ENDOCARDIAL SIGNALING PATHWAYS REGULATING CARDIAC INTERSTITIUM

A thesis presented

by

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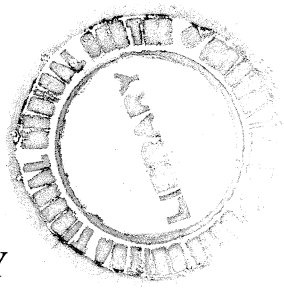

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CERTIFICATE

I, **Leena Kuruvilla,** hereby certify that I had personally carried out the work depicted in the thesis entitled ***"Endocardial signaling pathways regulating cardiac interstitium",*** except where external help sought and acknowledged.

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This is to certify that **Ms. Leena Kuruvilla**, in the Division of Cellular and Molecular Cardiology of this institute, has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the PhD degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. The work relating to her thesis entitled **"*Endocardial signaling pathways regulating cardiac interstitium*"** was carried out under my direct supervision.

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Leena Kuruvilla

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ABBREVIATIONS

Ang II	Angiotensin II
ACE	Angiotensin Converting Enzyme
$[Ca^{2+}]i$	Intra-endothelial Ca^{2+} concentration
BAECs	Bovine Aortic Endothelial Cells
bFGF	basic Fibroblast Growth Factor
BIM	Bis-indolylmaleimide
BSA	Bovine Serum Albumin
cADPR	cyclic ADP ribose
cAMP	cyclic Adenosine Mono Phosphate
cGMP	cyclic Guanosine Mono Phosphate
CTGF	Connective Tissue Growth Factor
DiI-Ac-LDL	1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate labeled Acetylated Low Density Lipoprotein
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
EECs	Endocardial Endothelial Cells
eNOS	Endothelial Nitric Oxide Synthase
ET	Endothelin
ET_A	Endothelin receptor type A
ET_B	Endothelin receptor type B
ERK 1/2	Extracellular Signal Regulated Kinase 1/2
FITC	Fluorescein Isothiocyanate
GMCSF	Granulocyte Monocyte Colony Stimulating Factor
GPCR	G protein Coupled Receptor
hTERT	Human Telomerase Reverse Transcriptase
$[^3H]$-Thymidine	Tritiated Thymidine
HRP	Horse Radish Peroxidase
ICAM-1	Intercellular Adhesion Molecule-1
IL	Interleukin

iNOS	Inducible Nitric Oxide Synthase
JNK	c-jun N terminal Kinase
LPS	Lipopolysaccharide
LVF	Left Ventricular Function
MAPK	Mitogen Activated Protein Kinase
MEK/MAPKK	Mitogen Activated Protein Kinase Kinase
Medium E199	Medium 199 with Earle's salts
MEM	Minimum Essential Medium
MMP	Matrix Metallo Proteinase
MVE	Microvascular Endothelium
MVECs	Microvascular Endothelial Cells
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
PBS	Phosphate Buffered Saline
PDE	Phospho Diesterase
PECAM	Platelet and endothelial cell adhesion molecule
PDGF	Platelet Derived Growth Factor
PG	Prostaglandin
PKC	Protein Kinase C
PKG	cGMP-dependent protein kinases
RAS	Renin-Angiotensin System
RAAS	Renin-Angiotensin Aldosterone System
SMC	Smooth Muscle Cells
SR	Sarcoplasmic Reticulum
TCA	Tri Chloroacetic Acid
TERT	Telomerase Reverse Transcriptase
TGF-β	Transforming Growth Factor β
TIMP	Tissue Inhibitor of Metalloproteinase
TNF-α	Tumor Necrosis Factor
VSMCs	Vascular Smooth Muscle Cells

SYNOPSIS

INTRODUCTION

The endothelium is recognized as a massive, regionally specific, multifunctional organ, reactive to blood constituents and mechanical forces exerted by blood flow. Two types of endothelial cells are recognized in the heart- the endocardial endothelial cells (EECs), which line the cavities of the heart and microvascular endothelial cells (MVECs), which line the blood vessels of the heart. Both EECs and MVECs are distinct cell populations with dissimilar embryological origin, cytoskeletal organization, receptor mediated functions, electrophysiological properties and growth characteristics in culture. Vascular endothelial cells play an important role in the modulation of vascular structure and vasomotor function. These cells secrete several factors, which have a paracrine influence on proliferation and extracellular protein turnover in smooth muscle cells and fibroblasts of the vessel wall. Similar to the vascular endothelium, the endocardial endothelium between the circulating blood and the cardiac muscle is strategically situated. Brutsaert *et al* (1988) demonstrated that EEC is an important modulator of subjacent cardiac muscle performance. Dysfunction of this interface can be a critical factor in various pathological conditions of the heart. Endocardial endothelial dysfunction may lead to cardiac failure in dilated cardiomyopathy and is possibly involved in the pathogenesis of endomyocardial fibrosis.

Given that endothelial cells stimulate connective tissue cells and endocardial endothelium regulate myocyte function, we hypothesized that EECs may have a modulator role in regulating the cardiac interstitial cells. Endocardial endothelial cells may secrete mitogenic factors and substances that bring about the proliferation of cardiac fibroblasts and enhance collagen synthesis in the fibroblasts.

OBJECTIVES OF THE STUDY

1) To isolate, characterize and immortalize endocardial endothelial cells
2) To determine whether endocardial endothelium has a role in regulating cardiac fibroblast growth and function
2) To identify endocardial endothelium derived factors, if any, which stimulate cardiac fibroblast proliferation and collagen synthesis
3) To delineate the signaling pathways involved in the endocardial endothelial regulation of cardiac fibroblast function
4) To ascertain the influence of pro-inflammatory cytokines TNF-α and bacterial lipopolysaccharide (LPS), as may occur in cardiac inflammatory states, on EEC-cardiac fibroblast interaction.

METHODOLOGY

Endocardial endothelial cells were isolated from freshly collected pig hearts. The cells were harvested after filling the ventricles with 0.1% collagenase (Type IA) in medium E199 and incubating the ventricles for 45 minutes. The cells were resuspended in complete medium (medium E199 supplemented with 20% fetal bovine serum (FBS), 1% endothelial cell growth factor, 100 U/ml benzyl penicillin and 100 μg/ml streptomycin) and seeded in gelatin coated culture dishes. Cultures were incubated in a CO_2 incubator (37^0C in 95% air-5% CO_2). Cells were allowed to grow and confluent cells were subcultured using 0.025% trypsin-0.02% EDTA mixture. The cells were verified as endothelial cells by their cobblestone appearance, positive staining for factor VIII antigen and incorporation of DiI-Ac-LDL.

To immortalize EECs, the cells were transfected with the mammalian expression vector LZRS-hTERT-IRES-GFP containing the full-length human telomerase reverse transcriptase (hTERT) carrying puromycin resistance gene. The cells were transfected with 2 μg of plasmid DNA using lipofectamine reagents and serum free OptiMEM medium for six hours. The cells were incubated in medium containing 0.5 μg/ml of puromycin for the selection of positive population. The selected clones of cells were expanded, maintained separately and used for subsequent experiments. The cells were characterized as EECs by their cobblestone morphology, positive staining for Factor VIII related antigen, capacity to uptake DiI-Ac-LDL and response to proinflammatory agents TNF-α and LPS.

Cardiac fibroblasts were isolated from 3- to 4-day-old Wistar rat pups. The heart tissue was minced and digested with collagenase and trypsin. The supernatants were centrifuged and cell pellet was resuspended in medium 199 with 10% FBS. The attached cells were grown to confluence and passaged. The cells were identified by their spindle morphology, positive staining for vimentin and negative staining for factor VIII antigen. All experiments were carried out on quiescent cells.

Endocardial endothelial cell conditioned medium was prepared by incubating the cells for 24 hours either with medium containing 0.4% fetal bovine serum, 10 ng/ml TNF-α or 1μg/ml bacterial lipopolysaccharide (LPS). Endocardial endothelium derived factors Endothelin-1 (ET-1), Transforming Growth Factor-β (TGF-β) and Angiotensin-II (Ang-II) in the conditioned medium were assayed using ELISA kits. Nitrite levels in the conditioned medium were measured using Griess reaction. To delineate the signaling pathways involved, cardiac fibroblast cultures were treated with specific inhibitors: 1

mmol/l BQ123 (selective ET_A receptor antagonist), 1 mmol/l PD 142893 (non selective ET_A/ET_B receptor antagonist), 1 mmol/l Bis-indolylmaleimide (BIM; PKC inhibitor), 10 mmol/l PD 098059 (MEK inhibitor) or 10 μg/ml neutralizing anti-TGF-β-antibody. Proliferation of cardiac fibroblasts was assayed by the incorporation of 1 μCi/ml [^{3}H]-Thymidine into a TCA-insoluble cell fraction and collagen synthesis as the percentage of total protein by the incorporation of [^{3}H]-Proline. Radioactivity was determined using a scintillation counter. To confirm whether the conditioned medium-mediated growth of cardiac fibroblasts occurred via PKC activation of ERK1/2, the effect of BIM on ERK1/2 phosphorylation was examined by Western blot analysis.

STATISTICAL ANALYSIS

The values are expressed as mean ± SD. Sample means were compared using Student's t-test and group means by one-way ANOVA where necessary. A level of $p<0.05$ was considered statistically significant.

RESULTS

Isolation, characterization and immortalization of endocardial endothelial cells

Cells isolated from porcine ventricles were characterized as endocardial endothelial cells by their cobblestone pattern, presence of Factor VIII related antigen in the Wiebel-Palade bodies and the uptake of DiI-acetylated-LDL.

The cells transfected with hTERT have been continuously passaged without evidence of altered morphology. The hTERT expressing clones have achieved a passage number 5 to 6 times that of parental cells. The transfected cells exhibit normal endothelial cobblestone morphology, Factor VIII related antigen demonstrated by immunofluorescence, contact

inhibition, retain the capacity to scavenge oxidized LDL and respond to proinflammatory agents in a manner similar to primary EECs, as assessed by their ability to release nitrite on treatment with TNF-α and LPS.

Effect of endocardial endothelial cell conditioned medium on cardiac fibroblast proliferation and collagen synthesis

Incubation of cardiac fibroblasts with EEC conditioned medium resulted in a 53% increase ($p<0.05$) in [^{3}H]-Thymidine incorporation into DNA when compared to fibroblasts grown in the absence of conditioned medium. The conditioned medium caused a 65% increase ($p<0.001$) in the collagen synthesis by fibroblasts.

Effect of TGF-β and ET-1 receptor inhibition on cardiac fibroblast proliferation

Cardiac fibroblasts incubated with conditioned medium containing neutralizing anti-TGF-β demonstrated an increase in DNA synthesis ($p<0.05$) compared to fibroblasts incubated without the antibody. Incubation of fibroblasts in conditioned medium after pretreatment with ET_A receptor antagonist decreased the [^{3}H]-Thymidine uptake to nearly the control values ($p<0.001$) whereas the nonselective ET_A/ET_B receptor antagonist decreased DNA synthesis levels by 1 fold.

Effect of PKC and MAPK inhibition on cardiac fibroblast proliferation

When MAPK was inhibited by pretreatment with PD 098059, the proliferation of fibroblasts was blocked ($p<0.001$) completely. PKC inhibitor BIM in the EEC conditioned medium also depressed ($p<0.001$) DNA synthesis in cardiac fibroblasts.

Effect of PKC inhibition on ERK1/2 phosphorylation

The EEC conditioned medium activated ERK1/2 in cardiac fibroblasts. The conditioned medium increased the phosphorylation of ERK1/2 whereas BIM significantly inhibited the conditioned medium-induced phosphorylation of ERK1/2.

Effect of TNF-α and LPS on EEC mediated proliferation and collagen synthesis of cardiac fibroblasts

TNF-α and LPS attenuated the EEC mediated collagen synthesis and proliferation in cardiac fibroblasts. The levels of ET-1 in the conditioned medium were decreased in response to these proinflammatory agents, whereas TGF-β and nitrite release was increased when compared to the control. Angiotensin levels were undetectable in the conditioned media.

CONCLUSION

Endocardial endothelial cells isolated from the porcine ventricles manifest features characteristic of these cells. It is also demonstrated that EECs are immortalized by the ectopic expression of hTERT. The immortalized cells are morphologically and functionally similar to the primary EECs.

