison, WI, USA). 3. Goat polyclonal antibody against TβRI, TβRII, Smad2, 3 and 4 antibodies were diluted 1:50 to 1:100 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), 4. Monoclonal anti-vimentin clone #V9 (Sigma-Aldrich, Oakville, ON, Canada, 1:100 dilution), Monoclonal anti-myosin MF-20 (Developmental Studies Hybridoma Bank, 1:100 dilution), monoclonal antibody against α SMA (Sigma, 1:400 dilution), human monoclonal antibody against smooth muscle myosin (SMM, Signma, 1:250 dilution), monoclonal antibody against factor VIII (von Willebrand factor, Sigma, rabbit, 1:250 dilution). Secondary antibodies are as follows: biotinylated anti-goat, anti-mouse and anti-rabbit IgG were diluted 1:20 (Amersham Life Sciences Inc. Canada). Texas Red-labeled streptavidin and FITC-labeled streptavidin were diluted 1:20 (Amersham Life Sciences Inc. Canada). Texas Red-labeled streptavidin was used for the detection of collagen type I and FITC-labeled streptavidin was used for the detection of all other proteins. All antibodies were diluted in PBS containing 1% BSA and 0.1% sodium azide.

3.7 RNA EXTRACTION

Total RNA was extracted from cardiac tissues by the procedure of Chomczynski and Sacchi as described previously (54) MCB (239). Briefly, cardiac tissue was rapidly excised, the atria were removed and the ventricular tissue was washed twice with a solution containing 10 mM 3-[N-morpholino] propanesulfonic acid (MOPS) and 10 mM sodium ethylenediaminetetraacetate (EDTA). Tissue samples were then quickly frozen and stored in liquid nitrogen (-196°C). Previously frozen ventricular tissues were ground with mortar and pestle while immersed in liquid nitrogen. Powdered samples were suspended in 4 ml Solution D [4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0) 0.5% N-lauroylsarcosine, 0.1 M 2-mercaptoethanol] and subjected to mechanical homogenization (Diamed, Toronto) (3 x 10 seconds). At this point, tissue homogenates were treated with 0.1 volumes of 2 M sodium acetate (pH 4.0), equal volumes of water-saturated phenol (pH 7.4) and 0.2 volumes of chloroform-isoamyl alcohol mixture (49:1) then mixed by inversion. After the mixture was cooled on ice for an additional 15 minutes, samples were centrifuged at 6,000 x g for 20 minutes at 4°C. The RNA-containing aqueous phase was transferred to a fresh tube, mixed with an equal volume of isopropanol and placed at -20°C for 60 minutes. RNA was sedimented at 10,000 x g for 20 minutes and resuspended in solution D and then again precipitated with an equal volume of cold isopropanol and placed at -20°C for 30 minutes. Then the ventricular RNA pellets were washed twice by repeated resuspended in 75% ethanol and sedimented. The ethanol solution was decanted and finally vacuum dried (duration of 30 seconds to 2 minutes, then visually assessed to avoid complete drying of the pellet). RNA was

dissolved in diethyl pyrocarbonate (DEPC)-treated water and the concentration of nucleic acid was calculated from the absorbance at 260 nm prior to size fractionation.

3.8 Northern Blot Analysis

Steady-state levels of mRNA were determined by Northern hybridization analysis. Twenty μg of total RNA was denatured in 50% formamide, 7% formaldehyde, 20 mM MOPS (pH 7.4), 2 mM EDTA (pH 8.0), 0.1% SDS and electrophoresed in a 1% agarose/formaldehyde gel to size fractionate the mRNA transcripts. The fractionated RNA was transferred (using capillary action) on to a 0.45 μm positively charge-modified nylon filter (NYTRAN Maximum Strength Plus, Schleicher and Schuell, Keene, NH, USA) filter. After 24 hours, the filter was removed and RNA was covalently cross-linked using UV radiation (UV Stratalinker 2400, Stratagene). Blots were prehybridized in a mixture of 50% formamide, 10x Denhardt's solution, 1% SDS, 0.2 mg/ml denatured salmon sperm DNA and 10 mM EDTA (pH 8.0), 25% "4 x RNA" solution [3 M NaCl, 0.6 M Tris-HCl (pH 7.5), 0.18 M NaH_2PO_4, 0.24 M Na_2PO_4, 0.1 M $Na_4P_2O_7$] at 42°C for 6-16 hours. Membrane was hybridized for 6 to 16 hours at 42°C in the presence of labeled probe with a specific activity > 10^9 cpm per μg DNA. The filter then was washed for a certain period of time in each of the following: 2 x SSC / 0.1% SDS (first wash), 0.5 x SSC / 0.1% SDS (second wash), and 0.1 x SSC / 0.1% SDS (third wash) using an INNOVA 4000 incubator (New Brunswick Scientific, Canada) oscillating at a rate of 60 rotations per minute. After washing, the membrane was exposed to x-ray film (Kodak X-OMAT) at -80°C with two intensifying screens.

The following inserts were separated from recombinant plasmids and used as cDNA probes in Northern blot analysis: human procollagen type α1(I) (Hf 677), type α1(III) (Hf 934), TGF-β1 and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from the American Type Culture Collection (Rockville, MD, USA). Decorin cDNA was graciously provided by Dr. Kevin L. Dreher, United States Environmental protection Agency, Research Triangle Park, NC, USA. Rat 18S rRNA (5'-ACGGTATCAGATCGTCTTCGAACC-3') was synthesized using the Beckman Oligo 1000 DNA synthesizer (240). The cDNA clones were prepared for hybridization to specific mRNA transcripts and subsequent autoradiography using a Random Primers DNA Labeling System (GIBCO BRL) radiolabeled α-^{32}P-dCTP. Results of autoradiographs from Northern blot analysis were quantified by densitometry (Bio-Rad imaging densitometer GS 670 Hercules, CA, USA).

3.9 Enzyme Immunoassay for Prolyl 4-Hydroxylase

Cardiac tissues from different groups were ground into powder under liquid nitrogen. Powdered tissues (20 mg/ml) were homogenized in 10 mM Tris-HCl buffer pH 7.8 containing 0.1 M NaCl, 0.1 M glycine, 0.1% Triton X-100, 20 mM EDTA, 10 mM N-ethylmaleimide, 1 mM Phenylmethylsulfonyl fluoride (PMSF), 1 mM P-hydroxymercuribenzoic acid and 1 mM Dithiothreitol (DTT). The homogenized samples were centrifuged 20,000 x g at 4°C for 30 minutes. The supernatants were transferred to fresh Eppendorff tubes and then used for prolyl 4-hydroxylase assays, employing an ELISA kit (Fuji Chemical Industries, Ltd. Toyama, Japan) (241). Briefly, this assay employs two monoclonal antibodies wherein the first is used as a capture antibody in

solid phase and the other antibody is linked to horseradish peroxidase. Myocardial samples were diluted 1:20 in distilled water prior to the total protein concentration assay.

3.10 Nuclear Isolation from Cardiac Fibroblasts

Nuclei of cardiac fibroblasts were isolated using the Nuclei EZ Prep Nuclear Isolation Kit (Sigma-Aldrich, Oakville, ON, Canada) according to the manufacturer's instructions. The purity and integrity of isolated nuclei was confirmed by flow cytometry and light microscopy following trypan blue staining (data not shown). Isolated nuclei were resuspended in 100 mM Tris (pH 7.4) containing 1 mM EDTA, 1 mM PMSF, 4 μM leupeptin, 1 μM pepstatin A, and 0.3 μM aprotinin. Phosphatase inhibitor (10 mM NaF,1 mM NaOV and 20 mM β-glycorophosphate) was also added to the solution. Samples were subjected to sonication 3 x 10 sec to further disrupt the nuclei and the nuclear protein concentration analysis was performed by BCA methods (242).

3.11 Protein Assay

Total protein concentration in cardiac samples was determined using the Bicinchoninic acid solution (BCA) Kit (Sigma, St. Louis USA) (242).

3.12 Western Blot Analysis

TGF-β_1, TβRI, TβRII, Smad2, Smad3, Smad4, and phosphorylated Smad2 proteins were detected using Western blot analysis. Cardiac tissues from sham-operated LV, remnant LV, border area and scar were homogenized in 100 mM Tris (pH 7.4) containing 1 mM EDTA, 1 mM PMSF, 4 μM leupeptin, 1 μM pepstatin A and 0.3 μM aprotinin. Samples were sonicated for 3 x 5 seconds. Crude membrane and cytosolic

fraction were isolated according to the method of Gettys *et al.*,(243). Briefly, samples were centrifuged for 3000 x g at 4°C for 10 minutes to remove unbroken cells and nuclei. The supernatant was further subjected to centrifugation for 48,000 x g for 20 minutes at 4°C. The subsequent crude membrane pellet was resuspended in the homogenizing buffer. For total cardiac Smad proteins assay, myocardium was homogenized with above buffer containing 0.1% Triton X-100. This homogenate was sonicated for 5 x 5 seconds to disrupt nuclear membrane. The homogenate was allowed to lyse for 15 min on ice to further disrupt the nuclear membrane. After centrifugation at 10,000 x g for 20 min at 4°C, the resultant supernatant was used for Smad proteins assay. Total protein concentration of membrane fractions was measured using the BCA method (242). Prestained high or low molecular weight markers (Bio-Rad, Hercules CA, USA) and 20 μg proteins from samples were separated on 10% SDS-PAGE. Separated proteins were transferred on to 0.45 μm polyvinylidene difluoride (PVDF) membrane. PVDF membrane was blocked overnight at 4°C or at room temperature for 1 hour in Tris-buffered saline with 0.1% Tween-20 (TBS-T) containing 5%-8% skim milk. After washing with TBS-T solution, membranes were probed with primary antibodies for 1 hour at room temperature. After washing, membrane was incubated with HRP-labeled secondary antibodies for 1 hour at room temperature. The target proteins were detected and visualized by enhanced chemiluminescence (ECL) or ECL "Plus" according to the manufacturer's instruction (Amersham Life Science Inc. Canada). Specific bands from autoradiographs derived from Western blots were quantified using a CCD camera imaging densitometer (Bio-Rad GS 670, Hercules, CA, USA).

Primary antibodies used in the current set of studies were as follows: 1. Rabbit polyclonal antibodies against TGF-β_1, Smad 4, TβRI and TβRII were from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against phosphorylated Smad 2 was obtained from Upstate Biotechnology (Lake Placid, NY). 2. Goat polyclonal antibodies against Smad 2, Smad 3 were purchased from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA, USA). The primary antibodies were diluted in TBS-T containing up to 5% skim milk (Smad 2, 3 and 4 at 1:500 and TGF-β_1, P-Smad 2, TβRI, TβRII at 1:250). Secondary antibodies were as follows: Horseradish peroxidase (HRP)-labeled anti-rabbit IgG and anti-goat IgG were diluted in 1:10,000 with TBS-T containing 1% skim milk.

3.13 ELISA Assay for Cardiac TGF-β_1

TGF-β_1 concentration was determined using a "sandwich" ELISA by the method as described by Danielpour with minor modifications (244). After excision, the heart was perfused with 5 ml cold PBS to flush out the remaining blood in the myocardial vascular lumen in order to eliminate contamination by TGF-β_1 from blood sources. Approximately 0.5 g heart tissue was homogenized in 4 ml cold acid-ethanol (93% ethanol, 2% HCl, 85 μg/ml PMSF and 5 μg/ml pepstatin A). Three samples were pooled in the case of border and scar tissues. After overnight extraction at 4°C by gentle rocking, extracts were subjected to centrifugation at 10,000 g for 10 minutes. The resulting supernatants were dialyzed extensively (3 x 100 volume) against 4 mM HCl at 4°C, using a 3,500 MW-cutoff Spectrapore dialysis membrane. Plate wells were coated with either soluble TβRII or PBS (control). Recombinant human TGF-β_1 was used to

generate a standard curve (triplicate). Standards and samples were incubated for 1 h at room temperature on a rotating platform, washed and incubated with chicken anti-TGF-β_1 antibody (< 5% cross-reactivity with TGF-β_2 and -β_3). Wells were washed again before incubation with phosphatase-linked, goat, anti-chicken antibody for 1 hour. After washing, the plates were incubated overnight at 4°C with phosphatase substrate in diethanolamine buffer (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD). The difference in optical density between 405 and 450 nm was measured on a V_{max} microplate ELISA reader (Molecular Devices, Menlo Park, CA). TGF-β_1 concentrations in the samples were calculated by a four-parameter regression equation (after correction for the background from control wells) with Molecular Device's Sofmax program. TGF-β_1 concentration was expressed as ng/g of tissue. This assay measured total cardiac TGF-β_1 since latent TGF-β_1 was activated during the acid-ethanol extraction step.

3.14 Infection of Adenovirus to Cardiac Fibroblasts

Cultured rat cardiac fibroblasts at a second passage were infected with adenoviral dorminant negative type II TGF-β type II receptor (Ad DN-TβRII) following the instruction of AdEasy™ Vector System kit. Ad DN-TβRII was from Dr. P. Cattini's laboratory (in collaboration). Briefly, Ad DN-TβRII was administered to cultured cardiac fibroblasts [Multiplicity Of Infection (MOI) = 25] for 24 hours. The efficiency of transfection is over 95% identified by Trypan Blue staining and no significant cytotoxicity to fibroblasts was noted. Adenovirus expressing β-gal gene (MOI=25) was used as a control. Infected fibroblasts were treated with angiotension (10^{-6} M) for 15 min.

The P-Smad 2 localization in cardiac fibroblasts was examined by double immunofluorescent staining as mentioned before.

3.15 Statistical Analysis

All values are expressed as mean ± SEM. The difference between the control and the experimental groups were calculated using the student's t-test. One way analysis of variance (ANOVA) followed by the Student-Newman-Keuls Test was used for comparing the differences among multiple groups at each time point (SigmaStat). Significant differences among groups were defined by a probability of less than 0.05. The Northern blot data in multiple time point study was expressed as a percentage of control according to the method of Fisher and Periasamy (245).

3.16 Reagants

Angiotensin II (angiotensin) was purchased from Sigma. Co. TGF-β_1 and TGF-β neutralizing antibody was purchased from R & D Systems Inc (Minneapolis, USA). Losartan (Los) was a kind gift from (Merck (Rahway, NJ). β-Gal staining Set was purchased from Roche Diagnostics Co. (Indianapolis, IN, USA).

