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Expression of IGF-I and TGFβ1 in Cultured Human Myocardium

Insights into the Role of Growth Factors in Hypertrophic Obstructive Cardiomyopathy

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of the Institute of Medical Science
University of Toronto

ABSTRACT

Expression of IGF-I and TGF-β1 in Cultured Human Myocardium
Insights into the Role of Growth Factors in Hypertrophic Obstructive Cardiomyopathy

Master of Science, 1998

Warren Todd Ball

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Objectives: The characterization of human myocardial explants in culture and the investigation of IGF-I and TGFβ1 gene expression in cultured hypertrophic obstructive cardiomyopathic (HOCM) tissue. **Methods:** Biopsies were cultured in either a mitogenic or a minimally mitogenic medium for one week. Phenotype and growth factor gene expression were evaluated by histology and RT-PCR, respectively. **Results:** Cultured myocardium maintained organized sarcomeres for one week. IGF-I gene expression decreased in HOCM explants ($p<0.05$); this change was partially prevented by the mitogenic medium ($p<0.05$). IGF-I mRNA levels remained elevated compared to non-HOCM controls ($p<0.05$). TGFβ1 gene expression increased in both HOCM and non-HOCM controls. The initial *in vivo* over-expression of TGFβ1 was lost after seven days of culture. **Conclusions:** Cultured myocardium maintains cardiac characteristics for one week. *In vivo* factors may contribute to the up-regulation of TGFβ1 and IGF-I in HOCM, however, an intrinsic factor may predispose HOCM myocardium to over-express IGF-I.

ACKNOWLEDGEMENTS

It has been said, "No (individual) is an island". I would certainly not be where I am today without the generous support of many individuals.

I am deeply indebted to my supervisor, Dr. Ren-Ke Li, for his unending support, advice and patience over the last two years. Dr. Li exemplifies hard work and dedication. He has taught me to think scientifically and has been a great friend and helped me achieve my dreams and goals. I promise to never forget the lessons learned under his guidance.

A significant amount of recognition is deserved of my Masters Committee members, Drs Mingyao Liu, Don Mickle, and Tom Parker, for their guidance throughout this thesis. Also, this project would not have existed without the myocardial biopsies so generously provided by Drs William G. Williams, R.J. Cusimano, and George Christakis.

The day-to-day work of conducting research can, at times, be an exercise of patience, perseverance, and blind faith. I am truly grateful to the camaraderie and counseling provided by everyone in the lab that has helped along the way: Guangming Li, Frank Merante, Laura Tumiati, Molly Mohabeer, Zhi-Qiang Jia, Eung Joong Kim, Shinji Tomita, Tetsuro Sakai, and Vania DeSouza. I wish you all the best in your future endeavors.

I wish to especially acknowledge my family and friends. My family has remained steadfast in their faith in my abilities and supported me with love; I truly appreciate their tolerance of my unpredictable schedule and of my periodical ranting and raving. To each and every one of my friends who shared in my frustrations (whether you liked it or not), shared in my triumphs (yes there were a few), and showered me with support and understanding from places as far away as Quito, Palo Alto and Portland and as close to home as London, Guelph, the GTA and especially Kingston, I send out a heart felt "Thank you".

It has been a tremendous experience studying cardiovascular research at the University of Toronto and The Toronto Hospital. I am grateful for the educational opportunities provided by the Cardiovascular Sciences Student Program and the Centre for Cardiovascular Research. The CCR, in particular deserves recognition for their financial support of my training.

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LIST OF ABBREVIATIONS

ABS	Adult bovine serum
ANF	Atrial natriuretic factor
ATP	Adenosine triphosphate
FBS	Fetal bovine serum
GAPDH	Glyceraldehye-3-phosphodehydrogenase
HCM	Hypertrophic cardiomyopathy
HOCM	Hypertrophic obstructive cardiomyopathy
IGF-I	Insulin-like growth factor-I
MHC	Myosin heavy chain
MLC	Myosin light chain
mRNA	Messenger ribonucleic acid
PBS	Phosphate buffered saline
RT-PCR	Reverse transcription polymerase chain reaction
TGFβ1	Transforming growth factor-beta-1
TM	Myocardium from explanted hearts from patients undergoing heart transplantation

INTRODUCTION

Cardiovascular disease represents the major cause of death, disability and illness in Canada. In the last two decades, research efforts have shifted from organ based physiology to the molecular mechanisms underlying disease pathology and, correspondingly, therapy targeting these newly discovered pathways. Experiments *in vivo* are limited by their inability to distinguish between a host of factors contributing to cardiac function and studies employing an isolated heart preparation are limited by its short-term viability (a few hours). The advent of primary cardiomyocyte culture has permitted the elucidation of many molecular signaling pathways in cardiac myocytes. However, cell culture models are limited in their ability to accurately reflect the *in vivo* environment.

Concerns with using isolated cell culture to investigate normal or pathological cardiac function arise because cardiomyocyte morphology and gene expression are altered in culture and because non-myocytes also contribute to the cardiac phenotype. Cardiomyocytes in culture experience a remodeling from the elongated *in vivo* structure to a spheroidal flat shape[1-4] and a breakdown of their characteristic myofibrillar contractile apparatus. In neonatal[5] and adult[3,4] cardiomyocytes, the presence of serum in the culture medium either maintains or induces, respectively, a pattern of gene expression typical of fetal or embryonic cells. Although they account for 80% of the heart's mass, cardiomyocytes represent only 25% of the total cardiac cell population[6] whereas fibroblasts, vascular smooth muscle cells, neurons and endothelial cells account for the majority of cells. It has been hypothesized that the changes observed *in vitro* are occurring because cardiomyocytes are removed from the three-dimensional orientation, cell-to-cell interactions, and contractile load found *in vivo*.

Cultured myocardial tissue explants maintain *in vivo* tissue geometry and thus more closely approximate the *in vivo* environment. However, since few reports on the culture of myocardial explants exist[7], the phenotype and gene expression of myocardial explants in culture remains to be characterized. The establishment of a viable tissue culture model will permit the investigation of a number of aspects of cardiovascular diseases, including: aberrant cell-to-cell interactions, molecular disease mechanisms and therapeutic interventions, such as receptor blockade or application of antisense oligodeoxynucleotide technologies. For example, hypertrophic obstructive cardiomyopathy (HOCM) is an abnormality whose pathology appears to involve multiple cell types: hypertrophy of cardiomyocytes and an increased production of extracellular matrix components by cardiac fibroblasts. Recent work has suggested that growth factors, particularly IGF-I and TGFβ1, may be involved in the development of HOCM[8-12]. However, *in vivo* factors, such as hemodynamic load and sympathetic or parasympathetic stimulation, are also associated with growth factor up-regulation. A myocardial tissue culture model permits the evaluation of growth factor expression in the absence of such potentially confounding factors and may therefore help clarify the impetus for growth factor activation in HOCM.

This introduction will briefly outline the morphological and molecular aspects of both normal cardiac growth and pathological hypertrophy. This thesis is interested in investigating the role of growth factors in the pathogenesis of HOCM; the current perspectives on this phenomenon are therefore presented. Finally, the aims and challenges in developing a myocardial tissue culture model for the investigation of cardiovascular disease are introduced.

CARDIAC GROWTH

Cardiac Development

At a cellular level, cardiac growth during embryonic and fetal development occurs via cardiomyocyte hyperplasia, with little change in the size of individual cells[13]. Early in neonatal life cardiomyocytes lose the ability to replicate; thus, hyperplastic growth ceases and subsequent cardiac growth to adult proportions occurs via an increase in the size, or hypertrophy, of a finite population of cardiomyocytes.[6,13,14] During the transitional phase from hyperplastic to hypertrophic growth cell growth, cardiac myocytes exit the cell cycle and undergo additional rounds of nuclear division without cytokinesis, resulting in bi-nucleated, and possibly polyploidic, myocytes[13]. The transition to hypertrophic growth is also characterized by rapid tissue remodeling of the neonatal ventricle, capillary angiogenesis and the formation of an extensive extracellular matrix[15].

Cardiac Hypertrophy

Unable to proliferate, adult mammalian cardiomyocytes undergo hypertrophy as an adaptive response to physiological or pathological stimuli[15]. Physiological hypertrophy may occur as part of normal developmental growth, as discussed above, or may be induced by aerobic exercise training. Physiological hypertrophy in athletes is usually reversible. Pathological hypertrophy, on the other hand, is associated with various disease states, is generally not easily reversed, and is an independent risk factor for cardiovascular morbidity and mortality[16].

Pathological cardiac hypertrophy may involve either the right or the left ventricle. Right ventricular hypertrophy manifests secondary to disorders such as pulmonary hypertension or tricuspid valve dysfunction. Left ventricular hypertrophy, however, is far more common; it may be classified as either concentric or eccentric. Concentric hypertrophy of the left ventricle is distinguished by an increase in the thickness of the ventricular wall with either no change in or even a decrease in chamber size. This form of hypertrophy typically presents in patients with an increased afterload, as in hypertension or isolated valvular aortic stenosis. Conversely, eccentric hypertrophy is defined by a proportionate increase in left ventricular wall thickness and internal chamber diameter. Patients presenting with an increased preload, or volume overload, as is present in mitral regurgitation, display eccentric hypertrophy. However, the distinction between these two types of hypertrophy is not always clear. The pressure overloaded heart can undergo a transition from compensatory concentric hypertrophy towards cardiac dilatation and failure which results in a morphological pattern of eccentric hypertrophy. Hypertrophy may also be classified as regional. This may occur following a myocardial infarction; the myocytes surrounding the infarcted region hypertrophy in an adaptive attempt at preserving cardiac function. HOCM also presents a unique form of hypertrophy that is remarkably regional and primarily affects the inter-ventricular septum. Its etiology, however, remains to be defined and is the focus of current research efforts, as will be presented in a later section.

Cardiac hypertrophy involves a progressive series of morphological and functional changes[15,17]. There is a transient breakdown or damage of muscle cells. This is followed by overall increases in myocyte metabolism, volume and mass, which include increases in mRNA, rRNA, and protein synthesis. These compensatory adaptations restore normal

cardiac function, or even produce hyperfunction. However, unchecked, pathological hypertrophy eventually progresses beyond the heart's adaptive ability and there is a gradual exhaustion of its ability to synthesize proteins. This results in a failure to renew mitochondria and myofibrils, producing myofibrillar damage, cellular atrophy and the replacement of degenerating muscle fibers by fibrous tissue, leading to cardiac failure. The mechanism(s) leading from adaptive hypertrophy to cardiac failure remain to be elucidated.[17]

REGULATION OF CARDIAC GENE EXPRESSION

Developmental Expression Patterns

The hallmark a cardiomyocyte is its typical striated appearance. Myocytes are made up of a large number of cross-banded strands call myofibrils that are themselves comprised of longitudinally aligned contractile units, termed sarcomeres. Electron microscopy has revealed that the sarcomere is made up of an inter-digitation of thick and thin filaments. The thick filament consists of the protein myosin, while the thin filament consists of two chains of globular actin molecules arranged in a double helix that is associated with a troponin-tropomyosin complex. A series of steric changes in the contractile proteins permits the interaction of actin and myosin and forms the basis for the excitation-contraction coupling sequence (ie force generation). Each of the sarcomeric proteins has multiple isoforms that exhibit differential expression patterns at different stages of cardiac development and growth, as reviewed by Roberts[18].

Each molecule of myosin consists of two myosin heavy chains (MHCs) and two pairs of myosin light chains (MLCs). Two isoforms of MHC are expressed in mammalian cardiac muscle, an α-MHC and a β-MHC. Throughout embryonic life, all species of mammals studied to date express predominantly the β-MHC isoform in the ventricles and the α-MHC in the atria[19]. Towards the end of gestation an isoform transition occurs; the α-MHC appears in the ventricles and the β-MHC in the atria. In rodents, by three weeks the ventricles express almost exclusively α-MHC[20]. In contrast, large mammals, including humans, exhibit only minimal increases in α-MHC expression following birth; β-MHC remains the predominant ventricular isoform throughout life[21]. Of the three MLC isoforms identified,

only MLC1 and MLC3 are expressed in the heart. MLC1 has distinct atrial and ventricular forms. In early embryonic life the ventricular and atrial forms of MLC1 are expressed in their appropriate cardiac chambers, however, atrial MLC expression is also found in the embryonic ventricles.

The α-actins have been the greatest studied of the thin filament proteins in the myocardium. The two sarcomeric actins actively expressed in the myocardium are skeletal α-actin and cardiac α-actin, although a third α-actin, smooth muscle α-actin, is found transiently in the embryonic myocardium. As was described for the MHCs, expression patterns of the actin isoforms differs between rodents and larger mammals. In the fetal rodent heart the skeletal α-actin isoform predominates, but a transition to predominantly cardiac α-actin occurs in the neonate. However, human ventricular myocardium exhibits a persistent and variable expression of skeletal α-actin, while cardiac α-actin gene expression is restricted to the atrium. The other two major thin filament proteins, tropomyosin and troponin also display differential expression throughout cardiac development. In rodents, β-tropomyosin represents the embryonic isoform, while α-tropomyosin is the only form present in the adult ventricles. In contrast, human myocardium retains approximately 20% β-tropomyosin expression. Little information exists concerning the expression of the troponin isoforms T, C, and I. Immunological analysis, however, has demonstrated two troponin T isoforms in the rat heart - a larger form in the fetal ventricle that is ultimately replaced by a smaller form in adult myocardium.

Hypertrophic Gene Expression Paradigm

At the molecular level, a highly specific program of changes in gene expression characterizes hypertrophy induced by pressure overload in rodent models[5,22,23]. The so-called early response genes (Egr-1, Hsp70, *c-fos*, *c-jun*, *c-myc*) are activated within 30-60 minutes after exposure to a hypertrophic stimulus[24]. This plasticity of expression is continued through a transition from adult to fetal protein isoforms of various sarcomeric proteins and enzymes. An induction of the β-MHC isoform and a repression of the α-MHC occurs in the adult ventricle[25,26]. In fact, there is some regional variability to this expression; a transmural gradient with greater endocardial β-MHC expression has been observed[27]. This reversion to fetal protein isoforms has been corroborated for other transcripts of the contractile apparatus such as: skeletal α-actin [25,28,29], β-tropomyosin [28], and atrial MLCs[30]. In addition, a more generalized re-expression of fetal isoforms occurs in cardiac hypertrophy, as given by the resurgence of some cardiac metabolic enzymes[31,32] and the sarcolemmal Na^+/K^+ ATPase[33]. The inhibition of the sarcoplasmic reticulum (SR) Ca^{2+} ATPase[34,35] and the re-expression of atrial natriuretic factor (ANF)[28,36] also mirror embryonic, rather than adult, modes of expression following over-load of the myocardium.

In the ventricular tissue of larger animals, including humans, where β-MHC already predominates, the MHC isoform transition is less dramatic. α-MHC expression is lost with time with some increase in β-MHC[21,37]. Unlike the clear re-induction of α-skeletal actin in pressure overload animal models, there is a heterogeneity of expression of the sarcomeric α-actins and no predictable re-expression pattern[38]. The hypertrophic human heart does exhibit

plasticity of gene expression that parallels that observed in rodent models[39,40]: atrial MLC1 and ANF are re-expressed in the adult ventricle and the SR Ca^{+2} ATPase is down-regulated.

In principle, these sarcomeric protein isoform shifts may eventually become unfavorable in conjunction with deteriorations in other cellular systems.[23] However, the up-regulation of some of these genes certainly initially appears to improve contractility. β-MHC diminishes myocardial contractility on the basis of slower cross-bridge cycling[41], which is proposed to be advantageous during high oxygen demand based upon the lower energetic cost of developed work[42]. Replacement of ventricular with atrial MLC species enhances the Ca^{2+} sensitivity of the contractile apparatus.[43] Contractile function is also improved by a switch to the skeletal α-actin from α-cardiac actin isoform.[44] However, the definitive effects on heart function resulting from changes in gene expression remains to be elucidated.

MYOCARDIAL GROWTH FACTORS

It has been shown that both normal and abnormal cardiac growth are dependent upon localized cell-cell interactions[45] that are mediated by a whole range of substances, such as cytokines, hormones and growth factors. The transmission of these signals into the cell involves three phases: (i) activation of cell receptors (ii) intracellular signal transmission, and (iii) initiation of cytosolic events, including activation of nuclear transcription factors, such as *Fos, Jun, JunB, Myc*, and *Egr-1*. As reviewed by Hefti[24], intracellular signaling was once thought to occur via a series of distinct and separate pathways. Further studies have shown that signaling switches and their intracellular transduction pathways likely form a complex biochemical network of multiple interacting pathways.

Early evidence suggested that circulating factors were unlikely to be the principal mediators of hypertrophy. There was an absence of hypertrophy in the right heart following aortic constriction[46], while right ventricular growth was induced following pulmonary artery banding[47]. Thus, the long-standing hypothesis[48] is that locally produced multifunctional polypeptide growth factors, acting via autocrine and/or paracrine mechanisms, are the regulators of myocardial differentiation and growth. In particular, endothelin, angiotensin II, myotrophin, fibroblast growth factors, transforming growth factors (TGFs), and insulin-like growth factors (IGFs), have been localized to the heart. These factors, through their spatial and temporal expression patterns in the heart, both during embryogenesis and in the adult, have been implicated in autocrine and paracrine signaling of myocyte differentiation, development and growth[49]. This thesis is focused on IGF-I and TGF-β1 as our lab has shown that these two growth factors are over-expressed in HOCM myocardium, as will be discussed

later; thus, a discussion of these factors follows.

Insulin-Like Growth Factors

Insulin and the IGFs (IGF-I and IGF-II) comprise a family of related polypeptides with wide-ranging effects on cellular growth and metabolism. This class of growth factors is responsible for mediating many of the actions of growth hormone on peripheral tissues.[50] Sharing structural and biological functional homology with pro-insulin, IGF-I an IGF-II are cleaved to release an active monomeric protein with 70 and 67 amino acids respectively. Although IGFs act in a similar endocrine manner to insulin following their synthesis in the liver[51], both isoforms are also produced by a variety of cell types throughout the body, including the heart[52-55]. This is suggestive of an autocrine and/or paracrine role in growth and differentiation[56,57]. IGF-II is more prominent during fetal development, while IGF-I persists during adult life and has a half life of approximately 12 hours[58].

Complex formation of IGFs with specific binding proteins is required for their transport through membranes and may control the interaction of the IGFs with their receptors.[24] In general, IGF-I acts through the IGF-I receptor and IGF-II acts by cross-reacting with both the insulin and the IGF-I receptors[59,60]; the physiological role of the type II IGF- receptor in IGF signal transduction is not currently clear.[59] IGF-I receptors are synthesized as single chains that become glycosylated and cleaved into α- and β-subunits. The functional receptor is a heterotetramer with two extracellularly oriented α-chains and two β-chains that extend through the membrane and contain tyrosine kinase activity.[24] Binding of IGF protein to this transmembrane tyrosine kinase produces an allosteric

interaction of the two halves and phosphorylation of its catalytic domains. Subsequently, intracellular insulin-receptor substrates become phosphorylated and serve as high-affinity binding sites for the docking and activation of signaling proteins, which, once bound, can be further regulated by phosphorylation or conformational changes to initiate multiple downstream responses. One such event is the activation of *Ras* by *Sos*, which leads to phosphorylation of the MAP kinase cascade and the ultimate activation (phosphorylation) of nuclear transcription factors, such as e*gr-1*, *fos*, *jun*, j*unB*, and *myc*.

Transforming Growth Factor β

Mammalian transforming growth factor-beta (TGF-β) is the prototype of a super-family of molecules sharing a homologous region of conserved cysteine residues[61-63]. TGFβ1, a 25000 kD homodimer with a unique NH_2-terminal sequence, was the first to be isolated and characterized. It is known that three mammalian TGF-β isoforms exist, TGF-β_1, -β_2, and -β_3, that share approximately 70% sequence homology at the amino acid level[64] and in most cell systems examined to date have indistinguishable biological activities[65]. However, their patterns of expression are quite distinct in both the embryo and the adult.[66-68] In most tissues TGF-β is secreted as a latent complex non-covalently associated with its processed latency peptide and an additional binding protein[69-71] and has been shown to have a half-life of approximately 14 hours[72]. The action of this growth factor may also be modulated through the interaction of complementary cell types[73].

TGF-β was originally identified as a factor that transformed the phenotype of fibroblasts and permitted their growth in soft agar culture[74]. However, the pleiotropic actions

of TGF-β are perhaps best illustrated by its potential ability to exert either positive or negative effects on cell growth, demonstrating the necessity to determine its biological role empirically in any given context. In the heart, TGF-β1 is synthesized by both cardiomyocytes and cardiac fibroblasts[75]. In the adult ventricular myocardium the preponderance of basal TGF-β1 is localized to the non-myocyte cell fraction.[75] It has been implicated as an inducer of cardiac muscle formation[67], differentiation[76], morphogenesis[45,66,77] and, as will be discussed below, hypertrophy.

The three mammalian forms of TGF-β bind to three types of receptors found on most mammalian cell types. The type-III receptor, however, has no known signaling function.[65] Type-I and type-II receptors are distantly related *Ser/Thr* kinases. As reviewed by Hefti et al[24], ligand binding to the primary type-II receptor enables it to bind and phosphorylate the type-I receptor, which then propagates the signal. Recently, Smad proteins were identified as the first family of putative TGF-β signal transducers.[78] Phosphorylated by activated receptors, Smads form complexes, move into the nucleus and, by associating with DNA binding proteins, activate gene transcription.

Growth Factors & Cardiac Hypertrophy

In addition to their role in cellular differentiation and growth, growth factors have been implicated in the development of hypertrophy secondary to a variety of cardiac diseases, specifically, infarction[79], progressive coronary artery occlusion[80], ischemia in the porcine heart[81], and pressure overload[34,82,83]. In addition, norepinephrine, AN-II, and isoproterenol,

agonists known to produce cardiac hypertrophy, have also been shown to up-regulate the expression of growth factors in the heart [84-86].

TGFβ1 has been associated with left ventricular mass secondary to pressure overload[65]. Directly linking growth factor signaling with cardiac hypertrophy, TGFβ1 has been shown to stimulate cultured rat cardiomyocytes to synthesize contractile proteins[87]. Parker *et al*[5,88] have provided evidence that TGF-β1 alters the expression of at least six myocardial genes in neonatal cardiac myocytes: the induction of β-MHC, skeletal α-actin, smooth muscle α-actin and ANF, and the down-regulation of α-MHC and of sarcoplasmic reticulum Ca^{2+} ATPase. Notably, this response profile mimics the genetic events described above following exposure of the heart to pressure overload. Although these results were observed in cultured neonatal rat cardiomyocytes, TGF-β1's ability to increase the fractional rate of β–MHC synthesis while suppressing that of α-MHC has also been shown in adult rabbit cardiac myocytes[89].

Clinical studies have demonstrated a positive correlation between left ventricular mass and IGF-I levels in patients with growth hormone deficiency[90] and in hypertensive patients[91]. In animal models, pressure or volume overload resulting in ventricular hypertrophy has also been associated with elevated IGF-I levels[82]. Decker[89] and others[92,93] have demonstrated the capacity of IGFs to stimulate cardiomyocytes hypertrophy *in vitro*. These studies showed that IGFs modulate protein turnover, resulting in an accumulation in total and contractile protein and physical cellular hypertrophy, independent of mechanical load.[89] Specifically, the greatest degree of cardiomyocyte growth, via increased protein synthesis, occurred in response to IGF-I. A study[56] involving both renin-dependent and

renin-independent models of pressure-induced ventricular hypertrophy in the rat also demonstrated an up-regulation of IGF-I mRNA and protein coincident with the onset of systolic hypertension and accelerated ventricular growth. However, despite continued pressure overload and hypertrophy mRNA levels returned to baseline, suggesting a role for IGF-I in the initiation of hypertrophy. *In vitro* experiments have also demonstrated the capacity of IGF-I to enhance the expression of β-MHC[94] and the muscle specific genes: skeletal α-actin, MLC2, and troponin I[92]. However, in contrast to the stimulatory effects of TGF-β and pressure overload, IGF-I down-regulates smooth muscle α-actin and ANF in adult rat cardiomyocytes[93,95]. This is interesting given the increase in cell size reported for neonatal rat cardiomyocytes treated with IGF-I[92] but not of treated adult rat cardiomyocytes[95].