Endocardial endothelial cells stimulate cardiac fibroblast proliferation and collagen synthesis and therefore have a significant role in the regulation of cardiac interstitium. ET-1 in the EE conditioned medium is responsible for the proliferation, through its ET_A receptor. Endogenous ET-1 from fibroblasts has no role in cardiac fibroblast proliferation. The EEC conditioned medium activates ERK in cardiac fibroblasts. The significant reduction in ERK phosphorylation on inhibition of PKC confirms that the signaling

system involved in the EEC induced proliferation of cardiac fibroblasts is through PKC dependent MAPK activation.

The attenuation of the EEC mediated proliferation of cardiac fibroblasts in response to TNF-α maybe due to the decreased secretion of the EEC-derived factor, ET-1 and the increased release of nitric oxide and TGF-β. The remnant proliferation in response to the conditioned medium from TNF-α may be due to the direct proliferative effect of TNF-α on cardiac fibroblasts, which is also mediated through PKC and MAPK.

SIGNIFICANCE OF THE STUDY

The important nature of the work is that the findings are novel and demonstrate that the endocardial endothelium, besides its physiological control over myocyte mechanics, has a significant role in the regulation of fibroblast proliferation and collagen synthesis in the cardiac interstitium. A critical balance in the complex interaction between EECs and cardiac fibroblasts could be of significance in ventricular remodeling in pathological conditions of the heart. The observation that TNF-α and LPS, which play a critical role in the initiation and continuation of inflammation and immunity, influence EEC-cardiac fibroblast interaction indicate influences exerted by TNF-α and LPS on ventricular remodeling in myocardial inflammation. The immortalized cells may be useful tools in extending studies on EECs.

I. INTRODUCTION

I.1. CELL SIGNALING IN THE HEART

The heart is a muscular pump composed of cardiac myocytes. Cardiac myocytes and coronary vasculature are central to the contractile function and viability of the heart where as the cardiac interstitium is vital for the structural integrity of the heart (Weber 1989). In the last decade, the endocardial endothelium which forms the innermost lining of the heart has been acknowledged to be imperative in the modulation of myocardial function. The importance of reciprocal cell-cell signaling involving cellular constituents of cardiac muscle and the cardiac endothelial cells has become a mainstay for research. The investigations have focused on cell-cell signaling in cardiac ontogeny as well as in the regulation of cardiac muscle function and adaptation in the developed heart. In spite of the identification of numerous peptide signaling factors that regulate and co-ordinate cardiac muscle growth, vasculogenesis and angiogenesis, the identity of specific peptide and non-peptide signaling factors involved in modulation of cell-cell communication among the different cell types of the heart remains unknown. Some of the paracrine/autocrine acting trophic factors synthesized and released by cardiac myocytes and/or endothelial cells include VEGF, IGF, acidic and basic FGF, ET, Ang and TGF. Inflammatory cells and activated endothelial cells produce cytokines such as IL-1 and TNF-α (Balligand J-L *et al.* 1997). Insights into the inter relationship between myocytes and non-myocytes link the processes of activation of signal transduction, stimulation of growth factors and second messengers by autocrine, intracrine and paracrine mechanisms.

I.2. CARDIAC ENDOTHELIAL CELL INTERACTION WITH MYOCYTES

The concept that endothelium is an essential structural and functional element of the cardiovascular system emerged after the pioneering discovery by Furchgott and Zawadski of the mandatory role of vascular endothelium in vasomotor tone (Furchgott and Zawadzki 1980). Subsequent studies provided more information on the regulation of vascular tone, permeability and vascular function in general, by endothelial cells. It is now well established that there exists cell-cell interaction between the cardiac endothelial cells and myocytes. Evidences have emerged to support the notion that locally produced humoral factors, possibly derived from microvascular or endocardial endothelium act as key agents in modulating cardiac function. The first report on the modulation of contractile state of cardiac myocytes by endothelial cells in the heart was based on studies employing EECs. The contractile performance of isolated cardiac muscle was intensely modified by the presence of intact EECs (Brutsaert *et al.* 1988; Brutsaert 1989). Later studies extended to the vascular endothelium in myocardial capillaries when Li *et al* demonstrated that selective removal of MVE causes changes similar to that seen on denudation of EE (Li *et al.* 1993). Release of locally active factors secreted by coronary endothelium that influence the function of subjacent cardiac muscle has also been established (McClellan *et al.* 1993; Ramaciotti *et al.* 1993). These studies recorded the existence of several diffusible factors that both up- and down-regulate cardiac contractile function in response to diverse stimuli. Moreover, the close proximity of both MVECs and EECs with adjacent myocytes permits the direct cellular communication and

signaling between both cell types. Further evidence of physiologically significant cell-cell interactions between the endothelial cell population and muscle cells of the heart transpired from pharmacological studies on factors considered to be of endothelial origin, such as endothelins, angiotensin and NO. Other factors of endocardial endothelial origin, which mediate its regulatory role on its neighboring cell types, include bradykinin, natriuretic peptides, prostaglandins, adenylpurines, myofilament desensitizing element and enzymes such as ACE, kinin ase and ECE. An imbalance in the secretion and breakdown of these factors can pilot a disturbance in cardiomyocyte function and alteration in the normal structural design of the ECM, characteristic of cardiovascular diseases.

I. 3. ENDOTHELIN, ANGIOTENSIN AND NITRIC OXIDE

Endothelins are a family of peptides distributed widely in several tissues and exhibit an array of biological actions. Endothelin-1, identified as a vasoconstrictor, is secreted by endothelial cells. Complex interactions exist between endothelin and other cardioactive factors such as NO and Ang II. Endothelin has been identified as a mitogenic agent in many cell types. In addition, it has been demonstrated to augment the production of cytokines and growth factors and to act synergistic to effects of TGF-β and PDGF.

Nitric oxide is synthesized by nitric oxide synthases in several cell types. Nitric oxide secreted in the heart mainly by endothelial cells which contain both eNOS and iNOS, is now recognized to be a ubiquitous inter- and intracellular signaling molecule with key roles in many physiological processes such as regulation

of vascular tone and endothelial proliferation, inhibition of platelet adhesion and aggregation, peripheral and central neurotransmission as well as macrophage cytotoxicity (Shah *et al.* 1996). Besides its vasodilator role, it also influences the cell growth and function.

Angiotensin II is a peptide hormone, which in the heart is released mainly by the MVE (Balligand *et al.* 1997). Angiotensin II acts as a paracrine factor regulating the growth and phenotype of vascular SMCs, cardiac fibroblasts and cardiac myocytes (Sadoshima and Izumo 1993; Ito *et al.* 1993; Lokuta *et al.* 1994). It is interesting to note that defined medium from cells that were serum starved for 48 hours after attaining confluence did not contain exogenous renin or angiotensin suggesting that the angiotensin release in the heart is due to de novo synthesis.

Based on reports from several studies over the last decade, the modulator effect of endocardial endothelium on myocytes is firmly established. However, less well characterized, is the role of EE on the cardiac ECM and its metabolism, despite the fact that the ECM comprises a major bulk of the myocardium in health and the bulk of abnormal depositions during cardiac diseases.

I. 4. CARDIAC INTERSTITIUM AND ITS COMPONENTS

Heart, like other organs, is composed of parenchyma and stroma. Cardiomyocytes, which are the specialized parenchymal cells of the heart, are supported by the stroma, made up of a complex network of collagen fibers and different cell types of mesenchymal origin including fibroblasts, vascular endothelial cells, smooth muscle cells, macrophages, pericytes and neurons which are bathed in a

gel-type ground substance composed of glycosaminoglycans and glycoproteins (Jugdutt 2004). The cardiac ECM plays a pivotal role in the maintenance and adaptation of the structure in the normal heart. It is also closely linked with the remodeling and related impairment of cardiac function that accompanies diseases such as heart failure and hypertension (Maisch 1995). Until recently it has been presumed that when the myocardium fails, it is due to systolic failure and the primary defect must lie within the myocytes. Alongside reports overturning the perception that the cardiac ECM is simply an inert substratum, the concept of myocardial failure took a broader stance as evidence mounted to indicate that abnormalities in any of the cellular components of the myocardium or its regulation may serve as primary mechanism for heart failure.

Endothelial cells and vascular SMCs in the cardiac interstitium are highly specialized and fixed anatomic elements while cardiac fibroblasts which constitute nearly two thirds of the cell population are multipotential cells that are free to move within the extra cellular space. These cells are responsible for the synthesis of type I and type III collagens, the major fibrillar collagens of the myocardial compartment. These collagens are the types involved in interstitial and perivascular fibrosis of the myocardium. Fibroblasts not being terminally differentiated are capable of reentering the cell cycle and undergoing hyperplastic growth. While growth of endothelial and vascular SMCs manifest as intimal and medial thickening of the vasculature, increased myocardial collagen synthesis and accumulation of fibrillar collagen, relative to its breakdown is indicative of fibroblast growth (Weber and Brilla 1991).

I. 5. ENDOCARDIAL ENDOTHELIAL CELLS AND FIBROBLASTS

Considering the above facts that endothelial cells stimulate connective tissue cells and EE regulate myocyte function, it was interesting to explore whether EECs have a modulator role in regulating the cardiac interstitial cells. Mediators secreted by endothelial cells, which determine the effect of EECs on underlying cardiac myocytes and effect of vascular endothelial cells on blood vessels also may be responsible for bringing about a regulatory effect of EECs on cardiac fibroblasts. One or more of these factors may stimulate or inhibit growth and function of cardiac fibroblasts, consequently affecting myocardial remodeling.

It is likely that inflammatory cytokines such as interleukins, TNF and interferons exert pharmacological actions on the function of cardiac interstitial components through their effects on endocardial endothelial cells. Endocardial endothelial cells may transduce or amplify signals generated in response to injury, causing alterations in the phenotype and function of surrounding and downstream cells such as cardiomyocytes and fibroblasts.

I. 6. OBJECTIVES OF THE PRESENT STUDY

The broad aim of the study was to explore the endocardial endothelial signaling pathways regulating cardiac interstitial cells.

The specific objectives were

1. To isolate, characterize and immortalize endocardial endothelial cells

2. To determine whether endocardial endothelium has a role in regulating cardiac fibroblast growth and function

3. To identify the endocardial endothelial cell derived factors, if any, which stimulate cardiac fibroblast proliferation and collagen synthesis

4. To delineate the signaling pathways involved in the endocardial endothelial cell regulation of cardiac fibroblast function

5. To ascertain the influence of pro-inflammatory cytokines TNF-α and LPS, as may occur in cardiac inflammatory states, on EEC cardiac fibroblast interaction

I.3. RESULTS AT A GLANCE

I.3.1. Isolated cells are characterized as endocardial endothelial cells

Cells isolated from the porcine ventricles were identified as endocardial endothelial cells by their cobblestone morphology at confluence, positive staining for Factor-VIII related antigen and their ability to scavenge DiI-Ac-LDL.

I.3.2. Endocardial endothelial cells are immortalized and characterized

Endocardial endothelial cells were immortalized by ectopic expression of human telomerase reverse transcriptase. The cells have attained a passage number 5 to 6 times that of parental cells, fail to express porcine TERT and express hTERT at the protein and transcript level. The cells retain the characteristics of the primary cells in that they demonstrate cobblestone morphology, stain positive for Factor-VIII related antigen, are capable of DiI-Ac-LDL uptake and respond to pro-inflammatory cytokines by releasing elevated levels of nitric oxide. These cells also have a

significant survival advantage over the primary cells because they require only a decreased serum percentage for their growth in culture.

I.3.3. Endocardial endothelial cells induce proliferation and collagen synthesis of cardiac fibroblasts

Conditioned medium from EECs enhanced the proliferation of cardiac fibroblasts, suggesting a proliferative potential of EECs. The EEC conditioned medium also augmented collagen synthesis in cardiac fibroblasts. Together, the data substantiates that EECs secrete factors into the conditioned medium that are both mitogenic and fibrogenic. Assays to explore the effect of EEC-derived factors in the conditioned medium and their inhibition on DNA synthesis by cardiac fibroblasts further proved that ET-1, through its ET_A receptor is the major factor mediating the observed proliferative effect. Data from further experiments substantiated that the signaling cascade induced by EEC conditioned medium, leading to the proliferation of cardiac fibroblasts involves MAPK activation dependent on PKC.