Chapter 4. RESULTS

4.1 General Observations: Cardiac Hypertrophy, Total Cardiac Collagen Concentration and Heart Failure

Hearts of experimental animals were characterized by significant cardiac hypertrophy as reflected by an increase in the mass of the remnant left ventricular tissue (LV) and also by increased LV to body mass (BW) ratio in experimental animals compared to control values (Table 1). These incidence and magnitude of left ventricular hypertrophy noted was comparable to our previous findings (105,246). Cardiac collagen concentration in surviving myocardium remote to infarct (i.e., remnant heart: 58.2 ± 5.1 μg/mg dry wt) and border + scar tissues (126.3 ± 10.8 μg/mg dry wt) were both significantly higher than that of control value (20.3 ± 3.2 μg/mg dry wt). Furthermore, cardiac collagen concentration in remnant heart treated with losartan (37.4 ± 3.4 μg/mg dry wt) was significantly reduced *vs.* values from nontreated tissues. Heart failure, reflected by an increase in left ventricular end-diastolic pressure (LVEDP) and a decrease in the maximum rate of isovolumic pressure development or decay ($\pm dP/dt_{max}$) relative to their controls, along with congested lungs, has been characterized in this model from our previous studies (247). Losartan treatment was associated with normalization of indices of cardiac hypertrophy and cardiac function (Table 1), in agreement with our previous findings (214).

4.2 Alteration of Total Cardiac TGF-β_1

Quantitative assessment of total cardiac TGF-β_1 protein concentration in control and remnant left ventricular tissues as well as border + scar tissues of the 8-week post-MI rats was carried out using ELISA. The results indicated that TGF-β_1 was increased by approximately 2.4-fold in border and scar tissues, compared to that from the control animals (Figure 1). There was no significant alteration of TGF-β_1 in samples from remnant left ventricle *vs.* control values.

4.3 Alteration of Cardiac TGF-β1, Decorin and Collagen Type I mRNA

Abundance

We addressed steady-state mRNA abundance of cardiac TGF-β_1, collagen type I and decorin in tissues taken from various left ventricular regions of the 8 weeks post-MI rats. Figure 2 shows a representative Northern blot with autoradiographic bands specific for TGF-β_1, collagen type I and decorin and GAPDH mRNAs from left ventricular samples of sham, remnant, as well as border + scar tissues. Estimation of the target gene mRNA abundance was calculated by the ratio of target gene to GAPDH signal. The ratios for TGF-β_1, decorin and collagen type I were significantly increased in the border and scar regions *vs.* values from remnant tissue and control (histograms in Figure 2).

4.4 Localization of Active TGF-β1 and Decorin in Cardiac Tissue Sections

Active TGF-β_1 was localized using immunofluorescent staining of frozen serial sections of control (sham-operated) left ventricle from age-matched rats, remnant (viable) left ventricular tissue remote to the site of infarction, as well as border + scar tissue samples (Figure 3). Active

TGF-β_1 protein was localized mainly to the interstitial space in these sections. Compared to sham-operated control samples, remnant tissues exhibited a relatively bright pattern of staining in the interstitial spaces suggesting the presence of high levels of TGF-β_1. Intense staining of active TGF-β_1 protein was noted in the border + scar tissue in the post-MI hearts as compared with that of sham hearts. Localization of immunoreactive decorin in cardiac sections taken from the control, remnant tissue remote to the infarct, and infarct scar was carried out (Figure 4), and the presence of decorin (brightly stained material) is noted in the interstitial space of control (Figure 4A) and remnant (Figure 4B) sections. Decorin staining appeared to be more prominent among the hypertrophied myocytes of the remnant heart compared to the control section. Compared to these sections, a more compact pattern of decorin staining (bright patches of specific staining) was noted in sections of the infarct scar (Figure 4C).

4.5 Quantification and Localization of Cardiac Smad 2, 3, and 4

Western analysis was used to determine cardiac Smad 2, 3, and 4 protein concentrations from the 8 week sham-operated control hearts and different regions of post-infarct myocardium. Quantitative densitometry scanning of specific cardiac Smad 2 (55 kDa band), Smad 3 (45 kDa band) and Smad 4 (62 kDa band) bands revealed that the concentrations of these proteins were significantly increased in border and scar tissues (lanes 7-9) when compared to the control values (lanes 1-3, Figure 5A). Furthermore, the total Smad 2 and Smad 3 proteins were increased in the remnant left ventricular samples (lanes 4-6) from experimental animals *vs* controls. Immunofluorescence staining patterns of Smad 2, Smad 3, and Smad 4 is shown in sections of remnant (viable) left ventricular tissue remote to the infarct (Figure 6A) and of the infarct scar (Figure 6B) from the 8

week post-MI rat heart. Double-staining of cellular nuclei in sections of the infarct scar demonstrate the relative cellularity present in this tissue (Figure 6B ii, iv and vi). We observed that in the remnant post-MI tissue sections, cardiac Smad 2, 3, and 4 proteins were localized mainly in the perivascular space (Figure 6A). We also observed marked localization of Smad 2, Smad 3 and Smad 4 proteins proximal to the nuclei of nonmyocyte cells from sections of the infarct scar.

4.6 Quantification and Localization of TβRI (ALK-5) and TβRII

Western blot analysis revealed that both TβRI and TβRII are detectable in the membrane fraction but not in the cytosolic fraction. Figure 7A (upper band) provides a representative blot illustrating the presence of a characteristic 53 kDa band for TβRI. It shows that there is a dramatic decrease of TβRI in the border and scar tissues. Figure 7A (bottom band) illustrates the bands specific for TβRII at 75 and 110 kDa (248). In contrast to TβRI, the major isoform of TβRII (75 kDa) was modestly decreased, but the 110 kDa isoform was markedly increased in the border + scar region. Figure 7B indicates relatively even loading of samples by amido black 10B staining of the same Western blot membrane. TGF-β1 receptors and their distribution in 8-week experimental and age-matched control tissues were localized using immunofluorescent techniques. In a representative photomicrograph (Figure 8), the staining pattern of immunoreactive TβRI (ALK-5) and TβRII appears as bright staining within and surrounding cardiac myocytes, as well as in the interstial space. The latter staining is likely due to the presence of nonmyocyte cells. Myocytes in the border region (lower portions of panels C and F) contain brightly stained material, which was taken to represent TβRI and TβRII immunostaining,

respectively. Notably the infarct scar (present in the upper portion of panel C) is marked by the appearance of very little TβRI immunoreactive material. In contrast to the results addressing TβRI, stronger staining of TβRII was present in the scar and border region compared with sham and remnant tissues.

4.7 Characterization of Myofibroblasts in the Infarct Scar at 8 Week After Myocardial Infarction

Viemntin, myosin and nuclear staining was noted among frozen sections of sham, remnant as well as scar and border tissues from 8 week post-MI rat heart (Fig. 9). From left to right, in samples of sham-operated and remnant myocardium, vimentin staining (top row, apparent as brightly staining network) was prominent in the intramyocytic spaces. Cardiac myocytes in these sections (Fig. 9 middle row) stained positive for MF-20 (myosin) and these tissues appear as homogeneously brightly stained secions. The infarct scar proper stained negatively for myosin.

As immunoreactive vimentin stains not only fibroblasts, but also myofibroblasts, endothelial cells and smooth muscle cells, we carried out a series of immunohistochemical experiments to determine the phenotypic distribution of these cells in the infarct scar. Representative scar sections were stained with primary antibodies for α-smooth muscle actin (α-SMA), factor VIII (von Willebrand factor), and smooth muscle myosin (SMM) and the results are shown in Fig. 10. In the 8 week infarct scar tissue, significant α-SMA staining (Fig. 10A) was noted in parallel to relatively low expression of SMM (Fig. 10C) and we took these combined findings to reflect the appearance of myofibroblasts. In the remnant cardiac tissue remote to the scar, α-SMA

staining was localized to the perivascular space of vessels (data not shown). Staining of the 8 week infarct scar sections with primary antibody against factor VIII was carried out to mark endothelial cell distribution. This tissue was characterized by a negative staining pattern for this antibody (Figure 10B).

4.8 Localization and Quantification of Cardiac Smads in Post-MI Heart

Immunofluorescent staining revealed that total Smad 2 protein was localized to the extracellular space proximal to nuclei as shown in Figure 11. Double staining with vimentin showed that Smad 2 was mainly localized to nonmyocytes proximal to the nuclei. We observed enhanced accumulation of Smad 2 proteins in the nuclei of cells from scar tissue (Figure 11). Western analysis was used to determine the protein concentration of cardiac Smad 2 and Smad 4 from different groups (Figure 12). Cardiac Smad 2 (55 kDa) protein concentration was significantly increased in remnant and scar tissues when compared to control values, while cardiac Smad 4 (62 kDa) protein concentrations was only significantly elevated in scar tissue *vs* the control. Losartan treatment was associated with a significant inhibitory effect on Smad 2 and Smad 4 accumulation in remnant tissue and infarct scar tissue, respectively (Figure 12).

4.9 Effect of Losartan on the Expression of Cardiac TGF-β_1

Using Western blot analysis, cardiac TGF-β_1 protein concentration was quantified in the control and the remnant left ventricular tissues as well as the border and scar tissues of the 8-week post-MI rats (Figure 13). The TGF-β_1 polyclonal antibody

recognized both the latency associated peptide (LAP) and active forms of TGF-β_1 at ~40 kDa and 25 kDa, respectively. Although the LAP dimer of ~80 kDa binds TGF-β_1 *per se,* we observed the monomeric LAP band due to reducing gel conditions. The active form of TGF-β_1 was increased in both remnant and scar tissues from post-MI heart, which was significantly attenuated by the administration of losartan. Conversely, the latent form of TGF-β_1 was decreased in both remnant and scar tissues and this decrease was partially prevented by losartan treatment (Figure 13). Previous studies have shown that TGF-β_1 can be released from latent complexes and can be activated by cleaving an inactive high molecular weight precursor complex (249). We observed that the conversion of TGF-β_1 from its latent to active form was augmented in remnant myocardium and infarct scar. Losartan treatment was associated with an inhibition of this conversion. Immunofluorescent staining revealed that the total TGF-β_1 localized to the extracellular space in normal tissue and remnant myocardium (Figure 14). Furthermore, the infarct scar stained brightly for total TGF-β_1, as did myocytes bordering the infarct scar region. Cardiac myocytes remote to the infarct scar expressed comparatively moderate levels of TGF-β_1 (Figure 14).

4.10 Total and Phosphorylated Smad 2 Distribution in Post-MI Heart

Immunofluorescence data indicated relatively moderate staining of phosphorylated Smad 2 (P-Smad 2) in myocytes of sham-operated, remnant and losartan-treated remnant tissues from the post-MI rat heart (Figure 15A, C and E). Compared to the control and remnant tissues, the scar and treated scar sections (Figure 15G and I,

respectively) were characterized by brightly stained regions and areas of punctate nuclear accumulation of P-Smad 2 were found in the scar (arrows) (Figure 15). This pattern was associated with cellular nuclei in the scar tissue (Figure 15H and J). Western analysis of cytosolic P-Smad 2 revealed a significant decrease in band intensity from the cytosolic remnant and scar tissue compared to the sham-operated control (Figure 16). These trends were normalized by losartan treatment. Our results suggest that a decreased P-Smad 2 in the cytosolic fraction may be associated with an increased P-Smad 2 in the nuclear fraction from the remnant and scar tissues of the 8 week post-MI hearts, and this is supported by the following experiments.

4.11 The Effect of Angiotensin on Smad 2 Activation in Cultured Cardiac Fibroblasts

In studies of quiescent and unstimulated cultured cardiac fibroblasts, the total Smad 2 localized to cellular nuclei and cytosol (Figure 17a - panel A), as did P-Smad 2 (Figure 6b -panel E). Total Smad 2 staining was elevated in intensity after stimulation with angiotensin (10^{-6} M) for 15 min *vs* the unstimulated cells (Figure 17a - panel C). Furthermore, angiotensin (10^{-6} M) stimulation for 15 min was associated with marked translocation of P-Smad 2 from the cytosol to the nuclei (Figure 17b - panel G). We further isolated the nuclei from the cultured cardiac fibroblasts from a normal rat heart stimulated with angiotensin (10^{-6} M) in the absence or prescence of losartan (10^{-6} M) 1 hour before stimulation. Western analysis revealed that angiotensin stimulation for 15 min was associated with a significant increase of P-Smad 2 protein, and this change was inhibited by AT_1 receptor blockade (Figure 18).

4.12 Angiotensin Stimulation of Smad 2 in Cardiac Fibroblasts in the Presence of TGF-β Neutralizing Antibody

In order to investigate the possible existence of a direct angiotensin-Smad 2 interaction, we performed a series of experiments designed to eliminate TGF-β_1 ligand-receptor interaction. First, we stimulated cardiac fibroblasts with angiotensin (10^{-6} M) in the presence of TGF-β_1 neutralizing antibody (TGF-β_1 NAb, 1.5 μg/ml), which has been widely used to inhibit TGF-β activity (250). TGF-β_1 NAb at this concentration is able to exert an inhibitory effect of over 95 % of TGF-β_1 activity. Cultured cardiac fibroblasts were treated with angiotensin (10^{-6} M) or TGF-β_1 (10 ng/ml) for 15 min in the absence or presence of TGF-β_1 NAb 1 hour before stimulation. In TGF-β_1 NAb and losartan (Los, 10^{-6} M) treated groups, Los was applied to fibroblasts 1 hour prior to angiotensin stimulation. Immunoreactive P-Smad 2 localization was examined using immunofluorescent staining. Compared to untreated control, TGF-β_1 stimulation was associated with brighter P-Smad staining, which was largely concentrated in the cellular nuclei (Fig. 19), indicating an activation of R-Smad 2 (phosphorylation/activation) by TGF-β_1, and this effect was blocked by TGF-β_1 NAb (Fig. 19). This result was confirmed in other experiments using confocal microscopy (data not shown), providing evidence that R-Smad 2 was present within the nucleus itself, and not in the perinuclear space. Angiotensin also exerted a stimulatory effect on R-Smad activation, similar to that of TGF-β_1, and this effect was inhibited by Los treatment (Fig. 19). However, the activation of R-Smad 2 by angiotensin was not blocked in the prescence of TGF-β_1 NAb (Fig. 19), suggesting that angiotensin can stimulate Smad 2 activation independent of TGF-β_1 receptor activation.

4.13 Stimulation of Angiotensin on Cardiac Fibroblasts Overexpressing Dominant negative TβRII

To add more evidence in support of the specificity of angiotensin- mediated phosphorylation of R-Smad2, cultured cardiac fibroblasts at second passage at a confluence of 70% were infected with an adenoviral dominant-negative TβRII (AdDNTβRII, MOI=25). AdDNTβRII is a kinase-defective mutant of TβRII, lacking of the function of normal TβRII. Overexpression of an AdDNTβRII has been shown to abrogate TGF-β_1 signaling mediated by TGF-β receptors (78). β-Gal adenovirus (AdβGal, MOI=25) was used as a control. The infection efficiency was over 95% after 24 hours infection examined by β-Gal staining (data not shown). There was no significant cytotoxicity to the cultured cardiac fibroblasts under our conditions. Twenty four hours after infection, fibroblasts were stimulated with angiotensin (10^{-6} M) for 15 minutes. Immunoreactive P-Smad 2 localization was examined using immunofluorescent staining. In AdβGal infected fibroblasts, a weak staining of P-Smad 2, which was sparsely localized at the perinuclear space (Fig. 20). Angiotensin stimulation was associated with a brighter staining for P-Smad 2, which was mainly localized in the nuclei. In AdDNTβRII infected fibroblasts, the pattern of P-Smad localization was similar to that of AdβGal infected cells in the absence of angiotensin. Similar to AdβGal infected cells, angiotensin stimulation of AdDNTβRII infected fibroblasts resulted in an increased staining of P-Smad 2, which was mainly localized to the nuclei (Fig. 20). Some staining was also seen in the cytoplasm and in focal adhesions. Our results indicated that

overexpressing AdDNTβRII in fibroblasts could not block the effect of angiotensin on the activation of R-Smad 2, suggesting that angiotensin stimulates Smad 2 activation independent of TGF-β_1 receptor activation.