In addition to their autocrine and paracrine actions on cardiomyocytes, peptide growth factors would also be expected to affect non-muscle components of the myocardium. In fact, both TGF-β1 and IGF-I have been shown to induce collagen production by fibroblasts[96,97]. This is in agreement with the development of interstitial fibrosis in myocardial hypertrophy[83], particularly in hypertrophic cardiomyopathy (see below)[98].

HYPERTROPHIC CARDIOMYOPATHY

Hypertrophic cardiomyopathy (HCM) is a primary cardiac disease characterized by left ventricular hypertrophy of unknown cause. Of significant clinical importance, HCM is the most common cause of sudden death in otherwise apparently healthy competitive athletes.[99] Originally described by two French pathologists in the mid 19th century[100,101] and a German pathologist[102] in the early 20th century, HCM came to the attention of modern medicine following the simultaneous reports of Brock[103] and Teare[104] in the 1950's. HCM displays a unique pathophysiology, heterogeneous expression and clinical spectrum; it is from this great diversity that much of the confusion surrounding this disorder arises, in terms of etiology, diagnosis, natural history, and clinical management.

Definition and prevalence

The most characteristic morphologic abnormality of HCM is a hypertrophied and non-dilated left ventricle in the absence of another cardiac or systemic disease that itself is capable of producing left ventricular hypertrophy, such as aortic stenosis or systemic hypertension[105]. While a symmetrical pattern of left ventricular hypertrophy may occur[106], the hypertrophy is almost always asymmetrical and primarily involves the ventricular septum, with mid-ventricular, apical and rarer types being far less common.[107] In addition, HCM patients typically present with atrial enlargement, thickening of the mitral valve leaflets, and areas of fibrosis in the ventricular wall.[106] Seventy-five percent of HCM hearts also show a characteristic fibrous plaque on the mural endocardium of the left ventricle in apposition to

the anterior mitral leaflet, presumably resulting from systolic contact between the mitral valve and the septum.[106] These pathological features are reflected in the distinctive histological appearance of HCM - striking cardiomyocyte hypertrophy accompanied by gross muscle bundle disorganization and a significant amount of extracellular matrix deposition.

Left ventricular hypertrophy is one of the most powerful predictors of cardiac morbidity and mortality.[108] In HCM patients, the pathophysiological features affecting clinical course can include:systolic dysfunction, left ventricular diastolic dysfunction, impaired coronary vasodilator reserve and myocardial ischemia reviewed by Wigle[107] and Maron[106]. In addition, hypertrophy of the interventricular septum may produce a pronounced obstruction to LV outflow tract, termed hypertrophic obstructive cardiomyopathy (HOCM). Severe symptoms unresponsive to pharmacological therapy can necessitate surgical correction to improve cardiac function.

The prevalence of HCM in the general population is estimated to range from 0.02% (1 in 5000)[109] to 0.2% (1 in 500)[110]. However, it is believed that many individuals in the community go undetected because they manifest no or only mild symptoms and therefore do not undergo the required diagnostic echocardiographic analysis.

Molecular Genetics

Molecular genetic risk factors are of great interest to cardiovascular physicians, since genes may be directly responsible for some cardiac diseases or may predispose an individual to cardiac disease through interaction with other genes or with the environment. Approximately 50% of HCM patients show a Mendelian autosomal dominant pattern of

familial transmission, currently referred to as familial hypertrophic cardiomyopathy.[110,111] The penetrance of the disease gene is incomplete during adolescence and increases with age to nearly complete penetrance in adulthood.[110,112]

HCM has widely been described as a heterogeneous disease of the sarcomere. To date, mutations in genes coding for six different sarcomeric proteins, namely, β-myosin heavy chain (β-MHC)[112-119], cardiac troponin T[114,120], myosin binding protein-C[121,122], α-tropomyosin[114,120,123], and the essential and regulatory myosin light chains[124] have been identified in patients with HCM. It is therefore believed that the hypertrophy in HCM may be compensatory in response to abnormalities resulting from these mutations. However, the mechanism by which mutant sarcomeric proteins produce the regional hypertrophy in HCM patients remains to be identified. Recent work has begun to explore the potential functional significance of these mutations to contractile function. Functional studies have shown that some mutant β-MHCs have abnormal function.[125-127] Since, in addition to their expression in the heart of HCM patients, mutant β-MHCs are expressed in slow-twitch skeletal muscle fibers, *in vitro* contractility assays with skeletal muscle have permitted an analysis of contractile properties of single slow-twitch human muscle fibers with distinct β-MHC mutations. This has demonstrated a heterogeneic effect on contractile function: a decreased maximum velocity of shortening and decreased isometric force generation in a mutation near the binding site of the essential light chain, a lowered force/stiffness ratio and depressed velocity of shortening in a mutation at the actin interface of myosin, and normal contractile properties in a mutation at the end of the ATP-binding pocket.[127] Support for the role of troponin T mutations in the pathogenesis of HCM has also been suggested by the

demonstration of abnormal function in two mutations expressed *in vitro*.[128,129] Conversely, a different troponin T mutation has been shown to result in an increase in the velocity of actin-troponin filaments in an *in vitro* motility assay. Rare mutations in cardiac MLCs have also been associated with an increase in the velocity of actin-myosin interaction.[124] Thus, sarcomeric mutations in HCM do not appear to act through a common mechanism (ie functional contractile impairment) that supports the compensatory hypertrophy hypothesis.[128 130-132]

Another unexplained observation of HCM is that first degree relatives (ie carrying identical mutations) manifest variable patterns of left ventricular wall thickening and clinical prognosis.[133] Furthermore, as previously mentioned, family studies have estimated that this autosomal dominant trait is only present in 50% of HCM patients, with the remaining HCM patients presumably representing sporadic occurrences.

Perhaps the most confounding feature of HCM is the remarkably asymmetrical pattern of hypertrophy. As sarcomeric mutations are present in all cardiac myocytes, it would be intuitive to expect any resulting impairment to contractile function to produce a response analogous to hypertrophy compensatory to load - a global, symmetric hypertrophy. Just as intriguing is that despite the presumed impairment in contractile function given by the sarcomere hypothesis, the left ventricular ejection fraction, a measure of systolic function, is normal or even increased in HCM patients.[107,134,135] Lastly, although hypertrophy of individual cardiomyocytes is characteristic of both HCM hypertrophy and that compensatory to load, myocardial fibrosis is more extensive in HCM myocardium[98].

A functional interaction between mutant sarcomeric genes and other genes regulating myocardial growth remains a possibility. Angiotensin II is one agonist known to invoke

myocardial hypertrophy. The angiotensin conversing enzyme (ACE) converts angiotensin I to angiotensin II in the heart. The ACE gene has insertion/deletion polymorphism, of which the genotype with two deletion alleles (DD genotype) has been associated with higher plasma ACE activity. The DD genotype has been associated with an increased likelihood of LV hypertrophy in hypertensive patients[136], and sudden death in patients with HCM[137,138]. Myocardial calcium kinetics[139] and sympathetic stimulation[140,141] have also been investigated because of the diastolic functional abnormalities in symptomatic HCM patients. While the contribution of multiple factors such as these may eventually contribute to our understanding of the heterogeneity of this abnormality, the predilection for severe regional hypertrophy of the interventricular septum remains to be explained. Clearly, the evidence available to date cannot account for a number of features of HCM and there must therefore be other factors involved in the development of this abnormality.

Growth Factors in HOCM Patients.

Li *et al* have recently shown that IGF-I and TGF-β1 mRNA[8], protein[9] and their respective receptors[9,142] are over-expressed in HOCM myocardium compared to that from hypertrophic myocardium compensatory to load (aortic stenosis) and to non-hypertrophic myocardium from patients with stable angina and from explanted hearts. Significant elevations of TGFβ1 mRNA have also been shown to accompany genetically determined hypertrophy in the cardiomyopathic Syrian hamster[143] and immunohistochemistry has demonstrated elevated levels of aFGF[10] in HOCM patients.

Evidence from experiments focusing on the signaling molecules downstream of growth factor receptors have strengthened the premise that myocardial growth factors are involved in the development of HOCM. Utilizing cultured cardiomyocytes, three independent signaling pathways downstream of growth factor receptors, *ras*[144], G_q[145], and gp130[146], have recently been implicated in the activation of key features of the hypertrophic response *in vitro*. Subsequently, transgenic mice expressing a ventricular MLC-*ras* fusion gene [147] were used to develop a sub-strain of MLC-*ras* transgenic mice that displayed a significantly enhanced ventricular hypertrophic phenotype.[11] This enhanced sub-strain displayed increased wall thickness when compared to both wild-type animals and a pressure-overload model. An increased systolic Doppler velocity was seen in the left ventricle of 69% of MLC-*ras* animals; no wild-type or pressure overload animals exhibited this phenomenon, which parallels that observed in patients with HOCM. Also, myocyte disarray was present in all MLC-*ras* animals and was also significantly more extensive than in over-load or wild-type myocardium. On a molecular level, while the increased cardiac mass in the MLC-*ras* sub-strain is not accompanied by activation of the full complement of embryonic gene isoforms observed in pressure-overload compensatory hypertrophy, a selective up-regulation of mRNA for ANP and BNP was observed. This phenomenon has also been documented in LV biopsies from human HOCM patients.[148] Finally, the nuclear proto-oncogenes c-*fos*, c-*jun*, and c-*myc*, the ultimate targets of growth factors signaling, have also been found to be elevated in HOCM patients[12].

Mounting evidence implicates growth factor signaling as a component in the pathological mechanism resulting in the HOCM phenotype. However, the impetus for the activation of the growth factor signaling remains to be identified.

TISSUE CULTURE

The current models employed for the investigation of cardiac function have limitations. Experiments on the *in vivo* heart are limited by their inability to distinguish between the host of factors contributing cardiac function. Also, long-term responses cannot be studied in isolated organ culture preparations, such as the Langendorff preparation, because the organs are not viable *ex vivo* for more than a few hours.

The advent of primary cardiomyocyte cell culture[149-152] permitted cellular cardiologists to dissect many of the molecular cardiac signaling pathways from confounding *in vivo* factors and attribute them to particular cell types. However, experience with cardiomyocyte culture has shown that the culture environment can have a dramatic effect on the phenotype of cultured cells. Adult ventricular cardiomyocytes in primary culture undergo significant morphological remodeling from the elongated *in vivo* structure to a spheroidal flat shape and an accompanying loss of the characteristic myofibrillar contractile apparatus within the first 1-3 days in culture[1-4]. In contrast, neonatal cardiomyocyte's striated structure is lost almost immediately in culture[4,153].

Whereas mammalian cardiomyocyte division *in vivo* is thought to stop shortly following birth[6,13], serum has been observed to be mitogenic for cultured cardiomyocytes[1,154]. However the degree of mitogenicity is dependent upon the serum type. Adult rat cardiomyocytes cultured in a 10% adult horse serum exhibit limited mitosis and division only during the first week of culture.[154] In contrast to this minimally mitogenic stimulus, 10% fetal bovine serum proved to be strongly mitogenic, permitting adult cardiomyocytes to divide for as long as 6 months, presumably because fetal serum should have more growth

factors than adult serum[1]. Further, cardiomyocytes division did not occur in the absence of fetal bovine serum[1].

Serum is also known to modulate the characteristics of cultured cells. In both neonatal[5] and adult[3,4] cardiomyocytes serum or growth factors are known to modulate the growth process and to maintain or induce, respectively, a pattern of gene expression typical of fetal or embryonic cells. Of interest to the study of cardiac hypertrophy, not only does serum induce a hypertrophic phenotype in cultured cells[155], but it induces changes to gene expression paralleling those invoked by hypertrophy *in vivo*. Serum increases neonatal cardiomyocyte cell volume and total protein[156], as well as expression of the proto-oncogene *c-myc*[157] and α- skeletal actin[157] and ANF[158] mRNA. In contrast, mitogen withdrawal permits neonatal cardiomyocytes to express cardiac-specific genes in a developmentally accurate pattern when compared to *in vivo* age matched controls.[157]. Adult cardiomyocytes also display a pattern of gene expression paralleling that described for myocardial hypertrophy. High serum concentrations invoke a shift to the fetal β-MHC[3] and an up-regulation of ANF[158]. Culture of cardiomyocytes in serum free conditions, however, maintains the ratio of α to β-MHC mRNA and the level of cardiac α-actin for 7 days[159]. However, a minimally mitogenic culture environment, 5% calf serum, has also been shown to maintain the differentiated characteristics of adult cardiomyocytes, maintaining α-MHC expression levels for up to one week[4].

It has been suggested that the dramatic morphological changes observed likely represent an adaptation to a new environment following the removal of *in vivo* factors, such as strict three-dimensional geometry, cell-to-cell interactions and the signaling capacity

provided therein and an environment requiring a significant amount of work (ie contraction).[4] While the significance of studying cardiomyocytes may be intuitively tied to their contractile function in the normal and pathological heart, the importance of non-myocyte cardiac cells also cannot be understated. Myocytes comprise approximately 80% of the heart mass, but represent only 25% of total cardiac cells[6], whereas fibroblasts, neurons and endothelial cells account for the majority of cells by number. Cardiac fibroblasts, representing 90% of the non-myocyte cell population[160], have the potential to dramatically affect alter the cardiac phenotype as they are responsible for the production of the extracellular matrix (ECM) proteins that constitute the intricate and highly organized collagen matrix serving to interconnect cardiac myocytes to one another and their neighboring capillaries.[160] Thus, if integrated function, or dysfunction, of whole organs is the goal, an organotypic model is required.

The utility of any *in vitro* model is dependent upon its stability over the time period required for evaluation of the desired variables. However, experience with myocardial tissue culture is limited to a single report by Ationu[7] demonstrating the maintenance of IGF-I expression in human ventricular TM explants cultured for one week in 10% fetal calf serum. To demonstrate the utility and appropriateness of a myocardial tissue culture model, both the phenotype and gene expression of cultured myocardial explants should be characterized in a range of culture environments.

HYPOTHESIS & OBJECTIVES

A tissue culture model of the myocardium may offer a representative model of the myocardium because it maintains normal three-dimensional geometry and cell-to-cell interactions between myocytes and non-myocytes found *in vivo*, characteristics not achieved in traditional cardiomyocyte culture models. IGF-I and TGFβ1 have been found to be over-expressed in HOCM myocardium. However, investigations into the role of these growth factors *in vivo* is confounded by the presence of multiple *in vivo* factors such as pressure or catecholamines, which are also known to modulate growth factor expression. A myocardial tissue culture model permits the evaluation of growth factor gene expression in the absence of such potentially confounding factors.

In light of the above rationale the following hypotheses were formulated:

Hypothesis 1:

A myocardial tissue culture model will preserve the cardiac phenotype *in vitro*.

Hypothesis 2:

IGF-I and TGFβ1 are involved in the development of hypertrophy in HOCM.

An investigation of these hypothesis was conducted with the following aims:

Specific Aims:

1. Examine the phenotypic changes (ie sarcomere and nuclear structure) of cultured human myocardium with time.
2. Evaluate the effects of serum on IGF-I and TGF-β1 gene expression over time in cultured human myocardium.

3. Evaluate IGF-I and TGF-β1 gene expression versus time in culture in normal (non-diseased) rat myocardium.

4. Observe the differential effects of a minimally mitogenic versus a mitogenic environment on IGF-I and TGF-β1 gene expression over time in cultured HOCM and non-HOCM myocardium.

5. Determine if the observed *in vivo* over-expression of IGF-I and TGF-β1 mRNA in HOCM myocardium persists in culture.

METHODS

SPECIMEN COLLECTION

Human myocardial left ventricular septal biopsies were obtained from: (i) adults with hypertrophic obstructive cardiomyopathy (HOCM) undergoing corrective sub-aortic septal myectomy and (ii) non-HOCM explanted hearts from patients undergoing heart transplantation (TM). The pathology of the TM hearts was either dilated or ischemic cardiomyopathy. For all biopsies, the patient's age and gender was recorded and is presented in Table 1. Permission was obtained from the Toronto Hospital's Human Experimentation Committee and from the patients. Rat myocardium was obtained from adult WKY rats, approximately 300-350g.

Table 1: Patient Age and Gender Demographics by Experimental Group

			Sex	
	Experiment	**Age (yrs)**	**Male**	**Female**
HOCM	2% ABS (n=7)	52.9±12.4	5	2
	10% FBS (n=6)	47.4±9.1	5	1
TM	2%ABS (n=6)	48.3±5.9	4	2
	10% FBS (n=6)	54.4±12.7	4	2

Upon explantation, a tissue sample was immediately frozen in liquid nitrogen, to serve as the *in vivo* (day 0) control, and the remainder of the tissue was placed into culture medium (see below) for tissue transportation to the tissue culture facility.

Tissue Culture

Tissue specimens were washed in phosphate-buffered saline (PBS) (NaCl 136.9 mM, KCl 2.7 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 1.5 mM, pH 7.3) and dissected into fragments for culture. A control sample was immediately placed in neutralized formalin for histology. The remainder of the myocardial fragments were placed in multi-well culture plates, 4-5 fragments per well, and covered with 1 ml of Iscove's Modified Dulbecco's Medium (Canadian Life Technoloiges Inc., Burlington, Ontario) with 100 U/ml penicillin and 100 μg/ml streptomycin, 0.1 mmol/L β-mercaptoethanol and supplemented with either 0%, 2%, or 10% fetal bovine serum or 2% adult bovine serum. Tissue was maintained in a humidified environment containing air and 5% carbon dioxide at 37°C. Culture medium was changed every two days.

Tissue collection for histology and molecular biology occurred on days 3, 5, and 7 of culture, while samples for histology were collected up until 21 days of culture. Samples were pooled from multiple wells, washed three times in PBS and placed immediately in either neutralized 10% formalin, for histology, 1% glutaraldehyde, 4% formaldehyde in 0.1 M phosphate buffer for electron microscopy or snap frozen in liquid nitrogen for molecular biological analysis.

GROWTH FACTOR MRNA QUANTIFICATION

RNA Isolation

RNA was extracted from frozen myocardial tissue using TRIzol reagent (BRL-Life Technologies), a monophasic solution of phenol and guanidine isothiocyanate based upon the single-step RNA isolation method developed by Chomczynski and Sacchi[161]. Total RNA was isolated as outlined by the manufacturer. Briefly, frozen myocardium (50 - 100 mg) was powdered under liquid nitrogen and homogenized in 1 ml TRIzol. Chloroform (0.2 ml per ml of reagent) was added, mixed and incubated for 3 minutes at room temperature. Samples were centrifuged at 12,000g for 15 minutes (at 4°C) and the extracted aqueous phase transferred to a new tube. Total RNA was precipitated for 1 hour at -20°C following addition of isopropyl alcohol (0.5 ml per ml of reagent). RNA precipitate was pelleted by centrifugation at 14,000g for 15 minutes and washed once with 75% ethanol (DEPC water). Dry isolated RNA was re-suspended in 15-20 μg DEPC treated H_2O and quantified using spectrophotometry, based upon its absorbence at 260 nm (A_{260}).

Evidence of RNA integrity was demonstrated by: (i) ethidium bromide visualization of sharp, non-degraded, 28S and 18S rRNA upon electrophoretic fractionation of RNA through a denaturing agarose/formaldehyde gel, and (ii) an A_{260}/A_{280} ratio of 1.5-2.0.

First Strand cDNA Synthesis

Total cellular RNA was reverse transcribed by using the Superscript II reverse transcription enzyme (BRL-Canadian Life) and oligo dT(20)[162]. Briefly, RNA (2-4 μg) was

combined with 1 μg oligo dT_{20} and DEPC H_2O (to 12 μl) and heated for 10 minutes at 70°C, cooled on ice, and briefly centrifuged. A mixture of 4 μl 5X RT-reaction buffer (250 mM Tris-HCl (pH 8.3), 275 mM KCl, 15 mM $MgCl_2$), 2 μl 0.1 mol/L DTT, 1 μl dNTPs (25 mmol/L each of dATP, dCTP, dGTP, dTTP; BRL-Canadian Life) and 200 units Superscript II was added. Samples were incubated at 42°C for 1 hour. The reaction was stopped by incubation at 70°C for 15 minutes and samples were collected by centrifugation and stored at -20°C.

Polymerase Chain Reaction (PCR)

PCR is a highly sensitive technique. Hence, small variations in RNA extraction, and quantification and in reverse transcription reaction efficiency may translate into larger differences in PCR products. Furthermore, the amount of amplified reverse transcription product generated from the same target sequence after the same number of cycles and under identical experimental conditions often differs from one PCR reaction to another, even when using a master mix of reaction components[163,164] To correct for these potential variations, a housekeeping gene is commonly used. The premise for this is that if the housekeeping gene standard mRNA is expressed at the same level in two samples, the ratio of PCR products generated from the target and housekeeping genes should be indicative of the relative level of expression of the target mRNA in those samples[165]. This form of semi-quantitative PCR, when combined with a Bio-Rad gel documentation and densitometric analysis system, as described below, has been shown to be capable of producing results that mirror those

produced by quantitative PCR (Mingyao Liu, University of Toronto, personal communication).

PCR primer sets corresponding to IGF-I and TGF-β1 were designed (Primer Designer, version 1.01) and, along with the housekeeping gene, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (Clontech), were synthesized (ACGT, Toronto, ON). PCR primers were designed to discriminate between genomic and cDNA sequences by selecting primers that span two exons. Thus, amplified contaminating genomic DNA, which contains introns in addition to exons, would appear as larger PCR product. Primer characteristics and their anticipated PCR product size are summarized in Table 2.

Table 2: PCR Primer Sequences of Housekeeping and Growth Factor Genes

cDNA and Primer	Position in Sequence	Sequence of Primer	PCR Product Size
GAPDH[166]			
Forward	584 - 606	5' - TGA CCA CAG TCC ATG CCA TCA CT- 3'	274 bp
Reverse	857 - 579	5' - GAC GCT TCC TTC ACC ACC TTC TT - 3'	
TGF-β1[167]			
Forward	1678 - 1699	5' - GCC CTG GAC ACC AAC TAT TGC T - 3'	161 bp
Reverse	1838 - 1817	5' - AGG CTC CAA ATG TAG GGG CAG G - 3'	
IGF-I[168]			
Forward	349 - 370	5' - TCT TCA GTT CGT GTG TGG AGA C - 3'	181 bp
Reverse	509 - 528	5' - ACA GAG CGA GCT GAC TTG GC - 3'	

The PCR reaction solution (total volume 50 μl) was mixed on ice and contained: 1 μl first-strand cDNA, 5 μl 10X PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl) and 1.5 μl of 50 mmol/L $MgCl_2$ (BRL-Canadian Life), 1 μl of 25 mmol/L dNTP, 1.25 units *Taq* DNA Polymerase (BRL-Canadian Life), 20 nmole primers (GAPDH, IGF-I, or TGFβ1.

Sample were transferred into a thermocycler (GeneAmp 2400, Perkin-Elmer) set to 95°C and the DNA was denatured for 10 minutes. Subsequently, amplification cycles (see below for description of number of cycles) were performed in the following conditions: denaturation (94°C, 30 seconds), annealing (55°C, 30 seconds), and primer extension (72°C, 1 minute). A final incubation at 72°C for 5 minutes was followed by storage at 4°C.