Pro-inflammatory agents TNF-α and LPS attenuated the proliferation and collagen synthesis of cardiac fibroblasts in response to EEC conditioned medium, suggesting that in inflammatory conditions of the heart, endocardial endothelium may act as a negative modulator to prevent fibroblast hyperplasia and unwarranted scarring response.

This study for the first time established that endocardial endothelial cells can be immortalized by ectopic expression of hTERT. The regulatory role of endocardial endothelium on the cardiac interstitium is noteworthy, considering its possible

contribution in the development of myocardial fibrosis and ventricular remodeling as well as in the pathogenesis of perivascular fibrosis and endomyocardial fibrosis.

II. REVIEW OF LITERATURE

II.1. CELL-CELL INTERACTION IN HUMAN PHYSIOLOGY – FOCUS ON CARDIOVASCULAR PHYSIOLOGY

Why did multicellular organisms take 2.5 billion years more to develop than the unicellular organisms? A plausible answer is that a multicellular organism needs elaborate signaling mechanisms that enable its cells to communicate with one another so as to coordinate their behavior for the benefit of the organism as a whole. The complexity of cellular organization in multicellular organisms warrants a role for cell-cell interactions in both cell and tissue function (Long *et al.* 1991).

Cells in a multicellular organism must communicate with one another, in order to direct and regulate growth, development and organization. Importance of the social controls becomes apparent when the controls fail as in cancer, leading to the death of the organism. Animal cells communicate by secreting chemicals that signal to distant cells, display cell surface chemicals that influence other cells in direct physical contact and communicate directly via porous cellular junctions called gap junctions. The signaling molecules include proteins, small peptides, amino acids, steroids, retinoids, fatty acid derivatives and even dissolved gases such as nitric oxide and carbon monoxide (Alberts *et al.* 1994). These signaling molecules are made available to the multitude of cells in the body mainly by the circulatory system. The circulatory system by itself is a collection of cells which coordinate with each other for the normal functioning of the organ as well as the organism as a whole.

The heart is the central organ in the circulatory system. Many invertebrates such as bivalves exhibit an open circulatory system where the blood flows freely in

the body cavity. In these animals, the blood collects in a series of specialized cavities where it is returned to the heart and is again released into the body. Invertebrates such as earthworm do not actually have a heart but has five aortic arches that can serve the same purpose. In fishes which have only one circuit, the heart has only two chambers – one atrium and one ventricle. The blood pumps through the gills and onto the body before returning to the heart. Amphibians and reptiles have a three chambered heart. Both atria empty into the single ventricle, mixing the oxygen-rich blood returning from the lungs and the deoxygenated blood from the tissues. The spiral valve in these species is essential to keep the mixing of the two types of blood to a minimum. Mammals and birds exhibit complete separation of the heart into four chambers – two ventricles and two atria.

A functioning cardiovascular system is required by the middle of the third week of gestation to satisfy the nutritional requirements of the developing human embryo. Cardiac development requires the integration of cell commitment, growth, looping, septation and chamber specification. Soon after gastrulation, cardiac progenitor cells within the anterior lateral plate mesoderm become committed to a cardiogenic fate in response to an inducing signal that originates from the endoderm. Right from the development of the heart, there exists a complex interplay between the several molecules released from the cardiac cell types, implicating that cross talk between these cells is important to the normal cardiac function. The pathways by which cells transduce signals from extra cellular mediators to effect changes in cell function are extraordinarily intricate. Alterations in the molecular mediators occur at the level of gene polymorphisms, altered gene expression or post-translational

modification and these contribute to pathophysiologic changes in cell and organ function that underlie cardiovascular disease.

The cell types of the heart include very specialized cardiac myocytes constituting one third of the total number of cells and the non-myocytes such as fibroblasts, endothelial cells and vascular smooth muscle cells, accounting for majority of the remaining two thirds. Myocytes are the cells responsible for the contractile ability of the heart and are the largest of the cardiac cell types. Endothelial cells which line the coronary and lymphatic vasculature and endocardium influence vasomotor reactivity of blood containing vessels. Vascular smooth muscle cells which are found in epicardial and intramyocardial coronary arteries and arterioles influence the reactivity and vasodilatory capacity of these vessels, in a manner similar to endothelial cells. Cardiac fibroblasts are the cells responsible for the production of degradation of structural proteins such as collagen and elastin in the myocardium. Macrophages and mast cells which are defenders against foreign proteins constitute a minor proportion of the cardiac cell types.

II.2. INTERACTION OF ENDOTHELIAL CELLS WITH OTHER VASCULAR CELLS

Endothelial cells form a continuous monolayer that lines the cavitary surface of all blood vessels and the heart. Endothelium covering the luminal surface of heart and blood vessels is in a unique position, separating the circulating blood from the other parts of the cardiovascular system. For a long time the endothelium was considered as "a layer of nucleated cellophane" of the blood vessel, with no major

functional properties. It is now recognized as a massive, regionally specific, multifunctional organ (Mantovani *et al.* 1997). Vascular endothelium has numerous important functions, including the regulation of vascular tone, permeability and blood flow, vascular growth and repair, anti-thrombogenicity and hemostasis, cytoprotection and immunological and inflammatory responses.

Furchgott and Zawadski in their classical study demonstrated that endothelial cells lining the lumen of blood vessels play an obligatory role in the vasoactive effect of acetylcholine (Furchgott and Zawadzki 1980). The mechanisms of vasoregulation by endothelium have since been elucidated. Endothelium-dependent vasorelaxation is mediated mainly by the release of NO and prostacyclin. Endothelin or constrictor prostanoids mediate endothelium dependent vasoconstriction. Besides chemical transmitters, changes in oxygen tension and blood flow alter vascular endothelial cell production of mediators and can induce a new level of vascular tone (Mebazaa *et al.* 1995). Endothelial cells and subjacent ECM constitute a heterogeneous structure with specific morphological and physiological properties in different parts of the vasculature (Andries *et al.*, 1995). A complex interaction exists between the endothelium and neighboring non-endothelial cells, mediated in part by soluble peptides and specific effector substances released by endothelial cells. Several studies have reported the regulatory role of vascular endothelial cells on neighboring non-endothelial cells such as myocytes, fibroblasts and smooth muscle cells.

II.2.1. Interaction of endothelial cells with myocytes

A study using cardiac MVECs and adult cardiac myocytes demonstrated that MVECs produce trophic factors that are highly effective in reorganization of the contractile apparatus, induction of protein synthesis, increase in survival of myocytes, induction of myofibrillogenesis and reexpression of fetal α-smooth muscle actin which is usually not expressed in adult myocytes (Kubin *et al.* 1999). Presence of endothelial cells markedly inhibit apoptosis and necrosis in cardiac myocytes and synchronize their contraction and connexin 43 expression, indicating that endothelial cells specifically promote cardiac myocyte survival and organization (Narmoneva *et al.* 2004). Endothelins released from endothelial cells have growth-enhancing and mitogenic effects in a number of cells and tissues (Rubanyi and Polokoff 1994), including neonatal rat cardiac myocytes. Agents produced by myocytes can likewise affect endothelial function. Cardiac myocytes may release factors depending on their metabolic and mechanical state. These factors may interact with endothelial cells causing them to release mediators of cardiac contractility (Ramaciotti *et al.* 1992). Some of the endothelial mediators may be produced within the cardiac myocytes themselves, often under pathological conditions.

II.2.2. Interaction of endothelial cells with fibroblasts

In two of the earliest reports on the mitogenic effect of endothelial cells, Gajdusek *et al* and DiCorleto demonstrated that aortic endothelial cells produce polypeptide growth factors, either related or distinct from PDGF, which supports growth of 3T3 cells, fibroblasts and SMCs (Gajdusek *et al.* 1980; DiCorleto 1984).

Endothelial cells injured by mechanical forces or by radiation release factors which induce growth or protein synthesis in fibroblasts (McNeil *et al.* 1989; Flavin *et al.* 1990). According to a report by Vlodavsky *et al*, bovine aortic and corneal endothelial cells synthesize a growth factor, probably bFGF which remains both cell-associated and is secreted into the subendothelial ECM deposited by these cells. This suggests that endothelium can store growth factors capable of autocrine growth promotion in two ways: by sequestering growth factor within the cell and by incorporating it in the ECM (Vlodavsky *et al.*1987-a). In addition, BAECs secrete growth factors into the conditioned medium, which are mitogenic for VSMCs and fibroblasts but not for endothelial cells, implying that these growth factors may act in a paracrine manner to stimulate the proliferation of neighboring cells (Vlodavsky *et al.*1987-b). Conditioned medium from these cells also activate collagen and total protein production in human embryonic lung fibroblasts (Villanueva *et al.* 1991). Reports exist to substantiate that a substance derived from endothelial cells signals an increase in cardiac fibroblast collagen synthesis and augmentation of collagenase activity. The report also indicates that neither aldosterone nor Ang II influences the response in cardiac fibroblast collagen metabolism to endothelial cell conditioned medium (Guarda *et al.* 1993).

In 1988, Yanagisawa and colleagues reported the isolation of a potent 21-amino acid vasoconstrictor peptide from the supernatant of cultured porcine aortic endothelial cells, which they termed "endothelin" (Yanagisawa *et al.* 1988). Among the three isoforms of endothelin, endothelin-1 is the only isoform secreted by endothelial cells and is the most potent vasoconstrictor identified to date. The endothelin converting enzyme-1 isoforms ECE-1α and ECE-1β responsible for

conversion of big ET-1 to ET-1 are located in the perinuclear region in the endothelial cells in the Weibel-Palade bodies (Russell *et al.* 1998). Recently, ECE-independent pathways such as chymase and non-ECE metalloprotease, contributing to ET-1 formation have been identified (Luŝcher and Barton. 2000). Two distinct sarcolemmal receptors exist for endothelin, viz., the ET_A and ET_B receptor. Only the ET_B receptor, which is important in the clearance of circulating ET-1, is located on the endothelium. These receptors are also present in high density on myocytes, fibroblasts and conduction tissue and are coupled to multiple subcellular signaling pathways which can stimulate phosphoinositide breakdown, decrease cAMP levels, enhance arachidonic acid metabolism and G inhibitory proteins.

ET-1 and ET-3 increase the synthesis of fibrillar collagens and fibroblast DNA synthesis in adult cardiac fibroblasts, which suggests that both ET_A and ET_B mediate these effects (Guarda *et al.* 1993; Turner *et al.* 2004). In addition to its vasoconstrictive and mitogenic effects, ET stimulates the production of extra cellular matrix proteins, cytokines and growth factors such as VEGF, bFGF and epiregulin and augments the effects of TGF-β and PDGF. However, different populations of fibroblasts can exhibit heterogeneous responses to ET-1 (Dawes *et al.* 1996).

Vascular endothelial cells are a source of two other potent multifunctional regulatory molecules, bFGF and TGF-β, which influence the growth of fibroblasts, SMCs and white blood cells (Hannan *et al.* 1988). TGF-β is capable of stimulating collagen synthesis without activating fibroblast proliferation. Endothelial cells increase the proliferation and collagen synthesis in fibroblasts in co-culture

experiments using human umbilical vein endothelial cell line and dermal fibroblasts (Denton *et al.* 1996). Microvascular endothelial cells respond to angiogenic stimuli by increasing the expression of collagenase and TIMPs to modulate the synthesis and deposition of matrix components (Herron *et al.* 1986; Herron *et al.* 1986).

II.2.3. Interaction of endothelial cells with smooth muscle cells

The postulation that endothelial cells affect SMC function is supported by reports that endothelial cell seeding of injured arterial surfaces can retard the development of intimal hyperplasia. Endothelial cells inhibit SMC collagen synthesis and down regulate SMC type I collagen gene expression (Powell *et al.* 1997), in a manner independent of TGF-β. Besides these effects, in a co-culture system, endothelial cells induce a more spindle shaped, filamentous SMC phenotype, inhibit hill and valley formation, increase SMC proliferation rate and ultimate cell density (Fillinger *et al.* 1997).

