THE UNIVERSITY OF MANITOBA

EVIDENCE FOR THE PARTICIPATION OF BASIC FIBROBLAST GROWTH FACTOR (bFGF) IN THE RESPONSE OF CARDIOMYOCYTES TO ISOPROTERENOL-INDUCED INJURY

by

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Abstract

Basic FGF is a multifunctional protein which promotes regeneration in several tissues. To investigate involvement in cardiac injury- repair, bFGF accumulation and localization was examined in hearts of rats injected with a single high dose of isoproterenol. The bFGF content of cardiac extracts was analyzed at 6 and 24 hours as well as 1,4 and 6 weeks by Western blotting of heparin-sepharose- bound fractions. The 18 kilodalton bFGF species showed an approximately 2-fold increase in extracts from treated animals compared to non- treated controls. A transient rise in a 21-23 kilodalton bFGF species was seen at 24 hours after treatment, temporally coinciding with a peak in the mitogenicity of cardiac extracts. To localize bFGF in vivo, immunofluorescent labelling with specific antibodies was used at 4-24 hours and 1-4 weeks after treatment. Simultaneous labelling for the proteins vinculin, desmin, or myosin was employed to identify and characterize viable and non-viable muscle cells. Labelling for alpha-smooth muscle actin or vimentin was employed to identify non-muscle interstitial cells. Necrotic myocytes, identified by a loss of vinculin, displayed a pronounced increase in cytoplasmic anti- bFGF staining compared to adjacent normal myocytes. This increase occurred prior to and likely promoted mobile cell migration in areas of necrosis. Viable myocytes adjacent to fibrotic regions displayed strong pericellular and nuclear anti-bFGF, and occasionally, were also stained by anti-vimentin and anti-alpha- smooth muscle actin antibodies, suggesting reexpression of an embryonic phenotype and

thus an attempt at regeneration. These data showing increased accumulation and distinct patterns of localization of bFGF in the hearts of isoproterenol- treated animals suggest that this growth factor plays a role in injury-
repair processes of the myocardium.

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LIST OF ABBREVIATIONS

Hormones, Cytokines and Growth Factors

IL-1	Interleukin 1
TNF	Tumour necrosis factor
PDGF	Platelet-derived growth factor
EGF	Epidermal growth factor
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor-II
TGF	Transforming growth factor
bFGF	Basic fibroblast growth factor
aFGF	Acidic fibroblast growth factor
ANF	Atrial natriuretic factor
ECGF	Endothelial cell growth factor

Units of Measurement

ng	nanogram	ml	millilitres
ug	microgram	mA	milliamperes
mg	milligram	wk	week or weeks
g	gram	hr	hour or hours
Da	dalton	min	minutes
kDa	kilodalton	sec	seconds
bp	basepair	mo	months
kbp	kilobasepair	yr	year or years
mM	millimolar	^{0}C	degrees celsius
M	molar	%	percent
uCi	microcurie		

DPM	disintergrations per minute
l	litres
um	micrometers

Miscellaneous

DNA	deoxyribonucleic acid	Na^+	sodium
cDNA	complementary deoxynucleic acid	K^+	potassium
RNA	ribonucleic acid	NaCl	sodium chloride
mRNA	messenger ribonucleic acid	HCl	hydrochloric acid
cRNA	complementary ribonucleic acid	H_2O	water
O_2	oxygen	H	hydrogen
pI	isoelectric point	a-	alpha
aa	aminoacid	B-	beta
ATP	adenosine triphosphate		
mdx	muscular dystrophy		
SDS	sodium dodecyl sulphate		
PAGE	polyacrylamide gel electrophoresis		
Inc	incorporated	c-	complementary
Co	company	mwt	molecular
Eds	editors		weight
IgG	immunoglobulin	Ca^{2+}	calcium
N-	amino-		
MHC	myosin heavy chain		
MLC	myosin light chain		
a-SKA	alpha-skeletal actin		
a-CaA	alpha-cardiac actin		
SR	sarcoplasmic reticulum		

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LITERATURE REVIEW:GROWTH FACTORS.

A.INTRODUCTION

Growth factors were so named because of their stimulatory effects on cell proliferation and DNA synthesis in vitro. In vitro, these peptides are believed to be important in many biological processes, including embryogenesis, neoplasia and wound healing.

There are five important general concepts common to most, but certainly not all, growth factors which participate in the above mentioned biological functions: (1) their multifunctional properties; (2) their relationship to oncogenes; (3) their mode of action by tyrosine kinase system; (4) their classification as competence or progression factors; and their role in the autocrine of cell growth [1]. Here, a review of growth factors which may play a role in cardiac growth, injury and repair, will be presented.

B. Interleukin I (IL-1).

Interleukin- 1 refers to a family of related proteins. Two major forms of IL-1 protein exist, that is IL-1a and IL-1B. Although both proteins show little homology to one another, they exert similar biological actions and bind to the same receptors [2]. Other growth factors that share homology to IL-1 include aFGF and bFGF [3]. Like bFGF and aFGF, IL-1 lacks a signal peptide sequence normally considered a prerequisite for protein secretion via the exocytotic pathway within a membrane bound compartment [4,5]. This raises the possibility that IL-1 may be released via "leakage" or "lysis" from dead, damaged or injured cells [6].

IL-1 is present in a number of cells which participate in the immune response and wound healing. These include activated monocytes/macrophages, B and T lymphocytes, neutrophils, human keratinocytes, kidney mesangial cells, brain astrocytes, large granular lymphocytes, epithelial cells, endothelial cells and vascular smooth muscle cells [7,8].

The receptors for IL-1 also exist in multiple forms. One form is found on T- cells, fibroblasts, and epithelial cells [9]. An alternate high affinity IL-1 receptor has been characterized on B-cells, neutrophils and macrophages [10,11]. These receptors are products of two different genes and bind both IL-1a and IL-1B.

Mechanistic events that occur once IL-1 binds to its receptor, vary depending on the cell type. For instance, in T-cells and fibroblasts, IL-1/IL-1R complexes are internalized into the nucleus. In mesangial cells, IL-1 may activate a tyrosine kinase receptor, which, in turn, may activate a phosphatidyl inositol kinase to produce phosphatidyl inositol-4- phosphate. This pathway would bypass the phospholipase- C- mediated hydrolysis of phosphatidyl inositol phosphates and thus no production of inositol phosphate intermediates. IL-1, in this case, may play a role in regulating substrate availability for phospholipases involved in second messenger production [12].

Various examples exists of proto-oncogene involvement in IL-1 signal transduction. For instance, c-jun induction by IL-1 is seen to precede activation of IL-2 gene expression. In addition, induction of c-myc and c-fos expression, which code for nuclear

proteins, occurs also upon IL-1 stimulation. Thus, it is possible that the mitogenic actions of IL-1 may be mediated by c-myc, c-fos and c-jun proto-oncogenes since expression of these genes usually occurs in proliferating cells [13]. In addition, growth factors and other cytokines may also mediate IL-1 activity [14].

Furthermore, IL-1 can exert its effects in an autocrine or paracrine type fashion and may thus stimulate its own production in other surrounding cells. For instance, during wound healing, macrophages, which invade damaged tissue regions in response to injury, release IL-1 into the surrounding milieu. IL-1 release, in turn, stimulates the synthesis and release of more IL-1 from neighboring macrophage and other cell types present at the site of injury. The newly synthesized IL-1 can then increase its own production or exert other biological actions [17].

Upon tissue injury, IL-1 plays an important role in the inflammatory response and subsequent wound healing events. This stems from IL-1's numerous biological effects on cells present at the site of injury. These include chemotaxis, mitogenesis, connective tissue production and clotting [15,16].

As a chemotactic factor, IL-1 can attract monocytes, neutrophils and leucocytes to the site of injury [15]. As previously mentioned, this action may be mediated by PDGF and bFGF which are also chemotactic for these cells [15,17].

IL-1 can act as a clotting agent by increasing endothelial procoagulant activity and promote the synthesis of plasminogen activator inhibitors [16].

IL-1 enhances wound repair and scar tissue formation by stimulating connective tissue cells to synthesize type I procollagen, collagenase, hyaluronic acid, fibronectin and prostaglandin E_2 [18-21].

Under pathological conditions of the heart, IL-1 can be seen to exhibit both advantageous and detrimental effects. Although no direct evidence exists, in infarcted tissue, for example, IL-1 could possibly participate in cardiac healing, probably by exerting its chemotactic, mitogenic and other wound healing actions on resident cell, as seen in other tissue injury sites [17]. As well, if IL-1 does exert induction of proto- oncogenes, such as c-fos, c-myc and c-jun, then it may either directly or indirectly influence regeneration of cardiomyocytes.

However, IL-1, like TNF-alpha, can have detrimental effects on cardiomyocytes, especially during ischemia and reperfusion. For instance, upon restoration of blood flow to ischemic cardiac tissues, adhering neutrophilic leukocytes and endothelial cells produce and release IL-1, TNF-alpha and oxygen derived free radicals (such as superoxides) [22,23,24]. IL-1 has been shown to enhance endothelial production of leucocyte adhesion molecules which, in turn, promote neutrophil adherence to endothelial cells [26]. The myocardial accumulation of polymorphonuclear leukocytes during reperfusion following ischemia is speculated to be detramental because these cells may generate oxygen free radicals (e.g. H_2O_2) which appear to be key mediators in ischemia-reperfusion injury [24,300].

In terms of cardioprotection, IL-1's detramental effects on the myocardium could be used as a way of inducing myocardial resistance to ischemia-reperfusion injury. Brown et al. has shown that rats pretreated with IL-1 36 hrs prior to ischemic insult exhibited a resistance to myocardial ischemia-reperfusion injury in a time dependent manner. They found that H_2O_2 generated by IL-1-recruited and -stimulated polymorphonuclear leukocytes may have been involved in subsequent increases in glucose-6-phosphate dehydrogenase activity and resistance to ischemia-reperfusion damage. This would indicate that IL-1, by inducing an early oxidant stress may in turn decrease a subsequent oxidant insult by increasing endogenous antioxidant and perhaps other defense mechanisms [25].

Tumour Necrosis Factor (TNF)

Tumour necrosis factor is a distinct monocyte-macrophage derived cytokine that shares identical physical properties with lymphotoxin and has similar biological activities to IL-1. TNF alpha first became known due to its cytostatic or cytocidal actions on tumour cells in vitro and its ability to induce haemorrhagic necrosis in tumour cells in vivo [27,28].

The biologically active TNF molecule exists as a monoglycosylated trimer protein with 17 kDa subunits of 157 amino acids. It is first produced in the cell as a 26 kDa propeptide. this 26 kDa form is biologically active as a transmembrane protein. After cleavage to a 17 kDa peptide, TNF is a biologically active extracellular molecule [29,30].

TNF exerts its cytotoxic and cell regulatory activities by interacting with a specific cell surface receptor which it shares with lymphotoxin [32]. Cells such as fibroblasts [301], monocytes [302], endothelial cells [303], astrocytes [304] and activated human peripheral T cells [31] possess both high-affinity (low abundancy) and low-affinity (high abundancy) receptor sites for TNF-alpha, in common with bFGF and IL-2 [304].

Tumour necrosis factor-alpha's actions are not species-specific and its amino acid sequence is highly conserved among species (e.g. murine and human proteins show 80% homology)[30].

Various cell types such as monocytes, macrophages, lymphocytes and transformed cell lines of haemopoietic and nonhaemopoietic origin can produce TNF [31].

TNF exerts its cytotoxic and cell regulatory activities by interacting with a specific cell surface receptor which it shares with lymphotoxin [32].

TNF-alpha exhibits a diversity with respect to its biological actions. It plays a role in the cells immunological response, inflammation, bone reabsorption, disease states, atherogenesis and wound healing [47].

As a growth factor, TNF is important in wound healing and atherogenesis. For instance, TNF- alpha is shown to be mitogenic for fibroblasts and various cell lines. This mitogenic action is synergistic with epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and insulin [34]. In the inflammatory and wound healing process, TNF-alpha, like IL-1, promotes fibroblast proliferation; collagen and collagenase biosynthesis; and prostaglandin E2 release [35,36].

Although TNF-alpha is an inhibitor of vascular endothelial cell growth in vitro, it has been shown to promote angiogenesis and new blood vessel formation in vivo [37,38] and to mediate smooth muscle cell proliferation in vitro [33].

As an inflammatory agent, TNF-alpha activates the endothelium of postcapillary venules. This results in enhancement of lymphocyte adhesion [39], an increase in adhesion molecule expression (e.g. ICAM-1) [40] and an increase in permeability [41]. TNF activates inflammatory cells such as monocytes [42], eosinophils [43] and neutrophils [44].

In pathological hearts, TNF-alpha action is similar to that of

IL-1. It is shown to mediate the damaging effects of cardiac ischemia-reperfusion injury. Neutrophilic leucocytes and endothelial cells, during reperfusion, produce and release TNF-alpha, IL-1 and oxygen-derived free radicals. TNF-alpha in turn promotes adhesion of cytotoxic neutrophils to endothelial cells allowing them to exert damage on the vasculature and surrounding cardiomyocytes [25]. TNF-alpha release also increases during myocardial infarction [45]. This results in the loss of endothelium-dependent relaxation due to a TNF-alpha mediated blockade of EDRF release from endothelium [46]. This induces vasoconstriction of the coronary vasculature, thus decreasing the blood flow and amount of O_2 supply to the ischemic region [25].

No direct evidence as of yet has been documented for any healing effects of TNF-alpha in the heart. However, any benificial effects TNF-alpha may have in the heart could stem from its ability to promote scar formation in wounds due to its ability to be chemotactic and mitogenic for a number of inflammatory cells.

Platelet-derived growth factor (PDGF)

PDGF, a polypeptide mitogen, was originally purified from human platelets in 1974. Structurally PDGF has been shown to be a dimer molecule composed of disulfide-bonded A and B chains. The molecular weight of the A and B chains was found to be roughly 14 and 17 kDa, respectively, with a 60% homology in amino acid sequence [48]. Genes for A and B chains are located on chromosome 7 and 22, respectively. Isoforms of PDGF have been characterized as either being homodimers or heterodimers: PDGF-AA; PDGF-AB; and PDGF-BB. These isoforms of PDGF exhibited different biological actions. For instance, only the PDGF-AB isoform was mitogenic and chemotactic for human fibroblasts [52,53].

The receptor for PDGF was found in all cells that show a mitogenic response for PDGF. These cells include fibroblasts, glial cells, and vascular smooth muscle cells. Two distinct PDGF receptor types have been shown to exist: type A (170 kDa) was found to bind all PDGF isoforms; and type B (180 kDa) binds only the PDGF-BB isoform with high affinity [49-51,54]. The receptors themselves were shown to contain a tyrosine-specific protein kinase, homologous to the oncogene product v-kit and v-fms.

PDGF, because of its mitogenic and chemotactic actions on vascular smooth muscle cells and activated macrophages, has been shown to be an important participant in atherosclerosis. PDGF has contributed to the formation of fatty streaks and fibrous plaques which cause the attenuation of blood flow in arteries [59,60].

In the heart PDGF has yet to be shown to play a direct role in

cardiomyocyte growth. However, during development, PDGF was seen as an important angiogenic factor in the formation of the embryonic vasculature, which supplies blood to the flowing cardiomyoblasts [55]. Under pathological conditions of the heart, PDGF may enhance cardiac wound healing by improving the blood supply to ischemic regions via its angiogenic actions. Cells which inhabit the injured myocardial region, such as activated endothelial cells, platelets and macrophages, have been previously shown to be a good source of PDGF [60]. Myocardial scar formation may also involve PDGF activity since PDGF has been previously demonstrated to induce connective tissue synthesis [56-58]. However, PDGF may also contribute to the myocardial injury following ischemia in that it has been previously shown to be a potent chemotactic factor for neutrophils. These neutrophils have been shown to be a rich source of cytokines, such as TNF-alpha and IL-1, and free radicals which can cause coronary vascular damage [33,25].

D. Epidermal Growth Factor (EGF)

Epidermal growth factor is a 6045 dalton polypeptide composed of 53 amino acids [61]. Transforming growth factor-alpha (TGF-alpha) and vaccinia virus growth factor are two growth factors which have been shown to possess EGF-like activity [62]. In particular, TGF-alpha, produced as a result of cell transformation, was shown to be structurally homologous to, but antigenically different from EGF. Never-the-less, TGF-alpha was shown to compete with EGF for the same receptor [62].

Many cells have been shown to respond to EGF and TGF-alpha. These peptides are thought to modulate the development of epidermis, breast and gut; acted as angiogenic factors; and mediated hypercalcaemia [63,64].

The EGF receptor has been demonstrated to be a 175 kDa molecular weight glycoprotein which exhibits an intrinsic tyrosine kinase activity which autophosphorylates the receptor [306]. This receptor consisted of an external ligand binding domain separated from the internal tyrosine kinase domain by a hydrophobic transmembrane [306]. The oncogene protein V-erb-B was shown to have some structural homology to the EGF receptor. However, the V-erb-B differed from the human EGF receptor in that the V-erb-B's extracellular domain was truncated. This, in turn, activated that tyrosine kinase constitutively, producing a continuous mitogenic signal for transformation [307].

EGF, in vitro and in vivo, was shown to play an important role in wound healing and in regulating muscle cell proliferation and

function. In vivo, EGF participated in the wound healing process by stimulating neovascularization, fibroblast proliferation and collagen accumulation [65]. This was seen in experiments where EGF was applied directly to wounds on the skin [65,66,67,68]; eye [72]; intestine [69]; gastric ulcers [70]; and perforated tympanic membrane [71]. In all cases, substantial healing was observed in layers of the epithelial, endothelial and keratinocytes cells.

In vitro experiments using human corneal endothelium showed that EGF promoted wound closure by: i) stimulating the migration of individual cells from the wound edge into the wound defect; (ii) inducing cellular elongation and (iii) promoting a diffuse distribution of actin filaments [73]. In addition, urokinase-type plasminogen activator (u-PA) was shown to be produced by human retinal endothelial cells upon EGF stimulation. Furthermore, EGF was shown to exert mitogenic action upon cells involved in wound healing, such as fibroblasts [76], keratinocytes [72,76], epithelial and endothelial cells [72]. As an angiogenic factor, EGF promoted the proliferation of vascular endothelial cells as seen during would healing [74,75]. Interstitial tissue, in particular collagen, was also found to be regulated by EGF. For example in skin wounds induced in diabetic rats, EGF treatment was shown to cause an increase in collagen synthesis, particularly type I collagen, and an increase in protease activity [77].

EGF has also been shown to participate in regulating muscle growth and function. For instance, EGF has been shown to act on the BC3H1 smooth muscle cell line, in synergy with bFGF, to prevent

cell differentiation and act as competent and progressive factors for smooth muscle cell proliferation [78].

In skeletal muscle, EGF and IGF-1 have been shown to act synergistically on satellite cells to increase protein synthesis [81]. Upon differentiation of skeletal myoblasts, a decrease in EGF and FGF receptors was observed, suggesting the EGF may play an important role in skeletal muscle cell growth [82].

In the heart EGF was shown to participate in promoting cardiomyocyte cell growth [83]. A GTP binding protein, coupled to the EGF ligand/receptor complex has been shown to mediate EGF's growth signal [83,84]. In addition, like the B-adrenoceptor agonist isoproterenol, EGF stimulated adenylate cyclase activity resulting in an increase in cAMP levels, which in turn elicited the biological growth response [85,86]. As in other cell systems, cAMP has been shown to act as a critical positive regulatory element during cell growth [87].

Other than regulating cardiomyocyte growth, EGF exerted a chronotropic effect on cardiac muscle cells. EGF, at a concentration range between 5 to 20 ng/ml, was able to produce a dose-dependent increase in the frequency of contraction as seen in 7 day chicken embryo cardiac cell aggregates. This chronotropic action was found to be mediated through an ionic transport mechanism which included: increased Ca2+ entry into the cell via voltage-dependent calcium channels; stimulation of Na+-H+ - exchanger and; stimulation of the Na+-Ca2+ exchanger [88].

Although no direct evidence has been shown for EGF's

participation in cardiac healing, EGF may still play a role in the healing process associated with myocardial ischemia and disease. For instance, in light of EGF's angiogenic actions in other wound systems, EGF may also induce collateral growth in ischemic and damaged heart tissue [89,78-80]. As well, scar tissue formation in the heart, following injury, may also be regulated by EGF since, in other wound systems, EGF increased fibroblast proliferation and collagen synthesis [77].

E. Insulin- Like Growth Factor (IGF)

Insulin-like growth factors (IGFs), also called somatomedins, are polypeptides with marked homologies to insulin, and possessing potent anabolic and mitogenic effects, both in vivo and in vitro [90]. Insulin growth factor I (IGF-1 or somatomedin C) and insulin growth factor II (IGF-II or somatomedin A) are members of a family of peptide hormones that mediate many, but not all, of the growth promoting actions of growth hormone (GH) [91]. These factors are required for normal fetal and postnatal growth and development as well as for the growth of cultured cells, especially during their progression through the G1 phase of the cell cycle [92].

IGF-I and IGF-II are synthesized in many, if not all, tissues, including liver, heart, kidney, pancreas, spleen, small intestine, colon, brain, and pituitary gland, although abundance of IGF-I and IGF-II synthesis in each tissue varies [93].

The physiological properties of IGF-I are different from those of IGF-II. IGF-I is more GH dependent and more mitogenic than IGF-II, which is more insulin like in its action and is present in the blood at levels three times greater than IGF-I [94].

In addition to their respective receptors, IGF-I and II are also known to bind to several carrier proteins present in the blood and extracellular fluid. These binding proteins have been shown to exist in different molecular forms depending fron where they are secreted. For instance, one type of IGF-I binding protein (25 Kda form) is shown to potentiate the effect of IGF-I on DNA synthesis in skeletal satellite cells [102]. Similar IGF-I binding protein

complex effects have been shown to occur in cultured human, chick and mouse embryo fibroblasts and in porcine aortic smooth muscle cells. In these tissues, the IGF-I binding protein is shown to increase IGF-I action by as much as five-fold [95].

Functionally, IGF's have been shown to play a pivotal role in wound healing. For instance, IGF-I in concert with PDGF-I, has been shown to have maximal effects on porcine skin wound healing. An increase in new connective tissue volume and collagen content and maturity occurred without inflammation [96]. IGF-I also has been demonstrated to be a potent angiogenic factor; acting in an autocrine fashion to stimulate fibroblast, smooth muscle cells and endothelial cells to form new blood vessels, as seen in wounded tissue [97]. IGF-I has also been implicated in the regeneration of nervous tissue as seen following hypophysectomy.

In smooth muscle cells, IGF-I has been shown to act as a potent regulator of muscle cell growth and differentiation. For instance, in cultured smooth muscle cells, IGF-I, in concert with bFGF and PDGF, stimulated DNA synthesis and cell proliferation via the enhancement of IGF-I binding to its receptor. This in turn, enhanced PDGF binding and IGF-I autostimulation of its own expression. This regulatory action of one growth factor influencing the receptor binding of another growth factor has been shown to important in the control of cell growth [98,99,100]. In addition, it has been shown that IGF-I's growth promoting ability in muscle cells can be correlated with an induction of c-Myc expression, when acting in concert with PDGF [101].

Furthermore, IGF and its binding protein and receptors have been shown to be developmentally regulated in skeletal muscle cells. For instance, in rats, a marked predominance of IGF-II receptors in fetal skeletal muscle has been observed (55 times more abundant than IGF-I receptors), which then declines during postnatal development. As well, IGF-I receptors was found to be abundant in the term fetus and then gradually declines to adult levels by four weeks of age [103]. In terms of gene expression, IGF-I transcripts were found to be highest in skeletal muscle during late fetal and early neonatal periods. In the heart, IGF-I mRNA levels were found also found to be developmentally regulated. At birth IGF-I mRNA levels were found to be initially low and then increase during postnatal development [104]. Similarly, IGF-II mRNA levels also display developmental regulation. For instance, IGF-II mRNA levels were found to be highest in skeletal muscle and heart of new born animals and then gradually decreased during adulthood [105].

In the heart, IGF-binding proteins play an important role in IGF-I localization and action. For instance, endothelial cell IGF binding proteins (ECBP), when complexed with IGF-I, become localized in cardiac muscle when they leave the microcirculation. When uncomplexed, ECBP was shown to become localized preferentially in the connective tissue of the heart [106]. This observation correlated with IGF-I and II binding sites located along the myocardial capillary endothelium [107] and along cardiomyocytes [108]. Similar to the situation seen in skeletal muscle, IGF-I

and II expression in the heart was shown to be growth hormone inducible. This IGF induction, in turn, was correlated with an increase in cardiomyocyte hypertrophy [109]. The mechanism by which IGF can help to promote cardiac hypertrophy is unknown. In addition, neonatal cardiomyocytes have been shown to express IGF-I and -II, with IGF-II being more abundant than IGF-I [111]. The mitogenic actions of IGF-I and -II on neonatal chick cardiomyocytes in culture have been shown by Kardami [110]. In culture, IGF-I and II were found to stimulate DNA synthesis and cell proliferation in chicken cardiac muscle. As well, Kohtz et al were able to demonstrate IGF-I and II's ability, in cooperation with bFGF, to induce cell proliferation in human fetal cardiac myoblast cultures [112].

Thus, IGF-I and II, in the heart, may act as potent mitogens, possibly playing a role in cardiac cell proliferation and growth during early neonatal and adult stages of development.

F. Transforming growth factor beta1(TGF beta1)

TGF-beta1 belongs to a large family of closely related genes and polypeptides. In addition, to TGF-beta1, originally purified from human platelets, there are TGF-beta2 [113], TGF-beta3 [114], TGF-beta4 [115], activins and inhibins [116], mullerian inhibiting substance [117], and bone morphogenetic proteins [118]. All these proteins have been shown to have, more or less, some degree of structural and functional homology to TGF-beta1.

Tissue distribution studies of TGF-beta1 show that it is ubiquitous. This has been demonstrated using immunohistochemical techniques [134]. The biological function of TGF-betas appear to be numerous. For instance, TGF-beta1 regulates the production of extracellular matrix components. TGF-beta1 has been shown to cause an increase in expression of collagen, fibronectin and proteoglycans [125]; integrins [126]; protease inhibitors (such as plasminogin activator inhibitor type I [127] and tissue-specific inhibitor of metalloprotease [137]); and a decrease in expression of proteases such as collagenase [137] and transin [128]. These actions of TGF-beta1 on extracellular matrix production have been shown to occur in concert with other growth factors, such as bFGF and EGF [47]. Thus TGF-beta1 is thought to play an important role in wound healing.

During wound healing, TGF-beta1 has been shown to be released from cells of the inflammatory response such as platelets [121], lymphocytes [122], activated monocytes/macrophages [123] and neutrophils [124]. It has been hypothesized that TGF-beta is

initially released by platelets [129] which, in turn, chemoattract monocytes/macrophages, T-lymphocytes and neutrophils to the wound site [130,131]. These inflammatory cells, in turn, has been shown to produce chemotactic and growth promoting factors for fibroblast cells. These factors included IL-1, PDGF, bFGF, TNF [130] and auto-induced TGF-beta1 [131]. Thus increased fibrosis and fibronectin/collagen remodelling was shown to be correlated with the increased TGF-beta1 levels and fibroblast proliferation [132,133,125]. In addition, TGF-beta1's wound healing actions have been shown to be regulated by other growth factors. EGF, for instance, inhibited TGF-beta1 induced fibrosis [132] and bFGF inhibited TGF-beta1 induced wound contraction [146].

As an angiogenic factor during wound healing, TGF-beta1 is shown to be unusual in that it inhibited the growth of vascular endothelial cells [135] and smooth muscle cells [136] but at the same time induced angiogenesis by eliciting microvascular endothelial cells to form tube-like structures with apparent tight junctions and subluminal basal lamina depositions [145].

In addition, depending on the target cell, TGF-beta1 has been shown to be a growth promoter or inhibitor; and/or a differentiation promoter or inhibitor [128,137,138-144,156].

TGF-beta1 has also been shown to play an important role during development. In synergy with bFGF, TGF-beta1 induced mesoderm formation in early xenopus embryos [147,148]. During organ development, TGF-beta1 acted as a instructive and permissive signal factor inducing embryonic mesenchyme cells to differentiate into

specific tissue types as well as inducing extracellular matrix formation [149]. In the mouse embryonic heart, TGF-beta1 has been localized in the developing endocardial cushion and valves [150]. In addition, the embryonic chick myocardial extracellular matrix, a potential source of bFGF and TGF-beta1, has been shown to induce atrioventricular valve primordia formation [151,152]. Thus TGF-beta1 may play a role in cardiac morphogenic events and in the induction of cardiac cell lineages.

In vascular tissues, TGF-beta1 has been demonstrated to be an important regulator of smooth muscle cell growth. It has been shown that TGF-beta1 inhibited this serum induced smooth muscle cell growth by increasing the cell cycle transit time, thus leading to smooth muscle cell hypertrophy and hyperploidy [136]. In addition, heparin and dextran sulfate were shown to potentiate TGF-beta1's inhibitory actions on smooth muscle cells by freeing TGF-beta1 from its complex with alpha-macroglobin [153]. In vivo, TGF-beta1 was shown to stimulate its own gene expression in arterial blood vessel smooth muscle cells following balloon denudation [158].

In skeletal muscle, TGF-beta1 was shown to block myoblast differentiation and suppress the induction of muscle specific genes, but was not able to induce mitogenesis [154]. Indeed TGF-beta1 has been shown to block the proliferative actions of bFGF on skeletal myoblasts [161]. However, once skeletal muscle cells have undergone terminal differentiation, TGF-beta1 can no longer exert any influence on muscle-specific gene expression [155]. As well,

receptors for TGF-beta1 were down regulated upon terminal differentiation [156]. Nevertheless, a few functional TGF-beta1 receptors remain after differentiation, probably to regulate TGF-beta1's actions on the extracellular matrix [157].

In cultured rat cardiomyocytes, TGF-beta1 along with bFGF has been shown to alter the expression of both MHC and actin genes in a pattern strongly resembling that observed after pressure overload hypertrophy in vivo. Despite inclusion of thyroxine in the serum-free medium the adult alpha-MHC expression was inhibited by TGF-beta1 by as much as two thirds with reciprocal induction of the "embryonic" B-MHC expression (approximately four-fold) [159]. Other than its effect on contractile proteins, TGF-beta1 has been shown to stimulate ANF expression in cultured ventricular myocytes and inhibit the sarcoplasmic reticulum Ca^{2+}- ATPase gene, consistent with the influence of a more generalized embryonic phenotype [160].

TGF-beta1 also has been shown to participate in regulating the proliferation of various cell types in the heart. For instance, TGF-beta1 was shown to stimulate cardiac fibroblast proliferation, while at the same time, cancel the proliferative actions of bFGF on endothelial and neonatal cardiomyocyte cells [161,110,162]. Cardiac fibroblasts, in particular, have been shown to be both a major source and target for TGF-beta1's autocrine mode of action. By exerting its influence on cardiac fibroblasts, TGF-beta1 was shown to regulate extracellular matrix protein production, e.g. collagen types I and III. This action would suggest that TGF-beta1 may play an essential role in cardiac wound healing [163]. In

addition TGF-beta1 has been shown to induce cultured adult cardiac fibroblasts to differentiate into cells that display cardiomyocyte characteristics such as the expression of muscle specific genes, loss of vimentin, and induction of myocyte morphological features [164]. During disease and ischemic conditions, TGF-beta1 levels, along with endothelial cell growth factor (ECGF) increase in adult hearts. Differences, however, in the topographic distribution of TGF-beta1 and EGF occurred following myocardial infarction. TGF-beta1 mRNA was shown, by in situ hybridization, to localize to ventricular myocytes, whereas the expression of EDGF was detected principally within coronary arteries following infarction. At the protein level, immunoperoxidase staining of infarcted hearts showed increased levels of TGF-beta1 along surviving myocytes bordering the infarcted tissue but none in the necrotic myocytes [165]. Thus it is believed that this increased TGF-beta1 expression may represent an adaptive response to cardiac injury participating in infarct healing and in the compensatory hypertrophy of surviving myocytes [160].

As well, during myocardial ischemia, TGF-beta1 has been shown to exert a cardioprotective role [25]. Researchers have shown that if TGF-beta1 is given before or immediately after ischemic injury: (i) a reduction in the amount of superoxide anions in the coronary circulation is observed; (ii) endothelial-dependent coronary relaxation factor levels are maintained; and (iii) a reduction in injury inducing exogenous TNF occurred.

G. Basic Fibroblast Growth Factor (bFGF).

Basic fibroblast growth factor (bFGF) is a member of a family of fibroblast growth factors which include: aFGF, int-2, hst/k-FGF, FGF-5 and FGF-6 [166,167]. This growth factor is synthesized and localized in most tissues of neurodermal, mesodermal and ectodermal origin (e.g. astrocytes, neuronal cells, muscle cells, chondrocytes, keratinocytes, platelets, macrophages, etc.) [166]. Basic FGF itself is a cationic peptide with a pI of 9.6 which makes it basic. Structurally, bFGF lacks an amino- terminal hydrophobic signal peptide sequence [167]. Thus bFGF is not secreted conventionally like other secreted proteins, i.e. via the golgi apparatus [168].

Basic FGF and aFGF are shown to share 55% amino acid homology [166]. Between species, bFGF is well conserved, e.g. bovine and human bFGF share 97% amino acid homology to one another [169,170].