PCR products were subsequently separated using agarose gel electrophoresis, visualized by ethidium bromide staining, photographed and densitometrically uantitated , as described below. For each of the growth factor and the housekeeping genes, PCR reactions were carried out at each of 20, 25, 30, 35, and 40 reaction cycles. The appropriate number of amplification cycles for each gene was determined by densitometric analysis to ensure that the amplified product fell within their respective amplification curves. For all three genes (IGF-I, TGFβ1, and GAPDH), 30 amplification cycles was selected. In addition, PCR products were initially confirmed to correspond to the targeted genes by sequencing (see below). Thenceforth, products were identified by their expectant molecular size (Table 2) when compared to a standard ladder. The GAPDH housekeeping gene was confirmed to be expressed at constant levels between HOCM and non-HOCM tissue and between each time point in culture and *in vivo* control samples (data not shown).

Sequencing of PCR Products

PCR products were confirmed to correspond to the targeted genes by cDNA sequencing, based upon a modified dideoxy chain termination sequencing procedure of Winship[169]. First, PCR products were isolated and purifed. Briefly, PCR products separated

by agarose gel electrophoresis were excised and suspended in 0.5 ml TE buffer by shaking overnight at 37°C. The supernatant was extracted with phenol and chloroform (1:1). DNA was precipitated overnight at -20°C with 1/10 volume of 3 M sodium acetate and 2 volumes of 100% ethanol. Following centrifugation, the DNA pellet was washed once with 70% ethanol, re-spun and dried. DNA was redissolved in 50 µl of H_2O. The amount of purified DNA to be used in the sequencing reaction was estimated by running on an agarose gel. Purified DNA (50 ng) was mixed with approximately 20 pmoles of the respective primers, dissolved in 6 µl annealing buffer (20 mM Tris pH 7.5, 25 mM $MgCl_2$, 50 mM NaCl in 10% DMSO) and annealed at 100°C for 3 minutes. To prevent renaturation, samples were immediately frozen in liquid nitrogen. On ice, 4 µl of reaction mix (1 µl of 0.1 M DTT, 1 µl S^{35} dATP (10µCi, 1000 Ci/mmol), 2 µl of 8:1 diluted Sequenase™, with Enzyme Dilution Buffer from Sequenase™ kit) to the annealing mixture (total volume, 10 ml). After mixing, each reaction was aliquoted (equal parts) into pre-warmed dideoxy mixes (2µl of either ATP, GTP, CTP, or TTP). The reaction was allowed to proceed for 5 minutes at 37°C at which point it was stopped with 5 µl stopping buffer. After being denatured at 100°C for 5 minutes, the sequencing products were fractionated through a 6% acrylamide gel (25 ml of a 46.7% urea solution, 5ml 10X TBE and 20ml 20% acrylamide). The gel was dried overnight and placed with X-ray film (Kodak) for an overnight exposure. Sequenced products were read and compared with the predicted cDNA base sequence.

Densitometric Analysis

The densitometric ratio of growth factors to GAPDH PCR product bands was determined using a computer image densitometer (BioRad Gel Doc 1000) as a semi-quantitative measure of relative gene expression. The ratio of the growth factor to GAPDH products was calculated. For time course studies, given that the samples were collected, cultured and analyzed by at different time points, absolute densitometric ratios cannot be compared because of anticipated inter-reaction variations. Therefore, to compare gene expression trends both within and among the sample groups, growth factor / GAPDH ratios were determined and standardized to day 0, or *in vivo*, values, which were set equal to 1.0. Data was expressed as average values ± SD. In view of the aforementioned limitations to semi-quantitative analysis, for the direct comparison between HOCM and TM myocardial growth factor levels, samples at each time point were analyzed within the same PCR reaction.

PROTEIN ANALYSIS

Protein Quantification

Myocardial tissue was homogenized in 1 ml solution containing 50 mM Tris-HCl (pH 7.4). Following 30 minutes incubation on ice, the homogenate was centrifuged at 14,000 x g for 15 minutes at 4°C. The supernatant was collected and total protein concentration measured using the Bio-Rad protein assay (Bio-Rad, Richmond, CA).

Chemiluminescent Slot Blot

IGF-I and TGF-β1 protein levels were determined using chemiluminescent slot blot analysis.[170] Forty micrograms of protein from HOCM or TM samples was loaded onto a 0.2 μm nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH) using the Minifold II Slot Blotting System (Schleicher & Schuell Inc., Keene, NH). Growth factor standards (IGF-I: 250, 125, 62.5, 31.25ng; TGF-β1: 40, 20, 10, 5ng, Sigma, Mississauga, ON) were loaded on the same membrane to confirm antibody specificity. After drying at room temperature for 10 minutes, subsequent reactions were performed at 37°C. The membrane was washed twice in TTBS buffer (50 mM Tris-HCl buffer, pH 7.4 and 0.1% Tween-20) for 5 minutes. Non-specific membrane binding sites were blocked by 30 minutes treatment with blocking buffer (5% milk, 95% TTBS). Mouse monoclonal antibodies against human IGF-I (Cederlane Laboratories, Hornby, ON) and TGF-β1 (R&D Systems, Minneapolis, MN) (1:3000 and 3:1000 diluted with 0.5 x blocking buffer, respectively) were added and incubated for 30 minutes. Unbound antibody was removed by two washings with TTBS, 5 minutes each, and the membrane was incubated with goat anti-mouse-HRP conjugated antibody (1:3000 dilution, Bio-Rad Laboratories, Hercules, CA) for 30 minutes. The membrane was washed twice with TTBS for 5 minutes each and IGF-I and TGF-β1 proteins were detected by chemiluminescence using the Boehringer Mannheim detection kit (Quebec, Canada). Densitometric analysis of the sample bands was performed with a Bio-Rad image analysis system (Bio-Rad Laboratories, Hercules, CA). Results were standardized to Day 0 *in vivo* protein levels, which were set equal to 1.0. Results are expressed as mean ± SD.

STATISTICS

Data is expressed as mean ± SD. Statistical analysis on time course studies was performed using two-way ANOVA to determine the effects of time and serum concentration on growth factor gene expression in culture within each tissue type (ie HOCM or non-HOCM). Subsequently Student's *t*tests were performed to compare growth factor expression at each time point while controlling for serum concentration and conversely to compare the effect of serum concentration on growth factor expression while controlling for time in culture. Direct comparison studies of growth factor expression between HOCM and non-HOCM myocardium were also analyzed by Student's *t*test. Significance was accepted at the 95% confidence limit, i.e. $p<0.05$ level.

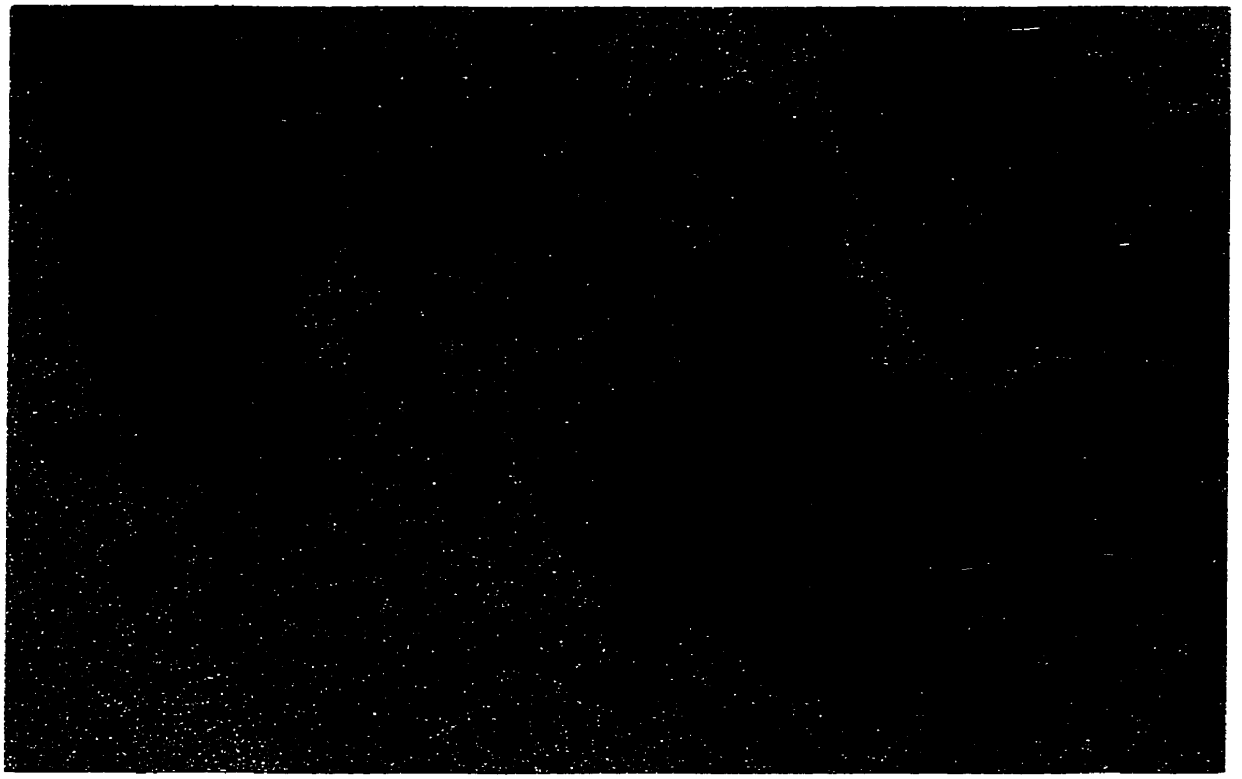
RESULTS

PHENOTYPE OF CULTURED HUMAN MYOCARDIUM

Since experience with cultured human myocardial explants is limited[7], tissue viability, phenotype and gene expression of cultured myocardium needs to be more fully characterized. While there is little concern with the diffusion of nutrients and wastes into and out of cells, respectively, in cell culture, it is of vital importance when culturing tissue fragments. Thus, the first parameter evaluated was the size of cultured explants. Myocardial tissue from explanted adult hearts from patients undergoing heart transplantation (TM) was cultured as fragments of approximately 1 mm^3 to 12 mm^3 for one week and evaluated by histology. As shown in (Figure 1 and Figure 2), cultured myocardium that was approximately 2 mm^3 appeared to survive best, staining strongly with a hemotoxylin and eosin stain and exhibiting normal nuclei. As the size of the cultured explants increased, the tissue survival decreased. While the central tissue area appeared necrosed, staining weakly and displaying few nuclei, areas of surviving myocardium could be identified around the perimeter of the larger explants, illustrating the limits of diffusion in providing appropriate nutrition and removal of wastes in this tissue culture model.

Cardiomyocytes are known to undergo dramatic morphological changes in primary cell culture. Most significantly, the myofibrillar contractile apparatus breaks down in cultured cardiomyocytes. The phenotype of HOCM explants cultured in 2% and 10% fetal bovine serum (FBS) or 2% adult bovine serum (ABS) for up to three weeks was evaluated by light microscopy and, for selected samples, electron microscopy. Cultured tissue continued

to exhibit organized sarcomeres after one week of culture when viewed with both light (Figure 3) and electron (Figure 4, Figure 5, Figure 6) microscopy. However, as seen by electron microscopy, after two and three weeks in culture organized sarcomeres could not be identified (two-week photomicrograph shown in Figure 7).



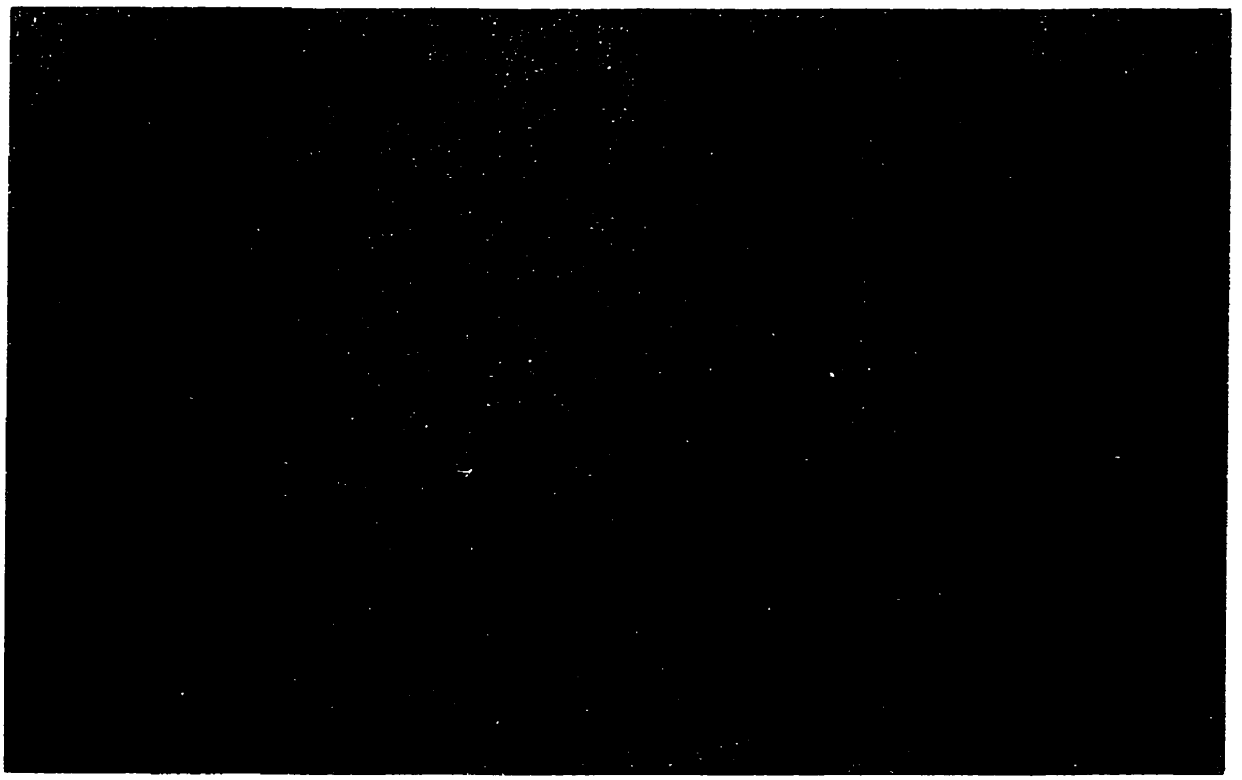


Figure 1: Effect of explant size on survival of human myocardium in culture. Photomicrographs of hemotoxylin and eosin stained hypertrophic obstructive cardiomyopathic explants cultured in 10% fetal bovine serum for seven days; magnification, 40x. A. Myocardium cultured as 2 mm^3 explants survived best staining strongly and homogeneously. B. Myocardium cultured as 12 mm^3 explants appeared necrosed, staining weakly, except for peripheral areas (←). This demonstrates the limitations of diffusion in adequately supporting the nutritional requirements of cultured myocardial explants.

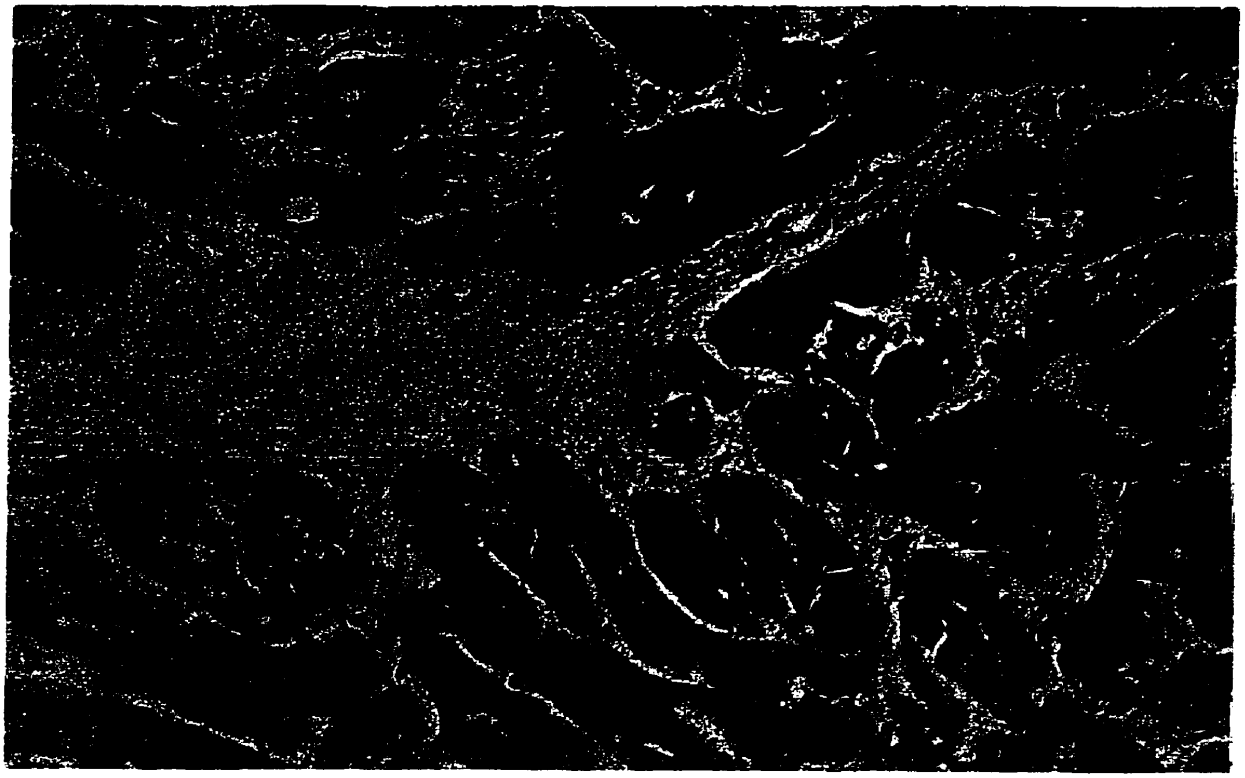
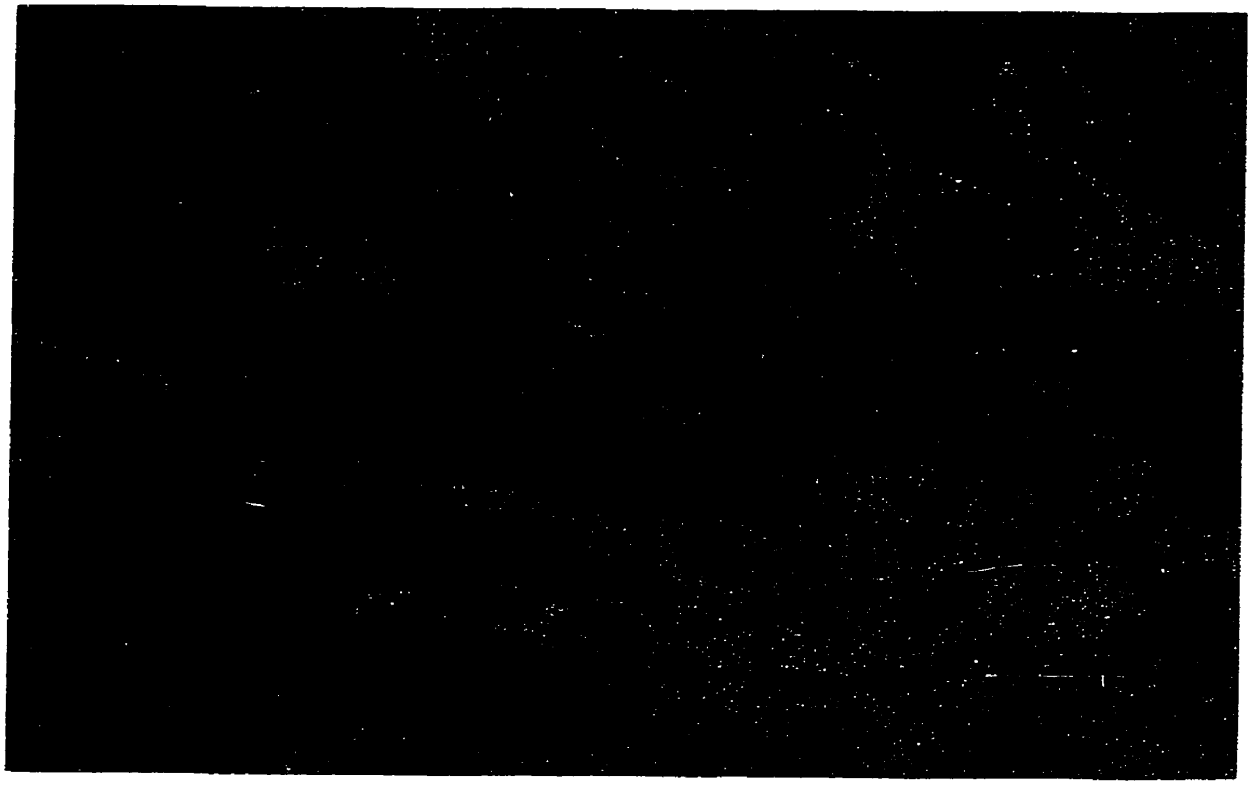


Figure 2: Cardiac phenotype in human myocardium cultured in 10% fetal bovine serum. Photomicrographs of hemotoxylin and eosin stained hypertrophic obstructive cardiomyopathic explants, magnification 200x. Myocardial explants cultured in 10% fetal bovine serum for three (A) and seven (B) days.



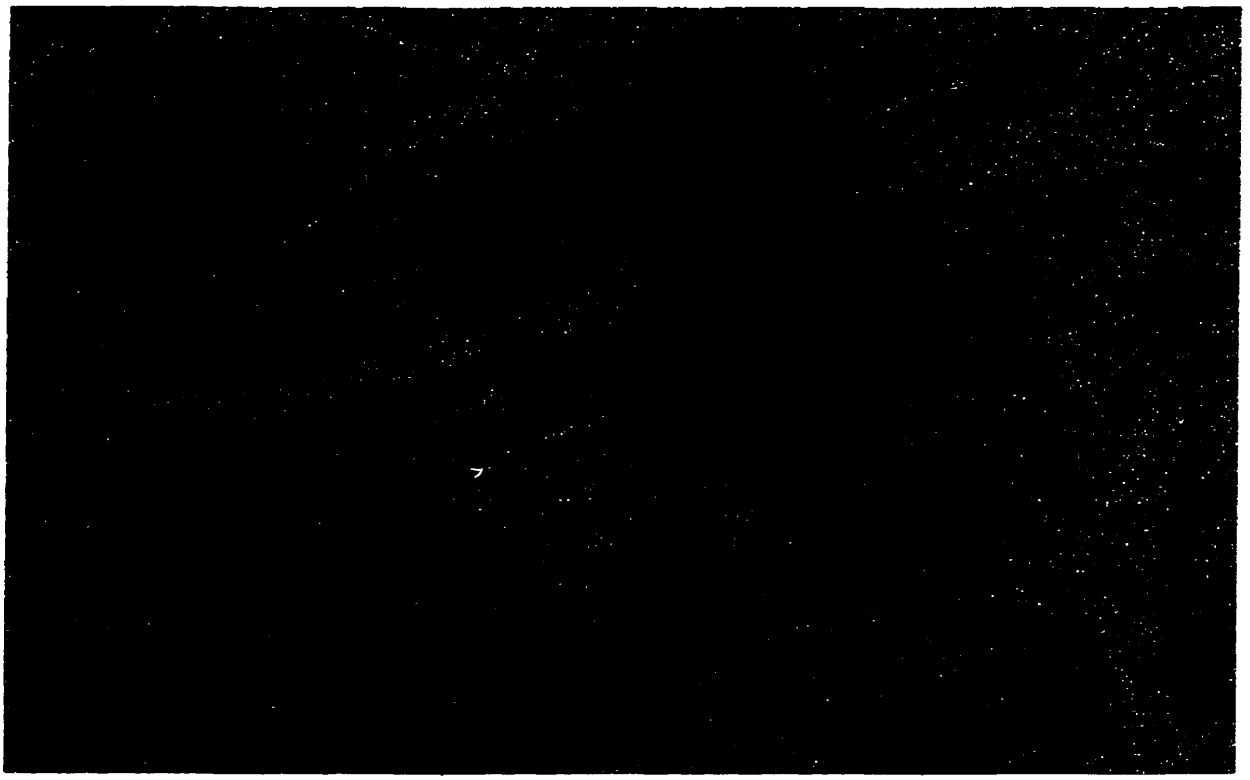


Figure 3: Cardiac phenotype in human myocardium cultured in 2% adult bovine serum. Photomicrographs of hemotoxylin and eosin stained hypertrophic obstructive cardiomyopathic explants, magnification 400x. A. *In vivo* control myocardium (day 0) illustrating normal appearance of myocardial sarcomeres (←). B. Myocardial explants cultured for seven days in 2% adult bovine serum. Cardiomyocytes continue to display organized sarcomeres (←) after seven days of culture.