A potential mechanism of protein regulation at the post translational level by the endothelium-derived factor, NO is through inhibition of PKC activity (Witte *et al.* 2000). Data from co-culture studies using coronary artery endothelium and coronary VSMCs suggest that NO has an inhibitory role on collagen type I and III production (Myers and Tanner 1998), a finding consistent with an integral role for the endothelium in modulating ECM metabolism. Endothelin not only produces sustained contraction of vascular smooth muscle *in vitro* but also enhances smooth muscle and cardiac cell growth (Kramer *et al.* 1992). When applied to aortic SMCs, ET-1 alone in

the absence of other growth promoting agents could induce MAPK activation, DNA synthesis and cell proliferation (Fujitani *et al.* 1995).

II.3. SIGNALING PATHWAYS AND MOLECULES INVOLVED IN INTERACTION OF ENDOTHELIAL CELLS WITH OTHER VASCULAR CELLS

Endothelium-derived factors such as endothelin, NO, angiotensin and TGF are privy to the interaction of endothelial cells with other cells of the vascular system. Majority of the actions of NO is through the activation of soluble guanylyl cyclase to augment cGMP content. ET-1 mediated growth responses are through various pathways that may involve PKC, depending on the cell type. Increased circulating levels of ET-1 would be deleterious to the myocardium because it will restrict coronary flow, promote hypertrophy of cardiomyocytes and stimulate fibroblast proliferation. ET-1 promotion of vasoconstriction and induction of cell growth, cell adhesion and thrombosis is predominantly via ET_A receptors (Luscher and Barton 2000). Yanagisawa *et al* demonstrated that 12-O-tetradecanoyl phorbol-13-acetate (TPA), a potent PKC activator caused a marked and immediate induction of preproET mRNA in human endothelial cells and that two TPA-responsive elements are located in the 5'-flanking region of the structural gene of preproET (Yanagisawa *et al.* 1989). These cis-acting nucleotide sequences are binding sites for c-jun and c-fos proto oncogene products (Rauscher *et al.* 1988), suggesting that activation of PKC by TPA is functionally coupled to activation of these transcription factors, in turn affecting the preproET (Emori *et al.* 1989).

On activation, PKC isozymes translocate to characteristic intracellular sites and act as upstream mediators of signaling. ERK is directly involved in signal transduction processes that lead to differentiation or proliferation of various cell types. It is recognized that all extra cellular mitogens ultimately activate MAPKs to initiate cell division, but there are distinct upstream pathways that cause the activation of MAPKs. ET-1 induced cell cycle progression is mediated at least partly by ERK, PKC and PI-3-kinase pathways which are involved at distinct points in the cycle (Suzuki *et al.* 1999). Many of the inhibitory actions of ET are via cGMP- mediated inhibition of phosphoinositol metabolism. Both receptor subtypes of ET-1 can couple to Gq and Gi proteins and the $\propto$ subunit of Gq may mediate a PKC-dependent, Ras-independent activation of MAPK. In contrast to previous reports that cardiac fibroblast proliferation stimulated by Ang II acts independently of PKC, the ET-1 mediated mitogenic effect requires PKC activation. This requirement for PKC in the ET-1 signaling pathway suggests that Gq is an upstream mediator of this response. The pathways downstream to PKC are not clearly identified in cardiac fibroblasts (Piacentini *et al.* 2000). ET-1 induces hypertrophy of cardiomyocytes through activation of PLC, PKC and ERK 1/2. PKC is thought to activate the p42 and p44 isoforms of MAPKs, leading to phosphorylation and activation of transcription factors c-fos and c-jun, with consequent expression of hypertrophic genes (Brunner *et al.* 2006).

Several factors such as inflammatory cytokines, hormones, pharmacological agents and hemodynamic factors affect the interaction of endothelial cells with other

vascular cells. Among these, the pro-inflammatory cytokines, TNF-∝ and IL-1 play a predominant role in pathological conditions. TNF-∝ and IL-1, which share many common activities, activate unique signaling mechanisms in cells. Increased phosphorylation of target proteins occurs in TNF-treated cells. This is likely to be due to activation of intracellular kinases, such as MAPK since the treatment with TNF produce a rapid increase in MAPK activity in human fibroblasts (Vietor *et al.* 1993). Besides activating PKC, PKA and MAPKs which are implicated in the actions of many hormones, TNF also activates JNK kinases. In bovine aortic endothelial cells, TNF activates JNK and ceramide activated protein kinase, augments Jun-b expression, elevates the production and secretion of prostacyclin and when protein synthesis is inhibited, induces cytotoxicity. The protein kinase inhibitor dimethylaminopurine abrogates or attenuates each of these responses (Marino *et al.* 1996). MAPK is activated by TNF in cells in which TNF has either mitogenic or growth inhibitory actions, suggesting that MAPK may be part of a more general signal transduction pathway involved in multiple TNF actions. It is construed that MAPK proteins represent the most abundant targets of enhanced tyrosine kinase activity resulting from TNF action (Vietor *et al.* 1993). Some of the endothelial cell types release NO for a prolonged period (Lamas *et al.* 1991; Estrada *et al.* 1995) and ET-1 for a shorter period in response to TNF-∝ (Barker *et al.* 2002; Golden *et al.* 1995; Zhao *et al.* 2005). In BAECs, ET-1 release is maximal over 1-8 hours and decline to or below the baseline values after 16 hours (Marsden and Brenner 1992).

II.4. CARDIAC ENDOTHELIUM

Cardiac endothelium has a significant role in the regulation of cardiac function and homeostasis. The heart contains two kinds of endothelial cells: the vascular endothelial cells, which line all coronary blood vessels, and the endocardial endothelial cells, which line the cardiac cavities. Although there is broad similarity in structure and function between these cell types, there are also significant differences. Endothelial diversity is reflected at the structural, functional and transcriptional level. This is not surprising given the fact that the immense EE surface is continuously exposed to all of the circulating blood, whereas MVE is reached by only 3-5% of it. There is intimate contact between the cardiac myocytes and cardiac endothelial cells. It is estimated that no cardiocyte is more than 2 to 3 μm away from a coronary vascular endothelial cell though the EE monolayer is in close contact with only a tiny proportion of the total myocardium (Shah *et al.* 1996). Both MVE and EE cells exert a paracrine influence on the contraction of the myocardium and these influences are mediated by several diffusible factors released by the endothelial cells. Both microvascular and EE cells produce common autacoids and share similar roles in signal transduction induced by neurotransmitters, hormones or mechanical stimuli. They are however two distinct cell populations with dissimilar embryological origin, cytoskeletal organization, receptor mediated functions and electrophysiological properties (Table 1). They also differ with respect to the release of prostanoids and have distinguishable growth rates in culture.

In the developing heart, EE is responsible for myocardial trabeculation, cushion formation and formation of Purkinje fibers. Endocardial endothelium is probably the first of the endothelium to form and originates from the cardiogenic plate, whereas the endothelium of the coronary blood vessels and its microvasculature develops later and originate from the mesothelial cells of the epicardium. Endocardial endothelium is formed by the process of vasculogenesis; by contrast, coronary blood vessels and capillaries are formed by angiogenesis.

The cytoskeleton in endothelial cells plays an important role in intracellular transport and secretion, regulation of transendothelial transport, cell adhesion and regulation of cell shape (Andries and Brutsaert 1993). In the heart, endothelial cell shape and organization of the actin filament system appears not only to be influenced by shear stress, but also by mechanical stress, caused by deformation of the heart wall. Features of the cytoskeleton such as the presence of stress fibers, vimentin filaments and microtubules also are different in EECs and MVECs. Vascular endothelial cells contain more actin filament bundles or stress fibers compared to the EE cells. These stress fibers are oriented mainly by shear-stress forces and are involved in maintenance of the integrity of the endothelium, by strengthening their cell surface and enhancing cell-substratum adhesion. Vimentin filaments and microtubules are closely packed and aligned parallel to the cell axis in the MVE whereas in EE cells these form an extensive filamentous network (Brutsaert *et al.* 1996; Brutsaert and Andries 1992). Cytoskeleton in endothelial cells may play a role in intracellular transport, regulation of cell shape, cell adhesion and cellular

mechanism. Accordingly, differences in cytoskeletal organization between vascular endothelial cells and EE cells may relate to differences in these properties.

	Endocardial endothelium	Microvascular endothelium
Location	Cavities of the heart	Capillaries of the heart
Origin	Cardiogenic plate (Vasculogenesis)	Mesothelial cells of epicardium (Angiogenesis)
Cytoskeleton	Stress fibers are more	Stress fibers are less
	Vimentin filaments and microtubules form a filamentous network	Vimentin filaments and microtubules are closely packed and aligned parallel to cell axis
Organelles	Larger Golgi apparatus and higher metabolic activity	Smaller Golgi apparatus and lower metabolic activity
	Vesicles are scarce	Vesicles are abundant
Cell junctions	Intercellular clefts are tortuous and deep with one or two tight junctions	Intercellular clefts are simple and shallow with few tight junctions
	Gap junctions are abundant	Gap junctions are probably lacking

Table 1: Differences between endocardial endothelial and microvascular endothelial cells

Gap junctions are present in areas with extensive overlap of endothelial cells and have been postulated to play a role in the regulation of cell proliferation (Brutsaert and Andries 1992). Gap junctions are low resistance channels and ions or second messengers can pass through them easily, activating the neighboring EE cells. Thus, binding of first messengers to receptors on a single EE cell brings about a signaling cascade, amplifying the cell response (Brutsaert *et al.* 1996). The probable lack of gap junctions in MVE implies a more local regulatory control of myocardial function. In addition, major differences are observed with respect to the various adhesion molecules – ICAM-1 and antigens of the major histocompatibility complex (MCHI, MCHII and DR) being more prominent in the MVE than in EE. Endothelial markers PAL-E and von Willebrand factor are found to be abundant in EE and hardly at all in MVE. Golgi bodies in EE by far exceed in size those in MVE, suggesting a higher metabolic synthetic capacity of EE. The MVE have an abundance of vesicles compared to the scarce number of vesicles in EE and this signifies a prominent vesicular transport and transcellular transport capacity in the former than in the latter (Brutsaert *et al.* 1998).

Genes commonly expressed in EE and MVE include GATA-GT2, oxidized LDL receptor 1 (OLR1), apolipoprotein E (APOE), creatine kinase brain type (CKB), parathyroid hormone receptor (PTHR) and UDP glucosyl transferase 1 (UGT1A7) all of which participate in the functional interaction of these endothelial cells with cardiac myocytes. A few genes, such as sortilin, macrophage metalloelastase and A5D3 protein gene have been classified as endocardial endothelial genes based on

their preferential expression in EE and their lower mRNA abundance in MVECs and aortic endothelial cells (Hendrickx *et al.* 2004).

Endothelial cell/cardiac myocyte interaction is now recognized to be important physiologically as well as in disease states. Several substances released or metabolized by cardiac endothelial cells have direct effects on cardiac myocyte function. Intercellular signaling between myocytes and MVECs regulates the abundance of precursor transcripts of endothelin, which has documented effects on myocyte function and gene expression (Nishida *et al.* 1993). Other factors of interest include NO, prostanoids, natriuretic peptides, Ang II, kinins, reactive oxygen species and adenyl purines. Cardiac endothelial cells also possess enzymatic activities such as ACE and kininase activity, which contribute to changes in local levels of Ang II and bradykinin.

Cardiac endothelial cell function can be affected by several factors including cytokines, pharmacological agents. Shear stress is also an important determinant of endothelial cell phenotype, function, gene expression.

II.5. ENDOCARDIAL ENDOTHELIUM - FUNCTIONAL MORPHOLOGY

In the mature heart, the inner surface of the ventricular chambers is extremely irregular and spongy, consisting of ridges and valleys formed by cord like structures, the trabeculae carneae and papillary muscles, which support the cardiac valves. Ventricular wall, trabeculae and papillary muscles consist of myocardium covered on the cavitary side by the endocardium. In all animal species, except in some

invertebrates, the luminal surface of the endocardium is lined by a monolayer of EE cells (Lannigan and Zaki 1966; Melax and Leeson 1967). Endocardial endothelial cells in vertebrates form one continuous layer of closely apposed cells. In the human ventricle, this monolayer of EE cells has a thickness of ~0.5μm and rests on a thin basement membrane, which consists of a continuous basal lamina and a subjacent layer of reticular fibers. Underneath the basement membrane is a fibroelastic layer with collagen and elastic fibers (Howse *et al.* 1970; Klein and Bock 1983; Lannigan and Zaki 1966), occasional smooth muscle fibers (Davies 1960; Lannigan and Zaki 1966), fibroblast-like cells, and myelinated and unmyelinated axons (Yokota 1984). The subendocardium lies below the endocardium and is characterized by the presence of thick collagen fibers and small blood vessels (Lannigan and Zaki 1966; Klein and Bock 1983).