Basic FGF is a heparin binding protein with a high affinity for heparin [168]. This heparin- bFGF interaction is shown to be important in: (i), protecting bFGF from degradation and protease actions [171]; (ii), facilitate bFGF binding to high affinity receptors [172]; (iii), aid in bFGF purification via heparin sepharose chromatography [8]; and (iv), stabilize the tertiary structure of bFGF[173].

Basic FGF also contains an inverse cell adhesion (R-G-D) sequence which facilitates bFGF's cell adhesive function [166]. The bFGF peptide also contains serine and threonine amino acids which

become phosphorylated by either protein kinase A or C depending on the bFGF's association with various ECM components. This phosphorylation influences bFGF's affinity for high affinity receptors [174]. Recently, Ca^{2+} has been found to stabilize bFGF's structure [175].

Different sizes of bFGF peptides have been identified ranging from the low molecular weight forms(14 to 18 kDa) to the high molecular weight forms (21.5 to 29kDa) [167]. These different species of bFGF differ in their localization in the cell and thus are thought to perform different functions. Shorter bFGF peptides have been found mainly in the cytoplasm and extracellular matrix [176,186,187]. It is speculated, that this type of cellular localization reflects a possible chemotactic role and/or regulatory role in cellular differentiation [176]. The longer bFGF peptides, are found to be preferentially targeted to the cell nucleus. This nuclear localization of bFGF is associated with proliferative cells suggesting a possible role in cell proliferation and growth [177]. Various reasons for these multiple bFGF forms exist. One reason is that the initiation of translation may occur at multiple sites. Three potential "CUG" codons for bFGF translation, at positions -302, -329 and -344, were found to exist at the 5' terminal end of the gene. This amino-terminal end extension of the bFGF gene results in higher molecular weight bFGF's (21.5 kDa, 22.5kDa, and 25 kDa) [178,179]. In addition, these amino terminal extended bFGF's contain a nuclear translocation sequence in the extended portions [180]. Another reason for the different molecular bFGF

forms could be the action of acid proteases. Acid activated proteases are induced during certain protein extraction procedures and can mediate the truncation of bFGF at the amino terminal end [175].

The bFGF gene is approximately 40 kbp long and is localized on chromosome 4 [167]. In cultured cells and tissues, the bFGF gene produces 5 stable transcripts [181].

Receptors for bFGF include low affinity and high affinity receptors. The low affinity receptor consists of heparin sulfate proteoglycans [182]. These receptors are localized on the cell surface, ECM and basement membranes [181,182]. The number of receptors ranges from 0.5 to 2.0 ($x10^6$) per cell [166,167]. These receptors are heparinase sensitive and are thought to function as a reservoir for bFGF in the vicinity of potential target cells. Low affinity receptors may also facilitate the binding of bFGF to high affinity receptors [182].

The high affinity receptors for bFGF are glycosylated single chain polypeptides with molecular weights ranging from 110 to 165 kDa [166,167] and its numbers ranging from 0.2 to 1.0 ($x10^5$) per cell depending on cell type [166,167]. These receptors are shown to be tyrosine kinases embedded in the cell membrane [167]. As of yet, six different isoforms of this receptor are known to exist. These receptors are shown to be encoded by a single Flg oncogene and that the different isoforms of the bFGF receptor are due to alternate RNA splicing of this single gene. This RNA splicing process has been shown to be highly regulated and tissue specific [308]. For

instance, it has been shown that mouse muscle and heart express two different forms of the bFGF receptor having either two (short form) or three (long form) Ig-like domains, whereas the developing brain and adult brain express only mRNA encoding the long form. Structurally, four of these isoforms have been shown to be transmembrane receptors with either two or three Ig-like domains, respectively. Within each group, one receptor differs from the other by the presence of a two-amino acid insertion. The fifth isoform has been shown to be a trucated, possibly secreted protein, containing the first IgG-like domain [308]. The sixth isoform, reported by Johnson et.al. [309], is also a truncated form of the bFGF receptor, which differs from the others in that it contains the acidic region of the receptor and the Ig-like domains II and III. The receptors themselves are linked to a G- protein encoded by the H-ras oncogene [183].

The question of the mechanism by which bFGF is secreted when it lacks a hydrophobic amino terminal signal sequence" still remains unanswered. Theories, however, about bFGF's mode of release vary. One theory speculates that bFGF is released upon cell lysis during tissue injury and cell death [185]. Other studies have shown that cell death may not be required for bFGF release. These studies have shown that minor damage to the cell, without cell death, can also induce bFGF leakage from the cell interior [184]. More theories of bFGF's mode of secretion range from evagination of the cell plasma membrane forming extracellular vesicles [168]; to bFGF 'piggy backing' on nascent heparin sulfate as a mode of

transportation out of the cell [168].

Some in vitro biological effects of bFGF include induction of the transform phenotype in such cells as NIH 3T3 [181] and BHK- 21 [181]. Basic FGF is also shown to play a role in cell proliferation. For instance, exogenous bFGF, added to cultures of vascular endothelial cells, reduces the average doubling time and shortens the G1 phase of the cell cycle [167]. Basic FGF is also shown to play a role in differentiation. In culture, bFGF is able to act as both a differentiating agent (eg. capillary endothelial cells [167]) or an inhibitor of differentiation (e.g. myoblasts, chondrocytes and adipocytes [167]). In myoblasts, in particular, bFGF when added to cultures, decreased MyoD1 transcription and prevented fusion [188,189]. In vitro, bFGF is also shown to promote cell adhesion [190,191]; induce mesoderm formation [192]; and promote angiogenesis [193]. As an angiogenic agent, bFGF is shown to control degradation of the basement membrane; promote endothelial cell migration; tube formation and proliferation; and increase plasminogen activator and collagenase [167].

In vivo effects of bFGF include: early embryonic mesoderm induction [192]; regeneration (e.g. newt limbs, lens tissue and cartilage) [166]; and neurotropic and neurotrophic actions (bFGF promotes neurite out growth, differentiation and survival) [166]. In terms of wound healing, bFGF, in vivo, stimulates proliferation and growth of all cell types involved in the wound healing processes: Capillary endothelial cells [47,196], vascular smooth muscle cells [194], fibroblasts [47,194] and specialized tissue

cells (chondrocytes [195] and skeletal myoblasts [197]). Thus bFGF is seen to participate in the healing process of many systems such as arterial endothelium damage [198], skin wounds [199], corneal epithelial wounds [200], CNS injury [201], and epidermal wounds [202]. More recently, bFGF mRNA synthesis and anti-bFGF immunoreactivity has been demonstrated in models of cortical brain injury [201]; and muscle injury (specifically in myoblasts, and in degenerating and regenerating myotubes [197].

In the heart, bFGF is found to be synthesized and stored by various cardiac cells. In cardiomyocytes, bFGF is found inside atrial and ventricle muscle cells and is associated with the nucleus, cytoplasm, cell membrane, basement membrane and gap junctions [191]. This would suggest an intimate involvement with physiological function. Cardiomyocytes in culture have been shown to synthesize and store bFGF in their cytoplasm and extracellular matrix [191,203]. In blood vessels, bFGF is shown to be accumulated in all cell layers [166]. In vitro studies has shown that vascular endothelial and smooth muscle cells actively synthesize and respond to bFGF [204]. In addition, interstitial cells of the heart, such as macrophages, mast cells and fibroblasts, are known to synthesize and accumulate bFGF.

Recently, Kardami et al [176,205] have identified different forms of bFGF in both culture and tissue sections. These species of bFGFs differentially localized and accumulated in the cardiomyocyte according to the stage of development of the myocyte. For instance, in adult 5 week old rats, the 18 kDa bFGF species predominated

[205,191,203]. In new born rats, the 22 and 24 kDa species were mainly detected. In addition, the 25kDa species was shown to be localized in the nucleus of proliferating cardiomyocytes [176,205]. From this data, Kardami et al speculated that a switch from the high to low molecular weight bFGF occurs from neonatal to adult stages of development [176]. Combined with data from recent literature and from her own laboratory's work a working hypothesis was made as to the role of these different molecular weight species in the heart: (1), high MW bFGF correlates with increased proliferation and nuclear localization, and is predominately associated with immature cardiac muscle cells [176,205,206]; (2), low molecular weight bFGF correlates with the regulation of differentiation and hypertrophic growth. This form is predominately associated with the adult cardiac muscle [203,205].

Biological effects of bFGF in the heart are thought to be mediated in either an autocrine and/or paracrine mode of action [168,207]. Recently, bFGF in the heart was shown to play a possible role in regulating cell differentiation. Kardami et al [203], found that atrial myocytes, which are surrounded by bFGF- rich endomysium are less differentiated than ventricular myocytes, which have less bFGF pericellularly. As well, Schneider et al [208] has shown that bFGF, when added to neonatal cardiac cell cultures, induces a reversal to an earlier program of contractile and non- contractile gene expression. Basic FGF, along with TGF-beta1 and aFGF, are shown to uniformly suppress expression of the muscle- specific genes in skeletal myoblasts and biochemically differentiated

myocytes. For instance, TGF-beta1 and bFGF is both found to selectively provoke embryonic B-MHC and a-SKA expression and inhibit adult a-MHC expression with no change in adult a-cardiac actin expression. Both peptides also stimulated ANF and inhibited sarcoplasmic reticulum Ca^{2+}-ATPase expression. Acidic FGF, however, produced unexpectedly distinct effects in cardiac muscle. Although alpha- and beta- MHC, ANF and SR Ca^{2+}-ATPase genes responded similarly to aFGF and bFGF, both a-SKA and a-CaA were suppressed. The mechanism for bFGF differentiation action in the heart is thought to be mediated by nuclear oncogenes. Basic FGF and aFGF, for instance, is shown in cardiac muscle to induce c-fos, c-jun and jun B. These nuclear oncogene proteins are thought to couple transmembrane signalling to growth and transcriptional control, and their induction prevents myoblast differentiation. More evidence for bFGF's role in cardiomyocyte differentiation comes from experiments showing that the bFGF receptor, encoded by a flg oncogene protein, is down regulated upon terminal differentiation in skeletal muscle cells. Thus, similar down regulation of the bFGF receptor may occur in the cardiomyocyte upon differentiation [183,82].

Basic FGF in the heart may also induce cellular growth and proliferation of cardiomyocytes. In culture, Kardami et al [110] and Claycomb et al [209] have shown that addition of bFGF to cardiomyocyte culture will stimulate proliferation and DNA synthesis. This was found to be inhibited by TGF-beta1 [110]. In addition, bFGF has also been shown to stimulate hypertrophic growth

in cardiomyocytes [210]. In conjunction with a more proliferative state, bFGF induces reexpression of fetal contractile gene proteins, such as B-MHC and a-SKA [211], and increases the rate of protein synthesis.

Basic FGF may also play a role in cell to cell recognition, adhesion and communication. Evidence for this possible function comes from Kardami et al [190]. They have shown that in cardiomyocytes, bFGF is localized along the intercalated disc regions. This would indicate that bFGF is either an integral part of, or exists in close association with cardiac gap junctions. Thus, bFGF may play a role in modulating gap junctional intercellular communication.

Basic FGF's angiogenic actions may play a role in cardiac healing and myocardial development. Basic FGF released by myocytes, may trigger capillary and vessel formation and cardiac innervation [212].

In myocardial development bFGF has been localized, in embryonic chicks, at first only in developing myocardial cells and appears subsequently in the extracellular matrix [213]. Functional studies have shown that bFGF, along with TGF-beta, aFGF, IGF-II and other growth factors may contribute to induction of the cardiac myocyte lineage or other components of the heart. Formation of valve primordia in chick embryos (epithelial- mesenchymal induction) is elicited by TGF-beta1 [214] or cardiac extracellular matrix containing FGFs [215].

In cardiac disease and wound healing, bFGF may play a

regenerative role since it has been localized in extracellular, cytoplasmic as well as nuclear sites of the myocyte, suggestive of an intimate involvement with physiological function [203,191,216,190]. As well, bFGF stimulates proliferation [110] and induces reversal to an earlier program of contractile gene expression in cultured cardiomyocytes [211]. Both of these properties accompany the regenerative response of other cell types [218]. In addition, an increase in cytoplasmic bFGF immunolabelling for necrotic myocytes, and an overall increase in bFGF accumulation was observed for cardiac muscle cells in the mdx mouse model of injury and regeneration [217] and in the coronary occlusion model of infarction [Padua,R.R. and Kardami,E., unpublished data].

H. Cardiomyocyte regeneration and the role of growth factors.

Irreversible myocyte death and injury in the adult mammalian heart can be caused by a variety of factors such as ischemia [219], excessive catecholamines [220], direct mechanical trauma or genetic defects (mdx mouse [221]). Irrespective of the causative agent, the damaged area becomes necrotic and eventually replaced by scar tissue mainly comprised of connective tissue cells and extra cellular matrix [219]. The remaining myocardium is faced with an extra work load and must adapt to maintain cardiac function. This adaptation usually takes the form of cardiomyocyte hypertrophy [222]. However, when the work load on the injured heart becomes too great for the heart to adapt, cardiac failure eventually ensues [223]. Unlike other cell types, such as hepatocytes, which can be stimulated to divide and replace the injured areas in the liver [224], it is generally believed that adult viable ventricular myocytes do not regenerate [218]. It is not clear whether this is the result of irreversible loss of the ability to divide or whether there are environmentally imposed restrictions on these cells. Cardiac myocytes soon stop dividing after birth [225] and any subsequent increase in functional demand is met by increases in cell mass (hypertrophy) but not cell number (hyperplasia) [218]. Within the last fifteen years, however, evidence has come to light that the adult cardiomyocyte is capable of limited dedifferentiation in an attempt to adapt and regenerate following increased work load and myocardial injury. For instance, it has been reported that in intact adult cardiomyocytes, following

myocardial infarction, certain structures (eg. endoplasmic reticulum, golgi apparatus, nucleoli, myofibrils) within the cell change their shape and appearance similar to that seen in the immature cardiac muscles [226]. Furthermore, Vrako et al [227] reported active cardiomyocyte processes occurring along the basal laminae following myocardial injury, with the possible goal of reestablishing myocyte connections. These myocytes again displayed characteristics similar to those seen in developing cardiomyocytes.

In addition, cardiomyocytes in long term aged animals and in culture are reported to continue the karyokinetic process producing multi-nucleated myocytes [228] and to actively synthesize DNA [209], respectively. This demonstrates that certain proliferative and mitotic mechanisms are still active or can be activated in cardiomyocytes.

More substantial evidence as to the ability of the cardiomyocyte to regenerate itself comes from data showing that certain myocytes, following injury, are able to express an earlier program of contractile gene expression similar to that seen in other cell types capable of regeneration [208]. It is shown that the cardiomyocyte in response to a variety of initiating signals (e.g. increased work load, tissue injury, catecholamines, thyroid hormone, growth factors and angiotensin)[229], can revert back to an earlier phenotype. These dedifferentiated muscle cells were shown to reexpress embryonic contractile genes such as B-MHC, MLC_1 and alpha- SKA. In addition, proteins normally abundant in the immature cardiomyocyte are shown to have increased expression, e.g.

ANF, SR-Ca^{2+} ATPase [208]. Furthermore, certain oncogenic proteins, thought to be the transducing signals for cardiomyocyte growth and proliferation, are reexpressed or increased in response to signals seen following injury and increased hemodynamic load. Some of the oncogene proteins reexpressed include: (i), flg, a bFGF receptor down regulated upon terminal myocyte differentiation [208,82]; (ii) c-myc, a nuclear oncogene protein associated with hyperplasia in neonatal cardiomyocyte, but unable to prevent differentiation [230,231]; and (iii) c-fos, c-jun, JunB, and JunD nuclear oncogene proteins associated with immature cardiomyocytes and whose expression is shown to be influenced by different growth factors (TGF-beta, bFGF and aFGF)[208].

As an initiation signal for cardiomyocyte regeneration, growth factors, e.g. bFGF, aFGF, TGF-beta, IGF are likely candidates since they are shown to be present in the heart and play a proliferating role in culture and during cardiomyocyte development [104,105,149,150,203,232,209]. In the heart, these growth factors exert a number of different functions and have been shown to: increase in the heart following injury and hypertrophy [109,165,233,217,212]; induce regeneration in other cell systems [97,125,132,133,146,234,235]; and induce the cardiomyocytes to reversibly express fetal contractile proteins or proteins associated with early development [104,105,159,208,229]. As well TGF-beta, bFGF and aFGF are thought to exert its actions on cells either in an autocrine or paracrine type manner[208,234,235,207]. Likely candidates for inducing myocyte regeneration include acidic

and basic FGF, TGF-beta and the IGF growth factors, which, in view of their ability to affect myocyte DNA synthesis in culture, are likely to play a similar role in vivo [209,208,235].

Thus, the cardiomyocyte is indeed capable of limited dedifferentiation which could be influenced by endogenous or exogenous growth factors in the heart. This cardiomyocyte reversal to an earlier fetal gene program could represent both an adaptive and regenerative response of the heart to myocyte damage.

I. Isoproterenol- induced cardiac injury.

1. Isoproterenol- induced alterations within the heart

In 1959, Rona et al [236] discovered that the synthetic catecholamine isoproterenol produces massive infarct-like necrosis in experimental animals, involving the apical 2\3 of both ventricles and intraventricular septum. In addition, there was a close correlation between the dose of isoproterenol and the degree of severity of necrosis [238].

Within 24 hrs after isoproterenol administration a number of structural and ultrastructural changes in the myocardium occur. Hypercontraction of the myofibrillar bands is shown to occur as early as 15 min following isoproterenol administration [239]. Capillaries filled with neutrophils, are shown to be dilated nearby the affected myocytes. Interstitial fibroblasts become enlarged and there is an infiltration of mast cells, histocytes and lymphocytes into the region of necrosis. By twelve to twenty-four hours, interstitial edema was prevalent with an abundance of macrophages, fibroblasts and neutrophils [239]. At this time point, the amount of necrotic myocytes are seen to peak [240].

By seventy two hours, fibrotic scar formation occurred in the region where necrotic myocytes are located [240]. Necrotic myocytes at this time point have mostly disappeared, probably due to phagocytosis from the interstitial cells [239]. By one week, necrotic myocyte lesion is completely replaced by a fibrotic scar containing predominately fibroblasts, macrophages and a few "mini-myocytes" thought to be encased in interstitial collagen [241]. At

this time point, collagen deposition was most prominent among the extracellular materials deposited during fibrosis. It is believed that collagen realignment and accumulation contributes to the necrotic process observed in the remaining myocytes within the scar [241], by encasing and disrupting the microcirculation to these myocytes which would lead to progressive ventricular dysfunction.

2. Structural Alterations within the cardiomyocyte

The earliest structural changes to be observed in the cardiomyocyte are myofibril contraction bands which occur within 5 min of initial isoproterenol injection [242]. Within three hours, extensive myofibril contraction bands are prevalent along with dilated sarcoplasmic reticula and mitochondria. Some shrunken mitochondria are also observed at this time point containing amorphous densities within its matrix [242,227]. Within twelve hours after isoproterenol treatment, phagocytosis of the necrotic foci, by leucocytes and macrophages is observed. In those necrotic myocytes that do remain, extensive alterations in their mitochondria is evident such as fragmentation of the cristae and loss of the dark granular bodies in their matrix [236]. Alterations in these necrotic myocyte cytoskeletal protein network (e.g. vinculin) [272] was also observed along with disruptions in the myofilament network [299]. As well, glycogen and lipid deposits were shown to accumulate in the cytoplasm of these necrotic muscle cells. Basal lamina structure, however, remains intact.

By three days, cell debris from small necrotic foci was mostly

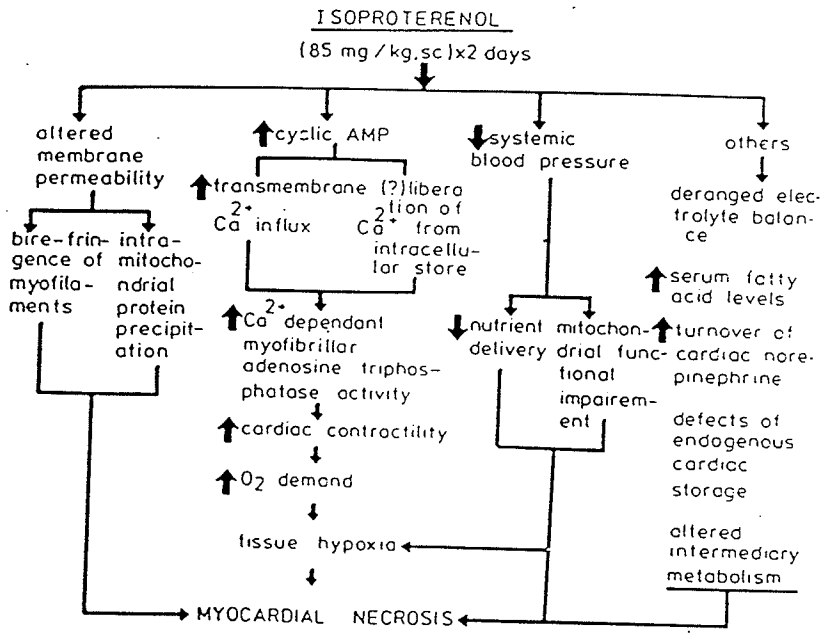
removed leaving behind numerous macrophages with phagocytosed mitochondria, cellular debris, interstitial fibres and activated fibroblasts [241]. At the border of the lesion, viable myocytes display stump ends, are edematous and show myofibrillar detachment and disarray with a loss of most of the intercalated disc structures [227]. In addition, sarcolemma alterations are visualized at this time point by the ability of myosin antibodies to leak into the muscle cell and stain myosin myofibrils [296]. By one week, cell debris is removed, basal laminae sheaths have collapsed and are incorporated into the scar, and interstitial fibroblasts and macrophages are present in large numbers and deposit extracellular materials into the scar region [241].

Along the scar region, certain myocytes, seen 10 days after isoproterenol treatment, display a dedifferentiated immature cardiac muscle phenotype characterized by: a lack of myofibril organization; cell processes extending from surviving myocytes in an apparent attempt to reestablish communication and contact; and a poorly differentiated cytoplasm resembling fetal immature cardiac cell [227].

3.Mechanism of isoproterenol induced cardiomyocyte necrosis:

Isoproterenol [1-(3,4- dehydroxyphenyl)-2-isopropylaminoethanol hydrochloride], a synthetic beta adrenergic compound is a potent cardiac stimulant, it has a strong inotropic and chronotropic action, resulting in greater oxygen demand by the heart. Isoproterenol, by means of peripheral vasodilation, reduces

systemic blood pressure which in turn reduces coronary blood flow [236]. A variety of mechanisms are believed to contribute, as well, to isoproterenol's detrimental effects on the cardiomyocyte, as summarized in the figure below:



From this figure, we see that isoproterenol administration to the heart is associated with a variety of biochemical changes which can lead to myocardial necrosis. One mechanism which is favoured to be the causative agent of isoproterenol induced myocardial necrosis is isoproterenol's beta- adrenergic overstimulation of cardiac

myofibrils. It is believed that Ca^{2+} overload may play a crucial role in this isoproterenol- induced necrotic event. Researchers have shown, using ^{45}Ca, that isoproterenol administration is followed by an increase in Ca^{2+} transport from the extracellular space to the cardiac muscle [243]. In association with an inotropic effect, increase of myocardial Ca^{2+} content will result in myofilament over stimulation, increase in contractile force and oxygen requirement as well as excessive ATP breakdown; each of these factors may contribute to cardiac muscle cell injury [244]. Thus Ca^{2+} may play an important role in inducing cardiomyocyte necrosis by initiating the breakdown of high energy phosphate required to maintain normal myocyte function and metabolism [245]. This process is shown to be cAMP mediated [221].

As well, isoproterenol is a depressor amine which induces strong peripheral vasodilation. This in turn, reduces systemic blood pressure, and, although the drug causes coronary vasodilation, the reduced systemic blood pressure presumably reduces coronary blood flow [236]. This would lead to depressed nutrient and oxygen delivery to the already overstimulated cardiomyocytes. In addition mitochondrial dysfunction would ensue, since less oxygen means less ATP synthesized by this organelle [246]. Thus, a combination of reduced ATP production and nutrient delivery and increased work load by the heart would eventually lead to tissue hypoxia and cell death [236].

Another mechanism by which isoproterenol may exert its detrimental effects on the cardiomyocyte is via the generation of

cytotoxic endogenous free radicals. In isoproterenol treated animals, there is a rise in endogenous catecholamine levels such as norepinephrine [247]. It has been proposed that under certain stressful conditions, such as ischemia, monoamine oxidase and catechol-O-methyltransferase, which are normally concerned with the metabolism of catecholamines, may either become saturated or defective. Thus catecholamines become more available, under in vivo situations, for oxidation reactions to produce adrenochrome and free radicals. It is believed that when adrenochrome is exposed to the blood, it is oxidized by blood cells to become adrenolutin [248,249]. This in turn produces coronary spasm, arrhythmias, ultrastructural damage and ventricular dysfunction [248].

Isoproterenol's detrimental effects on the cardiomyocyte is also associated with alterations in membrane permeability [250]. It is thought that with altered sarcolemmal membrane, there is also a change in sarcolemmal function which may compromise Ca^{2+} homeostasis and eventually lead to Ca^{2+} overload [250].

In addition, alterations in free fatty acid (FFA) metabolism and FFA serum accumulation is shown to associated with isoproterenol- induced cardiac injury. Researchers have shown that in ischemic and infarcted hearts, beta oxidation of FFA, which is how the myocardium normally obtains its ATP, is decreased due to a reduced availability of oxygen. This decrease in beta oxidation is associated with a concomitant increase in the intracellular concentration of long-chain acyl coenzyme A and long-chain acyl carnitine [251,252], produced by mobilization and incomplete beta

oxidation [251]. Beta-hydroxyl fatty acyl intermediates of incomplete beta-oxidation contribute to the observed increase in overall intracellular FFAs[251,252]. Excess intracellular FFAs may impair cardiac function by (1) direct adverse effects of the mitochondria itself [253], (2) the accumulation of amphipathic metabolites such as acyl CoA and long-chain acyl carnitine, which posses both hydrophobic and hydrophillic constituents which may alter cell membrane integrity and function thus contributing to electrophysiological derangements and cellular injury [254,255].

Isoproterenol- induced necrosis has also been associated with microcirculatory alterations. Research has shown that isoproterenol can indirectly influence platelet aggregation. This condition was further exasperated by the decrease in coronary blood flow due to peripheral dilation induced by isoproterenol [256].

Several other mechanisms thought to contribute to the development of myocardial injury induced by isoproterenol, which will not be discussed here, however mentioned, are increased intracellular acidity; changes in diet; malnutrition; stress susceptibility; inefficient oxygen utilization; deranged electrolyte milieu,; defects of endogenous catecholamine storage; an increased turnover of norepinephrine; an increased myocardial cAMP content and mechanical and dynamic hinderance of coronary circulation [257].

II. OBJECTIVES OF THIS STUDY:

1. To examine basic fibroblast growth factor localization and overall accumulation during the formation and healing of isoproterenol- induced lesions.

2. To relate bFGF localization and accumulation with cellular and subcellular changes seen during injured myocardial healing.

3. To elucidate the possible role of bFGF in the healing process following isoproterenol- induced myocardial injury.

III. Materials and Methods

A. Animals and Isoproterenol Treatment

Sprague-Dawley rats were used. Initial body weights at the start of the experiment were between 250-325 grams. All animals were housed together at 20^0 to 22^0C according to their study groups. They were maintained on an ad-libitum diet of rat chow [Agway Prolab animal diet. Rat, mouse, hamster 3000; Agway Inc., Country Foods Division] and tap water.

Isoproterenol hydrochloride [Winthrop Laboratories; New York, NY] was administered intraperitoneally (I.P.) at a dose of 40 mg/kg body weight in a carrier of 1.0 ml of normal saline. Age and initial weight-matched animals which served as controls did not receive any treatment.

Controls and treated rats were sacrificed at 4, 6, 9, 12 and 24 hours (short-term studies) and 1,4 and 6 weeks (long-term studies) after isoproterenol administration.

Individual body weight and heart weights were recorded for the statistical analysis and percent cardiac hypertrophy.

B. Standard Determination

1. Determination of percent hypertrophy

Percent hypertrophy of experimental hearts compared to controls were determined from measurements of the mean heart weight to body weight ratios. The formula employed for this calculation

was:

$$\frac{HW_{EX}/BW_{EX} - HW_C/BW_C}{HW_C/BW_C} \times 100 = \text{percent hypertrophy}$$

Where:

HW_{EX} = Mean heart weight of experimental rat subjects (g).

BW_{EX} = Mean body weight of experimental rat subjects (g).

HW_C = Mean heart weight of control rat subjects (g).

BW_C = Mean body weight of control rat subjects (g).

2. Statistical calculations

All statistical data, standard deviation, standard error and means were calculated using t-EASE statistical calculation software.

C. Analytical Procedures

1. Immunofluorescence of Tissue Sections

a. Anti-bFGF antibodies

Antisera S1 and S2 were raised in rabbits against a synthetic peptide containing residues [1-24] of the truncated 146 amino acid bovine brain bFGF [258] conjugated to keyhole limpet hemocyanin, as previously described [203,191]. These antisera recognize native or denatured bFGF specifically [191,176], and were used at 1:1000 to 1:2000 for immunofluorescence or 1:5000 for western blots. In addition, a polyclonal rabbit antiserum, that recognizes a peptide

sequence which is specific for three N-terminally extended forms of human bFGF [a generous gift from Dr. M. Klagsbrun, Department of Biochemistry, Harvard Medical School, Boston] was used to discriminate between 18 KDa bFGF and higher molecular weight rat.

b. Other marker antibodies and nuclear staining

Mouse monoclonal antibodies against smooth muscle alpha-actin, and vinculin were purchased from ICN Biomedicals Inc. [Cleveland, OH] and used at 1:400, 1:40 and 1:20 dilution, respectively. Monoclonal antibodies against vinculin [Sigma Chemical Co.; St. Louis, MO] and desmin [Dakopatts; Glostrup, Denmark] were used at 1:50 and 1:20 dilutions, respectively.

The secondary antibodies used were the following: Fluorescein-conjugated anti-rabbit IgG [Amersham Corp.; Arlington Heights, IL] raised in donkey and used at 1:20 dilution; Texas Red - conjugated anti-mouse IgG [Amersham Corp.], raised in goat and used at 1:20 dilution; Biotinylated - anti-rabbit IgG [Amersham Corp.] used at 1:50 dilution; and Fluorescein-Streptavidin [Amersham Corp.] used at 1:50 dilution.

Nuclear counterstain used was Bisbenzimide Hoechst dye 33342 [Boehring Diagnostics; La Jolla, CA] at a concentration of 1 ug/ml.

c. Immunofluorescence procedure

Cardiac tissue was obtained from the apex and middle portion of the heart immediately after animal sacrifice, frozen in a dry ice/ethanol bath and used immediately for cryosectioning. Transverse sections, 7 um thick were routinely obtained using a Letz kryostat [E. Leitz, Inc.; Wetzlar, FRG]. The sections were

collected onto gelatin-coated [Fisher Scientific; Fairlawn, NJ] slides and placed in humid chambers. The sections were then incubated overnight at 4^0C with the anti-[1-24] bFGF, at 1:1000 - 1:2000 dilution, and with one of the mouse monoclonal antibodies, which were used at the dilutions specified by the manufacturer. The carrier used for these antibodies was 1% (w/v) Bovine Serum Albumin or (BSA; Sigma Chemical Co.; St. Louis, MO), phosphate buffered saline (PBS), and, in some cases, 10% horse serum [Flow - ICN Biomedicals Inc.; Cleveland, OH] and 0.01% (w/v) sodium azide [Sigma Chemical Co.]. The PBS used consisted of 26.82 mM potassium chloride [Fisher Scientific], 14.7 mM potassium phosphate monobasic [Fisher Scientific], 0.59 M sodium chloride [Mallinckrodt Canada Inc.; Pointe-Claire, Quebec] and 81 mM sodium phosphate dibasic [Mallinckrodt Canada Inc.]. Sections were then washed gently with cold PBS (3 times) and incubated with the appropriate combination of secondary antibodies. If the primary antibodies had been raised in rabbit and mouse, sections were incubated for 1 hr, 20^0C, with biotinylated anti-rabbit IgG and Texas Red conjugated-anti-mouse IgG at 1:20 and 1:30 dilution, respectively, in 1% BSA-PBS. If the primary antibodies were raised in rabbit and sheep, sections were incubated with fluorescesin-conjugated anti-rabbit IgG and biotinylated anti-sheep-IgG, at 1:20 and 1:200 dilution, respectively. Sections incubated with biotinylated antibodies required a third incubation with either Fluorescein-Streptavidin (for rabbit antisera) or with Texas Red - streptavidin (for sheep antisera). Non-specific fluorescence was tested by incubating

sections with pre-immune rabbit serum at identical dilutions as the anti-bFGF sera used. A fluorescent image was considered to be bFGF-specific when it was obtained with the anti-bFGF but not with the non-immune sera.