Figure 4: Electron-micrograph of cardiomyocytes in hypertrophic obstructive cardiomyopathic myocardium, illustrating the myofibrillar sarcomeres characteristic of normal differentiated cardiomyocytes; magnification 35,040x.

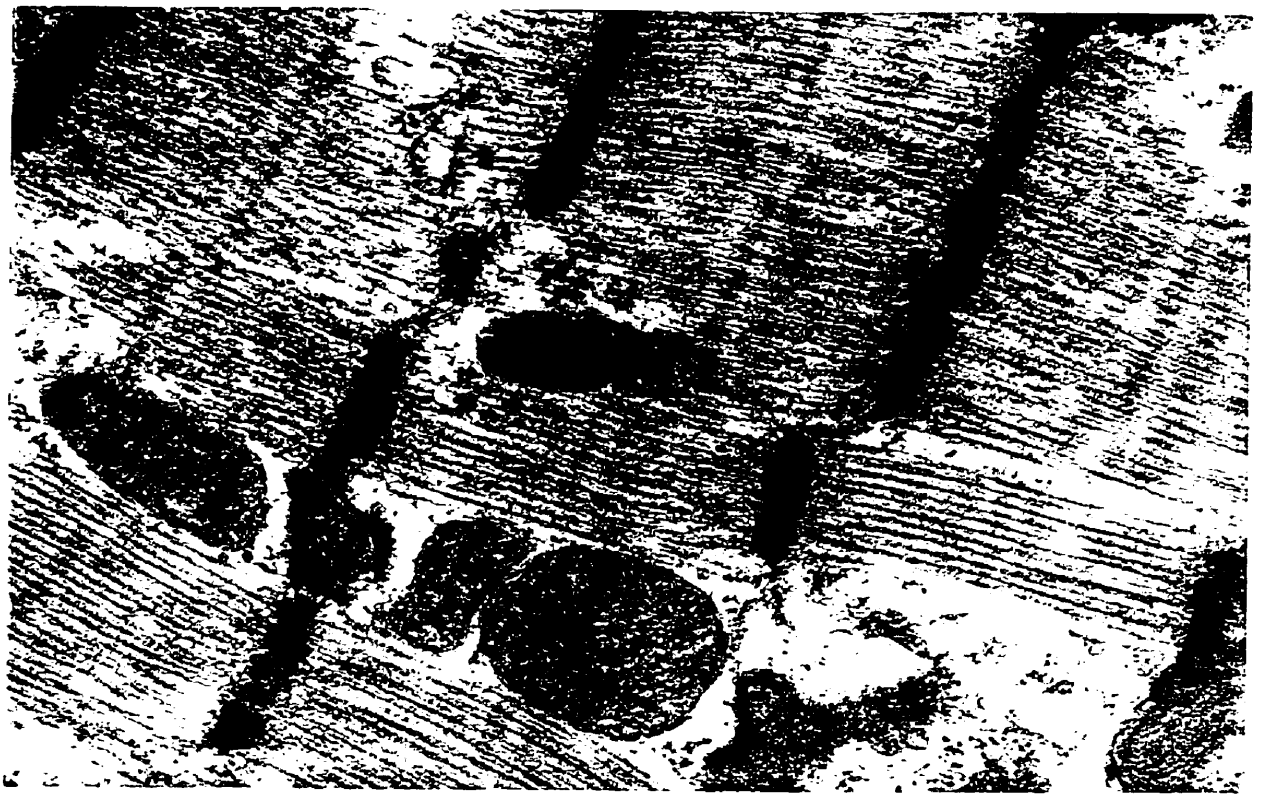


Figure 5: Electron-micrograph of a hypertrophic obstructive cardiomyopathic explant cultured for three days. Cultured myocardial explants continued to exhibit organized sarcomeres after three days of culture in 10% fetal bovine serum; magnification 35,040 x.

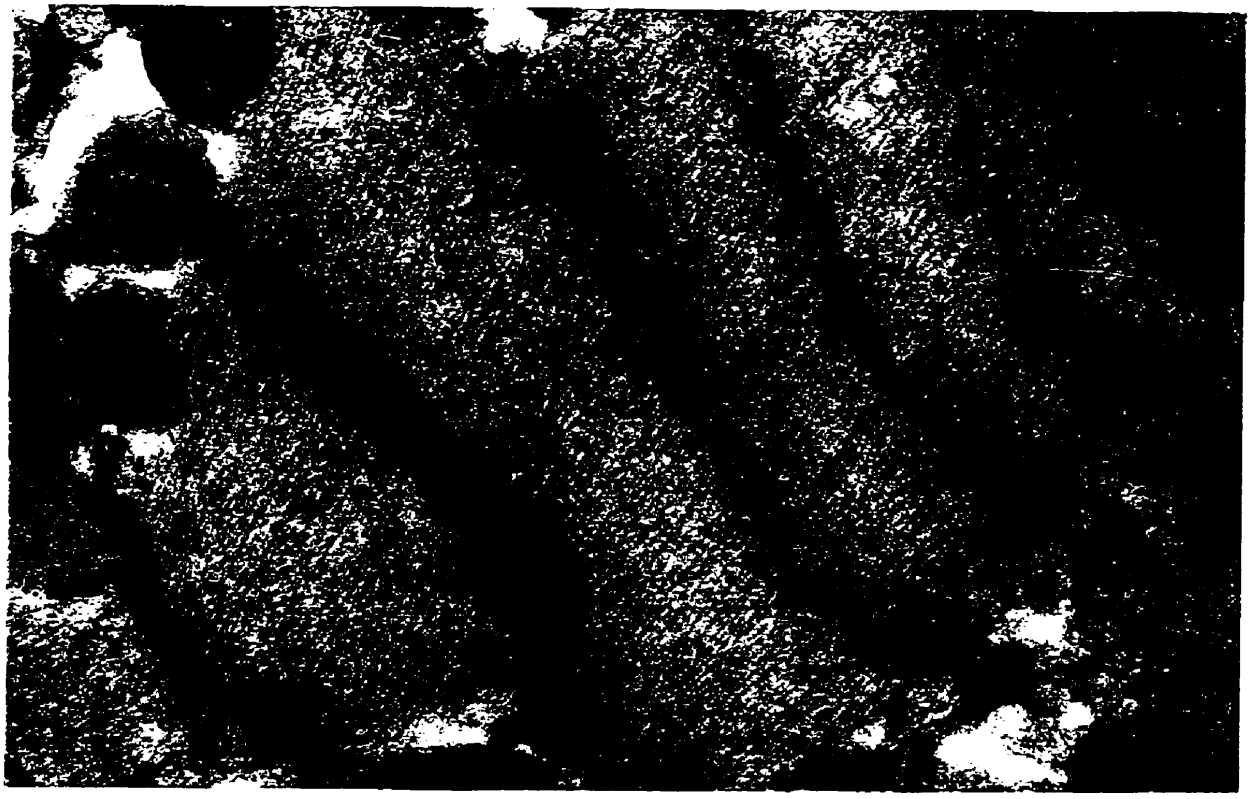


Figure 6: Electron-micrograph of a hypertrophic obstructive cardiomyopathic explant cultured for seven days. Cultured myocardium continued to exhibit organized sarcomeres after seven days of culture in 10% fetal bovine serum, magnification 35,040 x.

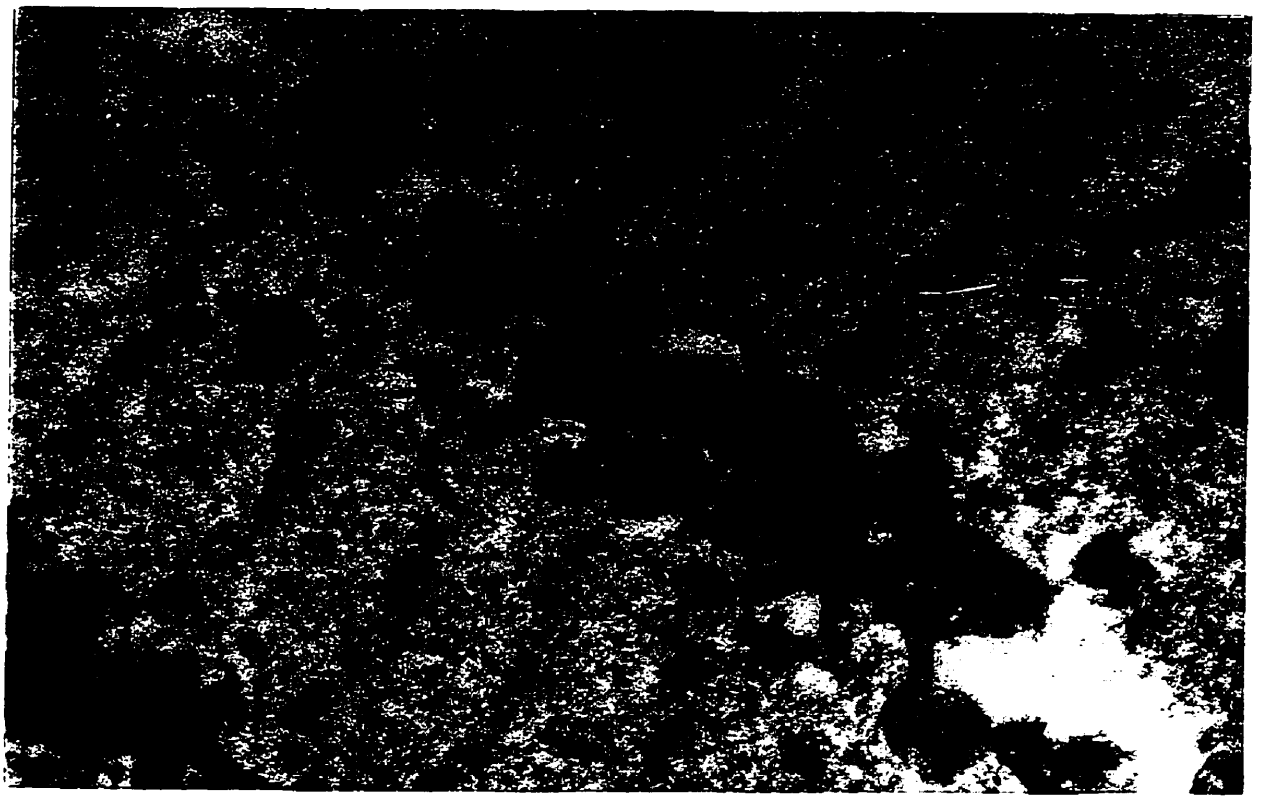


Figure 7: Electron-micrograph of a hypertrophic obstructive cardiomyopathic explant cultured for fourteen days. Organized sarcomeres could not be identified myocardial explants cultured for fourteen days in 10% fetal bovine serum, magnification 35,040 x.

Effects of Serum on IGF-I and TGF-β1 Gene Expression

The effect of serum on myocardial IGF-I and TGFβ1 gene expression was examined by RT-PCR in TM tissue cultured in 0%, 2% and 10% FBS or 2% ABS. Since histology showed that organized sarcomeres disappeared by two weeks of culture, gene expression was evaluated within one week of culture. RT-PCR results are shown in Figure 8. Electrophoretic fractionation of isolated mRNA showed sharp, non-degraded 28S and 18S rRNA bands (data not shown), demonstrating integrity of isolated myocardial RNA. PCR products were observed at the expected sizes upon agarose gel electrophoretic separation (GAPDH, 274bp; IGF-I, 181bp; TGF-β1, 274bp). There was no observable difference in TM IGF-I mRNA levels after seven days of culture in any of the culture environments. In contrast, TGF-β1 mRNA levels appeared to be elevated with increasing serum concentrations. This suggests that a minimally mitogenic culture environment, such as a 2% adult bovine serum supplemented medium, may maintain both IGF-I and TGFβ1 gene expression at *in vivo* levels. Similarly, our observation that serum increases TGFβ1 gene expression correlates with other work showing a serum induced embryonic pattern of gene expression in cultured adult cardiomyocytes and parallels that described secondary to pressure overload *in vivo*. Therefore, a mitogenic culture environment, such as a 10% FBS supplemented medium, may provide an *in vitro* hypertrophic stimulus.

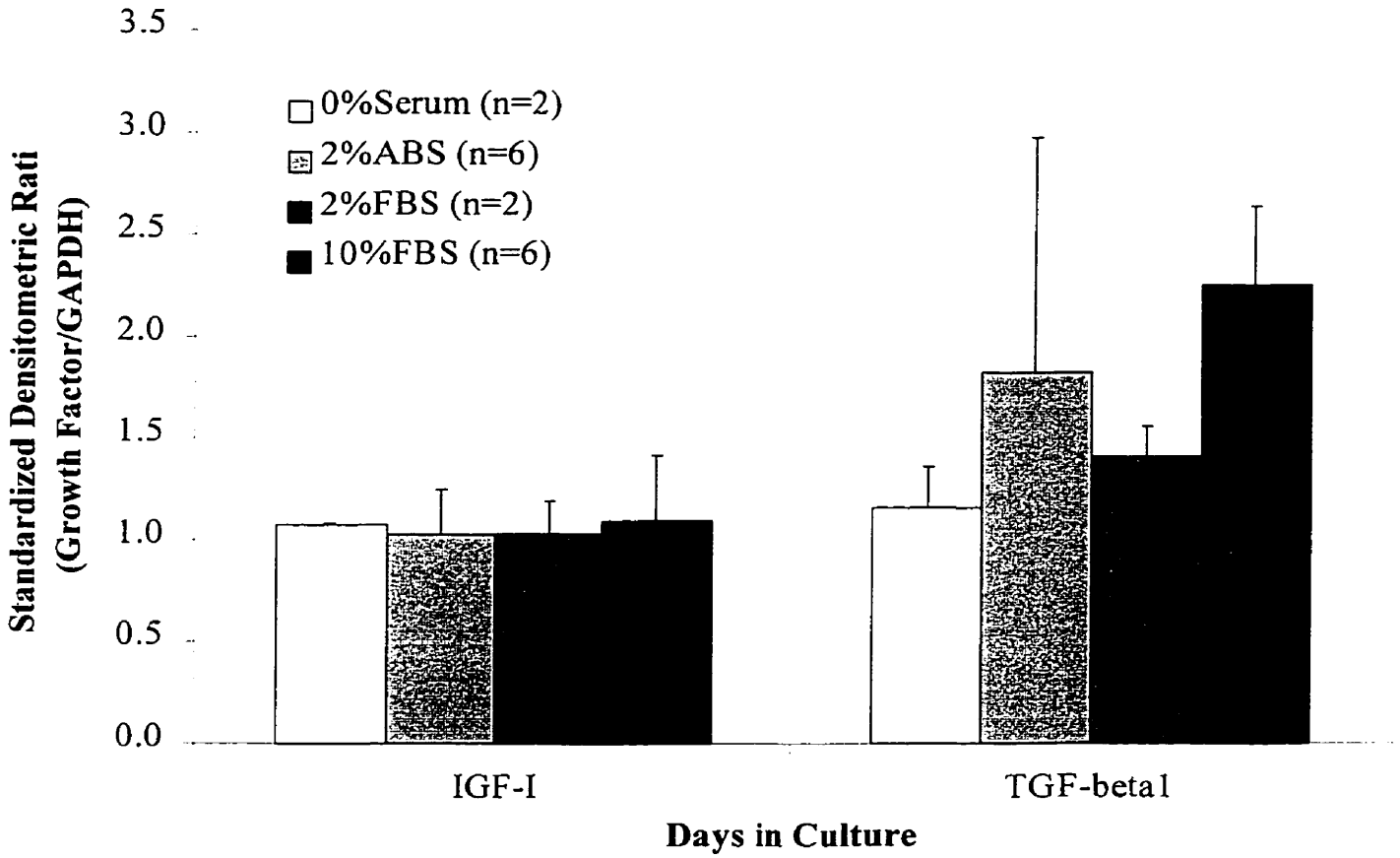

3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0%Serum (n=2)
2%ABS (n=6)
2%FBS (n=2)
10%FBS (n=6)
Standardized Densitometric Rati
(Growth Factor/GAPDH)
IGF-I
TGF-beta1
Days in Culture

Figure 8: Effect of serum concentration and type on IGF-I and TGF-β1 mRNA expression. Myocardium from explanted hearts from patients undergoing heart transplantation was cultured in medium containing 0%, 2% or 10% fetal bovine serum (FBS) or 2% adult bovine serum (ABS) for 7 days. Densitometric quantification of RT-PCR products was expressed as the ratio of growth factor / GAPDH, standardized to day 0 levels, which were set equal to 1.0; mean +/- SD.

GROWTH FACTOR EXPRESSION IN CULTURED NORMAL RAT MYOCARDIUM

Normal, non-diseased rat myocardium was cultured in 2% ABS-supplemented medium and gene expression evaluated after three, five and seven days of culture. In this minimally mitogenic environment, as predicted from our early observations, both IGF-I and TGF-β1 mRNA levels were maintained throughout the culture period relative to their *in vivo* levels (Figure 9).

EVALUATION OF GROWTH FACTOR EXPRESSION IN TM MYOCARDIUM

Having shown the stability of the cardiac phenotype in human myocardium and the maintenance of growth factor gene expression in cultured normal rat myocardial explants for one week, the response of human myocardium in culture over time was evaluated. TM myocardium was cultured in either a minimally mitogenic (2% ABS) or a mitogenic (10% FBS) culture environment and growth factor gene expression was examined after three and seven days of culture compared to *in vivo* control levels. There was no observable difference in gene expression patterns between the minimally mitogenic and mitogenic culture environments. TM IGF-I mRNA levels were maintained, relative to *in vivo* levels, in both culture environments at each time point up to seven days (90.7 ± 35.3% and 112.0 ± 33.8% of *in vivo* levels after seven days of culture in a minimally mitogenic or mitogenic culture environment, respectively; Figure 10B). In contrast, TGF-β1 mRNA levels appeared to increase with time in both the minimally mitogenic and the mitogenic culture environment (193.0 ± 137.3% and 225.4 ± 38.9% respectively after 7 days of culture; Figure 11B). A

preliminary evaluation of growth factor protein levels was conducted in a small sample of TM myocardium cultured in the minimally mitogenic environment. IGF-I protein levels were maintained throughout the one-week culture period (1.218 ± 1.125 at day 7, n=4) as was observed with the mRNA levels. TGF-β1 protein levels, however, on average remained constant during the culture period (1.30 ± 1.01 at day 7, n=4), in contrast to the apparent elevation of mRNA levels. Closer inspection of the individual sample expression patterns revealed a correlation between the mRNA and protein profiles. The samples that demonstrated a maintenance of TGFβ1 protein levels also maintained their mRNA expression levels.

EXAMINATION OF GROWTH FACTOR MRNA LEVELS IN CULTURED HOCM MYOCARDIUM

A host of potentially confounding factors that cannot feasibly be removed *in vivo*, such as hemodynamic load, contraction and sympathetic and parasympathetic stimulation, complicates the investigation of growth factor expression in HOCM. Evaluation of growth factor expression patterns in cultured HOCM explants in the absence of such factors may offer insight into the impetus for the activation of the growth factor signaling pathways that has been demonstrated in HOCM myocardium. HOCM tissue was cultured in either a minimally mitogenic (2% ABS) or a mitogenic (10% FBS) culture medium and growth factor gene expression evaluated after three and seven days of culture (Figure 10A and Figure 11A). Cultured HOCM myocardium displayed a significant decrease in IGF-I mRNA levels after only three days in a minimally mitogenic culture environment (69 ± 15% of *in vivo* levels,

$p<0.05$). This reduction in IGF-I mRNA levels continued throughout the one-week culture period (36 ± 20% of *in vivo* levels after 7 days of culture, $p<0.05$). A preliminary evaluation of HOCM protein levels in myocardium cultured in a minimally mitogenic culture environment revealed a similar decreased in IGF-I levels (35.6 ± 27.7% of *in vivo* levels after 7 days of culture, n=2). Although HOCM IGF-I mRNA levels were also significantly decreased after one week of culture in a mitogenic (10% FBS) environment (64 ± 15% of *in vivo* levels at 7 days of culture, $p<0.05$), there was a partial maintenance of gene expression when compared to levels in the minimally mitogenic environment ($p<0.05$ versus 2% ABS levels after 7 days of cultured). As was observed in cultured TM myocardium, HOCM TGF-β1 mRNA levels appeared to increase in both the minimally mitogenic and mitogenic environments (300.8 ± 226.8% and 143 ± 116.6% respectively after 7 days of culture); for the protein levels evaluated in sample cultured in the minimally mitogenic medium correlated with their respective mRNA levels (323% ± 303 at 7 days of culture, n=2). The clearly observable increases did not achieve statistical significance due to the high degree of inter-sample variation; some samples only displayed modest increases in TGFβ1 expression, while others exhibited a dramatic up-regulation of gene expression - up to 7 fold. In addition, the mitogenic culture environment appeared to suppress the up-regulation of TGFβ1 mRNA levels that occurred with time in culture.