Table 1. General and hemodynamic characteristics of age-matched sham, 8 week post-myocardial infarction (MI) and 8 week losartan-treated post-MI rats.

Parameters	Sham-operated control	Post-MI	Losartan-treated Post-MI
BW, g	514 ± 8	481 ± 9	486 ± 11
LVW, g	0.89 ± 0.03	1.02 ± 0.03*	0.91 ± 0.02 †
LV/BW, mg/g	1.74 ± 0.03	2.11 ± 0.05*	1.86 ± 0.04 †
Lung wet/dry wt ratio	3.42 ± 0.21	4.73 ± 0.23*	3.56 ± 0.19 †
LVEDP, mmHg	3.1 ± 0.6	12.8 ± 1.6*	8.8 ± 0.8 † *
LVSP, mmHg	132 ± 8	119 ± 11	121 ± 10
$+dP/dt_{max}$, mmHg/s	5612 ± 234	4481 ± 212*	5032 ± 224 †
$-dP/dt_{max}$, mmHg/s	5478 ± 229	3894 ± 218*	4652 ± 213 †

Table 1. Experimental animals (MI) were characterized by large left ventricular (LV) myocardial infarction (43 ± 4% of the total LV circumference); sham–operated animals were noninfarcted age-matched controls; BW, body weight; LVW, left ventricular weight; LVEDP, LV end-diastolic pressure; LVSP, LV systolic pressure; $+dP/dt_{max}$, the maximum rate of isovolumic pressure development; $-dP/dt_{max}$, the maximum rate of isovolumic pressure decay. The data depicted is the mean ± SEM of 8-10 experiments. *$P < 0.05$ *vs* sham-operated animals; † $P < 0.05$ *vs* untreated post-MI animals.

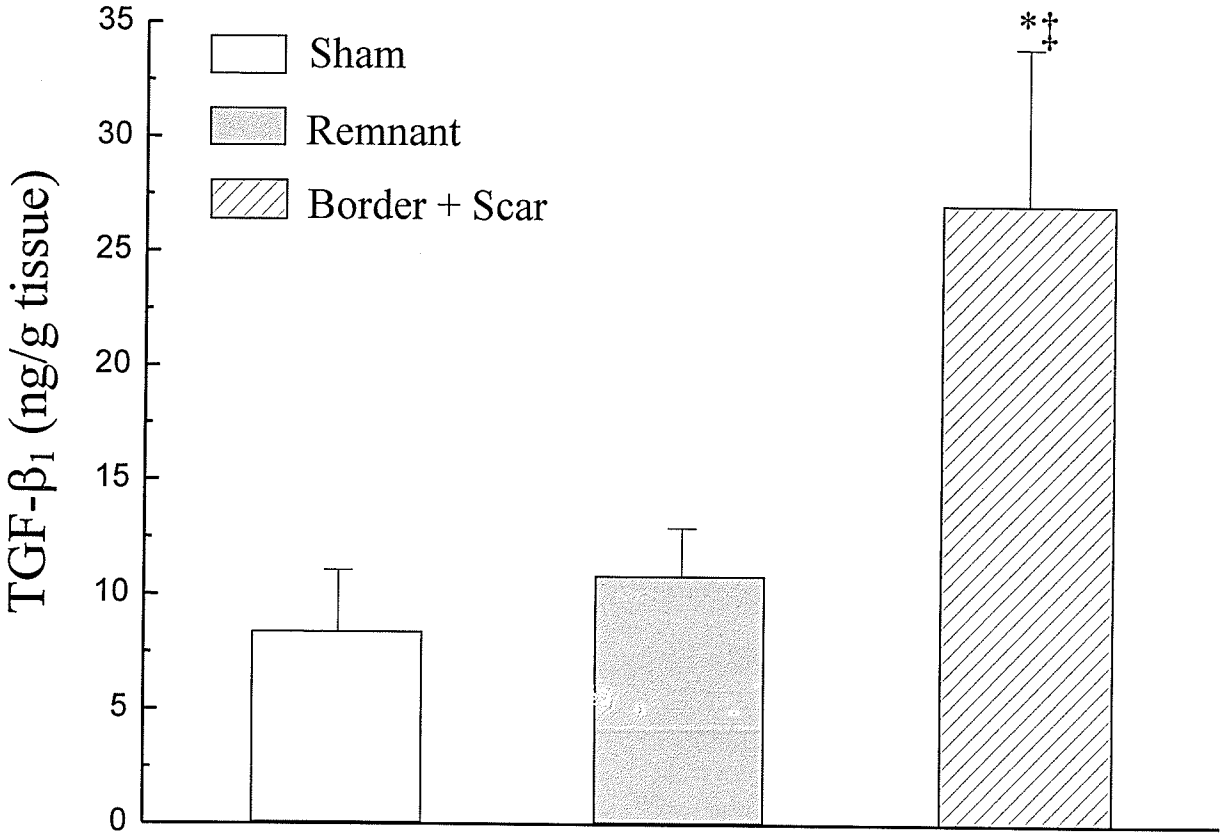

Figure 1. Cardiac transforming growth factor-β_1 (TGF-β_1) protein concentration in sham hearts, as well as remnant, border and scar tissues from 8 week post- MI as detected by enzyme-linked immunosorbent assay (ELISA). The data depicted is the mean $\pm$ SEM of 5 experiments. $^{*}P < 0.05$ and $^{‡}P < 0.05$ *vs* sham and remnant sample values, respectively.

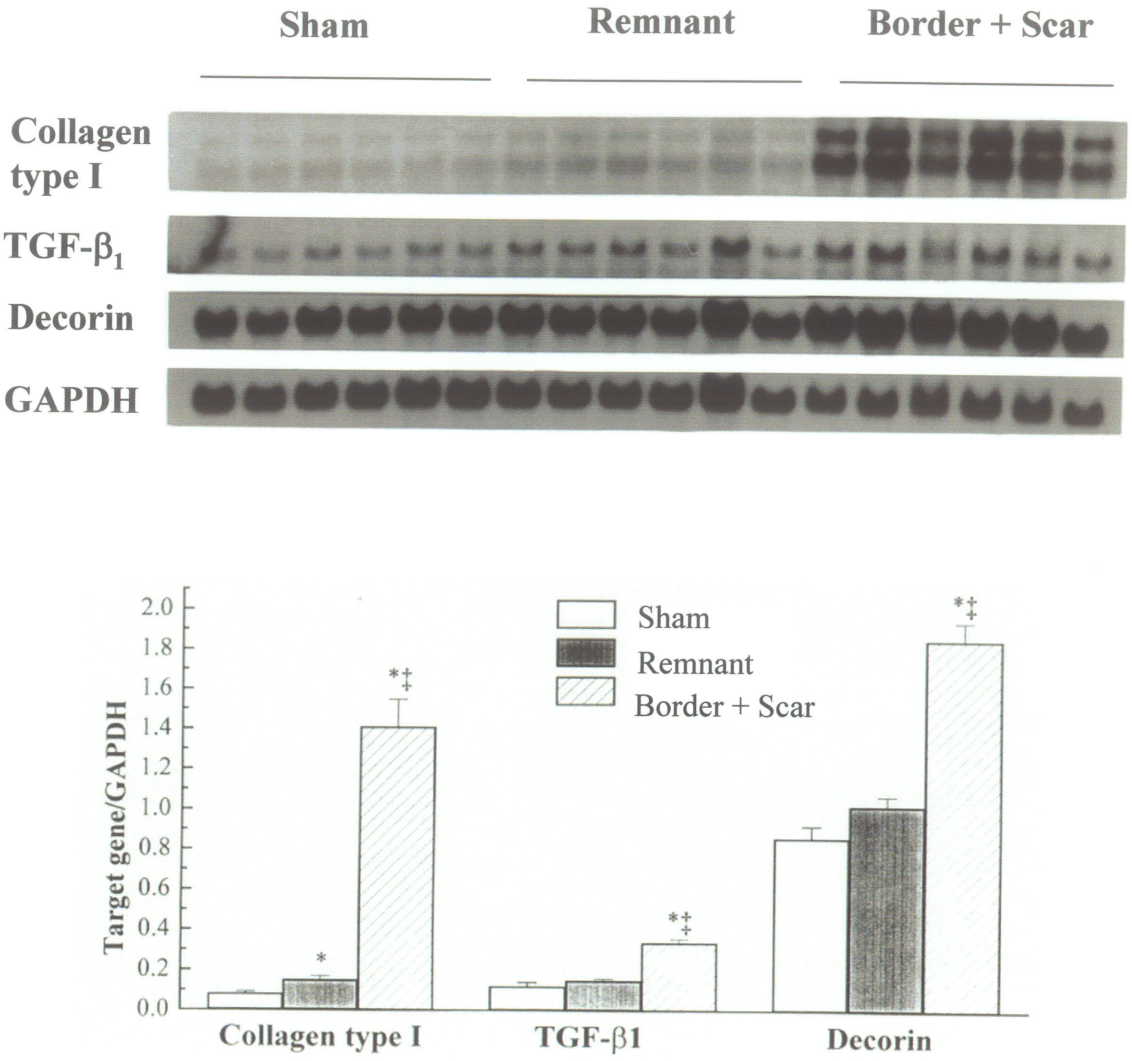

Figure 2. Upper panel. A representative autoradiograph from Northern blot analysis showing transforming growth factor-β_1 (TGF-β_1), collagen type I, decorin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) bands in sham, viable(remnant) as well as border and scar tissues from rat hearts 8 weeks post-MI. **Bottom panel**. Quantified data of target gene/GAPDH in sham, viable, as well as border and scar tissues. The data depicted is the mean $\pm$ SEM of 6 experiments. $^{*}P < 0.05$ and $^{‡}P < 0.05$ *vs* sham and viable sample values, respectively.

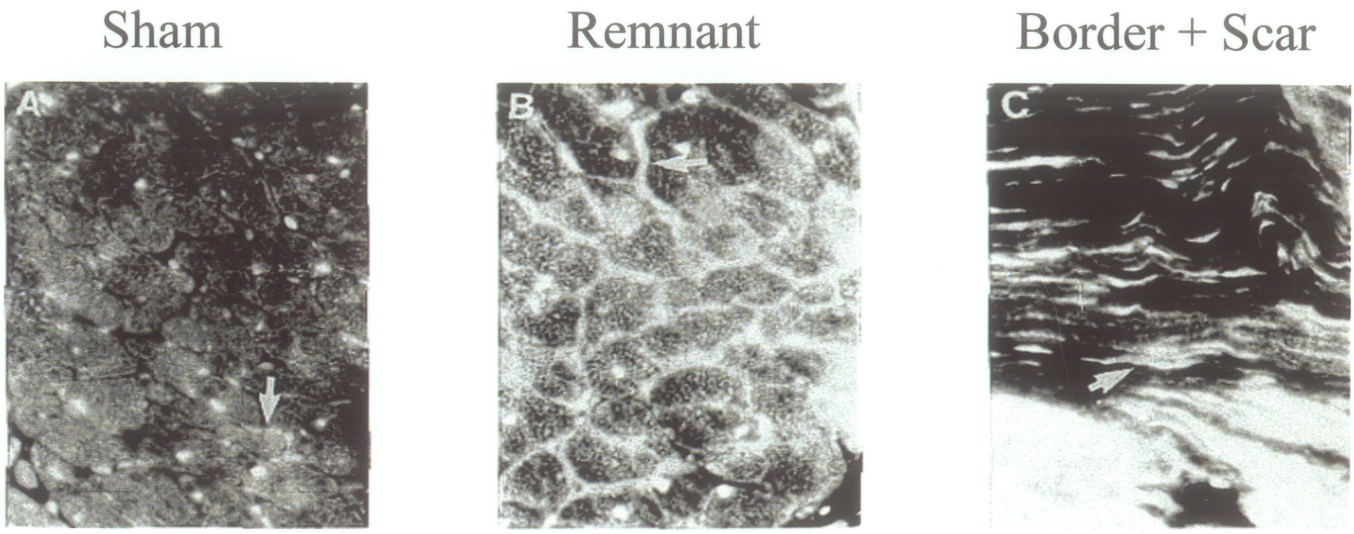

Figure 3. Immunofluorescent staining of active transforming growth factor-β_1 (TGF-β_1) in frozen tissue sections of sham-operated control hearts (A), remnant heart remote to the site of infarct (B) as well as border + scar tissues (C) from 8 week post-MI rat hearts. Active TGF-β_1 protein appears as brightly stained material in all sections; these proteins appear to be localized in the interstitial spaces between myocytes in sham and remnant sections (arrows). Magnification x 400.

Figure 4. Immunofluorescent staining of decorin in frozen tissue sections from sham-operated control hearts (A), as well as remnant tissue (B) and in the infarct scar (C) taken from 8-week post-MI rat heart. Immunoreactive decorin appears as brightly stained material; this protein is localized to the interstitial spaces in sham and remnant sections, and as relatively homogenous patches in the infarct scar. Magnification x 400.