After extensive washing with cold PBS, all sections were fixed in cold 95% ethanol [Commercial Alcohols Ltd.; Toronto, Ontario] for ten minutes, washed with PBS and immersed for thirty seconds in 1 ug/ml of Bisbenzimide hoechst dye 33342. Sections were then washed and mounted with glycerol [Sigma] PBS (9:1) containing 1 mg/ml p-phenylenediamene [Eastman Kodak Co.; Rochester, NY] sealed with colorless nail varnish and stored at -20^{0}C until observation.

d. Photography

A Nikon Labophot microscope [Diaphot:Nikon Inc; Garden City, NY] equipped with epifluorescence optics and appropriate filters (B-2A for fluorescein, G1-B for Texas Red, and DM400 for Hoechst 33342), plus phase contract optics was used for specimen observation. The UFX-IIA Nikon system and TX-400 black and white film (Kodak) were used to photograph selected fields.

2. Preparation of Extracts

All procedures were performed at 4°C unless otherwise specified. Immediately after the rats were sacrificed, heart tissue was trimmed of atria, large vessels and fat. Tissues were then weighed and ventricular muscles were minced with scissors. The minced tissues were then homogenized briefly with a polytron homogenizer [Brinkmann Instruments Co.; Rexdale, Ontario] at low

setting in three volumes over mass of extraction buffer (0.15 M ammonium sulfate [Mallinckrodt Inc.] pH 4.5, 1 mM phenylmethylsulphonyl fluoride, 5 ug/ml leupeptin and 5 ug/ml pepstatin. Residual tissue was removed by ultracentrifugation at 195,000 x g for 60 min at 4°C [ultracentrifuge and 42.1 rotor head: Beckman Instruments; Palo Alto, CA]. The supernatant collected and protein concentration was then determined using a Bradford colorimetric assay [Bio-Rad Laboratories; Richmond, CA], according to manufacturer's instructions.

3. Heparin-Sepharose Affinity Chromatography

Heparin-sepharose affinity chromatography was used for bFGF isolation from cardiac extracts, 100 ul of packed heparin-sepharose beads [prepared according to the manufacturer's instructions] were used per 100 mg of extracted protein. The pH of the crude extracts was brought to 7.0 with a dilute NaOH solution (0.01 N). Extracts were then made up to 0.6 M NaCl by adding solid NaCl (0.23 g NaCl/10 ml extract). Heparin-sepharose mini-columns [column tubes: Fisher Scientific; Fair Lawn, NJ] were equilibrated with column buffer (0.6 M NaCl, 10 mM Tris-HCl pH 7.0). Protein extracts were passed through the affinity column twice at room temperature followed by extensive washing of the column with column buffer. Affinity columns were then eluted with 1.1 M NaCl in 10 mM Tris-HCl pH 7.0, to remove acidic FGF and other heparin-bound proteins. Basic FGF was eluted from the heparin-sepharose columns either with 2 x volumes of 2.5 M NaCl, 10 mM Tris-HCl, pH 7.0, or by boiling of

the sepharose-beads in SDS/PAGE sample buffer [5]. The sample buffer used consisted of 10% mercaptoethanol [Biorad] 10% glycerol [Mallinckrodt], 1% SDS [Biorad] and 0.05 M Tris at pH 6.8. All of the results shown here were obtained by following the latter procedure which removes bFGF quantitatively from heparin-sepharose [5]. After boiling of the beads in two volumes (over mass) of SDS/PAGE sample buffer, the whole suspension was loaded onto the wells of the gel.

4. SDS-PAGE

The anionic detergent, sodium dodecyl (laurel) sulfate, is used routinely in PAGE. SDS will bind to the hydrophobic regions of proteins and separate many of them into component subunits. It will further give a large negative charge to the denatured polypeptides so that they can be easily separated using electrophoresis [ie. transport through a solvent by an electric field]. The mobility of many proteins in polyacrylamide gels, in the presence of SDS, is a linear function of the logarithms of their molecular weight [259].

To prepare the gel, the lower gel mixture (0.4 % N-N'-methylene-bis-acrylamide or BIS [Biorad], 15% acrylamide [Biorad], 3.5 mM SDS [Biorad], 0.375 M Tris-HCl, pH 8.8, 0.067% ammonium persulfate and 0.067% N,N,N',N'-tetra-methyl ethylenediamine or TEMED [Biorad] is carefully poured between two glass plates. The top of the gel is then layered carefully with d.d water. After the lower gel has polymerized, the overlay is removed and the upper gel

mixture (0.125 M Tris-HCl, pH 6.8, 3.5 mM SDS, 0.067% ammonium persulfate and 0.067% TEMED, 4.5% acrylamide and 0.12% Bis) is then poured on top of the lower gel. A comb is placed on top of the upper gel immediately after the gel is poured. Once the upper gel has polymerized, the Protean II electrophoresis apparatus [Biorad Laboratories] is assembled according to manufacturer's instructions. Samples are then loaded onto the wells, and electrophoresis migration buffer (25 mM Tris-HCl, 192 mM glycine [ICN] and 3.5 mM SDS [Biorad]) is placed in the upper and lower buffer chambers. The gel was run for one hour at 25 mA per gel at 18°C. As soon as samples have fully entered the upper gel, current is reduced to 5 mA/gel and run for 20 hrs at 18°C until completion. A Haake waterbath [Fisher Scientific Inc.] was used to maintain a constant temperature during the electrophoresis run and power was supplied using a Protean II electrophoresis constant power supply [ECPS 3000/150 model: Pharmacia Fine Chemical Co.].

5. Western Blotting (Protein transfer and immunodetection of bFGF)

a. Principle

The electrophoretic transfer of proteins on polyacrylamide gels onto immobilon-P membranes [Millipore, Ontario] was performed according to Burnette [260]. The Transblot cell electrophoretic apparatus [Biorad Laboratories] was assembled and utilized as per manufacturer's instructions.

Briefly, the technique of Western blotting involves the

transfer of proteins which have been electrophoretically separated on a acrylamide gel to a flexible membrane, which can the be used to examine antibody recognition of these proteins. To achieve transfer, electric current is passed through the gel from the cathode (negative charge) to the anode (positive charge). It was assumed that the negatively charged protein on the gel would move with the current, towards the anode where they would be trapped onto the flexible membranes (nitrocellulose or Immobilon-P).

b. Procedure for protein transfer

After completion of SDS/PAGE, polyacrylamide gel was carefully removed from the plates and the upper gel cut off. The lower gel was then trimmed, measured and placed in transfer buffer (0.325 M glycine, 0.1 M Tris-HCl, 0.2 M methanol) for 30 min. After, a sandwich setup was made in a shallow glass pan with the bottom of the pan covered in transfer buffer. First, the grey side of the plastic holder was placed at the bottom of the pan. Then a foam pad, presoaked in transfer buffer, was laid on top of the grey side of the holder. Next, two sheets of watman blotting paper [Whatman International Ltd.; Maidstone, England] presoaked in transfer buffer was placed on top of the foam pad. The gel was then gently laid on top of the watman paper and air bubbles removed. An Immobilon-P-membrane was prepared according to manufacturer's instructions, presoaked in transfer buffer and carefully placed over the gel. Next, two presoaked sheets of whatman blotting paper and another foam pad was placed on top of the immobilon-P membrane to complete the sandwich. The sandwich was then closed and placed

in the transblot cell with the grey side facing the black colored negatively charged cathodes and the white side facing towards the red colored positively charged anode. The transblot cell is maintained at 18°C during the transfer using a Haake waterbath. The transblot cell was connected to a power supply [model 200/7.0; Biorad Lab] and the transfer was run at 0.55 mA for 2 hrs.

c. Procedure for immunodetection of bFGF

Once the transfer was completed, the power was shut off, the sandwich disassembled and the immobilon-P membrane blocked in 1% BSA in PBS for 30 min at room temperature. This blocking agent served to saturate the non-specific protein binding sites. Afterwards, the blocking agent was poured off and a solution of anti-bFGF (S2) [1:5000] in TBST (10 mM Tris, 150 mM NaCl, 0.05% tween [Biorad Laboratories]) was added to the blot and incubated for 24 hrs at 4°C under gentle shaking motion. Several 15 min TBST washes followed to remove any non-specifically bound antibodies. Antigen-antibody complexes were then visualized by incubating the membrane with 0.1 uCi/ml of ^{125}I-protein A [Amersham Corp.; Oakville, Ontario] in TBST wash buffer for one hour at room temperature. Afterwards, the blot was washed 5 times with TBST and with PBS. The blots were then dried and packaged in plastic seal-a-meal bags.

d. Autoradiography

Once Western blot transfer and immunoblotting was completed, the sealed blots were placed, face up, between two intensifying screens [Lightning plus model; Dupont Cronex Co.]. An X-OMAT film

[Eastern Kodak Co.; Rochester, NY] was placed on top of the blot in the dark and the autoradiographic cassette was then incubated at -70°C for 7 days. Autoradiograms were then developed and scanned with a LBK 2202 Ultroscan laser densitometer [Sweden], as per manufacturer's instructions, to determine the relative intensity of the bands.

6. Procedure for Mason's trichrome staining

Cryosections on slides are initially fixed in primary fixative consisting of 10% (v/v) formalin [Mallinckrodt; Paris, Kentuckey] in 95% ethanol [Commercial Alcohols Ltd; Montreal, Que.] for approximately one hour. The formalin fixed sections then undergo a secondary fixation with Bouin's fixative solution for one hour at 56°C. Bouin's fixative solution consisted of 75% (v/v) picric acid [Sigma Chemical Co.; St. Louis,Mo.], 20% (v/v) formalin, and 5% (v/v) glacial acetic acid [Canlab; Toronto, Ont.]. Slides are then cooled to room temperature and washed in running water until the yellow stain of Bouin's fixative disappears. Next, slides are briefly rinsed in distilled water and are incubated in Weigert's iron hematoxylin solution, which is prepared by mixing, in equal parts, two different Weigert's iron solutions : Solution A consists of 1% (w/v) hematoxylin [Sigma chemical co.] in 95% ethanol. Solution B consists of 42.9 mM ferric chloride [Fisher Scientific Co.] in 1% (v/v) concentrated hydrochloric acid [Fisher Scientific Co.] and distilled water. Solutions A and B are kept separate prior

to use due to rapid degradation of the hematoxylin. Afterwards, slides are washed in running water for 5 min. followed by a brief rinse in distilled water. Next, slides are incubated in phosphomolybdic acid- phosphotungstic acid solution for 10 to 15 min. at room temperature followed by a brief rinse in distilled water. Phosphomolybdic- phosphotungstic acid solution consists of 2.5% (w/v) phosphomolybdic acid [Sigma Chemical Co.], and 2.5% (w/v) phosphotungstic acid [Sigma Chemical Co.] in distilled water. Slides are then placed in aniline blue staining solution for approximately 20 min. followed by a brief rinse in distilled water. Aniline blue solution consists of 2.5% (w/v) aniline blue [Fisher scientific Inc.] in 2% glacial acetic acid [Canlab] and distilled water. Aniline blue stained sections are then rinsed in distilled water followed by a 3 to 5 min. rinse in 1% acetic acid wash solution. Next, slides are washed one times in 95% ethanol, followed by two times in methanol [Malinkrodt Inc.] and two times in xylene [Malinkrodt Inc.] at approximately 3 min. per wash. Finally, sections are mounted with permount [Malinkrodt Inc.] and stored at room temperature.

7. Determination of extract mitogenic activity

Ventricular cardiac extracts to be tested for mitogenic activity were first equilibrated such that all extracts contain equivalent amounts of protein (approximately 30 mg total each). Protein concentration was determined using a Bradford colorimetric

assay [Bio-Rad Laboratories] according to manufacturer's instructions. Extracts were then dialysed against PBS to remove any salt and protease inhibitors for 6 to 8 hr at 4^0C. Extracts were then tested for mitogenic activity by adding them to cell cultures once at the time of plating, at a final concentration of 0.3mg/ml. Rabbit fetal chondrocytes were a gift from Dr. Friesen (University of Manitoba, Winnipeg, Manitoba, Canada) and were cultured as described [293,294]. For ^{3}H-thymidine incorporation assays, chondrocytes were plated in 96 well dishes at 5,000 cells/well in 10% FCS (Flow Laboratories, Mississauga, Ont.) and Ham's F-10 medium (Gibco laboratories). 24 hours after plating, medium was removed and replaced with HAM's F-10 medium (Gibco Laboratories) containing 1% BSA. Extracts were subsequently added, followed by 1 uCi/well of ^{3}H-thymidine 20 to 24 hrs later.(Amersham Corp., Arlington Heights, IL). Cells were maintained at 37^0C in a humidified atmosphere of 5% CO_2 in air. ^{3}H-thymidine incorporation was determined 6 to 8 hr later as described [295]. Basically, wells were washed three times in PBS and the cells were removed using a Puritan cotton-tipped applicator (Hardwood Products Company, Guilford, Maine). Applicators were attached to a plasticine platform and incubated in cold (4^0C) 10% trichloroacetic acid (Fisher Scientific Co.) followed by cold 5% trichloroacetic acid and cold 95% ethanol, each step for 15 min. Applicators were then dried, cotton tips cut off and placed into liquid scintillator vials (Kimble, Toledo, Ohio). 5 ml of cytoscint (ICN Biomedicals Inc) liquid scintillator cocktail was then added to each of the

vials and ^{3}H-thymidine incorporation was determined using a liquid scintillator (model LS 1701; Beckman, Instruments, Inc., Toronto, Ont.) [295].

IV. Results

A. Normal Muscle

1. Localization of bFGF

As previously described by Kardami et al [203,191], bFGF (S1) antiserum detects bFGF-like peptides predominately in association with the cellular periphery, including the intercalated disc region of the normal adult cardiomyocyte. This staining pattern is shown in Figure 1a. Here, the pericellular immunostaining reflects, most likely, the presence of bFGF along the basement membrane. In the cytoplasm, anti-bFGF antiserum S1 detects bFGF mainly along the Z-lines, which are dense structures that separate one sarcomere from the next. Muscle intermediate filaments show a similar cytoplasmic pattern of localization. Antiserum S1 also variably stains muscle nuclei, either intranuclearly or along the nuclear membrane. In comparison, S1 serum pre-absorbed with bFGF, as previously described by Kardami et al [203], was used as a control to differentiate positive anti-bFGF immunostaining from non-specific fluorescent labelling. This is seen in Fig. 1b.

Purified bFGF antibodies, derived from the anti-bFGF(S1) antiserum, are also used to examine bFGF distribution in the isoproterenol treated and control hearts. This anti-bFGF(S1 derived, affinity purified) was obtained by affinity chromatography as described by Kardami et al [191]. This antiserum was found to stain brightly the intercalated discs of cardiomyocytes Fig.1c,arrows), muscle and nonmuscle nuclei also stained with this antibody, in an overall punctate fashion (arrowheads). However,

this bFGF nuclear stain was somewhat weaker compared to bFGF staining along the intercalated discs.

2. Localization of anatomical markers

a. Vinculin

Vinculin is a cytoskeletal protein associated with the cytoplasmic face of both cell-cell and cell-extracellular matrix adherens-type junctions, where it is thought to function as one of several interacting proteins involved in anchoring F-actin to the membrane [261]. In normal mammalian cardiac muscle, vinculin is immunolocalized on the sarcolemma in a series of transverse, rib-like bands (costameres) which overlie the lines and repeat along the long axis of the cell with a periodicity corresponding to that of the underlying sarcomeres. In addition, vinculin is shown to be localized at the myofiber intercalated disk region and t-tubule sarcolemmal invaginations [262]. It has been suggested that vinculin may serve to mechanically couple the sarcolemma during fiber lengthening or shortening [262,263]. Vinculin immunostaining clearly delineates the myocytes and serves as a marker for normal cardiac muscle cells. Figure 2 a and b depicts anti-vinculin immunostaining of cryosections of adult rat ventricle in both transverse and longitudinal configurations, respectively. Arrows in (a) and (b) indicate the anti-vinculin staining of the myofiber sarcolemma. Arrowheads in (b) indicates the vinculin immunoreactivity in longitudinal myofibers in a costamere arrangement.

b. Desmin

Desmin (50-55 KDa) belongs to a multigene family of cytoskeletal proteins which form a morphologically and chemically distinct class of cytoplasmic structures - intermediate filaments. The presence of desmin is considered to be an exclusive feature of mature muscle [268]. In the mammalian heart desmin has been localized in the cytoplasm of Purkinje cells and in small cardiac vessels [261]. In adult cardiomyocytes, anti-desmin localization is seen at the Z-lines, intercalated discs, and just beneath the sarcolemma [261,268]. Figures 2 c and d depicts a cross section of the normal rat heart, showing both (c) transverse and (d) longitudinal configurations of the cardiomyocytes, respectively. The small arrows in (d) show anti-desmin immunoreactivity along the Z-line regions, the intercalated disk regions (arrowheads) and just beneath the sarcolemma. The transverse section, in (c), reveal a continuous network of desmin staining extending from the sarcolemma region into the cell interior and surrounding myofibrils.

c. Vimentin

Vimentin, a member of the intermediate filament multigene family, is predominately expressed in cells of mesenchymal origin, and particularly in undifferentiated cells including embryonic cardiomyocytes [264,265,266,268]. It is known to be growth regulated in different cell types and belongs to the early response competence gene family [267]. The role of vimentin during growth and differentiation, however, is poorly understood.

Figure 3 a and b depicts sections of the normal rat heart in either transverse or longitudinal configurations immunolabelled with anti-vimentin, respectively. This antibody characteristically immunostains cardiac non-muscle cells in the tight interstitial spaces between muscle cells, and this pattern of labelling serves as a marker for non- muscle cells.

d. Smooth muscle alpha-actin

Actin, the major component of muscle thin filament exists in multiple, tissue and stage-specific forms. The alpha-smooth muscle form is believed to be expressed very early in cardiac ontogenesis, eventually becoming fully replaced by alpha-sarcomeric-actin [269]. Consequently, alpha-smooth muscle actin is classified as a, "embryonic" sarcomeric actin. Recently, evidence that dedifferentiated adult cardiomyocytes re-express the alpha-smooth muscle-actin gene has been presented [270].

We have used a monoclonal antibody to alpha-smooth muscle-actin to examine its distribution in normal muscle. Normally, over 99% of myofibers are not labelled with this antibody. Occasionally, however, alpha-smooth muscle-actin positive fibers are seen (Figure 3c). Mostly, it is only the vessels and single interstitial cells (presumably smooth muscle cells or myofibroblasts) which are labelled by the anti-alpha-smooth muscle actin antibody (d).

B. Isoproterenol- induced cardiac injury

1.Clinical Symptoms

Within twenty minutes of a high- dose isoproterenol administration, irregular abdominal respiration is seen in the

treated rats, which assume a typical posture of lying on their sides or abdomens, with their head extended and respiring through their mouths. These symptoms decrease after 6 to 8 hrs, with a mortality rate of around ten percent.

By 24 hrs, the animals appear to be weak and immobile. In some of the animals, a bloody discharge from the nostrils is observed as well as edema of the tongue. At one week after high dose isoproterenol treatment, the animal would show signs of recovery with no external abnormalities present. Zero percent mortality rate is seen in these isoproterenol treated animals, after one week.

2. Morphological alterations that occur with isoproterenol-treated hearts

An examination of the 24 hr isoproterenol-treated hearts show an increase in mass of the ventricular portions as compared to their respective controls. This is illustrated in Table 1. Here, an assessment of the 24 hr isoproterenol-treated hearts, with respect to HW/BW ratios, reveals a 21.42 $\pm$ 4.27 % (mean±SEM) hypertrophy ($p < 0.05$) relative to the control hearts.

A histological examination of the experimental adult rat ventricular sections, 24 hrs after isoproterenol treatment, shows extensive necrosis of the myofibers, which is clearly evident using Masson's trichrome stain (fig.4) as well as phase contrast (fig. 5a). Figure 4 (a to c) depicts adult rat tissue sections at (a) control, (b) 9 hrs, and (c) 1 wk after isoproterenol injury. The arrows indicate the nuclei (black color) present among the

myofibers (red color). At 9 hrs, the lesions during early injury are distinguished by the increase in cellular infiltration (arrows) that usually occur among degenerating myofibers. At 1 wk after isoproterenol-induced cardiac injury, the scar tissue that replaces the degenerating myofibers are easily distinguished by the large number of non-muscle cells that infiltrate this region (arrows).

A characteristic profile of the subendocardial nuclear staining pattern was obtained from transverse cardiac sections of isoproterenol treated (fig.6) and control rats (data not shown). These sections were fixed in paraformaldehyde and stained with the fluorescent nuclear stain Hoechst 33342. This approach allows for an easy assessment of nuclear as well as cellular density and of cellular infiltration in cardiac tissue [203,217,191,278]. The areas indicated by an asterisks (fig.6) display dense accumulation of nuclei, representing, presumably, sites of cellular infiltration which occurred in response to IsP- induced myocyte necrosis.

3. Immunolocalization of bFGF, vinculin, desmin and vimentin after IsP injection

Cardiac sections from IsP- treated and control animals were processed for double immunofluorescnce staining for vinculin and bFGF. As a negetive control for anti-bFGF specificity, cardiac sections from Isp- treated and control animals were also processed for double immunofluorescence staining for vinculin and pre-immune serum. At 6 hr after injection, vinculin staining distinguished two groups of myocytes in the subendocardial region of the sections (Fig.7a): The first group presented the characteristic pericellular

vinculin localization pattern [272,263] which outlines each myocyte clearly (Fig.7a, curved arrows). The second group displayed greatly reduced or absent anti-vinculin staining along their margins (Fig.7b, arrowheads). These will be collectively referred to as vinculin- negative myocytes. Sections from control animals consisted exclusively of myocytes of the first group, as expected (Fig.2 c and d). Vinculin positive myocytes displayed mainly pericellular and nuclear bFGF localization (Fig.7b, curved arrows), similar to that of normal bovine, rat and mouse hearts as reported by Kardami et al [203,191,217]. A weak cytoplasmic staining for bFGF is also observed in normal striated muscles when antiserum S1 is used at higher concentrations [191,217]. Vinculin-negative myocytes were characterized by intense cytoplasmic anti-bFGF staining (Fig.,7b, arrowheads). This pattern of anti-bFGF localization was only observed in Isp-treated animals (experiment repeated 6 times). Each experiment included morphological observation of 40 sections per heart, and a minimum of three hearts. At 6 hr after treatment, all vinculin-negative myocytes stained intensely for bFGF. The reverse was also found to be true, i.e. myocytes showing strong cytoplasmic anti-bFGF labelling displayed reduced or absent anti- vinculin staining along their margins.

Nuclear counterstaining of the field shown in Fig.7a and b did not reveal significant changes in nuclear density in the areas of intense anti- bFGF staining (Fig.7c, compare area indicated by arrowheads with area indicated by curved arrows), indicating that

no significant cellular infiltration had occurred.

Morphological examination of sections (about 40 sections/time point) obtained at 6, 12, 24, 48 hr and 1 week indicated that groups of vinculin-negative myocytes were abundant 24 hr after treatment. Fig 7d and e show characteristic double immunofluorescnce labelling for vinculin and bFGF, respectively, at 24 hr. As was the case at 6 hr, intense cytoplasmic anti-bFGF staining was observed in all vinculin-negative myocytes and vice versa. These cells appeared more fragmented than at 6 hr, presumably undergoing phagocytosis. Nuclear staining of the field shown in Fig 7d and e revealed increased nuclear density in that area (Fig.7f, arrows) indicating cellular infiltration and/or proliferation.

The intense cytoplasmic anti-bFGF labelling of vinculin-negative myocytes elicited by anti-serum S1 was not seen when pre-immune rabbit serum was used (Fig.8). Omission of primary antiserum produced the same pattern as that seen with pre-immune serum.

Sections were examined for bFGF localization using an affinity- purified IgG preparation derived from S1, 6 and 24 hrs after treatment. These sections were doubly- labelled for either vinculin or for desmin (Fig.9). As was the case for the unfractionated S1, all vinculin negative myocytes presented bright anti- bFGF cytoplasmic stain (data not shown). Figure 9a and b show that at 6 hr after injection, no significant disruption or reduction in anti-desmin labelling is discerned in cardiomyocytes, including those with the intense anti- bFGF staining (Fig.9a,

arrowheads). By 24 hr however, the latter (Fig.9 c,e, arrows) had lost their anti-desmin label (Fig.9 d,f,arrows) to a significant degree. The loss of desmin, therefore occurs after the increase in cytoplasmic bFGF in damaged myocytes.

When the primary anti-bFGF antibody was replaced by pre-immune antiserum, at both 6 and 24 hrs after IsP- treatment, double fluorescence labelling revealed that in cardiomyocytes with disrupted anti-desmin staining, the intense cytoplasmic anti-bFGF staining was no longer evident (data not shown).

Most of these studies were performed on frozen, unfixed sections to preserve maximal immunoreactivity of the monoclonal antibodies used for double immunolabelling. Post fixation of sections with paraformaldehyde did not significantly affect immunostaining with anti-bFGF serum S1 and provided better preservation of nuclei and non-muscle cells (Fig. 3 and 10). Fig.10a shows bFGF localization at the site of a heavily infiltrated cardiac lesion, 24 hr after IsP injection. Viable myocytes, apparently separated at their intercalated discs are seen within the lesion, and display pericellular bFGF localization (Fig. 10a, curved arrows). Remnants of necrotic myocytes maintain their strong anti- bFGF labelling (Fig.10a,arrows) and are closely surrounded by tightly packed non-muscle cells. Cells infiltrating the lesion (Fig.10a, arrowheads) display nuclear and pericellular bFGF localization.

To examine the temporal relationship between loss of vinculin and increase in cytoplasmic bFGF staining, sections were obtained

at 2,3, and 4 hr after IsP- treatment. At 2 hr, the staining patterns of sections from IsP-treated animals for vinculin and bFGF were indistinguishable from that of controls (data not shown). Clusters of vinculin-negative myocytes were clearly visible 3-4 hr after IsP administration (Fig.11a, arrowheads). A fraction of these myocytes displayed strong cytoplasmic anti-bFGF labelling (Fig.11b, arrowheads). The rest of the vinculin-negative myocytes did not show changes in anti-bFGF staining intensity compared to controls (Fig.11b,arrows). This contrast with the observations seen at 6 hr and 24 hr after Isp administration. Here, all vinculin-negative myocytes were strongly labelled for bFGF. This would indicate that the loss in vinculin staining precedes the increase in cytoplasmic bFGF staining in injured cardiac myocytes.

4. Localization of bFGF and muscle markers 1-4 wks after isoproterenol treatment

In the later stages of isoproterenol-induced myocardial injury, there is a replacement of necrotic myocardial tissue with scar tissue. The scar tissue is known to consist of connective tissue cells (fibroblasts, cells of the immune response, endothelial and smooth muscle cells [219,258]) and extracellular matrix. Figure 12a, b and c depicts a ventricular section, 1 week after treatment, triple immunolabeled with (a) anti-bFGF (S1) antibodies, (b) anti-vinculin antibodies, and (c) nuclear stain. Thick arrows indicate the scar region where fibrotic tissue has fully replaced the IsP- induced cardiac lesions. Nonmuscle cells within this scar region, as characterized by lack of anti-vinculin

labelling (b) and high nuclear density (c), exhibited some bFGF immunolabelling. Myocytes with strong cytoplasmic anti-bFGF staining were no longer present. As well, increased pericellular bFGF, in association with myocytes adjacent to the fibrotic area compared to myocytes located further away, could be discerned (Fig. 12, small arrows). A large number of "mini-myocyte"-like structures were also observed in the scar region, which exhibited both the pericellular anti-vinculin and anti-bFGF labelling (Fig.12a, arrowheads), and the majority of which did not appear to contain a nucleus (Fig. 12c). It is concluded that these "mini-myocytes" likely represent residual fragments from degenerated myocytes. Nuclear counterstain of the regions shown in Fig.12c demonstrates the high nuclear (and therefore cellular) density of the scar.

When the primary antibody, antiserum S1, is replaced by pre-immune antiserum, double fluorescence labelling of cardiac sections, 1 wk after IsP treatment, revealed a lack of pericellular anti-bFGF immunoreactiveity in any of the vinculin positive myocytes in and along the scar region (data not shown).

5. Localization of vimentin

As shown, figure 13b, vimentin antibodies labelled interstitial cells (fibroblasts, endothelial cells) and not adult cardiomyocytes (Fig. 13b, arrows). Areas of cellular damage, clearly observed in sections 24 hr after IsP treatment by the enhanced cytoplasmic anti- bFGF staining (Fig.13a, arrowheads), contained an increased density of vimentin- positive cells (Fig.13 b, arrowheads) compared to intact areas (Fig.13 b, arrows). Figure

13 (c,d,e) shows an adult rat ventricular section, 4 weeks after isoproterenol treatment, triple stained for (c) bFGF, (d) vimentin and (e) nuclear DNA. The thick arrow indicates the area of the scar, which is characterized as vimentin-positive,(d), with a high nuclear density,(e). The small arrows in Fig.13c marks a cardiomyocyte at the edge of the scar which exhibits increased bFGF pericellular immunostaining compared to adjacent myocytes. This same myocyte displays strong cytoplasmic anti-vimentin immunostaining, Fig.13d, in contrast to the majority of cardiomyocytes. The nuclear counterstain in Fig.13e shows that there is no cellular infiltration in the vimentin- positive myocyte.

We have found that a small number of myocytes (0.1%) at the borders of cardiac injury re-express vimentin.

As expected, when the primary antibody antiserum S1 was substituted with a pre-immune serum, no bFGF immunoreactivity was observed in either vimentin- positive muscle cells or vimentin-negative muscle cells (data not shown).

6. Localization of alpha-smooth muscle-actin

As in control cardiac tissue sections (Fig. 3c and d), at 24 hrs after IsP treatment, little or no alpha-smooth muscle-actin-positive immunolabelling can be seen in myocytes in and along the regions of necrosis (Data not shown). By 4 wks, however, when the necrotic myocytes is replaced by fibrotic scar tissue, positive immunolabelling can be seen in the non-muscle cells of the scar and in some adult cardiomyocytes bordering the lesion. Figure 14

depicts an adult ventricular section, 4 wks after isoproterenol treatment. This section is triple immunostained with (a) anti-bFGF (S1), (b) anti-alpha-smooth muscle actin, and (c) Hoechst 33342 immunofluorescence dye. The thick arrow in (a) points to a cardiomyocyte at the edge of the scar which is immunoreactive for alpha-smooth muscle-actin (b). The arrow in (c) shows the respective nuclei of the immunoreactive alpha-smooth muscle-actin cardiomyocyte, indicating that this is indeed a muscle cell. Arrowheads in Figure 14b indicates the alpha-smooth muscle actin positive non-muscle cells present in the scar region.

When the primary antibody antiserum S1 was substituted with pre-immune serum, no bFGF immunoreactivity was observed at all in the section (data not shown).

7. Nuclear bFGF

Examination of cardiac ventricular sections of adult rat hearts 1 week after treatment shows strong nuclear anti-bFGF (S1) labelling in adult cardiomyocytes near the necrotic lesion. Figure 15 depicts a 1 wk isoproterenol treated ventricular section, triple immunolabelled with (a) anti-bFGF (S1), (b) Hoechst 33342 immunofluorescence dye and (c) anti-vinculin. Arrows in Fig.15a indicate myocyte nuclei exhibiting enhanced anti-bFGF immunolabelling compared to those indicated by arrowheads, situated away from the scar. Arrowheads in (b) confirm that the myocyte nuclei in (a) is indeed displaying the enhanced anti-bFGF (S1) nuclear staining. Anti-vinculin immunostaining in (c) confirms that the cells seen in (a) and (b) are indeed muscle cells.

Thus, from these results, it seems that the majority of myocytes located at the edges of the scar, exhibit increased nuclear bFGF localization compared to myocytes away from the scar.

8. Continued necrosis at 32 weeks after isoproterenol administration

Examination of ventricular sections, 32 wks after isoproterenol administration, shows that some myofibers within the scar region continue to undergo necrosis based on the increased cytoplasmic anti-bFGF staining (arrows in Fig.16). Anti-desmin counterstain of the field shown in Fig.16a demonstrates that the necrotic myofiber has lost its anti-desmin immunoreactivity. The nuclear counterstain, Fig. 16c, shows that this necrotic myofiber is indeed a muscle cell.

9. Basic FGF in cardiac extracts from isoproterenol-treated rats.

Cardiac protein extracts from control and isoproterenol-treated rats, at 6 and 24 hr, and at 1,4,and 6 weeks after treatment, were examined for bFGF- like peptides. These time points were selected to analyze the short term response (6-24 hr; before and/or during intense cellular infiltration and necrosis, edema) as well as the long term response (1-6 weeks; established fibrotic scar, hypertrophy) of the heart to IsP, at the level of bFGF accumulation. Protein yield was similar in extracts from all control as well as IsP treated rats, 42 ±2mg protein per g (wet weight) of tissue. Equivalent amounts of these extracts (ie. containing exactly the same amount of extracted protein) were

fractionated by heparin-sepharose affinity chromatography. Peptides which remained bound to heparin after elution with 1.1M NaCl were analyzed for their bFGF content by immunoblotting. Densitometry was used to obtain an estimate of the relative intensities of the immunoreactive bands, as previously described [203,217], arbitrarily assigning a value of 1.0 to the measurements from control extracts. Variation in the densitometric values between different control samples was less than 10% of the mean. Characteristic results are shown in Fig.17a. Overall immunoreactive bFGF showed a 2-fold increase in extracts from IsP-treated animals over equivalent controls, at 24 hr and 1-6 weeks after treatment. No such difference was detectable at 6 hr timepoint. A characteristic profile of bFGF in cardiac extracts is shown in the immunoblot of Figure 17b. Human recombinant bFGF (5 ng), migrating as a 18 kDa protein in our system, was used as a positive control (lane1). Each lane (Fig.17b, lanes 2-5) contains the heparin- bound fraction (approximately 3 ug total protein) from 40mg of extracted protein. Basic FGF in extracts from control rat hearts (Fig.17a and b, lanes 2 and 4) consisted primarily (90% of the total immunoreactivity) of an 18 kDa peptide and trace amounts of higher molecular weight, 21-23 kDa forms, at all time points examined. Similarly, the 18 kDa bFGF composed over 90% of total immunoreactivity in extracts from IsP-treated animals at 6 hr,1,4 and 6 weeks after injection (Fig.17a and b, lane5). At 24 hr after IsP injection, however, the 21-23 kDa bFGF levels were elevated, comprising about 27% of the total (Fig.17a and b,lane 3, arrow).