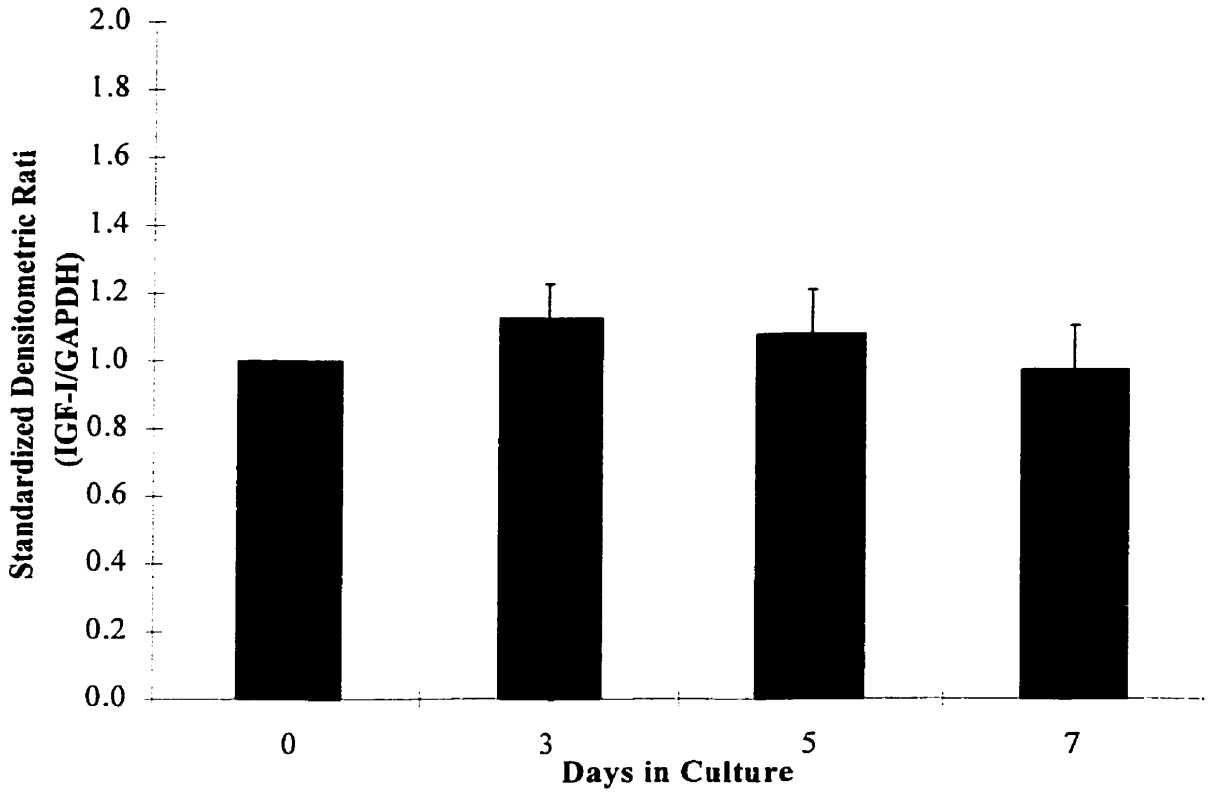
Direct Comparison of Growth Factor mRNA Levels in HOCM versus TM Myocardium

A direct comparison of growth factor mRNA levels between HOCM and TM myocardium cultured in a minimally mitogenic environment (2% ABS) was performed to determine the relative expression of IGF-I and TGFβ1 in the patient populations used throughout this study. HOCM IGF-I and TGF-β1 mRNA levels were found to be 2.79 and 1.67 times higher, respectively, than TM levels ($p<0.01$, each; Figure 12A and Figure 13A, respectively), which agrees with earlier findings[8].

The results presented above suggested a differential pattern of gene expression with time in culture in TM versus HOCM myocardium. However, inherent variations between different PCR reactions do not permit a direct comparison of TM versus HOCM gene expression at each specific time point in culture. Since this is necessary to determine if the over-expression of growth factors in HOCM myocardium persists in culture, PCR was performed on HOCM and TM samples simultaneously at each time point. IGF-I mRNA levels remained significantly elevated at both 3 and 7 days of culture in HOCM versus TM fragments (1.90 and 1.56 times, Figure 12B and C, respectively, $p<0.05$ for both), although the magnitude of the difference appeared to decrease from that observed *in vivo*. This does correlate, however, with the observed trend of decreased IGF-I mRNA levels in HOCM myocardium over the one-week culture period presented in the previous section. The expected IGF-I mRNA expression ratio (HOCM/TM) based upon these trends are 1.90 and 1.30 at days 3 and 7 respectively, which favourably compares with the directly calculated 1.90 and 1.56 ratios.

Conversely, cultured HOCM myocardium still expressed elevated TGF-β1 mRNA levels after 3 days in culture ($p<0.05$, Figure 13B), however, this phenomenon disappears by day 7 (Figure 13C). This likely occurs as a result of the increases in TGF-β1 gene expression in both cultured HOCM and TM myocardium, which in some cases, is dramatic (greater than seven fold).

A


Standardized Densitometric Rati
(IGF-I/GAPDH)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
3
5
7
Days in Culture

B

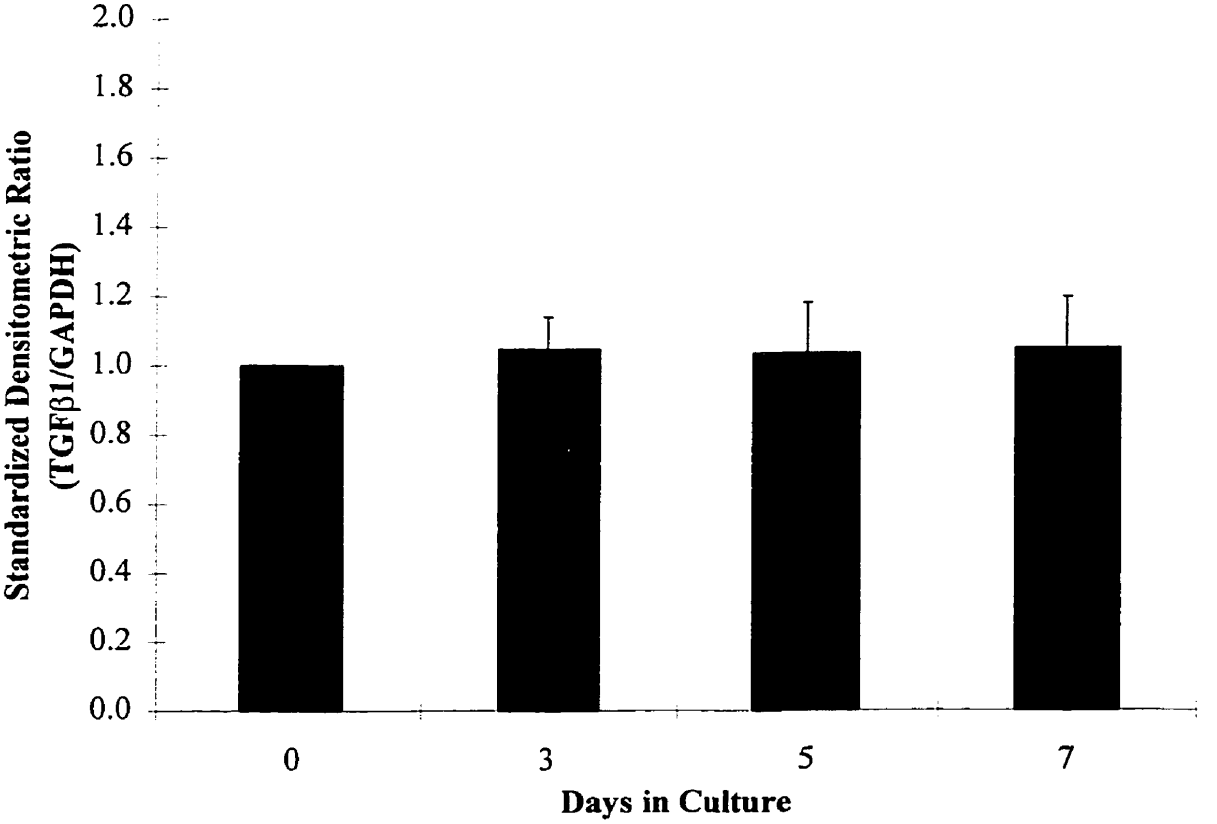
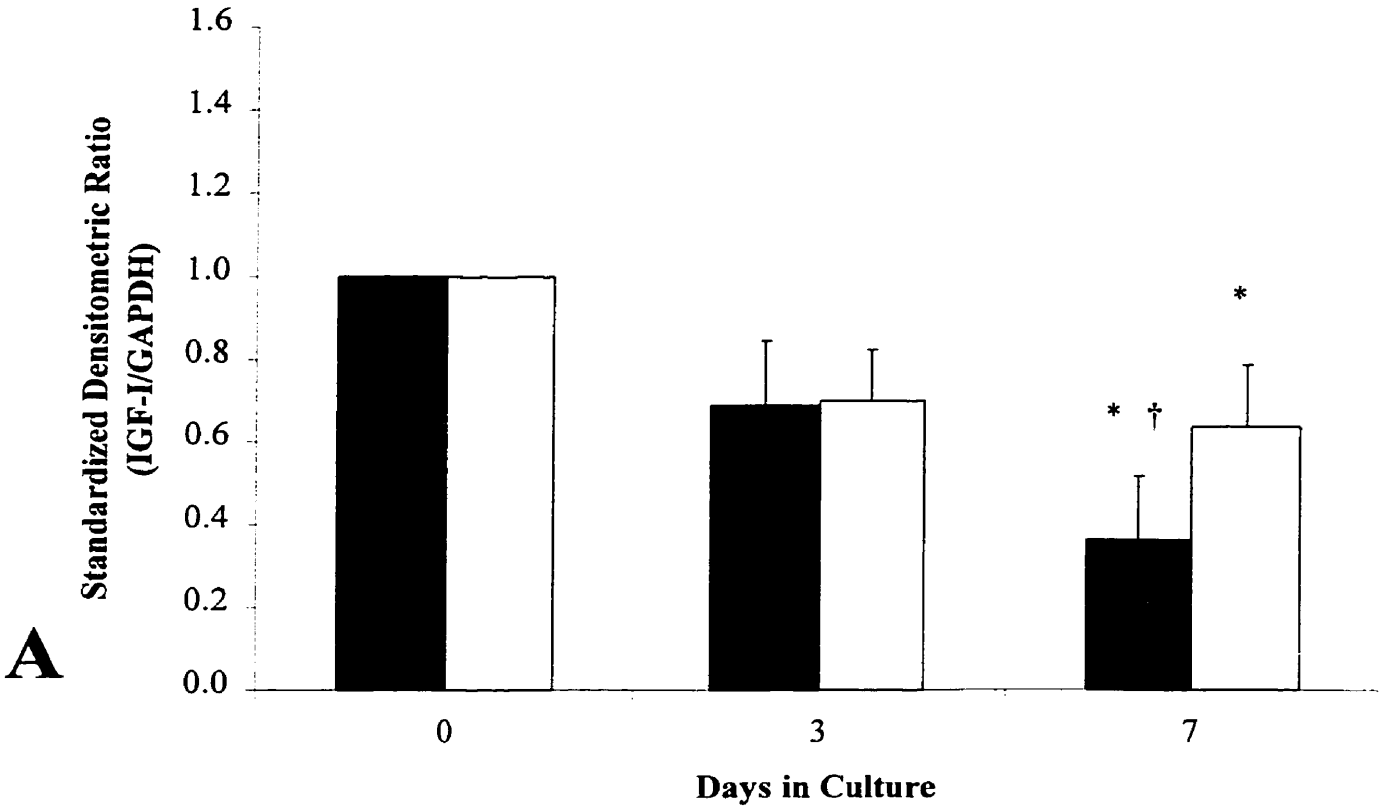

Standardized Densitometric Ratio
(TGFβ1/GAPDH)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
3
5
7
Days in Culture

Figure 9: Growth factor gene expression in cultured normal rat myocardium. Normal rat myocardium was cultured in 2% adult bovine serum-supplemented medium and mRNA levels of IGF-I (A) and TGFβ1 (B) were evaluated at 0, 3, 5 and 7 days of culture by semi-quantitative RT-PCR. Data is expressed as the densitometric ratio of growth factor / GAPDH, standardized to day 0 levels, which were set equal to 1.0; mean +/- SD, n=6.


A
Standardized Densitometric Ratio
(IGF-I/GAPDH)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
* †
*
0
3
7
Days in Culture

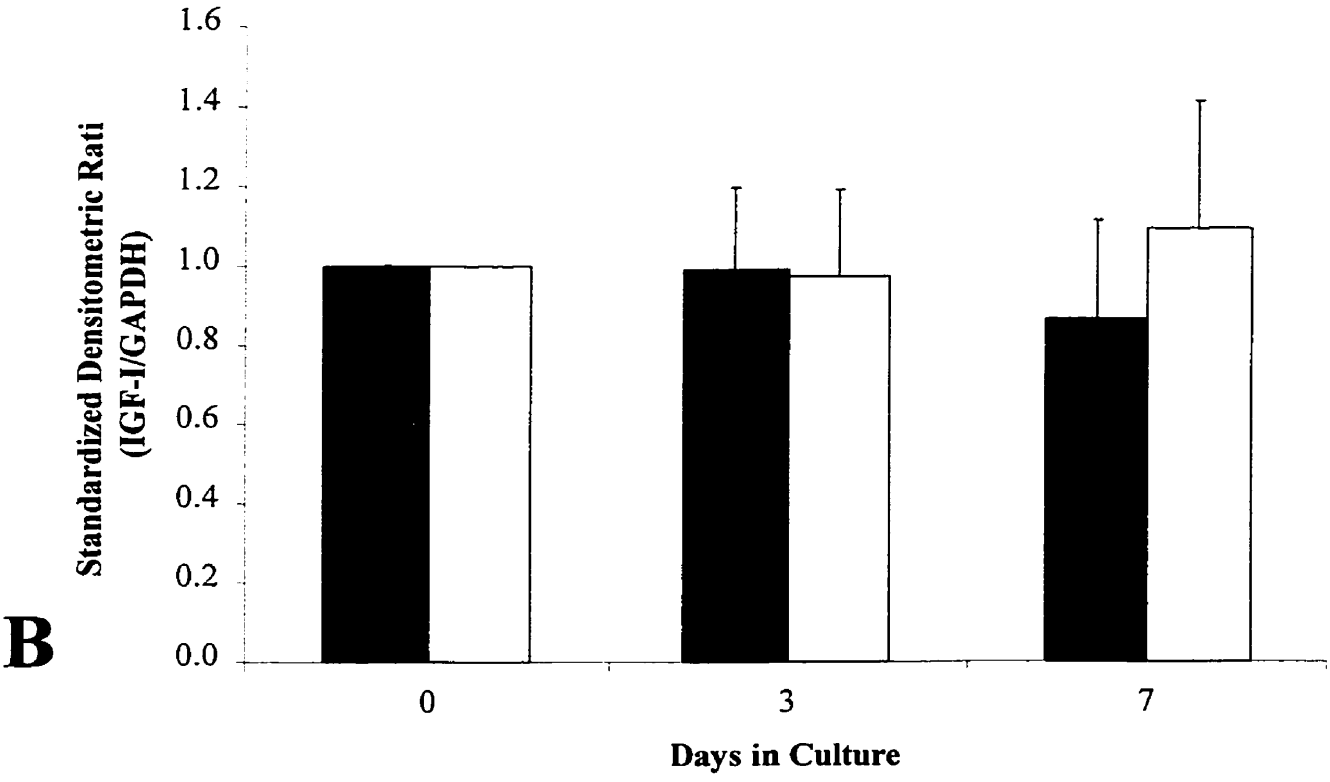
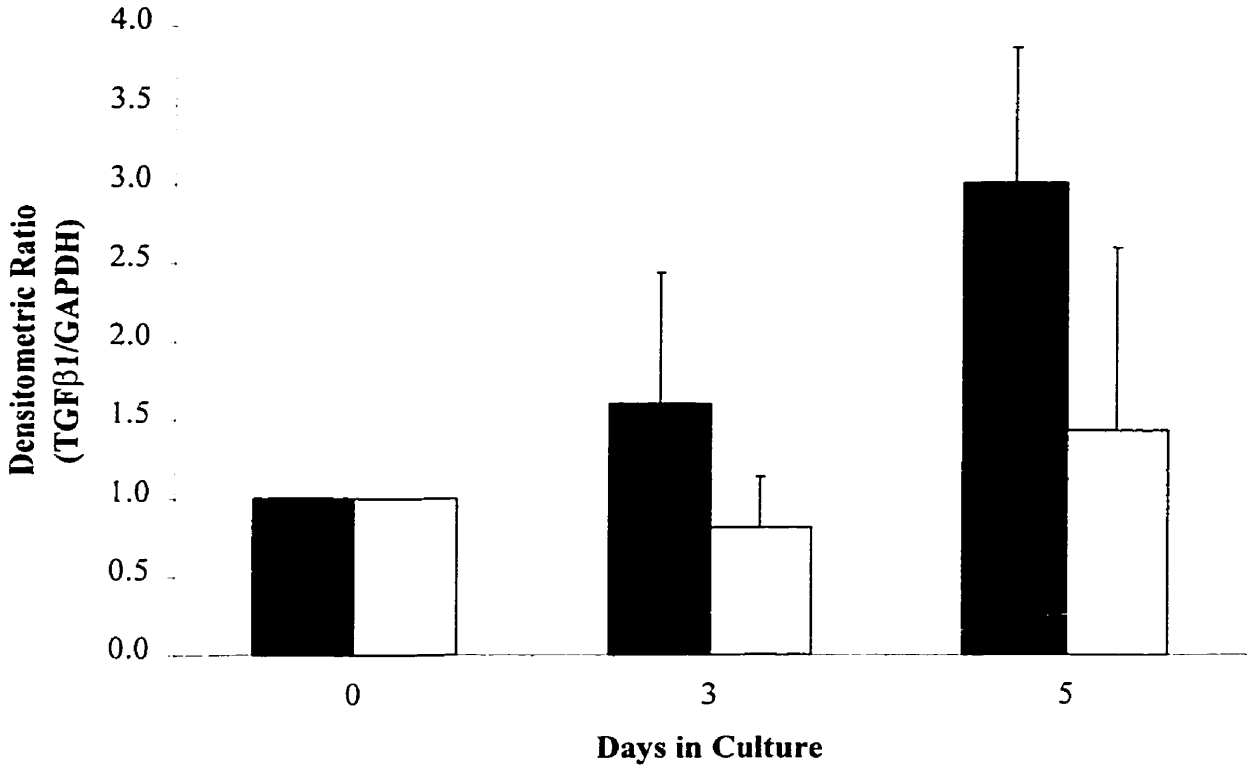

B
Standardized Densitometric Rati
(IGF-I/GAPDH)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
3
7
Days in Culture

Figure 10: IGF-I gene expression in cultured human myocardium. Hypertrophic obstructive cardiomyopathic myocardium (HOCM) (A) and that from explanted hearts from patients undergoing heart transplantation (TM) (B) was cultured in either a minimally mitogenic (2% adult bovine serum; black bars) or a mitogenic culture environment (10% fetal bovine serum, white bars). IGF-I mRNA levels were evaluated by semi-quantitative RT-PCR at days 0, 3 and 7 of culture. Data is expressed as the densitometric ratio of IGF-I / GAPDH, standardized to day 0 levels, which were set equal to 1.0; mean +/- SD; HOCM, n=7; TM, n=6; * $p<0.05$ versus day 0 (by two-way ANOVA), † $p<0.05$ versus 10% FBS at day 7 (Student's ttest).

A


Densitometric Ratio
(TGFβ1/GAPDH)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
3
5
Days in Culture

B

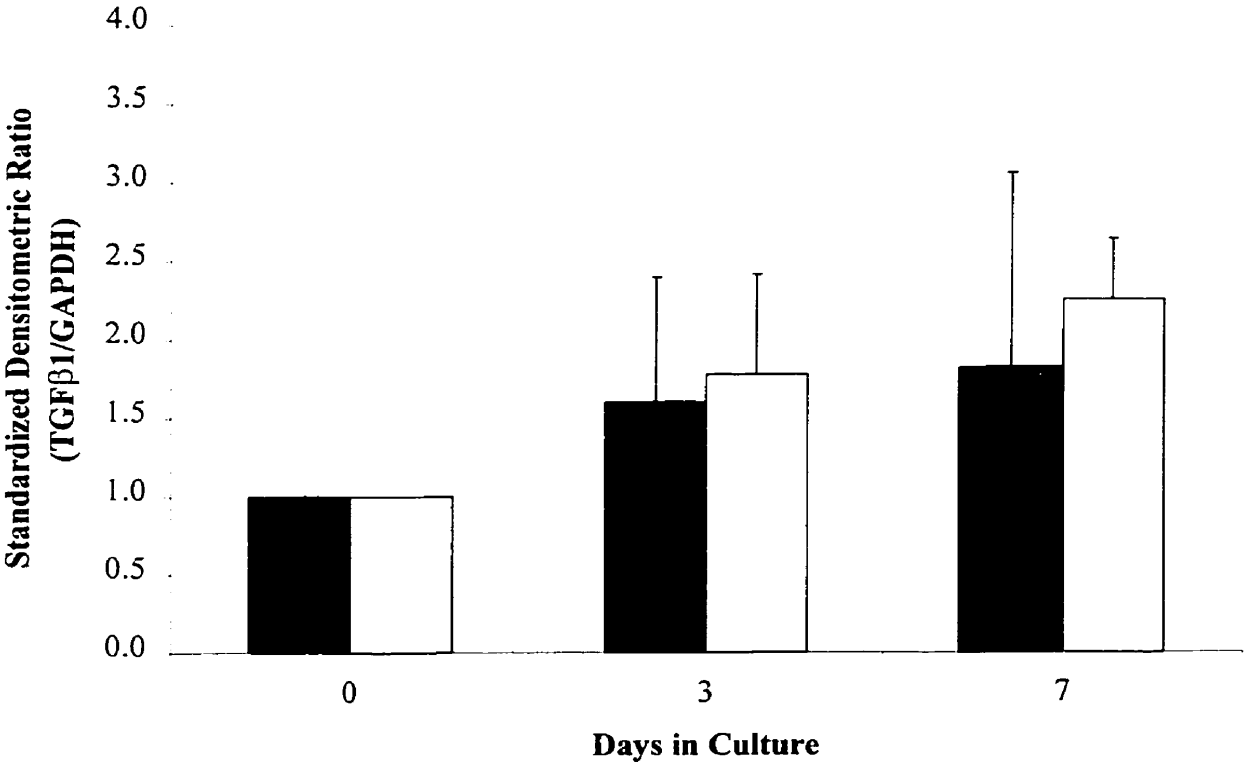
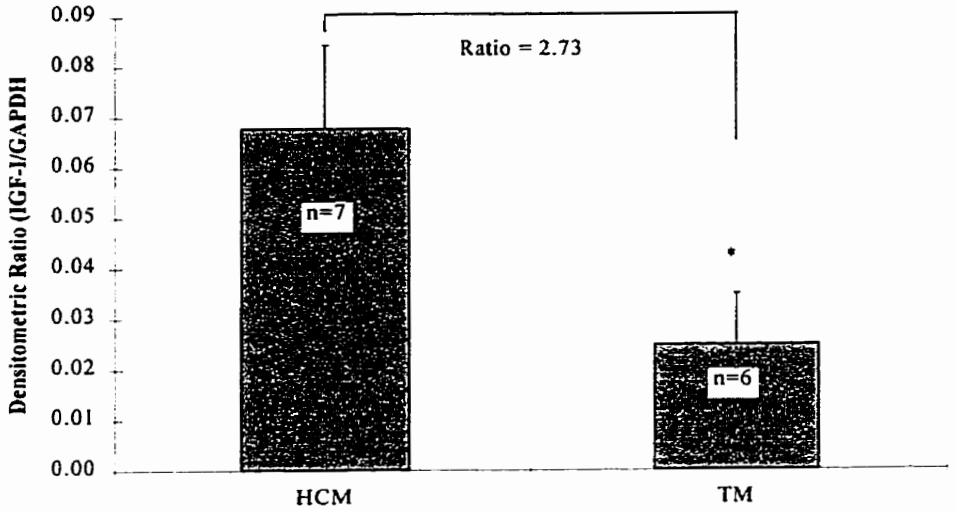
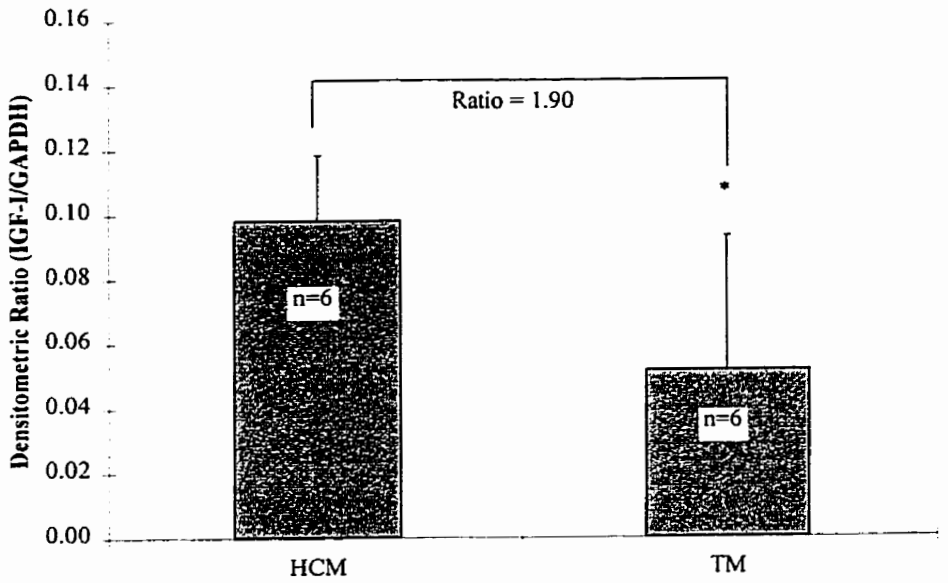

Standardized Densitometric Ratio
(TGFβ1/GAPDH)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
3
7
Days in Culture

Figure 11: TGFβ1 gene expression in cultured human myocardium. Hypertrophic obstructive cardiomyopathic (HOCM) myocardium (A) and that from explanted hearts from patients undergoing heart transplantation (TM) (B) was cultured in either a minimally mitogenic (2% adult bovine serum, black bars) or a mitogenic culture environment (10% fetal bovine serum; white bars). TGFβ1 mRNA levels were evaluated by semi-quantitative RT-PCR at days 0, 3 and 7 of culture. Data is expressed as the densitometric ratio TGFβ1 / GAPDH, standardized to day 0, mean ± SD; HOCM, n=7; TM, n=6; no significant differences (by two-way ANOVA and Student's *t*test).