Endocardium as a whole is of variable thickness, normally thicker in atria than in ventricles and in the atria, thicker in the left than the right. In the outflow tracts of ventricles, collagen and elastic fibers are more abundant than in the inflow tract, where the endocardium is more delicate, with fewer collagen, elastic and smooth muscle fibers. These regional differences may relate to differences in pressure, turbulence, or shear stress.

The surface architecture of EE cells consists of bulging nuclei, peripheral folds, a variable number of microvilli, small ruffle-like protrusions or microplicae and discrete membrane invaginations or pits (Edanaga 1975; Peine and Low 1975; Pexieder 1981). The number and distribution of these structures vary according to the

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site in the heart. These structures increase the surface area and enhance the interaction with the circulating blood by slowing down the blood flow over the surface. The surface structure of the EE with numerous microvilli provides a high ratio of EE surface area to ventricular volume. A large contact surface area also indicates an important sensory function for the EE (Brutsaert and Andries 1992).

At least four different types of endothelial junctions have been defined: tight junctions, gap junctions, adherence junctions and syndesmos (Dejana *et al.* 1995). Endocardial endothelial cells show extensive intercellular overlap and interdigitations between the junctional areas. In addition to the open clefts, a few junctional points obliterate the intercellular clefts, thus forming the tight junction. The endothelium forms a selectively permeable barrier to the passage of macromolecules from blood to the tissues and this permeability of the cells is regulated by zonula occludens or tight junctions (Andries and Brutsaert 1994). Tight junctions are always present on the luminal side of the EE and at this side the glycocalyx is better developed than at the basolateral side below the tight junctions. Intercellular clefts free of junctions are suggested to be the main route for transendothelial transport of macromolecules. The negatively charged fibrous matrix of the glycocalyx, which lines the long intercellular routes, might also influence endothelial permeability. Intercellular junctions are more complex and 3 to 5 times deeper in EE than in MVE, whereas the tight junctions have a similar structure in both endothelia (Brutsaert *et al.* 1998). Endocardial endothelial cells being larger than the MVE have longer intercellular clefts per unit endothelial surface area. In the EE, PECAM staining is typically confined to the border zone of the EE cells corresponding to the zone of cellular overlap and intercellular clefts. By

contrast in MVE, PECAM stain more diffusely over the entire cell surface. Hence, although present in both cell types, the pronounced differences in distribution of PECAM suggest profound differences in transendothelial permeability between these two cardiac sites (Andries *et al.* 1998). The difference in permeability between the EE and the MVE is possibly related to the distinctive electrochemical properties of the endocardium (Brutsaert *et al.* 1996).

II.6. ENDOCARDIAL ENDOTHELIAL REGULATION OF MYOCARDIAL CELL MECHANISMS

The paracrine influence of endothelial cells on various functions of the peripheral vasculature (e.g. regulation of vascular tone and repair, inhibition of platelet adhesion and aggregation) is well recognized. The balance between endothelium released vasodilators and vasoconstrictors is an important determinant of vascular tone. Several factors, including changes in O_2 tension and flow can alter vascular endothelial production of mediators and induce a new equilibrium in vascular tone (Mebazaa *et al.* 1995).

The initial suggestion that endocardium influences myocardial contractile function came from Brutsaert and coworkers who reported that selective denudation of EE in isolated feline cardiac papillary muscles resulted in a typical response, a shortening of the duration of isometric twitch contractions and a slight reduction in peak force without major reduction in the rate of force development (Brutsaert *et al.* 1988). Subsequent studies utilizing isolated ferret and rat papillary muscle preparations and cultured porcine ventricular EE cells demonstrated that the

influences on myocyte contractile function involved the release of diffusible factors by EE cells (Smith *et al.* 1991; Ramaciotti *et al.* 1992), i.e., a paracrine action. The presence of an intact EE helps to modulate LVF by prolonging ejection duration, increasing end diastolic volume and slightly increasing systolic peak performance (Kelly *et al.* 1996). Myocardial inotropic state depends primarily on the amount of transient intracellular calcium $[Ca^{2+}]_i$ and the affinity of the contractile proteins for the available calcium. Endocardial endothelium seems to modulate the affinity of the contractile proteins. Removal or damage of endocardium decreases the Ca^{2+} responsiveness of myofilaments (Wang and Morgan 1992)

Ventricular performance of the heart relies largely on autoregulatory mechanisms. Autoregulation is accomplished through intrinsic feedback by the loading conditions and the length of the muscle fibers (heterometric autoregulation) and through extrinsic feedback by the activity of neurohormonal system (homeometric autoregulation). Although the mechanisms of cardiac endothelial modulation of myocardial performance have not yet been fully elucidated, it is definite that cardiac endothelial release of several inotropic substances, such as ETs, PGs and NO are involved. Besides the autoregulatory control mechanisms, cardiac endothelial cells directly modulate underlying cardiomyocytes and mediate feedback through interaction with superfusing blood. Intracavitary autoregulation by EE and intracoronary autoregulation by MVE add a unique and independent type of controlling mechanism to the system (Brutsaert *et al.* 1996).

The EE is also known to influence myocardial growth. The concept that cardiac endothelial cells might be indispensable for myocardial growth was suggested by experiments with cardiac myocytes cocultured with endothelial cells. Only in the presence of cardiac endothelial cells could the cardiomyocytes maintain their adult phenotype; when vascular endothelial cells from aorta or fibroblasts were added, cultured cardiomyocytes continued to undergo dedifferentiation and reexpression of fetal proteins expressed in long-term monoculture of adult cardiac myocytes (Eid *et al.* 1990). The various receptors on EE for the circulating blood components such as ET, atrial natriuretic peptide, mineralocorticoid and ACE initiate the release of factors from the endocardium through receptor-mediated mechanisms. The distribution pattern of these receptors is non-uniform and differs between different populations of endothelial cells. The presence of ACE or kininase activity in the EE and MVE enables the modulation of local levels of Ang I and Ang II as well as bradykinin. Similar to endothelial modulation of vascular tone, myocardial contraction may be modulated by cardioactive agents released from the cardiac endothelium.

II.6.1. Mechanisms of signal transduction from endocardial endothelium to cardiomyocytes

Two mechanisms have been proposed for the signal transduction from EE to the underlying myocytes: (1) The EECs on stimulation may secrete factors that bring about a change in the contractile state of the cardiac myocytes (stimulus-secretion-contraction coupling). (2) EE may act as a physicochemical barrier, which controls the ionic constitution of the interstitial milieu of the myocytes and thus modulate cardiac performance (blood-heart barrier) (Brutsaert *et al.* 1996) (Figure 1).

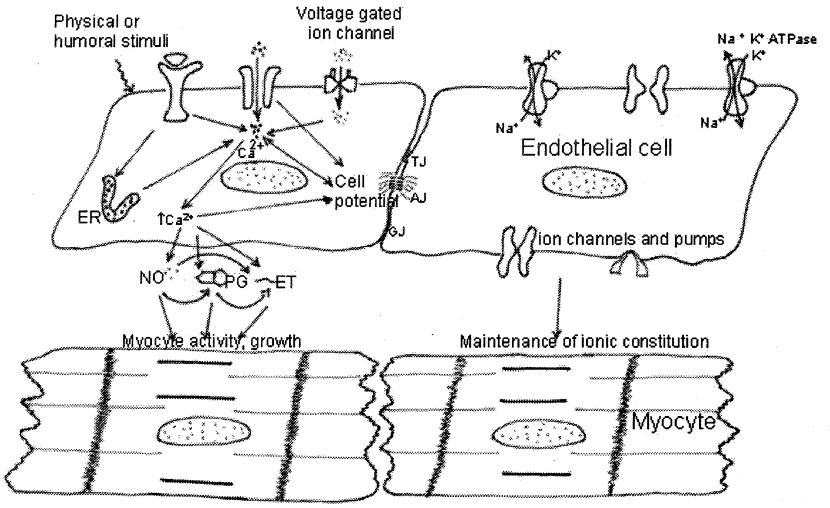

Figure 1 : Schematic diagram of pathways and mechanisms of endothelial regulation of myocardium. ER- endoplasmic reticulum, TJ- tight junction, AJ- adherens junction, GJ- gap junction, NO- Nitric oxide, PG- prostaglandins, ET-endothelin (Kuruvilla and Kartha 2003)

II.6.1.1. Stimulus-secretion-contraction coupling

Endocardial endothelial cells are able to respond to humoral and physical stimuli with the release of inotropic factors. A critical step in stimulus-secretion-contraction coupling is the increase in intra-endothelial Ca^{2+} concentration ($[Ca^{2+}]_i$) upon stimulation of the cells with agonists like acetylcholine, bradykinin, histamine, substance P or ATP (Manabe *et al.* 1995-a). Following a stimulus, the increase in $[Ca^{2+}]_i$ occurs in two phases, viz., release of Ca^{2+} ions from the intracellular sources resulting in an initial transient increase and then a sustained influx of Ca^{2+} from the extra cellular space. This influx which causes the $[Ca^{2+}]_i$ to remain elevated, depends

on the electrochemical gradient of Ca^{2+} and therefore on the resting membrane potential of the cells (Manabe *et al.* 1995 -b). The resting membrane conductance of non-stimulated EECs is influenced by a variety of ion channels, such as inwardly rectifying K^+ channels, voltage gated K^+ channels, Na^+/K^+ ATPase pump, outwardly rectifying Cl^- channels and non-selective cation channels (Fan and Walsh 1999; Fransen and Sys 1997). It is likely that any factor which influences these ion channels will also affect the release of substances from EECs.

Effluent from superfused cultured porcine EE cells caused vasodilation of endothelium-denuded pig coronary artery, which demonstrated that EE cells released an endothelium-derived-relaxing factor (Shah *et al.* 1991). In bioassay experiments with EE denuded ferret papillary muscles, effluent from cultured porcine EE cells reverse the changes in myocardial performance seen after EE removal suggesting the presence of positive inotropic and contraction prolonging mediator in the effluent (Smith *et al.* 1991). From these experiments it was postulated that mediators released by EE cells modulate the underlying cardiac muscle. The cardiac endothelium releases several chemical mediators such as NO, PGs and ET that influence the contraction of the underlying cardiac muscle in a manner similar to the release of relaxing and contracting factors by vascular endothelial cells. In addition, endothelial cells may benefit cardiac homeostasis through production of kinins which maybe responsible for some of the beneficial cardiovascular effects of ACE inhibitors (Scicli 1994). Many of these endothelial paracrine factors influence the contractile behavior of myocytes through changes in cardiac myofilament properties rather than by altering the cytosolic Ca^{2+} transients (Shah *et al.* 1996).

II.6.1.1.1. Role of Nitric Oxide

Nitric oxide, a highly reactive gas and a vital signaling molecule is generated from L-arginine by a family of nitric oxide synthases. All three isoforms of NOS may be expressed in the heart, in a cell-specific manner. Endothelial constitutive NOS or eNOS is expressed predominantly in coronary vascular and EE cells and in a much lower level, in the cardiac myocytes. There is considerable nonuniformity in eNOS distribution and expression in cardiac endothelium, with higher expression on endocardial and coronary arterial endothelium compared to cardiac venous and capillary endothelium (Andries *et al.* 1998). eNOS expression is under tight regulation and small amounts of NO are rapidly released following specific stimuli like moderate hypoxia and various receptor-dependent agonists. Inducible NOS or iNOS has been detected *in vivo* and *in vitro* in EE, infiltrating inflammatory cells, vascular smooth muscle, fibroblasts, MVE and cardiac myocytes. Neuronal NOS or nNOS is expressed in pre and post-ganglionic fibers in specialized cardiac conduction tissue such as the sinoatrial and atrioventricular nodes, in intrinsic cardiac neurons and sarcoplasmic reticulum (SR) of cardiac myocytes (Kelly *et al.* 1996; Shah and MacCarthy 2000).