These results would indicate that the higher molecular weight forms of bFGF may play a role in the early response to isoproterenol-induced cardiac injury, related possibly to the intrinsic cellular proliferation known to occur within the necrotic regions at this time point.

In addition, the information conveyed by Fig.17a represents the average of two complete experiments. There was an approximately 12% variation from the mean between measurements from individual experiments.

10. Determination of mitogenic activity of extracts

To compare relative bFGF content with mitogenic activity, extracts obtained at 6 and 24 hr and 1 week after IsP injection were tested for their ability to stimulate DNA synthesis of RFC's, a cell type which is highly sensitive to bFGF [203]. Extracts were added to RFCs maintained at low serum concentration, and ^{3}H-thymidine incorporation determined 24 hr later. Results were tested for significance using the T-EASE program (unpaired T-tests) and are summarized in Table 2. Insignificant changes in mitogenicity of cardiac extracts were observed at 6 hr after treatment while at 24 and 1 week cardiac extracts displayed a significant increase in activity compared to controls. This increase was most pronounced at 24 hr after treatment, at 40% over control value.

C. TABLES AND FIGURES

1.TABLES:

TABLE I: COMPARISON OF PERCENT CARDIAC HYPERTROPHY FOR IsP TREATED AND NON-TREATED RATS AT 24 hr AND 1 wk AFTER TREATMENT.

STATUS	n	BW(g)	HW($x10^{-2}$ g)	HW\BW($x10^{-4}$ g)	%Hyper
24hrs cont	4	343.50±8.89	128.00±5.93	37.23±1.10	
1wk cont	4	343.25±8.32	117.00±1.55	34.20±0.71	
24hrs isop	4	309.00±4.97	140.00±5.23	45.24±1.62	**21.42±4.27
1wk isop	4	312.25±9.86	110.00±3.37	35.26±0.57	*3.10±1.66

($p<0.05$) ± SEM.

* marginally significant from control group.

** significant from control group.

Results are means ± SEM of 4 animals for each group. *$P<0.05$ vs. non-treated controls. Rats were treated with IsP (40mg/kg body weight) and sacrificed after 24 hr and 1 wk after injection. Age-matched rats were used as controls and sacrificed at the same time as IsP- treated rats. Heart weights (HW) and body weights (BW) of the IsP- treated rats were measured and their HW/BW ratios were calculated and compared to their respective controls. This comparison is expressed as percent hypertrophy.

TABLE II: EFFECT OF CARDIAC EXTRACTS ON DNA SYNTHESIS OF RFCs. COMPARISON BETWEEN EXTRACTS FROM IsP-TREATED RATS AND AGE-MATCHED CONTROLS.

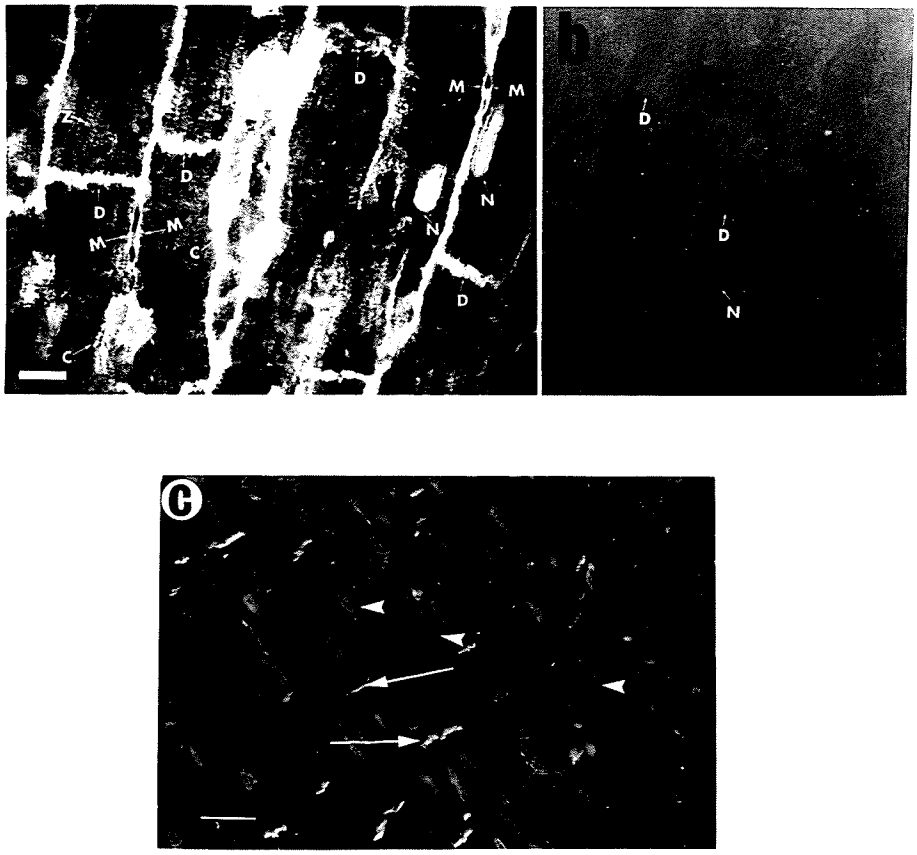
Status	n	X_{DPM}	(IsP-C/C)X100
6hr,C	5	6336±574	(-)10
6hr,IsP	4	5709±309	
24hr,C	5	6785±286	*40
24hr,IsP	5	9763±631	
1wk,C	8	6074±171	*20
1wk,IsP	7	7181±456	

(P<0.05) ±SEM.
* Significant.

Results are means ± SEM of 5 to 8 determinations for each group. * P< 0.05 vs. non- treated controls (C). Cardiac extracts from IsP-treated and age-matched control non-treated rats (at 6, 24hr and 1 wk after treatment) were added to RFCs cultures. ^{3}H-thymidine incorporation was then measured in RFC cultures and expressed as degradations per minute (DPM). A comparison of ^{3}H-thymidine incorporation, in RFCs treated with cardiac extracts from IsP-treated rats versus respective control extracts, are expressed as a percentage over control values.

2. FIGURES:

a. FIGURES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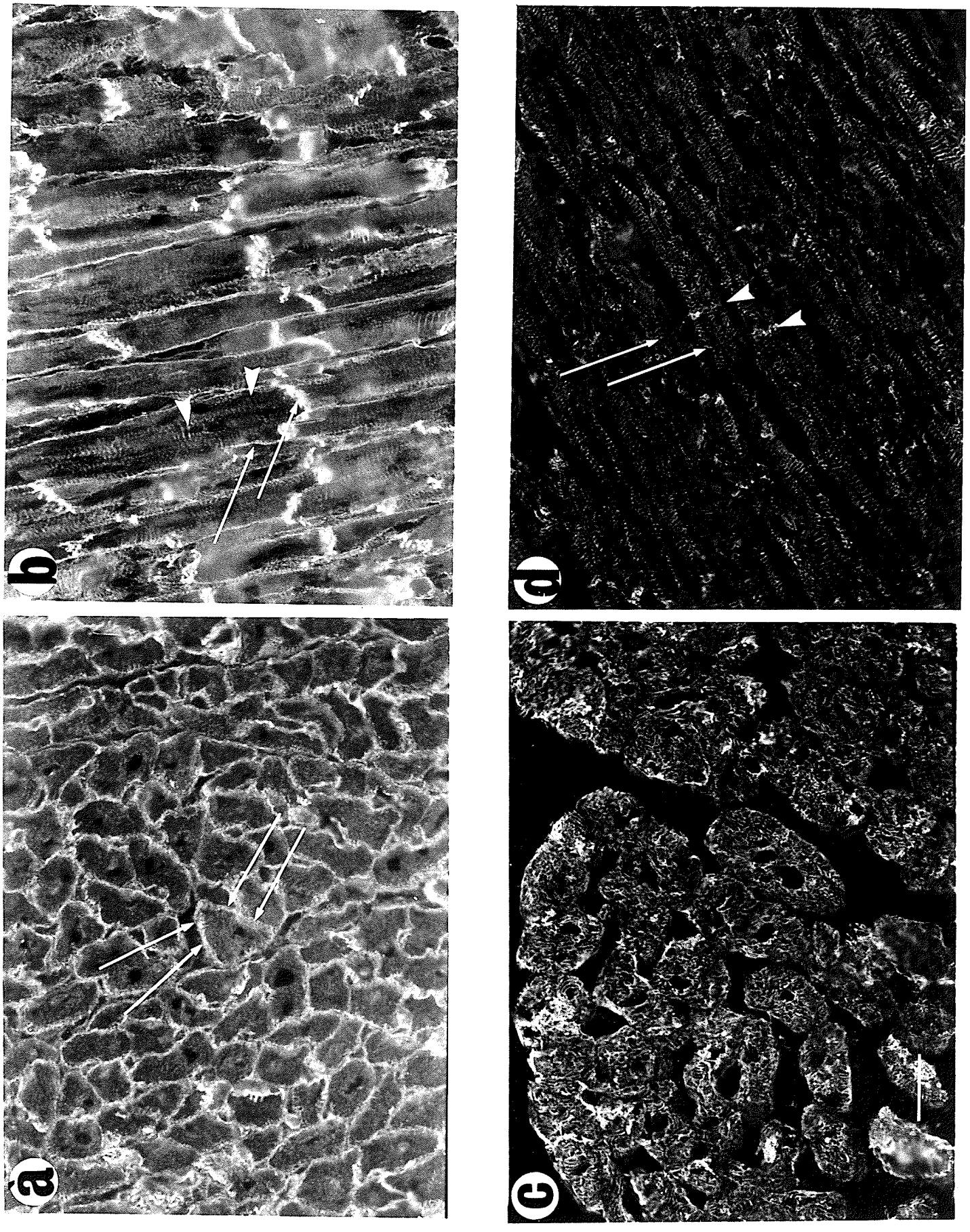
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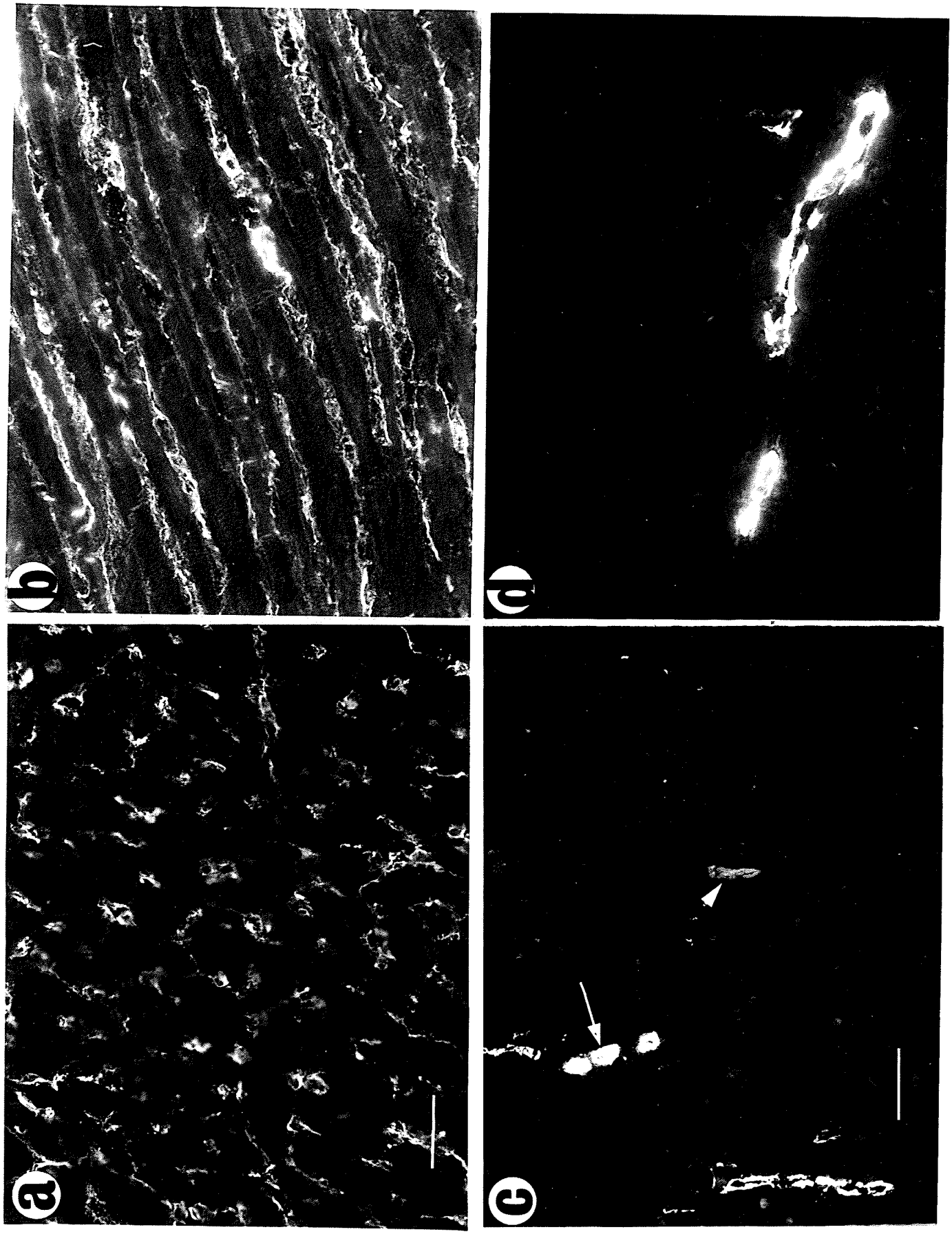
Figure 3



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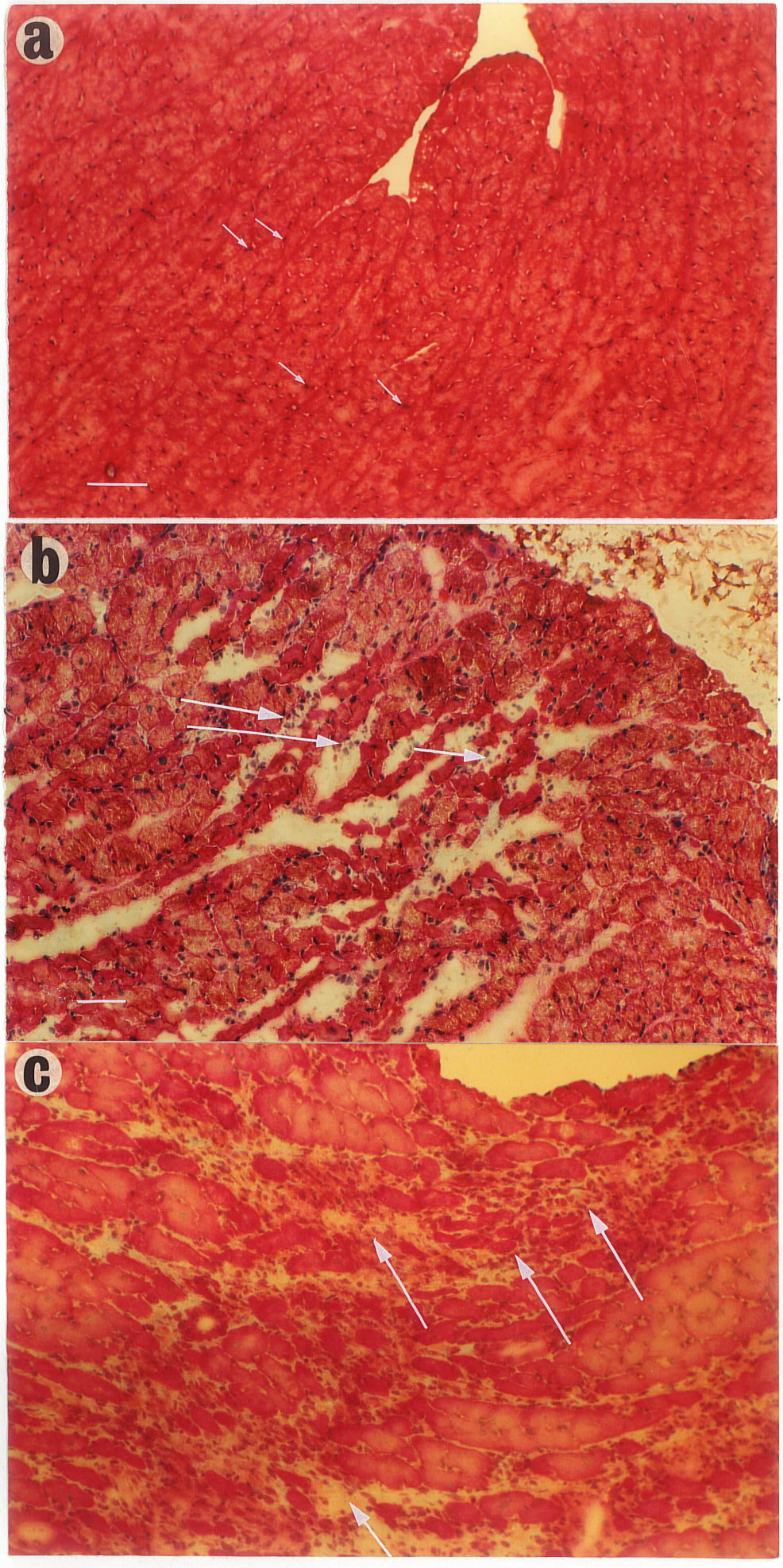

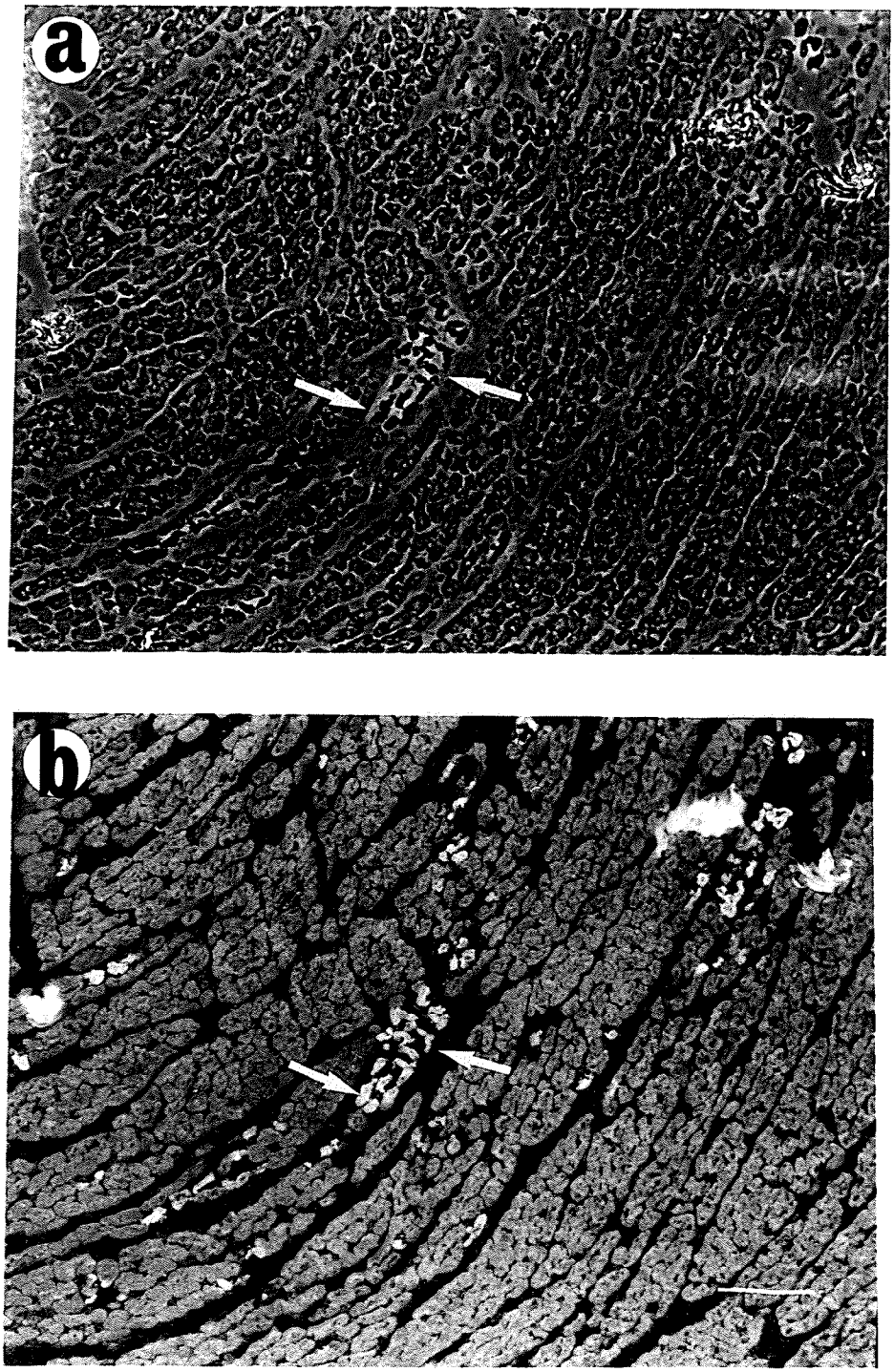
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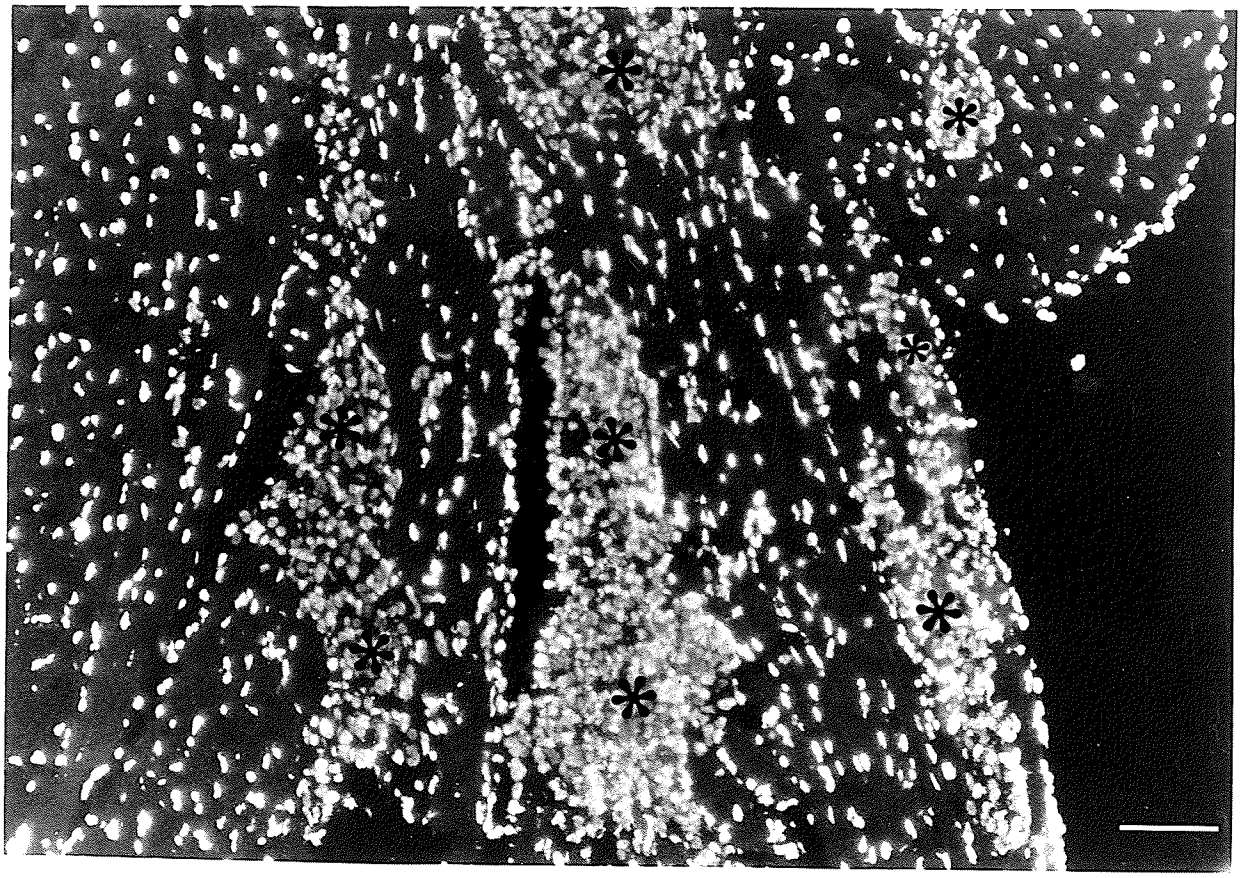