A


Densitometric Ratio (IGF-1/GAPDH)
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
Ratio = 2.73
n=7
n=6
HCM
TM

B


Densitometric Ratio (IGF-1/GAPDH)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Ratio = 1.90
n=6
n=6
HCM
TM

C

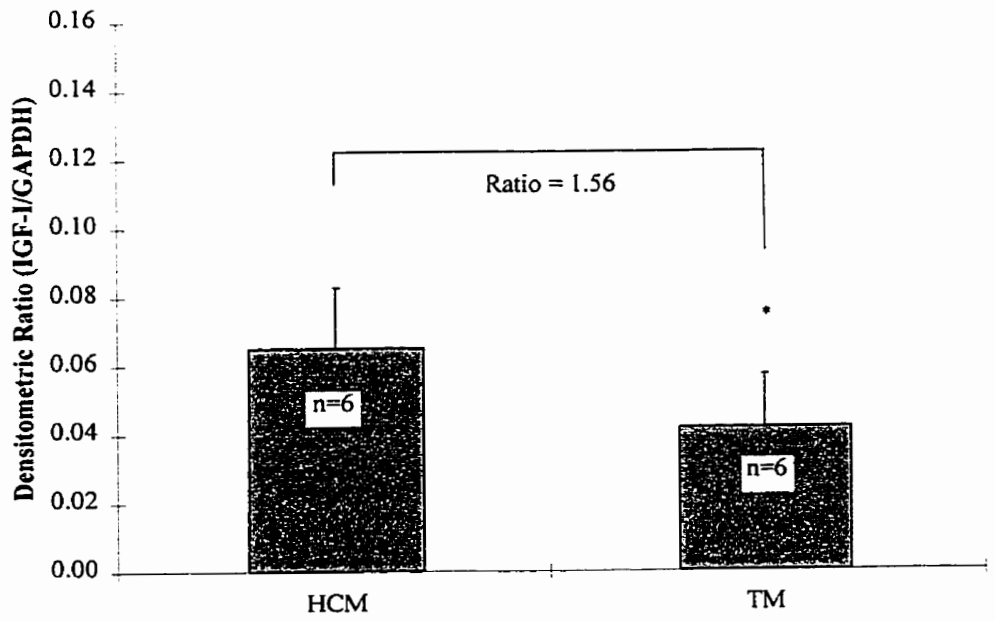
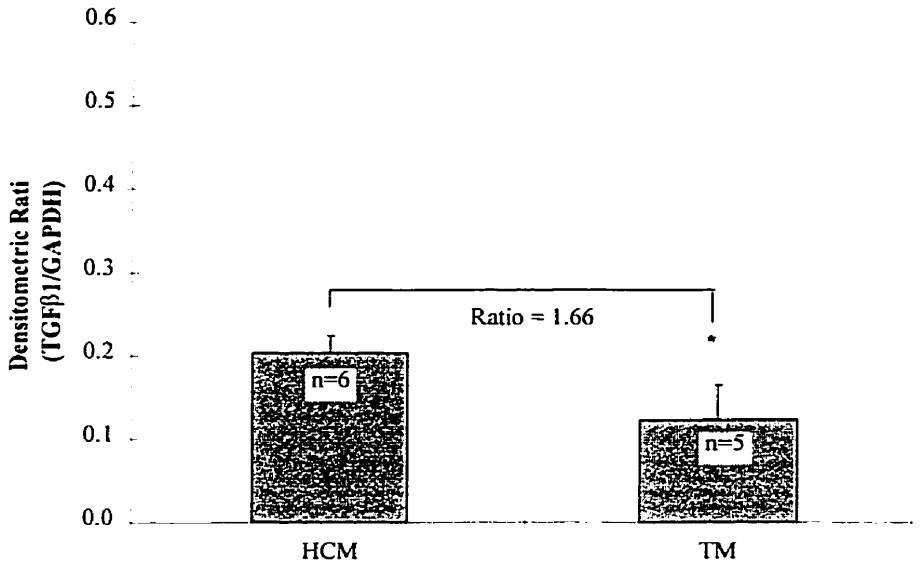
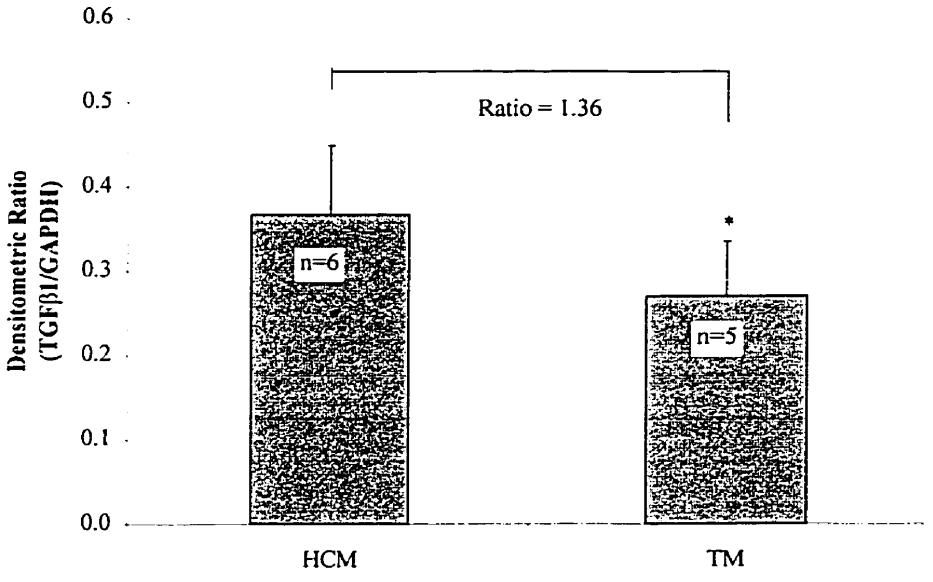

Densitometric Ratio (IGF-1/GAPDH)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Ratio = 1.56
n=6
n=6
HCM
TM

Figure 12: Relative IGF-I gene expression in hypertrophic obstructive cardiomyopathic (HOCM) versus non-HOCM control myocardium. HOCM myocardium and that from explanted hearts from patients undergoing heart transplantation was cultured in 2% adult bovine serum. IGF-I mRNA levels were evaluated by semi-quantitative RT-PCR at each time point: A. *in vivo*, B. after 3 days of culture and C. after 7 days of culture. Data is expressed as densitometric ratio of IGF-I / GAPDH; mean ± SD, n=6 each; * $p<0.05$ versus HOCM (Student's *t*test). The ratio of HOCM/non-HOCM IGF-I gene expression is also shown.

A


Densitometric Rati
(TGFβ1/GAPDH)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Ratio = 1.66
*
n=6
n=5
HCM
TM

B


Densitometric Ratio
(TGFβ1/GAPDH)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Ratio = 1.36
*
n=6
n=5
HCM
TM

C

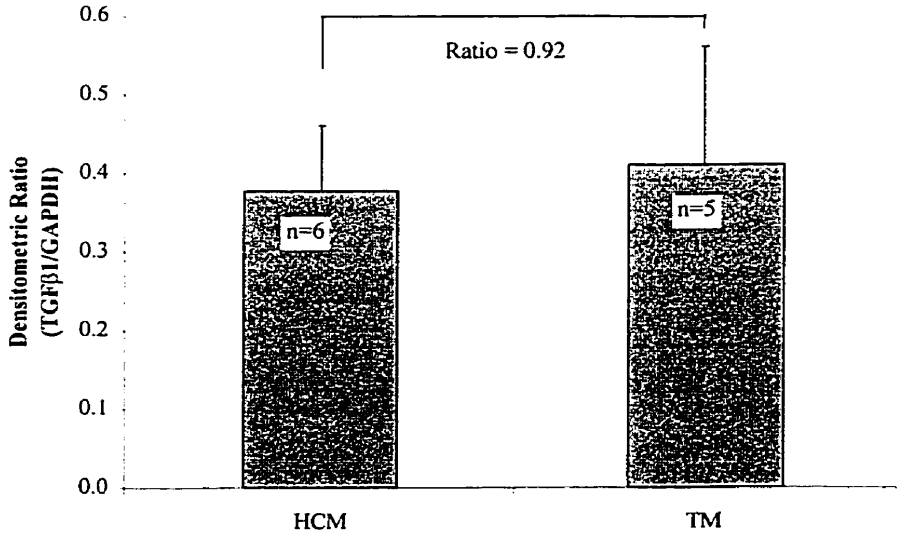

Densitometric Ratio
(TGFβ1/GAPDH)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Ratio = 0.92
n=6
n=5
HCM
TM

Figure 13: Relative TGF-β1 gene expression in hypertrophic obstructive cardiomyopathic (HOCM) versus non-HOCM control myocardium. HOCM myocardium and that from explanted hearts from patients undergoing heart transplantation was cultured in 2% adult bovine serum. TGF-β1 mRNA levels were evaluated by semi-quantitative RT-PCR at each time point: A. *in vivo*, B. after 3 days of culture and C. after 7 days of culture. Data is expressed as densitometric ratio of TGFβ1 / GAPDH; mean ± SD, n=6 each; * $p<0.05$ versus HOCM (Student's *t*test). The ratio of HOCM/non-HOCM IGF-I gene expression is also shown.

DISCUSSION

The significance of studying cardiomyocytes is intuitively connected to their contractile function in the heart. Primary culture of cardiomyocytes has permitted the elucidation of many of their molecular signaling pathways and their role in cardiac differentiation, development and growth. This technology has also produced important advances in understanding the molecular mechanisms involved in a variety of pathological conditions, such as ischemia-reperfusion injury[171] and the role of growth factors in cardiac hypertrophy[5,89,92,93]

Cardiomyocyte cell cultures, however, are limited in their ability to accurately reflect the *in vivo* environment. In culture, adult ventricular cardiomyocytes undergo a remodeling from the elongated *in vivo* structure to a spheroidal flat shape[1-4] and, most significantly, suffer a breakdown of their organized sarcomeres within the first 1-3 days in culture[4]. This loss of myofibrillar structures occurs despite the relatively long half-life of myosin, 5.5 days *in vivo*[172]. These structural changes have also been reported in cultured cardiomyocytes reported to remain highly differentiated, maintaining expression of the adult α-MHC isoform for 7 days[4]. Two possible explanations have been proposed for the changes observed in cultured cardiomyocytes: (i) the cells are released from strict *in vivo* three-dimensional interactions with both myocytes and non-myocytes and (ii) they are removed from an environment requiring a significant amount of work due to the hemodynamic load placed on the heart.[4] The influence of non-cardiomyocyte derived extracellular factors on a cardiomyocyte's properties has been demonstrated in other models, for example, cardiomyocytes lose their *in vitro* proliferative ability following transplantation into the

subcutaneous tissue of a rat[173]. Given that non-myocytes represent the majority of total cardiac cells[6,174], the importance of non-myocytes in normal and pathological cardiac function cannot be ignored. Cardiac fibroblasts, which comprise approximately 90% of the non-myocyte cell population[160], have the potential to dramatically alter the cardiac phenotype because they produce the extracellular matrix (ECM) proteins that form the scaffolding supporting the cardiomyocytes[160]. Therefore, if an investigation of integrated function, or dysfunction, of the myocardium is the goal, an *in vitro* model should reflect *in vivo* myocardial characteristics: cardiomyocytes, with their normal differentiated myofibrillar structures, in the presence of non-myocytes and the extracellular matrix.

CONDITIONS FOR MYOCARDIAL TISSUE CULTURE

Myocardial tissue culture maintains tissue architecture and cell-to-cell interactions between myocytes and non-myocytes and thus more closely approximates the *in vivo* myocardium. However, little work has been reported demonstrating the viability of myocardial tissue culture. Ationu et al[7] have shown that ventricular explants continue to synthesize IGF-I for nine days when cultured in 10% fetal calf serum. This thesis, in addition to similarly demonstrating the maintenance of IGF-I expression in cultured myocardial explants, extends previous work by characterizing (i) the explant size, (ii) the phenotype of explants cultured for up to three weeks and (iii) evaluating the effects of serum concentration on both IGF-I and TGFβ1 gene expression. After establishing the conditions

for myocardial tissue culture, this model was used to compare growth factor expression in cultured HOCM myocardium with that in non-HOCM myocardium.

We have shown that culture of adult human myocardial explants in a serum-supplemented environment is a viable *in vitro* model. The first achievement was demonstrating the explant size that maintained tissue viability in culture. As the tissue size increased, the central myocardium appeared necrosed while the periphery survived throughout one week of culture. This clearly demonstrated the reliance of cultured myocardium on diffusion to provide nutrients and remove wastes. Explants approximately 2 mm^3 survived best in culture and were therefore used as the standard for our myocardial tissue culture model. The most significant finding of this study is that adult myocardial explants not only survive in culture but continue to display organized sarcomeres for one week. This is a dramatic improvement over cell culture models, which only exhibit sarcomeres in adult cardiomyocytes for the first 1-3 days in culture[4]. Simpson et al[156] have identified the presence of cross-striations (sarcomeres) as an important indicator of a cardiomyocyte's differentiated phenotype. However, organized sarcomeres disappeared by two weeks of culture. This study supports the supposition that by maintaining tissue architecture and cell-to-cell interactions, short-term culture of myocardium should preserve the *in vivo* cardiomyocyte phenotype.

During the culture period, cells were observed to migrate away from the myocardial explants on the culture well surface. This occurred at higher rates in the mitogenic 10% FBS medium than in the minimally mitogenic 2% ABS environment. The migratory cell population was minute, compared to the myocardial explant size, and any unattached cells would have been washed away in the PBS washes prior to collection. Although there were

likely more fibroblasts than cardiomyocytes on the well surface, there was no observable difference between the number or composition of cells migrating from the HOCM versus the TM explants. Therefore, this factor would have been unlikely to affect the phenotype or gene expression of the myocardial explants in this tissue culture model.

Although serum is known to affect the gene expression of other hypertrophic markers in cultured cardiomyocytes, it has a differential effect on growth factor gene expression in this tissue culture model. Serum contains a variety of stimulatory factors and hormones and has been shown to be both a mitogenic and a hypertrophic stimulus in cultured cardiomyocytes. Adult rat cardiomyocytes cultured in 10% adult horse serum exhibit limited mitosis and division for only one week in culture.[154] In contrast to this minimally mitogenic stimulus, 10% fetal bovine serum is strongly mitogenic, permitting adult cardiomyocytes to divide for as long as 6 months[1]. This likely occurs because fetal serum has more growth factors and hormones than adult serum[1]. High concentrations of fetal serum can also dramatically affect gene expression in cultured adult cardiomyocytes, inducing a shift to the fetal β-MHC[3] and an up-regulation of α-skeletal actin[157] and ANF[158], as is observed in cardiac hypertrophy. Having defined the explant size and time period that retained the viability and phenotype of cultured myocardium, growth factor gene expression was evaluated in a range of culture conditions. Since the half-lives of IGF-I[58] and TGFβ1[72] are approximately 12 and 14 hours, respectively, changes in gene expression manifested by the culture environment would be expected to be readily apparent within the defined one week culture period. Utilizing the myocardial tissue culture model characterized in this thesis, a mitogenic culture environment (10% fetal bovine serum) did appear to induce TGF-β1 gene expression in TM

explants cultured for the defined one-week culture period. Surprisingly, IGF-I mRNA levels were maintained, relative to *in vivo levels*, throughout this one-week period in this culture environment. The observed differential pattern of IGF-I versus TGFβ1 gene expression reported in this study is interesting, although not unprecedented. An induction of both IGF-I and TGFβ1 expression occurs secondary to pressure overload but only TGFβ1 gene expression, is elevated in response to a volume overload stimulus[175].

Cell culture has also suggested that adult cardiomyocytes cultured in a minimally mitogenic environment can, in fact, retain some of their differentiated properties, specifically, expression of α-MHC[4]. As was shown in the mitogenic culture medium, TM IGF-I gene expression was maintained, relative to *in vivo* levels, throughout one week of culture in either a serum-free medium or in medium supplemented with 2% fetal bovine serum or 2% adult bovine serum. TM TGFβ1 gene expression also appeared to be maintained in each of these environments. However, in the 2% adult bovine serum only half of the samples maintained TGFβ1 expression levels; the others increased TGFβ1 gene expression. A degree of variability is a reasonable expectation in TM myocardium, given that this group can represent a variety of pathologies, most commonly ischemic or dilated cardiomyopathy. Ideally, base-line gene expression patterns should be determined in cultured normal myocardium. However, due to ethical constraints on obtaining normal human myocardium the evaluation of non-diseased myocardium is limited to animal sources. We found that both IGF-I and TGFβ1 mRNA levels were maintained after seven days of culture, relative to *in vivo* levels, in normal adult rat myocardium cultured in a minimally mitogenic environment (2% adult bovine serum. Therefore, on the basis of IGF-I and TGFβ1 gene expression, culture in a

minimally mitogenic environment appears to represent an environment similar to that found in normal conditions *in vivo*.

GROWTH FACTOR EXPRESSION PATTERNS IN CULTURED HUMAN MYOCARDIUM

The characteristics of pathological cardiac hypertrophy are cardiomyocyte hypertrophy[15] and increased interstitial fibrosis[83,98]. Increasing evidence implicates growth factors as regulators of these processes. Clinical studies have demonstrated a positive correlation between left ventricular mass and IGF-I levels in patients with growth hormone deficiency[90] and in hypertensive patients[91]. In animal models, pressure or volume overload resulting in ventricular hypertrophy is associated with elevated myocardial IGF-I[82] and TGFβ1[65] levels. *In vitro*, both IGF-I and TGFβ1 have been shown to directly stimulate hypertrophy in cardiomyocytes[5,89,92], and to stimulate cardiac fibroblasts to produce extracellular matrix components[96,97].

Investigations into the role of growth factors in cardiac hypertrophy *in vivo* are complicated by the contributions of factors such as contractile and hemodynamic load and circulating cytokines and hormones[24]. Furthermore, since growth factor signaling amongst myocytes and non-myocytes also appears to be an integral component in the hypertrophic response[24], cardiomyocyte cell culture models are limited in their applicability to the *in vivo* scenario. The myocardial tissue culture model characterized in this thesis provides a constant environment that maintains tissue geometry and cell-to-cell interactions. This model will permit the evaluation of growth factor expression in the absence of confounding *in vivo*

factors, such as load, while permitting the manipulation of the culture environment to parallel either a non-hypertrophic or a hypertrophic environment.

A significant finding of this study was that HOCM IGF-I mRNA and protein levels in cultured myocardium decreased compared to their *in vivo* levels. This decrease was significantly greater in the minimally mitogenic environment; the stimulation provided by the 10% FBS partially prevented the decrease in IGF-I gene expression. As was observed in the non-hypertrophic rat myocardium, TM IGF-I mRNA levels were maintained at days three and seven of culture in the minimally mitogenic culture environment; a corresponding maintenance of IGF-I protein expression was also observed in the small sample of cultured TM myocardium evaluated. However, culture in the mitogenic environment similarly maintained TM IGF-I mRNA levels at *in vivo* levels. This observed decreased in HOCM, but not TM, IGF-I gene expression suggest that an *in vivo* factor, not at work in the culture environment, may be partially contributing to the maintenance of expression of this growth factor in HOCM myocardium. Although hypertrophic stimuli can increase IGF-I expression[82] and serum was found to partially prevent the decrease in HOCM levels in this study, the stimulus provided by 10% FBS was not sufficient to provoke IGF-I gene expression in TM myocardium. IGF-I has already been shown to be expressed at relatively low levels in adult TM myocardium[8,9]. It is known that pathological hypertrophy may progress to heart failure. While it is thought that a gradual exhaustion of the heart's ability to synthesize proteins contributes to this process, the particular mechanism for this transition remains to be identified[17]. Therein may lie an explanation for the inability of serum to stimulate IGF-I gene expression in TM myocardium: this tissue has lost the ability to up-regulate IGF-I expression. Since growth factor signaling is an integral component of the

hypertrophic response[24], this would agree with the inability of failing hearts to generate or maintain a compensatory hypertrophic response and could contribute to an understanding of heart failure.

Unlike the differential IGF-I gene expression patterns between HOCM and TM myocardium in culture, both tissue types increased TGFβ1 gene expression throughout the culture period. Intriguingly, TGF-β1 gene expression increased not only in explants cultured in a mitogenic environment but also those cultured in the same minimally mitogenic environment that maintained normal rat growth factors levels. The reason for this is unknown. It would seem logical that it may be linked to the pathology of HOCM and TM hearts. One of the hallmarks of HOCM is an increase in extracellular matrix material[98], and given the pathology of TM myocardium, generally either dilated or ischemic cardiomyopathy, it would presumably also have an increased degree of interstitial fibrosis relative to normal rat myocardium. The majority of basal TGF-β1 mRNA in the adult myocardium is localized in the non-myocytes fraction[75], of which cardiac fibroblasts comprise approximately 90% of cells[160]. Since, cardiac fibroblasts are known to produce TGFβ1[75] and have also been shown to proliferate in response to serum[97], fibroblast proliferation may be the source of increased gene expression of this growth factor. However, given the two to three fold induction of TGFβ1 mRNA an observable increase in fibroblasts would be expected. This was not seen in the histological evaluation of cultured HOCM or TM myocardium. The ability of serum to stimulate existing cardiac fibroblasts to increase their synthesis of TGFβ1 is another possibility. If this were the mechanism of TGFβ1 up-regulation one would expect the mitogenic medium would stimulate more gene expression

than the minimally mitogenic medium. There was no observable difference, however, in TGFβ1 gene expression between the culture environments. In fact, expression of this growth factor in cultured HOCM myocardium appeared to be higher in the minimally mitogenic environment and lower in the mitogenic environment. Since increases in TGF-β1 mRNA levels in response to hypertrophic stimuli are found solely in the cardiomyocytes[176], these cells may be the source of the increased expression of this growth factor. However, the cause and the source of TGFβ1 up-regulation remains to be identified. As mentioned above, another interesting observation was that although, not statistically significant, the mitogenic culture medium appeared to maintain HOCM TGF-β1 mRNA levels closer to *in vivo* levels - in this case, a smaller increase in gene expression was observed than that in the minimally mitogenic environment. Taken together with HOCM IGF-I expression patterns, that a mitogenic environment maintained growth factor expression closer to *in vivo* levels, partially preventing a decrease in IGF-I gene expression while appearing to prevent an opposing increase in TGF-β1 gene expression, this suggests that part of the growth factor over-expression in this disorder may be the result of stimulatory factors found *in vivo* that do not exist in the culture environment.