The demonstration of Ca^{2+}-dependent NOS in EE cells supports the contribution of NO in EE modulation as myocardial relaxation factor. Smith *et al* were the first to report iNOS expression in porcine ventricular endocardial cells (Smith *et al.* 1993) as well as the effect of NO (initially identified as endothelium-derived-relaxing-factor) on myocardial signal transduction (Smith *et al.* 1991) by experiments utilizing substance P, a known stimulant of NO-release from vascular

endothelium. Substance P induces an abbreviation of twitch contraction similar in pattern to EE removal and an increase in myocardial cGMP in isolated ferret papillary muscles. This effect requires an intact EE and is inhibited by hemoglobin, an inhibitor of NO activity, indicating that it is mediated by release of NO from EE.

Shear stress and the mechanical deformation that occurs during cardiac cycle may be the most relevant physiological stimuli for the release of NO from coronary endothelial cells. Since there is an oscillation of these physical forces during cardiac cycle, the release and action of NO in the heart may also be cyclical, with the rise in levels occurring with ventricular relaxation (Shah and MacCarthy 2000). The finding that an increase in shear stress leads to an increase in $[Ca^{2+}]_i$ which in turn leads to NO release suggests that the signal transduction is similar to that of receptor–dependent activation. The increase in shear stress may enhance kinin release with subsequent release of NO from the endothelium. Being a potent vasodilator, endothelium derived NO can indirectly influence cardiac function secondary to changes in vascular tone. In addition, NO, synthesized by the activity of inducible and constitutive NOS in the cardiac endothelial cells or myocytes directly modulate myocardial contractility (Schulz *et al.* 1991; Schulz *et al.* 1992). After its production, the primary biologic function of NO is the activation of soluble guanylyl cyclase to increase the cGMP content (Moncada *et al.* 1991). However, several cGMP-independent actions of NO have also been identified (Stamler *et al.* 1992). cGMP-independent effects may be particularly relevant when large amounts of NO are produced by iNOS. Nitric oxide may react directly with mitochondrial respiratory

enzymes and with various nuclear proteins, causing cellular damage by these mechanisms. NO can also react with free radicals like superoxide to form peroxynitrite, which is a potent oxidant and is also highly toxic. The direct mechanism of NO-induced injurious effects may be the alteration of mitochondrial activity and consequent inhibition of aerobic energy producing processes in the heart (Tatsumi *et al.* 2000). Nitric oxide binding to cytochrome c oxidase is the main mechanism for inhibition of mitochondrial respiration (Trochu *et al.* 2000).

The response to NO is likely to depend on factors such as, the amount of NO released, the source of NO, the target tissue, the stimulus and whether the activated pathway is cGMP- dependent or cGMP-independent. An elevation in the $[Ca^{2+}]_i$ has been shown to be an absolute prerequisite for the acute increase in the synthesis of NO in endothelial cells induced by either receptor-dependent agonists like acetylcholine, ATP and bradykinin or by receptor-independent compounds like Ca2+ ionophores. The main target proteins for cGMP are PKGs, cGMP-regulated cyclic nucleotide PDEs and cGMP-regulated ion channels (Pfeilschifter *et al.* 2001). Signal transduction dependent on PKGs involves the phosphorylation of various targets, whereas PDEs modulate intercellular levels of cAMP. Actions of cGMP in the heart include either an increase or reduction in the sarcolemmal Ca^{2+} influx, altered sarcoplasmic reticular function by phosphorylation of SR proteins, reduction in myofilament Ca^{2+} sensitivity, alterations in action potential, modulation of cell volume and reduction in myocardial oxygen consumption (Shah and MacCarthy 2000). The inotropic properties of NO and cGMP depends on myocardial cGMP

content, which in turn is dependent on the integrity of the overlying endothelium and NOS activity. The mechanism of cGMP-mediated positive inotropic response is not clear. Studies on the subcellular actions of intracellular cGMP in the myocardium have led to suggestions that cGMP may increase intracellular calcium through the mediation of a second messenger, cyclic ADP ribose (cADPR) which stimulates calcium release through ryanodine receptor. cADPR can trigger Ca^{2+} release from sarcoplasmic reticulum in cardiac cells (Lesh *et al.* 1993). Alternatively, the stimulatory effects of cGMP on calcium could be through the inhibition of cAMP PDE, resulting in increased cAMP levels and stimulation of cAMP-dependent calcium channel.

Several studies have shown that submicromolar concentrations of NO may exert a small positive inotropic effect on basal contractile function whereas micromolar concentrations may induce negative inotropic effects primarily due to a reduction in myofilament responsiveness to Ca^{2+} (Brunner *et al.* 2001). The positive inotropic effect may be mediated by a cGMP-inhibited cyclic nucleotide phosphodiesterse (cGiPDE) and increase in intracellular cAMP levels but cGMP-independent mechanisms could also be involved. The negative inotropic effect is probably mediated by cGMP-dependent protein kinases.

In summary, EE probably regulates basal intracellular cGMP concentration in cardiomyocytes by basal release of NO. Tonic or basal release of NO from EE and consequent elevation in cGMP levels in cardiomyocytes by virtue of its positive inotropic response will thus help in preserving cardiac function in physiological

states. However, excess cGMP stimulation due to elevated levels of NO from nitrovasodilators or from inducible NOS in pathological states would cause a negative inotropic effect, contributing to deterioration of cardiac function (Sys *et al.* 1997).

Analogous to the potential for NO to inhibit vascular smooth muscle growth and proliferation (Myers and Tanner 1998), it can also influence cardiac myocyte growth and exhibits an anti hypertrophic effect (Calderone *et al.* 1998). In several cell types, high levels of NO activate apoptosis (Kim *et al.* 1999) and lower (physiological) levels promote cell survival.

II.6.1.1.2. Role of Endothelin

The first report of positive inotropic effect of ET-1 synthesized and released by EE cells was by Mebazaa *et al* (Mebazaa *et al.* 1993). Expression and release of biologically active ET-1 with strong contractile effects which could be blocked by the ET_A receptor antagonist FR139317 has been demonstrated by Opgaard *et al* in cultured porcine EE cells (Saetrum Opgaard *et al.* 2001).

The synthesis of ET-1 by EE, cardiac muscle and coronary endothelium suggests both paracrine and autocrine activity of endothelin in the regulation of cardiac function (Mebazaa *et al.* 1993; Evans *et al.* 1994; McClellan *et al.* 1994). Apart from its role as a vasoconstrictor, ET-1 acts as an apoptosis survival factor for endothelial cells via the ET_B receptor (Shichiri *et al.* 1997). Levels of ET below 1 nM cause increased contractile function, intracellular alkalinization and enhanced transcription of some genes and consequent myocyte hypertrophy (Kramer *et al.* 1992). There is abundant evidence that hypoxia is a potent stimulus for ET-1 release

as is shown by an increased gene expression induced by hypoxia. The other inducing factors for ET release include flow-induced shear stress and various pharmacological receptor-dependent agonists. Complex interactions exist between ET and the other relaxing and contracting factors released by the endothelium, which modify the overall inotropic response. Endothelin-1 induces the release of NO from endothelial cells and atrial natriuretic peptide from atrial tissue. Alternately, NO can inhibit ET production. Prostanoids blunt the vasoconstrictor effect of endothelin whereas Ang II stimulates the expression of ET-1 mRNA and release of ET-1 (Luscher and Haefeli 1993; Balligand *et al.* 1997). The endothelin that is released in response to Ang II may modulate gene expression in adult hearts, through its receptors. Hence, ET may represent one of the key mediators for Ang II thereby providing a potential mechanism for the transduction of Ang II in the transcriptional activation of cardiac specific genes. In this regard, ET-1 has been shown to produce cardiac hypertrophy and activate gene expression in cardiocytes (Ito *et al.* 1991; Shubeita *et al.* 1990; Wang *et al.* 1992).

At basal levels (plasma levels of ET are in femtomolar range), ET acts in an autocrine way. Rather than directly causing vasoconstriction, it may stimulate the release of the vasodilator mediators like NO and PG (Luscher and Haefeli *et al.* 1993). The concentrations of ET capable of exerting effects on the myocardium however produce marked vasoconstriction, counteracting the positive inotropic effect. The relative degree of these two activities may in turn depend upon whether the ET production occurs in the myocyte, the EE or the MVE.

The positive inotropic effect of ET has been partly attributed to the elevation of cytosolic Ca^{2+} transients and augmented response of the contractile proteins to calcium through the activation of sarcolemmal Na^{+}/H^{+} exchanger via protein kinase C and an increase in intracellular pH (Balligand *et al.* 1997). The increase in cytosolic calcium may be due to increased sarcolemmal Ca^{2+} influx through the calcium channels or because of calcium release from intracellular stores (Wang *et al.* 1991). ET_{B1} receptors induce vasodilatation through the release of NO and PGs, whereas ET_{B2} receptors induce direct vasoconstriction. ET-1 induced increase in myocardial distensibility mediated by ET_A receptor stimulation requires an intact EE as well as active endothelial ET_{B1} receptors (Bras-Silva and Leite-Moreira 2006). Exogenous ET-1 is a potent positive inotrope acting mainly via activation of ET_A receptors, though ET_B receptors also are present on cardiac myocytes. ET_{B1} stimulation induces a negative inotropic effect in the presence of an intact EE and mediated by NO and PGs (Leite-Moreira and Bras-Silva 2004). Inotropic effects of selective ET_B receptor stimulation depend on the functional integrity of the EE. Impaired response to ET_B receptor stimulation in heart failure could be an evidence of endocardial endothelial dysfunction (Bras-Silva *et al.* 2006). Activation of ET_B receptors inhibits ECE-1 expression and mediates reuptake of ET-1 by endothelial cells. Selective ET_A receptor antagonism was found to prevent and reverse some of the damage to the heart due to cardiac and vascular remodeling in DOCA-salt hypertensive rats and thus improve cardiac function (Allan *et al.* 2005). In addition to its inotropic effects, ET has also potent chronotropic effects. Basal release of ET from EE may be in part responsible

for the earlier onset of myocardial relaxation and decrease in peak isometric tension on selective denudation of EE (Evans *et al.* 1994; Shah 1995).

II.6.1.1.3. Role of Prostaglandins

Both coronary and EECs release prostanoids. Mebazaa *et al* demonstrated that pure, isolated EE cells are capable of sustained prostanoid production in response to classic endothelial stimuli. The production of prostacyclin was found to be ten times greater than that of PGE_2 and EECs produce much higher amounts of prostacyclin than vascular endothelial cells. Prostacyclin production is ten times greater than PGE_2 in EE (Mebazaa *et al.* 1993; Mebazaa *et al.* 1995). These results suggest that endocardial prostanoid production could modulate platelet and myocardial function locally and downstream vascular tone distally. Tissue cyclooxygenase responsible for prostaglandin production has been found to be twice as high in the endocardium as in the myocardium (Brutsaert 1989). Under experimental conditions of hypoxia, prostanoid production by EECs is reported to increase much more dramatically (34-fold) than that of vascular endothelial cells (Mebazaa *et al.* 1995). Cardiac myocytes themselves release prostanoids, during hypoxia and reoxygenation. Prostanoids may have indirect effects on myocardial contraction, especially under pathophysiological conditions because of their vasodilator and platelet anti-aggregatory action. The direct effects of prostanoids on myocardial function are still not clear. The subcellular actions of PGs at myocardial level are also not fully understood. The ability of the endothelial cells to release NO influences the inotropic activity of PGs. The presence of receptors for prostanoids on the cardiac myocytes suggests that prostanoids may be

coupled both to adenylyl cyclase/cAMP system and phosphoinositide pathway. Nevertheless, their inotropic effects are inconsistent. It has been suggested that prostanoids modulate inotropic response to other agonists (Meulemans *et al.* 1990).