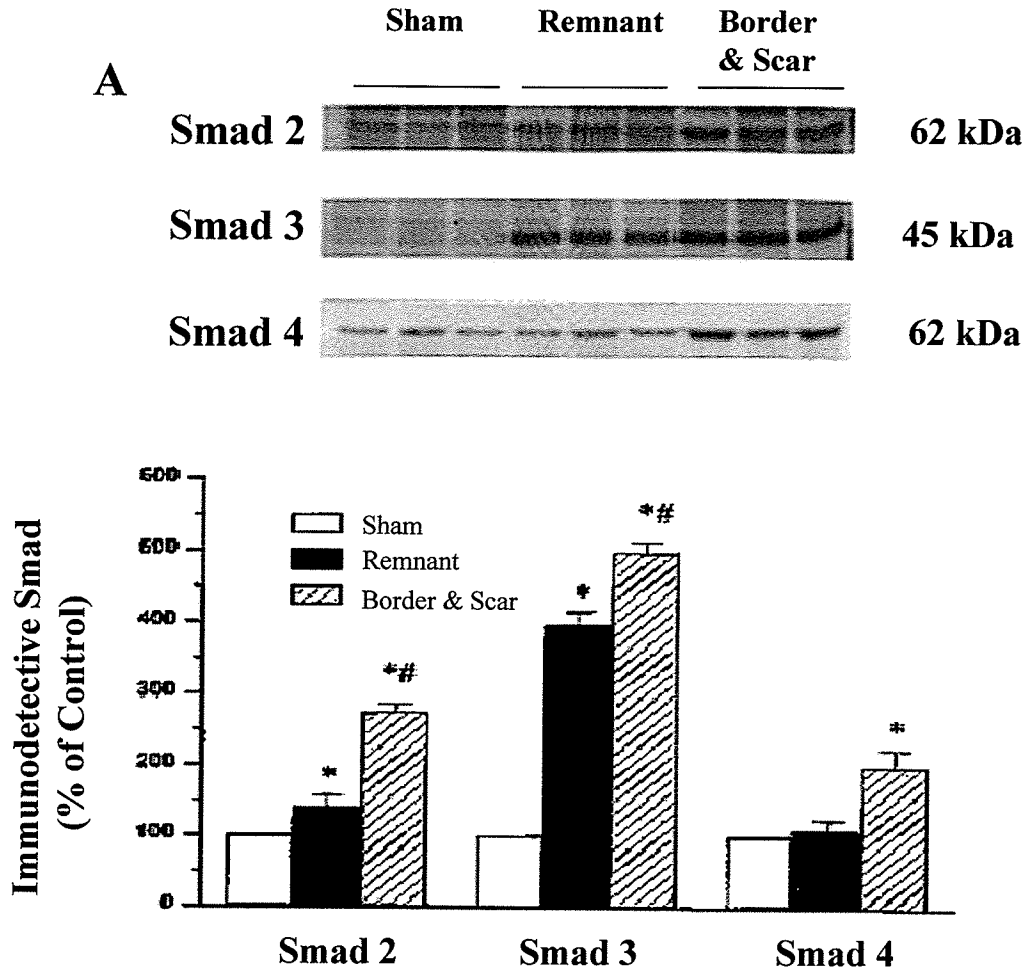

Figure 5. Western blot analysis for total Smad 2, Smad 3, and 4 in sections of age-matched sham-operated control hearts (lanes 1-3), as well as in remnant tissues (lanes 4-6) and border + scar tissues (lanes 7-9) from experimental rat hearts 8 weeks post-MI. **Panel A** is a series of representative Western blot autoradiographs indicating the 62 kDa, 45 kDa, and 62 kDa bands specific for Smad 2, 3, and 4. Similar results were obtained in 3 experiments. **Panel B** shows histograms for the quantified data of Smad protein expression (quantified by densitometric scanning), and is depicted as the mean $\pm$ SEM of 3 different experiments. *$P < 0.05$ and ‡$P < 0.05$ *vs* values from sham-operated control and remnant sample values, respectively.

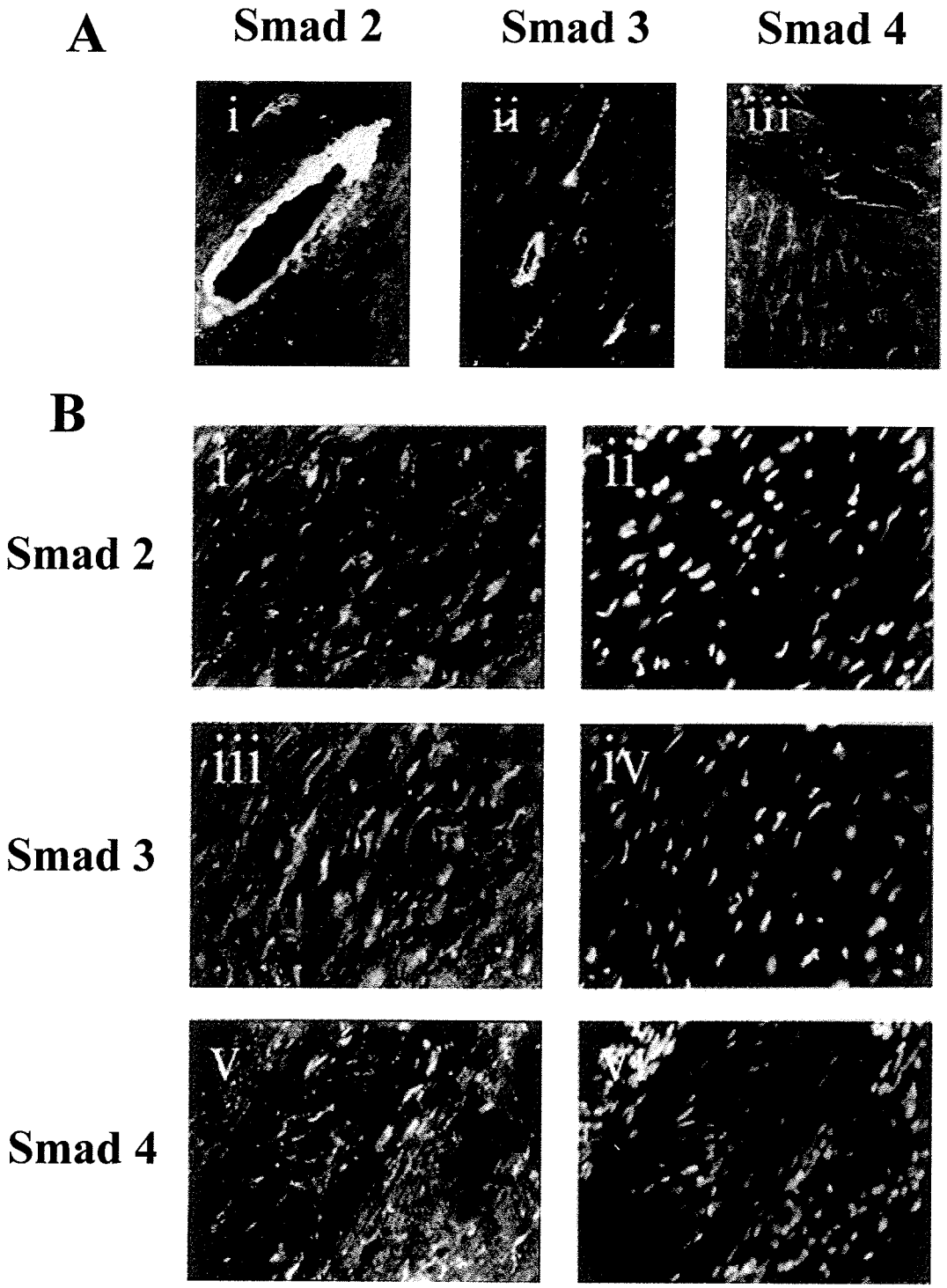

Figure 6. Smad localization in sections of remnant tissue and infarct scar from post-MI heart. Immunofluorescent staining of Smad proteins in remnant tissue remote to the site of infarct (**Panel A**) as well as infarct scar tissues (**Panel B**) from rat hearts 8 weeks after the induction of MI. **Panel A**: Immunoreactive total Smad 2 (Ai), Smad 3 (Aii), and Smad 4 (Aiii) proteins in the remnant tissue localized to the perivascular space. **Panel B**: In the infarct scar, immunofluorescent staining for Smad 2 (Bi), Smad 3 (Biii), and Smad 4 (Bv) is shown on the left; sections Bii, Biv, and Bvi depict nuclei (Hoechst 33342) from the identical fields to the immediate left, respectively. Magnification x 400.

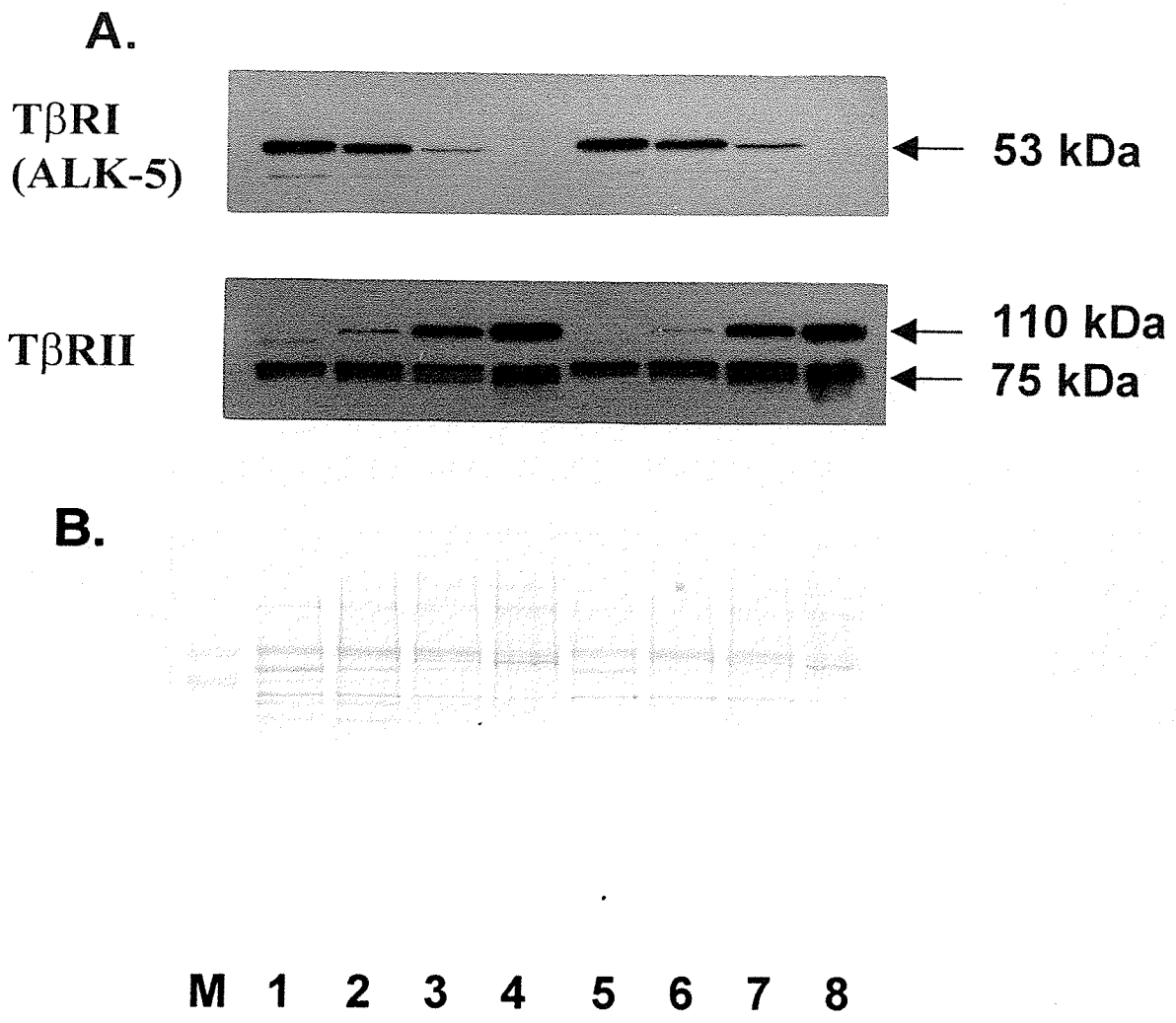

Figure 7. Western blot analysis of transforming growth factor-β receptor type I (TβRI, ALK-5) and transforming growth factor-β receptor type II (TβRII) protein concentration in sham, remnant, as well as border and scar tissues from 8 week experimental animals. **A.** Representative Western blots showing specific bands of TβRI (ALK-5, 53 kDa) and TβRII (75 kDa and 110 kDa).
Lanes 1 and 5 are sham, lanes 2 and 6 are remnant LV, lanes 3 and 7 are border tissue, and lanes 4 and 8 represent scar. **B.** Amido black staining of the PVDF membrane showing the loading of protein.

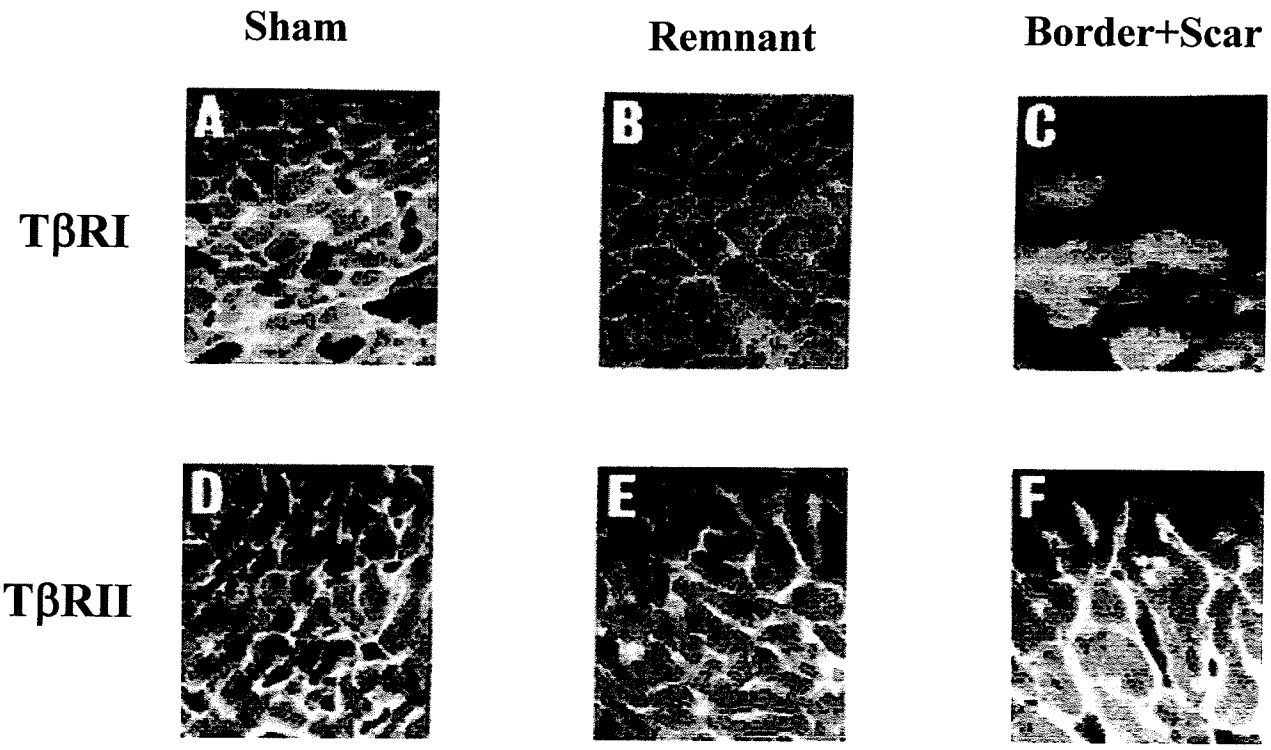

Figure 8. Immunofluoresent staining showing transforming growth factor-β receptor type I (TβRI) and transforming growth factor-β receptor type II (TβRII) in sham hearts (A and D), as well as remnant (B and E), border and scar (C and F) tissues from rat hearts 8 weeks post-MI. Immunoactive TβRI and TβRII proteins appear as brightly stained material. Magnification x 400.