Direct Comparison of Growth Factor Expression in HOCM versus non-HOCM Myocardium

Mounting evidence suggests that growth factors are involved in the mechanism producing the HOCM phenotype. IGF-I and TGFβ1 mRNA[8], protein[9] and their respective receptors[9,142] are over-expressed in HOCM myocardium compared to that from hypertrophic

myocardium compensatory to load and to non-hypertrophic myocardium. However, an up-regulation of these growth factors has also been shown to accompany hypertrophy compensatory to *in vivo* factors such as load or catecholamines[34,83]. Since such factors cannot be directly removed during *in vivo* experiments, an *in vitro* model is would be invaluable in helping to determine whether the marked increases in growth factor levels are a primary or secondary phenomenon in HOCM.

Myocardial tissue culture allows the investigation of growth factor expression patterns in HOCM myocardium in the absence of *in vivo* factors that may contribute to growth factor expression. The observed changes in gene expression presented above suggest that *in vivo* factors may contribute to growth factor up-regulation in HOCM patients. However, it is of practical interest whether growth factor gene expression in HOCM myocardium remains significantly elevated compared to controls or decreases to levels found in non-HOCM levels. Therefore, a direct comparison of gene expression was conducted in HOCM versus TM tissue. Analysis of *in vivo* control samples confirmed that IGF-I and TGFβ1 mRNA levels were significantly elevated in the HOCM versus TM patient populations used in this study, as has previously been reported[8]. While HOCM IGF-I mRNA was observed to decrease in culture, it remained significantly elevated compared to TM IGF-I levels after one week in a minimally mitogenic environment. The magnitude of the difference between HOCM and TM IGF-I mRNA levels also decreases with time. If the culture period was extended beyond one week, it is possible that the difference between HOCM and TM IGF-I levels may disappear. However, beyond one week there is concern over a change in the phenotype of cultured myocardium. Given the relatively short half life

of IGF-I (12 hours) compared to the one week culture period, a change in gene expression occurring following placement in the culture environment should be readily observable in culture. Since the elevation in IGF-I persists for one week, this suggests that there may be an intrinsic factor in HOCM myocardium predisposing it to over-express IGF-I. In contrast, the over-expression of TGF-β1 in HOCM versus TM myocardium disappeared in culture, despite having a similar half-life (14 hours) and being significantly elevated in HOCM myocardium after three days of culture.

FUTURE DIRECTIONS

This study has demonstrated that culture of myocardial tissue explants maintains their characteristic myofibrillar phenotype for as long as seven days, which has not been achieved in primary cardiomyocyte culture models. In addition, differential patterns of IGF-I and TGFβ1 mRNA expression have been observed in non-HOCM versus HOCM myocardium. This demonstrates the potential utility of myocardial tissue culture model and warrants a more detailed characterization of growth factor expression in cultured myocardial explants.

A change in myocardial mRNA levels is not necessarily indicative of a change in growth factor signaling - a change in functional protein levels is necessary. A preliminary evaluation of IGF-I and TGFβ1 protein levels in cultured myocardium has been reported in this study. However, the small sample sizes evaluated prevented firm conclusions from being reached. A more extensive evaluation of growth factor protein levels is required to make an accurate assessment as to whether protein expression patterns in culture parallel those of their respective mRNA.

To more fully characterize this tissue culture model, along with growth factor gene expression, the expression patterns of other markers of cardiac hypertrophy should be investigated in parallel with growth factor levels over time in culture. ANF would be an appropriate factor to study in human myocardium because it is not found in the normal adult myocardium, yet is re-expressed in cardiac hypertrophy. To further demonstrate the appropriateness of the culture environments used in this study it would be hoped that little or no ANF expression would occur in the minimally mitogenic environment, while an up-regulation would be induced by the mitogenic environment, as has been seen in other studies[158]. As an index of hypertrophy, if HOCM myocardium is intrinsically predisposed to hypertrophy as was seen with IGF-I, ANF expression should remained elevated in HOCM versus control myocardium in culture.

The observation that a mitogenic culture environment partially maintained HOCM growth factor expression at *in vivo* levels is interesting, given the ability of serum to modulate cardiac gene expression in a pattern paralleling that described in hypertrophy. The stimulation provided by the mitogenic 10% FBS medium employed in this model may be less than that found in HOCM patients. Thus, a higher concentration of serum, perhaps 20% FBS, should be evaluated to see if growth factors levels in cultured HOCM explants could be completely maintained relative to *in vivo* levels. This would further increase the utility of tissue culture to evaluate the HOCM phenomenon. This model could then be utilized to refine methods of blocking growth factor signaling in HOCM myocardium, either at the level of the receptors or by interfering with mRNA translation with antisense oligodeoxynucleotide technology, and stop or reverse HOCM hypertrophy.

To further clarify the role of growth factors in the etiology of HOCM, it should be determined if a relationship exists between increased growth factor levels in idiopathic HOCM and sarcomeric gene mutations. The predominant hypothesis is that sarcomeric mutations result in a compensatory hypertrophic response in the HCM heart. However, it remains to be explained why HCM hypertrophic is predominantly regional, rather that global and why individuals within the same family, and therefore carrying identical mutations, exhibit different patterns of left ventricular wall thickening and clinical prognosis. If sarcomeric mutations are found in patients exhibiting growth factor over-expression, it would be interesting to determine if specific mutations are associated with differing levels of growth factor up-regulation in HOCM myocardium. It would be also be of great interest to associate the diversity and occurrence of sarcomeric mutations with the variability of the growth factor expression patterns in culture. HOCM TGFβ1 mRNA levels in some patients only marginally increased while others increased as much as seven-fold, which resulted in high standard deviations and prevented statistical significance from being achieved. Also, one of the HOCM patients evaluated in each culture environment appeared to maintain IGF-I mRNA levels in culture. Given the heterogeneity in the HOCM phenotype such patients may represent a sub-population of patients with a different mechanism that produces growth factor over-expression and, resultantly, HCM hypertrophy completely independent of *in vivo* factors or of sarcomeric mutations. It has already been suggested that other genes regulating myocardial growth may contribute to the HCM phenomenon, as in the increased prevalence of the DD genotype for the angiotensin converting enzyme in association with an increased likelihood of HCM hypertrophy[137,138]. Further investigating the role of growth factors,

already identified as inducers of cardiomyocyte hypertrophy and the production of extracellular matrix by cardiac fibroblasts, in HOCM, and perhaps in other forms of HCM, may significantly contribute to an understanding of the phenotypic heterogeneity in these patients.

SUMMARY

This thesis characterizes the myocardial explant size and culture period that maintained tissue viability and the cardiac phenotype in tissue culture. It also evaluates IGF-I and TGFβ1 expression in HOCM and non-HOCM myocardium cultured in either a minimally mitogenic (2% adult bovine serum) or a mitogenic (10% fetal bovine serum) culture environment. Cardiomyocytes cultured as $2mm^3$ fragments survived best and continued to display organized sarcomeres for up to one week of tissue culture. IGF-I gene expression in non-HOCM myocardium was maintained, relative to *in vivo* levels, independent of the cultured environment. In contrast, TGFβ1 gene expression in non-HOCM myocardium appeared to increase in the mitogenic environment but not in the minimally mitogenic culture medium. Cultured HOCM explants exhibited a decrease in IGF-I gene expression in both the minimally mitogenic and the mitogenic culture environments; this down-regulation was partially prevented by the mitogenic medium. IGF-I mRNA levels remained significantly elevated in culture, however, compared to TM controls. In contrast, the expression of TGFβ1 in both HOCM and TM myocardium increases in culture, although the mitogenic medium appeared to prevent the increase in HOCM explants. Unlike IGF-I gene expression, the elevation of TGFβ1 mRNA levels in HOCM versus TM myocardium disappears in culture. This thesis demonstrates the potential utility of myocardial tissue culture for the evaluation of normal and pathological myocardium in the absence of confounding *in vivo* factors and helps elucidate the role of TGFβ1 and, particularly, IGF-I in HOCM.

REFERENCES

1. Li R-K, Mickle DAG, Weisel RD, Carson S, Omar SA, Tumiati LC, Wilson GJ, Williams WG: Human pediatric and adult ventricular cardiomyocytes in culture:assessment of phenotypic changes with passaging. *Cardiovasc Res* 1996;32:362-373

2. Claycomb WC, Palazzo MC: Culture of the terminally differentiated adult cardiac muscle cell:A light and scanning electron microscope study. *Dev Biol* 1980;80:466-482

3. Smith WR, Claycomb WC: Expression of α- and β- myosin heavy chain genes in the rat ventricular cardiac muscle cells. in Kedes LH, Stockdale FE (eds): *Cellular and molecular biology of muscle development.* Liss, New York, 1989, pp 359-368

4. Bugaisky LB, Zak R: Differentiation of adult cardiac myocytes in cell culture. *Circ Res* 1989;64:493-500

5. Parker TG, Packer SE, Schneider MD: Peptide growth factors can provoke "fetal" contractile protein gene expression in rat cardiac myocytes. *J Clin Invest* 1990;85:507-514

6. Zak R: Development and proliferative capacity of cardiac muscle cells. *Circ Res* 1974;34-35(suppl II):17-25

7. Ationu A, Hassall JS, Moules E, Nicholas DC, Burnstock G, Singer DRJ: Expression of insulin-like growth factor-1 by human ventricular explants in culture. *J Hum Hypertens* 1993;7:407-409

8. Li R-K, Li G, Mickle DAG, Weisel RD, Merante F, Luss H, Rao V, Christakis GT, Williams WG: Over-expression of transforming growth factor beta1 and insulin-like growth factor-I in patients with idiopathic hypertrophic cardiomyopathy. *Circulation* 1997;96:874-881

9. Li R, Li G, Mickle DAG, Weisel RD, Merante F, Ball WT, Christakis GT, Cusimano RJ, Williams WG: Elevated insulin-like growth factor-I and transforming growth factor β1 and their receptors in patients with idiopathic hypertrophic cardiomyopathy: a possible mechanism. *submitted for publication* 1998;

10. Tomita Y, Kusama Y, Seino Y, Munakata K, Kishida H, Hayakawa H: Increased accumulation of acidic fibroblast growth factor in left ventricular myocytes of patients with idiopathic cardiomyopathy. *Am Heart J* 1997;134:779-486

11. Gottshall KR, Hunter JJ, Tanaka N, Dalton N, Becker KD, Ross Jr. J, Chien KR: Ras-dependent pathways induce obstructive hypertrophy in echo-selected transgenic mice. *Proc Natl Acad Sci USA* 1997;94:4710-4715

12. Hengstenberg C, Maisch B: Increased nuclear proto-oncogene expression in hypertrophic cardiomyopathy. *Cardioscience* 1993;4:15-20

13. Clubb FJ, Bishop SP: Formation of binucleated myocardial cells in the neonatal rat. An index for growth hypertrophy. *Lab Invest* 1984;50:571-577

14. Bishop SP: Regulation of cardiac myocyte differentiation, hyperplasia, and hypertrophy during normal and stressed fetal and neonatal growth, in Anonymous*Heart growth and development.* 1995, pp 183-195

15. Zak R: *Growth of the Heart in Health and Disease.* New York, Raven Press Publishers, 1984, pp 1-480

16. Casale PN, Devereux R, Milner M, Zullo G, Harshfield GA, Pickering TG, Laragh JH: Value of echocardiographic measurement of left ventricular mass in predicting cardiovascular morbid events in hypertensive men. *Ann Med Interne* 1986;105:173-178

17. Tsatsul T, et al. Cytoskeletal role in the contractile dysfunction of hypertrophied myocardium. *Science* 1993;260:682

18. Roberts R, Towbin J, Parker T, Bies RD: Molecular biology of contractile and cytoskeletal proteins. *A primer of molecular biology.* New York, Elsevier Science Publishing Co. 1992, pp 129-149

19. Ng WA, Grupp I, Subramaniam A, Robbins J: Cardiac myosin heavy chain mRNA expression and myocardial function in the mouse heart. *Circ Res* 1991;69:1742-1750

20. Mahdavi V, Izumo S, Nadal-Ginard B: Developmental and hormonal regulation of sarcomeric myosin heavy chain gene family. *Circ Res* 1987;60:804-814

21. Tsuchimochi H, Kuro-o M, Koyama H, Kurabayashi M, Sugi M, et.al: Heterogeneity of beta-type myosin isozymes in the human heart and regulational mechanisms in their expression. *J Clin Invest* 1988;81:110-118

22. Parker TG, Schneider MD: Growth Factors, proto-oncogenes, and plasticity of the cardiac phenotype. *Annu Rev Physiol* 1991;53:179-200

23. Schaub MC, Hefti MA, Harder BA, Eppenberger HM: Various hypertrophic stimuli induce distinct phenotypes in cardiomyocytes. *J Mol Med* 1997;75:901-920

24. Hefti MA, Harder BA, Eppenberger HM, Schaub MC: Signaling pathways in cardiac myocyte hypertrophy. *J Mol Cell Cardiol* 1997;29:2873-2892

25. Schwartz K, de la Bastie D, Bouveret P, Oliviero P, Alonso S, Buckingham M: α-Skeletal muscle actin mRNAs accumulate in hypertrophied adult rat hearts. *Circ Res* 1986;59:551-555

26. Izumo S, Lompre A-M, Matsouka R, Koren G, Schwartz K, et.al: Myosin heavy chain messenger RNA and protein isoform transitions during cardiac hypertrophy. *J Clin Invest* 1987;79:970-977

27. Dool JS, Mak AS, Friberg P, Wahlander H, Hawrylechko A, Adams MA: Regional myosin heavy chain expression in volume and pressure overload induced cardiac hypertrophy. *Acta Physiol Scand* 1995;155:397-404

28. Izumo S, Nadal-Ginard B, Majdavi V: Proto-oncogene induction and reprogramming of cardiac gene expression produced by pressure overload. *Proc Natl Acad Sci USA* 1988;85:339-343

29. Schiaffino S, Samuel JL, Sassoon D, Lompre AM, Carner I, Marotte F, Buckinham M, Rappaport L, Schwartz K: Nonsynchronous accumulation of α-skeletal actin and β-myosin heavy chain mRNAs during early stages of pressure-overload-induced cardiac hypertrophy demonstrated by in situ hybridization. *Circ Res* 1989;64:937-948

30. Rovner AS, McNAlly EM, Leinwand LA: Complete cDNA sequence of rat atrial myosin light chain-1: Patterns of expression during development and with hypertension. *Nucleic Acids Res* 1990;18:1581-1586

31. Hammond GL, Nadal-Ginard B, Talner NS, Markert CL: Myocardial LDH isozyme distribution in the ischemic and hypoxic heart. *Circulation* 1979;53:637-643

32. Ingwall JS, Kramer MF, Fifer MA, Grossman W, Allen P: The creatine kinase system in normal and diseased human myocardium. *N Engl J Med* 1985;313:1050-1054

33. Zahler R, Gilmore-Hebert M, Benz EJ: Regional variations in Na^+ K-ATPase isoform gene expression in normal and hypertrophied dog heart. *Circulation* 1980;II-456

34. Komuro I, Katoh Y, Hoh E, Takaku F, Yazaki Y: Mechanisms of cardiac hypertrophy and injury: possible role of protein kinase C activation. *Jpn Circ J* 1991;55:1149-1157

35. de la Bastie D, Levitsky D, Rappaport L, Mercardier J-J, Marotte F, Wisnewsky C, Brovkovich V, Schwartz K, Lompré A-M: Function of the sarcoplasmic reticulum

and expression of its Ca^{2+}-ATPase gene in pressure overload-induced cardiac hypertrophy in the rat. *Circ Res* 1990;66:554-564

36. Seidmann CE, Wong DW, Jarcho JA, Bloch KD, Seidman JG: Cis-acting sequences that modulate atrial natriuretic factor gene expression. *Proc Natl Acad Sci USA* 1988;85:4104-4108

37. Mercadier JJ, Bouveret P, Gorza L, et.al: Myosin isoenzymes in normal and hypertrophied human ventricular myocardium. *Circ Res* 1983;53:52-62

38. Schwartz K, Schiaffino S, Allen PD, Wisnewsky C, Bouveret P, et.al: Expression of cardiac and skeletal actin genes in normal and failing human left ventricles. *J Mol Cell Cardiol* 1987;19:S34(Abstract)

39. Roberts R, Towbin J, Parker T, Bies RD: Molecular biology of cardiac growth and hypertrophy. *A primer of molecular biology*. New York, Elsevier Science Publishing Co. 1992, pp 151-166

40. Hirzel HO, Tuchschmid CR, Schneider J, Krayenbuehl HP, Schub M: Relationship between myosin isoenzyme composition, hemodynamics, and myocardial structure in various forms of human cardiac hypertrophy. *Circ Res* 1985;57:729-740

41. Schwartz K, Lecarpentier Y, Martin JL, Lompré AM, Mercadier JJ, Swynghedauw B: Myosin isoenzyme distribution correlates with speed of myocardial contraction. *J Mol Cell Cardiol* 1981;13:1071-1075

42. Nadal-Ginard B, Mahdavi V: Molecular basis of cardiac performance: plasticity of the myocardium generated through protein isoform switches. *J Clin Invest* 1989;84:1693-1700

43. Morano I, Hädicke K, Haase H, Böhm M, Erdmann E, Schaub M: Changes in essential myosin light chain isoform expression provide a molecular basis for isometric force regulation in the failing human heart. *J Mol Cell Cardiol* 1997;29:1177-1187

44. Hewett TE, Grupp IL, Grupp G, Robbins J: Alpha-skeletal actin is associated with increased contractility in the mouse heart. *Circ Res* 1994;74:740-746

45. Potts JD, Runyon RB: Epithelial-mesenchymal cell transformation in the heart can be mediated, in part, by transforming growth factor b. *Dev Biol* 1989;134:392-401

46. Rockman HA, Ross RS, Harris AN, Knowlton KU, Steinhelper ME, Field L, Ross J, Chien KR: Segregation of atrial specific and inducible expression of an ANF transgene in an *in vivo* murine model of cardiac hypertrophy. *Proc Natl Acad Sci USA* 1991;88:8277-8281

47. Rockman HA, Ono S, Ross RS, Jones LR, Karimi M, Bhargava V, Ross Jr. J, Chien KR: Molecular and physiological alterations in murine ventricular dysfunction. *Proc Natl Acad Sci USA* 1994;91:2694-2698

48. MacLellan WR, Brand T, Schneider MD: Transforming growth factor-β in cardiac ontogeny and adaptation. *Circ Res* 1993;73:783-791

49. Heldin CH, Claesson-Welsh L, Miy Azono K, Westermark B: Growth factors: In vivo function and mechanism of action, in Cummins P (ed): *Growth factors and the cardiovascular system*. Boston, Kluwer Academic Publishers, 1993, pp 1-15

50. Froesch ER, Schmid C, Schwander J, Zapf J: Actions of insulin-like growth factors. *Annu Rev Physiol* 1985;47:443-467

51. Schwander JC, Hauri C, Zapf J, Froesch ER: Synthesis and secretion of insulin-like growth factor and its binding protein by the perfused rat liver: dependence on growth hormone status. *Endocrinology* 1983;113:297-305

52. Engelmann GL, Boehm KD, Hasskell JF, Khairallah PA, Ilan J: Insulin-like growth factors and neonatal cardiomyocyte development: ventricular gene expression and membrane receptor variations in normotensive and hypertensive rats. *Mol Cell Endocrinol* 1989;63:1-14

53. Murphy LJ, Bell GI, Friesen HG: Tissue distribution of insulin-like growth factor-I and -II messenger ribonucleic acid in the adult rat. *Endocrinology* 1987;120:1279-1282

54. D'Ercole AJ, Stiles AD, Underwood LE: Tissues concentrations of somatodein-C: further evidence for multiple sites of synthesis and paracrine or autocrine mechanisms of action. *Proc Natl Acad Sci USA* 1984;81:935-939

55. Meuli C, Froesch ER: Binding of insulin and nonsuppressible insulin-like activity to isolated perfused rat heart muscle. *Arch Biochem Biophys* 1976;177:31-38

56. Donohue TJ, Dworkin LD, Lango MN, Fliegner K, Lango RP, Benstein JA, Slater WR, Catanese VM: Induction of myocardial insulin-like growth factor-1 gene expression in left ventricular hypertrophy. *Circulation* 1994;89:799-809

57. LeRoith D: *Insulin-like growth factors: molecular and cellular aspects*. Boca Raton, CRC Press, 1991,

58. Lewitt MS, Saunder H, Cooney GJ, Baxter RC: Effect of insulin-like growth factor-binding protein-1 on the half-life and action of administered insulin-like growth factor-I in rats. *J Endocrinology* 1993;136:253-260

59. Pimentel E: Insulin-like growth factors, in Pimentel E (ed): *Peptide growth factors*. Boca Raton, CRC Press Inc. 1994, pp 55-95

60. Blakesley VA, Scrimgeour A, Esposito D, Le Roith D: Signaling via the insulin-like growth factor-I receptor: does it differ from insulin receptor signaling? *Cyt & Grwth Factor Rev* 1996;7:153-159

61. Sporn MB, Roberts AB, Wakefield LM, Assoian RK: Transforming growth factor β: Biological function and chemical structure. *Science* 1986;233:532-534

62. Sporn MB, Roberts AB, Wakefield LM, de Crombrugghe B: Some recent advances in the chemistry and biology of transforming growth factor β. *J Cell Biol* 1987;105:1039-1045

63. Cheifetz S, Weatherbee JA, Tsang ML-S, Anderson JK, Mole JE, Lucas R, Massague J: The transforming growth factor β system, a complex pattern of cross-reactive ligands and receptors. *Cell* 1987;48:409-415

64. Roberts AR, Sporn MB: The transforming growth factors-betas, in Sporn MB, Roberts AR (eds): *Peptide Growth Factors and their Receptors. Handbook of Experimental Pharmacology*. Berlin, Springer-Verlag, 1990, pp 417-472

65. Brand T, Schneider MD: The TGFb superfamily in myocardium: ligands, receptors, transduction, and function. *J Mol Cell Cardiol* 1995;27:5-18

66. Akhurst RJ, Lehnert SA, Faissner A, Duffie E: TGF beta in murine morphogenetic processes: the early embryo and cardiogenesis. *Development* 1990;108:645-656

67. Dickson MC, Slager HG, Duffie E, Mummery CL, Akhurst RJ: RNA and protein localisations of TGFβ2 in the early mouse embyo suggest an involvement in cardiac development. *Development* 1993;117:625-639

68. Millan FA, Denhez F, Kondaiah P, Akhurst RJ: Embryonic gene expression patterns of TGFβ1, β1, and β3 suggest different developmental function *in vivo*. *Development* 1991;111:131-144

69. Sporn MB, Roberts AB: Transforming growth factor-β: recent progress and new challenges. *J Cell Biol* 1992;119:1017-1021

70. Long CS: Autocrine and paracrine regulation of myocardial cell growth *in vitro*; the TGFbeta paradigm. *Trends Cardiovasc Med* 1996;6:217-226

71. Kingsley DM: The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev* 1994;8:133-146

72. Zioncheck TF, Chen SA, Richardson L, Mora-Worms M, Lucas C, Lewis D, Green JD, Mordenti J: Pharmacokinetics and tissue distribution of recombinant human transforming growth factor $beta_1$ after topical and intravenous administration in male rats. *Pharm Res* 1994;11:213-220

73. Antonelli-Orldge A, Saunder KB, Smith SR, D'Amore PA: An activated form of transforming growth factor beta is produced by co-culture of endothelial cells and pericytes. *Proc Natl Acad Sci USA* 1989;86:4544-4548