II.6.1.1.4. Role of ACE, Ang II and Bradykinin

There exists a locally active renin-angiotensin system in the heart and except for ACE located on the luminal surface of endothelial cells, all other components of the system exist locally within the heart. In the normal heart, Ang II is synthesized locally through both ACE and an ACE-independent chymase pathway, which is expressed predominantly in cardiac endothelial cells. The cells responsible for the release of major part of the renin and angiotensin in the heart are the MVE (Balligand *et al.* 1997). Endothelial-myocardial signaling may in the adult heart substantially contribute to the growth-promoting properties of Ang II, much of which is from locally produced tissue Ang II. The growth-promoting effect of Ang II on cardiomyocytes has been shown to involve auto and paracrine release of ET-1 from cardiac endothelial cells (Ito *et al.* 1993; Ito *et al.* 1991; Rajagopalan *et al.* 1997). Angiotensin II is known to exert a modest positive inotropic effect in many species. A recent study reported that Ang II has a dose-dependent positive inotropic effect that depends to a great extent on ET_A receptor activation and intact EE (Castro-Chaves *et al.* 2006). The subcellular mechanism suggested is either an increase in cytosolic Ca^{2+} or most probably, an increase in myofilament response to Ca^{2+}. Besides the positive inotropic effect, it may also delay myocardial relaxation. In addition to the inotropic effect, direct cardiovascular effects of Ang II include a chronotropic effect on cardiac

muscle, effects on cardiac metabolism and vasoconstriction of blood vessels. Various experimental findings indicate that circulating Ang II mediates the pathological cell growth in remodeling of cardiac interstitium in the failing heart (Weber 1992). All these effects are seen with exogenous Ang II and the contribution of endogenous Ang II to the regulation of contractile function of the heart is not fully known. ACE inhibitors have beneficial effects on ventricular remodeling and diastolic dysfunction in heart failure and they can also regress left ventricular hypertrophy (Lindpaintner and Ganten 1991). These effects could be due to reduced Ang II levels or increased bradykinin levels. Endothelial cells themselves release bradykinin, the local levels of which are modulated by ACE or kininase activity on these cells. NO, prostanoids and other endothelial mediators release are induced by bradykinin through the stimulation of the bradykinin receptors (B2) on the endothelial cells. Enhanced activity of bradykinin has an additive effect on the cardioprotective, antihypertrophic and antiarrhythmic effects of ACE inhibitors. ACE inhibitors may also enhance the interaction of bradykinin with its receptor on endothelial cells. Bradykinin induces the release of NO, PGs, ATP through stimulation of B2 receptors on endothelial cells (Pelc *et al.* 1991; Yang *et al.* 1994).

II.6.1.1.5. Role of Myofilament Desensitizing Factor

Experiments using effluent from cultured EE and vascular endothelial cells on isolated endocardium-denuded ferret papillary muscle preparations and isolated cardiac myocyte preparations revealed the presence of an agent, which was referred to as "endocardin" (Smith *et al.* 1991). Subsequent studies demonstrated that the

endothelial cells release a potent negative inotropic substance in addition to ET-1. The combined activity of both these substances accounted for the "endocardin" effect. The novel, negative inotropic agent is a stable, low molecular weight, nonprotein and is termed "myofilament desensitizing agent" whose release may be regulated by coronary flow rate. The stimuli responsible for the release of this factor and its possible physiological role are not yet defined. The potency and rapidity of action of the factor, its reversibility and stability suggest its significant modulatory effect on myocyte function both locally and distally. The fact that these effects brought about by the factor is not accompanied by significant changes in cytosolic Ca^{2+} is indicative of a reduction in the relative myofilament response to Ca^{2+} ("desensitizing" them). The stability of the agent could help it to contribute to cardiac contraction-relaxation coupling and diastolic tonus (Shah *et al.* 1994).

Nitric oxide, bradykinin and myofilament desensitizing agent are factors most probably important in short-term regulation of myocardial functions. Endothelin and Ang II are probably involved in long-term regulation.

II.6.1.1.6. Role of Neuregulin

In mutant Zebrafish embryo lacking an EE tube, the developing myocardial tube remain somewhat smaller and dysmorphic, but is capable of initiating spontaneous contractions; however, contractility is reduced with distended atria and collapsed ventricles and the animals do not survive long (Stainier *et al.* 1995). The obligatory role of EE in myocardial cell maturation and function at subsequent stages of embryonic development was confirmed after the discovery of the neuregulin

growth factor signaling pathway in the EE. Neuregulin1 (NRG1) predominantly expressed in early embryogenesis is confined to the EE from where it is released as a paracrine growth signal essential for the proliferation, survival and maturation of the developing cardiac myocytes (Zhao *et al.* 1998; Zhao *et al.* 1999). It is demonstrated that ErbB2 signaling participates in the paracrine survival and growth controlling effects of NRG1 on cardiomyocytes *in vitro* (Lemmens *et al.* 2006). Besides NRG1 that governs the formation of ventricular trabecules the EE also express low levels of NRG3. Mice with loss of function mutation of NRG1 or either of the receptors ErbB2 and ErbB4 exhibit no trabeculation of the ventricles and die during embryogenesis. NRG may be another component in the cross-talk between cardiac endothelium and myocytes (Zhao *et al.* 1998).

II.6.1.2. Blood heart barrier

As in brain, the myocardium is also a highly excitable tissue, the ionic homeostasis of which is vital not merely to the function of the organ but also to the entire body. Endocardial endothelial cells are electrically highly active similar to the brain capillary endothelial cells. Intense transmembrane transport of ions through ion channels and the asymmetric localization of the non-selective cation channels and $\alpha 1$ isoform of Na^+ K^+-ATPase on the luminal membrane compared to the abluminal side of the EE as is seen in the blood brain barrier has led to the suggestion of the presence of a blood-heart barrier (Fransen *et al.* 2001). There might be transendothelial transport of ions regulating the ionic composition of the extra cellular fluid in the heart, which is crucial for myocyte electrical activity. Ion channels on the luminal side of the membrane are different from that seen on the abluminal side. The unique

position of the EE between the cardiac myocytes and blood makes it most probable to maintain the specific ionic environment of the cardiac myocytes. The transendothelial electrical resistance of near-confluent monolayers of cultured porcine EE is measured to be 50-80 ohm cm^2, which is much higher than that in other endothelia, suggesting that EE may function as an active barrier between the cardiac interstitium and the superfusing blood (Brutsaert *et al.* 1998; Brutsaert 1989). The syncytial character of EE is essential to the establishment of a putative barrier with unidirectional transcellular transport of ions (Brutsaert 2003). Location of tight junctions always on the luminal side, presence of complex intercellular clefts and numerous gap junctions in the EEC could be further adaptations for its barrier function (De Keulenaer *et al.* 1995). Besides its sensory function and paracrine modulation of myocardial performance, an active EEC layer as blood-heart barrier could be of significance for the ionic homeostasis of the interstitium surrounding the myocardium. Further investigations are warranted to delineate the blood-heart barrier.

II.7. CARDIAC INTERSTITIUM

Myocardium comprises of cardiac myocytes surrounded by an extra cellular space. Myocytes occupy three quarters of the structural space of the myocardium and the remainder of the space is occupied by the extra cellular space or the cardiac interstitium. Cardiac interstitium is the structural component that supports and tethers the myocytes and vessels. It maintains a defense mechanism and aids in nutrition and lubrication of myocytes. Besides myocytes, the cell types present in the cardiac interstitium include endothelial cells lining the coronary and lymphatic vasculature,

VSMCs, cardiac fibroblasts, mast cells and macrophages. Capillaries, lymphatic vessels, arterioles, venules and adrenergic neurons are some of the other components of cardiac interstitium. The ECM also contains cytokines and several proteins including collagen, fibronectin, proteoglycans, laminin, growth factors and proteases such as collagenases. Recently it has been demonstrated that fibrillin is also a constituent of the myocardial interstitium that is regulated during development of cardiac fibrosis (Bouzeghrane *et al.* 2005). Collagen types I and III are the major components of the fibrillar connective tissue (Weber 1989; Weber and Brilla 1991). These collagens are also involved in the interstitial and perivascular fibrosis of the myocardium and the replacement scarring that follows cell death. Eghbali *et al* found that cardiac fibroblasts contain the mRNA for fibrillar collagens (Eghbali *et al.* 1989). Fibroblasts also synthesize collagenase enzyme which degrades collagen and thus maintains the collagen concentration relative to collagen synthesis. Cardiac fibroblasts, which constitute the majority of nonmyocyte cells in the heart, reside in the interstitium where they are exposed to systemic hormones, neurotransmitters and growth factors that are either present in the myocardium or gain access to it through the circulating blood. Growth factors and cytokines influence collagen metabolism indirectly by modulating the expression of MMPs and their inhibitors, the TIMPs. Composition of the ECM is maintained by a balance between the components and depends on the rate of synthesis and break down of each component.

Growth responses to cardiovascular diseases could be 1) nonmyocyte cell growth without myocyte hypertrophy 2) myocyte hypertrophy without nonmyocyte cell growth or 3) simultaneous disproportionate or equal growth of these cells (Weber

and Brilla 1991). Myocyte growth is expressed as an increase in the size of the cell or hypertrophy with an increment in the myocardial mass and wall thickness whereas nonmyocyte cell growth is expressed as a structural remodeling of the interstitium.

The fact that hypertrophy of myocytes and enlargement of the heart in many pathologic states is accompanied by hyperplasia of cardiac fibroblasts and an enhanced collagen deposition, increased the interest in factors controlling fibroblast growth. Normally fibroblasts remain in a quiescent state, out of the cell cycle. Mitogenic agents and growth factor interactions can induce proliferation of fibroblasts. Fibroblast growth is indicated as accumulation of fibrillar collagen. Increased fibrillar collagen accumulation is a major determinant of impaired stiffness and pump dysfunction in the heart and its continued accumulation leads to ventricular dysfunction that first appears during diastole and later during systole (Capasso *et al.* 1990). Necrosis or apoptosis of myocytes consequent to myocardial ischemia or senescence leads to reparative fibrosis, which is mainly interstitial. On the other hand, reactive fibrosis occurs as a reaction to inflammation, in the absence of cell loss and is mainly perivascular. Both kinds of fibroses can alter the structural integrity in the heart and result in cardiac dysfunction.

Cardiac fibroblasts can undergo three major, but not necessarily interrelated changes during cardiac remodeling: (1) transform to myofibroblasts by expressing smooth muscle α-actin and exhibiting contractility (2) proliferate and (3) produce extra cellular matrix components. In response to myocardial injury, fibroblasts are activated and participate actively in the wound healing response. Hemostasis,

infiltration of immune and inflammatory cells, degradation and phagocytosis of necrotic myocytes, repopulation of cardiac fibroblasts by chemotaxis and hyperplasia, reconstruction of a granulomatous scar and subsequent ECM remodeling to produce a mature scar are the sequential steps in response to injury. The initial phase of response to injury is characterized by net ECM degradation due to elevated MMP expression whereas the later phase is dominated by net ECM deposition due to increased collagen synthesis (Brown *et al.* 2005). Whereas collagen deposition in non-infarcted areas is predominantly by fibroblasts, in the infracted areas, myofibroblasts also contribute to collagen deposition. Collagen deposition is thus controlled by hormonal factors, growth factors, regulatory proteins and/or hemodynamic factors. The renin-angiotensin-aldosterone system is one of the main hormonal regulators of fibrosis. A major source of cytokine and growth factor production in the area of healing post-infarction are non-myocytes such as myofibroblasts and endothelial cells. These factors which include TGF-β, Ang II, aldosterone, PDGF, TNF-α and ILs are known to influence proliferation and collagen synthesis in cardiac fibroblasts.