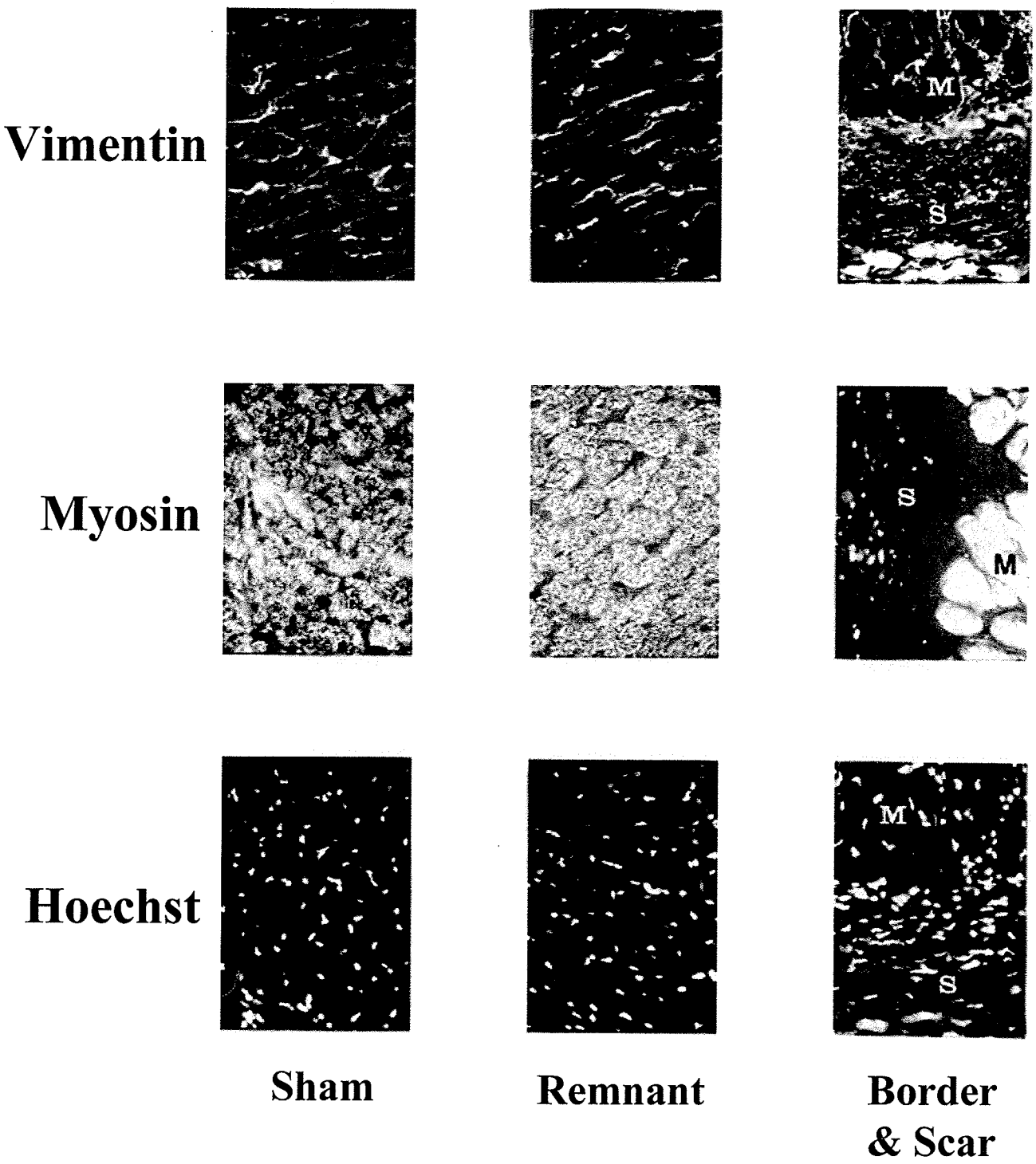

Figure 9. Representative frozen cardiac tissue sections stained for immunoreactive vimentin, myosin (MF-20), and cell nucleus (Hoechst) staining patterns in sham, viable as well as border and scar tissue from animals at 8 weeks after myocardial infarction. "M" depicts the myocytes occupying the border region; "S" depicts the scar region of the field. The sections shown within the tissue regions are not derived from serial preparation. Magnification, x 400.

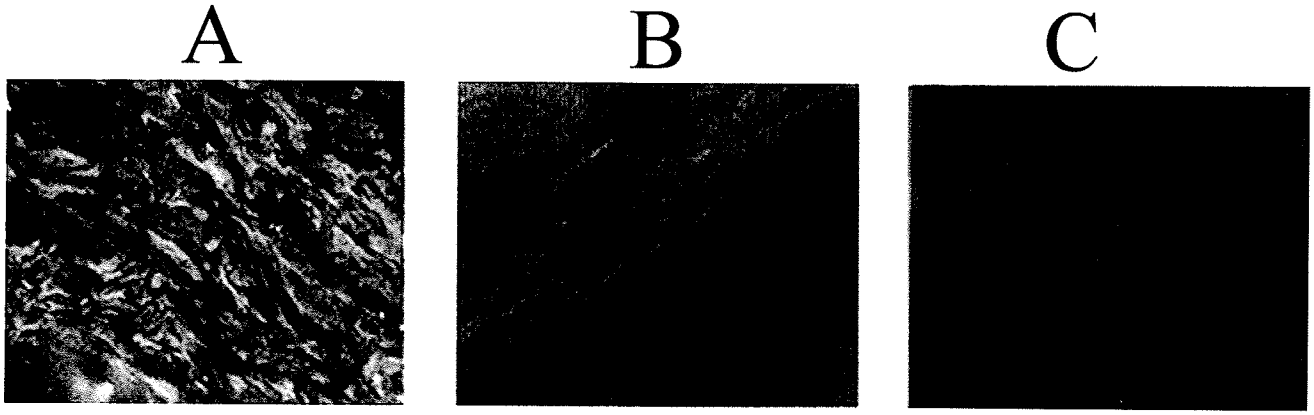

Figure 10. Characterization of myofibroblasts in the infarct scar at 8 weeks after myocardial infarction. **Panel A**. Frozen sections of infarct scar were stained with immunoreactive αsmooth muscle actin (αSMA); **panel B**. factor VIII (von Willebrand factor); **panel C**. smooth muscle myosin (SMM). Conditions for fluorescence were optimized in each field. The relatively low level of specific staining for smooth muscle myosin and negative staining pattern for factor VIII indicate minimal numbers of smooth muscle cells and endothelial cells in the 8 week infarct scar. Magnification, x 400.

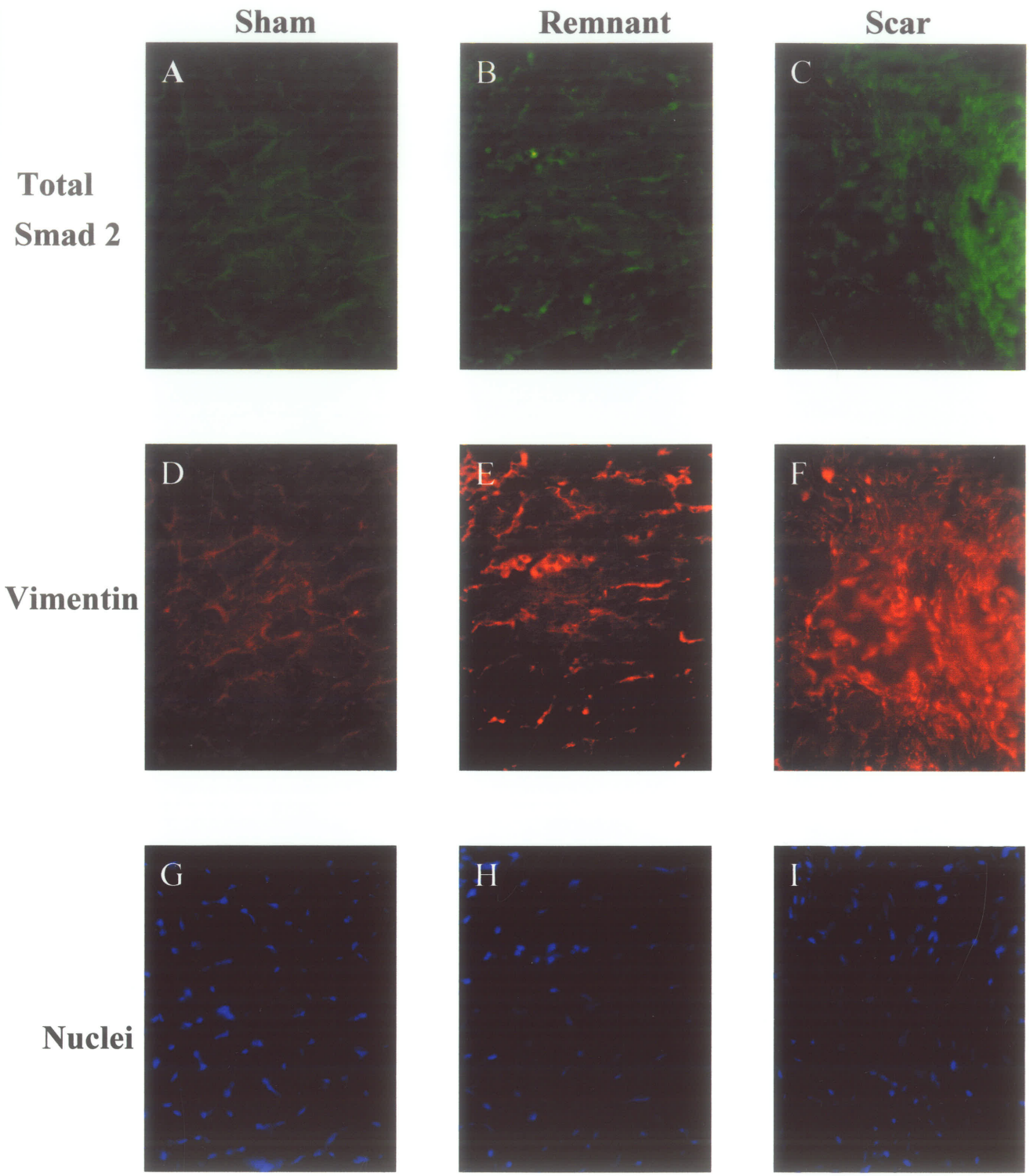

Figure 11. Immunoreactive total Smad 2 in the left ventricular myocardium from sham operated rat heart, viable (remnant) tissue and infarct scar. Experimental animals were harvested 8 weeks following surgery. The same fields as in A, B, C were double stained for vimentin (D, E and F) and for nuclei (G, H and I). In all sections, relatively bright staining of total Smad 2 protein was localized to nonmyocytes, while myocytes stained less intensely. Vimentin is a common marker of nonmyocyte cells, including smooth muscle (desmin-positive) cells. Magnification x400.

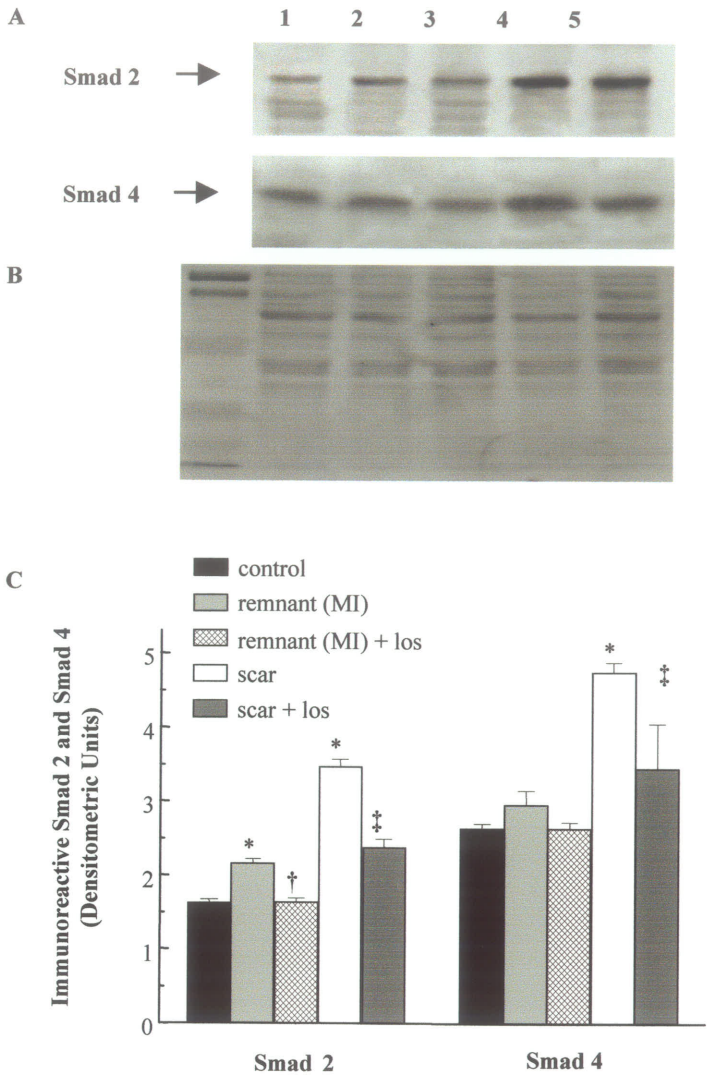

Figure 12. Western blot analysis of total Smad 2 and Smad 4 in 8 week post-MI heart tissue. **Panel A:** Representative Westerns for Smad 2 and Smad 4 from sham-operated control hearts (lane 1) as well as remnant tissue (lane 2), remnant tissue with 8 week losartan treatment (lane 3), infarct scar tissue (lane 4) and infarct scar tissue with 8 week losartan treatment (lane 5) from 8 week post-MI left ventricular samples. **Panel B:** Membrane from Panel A stained with Coomassie Blue to verify relatively even protein loading. **Panel C:** Histographic representation of quantified data of Smad 2 and Smad 4 expression in respective groups in A (quantified by densitometric scanning). The data depicted is the mean ± SEM of 4-6 experiments. $P \leq 0.05$ is expressed by * *vs* sham, † *vs* remnant and ‡ *vs* scar values.