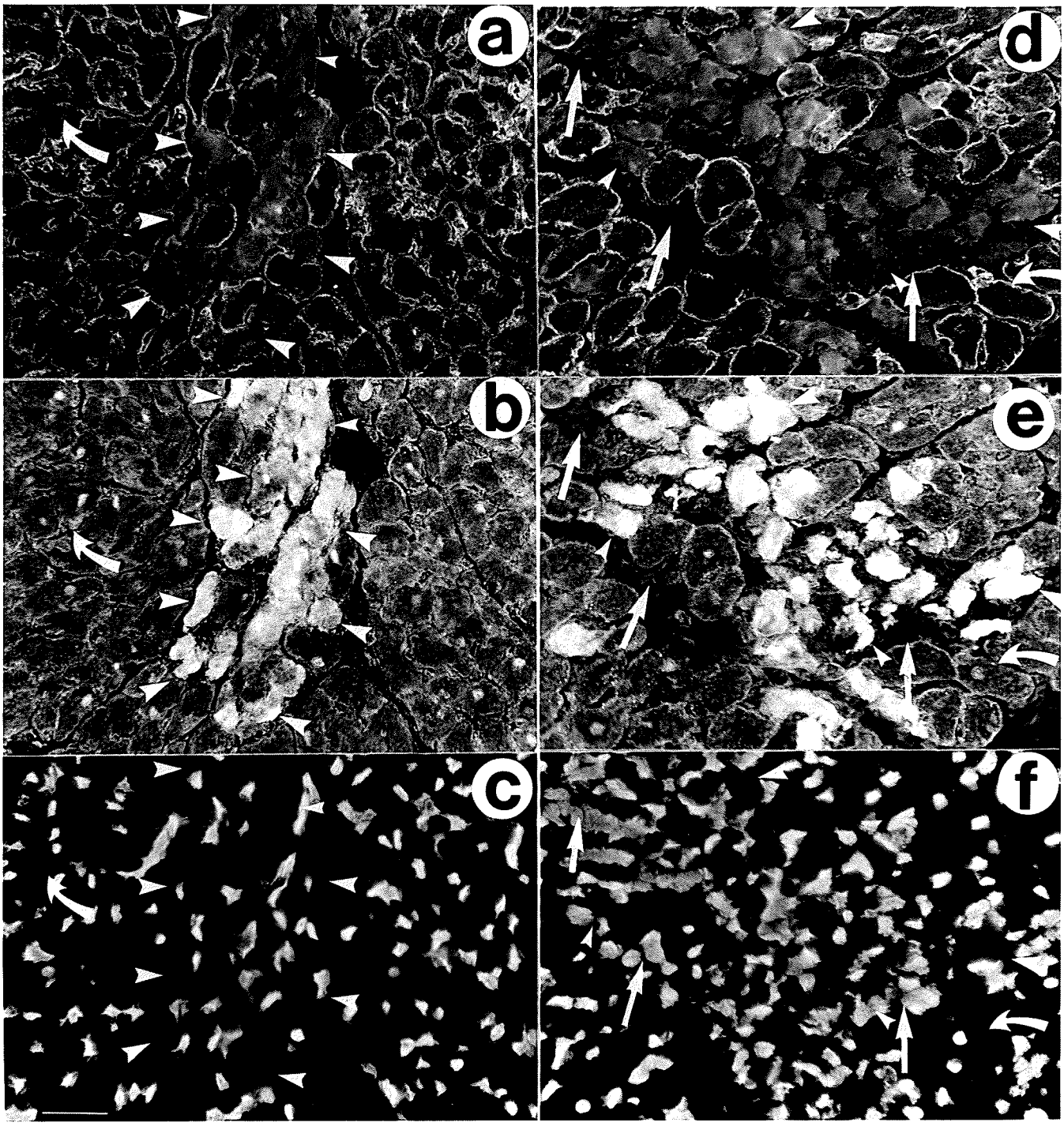
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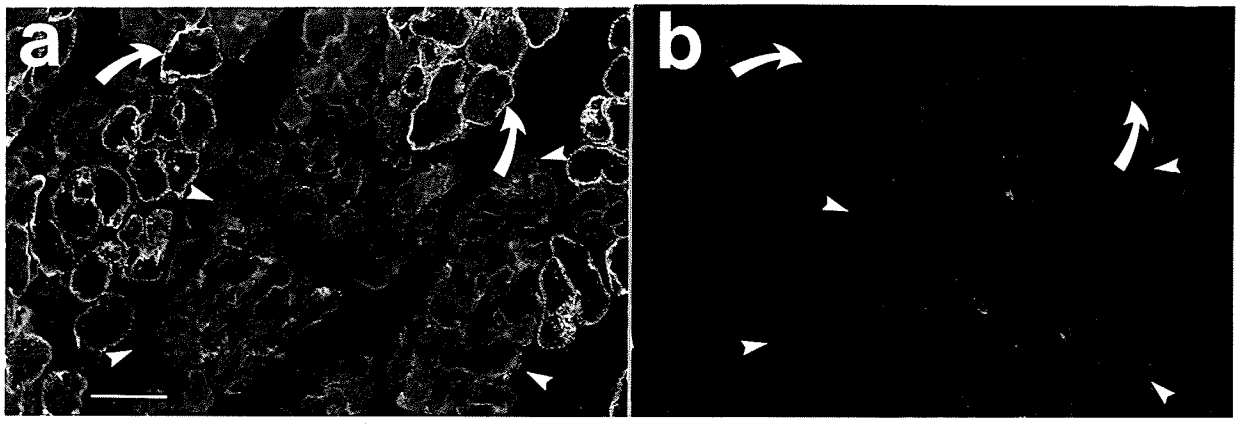
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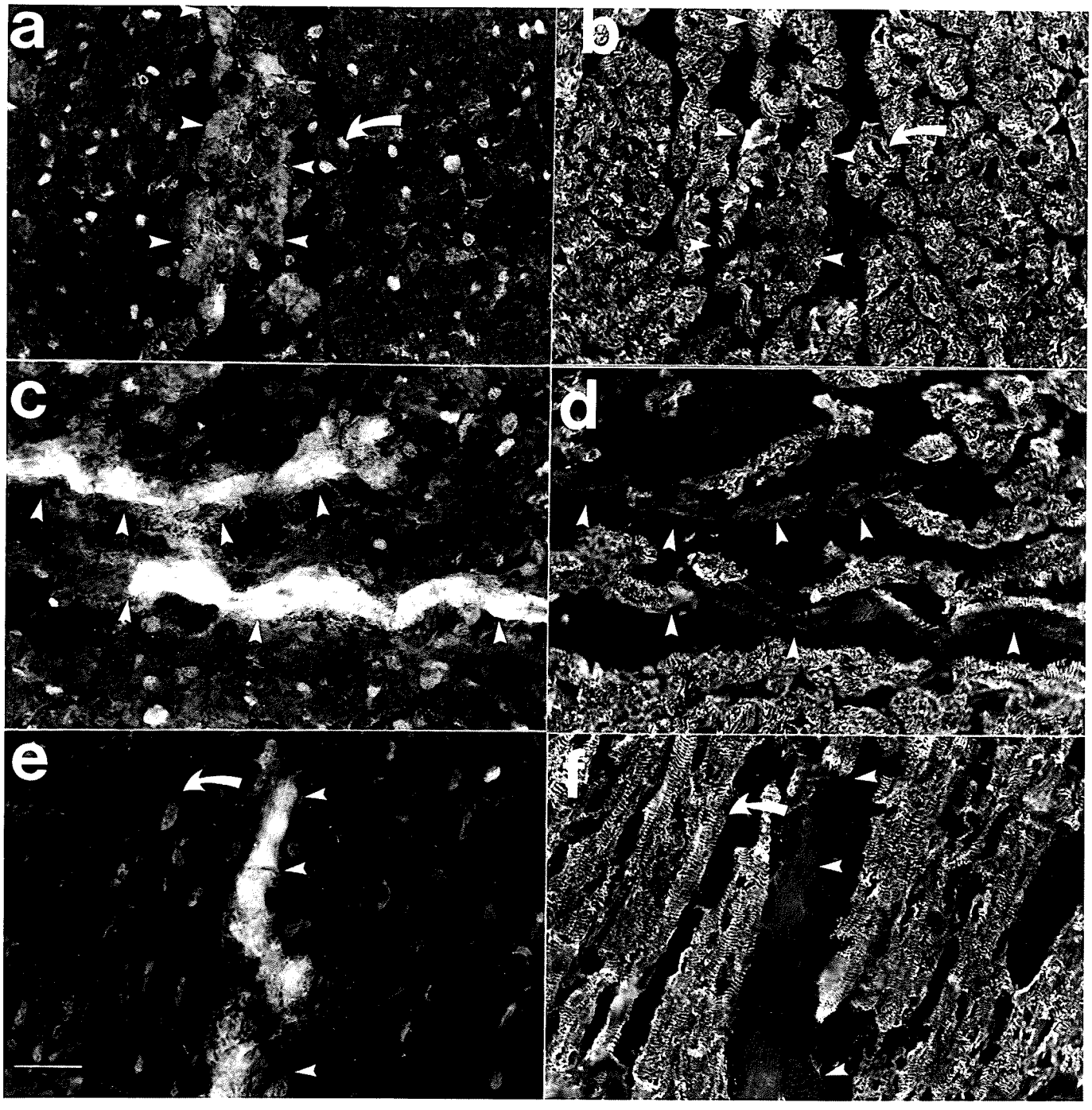
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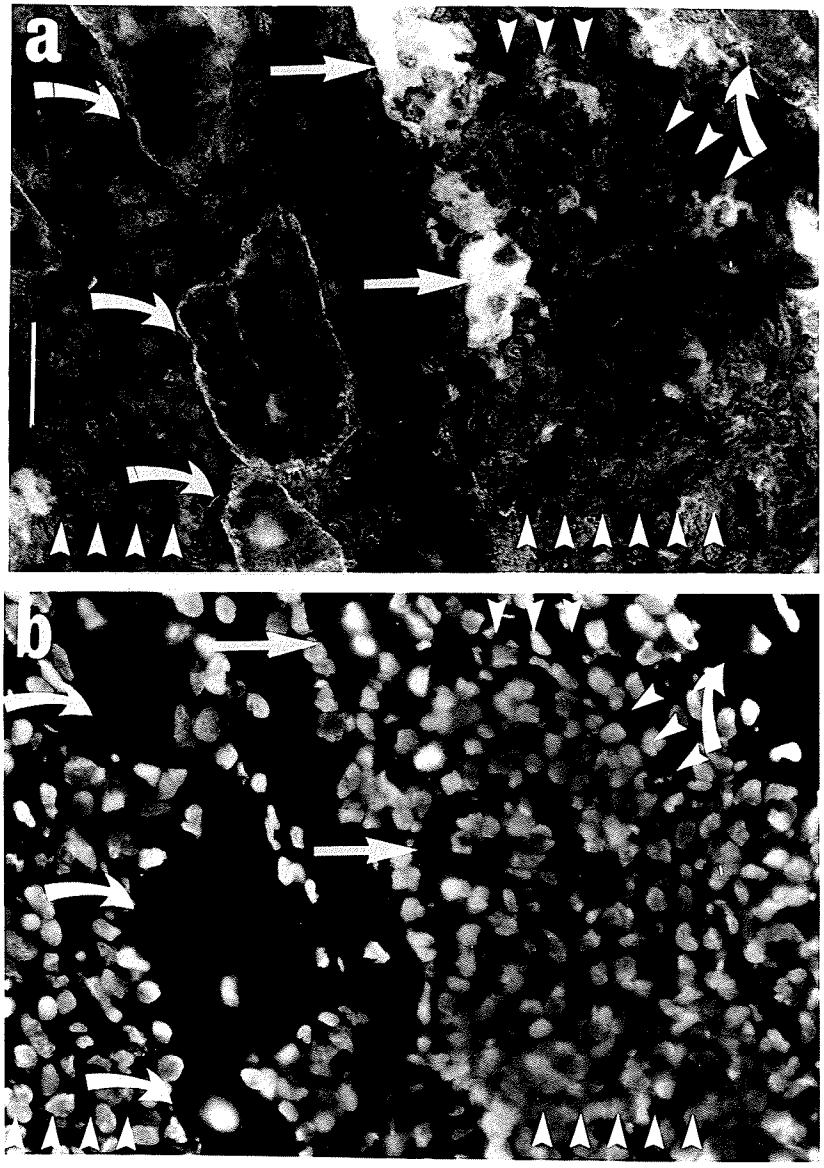
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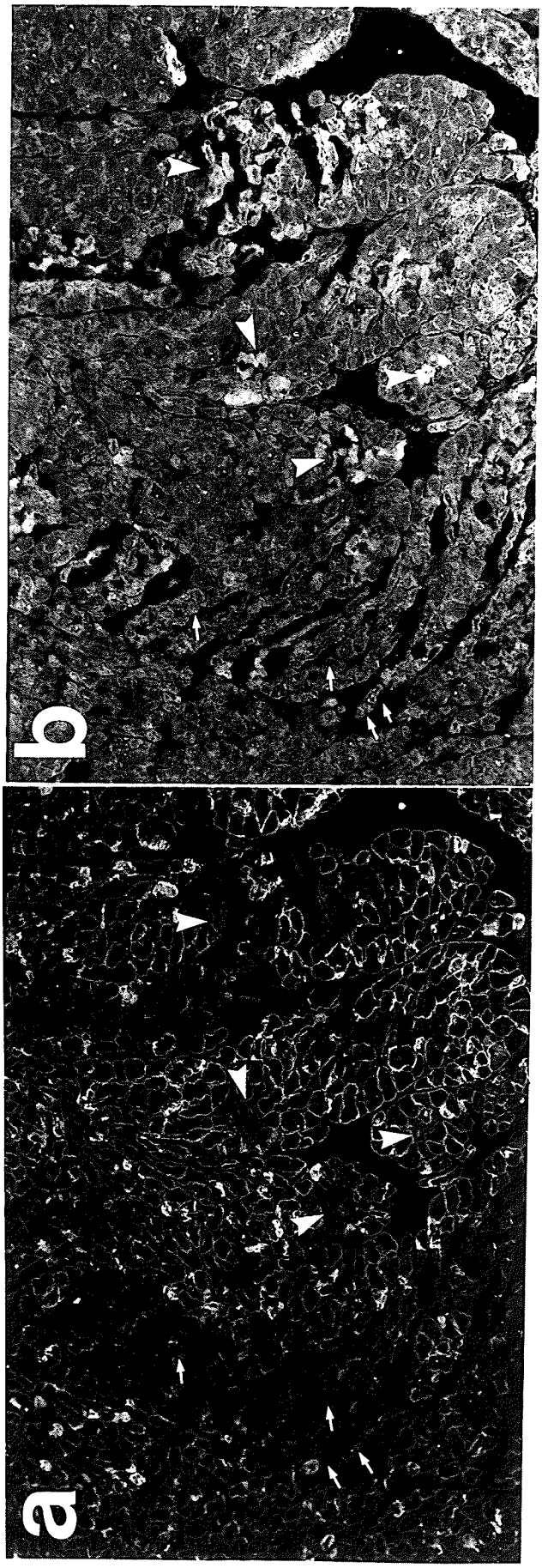
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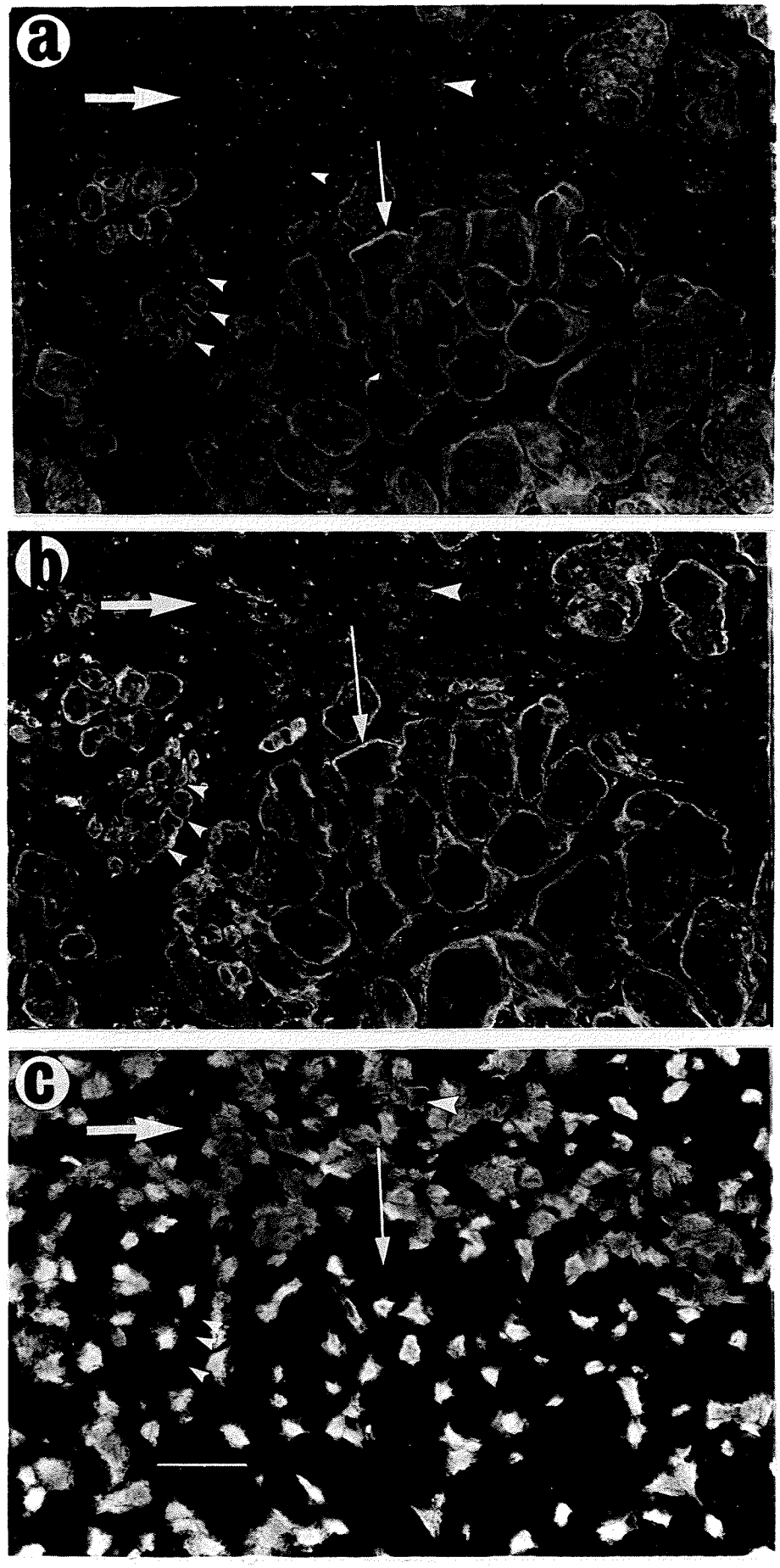
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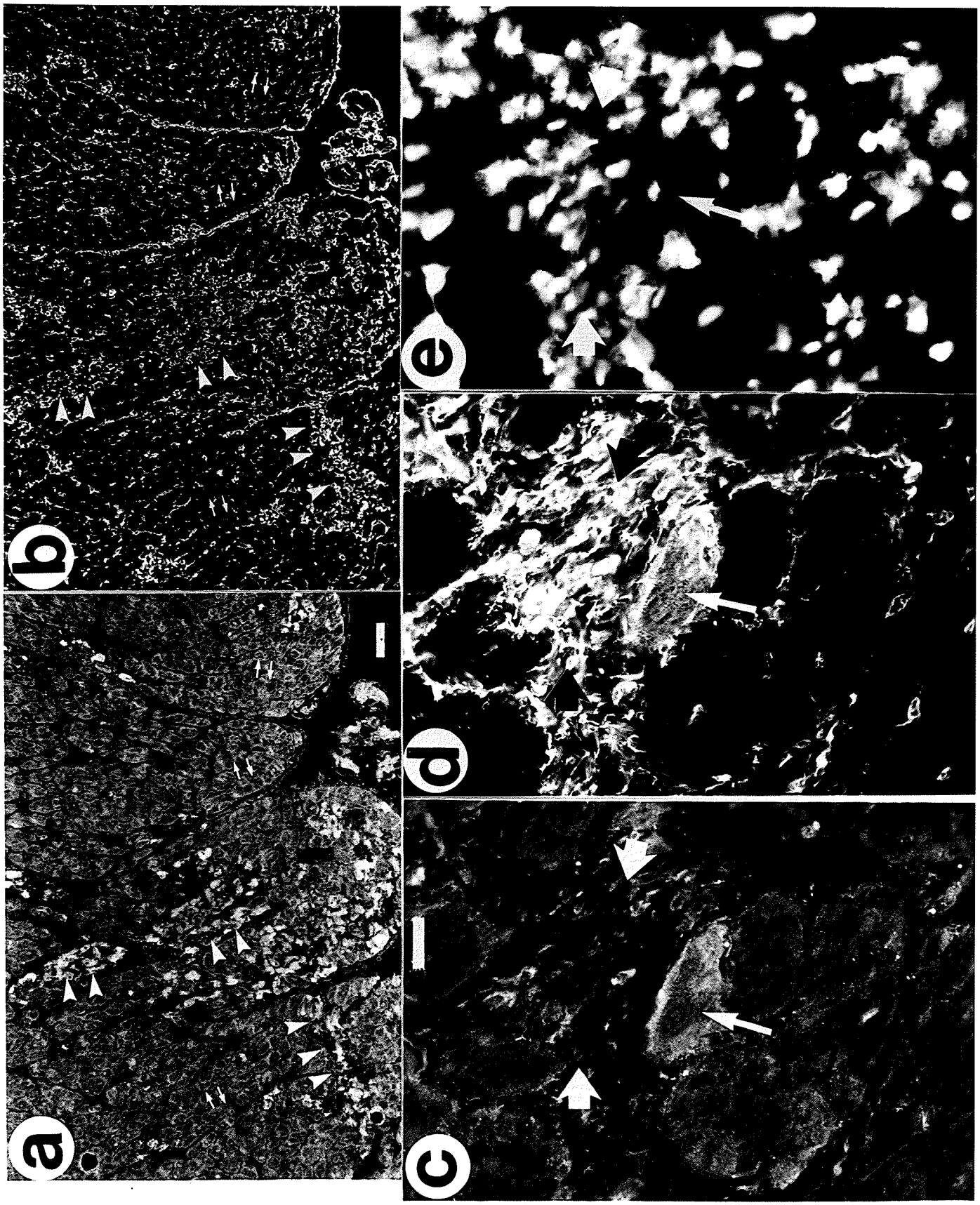
Figure 13



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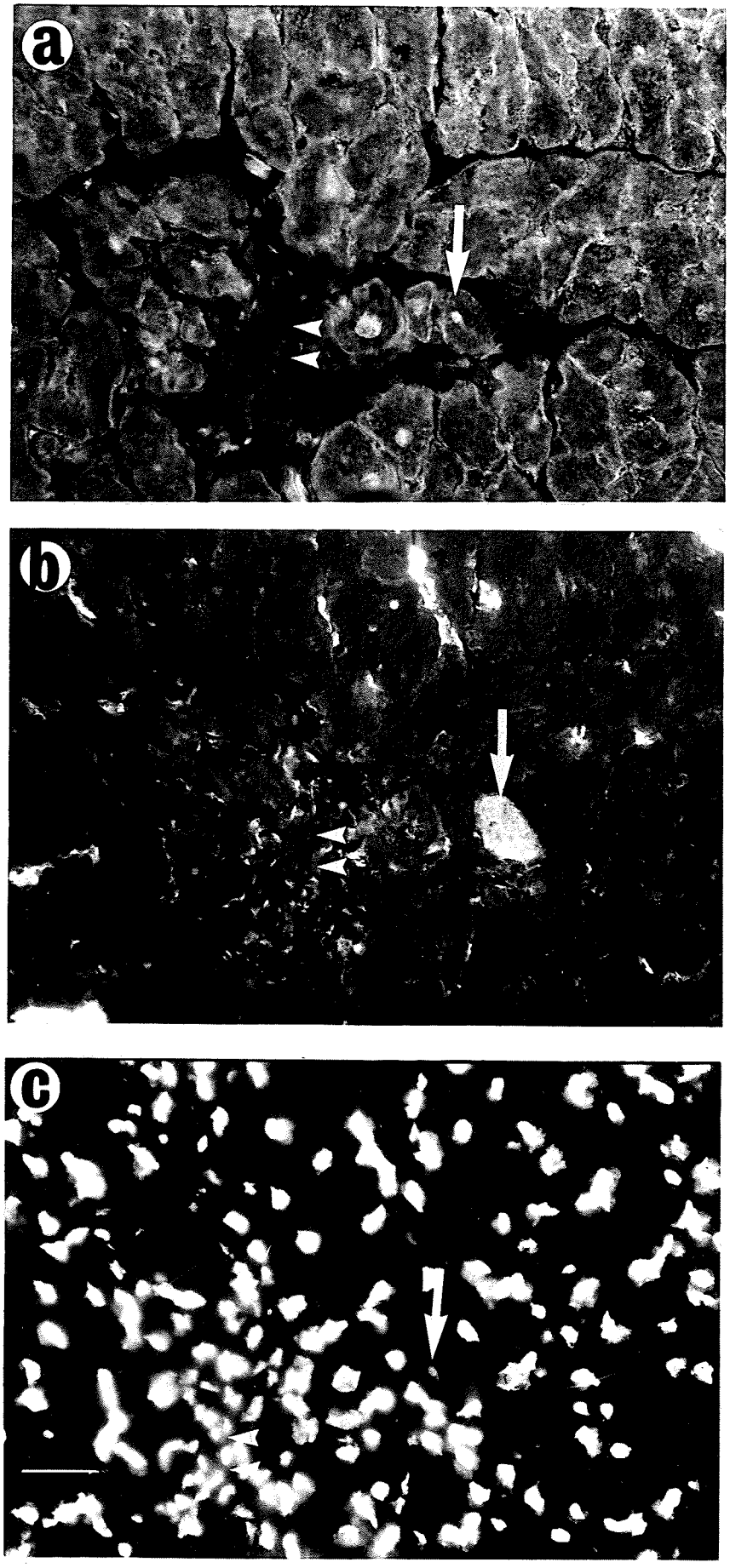


Figure 15

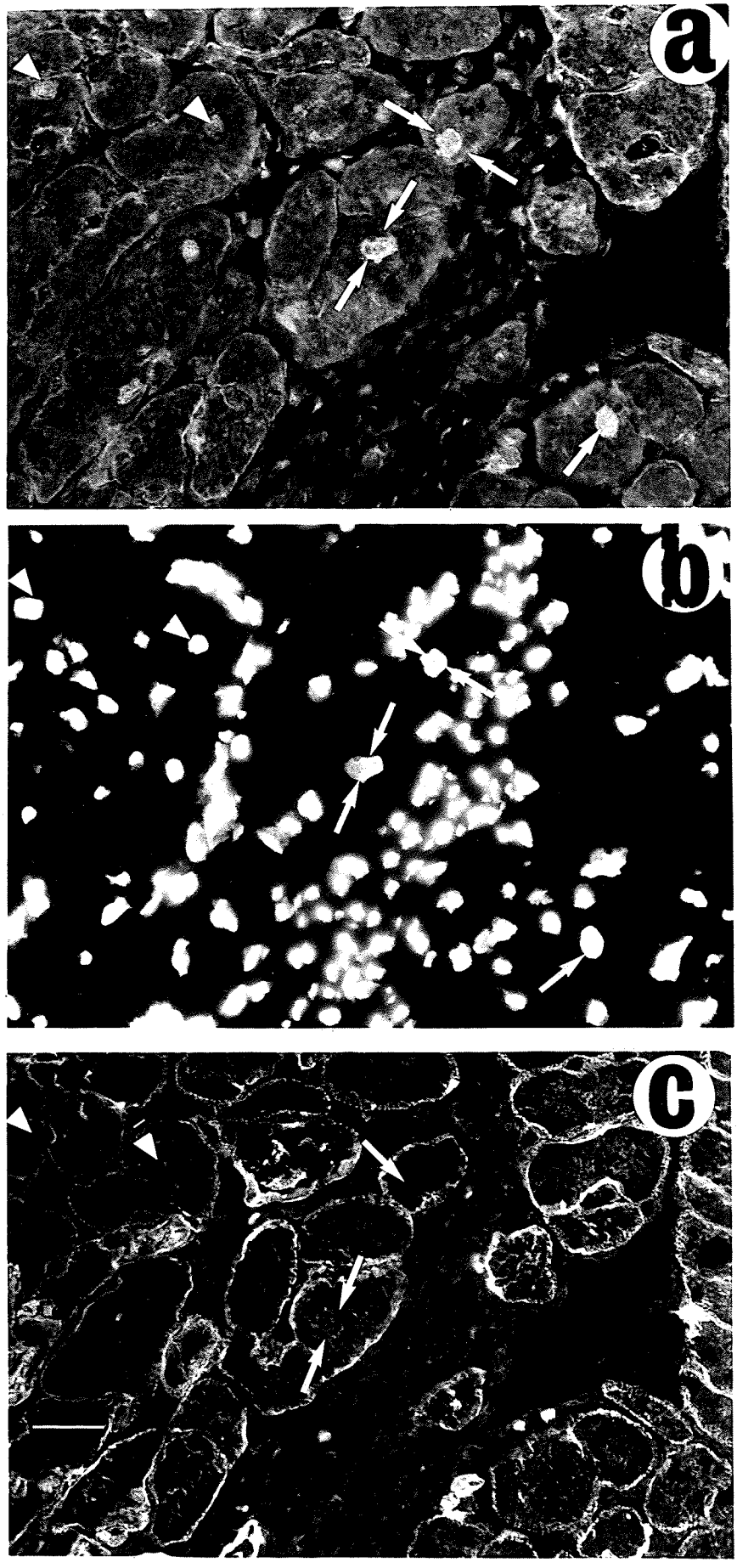
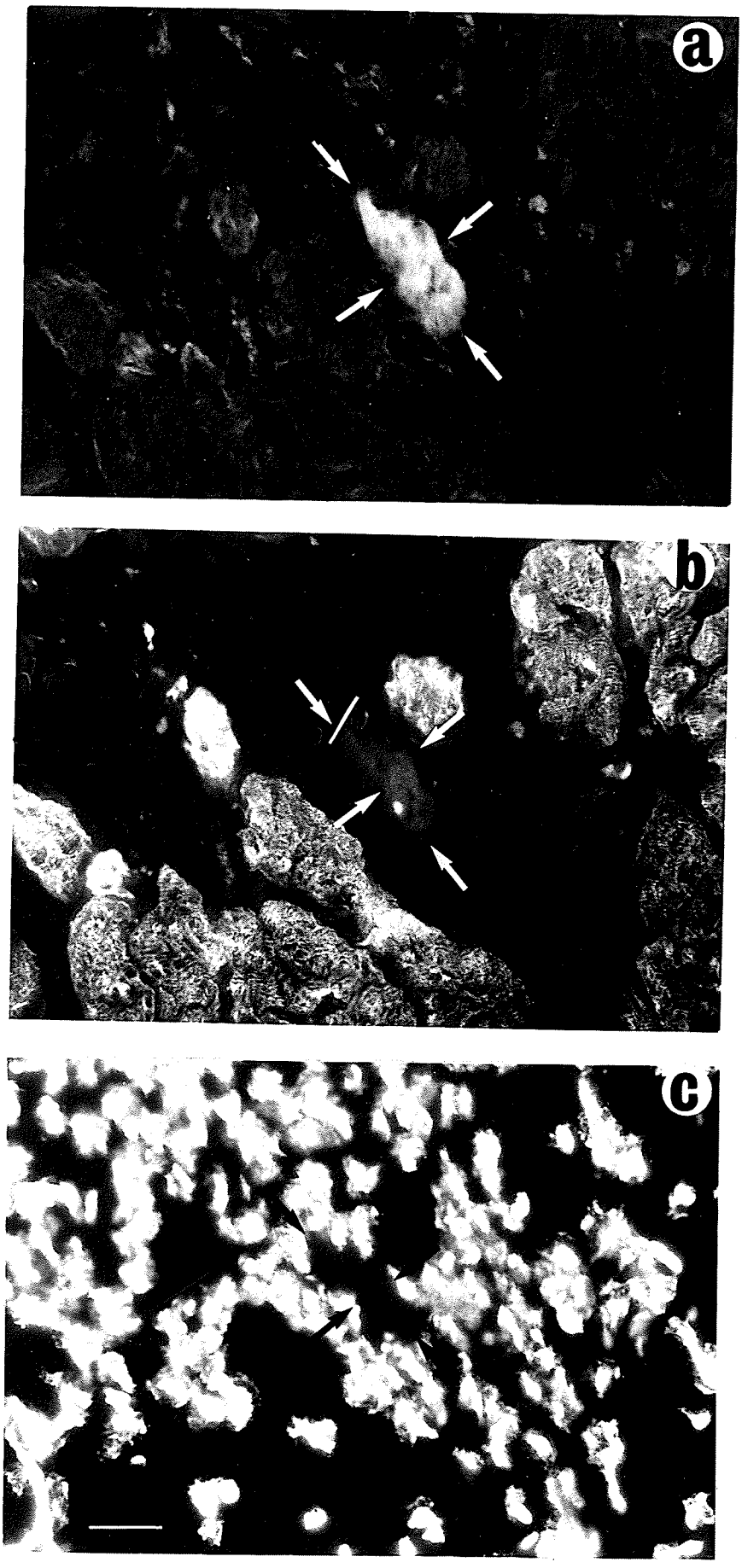
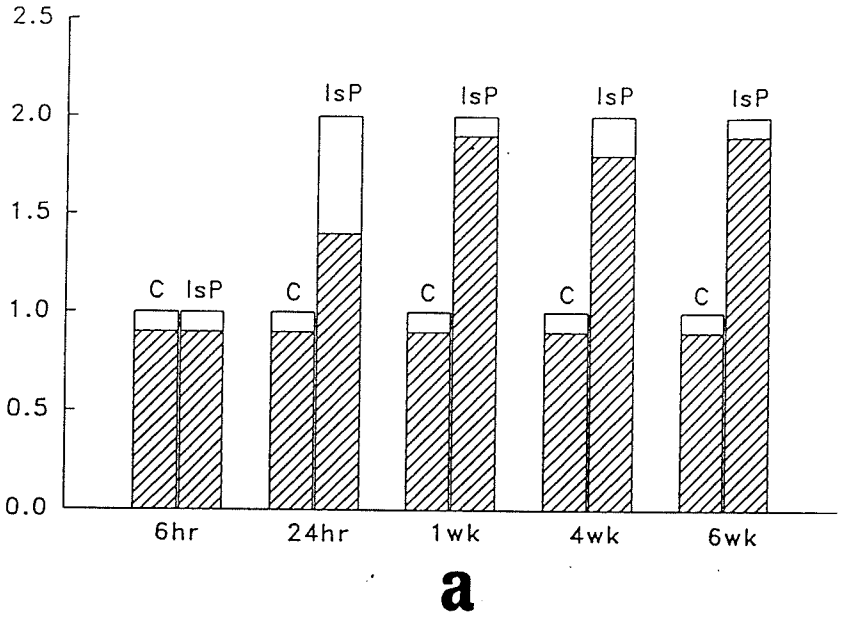


Figure 16





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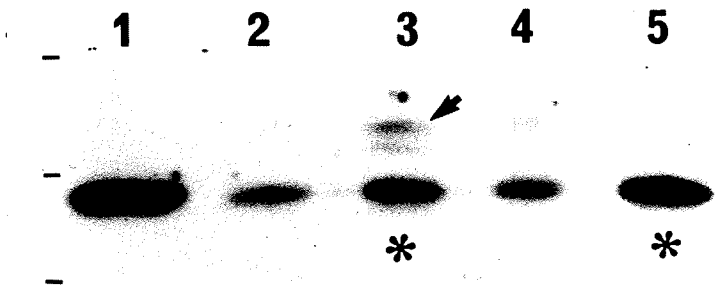

b

b. FIGURE LEGENDS

Figure 1:Micrograph of transverse cardiac section of a normal adult rat (a,b). Double fluorescence staining for bFGF and with pre-immune rabbit serum (1:2000), respectively. (a): C= capillaries, D= intercalated discs, M= basement membrane, N= muscle nucleus, Z= muscle Z-lines. (bar =10um). (c).Indirect immunofluorescence labelling with anti- bFGF(S1 derived, affinity purified) antiserum. Arrows point to intercalated discs, arrowheads point to nucleus. (bar =20um).

Figure 2:Micrographs of transverse,(a,c), and longitudinal,(b,d), cardiac section of a normal adult rat. (a,b). Indirect immunofluorescence labelling for vinculin. Arrows point to cell margins, arrowheads point to Z-line myofibrils. (c,d). Indirect immunofluorescence for desmin. Small arrows point to z-line myofibrils, arrowheads point to intercalated discs. (Bar =20 um).

Figure 3:Micrograph of a cardiac section of a normal adult rat (a,b) Indirect immunofluorescence labelling for vimentin in transverse,(a), and longitudinal, (b), sections respectively. (Bar =20 um). (c,d). indirect immunofluorescence labelling for alpha-smooth muscle actin at ,(a), high magnification (bar =50 um), and at, (b), low magnification (bar =20 um). Arrow points to blood vessel, arrowhead points to alpha- smooth muscle actin positive myocyte.

Figure 4:Micrographs of transverse cardiac sections. (a,b). Mason's trichrome staining at control (a), 9hrs (b), and at 1 week (c), after isoproterenol treatment. Arrows indicate nucleus. (bar =50 um).

Figure 5:Micrograph of a transverse cardiac section at 24 hr after isoproterenol treatment. (a,b). Phase contrast view and indirect immunofluorescence labelling for bFGF, respectively. Arrows indicate myocytes with disrupted cellular morphology and increased bFGF staining.(bar =50 um).

Figure 6:Micrograph of section cut across the middle of and perpendicular to the long axis of the heart, 24 hr after IsP injection, stained for nuclear DNA with the fluorescent stain Hoechst 33342. Asterisks mark areas of high nuclear (cellular) density. (bar=50 um).

Figure 7:Micrographs of transverse cardiac sections. (a,b,c): Triple fluorescence labelling for vinculin, bFGF and nuclear DNA, respectively, at 6 hr after IsP treatment.(c,d,e): Triple fluorescence labelling for vinculin, bFGF and nuclear DNA, respectively, at 24 hr after IsP treatment. Antiserum S1 was used for bFGF localization at 1:2000 dilution. Curved arrows indicate myocytes with intact vinculin staining along their margins and the anticipated (nuclear, pericellular) anti-bFGF labelling. Straight

arrows indicate infiltrating cells. (bar =20 um).

Figure 8:Micrograph of a transverse cardiac section at 24 hr after IsP treatment. Double fluorescence labelling for vinculin and bFGF, respectively. Antiserum S1 was used for bFGF localization at 1:2000. Arrowheads indicate myocytes with reduced vinculin and increased bFGF staining.(bar =20 um).

Figure 9:Micrographs of cardiac sections. (a,b). Double immunofluorescence labelling for bFGF and desmin, respectively, at 6 hr after IsP injection. (c,d). Double immunofluorescence labelling for bFGF and desmin, respectively, at 24 hr after IsP injection. (e,f), same as (c,d). Affinity-purified anti- bFGF IgG was used. Curved arrows indicate myocytes with the expected bFGF (nuclear,weak cytoplasmic) and desmin staining. Arrowheads indicate myocytes with increased cytoplasmic bFGF labelling.
(bar =20 um).

Figure 10:Micrographs of transverse cardiac sections at 24 hr after IsP treatment. (a,b). Double fluorescence for bFGF and nuclear DNA, respectively. Curved arrows indicate normal myocytes within a lesion. Antiserum S1 was used for bFGF localization at 1:2000.Arrowheads mark areas of high cellular density. Straight arrows point to residues of necrotic myocytes which stain intensely for bFGF. (bar= 20 um).

Figure 11:Micrographs of transverse cardiac sections at 4 hr after IsP treatment. (a,b). Double fluorescence labelling for vinculin and bFGF, respectively. Antiserum S1 was used for bFGF localization at 1:2000. Arrowheads indicate myocytes with reduced vinculin and increased bFGF staining. Small arrows point at myocytes with reduced vinculin but unchanged anti-bFGF staining.(bar= 50 um).

Figure 12:Micrographs of cardiac sections at one week after isoproterenol treatment. (a,b,c). Triple fluorescence labelling for bFGF, vinculin and nuclear DNA, respectively. Antiserum S1 was used for bFGF localization at 1:2000 dilution. Thick arrows indicates region of fibrotic scar. Thin arrows point to myocytes with enhanced pericellular bFGF staining. Small arrowheads point to mini myocytes. Large arrowheads point to bFGF stained nonmuscle cells. (bar = 20um).

Figure 13:Micrographs of transverse cardiac sections. (a,b). Double-fluorescence labelling for bFGF and vimentin, respectively, 24 hr after IsP administration. (c,d,e). Triple fluorescence labelling for bFGF, vimentin and nuclear DNA, respectively. Arrowheads in (a,b) point at regions of necrosis (a) and increased density of vimentin- positive cells. (b). Small arrows in (a,b) mark non-necrotic areas (a) with normal density of vimentin-positive cells (b). Thick arrow in (c,d,e) point at fibrotic region. Long arrow in (c,d,e) marks a cardiomyocyte which displays

increased anti-bFGF staining (c), stains positive for vimentin (d) and contains one nucleus (e). (bar in a,b=50 um) (bar in c,d,e=10 um).