74. Delarco JE, Todaro GJ: Growth factors from murine sarcoma virus-transformed cells. *Proc Natl Acad Sci USA* 1978;75:4001-4005

75. Eghbali M: Cellular origin and distribution of transforming growth factor beta 1 in the normal rat myocardium. *Cell Tiss Res* 1989;256:553-558

76. Muslin AJ, Williams LT: Well-defined growth factors promote cardiac development in axolotl mesodermal explants. *Development* 1991;146:242-245

77. Lyons KM, Pelton RW, Hogan BL: Patterns of expression of murine Bgr-1 and MBP-2a RNA suggest that transforming growth factor-beta-like genes co-ordinately regulate aspects of embryonic development. *Genes Dev* 1989;3:1657-1668

78. Massgué J, Hata A, Liu F: TGF-beta signaling through the Smad pathway. *Trends Cell Biol* 1997;7:187-192

79. Thompson NC, Bazoberyy F, Speir EH, Casscells E, Ferrans VJ: Transforming growth factor beta-1 in acute myocardial infarction in rats. *Growth Factors* 1988;1:91-99

80. Wünsch M, Sharma HS, Markert T, Bernotat-Danielowski S, Schott RJ, Kremer P, Bleese N, Schaper W: In situ localization of transforming growth factor beta 1 in porcine heart: Enhanced expression after chronic coronary artery constriction. *J Mol Cell Cardiol* 1991;23:1051-1062

81. Wünsch H, Sharma HS, Bernotat-Danielowski S, Schott RJ, Schaper J, Bleese N, Schaper W: Expression of transforming growth factor beta-1 (TGF-β1) in collateralized swine heart. *Circulation* 1989;80:II-453

82. Hanson MC, Fath KA, Alexander RW, Delafontaine P: Induction of cardiac insulin-like growth factor I gene expression in pressure overload hypertrophy. *Am J Med Sci* 1993;306:69-74

83. Villarreal FJ, Dillmann WH: Cardiac hypertrophy-induced changes in messenger RNA levels for TGF-beta 1, fibronectin, and collagen. *Am J Physiol* 1992;262:H1861-1866

84. Bhambi B, Eghbali M: Effect of norepinepyhrine on myocardial collagen gene expression and response of cardiac fibroblasts after norepinephrine treatment. *Am J Pathol* 1991;139:1131-1142

85. Sadoshima J, Xu YH, Slayter HS, Izumo S: Autocrine release of angiotensin-II mediates stretch-induced hypertrophy of cardiac myocytes *in vitro*. *Cell* 1993;75:977-984

86. Long CS, Hartogenesis WE, Simpson PC: β-Adrenergic stimulation of cardiac non-myocytes augments the growth-promoting activity of non-myocyte conditioned medium. *J Mol Cell Cardiol* 1993;25:915-925

87. Villarreal FJ, Lee AA, Dillmann WH, Giordano FJ: Adenovirus-mediated overexpression of human transforming growth factor-β1 in rat cardiac fibroblasts, myocytes and smooth muscle cells. *J Mol Cell Cardiol* 1996;28:735-742

88. Parker TG, Chow K-L, Schwartz RJ, Schneider MD: Differential regulation of skeletal α-actin transcription in cardiac muscle by two fibroblast growth factors. *Proc Natl Acad Sci USA* 1990;87:7066-7070

89. Decker RS, Cook MG, Behnke-Barclay M, Decker ML: Some growth factors stimulate cultured adult rabbit ventricular myocyte hypertrophy in the absence of mechanical loading. *Circ Res* 1995;77:544-555

90. Amato G, Carella C, Fazio S, Montagna GL, Cittadini A, Sabatini D, Mariano-Mone C, Sacca L, Bellastella A: Body compostion, bone metabolism, and heart structure and function in growth hormone (GH)-deficient adults before and after GH replacement therapy at low dose. *J Clin Endocinol Metab* 1993;77:1671-1676

91. Diez J, Laviades C: Insulin-like growth factor-1 and cardiac mass in essential hypertension: comparative effects of captopril, lisinopril and quinapril. *Journal of Hypertension* 1994;12:s31-s36

92. Ito H, Hiroe M, Hirata Y, Tsujino M, Adachi S, Shichiri M, Koike A, Nogami A, Marumo F: Insulin-like growth factor-I induces hypertrophy with enhanced

expression of muscle specific genes in cultured rat cardiomyocytes. *Circulation* 1993;87:1715-1721

93. Donath MY, Zapf J, Eppenberger-Eberhardt M, Froesch ER, Eppenberger HM: Insulin-like growth factor-1 stimulates myofibril development and decreases smooth muscle α-actin of adult cardiomyocytes. *Proc Natl Acad Sci USA* 1994;91:1686-1690

94. Florini JR, Ewton DZ: Induction of gene expression in muscle by the IGFs. *Growth Regul* 1992;2:23-29

95. Harder BA, Schaub MC, Eppenberger HM, Eppenberger-Eberhardt M: Influence of fibroblast growth factor (bFGF) and insulin-like growth factor (IGF-I) on cytockeletal and contractile structure and on atrial natriuretic factor (ANF) expression in adult rat ventricular cardiomyocytes in culture. *J Mol Cell Cardiol* 1996;28:19-31

96. Eghbali M, Tomek R, Sukhatme VP, Woods C, Bhambi B: Differential effects of transforming growth factor-β1 and phorbol myristate acetate on cardiac fibroblasts. Regulation of fibrillar collagen mRNAs and expression of early transcription factors. *Circ Res* 1991;69:483-490

97. Butt RP, Laurent GJ, Bishop JE: Collagen production and replication by cardiac fibroblasts is enhanced in response to diverse classes of growth factors. *Eur J Cell Biol* 1995;66:330-335

98. Factor SM, Butany J, Sole MJ, Wigle D, Williams WG, Rojkind M: Pathologic fibrosis and matrix connective tissue in the subaortic myocardium of patients with hypertrophic cardiomyopathy. *J Am Coll Cardiol* 1991;17:1343-1351

99. Maron BJ, Epstein SE, Roberts WC: Causes of sudden death in competitive athletes. *J Am Coll Cardiol* 1986;7:204-214

100. Louville H: Rétrécissement cardiaque sous aortique. *Gas Med Paris* 1869;24:161-163

101. Hallopeau L: Rétrécissement ventriculo-aortique. *Gaz Med Paris* 1969;24:683-684

102. Schmincke A: Ueber linksseitige muskulose Conusstenosen. *Dtsch Med Wochenschr* 1907;33:2082

103. Brock RC: Functional obstruction of the left ventricle. *Guys Hosp Rep* 1957;106:221-238

104. Teare RD: Asymmetrical hypertrophy of the heart in young adults. *Br Heart J* 1958;20:1-8

105. Post GP, Brown JH: G protein-coupled receptors and signaling pathways regulating growth responses. *FASEB J* 1996;10:741-749

106. Maron BJ, Roberts WC: Hypertrophic cardiomyopathy, in Schlant RC, Alexander RW (eds): *The Heart Arteries and Veins* (8th edition). NY, McGraw-Hill Inc. Health Professions Division, 1994, pp 1621-1635

107. Wigle ED, Rakowski H, Kimball BP, Williams WG: Hypertrophic cardiomyopathy: clinical spectrum and treatment. *Circulation* 1995;92:1680-1692

108. Levy D, Garrison RJ, Savage DD, Kannel WB, Castelli WP: Prognostic implications of echocardiographically determined left ventricular mass in the Framingham Heart Study. *N Engl J Med* 1990;322:1561-1566

109. Codd MB, Sugrue DD, Gersh BJ, Melton III LJ: Epidemiology of idiopathic dilated and hypertrophic cardiomyopathy. A population-based study in Olmsted county, Minnesota, 1975-1984. *Circulation* 1989;80:564-572

110. Clark CE, Henry WL, Epstein SE: Familiar prevalence and genetic transmission of idiopathic hypertrophic subaortic stenosis. *N Engl J Med* 1973;289:709-714

111. Maron BJ, Nichols PFI, Pickle LW, Wesley YE, Mulvihill JJ: Patterns of inheritance in hypertrophic cardiomyopathy assessment by M-mode and two-dimensional echocardiography. *Am J Cardiol* 1984;53:1084-1094

112. Epstein ND, Cohn GM, Cyran F, Fananapazir L: Differences in clinical expression of hypertrophic cardiomyopathy associated with two distinct mutations in β-myosin heavy chain gene. *Circulation* 1992;86:345-352

113. Jarcho JA, McKenna W, Pare JAP, Solomon SD, Holcombe RF, et.al. Mapping a gene for familial hypertrophic cardiomyopathy to chromosome 14q1. *N Engl J Med* 1989;321:1372-1378

114. Marian AJ, Roberts R: Recent advances in the molecular genetics of hypertrophic cardiomyopathy. *Circulation* 1995;92:1336-1347

115. Geisterfer-Lowrance A, Kass S, Tanigawa G, Vosberg HP, McKenna W, Seidman CE, Seidman JG: A molecular basis for familial hypertrophic cardiomyopathy: a b-myosin heavy chain gene missense mutation. *Cell* 1990;62:999-1006

116. Solomon SC, Jarcho JA, McKenna W, Geisterfer-Lowrance A, Germain R, Salerni R, Seidman JG, Seidman CE: Familial hypertrophic cardiomyopathy is a genetically heterogeneous disease. *J Clin Invest* 1990;86:993-999

117. Watkins H, Rosenzweig A, Hwang DS, Levi T, McKenna W, Seidman CE, Seidman JG: Characteristics and prognostic implications of myosin missense mutations in familial hypertrophic cardiomyopathy. *N Engl J Med* 1992;326:1108-1114

118. Hejtmancik JF, Brink PA, Towbin J, Hill R, Brink L, Tapscott T, Trakhtenbroit A, Roberts R: Localization of gene for hypertrophic cardiomyopathy to chromosome 14q1 in a diverse US population. *Circulation* 1991;83:1592-1597

119. Fananapazir L, Epstein ND: Genotype-phenotype correlations in hypertrophic cardiomyopathy: insights provided by comparisons of kindreds with distinct and identical b-myosin heavy chain gene mutations. *Circulation* 1994;89:22-32

120. Thierfelder L, MacRae C, Watkins H, McKenna W, Vosber HP, Seidman JG, Seidman C: α-Tropomyosin and cardiac troponin T mutations cause familial hypertrophic cardiomyopathy: a disease of the sarcomere. *Cell* 1994;77:701-712

121. Watkins H, Conner D, Thierfelder L, Jarcho JA, MacRae C, McKenna WJ, Maron BJ, Seidman JG, Seidman CE: Mutations in the cardiac myosin binding protein-C gene on chromosome 11 cause familial hypertrophic cardiomyopathy. *Nat Genet* 1995;11:434-437

122. Bonne G, Carrier L, Bercovici J, Cruaud C, Richard P, Hainque B, Gautel M, Labeit S, James M, Beckmann J, Weissenbach J, Vosberg H, Fiszman M, Komajda M, Schwartz K: Cardiac myosin binding protein-C gene splice acceptor site mutation is associated with familial hypertrophic cardiomyopathy. *Nat Genet* 1995;11:438-440

123. Theirfelder L, MacRae C, Watkins H, McKenna W, Vosberg HP, Seidman JG, Seidman C: Familial hypertrophic cardiomyopathy is a disease of the sarcomere. *Circulation* 1994;90:I-519

124. Poetter K, Jiang H, Hassanzadeh S, Master S, Chang A, Dalakas MC, Rayment I, Sellers JR, Fananapazir L, Epstein ND: Mutations in either the essential or regulatory light chains of myosin are associated with a rate of myopathy in human heart and skeletal muscle. *Nat Genet* 1996;13:63-69

125. Cuda G, Fananapazir L, Zhu WS, Sellers JR, Epstein ND: Skeletal muscle expression and abnormal function of beta-myosin in hypertrophic cardiomyopathy. *J Clin Invest* 1993;91:2861-2865

126. Cuda G, Fananapazir L, Epstein ND, Sellers JR: The *in vitro* motility activity of β-cardiac myosin depends on the nature of the b-myosin heavy chain gene mutation in hypertrophic cardiomyopathy. *J Muscle Res Cell M* 1997;18:275-283

127. Lankford EB, Epstein ND, Fananapazir L, Sweeney HL: Abnormal contractile properties of muscle fibres expressing b-myosin heavy chain gene mutations in patients with hypertrophic cardiomyopathy. *J Clin Invest* 1995;95:1409-1414

128. Lin D, Bobkova A, Homsher E, Tobacman LS: Altered cardiac troponin T in vitro function in the presence of a mutation implicated in familial hypertrophic cardiomyopathy. *J Clin Invest* 1996;97:2842-2848

129. Watkins H, Seidman CE, Seidman JG, Feng HS, Sweeney HL: Expression and functional assessment of a truncated cardiac troponin T that causes hypertrophic cardiomyopathy: evidence for a dominant negative action. *J Clin Invest* 1996;98:2456-2461

130. Marian AJ, Zhao G, Seta Y, Roberts R, Yu Q: Expression of a mutant ($Arg^{92}Gln$) human cardiac troponin T, known to cause hypertrophic cardiomyopathy, impairs adult cardiac myocyte contractility. *Circ Res* 1997;81:76-85

131. Golitsina N, An Y, Ludwig G, Thierfelder L, Iizuka K, Seidman JG, Seidman CE, Hitchcock-DeGregori SE: Effects of two familial hypertrophic cardiomyopathy-causing mutations on a-tropomyosin structure and function. *Biochemistry* 1997;36:4637-4642

132. Thompson CH, Kemp GJ, Taylor DJ, Conway M, Rajagopalan B, O'Donoghue A, Styles P, McKenna WJ, Radda GK: Abnormal skeletal muscle bioenergetics in familial hypertrophic cardiomyopathy. *Heart* 1997;78:177-181

133. Cirò E, Nichols PF, Maron BJ: Heterogeneous morphologic expression of genetically transmitted hypertrophic cardiomyopathy: Two dimensional echocardiographic analysis. *Circulation* 1983;67:1227-1233

134. Pollick C, Kimball B, Henderson M, Wigle ED: Disopyramide in hypertrophic cardiomyopathy I. Hemodynamic assessment after intravenous administration. *Am J Cardiol* 1988;62:1248-1251

135. Villari B, Hess MO, Kaufmann P, Krogmann ON, Grimm J, Krayenbuehl HP: Effect of aortic valve stenosis (pressure overload) and regurgitation (volume overload) on left ventricular systolic and diastolic function. *Am J Cardiol* 1992;69:927-934

136. Schunkert H, Hense H, Holmer SR, Stender M, Perz S, Keil U, Lorell BH, Riegger GA: Association between a deletion polymorphism of the angiotensin-converting-enzyme gene and left ventricular hypertrophy. *N Engl J Med* 1994;330:1634-1638

137. Iwai N, Ohmichi N, Nakamura Y, Kinoshita M: DD Genotype of the angiotensin-converting enzyme gene is a risk factor for left ventricular hypertrophy. *Circulation* 1994;90:2622-2628

138. Marian AJ, Yu QT, Workman R, Greve G, Roberts R: Angiotensin-converting enzyme polymorphism in hypertrophic cardiomyopathy and sudden cardiac death. *Lancet* 1993;342:1085-1086

139. Wagner JA, Sax FL, Weisman HF, Porterfied J: Calcium-antagonist receptors in the atrial tissue of patients with hypertrophic cardiomyopathy. *N Engl J Med* 1989;320:755-761

140. Golf S, Myhre E, Abdelnoor M, Andersen D, Hansson V: Hypertrophic cardiomyopathy characterized by beta-adrenoreceptor density, relative amount of beta-adrenoceptor subtypes and adenylate cyclase activity. *Cardiovasc Res* 1985;19:693-699

141. Shimizu M, Sugihara N, Kita Y, Shimizu K, Horita Y, Nakajima K, Taki J, Takeda R: Long-term course and cardiac sympathetic nerve activity in patients with hypertrophic cardiomyopathy. *Br Heart J* 1992;67:155-160

142. Toyozaki T, Hiroe M, Hasumi M, Horie T, Hosoda S, Tsushima T, Sekiguchi M: Insulin-like growth factor I receptors in human cardiac myocytes and their relation to myocardial hypertrophy. *Jpn Circ J* 1993;57:1120-1127

143. Sakata Y: Tissue factors contributing to cardiac hypertrophy in cardiomyopathic hamsters (BIO14.6): Involvement of transforming growth factor beta-1 and tissue renin-angiotensin system in the progression of cardiac hypertrophy. *Hokkaido Igaku Zasshi* 1993;68:18-28

144. Thorburn A, Thorburn J, Chen S-Y, Powers S, Shubeita HE, Feramisco JR, Chien KR: HRas-dependent pathways can activate morphological and genetic markers of cardiac muscle cell hypertrophy. *J Biol Chem* 1993;268:2244-2249

145. LaMorte VJ, Thorburn J, Absher D, Spiegel A, Brown JH, Chien KR, Feramisco JR, Knowlton KU: Gq- and Ras- dependent pathways mediate hypertrophy in neonatal rat ventricular myocytes following alpha-1 adrenergic stimulation. *J Biol Chem* 1994;269:13490-13496

146. Pennica D, King KL, Shaw KJ, Luis E, Rullamas J, Luoh SM, Darbonne WC, Knutzon DS, Yen R, Chien KR, Baker JB, Wood WI: Expression cloning of cardiotrophin 1: a cytokine that induces cardiac myocyte hypertrophy. *Proc Natl Acad Sci USA* 1995;92:1142-1146

147. Hunter JJ, Tanaka N, Rockman HA, Ross Jr. J, Chien KR: Ventricular expression of a MLC-2v-*ras* fusion gene induces cardiac hypertrophy and selective diastolic dysfunction in transgenic mice. *J Biol Chem* 1995;270:23173-23178

148. Hasegawa K, Fujiwara H, Doyama K, Miyamae M, Fujiwara T, Suga S, Mukoyama M, Nakao K, Imura H, Sasayama S: Ventricular expression of brain natriuretic peptide in hypertrophic cardiomyopathy. *Circulation* 1993;88:372-380

149. Dulbecco R: Production of plaques in monolayer tissue cultures by single particles of an animal virus. *Proc Natl Acad Sci USA* 1952;38:747-752

150. Moscona A: Cell suspensions from organ rudiments of the early chick embryo. *Exp Cell Res* 1952;3:535-539

151. Cavanaugh MW: Pulsation, migration and division in dissociated chick embryo heart cell *in vitro*. *J Exp Zool* 1955;128:573-589

152. Harary I, Farley B: In vitro studies of single isolated beating heart cells. *Science* 1960;131:1674-1675

153. Atherton B, Meyer D, Simpson D: Assembly and remodeling of myofibrils and intercalated discs in cultured neonatal rat heart cells. *Journal of Cellular Science* 1986;86:233-248

154. Ng AC, Cheng M, Flschman DA, Zak R: Long-term culture of adult mammalian cardiac myocytes: Electron microscopic and immunofluorescent analysis of myofibrillar structure. *J Mol Cell Cardiol* 1981;15:301-317

155. Simpson P, McGrath A, Savion S: Myocyte hypertrophy in neonatal rat heart cultures and its regulation by serum and catecholamines. *Circ Res* 1982;51:787-801

156. Simpson P, Savion S: Differentiation of rat myocytes in single cell culture with and without proliferating nonmyocardial cells: cross-striations, ultrastructure, and chronotropic response to isoproternol. *Circ Res* 1982;50:101-116

157. Ueno H, Perryman B, Roberts R, Schneider MD: Differentiation of cardiac myoctyes after mitogen withdrawal exhibits three sequential states of the ventricular growth response. *The Journal of Cell Biology* 1988;107:1911-1918

158. Claycomb WC: Atrial natriuretic factor mRNA is developmentally regulated in heart ventricles and actively expressed in cultured ventricular cardiac muscle cells of rat and human. *Biochem J* 1988;255:617-620

159. Ellingsen O, Davidoff AJ, Prasad SK, Berger H, Springhorn JP, Marsh JD, Kelly RA, Smith TW: Adult rat ventricular myocytes cultured in defined medium: phenotype and electromechanical function. *Heart Circ Physiol* 1993;34:H747-H754

160. Eghbali M, Weber KT: Collagen and the myocardium: Fibrillar structure, biosynthesis and degredation in relation to hypertrophy and its regression. *Mol Cell Biochem* 1990;96:1-14

161. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium-thiocyanate-phenol-choloroform extraction. *Anal Biochem* 1987;162:156-159

162. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laboratory Manual.* cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 1998, pp 14.5-14.20

163. Gilliland G, Perrin S, Blanchard K, Bunn HF: Analysis of cytokine mRNA and DNA: Detection and quantitation by competitive polymerase chain reaction. *Proc Natl Acad Sci USA* 1990;87:2725-2729

164. Kellogg DE, Sninsky JJ, Kwok S: Quantitation of HIV-1 proviral DNA relative to cellular DNA by the polymerase chain reaction. *Anal Biochem* 1990;189:202-208

165. Horikoshi T, Danenberg KD, Stadlbauer THW, Volkenadndt M, Shea LCC, Aigner K, Gustavsson B, Leichman L, Frosing R, Ray M, Gibson NW, Spears CP, Danenberg PV: Quantitation of thymidylate synthase, dihydrofolate reductase, and DT-diaphoroase gene expression in human tumors using the polymerase chain reaction. *Cancer Res* 1992;52:108-116

166. Ercolani L, Florence B, Denaro M, Alexander M: Isolation and complete sequence of a functional human glyceraldehyde-3-phosphatedehydrogenase gene. *J Biol Chem* 1988;263:15335-15341

167. Derynck R, Jarret JA, Chen EY, Eaton DH, Bell JR, Assoian RK, Roberts AB, Sporn MB, Goeddel DV: Human transforming growth factor-β complementary DNA sequence and expression in normal and transformed cells. *Nature* 1985;316:701-705

168. Steenbergh PH, Koonen-Reemst AMCB, Cleutjens CBJM, Sussenbach JS: Complete nucleotide sequence of the high molecular weight human IGF-I mRNA. *Biochem Biophys Res Commun* 1991;175:507-514

169. Winship PR: An improved method for directly sequencing PCR amplified material using dimethyl sulphoxide. *Nucleic Acids Res* 1989;17:1266

170. Hawkes R, Hiday E, Gordon J: A dot-immunobinding assay for monoclonal and other antibodies. *Anal Biochem* 1982;119:142-147

171. Tumiati LC, Mickle DAG, Weisel RD, Williams WG, Li R: An in vitro model to study myocardial ischemic injury. *J Tiss Cult Meth* 1998;16:1-9

172. Martin AF, Rabinowitz M, Blough R, Prior G, Zak R: Measurements of half-life of rat cardiac myosin heavy chain with leucyl-fRNA used as a precursor pool. *J Biol Chem* 1977;252:3422-3429

173. Li R-K, Mickle DAG, Weisel RD, Zhang J, Mohabeer MK: In vivo survival and function of transplanted rat cardiomyocytes. *Circ Res* 1996;78:283-288

174. Van der Laarse A, Treslong CHFB, Vliegen HW, Riogliarth L: Relationship between ventricular DNA content and numbers of myocytes and non-myocytes in hearts of normotensive and spontaneously hypertensive rats. *Cardiovasc Res* 1987;21:223-229

175. Calderone A, Takahashi N, Izzo NJ, Thaik CM, Colucci WS: Pressure- and volume-induced left ventricular hypertrophies are associated with distinct myocyte phenotypes and differential induction of peptide growth factor mRNAs. *Circulation* 1995;92:2385-2390

176. Takahashi N, Calderone A, Izzo NJJ, Mäki TM, Marsh JD, Solucci WS: Hypertrophic stimuli induce transforming growth factor-β_1 expression in rat ventricular cardiomyocytes. *J Clin Invest* 1994;94:1470-1476