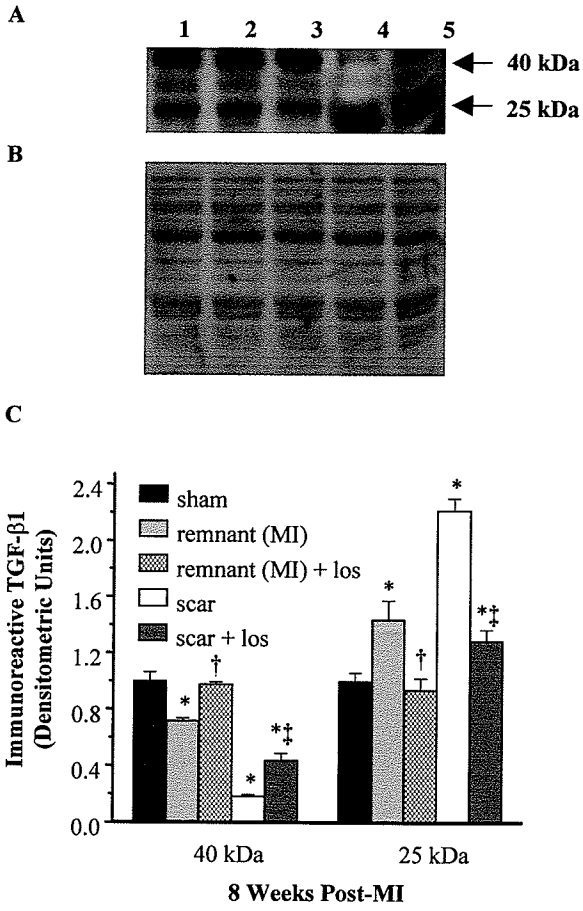

Figure 13. Western blot analysis of TGF-β_1 in 8 week post-MI heart tissue. **Panel A:** Representative Western of latent (40 kDa) and active TGF-β_1 (25 kDa) protein in from sham-operated control hearts (lane 1) as well as remnant tissue (lane 2), remnant tissue with 8 week losartan treatment (lane 3), infarct scar tissue (lane 4) and infarct scar tissue with 8 week losartan treatment (lane 5) from 8 week post-MI rat heart left ventricular samples. **Panel B:** Membrane from panel A stained with Coomassie blue to verify relatively even protein loading. **Panel C:** Histograms of quantified data from multiple samples from the groups in A. The data depicted is the mean $\pm$ SEM of 4-6 experiments. $P \leq 0.05$ is expressed by * *vs* sham, † *vs* remnant and ‡ *vs* scar values.

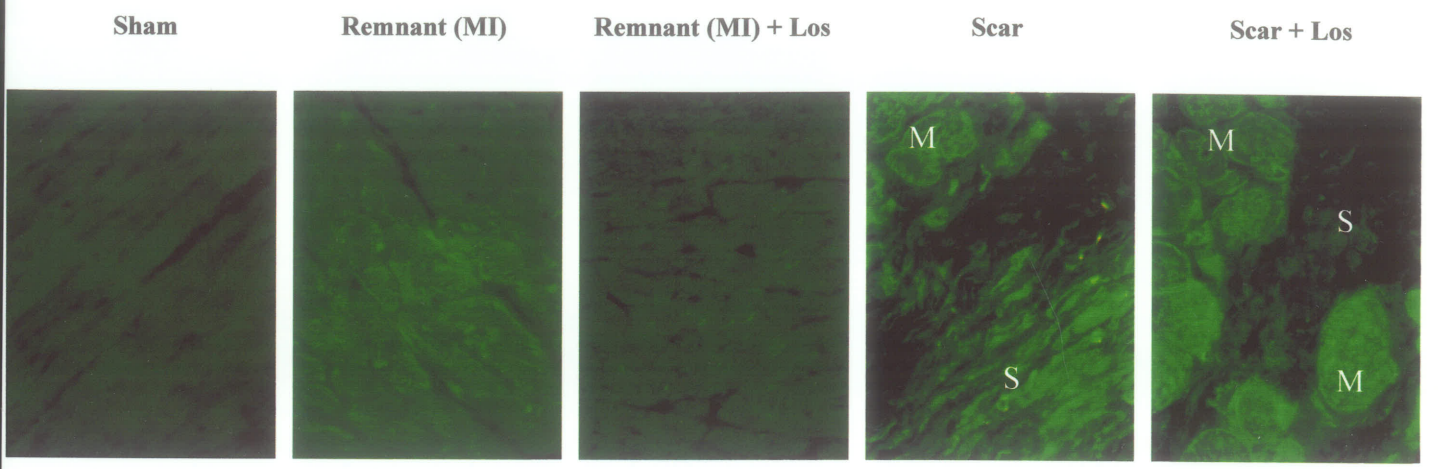

Figure 14. Localization of total TGF-β_1 in sham-operated control hearts (sham), remnant tissue, remnant tissue with 8 week losartan treatment (remnant + los), infarct scar tissue (scar) and infarct scar tissue with 8 week losartan treatment (scar + los). Experimental animals were harvested 8 weeks after surgery. Immunoreactive TGF-β_1 protein appears as brightly stained material. Scar tissue and remnant myocytes bordering the infarct scar are marked by S and M, respectively. Magnification x 400. Los; losartan (15 mg/kg/day).

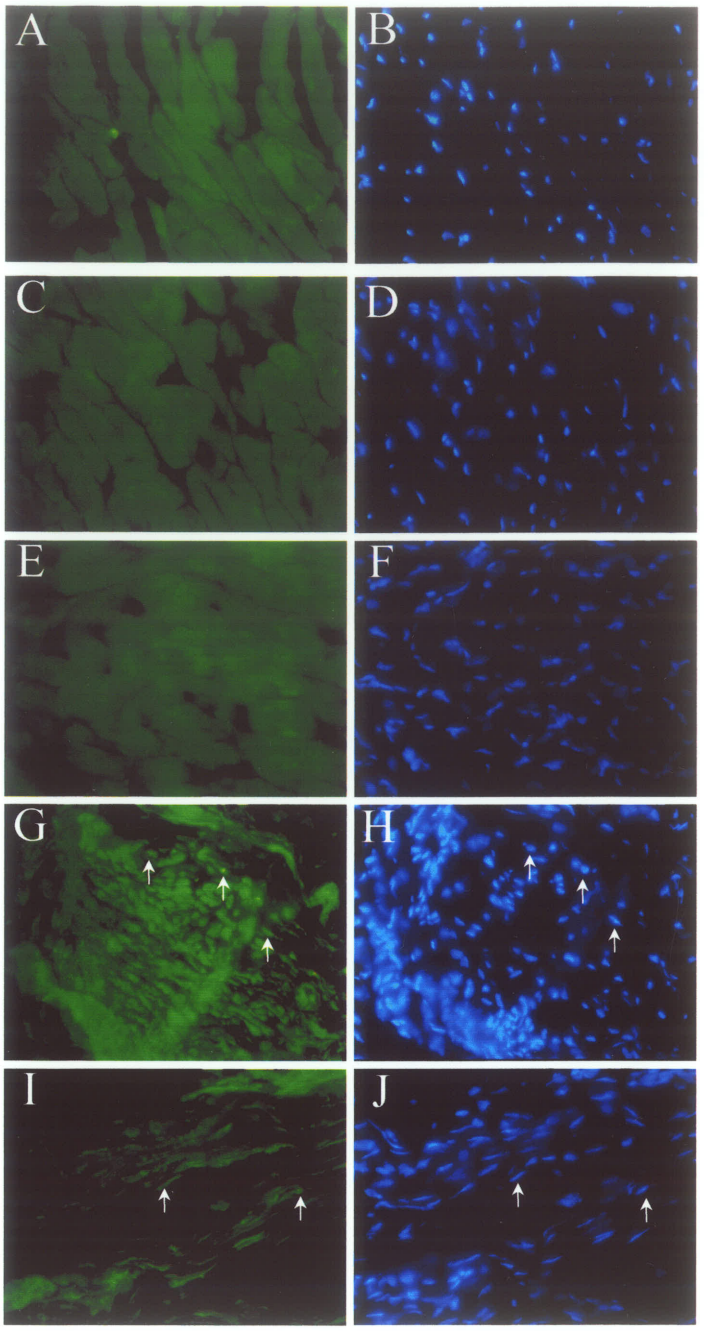

Figure 15. Phosphorylated Smad 2 localization in untreated and losartan-treated sections of remnant tissue and infarct scar in 8 week post-MI heart. Immunoreactive phosphorylated Smad 2 in sham-operated control hearts (A) as well as remnant tissue (C), remnant tissue with 8 week losartan treatment (E), infarct scar tissue (G) and infarct scar tissue with 8 week losartan treatment (I) from 8 week post-MI rat left ventricle. Fields depicted in panels A, C, E, G and I were stained for cellular nuclei with Hoescht 33342 and are shown in panels B, D, F, H and J, respectively. Arrows indicate focal bright staining for nuclei and phosphorylated Smad 2 in identical cells from corresponding panels. Magnification x 400.

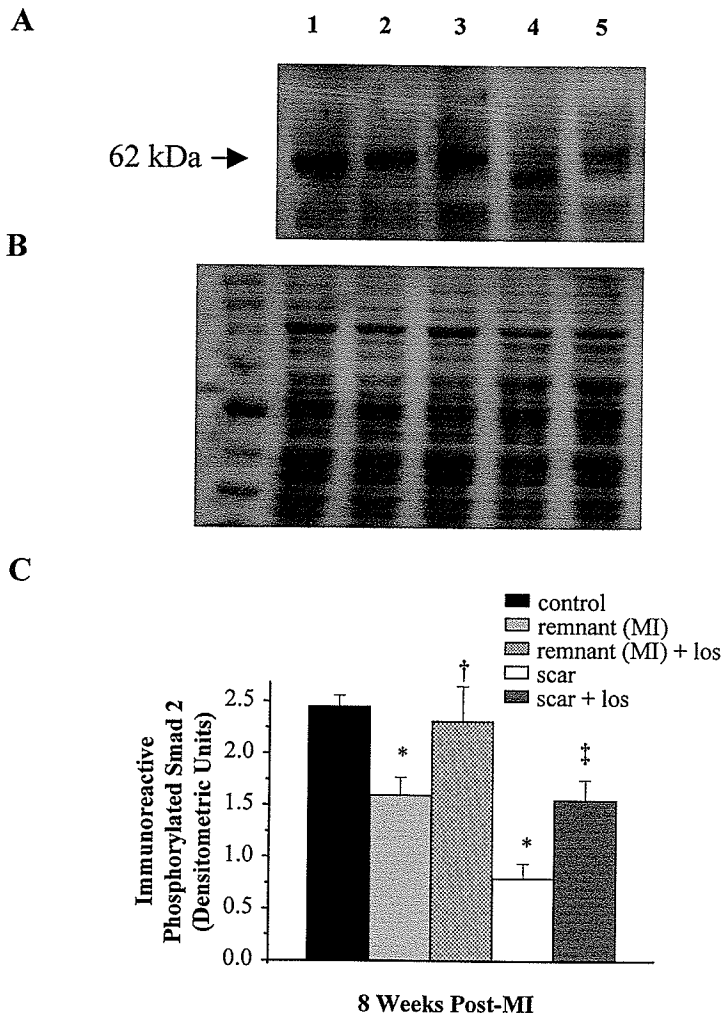

Figure 16. Western blot analysis of phosphorylated Smad 2 in 8 week post MI hearts. **Panel A.** Representative Western blot of cardiac tissue cytosolic fractions probed for phosphorylated Smad 2 in sham left ventricular tissue (lane 1), remnant myocardium (lane 2), remnant tissue treated for 8 weeks with losartan (3), scar tissue (lane 4) and from scar tissue with 8 week losartan treatment (lane 5). Experimental animals were harvested 8 weeks after surgery. **Panel B.** Membrane from Panel A stained with Coomassie blue to verify relatively protein loading. **Panel C.** Histograms for quantified data from multiple samples from the groups in **A** (quantified by densitometric scanning). The data depicted is the mean $\pm$ SEM of 4-6 experiments. $P \leq 0.05$ is expressed by * *vs* sham † *vs* remnant and ‡ *vs* scar values.

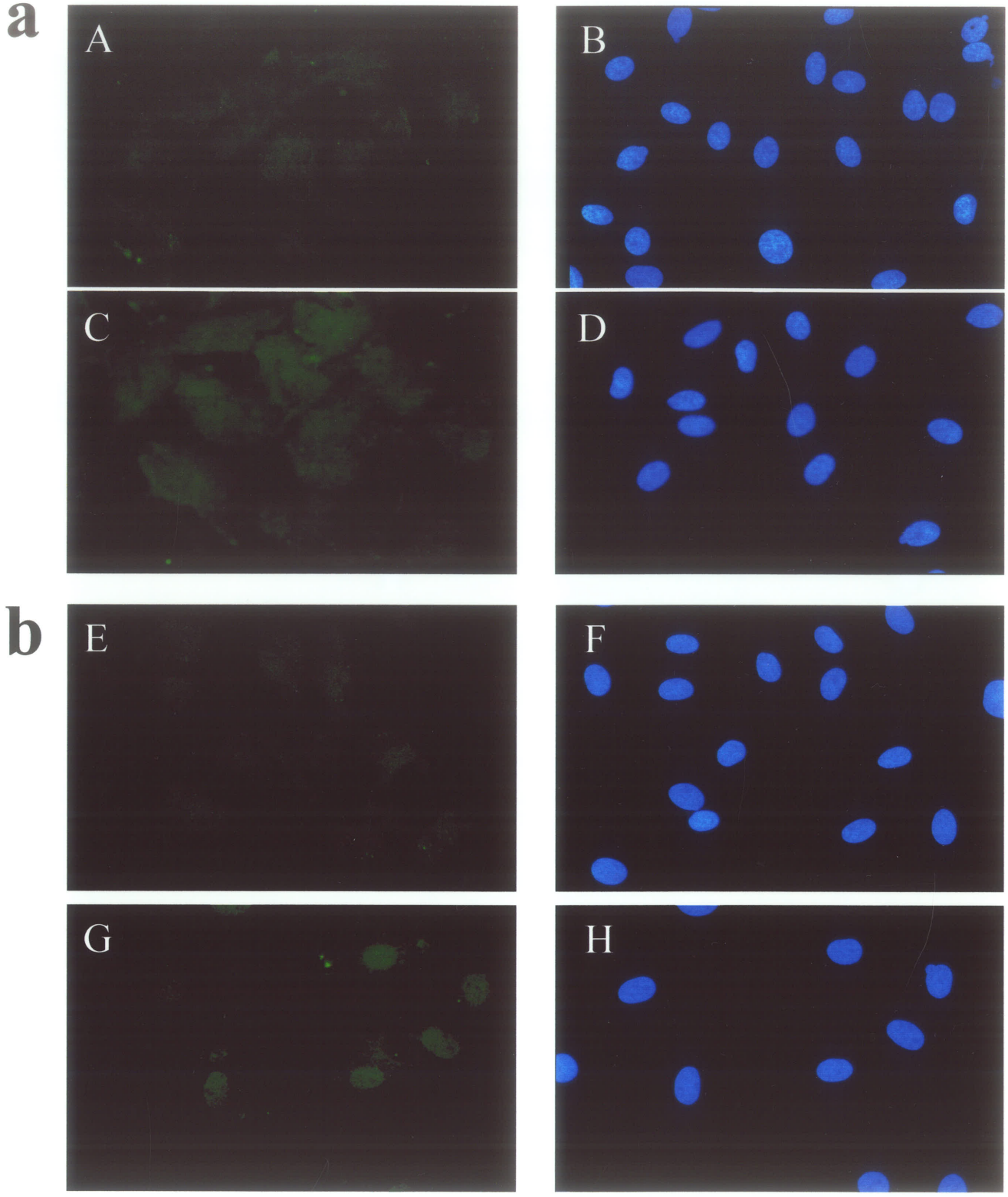

Figure 17. Immunofluorescent staining of total Smad 2 (**Section a**) and phosphorylated Smad 2 (**Section b**) from cultured cardiac fibroblasts stimulated by angiotensin II (10^{-6} M) for 15 min. Panels A and E represent untreated fibroblasts and panels C and G show angiotensin II treated fibroblasts. Panels B, D, F and H represent nuclei (Hoechst 33342 staining) of identical sections corresponding to panels A, C, E and G, respectively. Magnification x 400.