Figure 14:Micrograph of transverse cardiac sections at 4 weeks after IsP treatment. (a,b,c). Triple fluorescence labelling for bFGF, alpha- smooth muscle actin and nuclear DNA, respectively. Antiserum S1 was used for bFGF localization at 1:2000 dilution. Thick arrow points to a myocyte with increased alpha- smooth muscle actin staining and enhanced bFGF nuclear stain. Arrowheads point to nonmuscle cells in fibrotic scar which display alpha- smooth muscle immunostaining.(bar =20 um).

Figure 15:Micrographs of transverse cardiac sections at 1 week after IsP treatment. (a,b,c). Triple fluorescence labelling for bFGF, nuclear DNA and vinculin respectively. Antiserum S1 was used for bFGF localization at 1:2000 dilution. Arrows point to myocytes with enhanced bFGF nuclear staining. Arrowheads point to myocytes with normal bFGF nuclear staining. (bar =10 um).

Figure 16:Micrographs of transverse cardiac section at 32 weeks after isoproterenol treatment. (a,b,c). Triple fluorescence labelling for bFGF, desmin and nuclear DNA. S1 antiserum was used for bFGF localization at 1:2000 dilution. arrows point to a myocyte with increased cytoplasmic bFGF and decreased desmin staining. (bar =10 um).

Figure 17:(a). Relative bFGF content of extracts from control (c) and IsP-treated (IsP rats, at various time points after treatment. Verticle axis, normalized densitometric measurements from autoradiograms of immunoblots. Each column represents total bFGF (18 and 21-23 kDa species). Shaded area in each column indicates contribution from the 18 kDa bFGF species. (b). Analysis of bFGF in cardiac heparin-bound fractions by immunoblotting. Lane 1, recombinant human bFGF (5 ng). Lanes 2 and 4, bFGF in extracts from non-treated rat hearts, serving as age-matched controls for lanes 3 and 5, respectively. Lanes 3 and 5, bFGF in cardiac extracts from IsP- treated rats, at 24 hr and 1 week after treatment. Arrow in lane 3 indicates the position of 21-23 kDa bFGF. Small vertical bars denote migration of molecular weight markers (top, 31 kDa; middle,21 kDa; bottom, 14.4kDa).

V. Discussion.

The purpose of this study was to examine a potential role for bFGF in the response of cardiac myocytes to isoproterenol-induced injury. This study was conducted using three different approaches: (i) measuring biological activity of cardiac extracts; (ii) analyzing and characterizing BFGF in cardiac extracts by immunoblotting and densitometry; and, (iii) localizing BFGF distribution in cardiac tissue sections via indirect immunofluorescence. Thus far, we were able to demonstrate increased accumulation and distinct patterns of localization in cardiac myocytes from isoproterenol-treated animals.

V(A). Technical Considerations

Several technical aspects about the various procedures of extracting and immunoblotting and immunofluorescence need to be considered and discussed to appreciate the conclusions drawn from the results.

V(A1). Determination of bFGF accumulation

To obtain an estimate of overall bFGF accumulated by rat hearts, we used, (a) two cycles of tissue extraction followed by (b) heparin-sepharose affinity fractionation, and (c) Western blotting with specific anti-bFGF IgG. This procedure of tissue extraction resulted in maximum yields of soluble bFGF, as previously described [217]. Further extraction of tissue residues or use of different extraction buffers did not change the relative yields in bFGF from the various cardiac extracts tested. Heparin is widely used for the fractionation of FGF-like growth factors

from extracts, serving also to protect bFGF from degradation [181,175]. This protection is partially lost upon elution of bFGF with high salt [175]. This partial loss may be attributed to proteases, previously found to co-elute with bFGF at high salt [175], which attack the bFGF at its N-terminal ends. Thus according to these data, while the overall yield in bFGF may be comparable, the composition in bFGF species differs; bFGF is composed of 18 kDa or 18-25 kDa species, depending on whether protease inhibitors are absent or present, respectively, in the elution step [175]. Thus, extensive use of protease inhibitors, in all steps of the cardiac extraction procedure, ensured a more realistic estimation of bFGF yield as well as composition. Storage of extracts or fractions was also avoided whenever possible. To maximize yield, heparin-binding proteins were eluted directly by boiling the heparin-sepharose beads in standard electrophoresis buffer and processed immediately for immunoblotting. The antibodies used in the immunoblotting procedure have been previously shown to be highly sensitive for bFGF [175,190]. This made possible speedy analysis of the bFGF content of relatively small amounts of heart extracts (3 - 10 ml, 40 - 100 mg total extracted soluble protein bFGF content in the nanogram range [203,217]).

V(A2). ^{3}H-thymidine incorporation

It has been previously shown that bFGF is a potent mitogen for RFCs [203]. In addition, Kardami et al. has shown that cardiac extract stimulated DNA synthesis in RFCs and that this activity is

largely due to the presence of bFGF [203]. Consequently, increased mitogenic activity of extracts towards RFCs suggests increased bFGF content. This is directly shown by immunoblotting analysis [203]. It is unlikely that the differences in bFGF content, composition and mitogenicity between extracts from control and isoproterenol-treated animals reflect increased "extractability" of hearts from isoproterenol-treated animals for three reasons: (i) the overall protein yields were similar, (ii) repeated extractions of tissue does not alter relative yields [217], and (iii) immunolocalization data confirm the bFGF in hearts of isoproterenol-treated animals. Furthermore, it is unlikely that the differences observed between isoproterenol-treated rat heart extracts and age-matched control rat heart extracts reflect a differential response of individual animals to isoproterenol treatment since pooled extracts from several hearts were used to minimize any variations.

V(A3). Localization of bFGF and other proteins by indirect immunofluorescence

Basic FGF is believed to act locally, in a paracrine and/or autocrine manner. Therefore, localization of bFGF in tissue sections by immunocytochemistry is an approach favoured by many researchers to examine potential cellular sites of synthesis, storage and action of this growth factor. Unfractionated anti-bFGF serum (S1) as well as affinity purified anti-bFGF IgG used for immunolocalization of bFGF, have been shown to be specific for bFGF in several previous studies [203,191,278,279,206]. Commercially obtained monoclonal antibodies to vinculin, desmin and vimentin

were considered specific since they displayed the previously described patterns of localization.

V(A4) Identification of degenerating myocytes

Vinculin is a component of the cytoskeletal attachment complex between the plasma membrane and the 2-line of the underlying myofibrils [263]. Myocytes with decreased anti-vinculin staining along their margins were considered to be undergoing necrosis known to occur after high doses of isoproterenol administration, based on the following criteria: (a) Lethal myocyte injury induced by myocardial ischemia is accompanied by a loss of anti-vinculin staining along the lateral margin of myocytes [272]; (b) vinculin negative myocytes were observed only in animals injected with isoproterenol; (c) vinculin- negative myocytes were observed only at 6 - 24 hr and not 1 week after isoproterenol injection; (d) vinculin-negative myocytes were associated with areas of cellular infiltration at 24 h after injection.

V(A5) Isoproterenol Cardiotoxicity

To judge the authenticity of the isoproterenol effects in our system, morphological observations of tissue sections and percent of hypertrophy measurements were examined in comparison to previously characterized results of the model of isoproterenol injury in rat hearts [258,280,281]. Mason's Trichrome stain method, which stains myocytes red, collagen blue and nuclei black, was performed on sections of control and isoproterenol-treated rats at 9 hrs and 1 weeks after injection. We identified necrotic myocyte lesions in the isoproterenol-treated rat by their deep red

stain (Fig.4, arrow) along the apical subendocardial portions of the heart. Similar deep red staining of necrotic myocytes were observed by Rona et al. [280] using Cason's trichrome stain. At 1 week after isoproterenol induced injury, the necrotic cardiomyocyte lesion was replaced by a fibrotic scar tissue characterized by an increase in nuclear density. Similar fibrotic scar formation in isoproterenol-treated rat hearts at this time point was also described by Rona et al. [281]. Our phase contrast microscopic examination of the isoproterenol treated rat cardiac tissue section, 24 hrs after injection, revealed a disruption of the myofiber structures compared to the surrounding intact myofibers. Again, this region of myocyte structural disruption was localized predominately along the apical and subendocardial portions of the isoproterenol treated rat heart. In addition, a measurement of the percent hypertrophy of the isoproterenol treated rat hearts showed a similar increase of heart weight to body weight ratios at 24 hrs after isoproterenol-induced injury and a similar decrease to control animal heart weight to body weight ratio levels after 1 week isoproterenol treatment, as observed by Rona et al. [281]. Thus, our data confirmed the previously shown cardiotoxic effects of high dose isoproterenol administration.

V(B) Increased bFGF Accumulation in Hearts from Isoproterenol Treated Animals

Immunobiochemical analysis of cardiac fractions showed that the overall levels of extracted bFGF were doubled in hearts from isoproterenol treated animals compared to controls, at 24 hrs and

1 to 6 weeks after treatment. Increased bFGF was also indicated by the increased mitogenicity of extracts from treated animals at 24 hrs and 1 wk after treatment; by the intense anti-bFGF staining of necrotic myocytes; as well as by the strong pericellular anti-bFGF staining of myocytes near the fibrotic scar regions.

Immunofluorescence localization allows detection of changes in bFGF intensity in a small number of groups of myocytes as early as 6 hrs from treatment. At this time point no change in bFGF content was seen by the other two approaches which, since they reflect changes in the whole heart, are probably not sensitive enough to detect early increases in bFGF.

Interestingly, although their overall bFGF content was similar, the mitogenic activity is higher in extracts obtained at 24 hrs compared to those obtained 1 week after treatment. Elevated mitogenicity could be the result of the relative increase in the 21 - 23 kDa bFGF species as measured in hearts 24 hrs after isoproterenol treatment, although there is no evidence to indicate that the 21 - 23 kDa rat bFGF may be more biologically active than the 18 kDa. It is possible that other growth promoting or inhibiting factors could be present in the crude extracts obtained at 24 hrs after treatment which could contribute or regulate the increased mitogenic effect. Indeed, TGF-beta1 and ECGF synthesis has been shown, by in situ hybridization, to increase in ventricle myocytes following myocardial infarction [165]. In addition, in culture TGF-beta1 has been shown to inhibit bFGF's mitogenic effect on neonatal cardiomyocytes in culture [203].

V(C) What is the Significance of the 21-23 kDa bFGF Species?

Limited information exists as to the functional significance of the different forms of bFGF. The NH_2-extension of bFGF confers a nuclear localization signal [274,180], and increased nuclear bFGF localization has been reported for proliferative compared to growth arrested cells [206]. In addition, it has been reported by Baldin et al [282] that the nuclear and nucleolar localization of exogenously added bFGF in adult bovine aortic endothelial cells occurred only when the cells were growing or undergoing G1 --> S transition. Burgess et al. [180] demonstrated that the only forms of bFGF that contain sites of potential arginine methylation (apparent MW 22,000 and 24,000) were observed in their nuclear fraction of transfected NIH 3T3 cells. Recently, Kardami et al. [205] found that the immature rat cardiac muscle, which retains a proliferative phenotype, expresses predominately the 21-23 kDa bFGF species, likely in association with the nucleus. In contrast, bFGF from adult rat cardiac muscle consists primarily of the 18 kDa species [271,190,175], a finding which is reproduced in our experiments for control as well as isoproterenol treated hearts 1, 4 and 6 weeks after isoproterenol treatment. It is intriguing that the 21-23 kDa bFGF's showed a transient increase 24 hrs after isoproterenol treatment in cardiac extracts. This would be consistent with a reexpression of a more immature phenotype as an early response to isoproterenol-induced injury. The site(s) of accumulation of the 21-23 kDa bFGF cannot be discerned using antiserum S1 which recognizes the 18 as well as the higher

molecular weight forms of bFGF [176]. However, since the increase in 21-23 kDa bFGF occurred at a time of intense cellular infiltration/proliferation, in hearts with a high abundance of necrotic myocytes [241], it is suggested that the 21-23 kDa species are associated with either or both of the above mentioned events. Stimulation of fibroblast DNA synthesis which was observed 24 hrs after isoproterenol injection in areas of myocyte necrosis, is likely to involve higher levels of endogenous 21-23 kDa bFGF [241,281].

V(D) Possible Mechanisms Responsible for Increased bFGF

A recent paper by Frautchy et al. [201] demonstrated elevated bFGF mRNA synthesis and anti-bFGF immunoreactivity in cortical brain injury. Similarly, Guthridge et al. [197] demonstrated in the in vivo muscle injury model increased bFGF mRNA synthesis in myoblasts and in degenerating and regenerated myotubes. Guthridge et al. speculated that these myoblasts and myotubes may serve as a source of bFGF to activate quiescent satellite cells and simulate their proliferation during muscle regeneration. In light of this reports, it can be suggested that the increase in bFGF reported here is essentially the result of de novo bFGF synthesis. A local decrease in bFGF degradation, which could theoretically result in increased bFGF, is unlikely since high levels of proteolytic activity generally accompany necrosis [284].

The timing and localization pattern of the change provides some information as to what triggered the bFGF increase. Irreversible myocyte injury, characterized by vinculin loss from

myocyte margins, apparently preceded and probably caused the changes in bFGF. In agreement, similar increases in bFGF accumulation and immunostaining have been found in mdx model of muscle injury, in association with degenerative lesions [217] and in myocardial infarcts induced by coronary occlusion (Padua and Kardami, in preparation).

Irreversible injured myocytes may synthesize new bFGF or alternatively, internalize bFGF synthesiszed and released by adjacent viable cells (myocytes and/or non-myocytes). It is possible that bFGF (a basic protein) may have entered the damaged myocytes via permeabilized cell membrane, driven perhaps by increased acidity of the cytoplasm [285].

A question which must be addressed is where does this increased pericellular bFGF come from? We found that fibroblast-like non-muscle cells, which inhabit the scar region 1 week after isoproterenol treatment, display some pericellular anti bFGF immunoreactivity. Fibroblast cells and macrophages cells, which are known to inhabit the scar region at this time point [280,281] have been previously shown to proliferate in wounds and actively synthesize bFGF [167]. Thus these cells could be a potential source of bFGF for border myocytes. Alternatively, dedifferentiated cardiomyocytes along the border of the scar would also be a potential source of the increased pericellular bFGF since immature cardiomyocytes, in culture, have been shown to synthesize bFGF [176].

V(E) Localization of anatomical markers

Unlike vinculin loss, desmin loss from irreversible myocyte injury was seen to occur after the increase in anti-bFGF cytoplasmic staining of necrotic myocytes. This would suggest that cytoskeletal disruption, as characterized by vinculin loss from the injured myocyte margins, occurs prior to intermediate filament disruption. This desmin intermediate filament loss was not apparently completed in necrotic myocytes until 24 hrs after isoproterenol treatment. In contrast complete vinculin loss from the cell margins of injured cardiomyocytes was detected as early as 4 hrs after isoproterenol treatment. Thus, monitoring vinculin changes in the myocardium after isoproterenol injury was a useful tool for characterizing the temporal relationship between the appearance of bFGF accumulation and the appearance of injured necrotic myocytes.

V(F). Possible Functional Significance of Increased bFGF in the Heart After Isoproterenol Treatment

Numerous studies have shown bFGF to be a potent chemoattractant for endothelial cells and fibroblasts, a mitogen for many cell types including cardiac myocytes and non-muscle cells [181,110], to affect differentiation and to protect cells from degeneration [286,287]. All of these properties are of relevance to the injury-repair response of the heart and are addressed individually.

Fibroblast-like cells (identified by vimentin staining) were increased in areas of necrosis 24 hrs after isoproterenol

administration. This increase in vimentin positive cells corresponds to local increases in bFGF accumulation seen in necrotic cells at 24 hrs. Thus it seems likely that local increase could have provided a signal for these fibroblast-like cells to infiltrate the necrotic region and participate in the repair process in which local bFGF would be expected to promote their proliferation. Macrophages, which are abundant in necrotic areas at this time point [176] may also respond to the local increases in bFGF since bFGF has been previously shown to promote growth and chemotaxis of macrophage [181].

Viable cardiomyocytes could also be targets for the action of bFGF. Rose et al. [288] demonstrated the presence of low affinity bFGF receptors in adult cardiomyocytes. In addition, bFGF stimulated DNA synthesis in cultured adult cardiomyocytes [209], perhaps because these cells have undergone dedifferentiation. Runyanchev and his colleagues have reported that cardiomyocytes near scar sites also display signs of dedifferentiation [289] and could therefore be responsive to bFGF and other proliferation-affinity myocytes. Dedifferentiation itself may have been facilitated by increased bFGF since Schneider and coworkers have shown re-induction of fetal contractile gene expression by this factor [208]. However, other growth factors present in the heart may also participate in the dedifferiation seen in intact myocytes near the scar. TGF-beta1 for instance, which has been shown to increase in viable myocytes following myocardial infarction [165], can re-induce, along with bFGF and aFGF, fetal contractile gene

expression in cultured cardiomyocytes [208].

The heart possesses endogenous mechanisms which become activated by various stress factors and increase cardiomyocyte resistance to injury [290]. Basic FGF was shown to protect neurons and retinal cells from retrograde degradation and promote survival of cells in culture [286,287]. Thus a similar protective role for bFGF on surviving intact cardiomyocytes maybe envisioned to occur in isoproterenol-damaged hearts. Finally, bFGF my also be involved in the development of hypertrophy which occurs after isoproterenol administration [258]. Indeed exogenous bFGF has been shown to increase protein synthesis in cardiomyocytes in culture as well as growth and DNA synthesis [208].

V(G). Reexpression of proteins specific for the immature cardiomyocyte phenotype

Vimentin, a member of the intermediate filament multigene family, is predominately expressed in cells of mesenchymal origin, and particularly in undifferentiated cells [291]. Upon their commitment to differentiation, different cells replace vimentin entirely or partially with their cell type specific intermediate filament. Desmin is the major intermediate filament component in adult cardiomyocytes [291,261]. In our system, antibodies to desmin clearly stained cardiomyocytes while those to vimentin stained interstitial cells and did not recognize myocytes in the adult heart, in agreement with previous reports [292]. Anti-vimentin labelling was therefore used to localize interstitial cells.

We found that cells populating the areas of fibrosis, several weeks after isoproterenol injection, were highly reactive towards the anti-vimentin antibodies, a finding in agreement with their broad identification as fibroblast-like [292,176]. At this point, myocytes near the scar had increased pericellular bFGF and a small fraction of these myocytes were also vimentin-positive. It is suggested that these myocytes may have been stimulated by increased local bFGF to re-express a more immature phenotype, perhaps as a first step towards a regenerative response.

Similarly, alpha-smooth muscle actin, a major component of the muscle thin filament is expressed only in the immature cardiomyocytes. Its expression is not observed at all in the adult myocardium except along structures with smooth muscle as a major component (vessels and interstitial cells such as myofibroblasts and the rare myocyte), as seen by our results. At 4 weeks after isoproterenol-induced injury, however, we find that a few myocytes along the border of the scar display both enhanced nuclear bFGF and anti- alpha-smooth muscle actin labelling. This would be indicative of a dedifferentiated cardiac muscle state and may be induced by the bFGF accumulated at the nucleus of this myocyte. Indeed, as was previously mentioned, bFGF has been shown to induced reexpression of the fetal contractile proteins in adult cardiomyocytes [208].

V(H). Nuclear bFGF localization

In addition to increased pericellular bFGF an increase in nuclear bFGF staining was observed in intact myocytes along the

border of the scar region. Although the anti- bFGF (S1) antiserum does not distinguish the high molecular weight bFGF species from the low bFGF species, it has been previously shown that nuclear localization of bFGF was predominately with the high molecular weight bFGF species which are methylated at their NH_2-terminal arginine residues [282,180]. In addition, nuclear localization of bFGF has been associated with proliferating cells [206,282]. In culture, Kardami et al. [176] has demonstrated nuclear localization of bFGF in proliferating cardiomyocytes using unfractionated anti-bFGF (S1). Similarly, they have previously detected nuclear localization of bFGF in immature cardiomyocytes [205]. Thus the increased nuclear anti-bFGF staining in cardiomyocytes which border the scar region, 1 week after isoproterenol injection, may be part of a dedifferentiated, more proliferative cardiac muscle phenotype. This would be consistent with the previously mentioned observations by Rumyantsev [289] and Parker et al. [208] that dedifferentiated cardiac muscles can be detected along the myocardial scar region. Whether the increased nuclear bFGF suggested by our results (Fig. 15) represent high molecular weight or low molecular weight forms is uncertain because our antibody recognizes the 18 kDa and 21.5 and 23 kDa forms. Nevertheless, since it has been previously shown that the high molecular weight forms of bFGF are associated with the nucleus [274,282,180], it is likely our antibody is detecting the 21.5 and 23 kDA bFGFs in the nuclei.

V(I).Future Studies

In our experiments, we localized and characterized bFGF in the

heart following isoproterenol- induced injury. We found that there was an accumulation of bFGF in necrotic myocytes at approximately 4 to 24 hr after isoproterenol administration. The question raised by such a finding is whether this observed increase is due to de novo synthesis of bFGF in necrotic myocytes and/or surrounding viable muscle and nonmuscle cells. If the latter is the case, how does newly synthesized bFGF enter the necrotic myocytes. One possible method of ascertaining whether the increased bFGF is due to de novo synthesis is through the use of in situ hybridization on cardiac tissue sections. This technique employs the use of cRNA probes to hybridize with the bFGF message that would be synthesized by either muscle or nonmuscle cells in response to isoproterenol-induced injury. Recent findings by Frautchy et al. [201] and Guthridge et al. [197] in injured brain and skeletal muscle tissue, respectively, would suggest that damaged cardiomyocytes, like in brain and skeletal muscle, may synthesize the increased bFGF seen in necrotic cardiomyocytes in our model.

As well, findings from our western blot data show that, in addition to an increase in the 18 kDa bFGF species seen in our cardiac extracts at 24hr, 1,4, and 6 wk after IsP administration, there was a transient increase in the 21.5 and 23 kDa bFGF species in extracts 24 hr after IsP treatment. This observation could not be confirmed in our immunofluorescence experiments mainly because our antibody is not able to differentiate the low bFGF molecular forms from the higher molecular forms. Thus one future study would be to raise antibodies against the amino-terminal extended end of

the high molecular bFGF forms and use this antibody to localize their distribution in isoproterenol- injured hearts. Indeed, since the amino acid sequence of these amino-terminal extensions has already been elucidated, manufacturing of a synthetic peptide from these sequences is possible and raising antibodies against this fragment can be performed using procedures already established in our lab [208,176,150,107]. The importance of such a study stems from the suspected mitogenic role high molecular weight bFGFs may play on cardiomyocytes [205] as our ^{3}H-thymidine assay results may indicate. Reexpression of these bFGF species would most likely be very beneficial in the cardiac healing and regeneration process.

Another question that remains to be elucidated, as well, is what role would other growth factors play in response to isoproterenol- induced cardiac injury. Such growth factors would include IGF-I and -II, TGF- beta and aFGF. Studies have shown, that these growth factors are present in the heart [208,176,150,107] and exert either a mitogenic or differentiating action on the cells present there [208]. TGF-beta for instance has been shown to attenuate bFGF's mitogenic action in the heart [110], increase in viable myocytes which border cardiac scar regions following myocardial infarction [165], induce formation of myofibroblast in culture [164], and activate fetal cardiac gene expression following administration in cardiac cell cultures [208]. Thus the possibility one or more of these factors may be acting to regulate bFGF action and regulate the cardiac healing process is likely. Future studies to elucidate such a role would utilize similar experiments used in

our study of bFGF. In addition, other techniques such as in situ hybridization and gene therapy (i.e. injection of different growth factor genes at or near the damaged areas of the heart) would also be employed to help understand the role these growth factors may play during cardiac healing.

The next logical step following the characterization of endogenous bFGF in the heart following isoproterenol administration would be to examine exogenous effects of bFGF administration to damaged hearts. Due to the difficulties in locating damaged myocyte areas in the isoproterenol-induced injury model, the coronary occlusion model of injury would be employed. This model gives us a definite location of the infarcted myocytes, thus enabling us to efficiently administer bFGF peptides or genes to myocardial regions adjacent to the scar and monitor its effects. Once bFGF administration has taken place, cardiac healing could be monitored histologically and functionally, at different time points, to ascertain the effects of the exogenous bFGF. Methods employed would take advantage of already established techniques such as: (i),in vivo protein sponge administration [166] of a growth factor onto a wounded area; and (ii),the transfection of genes via the injection of plasmids, with promoter regions specially constructed to increase muscle cell specificity, to regions of the heart adjacent to the damaged area [297,298].

V(J). Overview

We examined the distribution and overall accumulation of bFGF in hearts of isoproterenol treated rats at different time points

following initial administration. We found that hearts of isoproterenol treated rats show an increase in bFGF over that of age-matched controls as determined by: i), immunoblot analysis of cardiac extracts, ii), ^{3}H- thymidine assay; and iii), indirect immunofluorescence analysis of cardiac tissue sections.

From our immunoblot studies, we found a transient increase in the high molecular weight bFGF species (21.5 and 23 kDa) in cardiac extracts 24 hr after isoproterenol- induced injury. This three fold increase, over that of controls and other cardiac extracts from isoproterenol- treated rats at different time points (6 hr,and 1, 4, 6 wk), coincided with the time of intense cellular infiltration observed in the scar site. This increased bFGF may represent either bFGF in proliferative cells or bFGF in cells at the edge of the scar.

In addition, our ^{3}H- thymidine assay showed that, in RFCs cells treated with cardiac extracts from rats, 24hr and 1 wk after isoproterenol treatment, there was a 4 fold and 2 fold increase in ^{3}H-thymidine incorporation, respectively, compared to extracts from their age- matched controls and from isoproterenol treated rats 6 hr after administration. The higher mitogenic activity in extracts observed at 24 hr, compared to 1 wk after treatment, could be the result of the relative increase in the 21.5 and 23 kDa bFGF species observed in our immunoblot assay. Furthermore, we believe that the transient increase in the 21.5 and 23 kDa bFGF species in our extracts, 24 hr after isoproterenol treatment, could reflect a cardiomyocyte reexpression of a more immature phenotype as an early

response to isoproterenol- induced injury. As well, the increase in 18 kDa bFGF species seen in our immunoblots, was observed to correlate with the hypertrophic response of cardiomyocytes as well as with the time of scar formation.

From our indirect immunofluorescence experiments (at acute time points of 2,4,6,24 hr after isoproterenol administration), we showed that degenerating myocytes exhibited an increase in cytoplasmic bFGF. This increase in bFGF labelling occurred just prior to cellular infiltration, which was shown to peak at approximately 24 hr after isoproterenol treatment. This would suggest that bFGF may play some sort of chemotactic role in promoting cellular infiltration to the injured cardiac region. As well, we found that irreversible myocyte injury, characterized by vinculin loss from myocyte margins (at 4 hr after isoproterenol treatment), apparently preceded and probably caused the changes in bFGF distribution and accumulation, observed at 6 and 24 hr after isoproterenol- induced cardiac injury. Thus, in light of recent experiments on bFGF distribution and synthesis in injury models of the brain [201] and skeletal muscle tissue [197], we believe that: (i), irreversibly, injured cardiomyocytes may synthesize new bFGF, or, (ii), alternately bFGF may be synthesized and released by adjacent viable cells such as myocytes and/or nonmuscle cells. If the latter is the case, it is possible that bFGF (a basic protein) may have entered the damaged cardiomyocytes via the permeablized cell membrane, driven perhaps by increased acidity of the cytoplasm.

At long term time points (1, 4 and 6 wks) after isoproterenol induced injury, immunofluorescence analysis showed that cells populating the area of fibrosis were highly reactive towards anti-vimentin and anti-α-smooth muscle actin. These nonmuscle cells displayed anti-bFGF labelling and may thus represent a source of bFGF to viable cardiomyocytes which surround the fibrotic scar. We also found that myocytes near the scar displayed an increase in pericellular and nuclear bFGF labelling. A small fraction of these cells were also α-smooth muscle actin and vimentin positive. We speculate that these myocytes may represent an immature cardimyocyte phenotype, reexpressed by the local increase in bFGF observed in these cells. It is also possible that the enhanced bFGF in these border myocytes may function in protecting these cells from further degradation during wound healing.

Thus, from our data, we can conclude that bFGF, which was found to promote regeneration in other wound injury models, may play an important role in the repair response that takes place in the heart by either: (a), promoting primarily infiltration and proliferation of motile cells in the injured cardiac region; and (b), promoting the reexpression of an immature regenerative phenotype in cardiomyocytes near the scar region. This data about the role of bFGF in the cardiac response to isoproterenol- induced injury, does not reflect a specific isoproterenol induced event since similar observations have been seen with the mdx model of injury [217] and the coronary occlusion model of injury. In addition, it is possible that other local factors, such as

transforming growth factor- beta which has been shown to be expressed and increase in the heart after ischemic injury [165], may act in synergy or to counteract the bFGF- induced effects observed in our model. Thus further studies are necessary to elucidate this possible relationship. Furthermore, because our antibody detected both forms of bFGF (high, 21.5 and 23 kDa, and low, 18 kDa, species) antibodies specific for the high molecular weight forms would be of tremendous assistance in understanding bFGF's function in the cardiac injury response process.

References

1. Rothe, M. and Falanga, V. (1989) Growth factors: Their biology and promise in dermatologic diseases and tissue repair. Arch. Dermatol. 125: 1390-1398.

2. Dinarello, C.A. (1986) Multiple biological properties of recombinant interleukin-I (beta). Immunobiology 172: 301-315.

3. Thomas, K.A., Rios-Candelore, M., Gimenez-Gallego, G. et al. (1985) Pure brain-derived acidic fibroblast growth factor is a potent angiogenic vascular endothelial cell mitogen with sequence homology to interleukin I. PNAS 82: 6409-6413.

4. Auron, P.E., Webb, A.C., Rossenwasser, C.J., Mucci, S.F., Rick A., Wolff, S.M. and Dinarello, C.A. (1984) Nucleotide sequence of human monocyte interleukin-1 precursor cDNA. PNAS 81: 7907-7911.

5. Lomedico, P.T., Gabler, U., Hellman, C.P., Dukovich, M., Giri, G., Pan, Y.C.E., Collier, K., Seminonow, R., Chua, A.O. and Mizel, S. (1984) Cloning and expression of murine interleukin-1 cDNA in Escherichia coli. Nature (Lond.) 312: 458-462.

6. Young, P.R., Hazuda, D.J. and Simon, P.L. (1988) Human interleukin-1 is not secreted from hamster fibroblasts when expressed from a transfected cDNA. J. Cell. Biol. 107: 447-456.

7. Oppenheim, J., Kovacs, E., Matsushima, K. and Durum, S.K. (1986) There is more than one interleukin-1. Immunol. Today 7: 45-56.

8. Dinarello, C. (1984) Interleukin 1. Rev. Infec. Disease 6: 51-95.

9. Sims, J.E., March, C.J., Cosman, D., Widmer, M.B., MacDonald, H.R., McMahan, C.J., Grubin, C.F., Wignal, J.M., Jackson, J.L., Call, S.M., Friend, D, Alpert, A.R., Gillis, S., Urdal, D.L. and Dower, S.K. (1988) cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily. Science 241: 585-589.

10. Chizzonite, R., Truit, T., Kilian, P.L., Stern, A.S., Nunes, P., Parker, K.P., Kaffka, K.L., Chua, A.O., Lugg, P.K. and Gubler, U. (1989) Two high affinity interleukin 1 receptors represent separate gene products. PNAS 86: 8029-8033.

11. Bomsztyk, K., Sims, J.E., Stanton, T.H., Slack, J., McMahan, C.J., Valentine, M.A. and Dower,S.K. (1989) Evidence for different interleukin 1 receptors in murine B- and T-cell lines. PNAS 86: 8034-8038.

12. Lovett, D.H., Martin, M., Bursten, S., Szamel, M. and Resch, K. (1989) Interleukin-1 and the glomerular mesangium. III. IL-1-dependent stimulation of mesangial cell protein kinase activity. Kidney Int. 34: 26-35.

13. Corkey, B.E., Geschwind, J.F., Deeney, J.T., Hale, D.E., Douglas, S.D. and Kilpatric, L. (1991) Ca^{2+} responses to interleukin 1 and tumor necrosis factor in cultured human skin fibroblasts. J. Clin. Invest. 87: 778-786.

14. Raines, E.W., Dower, S.K. and Ross, R. (1989) Interleukin-1 mitogenic activity for fibroblasts and smooth muscle cells is

due to PDGF-AA Science 243: 393-395.

15. Martinet, N., Harne, L.A. and Grotendorst, G.F. (1988) Identification and characterization of chemoattractants for epidermal cells. J. Invest. Dermatol. 90: 122-126.

16. Nachman, R.L., Hajjar, K.A., Silverstein, R.L. and Dinarello, C.H. (1986) Interleukin-1 induces endothelial cell synthesis of plasminergic activator inhibition. J. Exp. Med. 163: 1595-1600.

17. Ross, R. (1987) Platelet-derived growth factor. Ann. Rev. Med. 38: 71-79.

18. Krane, S.M., Dayer, J.M., Simon, L.S. and Byrne, L.S. (1985) Mononuclear cell-conditioned medium containing mononuclear cell factor (MCF), homologous with interleukin 1, stimulates collagen and fibronectin synthesis by adherent rheumatoid synovial cells: effects of prostaglandin E2 and indomethacin. Collagen Relat. Res. 5: 99-117.

19. Postlethwaite, A.E., Lachman, L.B., Mainardi, C.L. and Kang, A.H. (1983) Interleukin 1 stimulation of collagenase production by cultured fibroblasts. J. Exp. Med. 157: 801-806.

20. Postlethwaite, A.E. and Kan, A.H. In: Inflammation: Basic Principles and Clinical Correlates (Galllin, J.I., Golstein, J.M and Synderman, R., eds.), New York, Raven Press, 1988, pp. 577-597.

21. Estes, J.E., Pledger, W.J. and Gillespie, G.Y. (1984) Macrophage- derived growth factor for fibroblasts and

interleukin-1 are distinct entities. J. Leuk. Biol. 35: 115-129.

22. Martin, M. and Resch, K. (1988) Interleukin 1: more than a mediator between leukocytes. Trends Pharmacol. Sci. 9: 171.

23. Shanahan, W.R. Jr., Hancock, W.W. and Korn, H.H. (1989) Expression of IL-1 and tumor necrosis factor by endothelial cells: role in stimulating fibroblasts PGE2 synthesis. J. Exp. Pathol. 4: 17.

24. Zweier, J.L. (1988) Measurement of superoxide- derived free radical mechanism of reperfusion injury. J. Biol. Chem. 263: 1353.

25. Lefer, A.M., Tsao, P., Aoki, N. and Palladino, M.A. Jr. (1990) Mediation of cardioprotection by transforming growth factor B. Science 249: 61-64.

26. Bevilacqua, M.P., Pober, J.S., Wheder, M.E., Cotran, R.S. and Gembrone, M.A. Jr. (1985) Interleukin-1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leucocytes, monocytes, and related leucocyte cell lines. J. Clin. Invest. 26: 2003-2011.

27. Carswell, E.A., Old, L.J., Kassel, R.J., Grun, S. Fiore, N. and Williamson, B. (1975) An endotoxin-induced serum factor that causes necrosis of tumours. PNAS 72: 366-370.

28. Speis, T., Morton, C.C., Nedospasov, S.A., Fiers, W., Pious, D. and Strominger, J.L. (1986) Genes for the tumor necrosis factors alpha and beta are linked to the human major

histocompatability complex. PNAS 83: 8699-8702.

29. Kriegler, M., Perez, C. DeFay, K., Albert, I. and Lu, S.D. (1988) A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: Ramifications for the complex physiology of TNF. Cell 53: 43-53.

30. Pennica, D., Nedwin, G.E., Hayflick, J.S. et al. (1984) Human tumor necrosis factor: Precursor structure, expression and homology to lymphotoxin. Nature 312: 724.

31. Balkwill, F. (1989) Tumour necrosis factor. British Medical Bulletin. 45 (2): 389-400.

32. Aggarwall, B.B., Eessalu, T.E., and Hass, P.E. (1985) Characterization of receptors for human tumour necrosis factor and their regulation by gamma interferon. Nature 318: 665-667.

33. Hajjar, K.A., Hajjar, D.P., Silverstein, R.L. and Nachman, R.L. (1987) Tumor necrosis factor-mediated release of platelet-derived growth factor from cultured endothelial cells. J. Exp. Med. 166: 235-245.

34. Vilcek, J., Palombella, V.J., Zhang, Y., Lin, J.X., Feinman, R., Reis, L.F.L. and Le, J. (1988) Mechanism and significance of the mitogenic and antiviral actions of TNF. Ann. Inst. Pasteur/Immunol. 139: 307-311.

35. Beutler, B.A. and Cerami, A. (1986) Cachectin and tumour necrosis factor as two sides of the same biological coin. Nature 320: 584-588.

36. Dayer, J., Beutler, B. and Cerami, A. (1986)

Cachectin/tumour necrosis factor stimulates collagenase and prostaglandin E2 production by human synovial cells and dermal fibroblasts. J. Exp. Med. 162: 2163-2168.

37. Frater-Schroder, M., Risau, W, Hallmann, R., Gautschi, P. and Bohlen, P. (1987) Tumor necrosis factor type alpha, a important inhibitor of endothelial cell growth in vitro, is angiogenic in vivo. PNAS 84: 5277-5281.

38. Leibovich, S.J., Polverini, P.J., Shepard, H.M., Wiseman, D.M., Shively, V. and Nuseir, N. (1987) Macrophage- induced angiogenesis is mediated by tumor necrosis factor- alpha. Nature 329: 630-632.

39. Cavender, D.E., Edelbaum, D. and Ziff, M. (1989) Endothelial cell activation induced by tumor necrosis factor and lymphotoxin. Am. J. Pathol. 134: 551-560.

40. Pober, J.S., Lapierre, L.A., Stolpen, A.H., Brock, T.A., Springer, T.A., Friers, W., Bevilacqua, M.P., Mendrick, D.L. and Gimbrone, M.A. Jr. (1987) Activation of cultured human endothelial cells by recombinant lymphotoxins: Comparison with tumor necrosis factor and interleukin 1 species. J. Immunol. 138: 3319-3324.

41. Shijo, K., Tsuda, S., Hayami, T., Asahi, T. and Kawaharada, H. (1989) Increase in permeability of human endothelial cell monolayer by recombinant human lymphotoxin. Biochem. Biophys Res. Commun. 162: 1431-1437.

42. Sica, A., Wang, J.M., Colotta, F., Dejane, E., Mantovani, A., Oppenhein, J.J., Larsen, C.G., Zachariae, C.D.C. and

Matsushima, K. (1990) Monocyte chemotactic and activating factor gene expression induced in endothelial cells by IL-1 and tumor necrosis factor. J.Immunol. 144(8): 3034-3038.

43. Silberstein, D.S. and David, J.R. (1986) Tumor necrosis factor enhances eosinophil toxicity to schistosoma mansoni larvae. PNAS 83: 1055-1059.

44. Shalaby, M.R., Aggarwal, B.B., Rinderknect, E., Svedersky, L.P., Finkle, B.S. and Palladino, M.A. (1985) Activation of human polymorphonuclear neutrophil functions by interferon gama and tumor necrosis factor. J. Immunol. 135: 2069-2073.

45. Maury, C.P.J. and Teppo, A.M. (1989) Circulating tumour necrosis factor- alpha (cachectin) in myocardial infarction. J. Intern. Med. 225: 333.

46. Aoki, N., Siegfried, M. and Lefer, A.M. (1989) Anti-EDRF effect of tumour necrosis factor in isolated, perfused cat carotid arteries Am. J. Physiol. 256: H1509.

47. Wahl, S.M., Wong, H., and McCartney-Francis, N. (1989) Role of growth factors in inflammation and repair. J. of Cell. Biochem. 40: 193-199.

48. Ross, R., Raines, E.W. and Bowen-Pope, D.F. (1986) The biology of platelet derived growth factor. Cell 46: 155-169.

49. Heldin, C.H., Backstrom, G., Ostman, A. et al. (1988) Binding of different dimeric forms of PDGF to human fibroblasts: Evidence of two separate receptor types. EMBOJ 7: 1387-1394.

50. Escobedo, J.A., Navankasatussas, S., Cousens, L.S., Couglin, S.R., Bell, G.I. and Williams, L.T. (1988) A common PDGF receptor is activated by homodimeric A and B forms of PDGF. Science 240: 1532-1534.

51. Calaesson-Welsh, L., Hammacher, A., Westermark, B., Heldin, C.H. and Nister, M. (1989) Identification and structural analysis of the A type receptor for PDGF. Similarities with the B type receptor. J. Biol. Chem. 264 (3): 1742-1747.

52. Stroobant, P. and Waterfield, M.D. (1984) Purification and properties of porcine platelet derived growth factor. EMBOJ 3: 2963-2967.

53. Nister, M., Hammacher, A., Mellstrom, K. et al. (1988) A glioma-derived PDGF A chain homodimer has different functional activities than a PDGF AB heterodimer purified from human platelets. Cell 52: 791-799.

54. Hart, C.E., Forstrom, J.D., Kelly, R.A. et al.(1988) Two classes of PDGF receptors recognize different isoforms of PDGF. Science 240: 1529-1531.

55. Sufert, R.A., Schwartz, S.M. and Bowen-Pope, D.F. (1984) Developmentally regulated production of platelet-derived growth factor-like molecules. Nature 311: 669-671.

56. Scher, C.D., Shephard, R.C., Antoniades, H.N. and Stiles, C.D. (1979) Platelet-derived growth factor and the regulation of the mammalian fibroblast cell cycle. Biochim. Biophys. Acta ; 560: 212-241.

57. Narayanan, A.S. and Page, R.C. (1983) Connective tissues of

the periodontium: a summary of current work. Coll.Relat.Res. 3(1): 33-64.

58. Bauer, E.A., Cooper, E.W., Huang, J.S., Altman, J. and Deuel, T.F. (1985) Stimulation of in vitro human skin collagenase expression by platelet- derived growth factor. PNAS 82: 4132-4136.

59. Ross, R. and Glomset, J.A. (1976) The pathogenesis of atherosclerosis. N. Engl. J. Med. 295: 369-377, 420-425.

60. Ross, R.(1986) The pathogenesis of atherosclerosis: An update. N. Engl. J. Med. 314: 488-500.

61. King, L.E. and Carpenter, G.F. Epidermal growth factor. In: Biochemistry and Physiology of the Skin (Gold-smith, L, ed.), New York, Oxford University Press Inc., 1983; 269-281.

62. Brown, J.P., Twardizik, D.R., Marquardt, H. et al. (1985) Vaccinia virus encodes a polypeptide homologous to epidermal growth factor and transforming growth factor. Nature 313: 491-492.

63. Derynck, R. (1988) Transforming growth factor alpha. Cell 54: 593-595.

64. Bugess, A.W., Lloyd, C.J., Smith, S. et al.(1988) Murine epidermal growth factor: Structure and function. Biochemistry 27: 4977-4985.

65. Buckley, A., Davidson, J.M., Kamerath, C.D., Wolt, T.B. and Woodward, S.C. (1985) Sustained release of epidermal growth factor accelerates wound repairs. PNAS (USA) 82: 7340-7344.

66. Brown, G.L., Curtsinger, L. III, Brightwell J.R., Ackerman, D.M., Tobin, G.R., Polk, H.C. Jr. et al. (1986) Enhancement of epidermal regeneration by biosynthetic epidermal growth factor. J. Exp. Med. 163: 1319-1324.

67. Chernoff, E.A. and Robertson, S. (1990) Epidermal growth factor and the onset of epithelial epidermal wound healing. Tissue Cell. 22 (2): 123-135.

68. Nanney, L.B. (1990) Epidermal and dermal effects of epidermal growth factor during wound repair. J. Invest. Dermatol. 94 (5): 624-629.

69. Kingsnorth, A.N., Vowles, R. and Nas, J.R. (1990) Epidermal growth factor increases tensile strength in intestinal wounds in pigs. Br. J. Surg. 77 (4): 409-412.

70. Itok, M., Imai, S., Joh, T., Yokoyama, Y., Tasue, N., Iwai, A., Matsusako, K., Endoh, K., Kawai, T. and Takeuchi, T. (1990) Effect of epidermal growth factor in combination with sucralfate or omepazole on the healing of chronic gastric ulcers in the rat. J. Clin. Gastroenterol. 12 (Suppl. 1): 5187-5191.

71. O'Daniel, T.G., Petitjan, M., Jones, S.C., Zogg, J., Martinez, S.A., Nolph, M.B. and Schultz, G.S. (1990) Epidermal growth factor binding and action on tympanic membranes. Ann. Otol. Rhinol. Laryngol. 99 (1): 80-84.

72. Tripathi, B.J., Kwait, P.S. and Tripathi, R.C. (1990) Corneal growth factors: A new generation of opthalmic pharmaceuticals. Cornea 9 (1): 2-9.

73. Joyce, N.C., Meklir, B. and Neufeld, A.H. (1990) In vitro pharmacologic separation of corneal endothelial migration and spreading responses. Invest. Ophthalmol. Vis. Sc. 31 (9): 1816-1826.

74. Grotendorst, G.R., Sona, Y., Takehara, K. and Charette, M. (1989) EGF and TGF-alpha are potent chemoattractants for endothelial cells and EGF-like peptides and are present at sites of tissue regeneration. J. Cell. Physiol. 139 (3): 617-623.

75. Schreiber, A.B., Winkler, M.E. and Derynch, R. (1986) Transforming growth factor-alpha: A more potent angiogenic mediator than epidermal growth factor. Science 232: 1250-1253.

76. Wang, D.J., Huang, N.N. and Heppel, L.A. (1990) Extracellular ATP shows synergistic enhancement of DNA synthesis when combined with agents that are active in wound healing or as neurotransmitters. Biochem. Biophys. Res. Commun. 166 (1): 251-258.

77. Hennessey, P.J., Black, C.T., and Andrassy, R.J.(1990) EGF increase short-term type I collagen accumulation during wound healing in diabetic rats. J. Pediatr. Surg. Aug; 25 (8): 893-897.

78. Wang, Y.C. and Rubenstein, P.A. (1988) Epidermal growth factor controls smooth muscle alpha-isoactin epxression in BC3H1 cells. J. Cell. Biol. 106 (3): 797-803.

79. Hirosumi, H., Ouchi, Y., Watanabe, M., Kusunoki, J.,

Nakamura, T. and Orimo, H. (1989) Effects of growth factors on cytosalic free calcium concentration and DNA snythesis in cultured rat aortic smooth muscle cells. Tohoku J. Exp. Med. 1989; 157 (4): 289-300.

80. Ouchi, Y., Hirosumi, J., Watanabe, M., Hattori, A., Nakamura, T. and Orimo, H. (1988) Inhibitory effects of transforming growth factor-beta on epidermal growth factor-induced proliferation of cultured rat aortic smooth muscle cells. Biochem. Biophys. Res. Commun. 157 (1): 301-307.

81. Roe, J.A., Harper, J.M. and Buttery, P.J. (1989) Protein metabolism in ovine primary muscle cultures derived from satellite cells - Effects of selected peptide hormones and growth factors. J. Endocrinol. Aug; 122 (2): 565-571.

82. Olwim, B.B. and Hauschka, S.D. (1988) Cell surface fibroblast growth factor and epidermal growth factor receptor are permanently lost during skeletal muscle terminal differentiation in culture. J. Cell. Biol. 107 (7): 761-769.

83. Nair, B.G., Rashed, H.M. and Patel, T.B. (1989) Epidermal growth factor stimulates rat cardiac adenylate cyclase through a GTP-binding regulatory protein. Biochem. J. 264: 563-571.

84. Nair, B.G., Parikh, B., Mulligan G. and Patel, T.B. (1990) Gs alpha mediates epidermal growth factor-elicited stimulation of rat cardiac adenylate cyclase. J.Biol.Chem. 265(34): 21317-21322.

85. Van Patten, S.M., Heisermann, G.J., Cheng, H.C. and Walsh, D. (1987) Tyrosine kinase catalyzed phosphorylation and inactivation of the inhibitor protein of the cAMP-dependent protein kinase. J. Biol. Chem. 262: 3398-3403.

86. Balk, S.D., Riley, T.M., Gunther, H.G. and Morisi, A.(1985) Heparin- treated, v-myc-transformed chicken heart messenchymal cells assume a normal morphology but are hypersensitive to epidermal growth factor (EGF) and brain fibroblast growth factor (bFGF); cells transformed by the v-Ha-ras oncogene are refractory to EGF and bFGF but are hypersensitive to insulin-like growth factors. PNAS 82: 5781-5785.

87. Boynton, A.L. and Whitfield, J. (1983) Calmodulin and cyclic AMP-dependent protein kinases mediate calcium-induced stimulation of DNA synthesis by rat liver cells. Adv. Cyclic Nucleotide Res. 15: 193-294.

88. Rabkin, S.W.(1990) The effect of alteration of extracellular Na+ or Ca2+ and inhibiton of Ca2+ entry, Na+-H+ exchange, and Na+-Ca2+ exchange by diltiazem, amiloride, and dichlorobenzamil on the response of cardiac cell aggregates to epidermial growth factor. Exp. Cell. Res. 188(2): 262-6.

89. Kelvin, D.J., Semard, G. and Connolly, J.A. (1989) FGF and EGF act synergistically to induce proliferation in BC3H1 myoblasts. J. Cell. Physiol. 138 (2): 267-272.

90. Herington, A.C., Cornell, H.J. and Kuffer, A.D. (1983)

Recent advances in biochemistry and physiology of insulin-like growth factor/somatomedin family. Int. J. Biochem. 15: 1201.

91. Phillips, L.S. and Vassilopoulow-Sellin, R. (1980) Somatomedins. N. Engl. J. Med. 302: 371.

92. Campisi, J. and Pardee, A.B. (1984) Post-transcriptional control of the onset of DNA synthesis by an insulin-like growth factor. Mol. Cell. Biol. 4: 1807.

93. Bell, G.I., Gerhard, D.S., Fong, N.M., Sanchez-Pescador, R. and Rall, L.B.(1985) Isolation of the human insulin-like growth factor genes: Insulin-like growth factor II and insulin genes are contiguous. Proc. Natl. Acad. Sci. USA 82: 6450.

94. Russel, W.E., VanWyk, J.J. and Pledger, W.J.(1984) Inhibition of the mitogenic effects of plasma by a monoclonal antibody to somatomedin C. PNAS 81: 2389.

95. Basby, W.H., Klapper, D.G. and Clemmons, D.R. (1988) Purification of a 31000 dalton insulin like growth factor binding protein from human amniotic fluid. J. Biol. Chem. 263: 14205-14210.

96. Lynch, S.E., Colvin, R.B. and Antoniades, H.N. (1989) Growth factors in wound healing. Single and synergistic effects on partial thickness porcine skin wounds. J.Clin.Invest. 84(2): 640-646.

97. Hansson, H.A., Brandsten, C., Lossing, C. and Petruson, K. (1989) Transient expression of insulin-like growth factor I

immunoreactivity by vascular cells during angiogenesis. Exp.Mol.Pathol. 50(1): 125-138.

98. Pfeifle, B., Boeder, H. and Ditschuneit, H. (1987) Interaction of receptor of insulin-like growth factor I, platelet-derived growth factor, and fibroblast growth factor in rat aortic cells. Endocrinology 120 (6) 2251-2258.

99. Murphy, L.J., Ghahary, A. and Chakrabarti, S. (1990) Insulin regulation of IGF-I expression in rat aorta. Diabetes 39 (6): 657-662.

100. Cercek, B., Fishbein, M.C, Forrester, J.S., Helfant, R.H. and Fagin, J.A. (1990) Induction of insulin-like growth factor I messenger RNA in rat arota after ballon denudation. Circ. Res. 66 (6): 1755-1760.

101. Banskoter, N.K,, Taub, R., Zellner, K. and King, G-L. (1989) Insulin , insulin-like growth factor I and platelet-derived growth factor interact additively in the induction of the proto-oncogene-c-myc and cellular proliferation in cultured bovine aortic smooth muscle cells. Mol. Endocrinol. 3 (8) 1183-1190.

102. Allen, R.E. and Boxhan, L.K.(1989) Regulation of skeletal muscle satellite cell proliferation and differentation by transforming growth factor-beta, insulin-like growth factor I, and fibroblast growth factor. J. Cell. Physiol. 138 (2): 311-315.

103. Alexandrides, T., Moses, A.C. and Smith, R.J. (1989) Developmental expression of receptors for insulin, insulin-

like growth factor I (IGF-I), and IGF-II in rat skeletal muscle. Endocrinology 124 (2) 1064-1076.

104. Adamo, M., Lowe, W.L. Jr., LeRoeth, D. and Roberts, C.T. Jr. (1989) Insulin-like growth factor I messenger ribonucleic acids with alternative 5'-untranslated regions are differentially expressed during development of the rat. Endocrinology 124 (6): 2737-2744.

105. Beck, P., Samani, N.J., Byrne, S., Morgan, K., Gebhard, R. and Brammar, W.J. (1988) Histochemical localization of IGF-I and IGF-II mRNA in the rat between birth and adulthood. Development 104 (1): 29-39.

106. Bar, R.S., Boes, M., Dake, B.L., Sandra, A., Bayne, M., Cascieri, M. and Booth, B.A. (1990) Tissue localization of perfused endothelial cell IGF binding protein is markedly altered by association with IGF-I. Endocrinology 127 (6): 3243-3245.

107. Bar, R.S., Boes, M. and Sandra, A. (1988) IGF receptors in myocardial capillary endothelium: Potential regulation of IGF-I transport to cardiac muscle. Biochem. Biophys. Res. Commun. 152 (1): 93-98.

108. Eaglemann, G.L., Boehm, K.D., Haskell, J.F., Khairallah, P.A. and Ilan, J. (1989) Insulin-like growth factors and neonatal cardiomyocyte development: Ventricular gene expression and membrane receptor variations in normotensive and hypertensive rats. Mol. Cell. Endocrinol. 63: 1-14.

109. Turner, J.D., Rotwein, P., Novakofski, J. and Bechtel, P.J. (1988) Induction of mRNA for IGF-I and II during growth hormone-stimulated muscle hypertrophy. Am. J. Physiol. 255 (4 pt 1) E513-E517.

110. Kardami, E. (1990) Stimulation and inhibition of cardiac myocyte proliferation in vitro. Mol. and Cell. Biochem. 92: 129- 135.

111. Englemann, G.L., Boehm, K.D., Haskell, J.F., Khairallah, P.A., Ilan, J. (1989) Insulin- like growth factors and neonatal cardiomyocyte development: Ventricular gene expression and membrane receptor variations in normotensive and hypertensive rats. Mol. Cell. Endocrinology. 63: 1-14.

112. Kohtz, D.S., Dische, N.R., Inagami, T., Goldman, B. (1989) Growth and partial differentiation of presumptive human cardiac myoblasts in culture. J. Cell. Biol. 108: 10067-78.

113. Cheifetz, S., Weatherbee, J.A., Tsang, M.L-S., Anderson, J.K., Mole, J.E., Lucas, R. and Massague, J. (1987) The transforming growth factor-beta system, a complex pattern of cross-reactive ligands and receptors. Cell 48: 409-415.

114. Graycar, J.L., Miller, D.A., Arrick, B.A., Lyons, R.M., Moses, H.L. and Derynck, R. (1989) Human transforming growth-beta 3: recombinant expression purification, and biological activities in comparison with transforming growth factors-beta 1 and -beta 2. Mol. Endocrinol. 3 (12): 1977-

1986.

115. Jakowlew, S.B., Dillard, P.J., Sporn, M.B. and Roberts, A.B. (1988) Complementary deoxyribonucleic acid cloning of a messenger ribonucleic acid encoding transforming growth factor beta 4 from chicken embryo chondrocytes. Mol. Endocrinol. 2: 1186-1195.

116. Mason, A.J, Hayflick, J.S., Ling, N., Esck, F., Ueno, N., Ying, Y., Guillemin, R., Niall, H. and Seeburg, P.H. (1985) Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor- beta. Nature 318: 659-663.

117. Cate, R.L., Mattalianok, R.J., Hession, C. et al. (1986) Isolation of the bovine and human genes for Mullerian inhibiting substance and expression of the human gene in animal cells. Cell 45: 685-698.

118. Wozney, J.M., Rosen, V., Celeste, A.J., Mitsock, L.M., Whitter, M.J., Kriz, R.W., Herwick, R.M. and Wang, E.A. (1988) Novel regulators of bone formation: molecular clones and activities. Science 242: 1528-1534.

119. Cheifetz, S., Rike, B. and Massague, J. (1986) Cellular distribution of type I and type II receptors for transforming growth factor B. J. Biol. Chem. 261: 9972-9978.

120. Hsuan, J.J. (1989) Transforming growth factors B. British Med. Bull. 45 (2) 425-437.

121. Postlethwaite, A.E., Keski-Oja, J., Moses, H.L. and Kang,

A.H. (1987) Stimulation of the chemotactic migration of human fibroblast by transforming growth factor B. J. Experimental Med. 165: 251-256.

122. Kehrl, J.H., Wakefield, L.M., Roberts, A.B., Jakowlew, S., Alvarez-Mon, M., Derynck, R., Sporn, M.B. and Fauci, A.S. (1986) Production of transforming growth factor B by human T lymphocytes and its potential role in the regulation of T cell growth. J. Exp. Med. 163: 1037-1050.

123. Sporn, M.B., Roberts, A.B., Wakefield, L.M. and deCrombrugghe, B. (1987) Some recent advances in the chemistry and biology of transforming growth factor-beta. J. Cell. Biol. 105: 1039-1045.

124. Grotendorst, G.R., Smale, G. and Pencev, P. (1989) Production of transforming growth factor beta by human peripheral blood monocytes and neutrophils. J. Cell. Physiol. 140: 396-402.

125. Ignotz, R.A. and Massague, J. (1986) Transforming growth factor B stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J. Biol. Chem. 261 (9): 4337-4335.

126. Ignotz, R.A. and Massague, J. (1987) Cell adhesion protein receptors as targets for transforming growth factor- beta action. Cell 51: 189-197.

127. Laiho, M., Saksela, O., Anderson, P.A. and Keski-Oja, J. (1986) Enhanced production and extracellular deposition of the endothelial-type plasminogen activator inhibitor in

cultured human lung fibroblasts by transforming growth factor- beta. J. Cell. Biol. 103: 2403-2410.

128. Matrisian, L.M., Leroy, P., Ruhlmann, C., Gesnel, M. and Breathnach, R. (1986) Isolation of the oncogene and the epidermal growth factor- induced transin gene: complex control in rat fibroblasts. Mol. Cell. Biol. 6: 1679-1686.

129. Assoian, R.K. and Sporn, M.B. (1986) Type beta transforming growth factor in human platelets: release during platelet degranulation and action on vascular smooth muscle cells. J. Cell. Biol. 102: 1217-1223.

130. Wahl, S.M., Hunt, D.A., Wakefield, L.M., McCartney-Francis, N., Wahl, L.M., Roberts, A.B. and Sporn, M.B. (1987) Transforming growth factor B induces chemotaxis and growth factor production. PNAS 84: 8788-8792.

131. Allen, J.B., Manthey, C.L., Hand, A.R., Ohura, K., Ellingsworth, L. and Wahl, S.M. (1990) Rapid onset synovial inflamation and hyperplasia induced by transforming growth factor B. J. Exp. Med. 171: 231-247.

132. Roberts, A.B., Sporn, M.B., Assoian, R.K., Smith, J.M., Roche, N.S., Wakefield, L.M., Heine, U., Liotta, L.A. et al. (1986) Transforming growth factor type B: Rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. PNAS 83: 4167-4171.

133. Kulozik, M., Hogg, A., Lankat-Buttgereit, B. and Krieg, T. (1990) Co-localization of transforming growth factor B2 with alpha 1 (1) procollagen mRNA in tissue sections of

patients with systemic sclerosis. J. Clin. Invest. 86: 917-922.

134. Flanders, K.C., Thompson, N.L., Cissel, D.S. et al. (1989) Transforming growth factor B1: Histochemical localization with antibodies to different epitopes. J. Cell. Biol. 1081: 653-660.

135. Baird, A. and Durkin T. (1986) Inhibition of endothelial cell proliferation by type B transforming growth factor: Interactions with acidic and basic fibroblast growth factor. Biochem. Biophys. Res. Commun. 138 (1): 476-482.

136. Owens, G.K., Geisterfer, A.A.T., Yang, Y.W. and Komoriya, A. (1988) Transforming growth factor B-induced growth inhibition and cellular hypertrophy in cultured vascular smooth muscle cells. J. Cell. Biol. 107: 771-780.

137. Moses, H.L., Branunn, E.L., Proper, J.A. and Robinson, R.A. (1981) Transforming growth factor production by chemically transformed cells. Cancer Res. 41: 2842-2848.

138. Leof, E.B., Proper, J.A., Goustin, A.S., Shipley, G.D., DiCorletto, P.E. and Moses, H.L. (1986) Induction of c-sis mRNA and activity similar to platelet-derived growth factor by transforming growth factor beta: a proposed model for indirect mitogenesis involving autocrine activity. PNAS 83: 2453-2457.

139. Tucker, R.F., Shipley, G.D., Moses, H.L. and Holley, R.W. (1984) Growth inhibitor from BSC-1 cells closely related to platelet type beta transforming growth factor. Science 226:

705-707.

140. Shipley, G.D., Childs, C.B., Volkenant, M.E., Moses, H.L. (1984) Differential effects of epidermal growth factor, transforming growth factor and insulin on DNA and protein synthesis and morphology in serum-free cultures of AKR-2B cells. Cancer Res. 44(2): 710-716.

141. Shipley, G.D., Pittelkow, M.R., Will, J.J., Jr., Scott, R.E., Moses, H.L. (1986) Reversible inhibition of normal human prokeratinocyte proliferation by type beta transforming growth factor- growth inhibitor in serum free medium. Cancer Res. 46(4 pt 2) pp. 2068- 2071.

142. Coffey, R.J. Jr., Sipes, N.J., Bascom, C.C., Graves-Deal, R., Pennington, C.Y., Weissmann, B.E. and Moses, H.L. (1988) Growth modulation of mouse keratinocytes by transforming growth factors. Cancer Res. 48: 1596-1602.

143. Masui, T, Wakefield, L.M., Lechner, J.F., Laveck, M.A., Sporn, M.B. and Harris, C.C. (1986) Type beta transforming growth factor is the primary differentiation- inducing serum factor for normal human bronchial epithelial cells. PNAS 83: 2438-2442.

144. Ignotz, R.A. and Massague, J. (1985) Type beta transforming growth factor controls the adipogenic differentiation of 3T3 fibroblasts. PNAS 82: 8530-8534.

145. Merwin, J.R., Anderson, J.M., Kocher, O., VanItallie, C.M. and Machi, J.A. (1990) Transforming growth factor beta 1 modulates extracellular matrix organization and cell-cell

junctional complex formation during in vitro angiogenesis. J. Cell. Physiol. 142: 117-128.

146. Finesmith, T.H., Broadly, K.N. and Davidson, J.M. (1990) Fibroblasts from wounds of different stages of repair vary in their ability to contract a collagen get in response to growth factors. J. Cell. Physiol. 144: 99-107.

147. Kimelman, D. and Kirschner, M. (1987) Synergistic induction of meosderm by FGF and TGF-B and the identification of an mRNA coding for FGF in the early xenopus embryo. Cell 51: 869-877.

148. Slack, J.M.W. (1989) Peptide regulating factors in embryonic development. Lancet 1: 1312-1315.

149. Heine, U.I., Munoz, E.F., Flanders, K.C. et al. (1987) Role of transforming growth factor B in the development of the mouse embryo. J. Cell. Biol. 105: 2861-2876.

150. Thompson, N.L., Flanders, K.C., Smith, J.M., Ellingsworth, L.R., Roberts, A.B. and Sporn, M.B. (1989) Expression of transforming growth factor beta 1 in specific cells and tissues of adult and neonatal mice. J. Cell. Biol. 108: 661-669.

151. Krug, E.L., Mjaatvedt, C.H. and Markwald, R.R. (1987) Extracellular matrix from embryonic myocardium elicts and early morphogenic event in cardiac endothelial differentiation. Dev. Biol. 120: 348-355.

152. Potts, J.D. and Runyon, R.B. (1989) Epithelial-mesenchymal cell transformation in the heart can be mediated, in part by

transforming growth factor B. Dev. Biol. 134: 392-401.

153. McCaffrey, T.A., Falcone, D.J., Brayton, C.F., Agarwal, L.A., Welt, F.G.P. and Weksler, B.B. (1989) Tranforming growth factor B activity is potentiated by heparin via dissociation of the transforming growth factor B/alpha 2-macroglobin inactive complex. J. Cel. Biol. 109: 441-448.

154. Florini, J.R., Robert, A.B., Ewton, D.Z., Falen, S.L., Flanders, K.C. and Sporn, M.B. (1986) Transforming growth factor B. A very potent inhibitor of myoblast differentiation, identical to the differentiation inhibitor secreted by Buffalo rat liver cells. J. Biol. Chem. 261: 16509-16513.

155. Olson, E.N., Sternberg, E., Hu, J.S., Spizz, G. and Wilcox, C. (1986) Regulation of myogenic differentiation by type B transforming growth factor. J. Cell. Biol. 103: 1799-1805.

156. Schneider, M.D. and Olson, E.W. (1988) Control of myogenic differentiation by cellular oncogenes. Mol. Neurobiol. 2: 1-39.

157. Massague, J., Cheiftz, T., Endo, S., Nadal-Ginard, B. (1986) Type B transforming growth factor is an inhibitor of myogenic differentiation. PNAS 8206-8210.

158. Reidy, M.A., Fingerle, J., Au, Y.P. and Majesky, M.W. (1989) Correlation of mRNA expression of cellular mitogens and smooth muscle cell proliferation in arteries denuded of endothelium. J. Cell. Physiol. 13E: 194.

159. Parker, T.G., Packer, S.E. and Schneider, M.D. (1990)

Peptide growth factors can provide "fetal" contractile protein gene expression in rat cardiac myocytes. J. Clin. Invest. 85: 507-514.

160. Schneider, M.D. and Parker, T.G. (1990) Cardiac myocytes as targets for the action of peptide growth factors. Circulation 81 (5): 1443-1456.