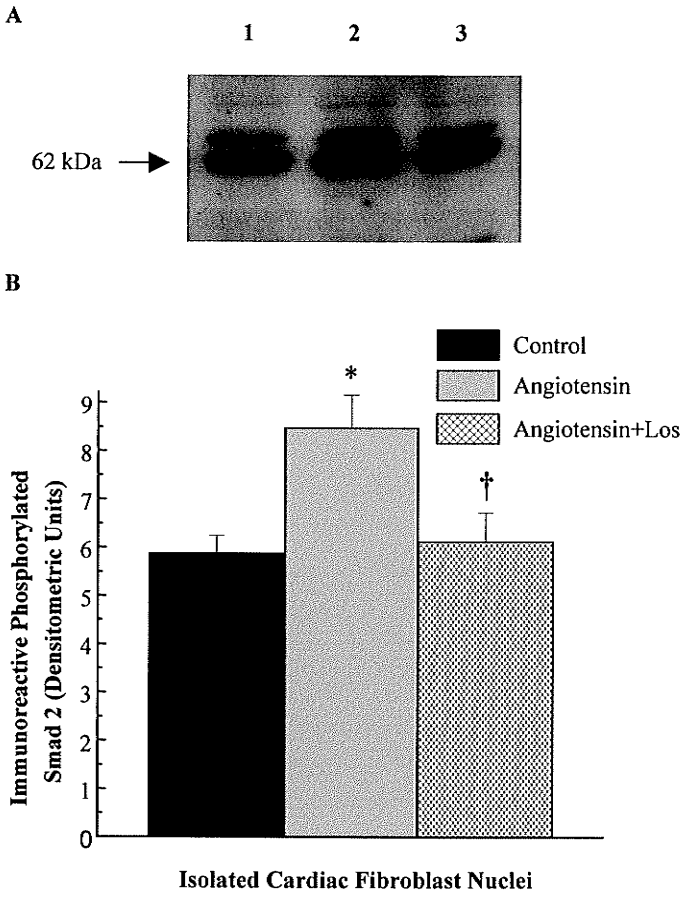

Figure 18. Western blot analysis of phosphorylated Smad 2 in cardiac fibroblast nuclei **Panel A.** Representative Western blot for phosphorylated Smad 2 in nuclei isolated from cultured cardiac fibroblast cells. The 62 kDa band shown represents nuclear phosphorylated Smad2 from untreated control cells (lane 1), 15 minute angiotensin II (angiotensin,10^{-6} M) stimulated cells (lane 2), and 15 minute angiotensin (10^{-6} M) stimulated cells with losartan (10^{-6} M) treatment (lane 3). **Panel B.** Histographic representation of quantified data from multiple samples from the groups in **A**. The data depicted is the mean ± SEM. of 3 experiments. $P < 0.05$ is expressed by * *vs* untreated control and † *vs* Angiotensin.

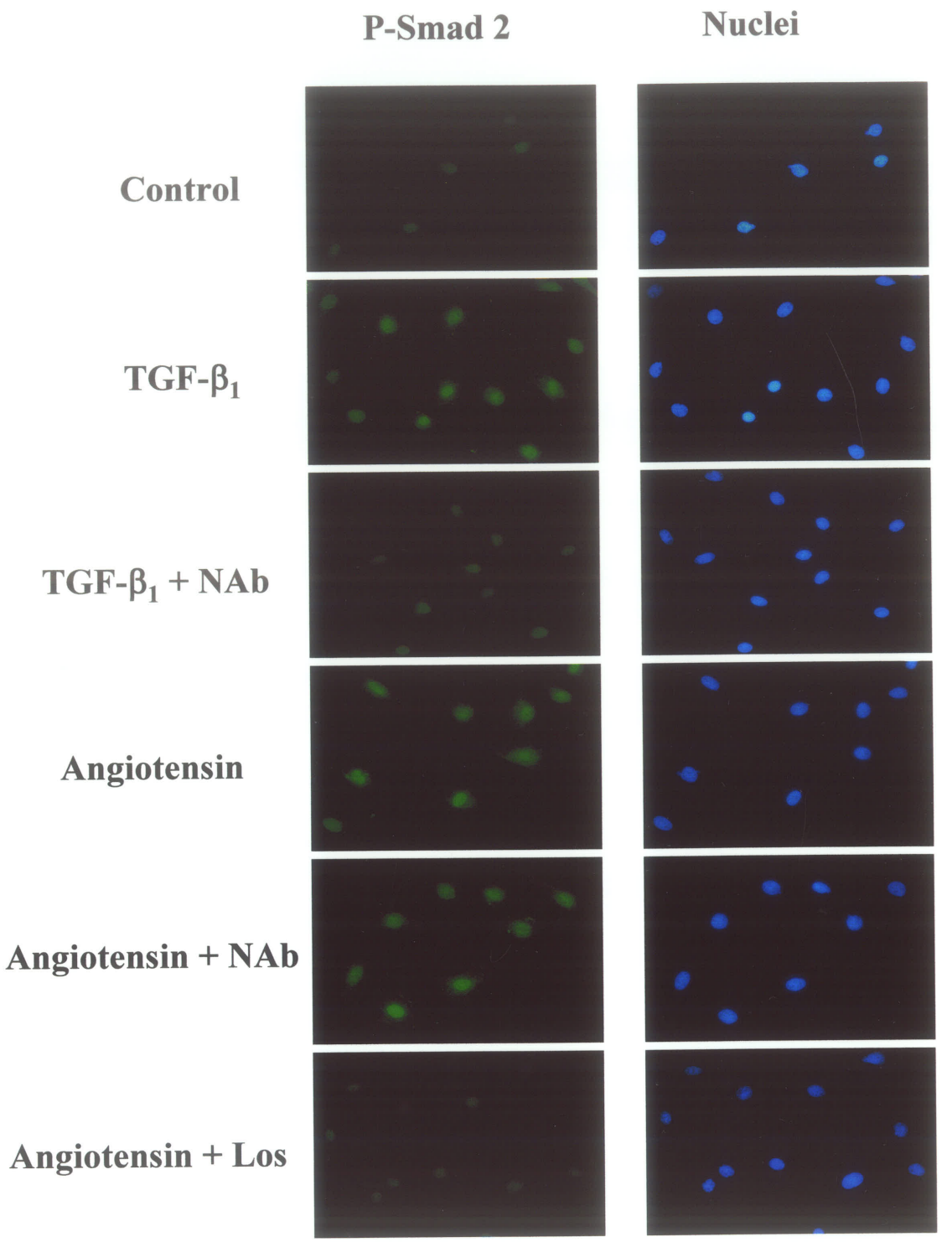

Figure 19. Effect of angiotensin II (angiotensin) and TGF-β_1 on the nuclear accumulation of phosphorylated Smad 2 in cultured cardiac fibroblasts. The left panels show staining of immunoreactive P-Smad 2 and the right panels show nuclear staining (Hoescht 33342). Indicated treatments were performed as follows: cells were treated with angiotensin (10^{-6} M) or TGF-β_1 (10 ng/ml) for 15 min in the presence or absence of TGF-β neutralizing antibody (NAb, 1.5 μg/ml). In the losartan treated group, losartan (Los, 10^{-6} M) was administered 1 hour before angiotensin treatment. Magnification x400.

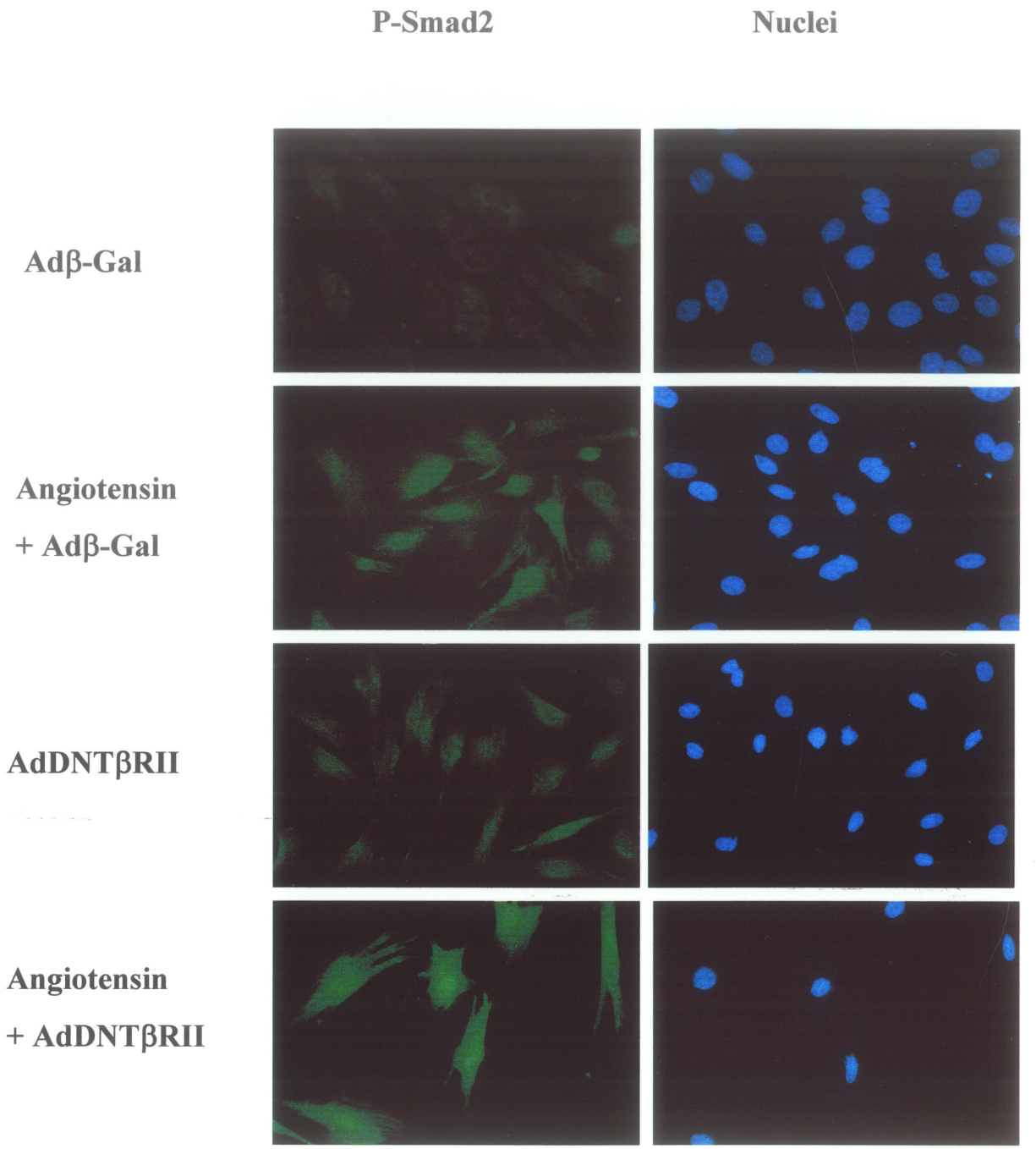

Figure 20. Effect of angiotensin II (angiotensin) on the localization of phosphorylated Smad 2 (P-Smad 2) in cardiac fibroblasts infected with adenoviral dominant-negative type II TGF-β_1 receptor (AdDNTβRII). Panels on the left depict cells immunostained for phosphorylated Smad 2, and right panels show nuclear staining (Hoechst 33342) of the corresponding fields of view. Cultured cardiac fibroblasts were infected with AdDNTβRII or β–Gal control adenovirus (Adβ Gal) at MOI=25 and treated with angiotensin II (15 min, 10^{-6} M) where indicated. Magnification x400.

Chapter 5. DISCUSSION

5.1 Experimental Model of Congestive Heart Failure

Animals with a relatively large infarct 8 weeks post-MI were considered to be in the moderate heart failure group based on current data and previous observations (246,247). Using this model, we have previously observed significant elevation in the deposition of cardiac collagen, in addition to the persistence of myofibroblasts in the remnant myocardium and scar tissue (247,251). Chronic scar remodeling has been shown to play a role in the functional preservation of the infarcted ventricle (252).

Elevated LVEDP, decreased time to peak pressure development and decay ($\pm dP/dt_{max}$) and the presence of pulmonary congestion was confirmed in 8 week experimental animals (due to a flattening of pressure wave). Although these were taken to reflect significant disruption of normal cardiac function, these animals did not display overt dyspnea, cyanosis, or marked lethargy and thus were considered to be in "moderate heart failure", as previously demonstrated (28,230). This classification is based on our observations of the development of post-MI heart failure in rats with relatively large MI ($\geq$ 40% LV free wall) and provides an arbitrary classification system to facilitate the comparison of differently timed experimental groups (28,230). The incidence of cardiac hypertrophy in 8 week post-MI rat hearts, as indicated by increased LV weight and the ratio of LV to body weight, was also apparent when compared to the non-infarcted controls. Significantly elevated deposition of cardiac collagens in the remnant tissue (cardiac fibrosis) and scar tissues (in chronic phase healing of the infarct scar) in post-MI

hearts was confirmed in this investigation and was similar to the patterns of fibrosis observed in our previous studies (28,53). As the mechanical properties of the infarct scar are critical for cardiac function after MI (253), any alteration in its structure may influence cardiac function. Thus, we chose to examine this stage of MI-associated heart failure to provide insights into the events associated with ongoing wound healing in various regions of the infarcted heart, including the infarct itself.

5.2 Fibroblasts, Myofibroblasts and Cardiac Fibrosis

Following MI, fibroblasts arrive at the site of repair where they undergo phenotypic transformation to myofibroblasts, a process inducible by TGF-β_1 (254). Myofibroblasts express α-smooth muscle actin (α-SMA), providing contractility and chronic mechanical tension to the remodeling scar (254). As vimentin is expressed by cardiac nonmyocytes but not by myocytes, we used immunoflourescent staining for the detection of this protein as a first step to highlight the distribution of these broad classes of cells in infarct scar tissue. We used vimentin as a marker of nonmyocytes and MF-20 (myosin) as a marker for myocytes. Our results showed that the infarct scar was populated with cells which stained positively for vimentin and negative for myosin, thus indicating the absence of myocytes in the infarct zone (Fig. 9). Phenotyping of the vimentin-positive cells of the infarct scar revealed significant positive staining for α-SMA (Fig. 10), which is not expressed by fibroblasts. The relatively low level of specific staining for smooth muscle myosin (SMM) and negative staining pattern for factor VIII indicates minimal numbers of smooth cells and endothelial cells in the 8-week post-MI infarct scar (Fig. 10). These results indicated that myofibroblasts are the predominant cell type in 8 week post-MI scar tissue in rats, which is in consistence with previous studies

(34,43). It has been known that myofibroblasts have a high synthetic capacity for fibrillar collagens and express cytokines including angiotensin and TGF-β_1 (30). These cells also express angiotensin receptors as well as TGF-β_1 receptors which potentiate fibroproliferative behavior (255). Thus our results, together with other other studies(33,34,43), sguuest that and are major players in the formation of infarct scar structure in the post-MI heart.

5.3 Activation of the TGF-β Signaling Pathway in Post-MI Heart

TGF-β contributes to an array of biological functions including regulation of ECM production, wound repair and growth inhibition (16,256). These phenomena are mediated through transmembrane TGF-β receptors (TβRI and TβRII) that display serine/threonine kinase activity (24). In experimental left ventricular hypertrophy, the selective increase in expression of TGF-β_1 *vs.* either TGF-β_2 or TGF-β_3 subtypes suggests that the former sub-type is important in the pathogenesis of cardiac disease associated with hypertrophy (17). TGF-β receptor activation occurs upon the binding of TGF-β to TβRII, which then recruits and phosphorylates TβRI (26). It is now clear that phosphorylated Smad 2 (or Smad 3) proteins mediate the TGF-β_1 signal transduction via binding of both Smad 4 (24,25,83) and FAST-1, a eukaryotic nuclear transcription factor (257). The C-terminal region of FAST-1 protein binds specifically to Smad 2 (258) and the heterotrimeric complex is required for initiation of TGF-β_1 mediated gene transcription. The phosphorylated TβRI is activated and phosphorylates cytosolic Smad 2 or possibly Smad 3 (24,80). Phosphorylated Smad 2 (and/or Smad 3) then form(s) a heterotrimeric complex with Smad 4 and this complex accumulates in the nucleus leading to the activation of target gene expression (24,25,83).

Interstitial fibrosis and attendant decreases in compliance of the surviving myocardium are believed to contribute to the occurrence of cardiac dysfunction (1) and it has become clear that the scar size is a reliable marker for the development of heart failure post-MI (259). Although gross morphological examination of experimental hearts has indicated that scar formation is completed 3 weeks after MI (260), more detailed investigation suggests that the scar is not quiescent even 8 weeks after MI (28). As opposed to interstitial fibrosis of the remnant heart, normal fibrosis in the healing of the infarct scar may help to preserve ventricular function (253). Although TGF-β stimulates ECM production which is involved in the development of heart failure in post-MI heart (261), alteration of downstream Smad proteins in this pathology is unknown. The present study represents a first step in this regard and our results support the hypothesis that elevated expression of TGF-β_1 (both mRNA and protein) is positively correlated to the increased cardiac Smad protein expression, chronic phase healing of the infarct scar and overt fibrosis of the remnant myocardium. Thus, it may be possible that activation of Smads 2, 3 and 4 expression in post-MI heart is contributory to the ongoing scar remodeling. Although the precise significance of the predominance of Smad proteins in the cellular nuclei within the infarct scar is unclear, they may participate in the stimulation of expression of the matrix genes i.e., fibrillar collagens. We suggest that by this mechanism, scar structure is chronically influenced in heart failure after MI. It is likely that balanced chronic infarct remodeling and marginal compensation of cardiac function is not maintained in the presence of large MI, as the functioning of experimental hearts rapidly deteriorates to overt decompensation by 16 weeks (230). In the infarct scar, myofibroblasts have been shown to be the predominant cell type in post-MI scar tissue in rat heart (36,40) and are likely candidate cells for the bulk of Smad protein expression in this tissue (34,43).

It has been reported that angiotensin concentration and AT_1 receptor density in myofibroblasts are significantly increased in the scar tissue post-MI (35,200,262). As angiotensin has been implicated in the stimulation of cardiac fibroblast proliferation (154,168,188), the net proliferation of myofibroblasts may depend upon a balance between TGF-β signaling and other trophic factors, i.e., angiotensin, during post-MI wound healing.

Decorin is a proteoglycan which is expressed in heart and is known to sequester TGF-β_1 in the extracellular matrix of different organs (263). The current finding, with regard to decorin, is the first to describe altered expression of this proteoglycan in experimental heart failure. The significance of increased steady-state abundance of decorin mRNA and altered localization of immunoreactive decorin in 8 week post-MI hearts is unclear. Decorin may act as an effector molecule in a negative feedback loop that regulates TGF-β_1 (264). Thus, increased expression of decorin may lead to inhibition of the action of TGF-β_1 *de facto* by binding this cytokine in the extracellular matrix (265).

The significance of differential regulation of TβRII (increased) and TβRI (decreased) receptors in border and infarct scar, is not easily explained by the current model of TGF-β receptor signaling. As TβRI may act as a downstream component of TβRII (26,64,266), reduced expression of either receptor subtype may simply confer a loss of TGF-β_1 responsiveness in target cells. On the other hand, recent evidence supports the view that the antiproliferative and fibrotic effects of TGF-β1 may be modulated independently in smooth muscle cells by selective changes in the TβRI/TβRII ratio (267). Massague has speculated that the type II receptor may confer the signal independently of the type I receptor by phosphorylating as yet unknown substrates (64). The downregulation of TβRI may be due to the reciprocal regulation by stimulation of high concentrations of TGF-β_1 in the scar and border tissues. This view is supported by the

demonstration that pre-exposure of osteoblasts to TGF-β_1 is associated with decreased receptor density (268). Conversely, TGF-β-mediated upregulation of TβRII receptors in vascular smooth muscle cells has been observed (269) and this autostimulatory mechanism may partially explain the expression in remnant border and infarct scar tissues in post-MI hearts.

This data indicate that increased Smad protein expression as well as increased TGF-β_1 expression in infarct scar tissue in the chronic phase of post-MI hearts is positively correlated to elevated deposition of cardiac collagens. We suggest that overexpression of Smad proteins are involved in ongoing extracellular matrix remodeling of the infarct scar and remnant myocardium in post-MI hearts by cardiac myofibroblasts and that these changes are involved in the progression of heart failure. It follows that blockade of TGF-β signaling may inhibit TGF-β_1-mediated fibrosis in post-MI heart. In fact, our previous studies have shown that a continued increase of TGF-β_1 expression as well as Smad 2 activation was accompanied by a decreased expression of Smad 7 in the infarct scar at 2 week and 4 weeks post-MI hearts (70), suggesting a loss of inhibitory effect on TGF-β_1 signaling in normal wound repair. In addition, overexpression of Smad 7 in cultured cardiac fibroblasts resulted in a decreased collagen expression (70). Thus, the direct regulation of cardiac Smad protein expression and activation may represent a novel therapeutic approach for modulating fibrotic events in post-MI hearts.

5.4 Crosstalk Between Angiotensin and TGF-β_1 in Post-MI Heart

Angiotensin has been shown to stimulate cardiac fibrosis in several different models of heart failure (214,216,270,271). Furthermore, angiotensin stimulates collagen production in cultured cardiac fibroblasts (272). Its expression and AT_1 receptor density in myofibroblasts of the infarct scar are significantly increased (273,274). We have

demonstrated that AT_1 blockade is associated with partial attenuation of cardiac fibrosis in post-MI rats (275,276), however, the precise mechanism of the antifibroproliferative effect of this therapeutic intervention is unclear. Mounting evidence supports the existence of putative crosstalk between angiotensin and TGF-β_1 at the level of ligand expression in cultured cells including adult primary cardiac fibroblasts (217,218). Furthermore, AT_1 receptor blockade has been shown to be associated with increased steady-state abundance of TGF-β_1 mRNA observed in the 4 week post-MI rat heart (219). These findings support the hypothesis that AT_1 modulation of TGF-β_1 ligand may occur in cardiac fibroblasts. Nevertheless, a role of angiotensin at the post-receptor levels of TGF-β_1 signaling has not been identified.

TGF-β is secreted as an inactive precursor complex containing a signal peptide, the active TGF-β_1 molecule, and the cleaved propeptide known as the latency associated peptide (LAP) (277). Following removal of the signal peptide, the gene product undergoes proteolytic cleavage to produce mature TGF-β_1 (residues 279-390) and LAP (residues 30-278) (249,277). We found that the active form of TGF-β_1 (25 kDa) was significantly elevated in remnant (viable) and scar tissues, whereas the LAP (~40 kDa in monomeric form as seen in a reducing gel) latent form of TGF-β_1 was decreased *vs.* the control in heart failure. This indicates a redistribution in expression of active TGF-β_1/LAP ratio in the remnant myocardium and infarct scar. As losartan treatment led to a normalization of this trend, AT_1 activation may play a role in relative activation of TGF-β_1 in experimental hearts and thus regulate the bioavailability of the active TGF-β_1 molecule.

These results indicate that elevated Smad expression in experimental heart failure is normalized by long-term AT_1 receptor blockade and that these changes are paralleled by modulation of fibroproliferative events in these hearts. Furthermore, AT_1 activation is associated with augmented nuclear accumulation of phosphorylated Smad 2 in failing hearts and with angiotensin stimulation of cultured cardiac fibroblasts. The current results also provide a link between angiotensin receptor activation and potentiation of Smad protein function in cardiac fibroblasts.

5.5 Angiotensin Activates R-Smad 2 in Cultured Cardiac Fibroblasts Independent of Activation of TGF-β Receptors

It is clear that the phosphorylation and subsequent nuclear translocation of Smad 2 is required for regulation of transcription in TGF-β signaling in mammalian cells. Our data has shown that P-Smad 2 is upregulated in the infarct scar 8 weeks after MI. However, the effect of angiotensin on the activation of R-Smad 2 (phosphorylation/nuclear translocation) in cardiac fibroblasts and post MI heart has not been reported. In this study we noted an increased total Smad 2 and a decreased P-Smad 2 in cytosol sections from remnant and scar tissues post-MI. This suggesting an increased nuclear accumulation of P-Smad 2 and these trends were normalized by AT_1 receptor blockade (losartan 15 mg/kg/day for 8 weeks). Our *in vitro* study demonstrated that angiotensin (10^{-6} M) stimulation of cultured adult rat cardiac fibroblasts was associated with an elevation of total Smad 2 protein. Furthermore, the presence of angiotensin caused an increased nuclear accumulation of P-Smad 2 in fibroblasts, as indicated by immunofluorescent staining and Western analysis. The protein level of P-Smad 2 in

nuclei isolated from cardiac fibroblasts increased following angiotensin stimulation, an effect that was blocked by AT_1 receptor blockade. Taken together, these results indicate a possible link between angiotensin and the phosphorylation and nuclear translocation of Smad 2. The molecular mechanism underlying this link is not yet clear and it is currently unknown whether this action is dependent or independent of TGF-β_1 ligand. It has been reported that Smad 2 activation may not be restricted to TGF-β receptors (278) and our data suggest a direct role for angiotensin in this regard. Recently, Janus N-terminal kinase (JNK) activity has been shown to cause phosphorylation of the C-terminal tyrosines on receptor-activated Smads (279). Furthermore, AT_1 activation causes a rapid increase (5 min after stimulation) in JNK activity in cardiac cells in dose-dependent manner (90). Additionally, Smad nuclear accumulation can by inhibited by Ras-activated Erk kinases (280), which can be activated by angiotensin in cardiac fibroblasts (281,282). Thus, it is reasonable to hypothesize that an angiotensin-mediated pathway for activation of cardiac R-Smad 2 proteins that is independent of TGF-β_1 receptor activation exists.

In order to prove the existence of a direct angiotensin-Smad 2 interaction, we first stimulated cardiac fibroblasts with angiotensin (10^{-6} M) in the presence of TGF-β_1 neutralizing antibody (1.5 μg/ml). We found out that TGF-β_1 neutralizing antibody blocked the effect of TGF-β_1 on the activation of cardiac R-Smad 2. However, activation of R-Smad 2 by angiotensin could not be blocked by TGF-β_1 neutralizing antibody. These results suggested that angiotensin stimulates R-Smad 2 activation independent of TGF-β_1 receptor activation.

To further support this conclusion, cultured rat cardiac fibroblasts were infected with adenoviral dominant-negative TGF-β type II receptor (Ad DN-TβRII) and then

stimulated with angiotensin (10^{-6} M). Fibroblasts infected with adenoviral β-Gal was used as control. The immunoreactive P-Smad 2 localization was examined using immunofluorescent staining. The efficiency of infection under our conditions was over 95% and no significant cytotoxicity was found. Our results showed that the increased nuclear accumulation of P-Smad 2 by angiotensin stimulation in control group, and this effect of was not abrogated in DN-TβRII infected cardiac fibroblasts. These results further support that activation of R-Smad 2 by angiotensin is independent of TGF-β receptors activation. The precise mechanism for this action is unclear. It has been reported that Smad 2 can be activated by JNK in primary bovine aortic endothelial cells and by ERK in cell line (280,283). It has also been shown that angiotensin stimulates the activation of MAP kinases in cardiac fibroblasts (281,282,284), suggesting a link between angiotensin and Smad 2 activation through MAP kinases. Whether R-Smad 2 can be activated by these MAP kinases in cardiac fibroblasts is unknown and it is also unclear that whether these MAP kinases play a role in TGF-β_1 signaling in cardiac fibroblasts. Nevertheless, our studies indicate that R-Smad 2 is activated in post-MI rat hearts and may specificlly mediate angiotensin and TGF-β_1 signaling, which may be involved in the pathogenesis of cardiac fibrosis and subsequent heart failure. Modulation of Smad 2 mediated signaling may provide a therapeutic target for the prevention of cardiac fibrosis and heart failure and further investigation is required.

Chapter 6. CONCLUSIONS

1. Increased R-Smad 2 protein expression as well as increased TGF-β_1 expression in infarct scar tissue in the chronic phase of infarction is positively correlated to elevated deposition of cardiac collagens in the infarct scar and the remnant heart.

2. Overexpression of Smad proteins may be involved in ongoing extracellular matrix remodeling of the infarct scar and in remnant regions in post-MI hearts by cardiac myofibroblasts, and these changes are involved in the progression of heart failure.

3. AT_1 blockade is associated with i) altered TGF-β_1 ligand processing in post-MI hearts, ii) normalization of both increased Smad 2 expression in remnant myocardium and infarct scar and increased Smad 4 expression in infarct scar. Furthermore, these events are positively correlated to normalized cardiac function and significant reduction in cardiac fibrosis in treated experimental hearts.

4. Angiotensin may elevate Smad 2 expression and nuclear accumulation in cultured adult cardiac (myo)fibroblasts, suggesting a link between angiotensin receptor activation and potentiation of Smad protein function in these cells.

5. Angiotensin activates Smad 2 in cultured cardiac fibroblasts through a TGF-β_1 ligand-independent pathway.

In summary, our studies indicate that R-Smad 2 is activated in post-MI rat hearts and may specifically mediate both angiotensin and TGF-β_1 signaling, which may be involved in the pathogenesis of cardiac fibrosis and subsequent heart failure. Modulation of Smad 2 mediated signaling may provide a therapeutic target for the prevention of cardiac fibrosis and heart failure and further investigation of this possibility is warranted in this regard.

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