161. Florini, J.F. (1987) Hormonal control of muscle growth. Muscle and Nerve 10: 577-598.

162. Kardami, E. and Fandrich, R.R. (1989) Heparin-binding mitogens in the heart: In search of origin and function. In: Molecular Biology of Muscle Development, UCLA Symp., New York, New Ser. Alan R. Liss, Inc., 315-325.

163. Eghbali, M., Lomek, R. Sukhatme, V.P., Woods, C. and Bhambi, B. (1991) Differential effects of transforming growth factor B1 and phorbal myrostate acetate on cardiac fibroblasts: Regulation of fibrillar collagen on RNAs and expression of early transcription factors. Circ. Res. 69: 483-490.

164. Eghbali, M., Tomek, R., Woods, C. and Bhanbi, B. (1991) Cardiac fibroblasts are predisposed to convert into myocyte phenotype: Specific effect of transforming growth factor B. PNAS. 88: 795-799.

165. Thompson, N.C., Bazoberry, F., Speir, E.H., Casscells, W., Ferrans, V.J., Flanders, K.C., Kondaiah, P., Geiser, A.G and Sporn, M.B. (1988) Transforming growth factor beta-1 in acute myocardial infarction in rats. Growth Factors 1: 91-

99.

166. Gospodarowicz, D., Neufeld, G. and Schweigerer, L. (1986) Review: Fibroblast growth factor. Mol. Cell. Endocrin. 46: 187-204.

167. Klagsbrun, M.(1989) The fibroblast growth factor family: Structural and biological properties. Prog. in Growth Factor Research 1: 207-235.

168. D'Amore, P.A. (1990) Modes of FGF release in vivo and in vitro. Cancer and Metastasis Reviews 9: 227-238.

169. Abraham, J.A., Whang, J.L., Tumolo, A., Mergia, A., Freedman, J., Gorpodarowizy, D. and Fiddes, J.C. (1986a) Human basic fibroblast growth factor: Nucleotide sequence and genomic organization. EMBOJ 5: 2523-2528.

170. Abraham, J.A., Mergia, A., Whang, J.L., Tuomolo, A., Friedman, J., Hjerrild, K.A., Gospodarowicz, D. and Fiddes, J.C.(1986b) Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor. Science 233: 545-548.

171. Gospodarowicz, D. and Cheng. J. (1986) Heparin protects basic and acidic FGF from inactivation. J. Cell. Physiol. 128: 475-484.

172. Yayon, A., Klagsbrun, M., Esko, J.D., Leder, P. and Ornitz, D.M. (1991) Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell 64: 841-848.

173. Sakaguchi, K., Yanagishita, M., Takeuchi, Y., and Aurback,

G.D. (1991) Identification of heparin sulfate proteoglycan as a high affinity receptor for acidic fibroblast growth factor (aFGF) in parathyroid cell line. J. Biol. Chem. 266 (11): 7270-7278.

174. Feige, J.J., Bradley,J.D., Fryburg, K., Farris, J., Cousens, L.C. (1989) Differential efffects of heparin, fibronectin, and laminin on the phosphorylation of basic fibroblast growth factor by protein kinase C and the catalytic subunit of protein kinase A. J. Cell. Biol. 109 (No. 6, pt. 1): 3105-3114.

175. Doble, B.W., Fandrich, R.R., Liu, L. and Kardami, E. (1990) Calcium protects pituitary basis FGF from proteolysis by copurifying proteases. Biochem. Biophys. Res. Commun. 173: 1116-1122.

176. Kardami, E., Liu, L and Doble, B.W. (1991) Basic fibroblast growth factor in cultured cardiac myocytes. N.Y. Acad. Sci. 638: 244-255.

177. Bouche, G., Gas, N. Prats, H., Baldin, V., Tauber, J.P., Teissie, J. and Amalric, F. (1987) Basic fibroblast growth factor enters the nucleolus and stimulates the transcription of ribosomal genes in ABAE cells undergoing Go --> G1 transition. Proc. Natl. Acad. Sci. 84: 6770-6774.

178. Florkiewicz, R.Z. and Sommer, A. (1989) Human basic fibroblast growth factor gene encodes four polypeptides: Three initiate translation from non-AUG codons. Proc. Natl. Acad. Sci. 86: 3978-3981.

179. Prats, H., Kaghad, M., Prats, A.C., Klagbrun, M. et al. (1989) High molecular mass forms of basic fibroblast growth factor are initiated by alternative CUG codons. Proc. Natl. Acad. Sci. 86: 1836-1840.

180. Burgess, W.H., Bizik, J., Mehlman, T., Quarto, N. and Rifkin, D.B. (1991) Direct evidence for methylation of arginine residues in high molecular weight forms of basic fibroblast growth factor. Cell Regulation 2: 87-93.

181. Rifkin, D.B. and Mascatelli, D. (1989) Recent developments in the cell biology of basic fibroblast growth factor. J. Cell. Biol. 109: 1-6.

182. Saksela, O., Moscateli, D., Sommer, A. and Rifkin, D.B. (1988) Endothelial cell-derived heparin sulfate binds basic fibroblast growth factor and protects it from proteolytic degradation. J. Cell. Biol. 107: 743-751.

183. Safrun, A., Avivi, A., Orr-Urtereger, A., Neufeld, G., Lonai, B., Lonai, P., Givol, D. and Yarden, Y. (1990) The murine flg gene encodes a receptor for fibroblast growth factor. Oncogene 5: 635-643.

184. McNeil, P.L. and Ito, S. (1989) Gastrointestinal cell plasma membrane wounding and resealing in vivo. Gastroenterology 96: 1238-1248.

185. Gajdusek, C.M. and Carbon, S. (1989) Injury-induced release of basic fibroblast growth factor from bovine aortic endothelium. J. Cell. Physiol. 139: 570-579.

186. Folkman, J., Klagbrun, M., Sasse, J., Wadzinski, M., Ingber,

D. and Vlodavsk, I. (1988) A heparin-binding angiogenic protein-basic fibroblast growth factor is stored within basement membrane. Am. J. Pathol. 130: 393-400.

187. DiMario, J., Buffinger, N., Yamada, S. and Strohman, R.C. (1989) Fibroblast growth factor in the extracellular matrix of dystrophic (mdx) mouse muscle. Science 244: 688-690.

188. Vaidya, T.B., Rhodes, S.J., Taparowsky, E.J. and Konieczny, S.F. (1989) Fibroblast growth factors and transforming growth factor B repress transcription of the myogenic regulatory gene myo D1. Mol. Cell. Biol. 9: 3576-3579.

189. Clegg, C.H., Linkhart, T.A., Olwin, R.B. and Hauschka, S.D. (1987) Growth factor control of skeletal muscle differentiation: Commitment to terminal differentiation occurs in G1 phase and is repressed by fibroblast growth factor. J. Cell. Biol. 105: 949-956.

190. Kardami, E., Stoski, R.M., Doble, B.W., Yamamsto, T., Hertzberg, E.L. and Nagy, J.I. (1991) Biochemical and ultrastructural evidence for the association of basic fibroblast growth factor with cardiac gap junctions. J. Biol. Chem. 266 (29): 19551-19557.

191. Kardami, E., Murphy, L.J., Liu, L, Padua, R.R. and Fandrich, R.R. (1990) Characterization of two preparations of antibodies to basic fibroblast growth factor which exhibit distinct patterns of immunolocalization. Growth Factors 4: 69-80.

192. Kimmelman, D., Abraham, J., Haparanta, T., Palisi, T. and

Kischner, M. (1988) The presence of fibroblast growth factor in the frog egg: Its role as a natural mesoderm inducer. Science 242: 1053-1056.

193. Folkman, J. and Klagsbrun, M. (1987) Angiogenic factors. Science 235: 442-447

194. Linder, V. and Reidy, M.A. (1991) Proliferation of smooth muscle cells after vascular injury is inhibited by an antibody against basic fibroblast growth factor. Proc. Natl. Acad. Sci. 88: 3739-3743.

195. Cuevas, P., Burgos, J. and Baird, A. (1988) Basic fibroblast growth factor (FGF) promotes cartilage repair in vivo. Biochem. Biophys. Res. Commun. 156 (2): 611-618.

196. McNeil, P.L, Muthukrishman, L., Warder, E. and D'Amore, P.A. (1989) Growth factors are released by mechanically wounded endothelial cells. J. Cell. Biol. 109: 811-822.

197. Guthridge, M., Wilson, M., Cowling, J., Bertolini, J. and Hearn, M.T.W. (1992) The role of basic fibroblast growth factor in skeletal muscle regeneration. Growth Factors 6: 53-63.

198. Linder, V., Reidy, M.A. and Fingerle, J. (1989) Regrowth of arterial endothelium: Denudation with minimal trauma leads to complete endothelial cell regrowth. 61 (5): P556-P563.

199. Greenhalgh, D.G., Srugel, K.H., Murry, M.J. and Ross, R. (1990) PDGF and FGF stimulate wound healing in the genetically diabetic mouse. Am. J. Pathol. 136 (6): 1235-1246.

200. Assouline, M., Huchinson, C., Morton, K., Mascarelli, F., Jeanny, J-C., Fayein, N., Pouliquen, Y. and Courtois, Y. (1989) In vivo binding of topically applied human bFGF on rabbit corneal epithelial wound. Growth Factors 1: 251-261.

201. Frautschy, S.A., Walicke, P.A. and Baird, A. (1991) Localization of basic fibroblast growth factor and its mRNA after CNS injury. Brain Res. 553: 291-299.

202. Fourtanier, A.Y., Jose Courty, D.P., Muller, E., Courtois, Y., Prunieras, M. and Barritault, D. (1986) Eye-derived growth factor isolated from bovine retina and used for epidermal wound healing in vivo. J. Inves. Derm. 87 (1): 76-80.

203. Kardami, E. and Fandrich, R.R. (1989) Basic fibroblast growth factor in atria and ventricles of the vertebrate heart. J. Cell. Biol. 109: 1865-1875.

204. Gay, C.G. and Winkles, J.A.(1991) Interluekin 1 regulated heparin-binding growth factor 2 gene expression in vascular smooth muscle cells. Proc. Natl. Acad. Sci. 88: 296-300.

205. Padua, R.R., Doble, B.W. and Kardami, E. (1991) Basic fibroblast growth factor in development and after isoproterenol-induced injury in the rat heart. J. Mol. Cell. Cardiol. 23 (Supple. III): S90206.

206. Cattini, P.A., Nickel, B.E., Klassen, M.E. and Kardami, E. (1991) Immunolocalization of basic fibroblast growth factor (bFGF) in growing and growth-inhibited placental cells:

Possible role for bFGF in placental cell development. Placenta 12: 341-352.

207. Mignatti, P., Morinmoto, T. and Rifkin, D.B. (1991) Basic fibroblast growth factor released by single, isolated cells stimulates their migration in an autocrine manner. Proc. Natl. Acad. Sci. 88: 11007-11011.

208. Parker, T.G. and Schnieder, M.D. (1991) Growth factors, proto-oncogenes and plasticity of the cardiac phenotype. Ann. Rev. Physiol. 53: 179-200.

209. Claycomb, W.C. and Moses, R.L. (1988) Growth factors and TPA stimulate DNA synthesis and alter the morphology of cultured terminally differentiated adult rat cardiac muscle cells. Dev. Biol. 127: 257-265.

210. Simpson, P.C., Henrich, C.J., Karns, L.R., Long, C.S., Ordahl, C.P. et al. (1989) Growth factors in cardiac myocyte hypertrophy and gene expression. J. Mol. Cell. Cardiol. Supple. III, 21: S.25 (Abstr.)

211. Parker, T.G., Packer, S.E. and Schneider, M.D. (1990) Peptide growth factors can provide fetal contractile protein gene expression in rat cardiac myocytes. J. Clin. Invest. 85: 507-514.

212. Chiba, M., Bazoberry, F., Speir, E.H., Sasse, J., Nesbitt, C.P. et al. (1989) Role of basic fibroblast growth factor in angiogenesis, healing and hypertrophy after rat myocardial infarction. Circulation 80: II, 452 (Abstr.)

213. Parlow, M.H., Bolender, D.L. and Lough, J. Localization of

bFGF as particulate inclusions in developing heart cells of the early embryo. J. Cell. Biochem. Suppl. 14, E:81 (Abstr.)

214. Potts, J.D. and Runyon, R.B. (1989) Epithelial-mesenchymal cell tranformation in the heart can be mediated, in part, by transforming growth factor B. Dev. Biol. 134: 392-401.

215. Krug, E.L., Mjaatvedt, C.H. and Markwald, R.R. (1987) Extracellular matrix from embryonic myocardium elicits an early morphogenic event in cardiac endothelial differentiation. Dev. Biol. 120: 348-355.

216. Gonzalez, A.M., Buscaglia, M, Ong, M. and Baird, A. (1990) Distribution of basic fibroblast growth factor in the 18 day rat fetus: Localization in the basement membranes of diverse tissues. J. Cell. Biol. 110: 753-765.

217. Anderson, J., Lui, L. and Kardami, E. (1991) Distinctive basic fibroblast growth factor distribution in regenerating and degenerating dystrophic (mdx) striated muscle. Dev. Biol. 147: 96-109.

218. Zak, R. Factors controlling cardiac growth In: Growth of the heart in health and disease. (R.Zak,Ed.). pp. 165-185. Raven press., New York, N.Y. 1984.

219. Jennings, R.B., Reimer, K.A. (1981) Lethal myocardial ischemic injury. Am. J. Pathol. 102: 241-245.

220. Rona, G. (1985) Catecholamine cardiotoxicity. J. Mol. Cell. Cardiol. 17: 291-306.

221. Bridges, L.R. (1986) The association of cardiac muscle

necrosis and inflammation with the degenerative and persistant myopathy of mdx mice. J.Neurol.Sci. 72: 147-157.

222. Morgan, H.E. and Baker, K.M. (1990) Cardiac hypertrophy: mechanical, neural and endocrine dependence. Circ. 83(1): 13-25.

223. Dhalla, N.S., Heyinger, C.E., Beamish, R.E., Innes, I.R. (1987) Pathological aspects of myocardial hypertrophy. The Canadian J. of Cardiol. 3(4): 183-196.

224. Rouslahti, E., Pihko, H., Seppela, M. (1974) Alpha feto- protein; immunochemical purification and chemical properties. Expression in normal state and in malignant and non-malignant liver disease. Transplant. Rev. 29: 38-48.

225. Clubb, F.J., Bishop, S.P. (1984) Formation of binucleated myocardial cells in the neonatal rat. An index for growth hypertrophy. Lab. Invest. 50: 571-577.

226. Rumyantsev, P.P. Some comparative aspects of myocardial regeneration. In: Muscle regeneration. A.Muuro et al. (Eds.)Raven Press, New York, N.Y., (1979) pp. 335-355.

227. Vracko, R., Thorning, D., Fredrickson, R.G., and Cummingham, D. (1988) Myocyte reaction at the borders of injured and healing myocardium. Lab. Invest. 59(1): 104-115.

228. Olivetti, G., Ricca, R., and Anversa, P. (1987) Hyperplasia of myocyte nuclei in long term cardiac

hypertrophy in rats. J. Clin. Invest. 80: 1818-1821.

229. Nadal-Ginard, B., and Madavi, V. (1989) Molecular basis of cardiac performance: Plasticity of the myocardium generated through protein isoform switches. J.Clin. Invest. 84: 1693-1700.

230. Jackson, T., Allard,M.F. Sreenan, C.M., Doss, L.K., Bishop, S.P. Swain, J.L. (1990) The c-myc proto-oncogene regulates cardiac development in transgeneic mice. Mol. Cell. Biol. 10:3709-3716.

231. Schneider, M.D., Prryman, M.B., Payne, P.A., Spizz, G., Roberts, R., et al. (1989) Autonomous myc expression in transfected muscle cells does not prevent myogenic differentiation. Mol. Cell. Biol. 7: 1973-1977.

232. Roberts, A.B., Anzano, M.A. Lamb, L.C., Smith, J.M. and Sporn, M.B. (1981) New class of transforming growth factors potentiated by epidermal growth factor: isolation from non-neoplastic tissues Proc. Nat'l. Acad. Sci. 78: 5339- 5334.

233. Wunsch, H., Sharma, H.S., Bernotat- Danielowski, S., Schott, R.J., Schaper, J., et al. (1989) Expression of transforming growth factor beta-1 (TGF-B1) in collateralized swine heart. Circulation 80:II-453 (Abstr.).

234. Kan,M., Huang, J., Mansson, P.E., Yasumitsu, H., Carr, B., and McKeehan, W.L. (1989) Heparin- binding growth factor type 1 (acidic fibroblast growth factor): a

potential biphasic autocrine and paracrine regulation of hepatocyte regeneration. Proc. Nat'l. Acad. Sci. 86: 7432-7436.

235. Ueno, H., Perryman, M.B., Roberts, R., Schneider, M.D. (1988) Differentiation of cardiomyocytes following mitogen withdrawl exhibits three sequential stages of the ventricular growth response. J.Cell. Biol. 107: 1911-1918.

236. Kahn, D.S., Rona, G., and Chappel, C.I. (1969) Isoproterenol- induced cardiac necrosis. Ann. of NY. Acad. of Sci. 156: 285-293.

237. Rona, G., Zsoter, R., Chappel, C.I., Gaudry, R. (1959) Myocardial lesions, circulatory and electrocardiographic changes produced by isoproterenol in the dog. Rev. Canad. Biol. 18: 83-94.

238. Chappel, C.I., Rona, G., Balazs, T., Gaudry, R. (1959) Severe myocardial necrosis produced by isoproterenol in the rat. Arch. Int. Pharmacdyn. Therapie, Bruxelles. 122:123-128.

239. Caspo, Z., Dusek, J., and Rona, G. (1972) Early alterations of the cardiac muscle cell in isoproterenol- induced necrosis. Arch. Path. 93: 356-365.

240. Benjamin, I.J., Jalil, J.E., Tan, L.B., Cho, K., Weber, K.J., Clark, W.A. (1989) Isoproterenol- induced myocardial fibrosis in relation to myocyte necrosis.

Circ. Res. 65(3): 657-670.

241. Jalil, J.E., Janicki, J.S., Peck, R., Abrahams, C. and Weber, K.T. (1989) Fibrosis- induced reduction of endomyocardium in the rat after isoproterenol treatment. Circ. Res. 65(2): 258-264.

242. Jennings, R.B., and Ganote,C.E. Ultrastructural changes in acute myocardial ischemia. In: Effect of acute myocardial ischemia on myocardial function. ed. Oliver, M.F., Julian, D.G., and Donald, K.W., Churchill Livingstone, (Edinburgh, London) p.50 (1972).

243. Fleckenstein, A., Janke, J., Doring, H.J., Pachinger, O. Ca^{2+} overload as the determinant factor in the production of catecholamine- induced myocardial lesions. In: Recent advances in studies on cardiac structure and metabolism, cardiomyopathies, E. Bajusz et al. (Eds.), vol.2, pp 455-466, Baltimore: University Park Press. (1973).

244. Gotzsche, O. (1983) Decreased myocardial calcium uptake after isoproterenol in streotozotocin- induced diabetic rats. Lab. Invest. 48: 156-161.

245. Fleckenstein, A. (1983) Calcium antagonism in heart and smooth muscle. Experimental Facts and therapeutic prospects. New York, Chichester, Brisbane, Toronto, Singapore. J. Wiley and Sons.

246. Jennings, R.B., and Ganote, C.E. (1976) Mitochondrial structure and function in acute myocardial ischemic

injury. Circ. Res. 38: 80.

247. Mueller, R.A., Thoenen, H. (1968) Cardiac catecholamine synthesis, turnover, and metabolism with isoproterenol-induced myocytolysis. Cardiovas. Res. 23: 771-778.

248. Ganguly, P.K. (1989) Catecholamines and cardiovascular disorders: pathophysiologic considerations. Am. Heart. J. 118(4): 868-872.

249. Dhalla, K.S., Ganguly, P.K., Rupp, H., Beamish, R.E., and Dhalla, N.S. (1989) Measurement of adrenolutin as an oxidation product of catecholamines in plasma. Mol. Cell. Biochem. 87: 85-92.

250. Rona, G., Boulet, M., Heittner, I. Membrane permeability alterations as manifestation of early cardiac muscle cell injury. In: Recent advances in studies on cardiac structure and metabolism. Pathophysiology and morphology of myocardial cell aclterations, A. Fleckenstein, G. Rona (Eds.), vol.6, pp. 439-451. Baltimore: University Park Press (1975).

251. Leidtke, A.J. (1981) Alterations of carbohydrate and lipid metabolism in the acutely ischemic heart. Prog. Cardiovas. Dis. 23: 321.

252. Whitmen, J.T., Idell-Wenger, J.A., Rovetto, M.J., and Neely, J.R. (1978) Control of fatty acid metabolism in ischemic and hypoxic hearts. J.Biol.Chem. 253: 4305.

253. Leidtke, A.J., Nellis, S., and Neely, J.R. (1978) Effects of excess free fatty acids on mechanical and

metabolic function in normal and ischemic myocardium in swine. Circ. Res. 43: 652.

254. Corr, P.B., Gross, R.W. and Sobel, B.E. (1984) Amphipathic metabolites and membrane dysfunction in ischemic myocardium. Circ.Res. 55: 135.

255. Sobel, B.E., Corr, P.B., Robinson, D.K., Goldstein, R.A., and Witkowski, F.X., et. al. (1978) Accumulation of lysoposphoglycerides with arrhythmogenic properties in ischemic myocardium. J.Clin.Invest. 62: 546.

256. Kammermeier,H., and Ober, M. (1985) Essential contribution of thrombocytes to the occurence of catecholamine- induced cardiac necrosis. J.Mol.Cell.Cardiol. 17:371-376.

257. Rona, G. (1985) Catecholamine Cardiotoxicity. J. Mol. Cell. Cardiol. 17: 291-306.

258. Esch,F., Baird,A., Ling,N., Ueno,N., Hill,F., Denoroy,L., Klepper,R., Gospodarowiciz,D., Bohlem,P., and Guilleman,R. (1985) Primary structure of bovine pituitary basic fibroblast growth factor (FGF) and comparison with the amino-termional sequence of bovine brain acidic FGF. Proc. Natl. Acad. Sci. USA. 82: 6507-6511.

259. Laemmli,U.K. (1970) Cleavage of structural proteins during ther assembly of the head of bacteriophage T4. Nature (London) 227: 680- 685.

260. Burnette,W.N. (1981) 'Western blotting':

electrophoretic buffer of proteins from sodium dodecyl sulphate- polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. 112: 195-203.

261. Asijee,G.M., Sturk,A., Bruin,T., Wilkinson,J.M., Ten Cate,J.W. (1990) Vinculin is a permanent component of the membrane skeleton and is incoreporated into the (re)organising cytoskeleton upon platelet activation. Eur.J.Biochem. 189: 131- 136.

262. Pardo,J.V., Siliciano,J.D., and Craig.S.W. (1983) Vinculin is a component of an extensive network of myofibril- sarcolemma attachment regions in cardiac muscle fibers. J. Cell. Biol. 97: 1081- 1088.

263. Pardo,J.V., Siliciano,J.D., and Craig,S.W. (1983) A vinculin- containing cortical lattice in skeletal muscle: Transverse lattice elements ("costameres") mark sites of attachment between myofibrils and sarcolemma. Proc. Natl. Acad. Sci. USA 80: 1008-1012.

264. Capetanaki,Y., Kuisk,I., Rothblum,K., and Starnes,S. (1990) Mouse vimentin:structural relationship to fos, jun, CREB and tpr. Oncogene 5: 645- 655.

265. Tokuyasu,K.T., Maker,P.A., and Singer,S.J. (1985) Distribution of vimentin and desmin in developing chick myotubes in vivo II. Immunoelectron microscopic study. J.Cell. Biol. 100: 1157- 1166.

266. Osinska,H.E., and Lamanski,L.F. (1989) Immunofluorescent localization of desmin, vimentin in developing cardiac muscle of syrian hamster. The Anatomical Record 223: 406- 413.

267. Sax,C.M., Farrell,F.X., Zehnewr,Z.E. (1989) Down-regulation of vimentin gene expression during myogenesis is controlled by a 5'- flanking sequence. Gene 78: 235- 242.

268. Nag,A.C., Krehel,W., and Cheng,M. (1986) Distributions of vimentin and desmin filaments in embryonic cardiac muscle cells in culture. Cytobios. 45: 195- 209.

269. Ruzicka.D.I., and Schwartz,R.J. (1988) Sequence activation of alpha- action genes during avian cardiogenesis: Vascular smooth muscle alpha- actin gene transcripts mark the onset of cardiomyocyte differentiation. J. Cell. Biol. 107: 2575-2586.

270. Eppenberger- Eberhardt,M., Flamme,I., Kurer,V., and Eppenberger,H.M. (1990) Reexpression of alpha- smooth muscle actin isoform in cultured adult rat cardiomyocyte. Dev. Biol. 139: 269- 278.

271. Kartenbeck,J., Franke,W.W., Moser,J.G.,m Stoffels,U. (1983) Specific attachement of desmin filaments to desmosomal plaques in cardiac myocytes. EMBO. J. 2(5): 735- 742.

272. Steenbergen,C., Hill,M.L., Jennings,R.B. (1987) Cytoskeletal damage during myocardial ischemia: changes in vinculin

immunofluorescence staining during total in vitro ischemia in canine heart. Circ. Res. 60: 478-486.

273. Rouslahti,E., Pihko,H., Seppalla,M. (1974) Alpha-fetoprotein; immunochemical purification and chemical properties. Expression in normal state and in malignant and non-malignant liver disease. Transplant Rev. 29: 38- 48.

274. Quarto,N., Finger,F.P., Rifkin,D.(1991) The NH_2-terminal extension of high molecular weight bFGF is a nuclear targeting signal. J. Cell. Physiol. 147: 311-318.

275. Moscatelli,D., Joseph- Silverstein,J., Manejias,R., and Rifkin,D.B. (1987) Mr. 25,000 heparin- binding protein from guinea pig brain is a higher molecular weight form of basic fibroblast growth factor. Proc. Natl. Acad. Sci. USA. 84: 5778- 5782.

276. Sommer,A., Moscatelli,D., and Rifkin,D.B.(1989) An amino- terminally extended and post- translationally modified form of a 25 kD. basic fibroblast growth factor. Biochem. Biophys. Res. Commun. 160: 1267- 1274.

278. Kardami,E. (1990) A potential new role of bFGF in host-parasite interactions. Growth Factors 4: 61- 68.

279. Kardami,E., Pearson,T., Bacroft,R., Fandrich,R.R. Identification of basic fibroblast growth factor- like proteins in african trypanosomes and leishmania. Mol.Biochem. Parasitol. In press.

280. Rona,G., Chappel,C.I., Balazs,T., and Gaudry,R. (1959) An infarct- like myocardial lesion and other toxic manifestations produced by isoproterenol in the rat. A.M.A. Arch. of Path. 67: 443- 455.

281. Rona,G., Kahn,D.S., Chappel,C.I. (1961) Study on the healing of cardiac necrosis in the rat. Am. J. Pathol. 39(4): 473- 489.

282. Baldin,V., Roman,A.M., Bosc- Bierne,I., Amalric,F., and Bouche,G. (1990) Translocation of bFGF to the nucleus is G_1 phase cell cycle specific in bovine aortic endothelial cells EMBO. J. 9: 1511-1517.

283. Amalric,F., Baldin,V., Bosc- Bierne,I., Buglen,B., Gouderc,B., Guyader,M., Patry,V., Prats,H., Roman,A.M. Bouche,G. (1991) Nuclear translocation of basic fibroblasst growth factor. Ann. N.Y. Acad. Sci. 638: 127- 128

284. Wildethal,K. (1978) Liposomal alterations in ischemic myocardium: Result or cause of myocardial damage? J. Mol.Cell. Cardiol. 10: 595.

285. Vanheel,B., Leybaert,L., De Hemptinne,A., Leusen,I.(1989) Simulated ischemia and intracellular pH in isolated ventricular muscle. Am. J. Phisiol. 257: C365- C376

286. Lavail,M.M., Factorovich,E.G., Hepler,J.M., Pearson,K.L., Yasumura,D., Mathes,M.T., Steinberg,R.H. (1991) Basic fibroblast growth factor protects

photoreceptors from light- induced degeneration in albino rats. Ann. NY. Acad. Sci. 368: 341-347.

287. Westermen,R., Grothe,C., Unsicker,K. (1990) Basic fibroblast growth factor (bFGF), a multifunctional growth factor for neuroectodermal cells. J.Cell. Sci. Suppl. 13: 97- 117.

288. Ross,C.R., Hale,C.C. (1990) Detection of the basic fibroblast growth factor- low affinity binding site in cardiac sarcolemmal vessicles. Life. Sci.46:1809- 1815.

289. Rumyantsev,P. In "Growth and hyperplasia of cardiac muscle cells". Edited by Bruce M. Carlson. Cardiology vol.3, Soviet Medical Reviews Supplement Series, Harwood Academic Publishers, 1991.

290. Li,G., Vasquez,J., Gallagher,K., Lucchesi,B. (1990) Myocardial protection with preconditioning. Circulation 82: 609.

291. Steinent,P.M., Roop,D.R. (1988) Molecular and cellular biology of intermediate filaments. Ann. Rev. Biochem. 57: 593- 625.

292. Schaper,J., Forede,R., Hein,S.T., Buck,A., Hashizume,H., Speiser,B., Friedl,A., Bleese,N. (1991) Impairment of the myocardial ultrastructure and changes in the cytoskeleton in detailed cardiomyopathy. Circulation 83: 504- 514.

293. Kellett,J.G., Tanaka, T., Rowe, J.M., Shiu,R.P.C., and Friesen,H.G. (1981) The characterization of growth

factor activity in the human brain. J.Biol. Chem. 256:54-58.

294. Too,C., Murphy, P.R., Hamel,A., and Friesen,H.G. (1987) Further purification of human pituitary- derived chondrocyte growth factor: heparin binding and cross-reactivity with anti-serum to basic FGF. Biochem.Biophys.Res.Commun. 144: 1128-11343.

295. Gospodarowicz,D., Bialecki,H., Greenburg,G. (1978) Purification of fibroblast growth factor activity from bovine brain. J.Biol.Chem. 253: 3736-3743.

296. Nolan,A.C., Clark,W.A.,jr., Karwoski,T., and Zak,R. (1983) Patterns of cellular injury in myocardial ischemia determined by monoclonal Antimyosin. Proc. Natl. Acad. Sci. USA 88: 6046- 6050.

297. Lin,H., Parmacek,M.S., Morle,G., Bolleny,S., Leiden,J.M. (1990) Expression of recombinant genes in myocardium in vivo after direct injection of DNA. Circulation. 82: 2217-2221.

298. Kitois,R.N., Buttrick,P.M., McNally,E.M., Kaplan,M.L., Lemwand,L.A. (1991) Hormonal modulation of a gene injected into rat heart in vivo. PNAS. 88: 4138-4142.

299. Iwai,K., Hori,M., Kitabatake,A., Kurihara,H., Uchida,K., Inoue,M., and Kamada,T. (1990) Disruption of microtubules as a early sign of irreversible ischemic injury: Immunohistochemical study of in situ canine hearts. Circ.Res. 67(3): 694-706.

300. Brown,J.M., White,C.W., Terda,L.S., Grosso,M.A., Shanly,P.F., Mulvin,D.W., Banerjee,A., et.al. (1990) Interleukin 1 pretreatment decreases ischemia/ reperfusion injury. Proc.Nat'l.Acad.Sci. USA. 87: 5026-5030.

301. Munker,R., Gassen,J., Ogawa,M., and Koeffer,H.P. (1986) Recombinant human TNF induces production of GM-CSF. Nature. 323: 79-82.

302. Oster,W., Linderman,A., Horn,S., Mertelsmann,R., and Herman,F. (1987) TNF-alpha but not TNF-beta induces secretion of colony stimulating factor for macrophages (CSF-1) by human monocytes. Blood. 70: 1700-1706.

303. Nawroth,P.P., and Stern,D.M. (1986) Modulation of endothelial cell homostatic properties by tumor necrosis factor. J.Exp.Med. 163: 740-745.

304. Lieberman,A.P., Pitha,P.M., Shin, H.S. and Shin,M.L. (1989) Production of tumor necrosis factor and other cytokines by astrocytes stimulated with lipopolysaccharide or neurotropic virus. Proc.Natl.Acad.Sci. USA. 86:6348-6352.

305. Inamura,K., Spriggs,D., and Kufe,D. (1987) Expression of tumor necrosis factor receptors on human monocytes and internalization of receptor bound ligand. J.Immunol. 139: 2989-2992.

306. Carpenter,G. (1987) Receptors for epidermal growth factor and other polypeptides mitogens.

Ann.Rev.Biochem. 56: 881-915.

307. Macara,I.G. (1989) Oncogenes and cellular transduction. Physiological Reveiws. 69(3): 197-220.

308. Eisemann,A., Ahn,J.H., Graziani,G., Tronick,S.R., and Ron,D. (1991) Alternate splicing generates at least five different isoforms of the human basic FGF receptor. Oncogene. 6: 1191-1202.

309. Johnson,D.E., Lu,P.L., Lu,J., and Williams,L.T. (1990) Diverse forms of a receptor for acidic and basic fibroblast growth factors. Mol and Cell. Biol. 10(9): 4728-4736.