II.7.1. Transforming Growth Factor-β

Transforming Growth Factor-β is a humoral factor known for its ability to strongly and ubiquitously stimulate the production and deposition of ECM proteins. High levels of this factor occur in cardiac fibrosis. It plays a causal role in myocardial fibrosis and diastolic dysfunction through fibroblast activation in pressure-overloaded hearts (Kuwahara *et al.* 2002). In cultured human cardiac fibroblasts, TGF-β is reported to increase type I collagen and TIMP mRNA and to suppress bFGF-induced

collagenase expression at the level of transcription (Eghbali 1989). Treatment of cardiac fibroblasts with TGF-β has an inhibitory effect on their DNA synthesis and pretreatment of the cells with TGF-β1 almost completely reverse the stimulatory effects of bFGF (Sigel *et al.* 1996). TGF-β is demonstrated to induce the phenotypic conversion of cardiac fibroblasts to myofibroblasts (Eghbali *et al.* 1991). It acts as an effective stimulus for matrix deposition by increasing the expression of collagen, diminishing the expression and activity of collagen breakdown enzymes such as MMP-2 and MMP-9 and augmenting the expression of MMP inhibitors, the TIMPs. TGF-β also suppresses the activity and expression of NOS, consequently increasing collagen synthesis and deposition (Vodovotz Y 1997). Three types of cell surface receptors: Types I, II and III on cardiac fibroblasts mediate the cellular actions of TGF-β. Based on the observations that TGF-β specifically induces connective tissue growth factor (CTGF) expression in both cardiac fibroblasts and myocytes and that there is significant upregulation of CTGF in heart samples from cardiac ischemic patients, it is likely that CTGF is an important mediator of TGF signaling in the heart (Chen *et al.* 2000). Contrary to the conventional observations, Petrov *et al* suggested that TGF-β1 actually stimulates the differentiation of fibroblasts to myofibroblasts and does not stimulate collagen production of usual cardiac interstitial fibroblasts. The augmented collagen production in myofibroblasts compared with fibroblasts is apparently an inherent property of the myofibroblasts and cannot be regulated by TGF-β1 (Petrov *et al.* 2002; Lijnen and Petrov 2002).

II.7.2. Angiotensin II

Angiotensin is an octapeptide hormone and neurotransmitter which is an important component of the renin-angiotensin system. It affects cell growth and differentiation in addition to the well known functions such as regulation of biosynthesis and secretion of aldosterone and vascular smooth muscle contraction. The long-term and short-term cardiac effects of Ang II are brought about by its direct action through Ang II receptors on cardiac cells and its indirect actions through release of other factors and possibly via cross-talk with intracellular signaling pathways of other cardiac hormones (Dostal *et al.* 1997). Cultured neonatal cardiac fibroblasts express abundant receptors that can couple Ang II stimulation to second messenger generation and proliferative growth responses (Booz *et al.* 1994). The mRNA levels for AT2 receptor are drastically repressed immediately after birth (Matsubara *et al.* 1994). Cardiac fibroblasts from adult rats express higher mRNA levels of AT1 compared to those from neonates. Brilla *et al* addressed the question whether the effector hormones of RAAS, namely Ang II and aldosterone would directly influence collagen synthesis and degradation and/or growth of cultured cardiac fibroblasts. Their results indicate that aldosterone has an effect on cardiac fibroblast metabolism similar to that of TGF-β, which is a known stimulator of collagen synthesis (Brilla *et al.* 1994). Moreover, ACE inhibition prevents myocardial fibrosis in diseased hearts, implying that angiotensin stimulates cardiac fibroblasts to synthesize collagen and deposit ECM (Booz and Baker 1995). Results obtained from cell count experiments indicate that under serum-free conditions, Ang II does not stimulate neonatal and adult cardiac fibroblast cell mitosis in any significant manner

(Villarreal *et al.* 1993). Following activation of AT1 receptors by Ang II, cardiac fibroblasts release at least two paracrine effectors, ET-1 and TGF-β. Ang II is reported to stimulate TGF-β gene expression, secretion of the peptide and its conversion to the active form. This suggests that the ability of Ang II to induce fibroblast growth and collagen synthesis may be mediated indirectly by increased TGF-β production, entailing a paracrine function for cardiac fibroblasts in the myocardium (Booz and Baker 1995). However, taking into account all the available *in vivo* and *in vitro* data, Bouzegrhane & Thibault argue that Ang II may not fulfill the criteria of a true growth factor (Bouzegrhane and Thibault 2002). They point out that Ang II can effectively synthesize pro-fibrotic substances such as collagen and TGF-β in adult cardiac fibroblasts and can partially differentiate these cells into myofibroblasts. There is however no evidence that this peptide has a direct action on the cell cycle. There is also no *in vivo* experimental evidence to suggest a role for Ang II in the growth of cardiac fibroblasts. However, this does not exclude the option that Ang II may induce another growth factor which can trigger cell division.

II.7.3. Aldosterone

Studies have demonstrated that aldosterone is locally produced in the heart and mineralocorticoid receptors are expressed on cardiac cells (Weber 2001). In contrast to Ang II which in low concentrations does not alter total collagen synthesis, aldosterone enhances collagen synthesis in adult rat cardiac fibroblasts in a dose-dependent manner. In contrast to the known inhibitory effects of glucocorticoids on collagen synthesis, aldosterone augments collagen synthesis and accounts for collagen

accumulation in cardiac tissue if RAAS is activated. Aldosterone conversely does not affect collagenase activity or the mitogenic potential (Brilla *et al.* 1994).

II.7.4. Endothelin-1

In the heart, ET-1 is expressed by endothelium, cardiac myocytes and fibroblasts. Both ET-1 and ET-3 were reported to increase the synthesis of type I and III collagens, while ET-1 but not ET-3 reduced collagenase activity. Cardiac fibroblasts express a mixed population of ET_A and ET_B receptor subtypes. ET-1 increases cell proliferation, MMP activity and net collagen synthesis in cultured cardiac fibroblasts (Guarda *et al.* 1993). Endothelin may have a role both in the initiation of fibrosis, by its pro-inflammatory effects and its role on myofibroblast formation and in maintenance of fibrosis, by its effect on collagen production, fibroblast proliferation and inhibition of MMPs (Clozel and Salloukh 2005). ET_A receptor antagonists were found to prevent TGF-β and Ang II dependent increase in cardiac collagen deposition induced by ET-1 over expression in the heart (Ammarguellat *et al.* 2001; Cheng *et al.* 2003). In contrast to results observed in other species, ET-1 in human cardiac fibroblasts is a pro-fibrotic but anti-proliferative factor, properties akin to those of TGF-β. The pro-fibrotic effects are mediated by ET_A receptor subtype (Hafizi *et al.* 2004).

II.7.5. Nor-epinephrine

Cultured adult cardiac fibroblasts when treated with norepinephrine augment mRNA levels of cytoskeletal actin and [^{3}H]-Thymidine incorporation into cell nuclei, suggesting that norepinephrine is capable of inducing either hypertrophy or

hyperplasia in cardiac fibroblasts. Though norepinephrine has no direct effect on collagen type I biosynthesis in cultured cardiac fibroblasts, intravenous administration in a sustained-release manner increase the abundance of mRNA for pro alpha 2 (I) collagen (Bhambi and Eghbali 1991). The nor-epinephrine induced effects are inhibited by β-blockers implying the involvement of β-adrenergic receptors in mediating these changes in cardiac fibroblasts (Long *et al.* 1993). There is also evidence that norepinephrine has an autocrine/paracrine effect on cardiac fibroblasts, resulting in induction of TGF-β expression (Fisher and Absher 1995).

II.7.6. Tumor necrosis factor-α

Tumor necrosis factor-α and IL-1β are inflammatory cytokines found to be enhanced in failing myocardium. Both cytokines decrease collagen synthesis and increase MMP activity (Siwik *et al.* 2000; Solis-Herruzo *et al.* 1988). The decrease in collagen synthesis occur without an alteration in either the cell number or overall protein synthesis, indicating that the reduction in collagen content is due to reduced synthesis at the cellular level. Results from a study using transgenic mice with cardiac restricted over expression of TNF specified the role played by time-dependent changes in the balance between MMP activity and TIMP levels in the spectrum of changes that occur in ECM during LV structural remodeling. Total MMP activity increased in early stages of LV remodeling without any accompanying increase in TIMP-1 levels, causing a decrease in total myocardial fibrillar collagen. As the mice aged, MMP activity decreased with increased TIMP-1 activity associated with increased total collagen content (Sivasubramanian *et al.* 2001). These findings explain

the development of LV dilatation and myocardial fibrosis, which is the hallmark of end-stage remodeling process. TNF-α is produced by a number of cells including inflammatory cells, fibroblasts, smooth muscle cells and endothelial cells. TNF-α participates in signal transduction cascade that regulates inflammatory and immunological responses. Some of the actions of TNF-α were mediated by the production of IL-1, IL-6, MCP-1, PDGF, TGF-β, NOS or GMCSF. Tumor necrosis factor can induce NO production and expression of class I MHC molecules, which I turn increase the permeability of endothelial cells (Nicoletti and Michel 1999). Deficiency of TNF-α receptor function increase infarct size after myocardial ischemia (Kurrelmeyer *et al.* 2000). TNF-α is critical to protection from lethal encephalomyocarditis virus by inducing adhesion molecule expression and recruitment of leukocytes to inflammatory sites (Wada *et al.* 2001). These studies indicate that TNF-α is cytoprotective in myocardial ischemia and acute viral myocarditis. However, several studies have also implicated this pro-inflammatory cytokine in the pathogenesis of heart failure accompanied with viral myocarditis, coronary heart disease, dilated cardiomyopathy (Bristow 1998; Mann DL 2001), myocardial dysfunction and remodeling. Inflammatory reactions accompanied by endothelial activation also play a crucial role in the propagation of congestive and ischemic heart disease (Wilhelmi *et al.* 2005). TNF-α effectively increases proliferation and fibronectin expression in cardiac fibroblasts isolated from infarcted and non-infarcted areas of the heart. The mitogenic effect of TNF and LPS on chick embryo cardiomyocytes was found to involve ERK activation and NOS activity, as well as to an increased intracellular cGMP levels (Tantini *et al.* 2003).

II.7.7. Other factors influencing cardiac fibroblast function

In addition to the above factors, PGE_2 was found to decrease cardiac fibroblast collagen synthesis in a concentration-dependent factor. Also, MMP1 activity was greatly increased by PGE_2 incubation in cardiac fibroblasts, causing a net reduction in collagen deposition in the myocardium (Brilla *et al.* 1995). Insulin-like growth factor-1 produced in and released from cardiac fibroblasts stimulates collagen synthesis (Horio *et al.* 2005).

The role of EECs in the regulation of cardiac interstitium or its components is still unclear. This lacunae remains despite the enormous amount of data relating to the modulator role of vascular endothelial cells on other cell types such as myocytes, vascular smooth muscle cells and fibroblasts. Mediators released by EECs may exert a paracrine effect on the cardiac interstitial cells, influencing their function during normal physiological or pathological conditions. It is also interesting to explore whether these humoral factors have an altered effect on the interstitial cells during inflammatory states of the heart.

III. MATERIALS AND METHODS

III.1. MATERIALS

III.1.1. Fine Chemicals

Medium-199, Dulbecco's Modified Eagle's medium, Hank's balanced salt solution, bovine serum albumin, collagenase type IA, collagenase type VII, trypsin, deoxyribonuclease, N-[2-hydroxyethyl]piperazine-n'-[2-ethanesulfonic acid] (HEPES), fetal bovine serum, ethylene diamine tetraacetic acid, glucose, monoclonal anti-vimentin antibody, anti-mouse IgM antibody, anti-human von Willebrand antibody, monoclonal anti-desmin antibody, SIGMA FAST^{TM} (Fast Red TR/Naphthol AS-MX, Alkaline Phosphatase substrate tablets set), TNF-α, bacterial LPS, Griess reagent, BQ123, PD 142893, Bisindolylmaleimide I, PD 098059, Anti-TGF-β antibody, L-Glutamine, TRI reagent, sodium dodecyl sulfate, Tris base, agarose, sodium acetate and immunostaining kit for desmin were purchased from Sigma-Aldrich, MO, USA. Lipofectamine, Plus reagent and Opti-MEM were procured from Gibco Invitrogen Corporation, NY, USA. Antibody to cytokeratin (AE1/AE3) was purchased from Dako, Denmark. Phospho ERK1/2 antibody was purchased from Cell Signaling Technology, Inc., MA, USA and ERK2 antibody was from Santa Cruz Biotechnology Inc., CA, USA. Antibody to the Telomerase catalytic unit was procured from Novocastra Laboratories, New castle upon Tyne, UK. DiI-Ac-LDL and FITC-labeled anti-goat secondary antibody was purchased from Molecular Probes, Netherlands. RT-PCR reagents were procured from Promega Corporation, Madison, WI, USA.

III.1.2. Routine Chemicals

Calcium chloride, magnesium chloride, potassium chloride, potassium dihydrogen phosphate, 1,4-bis[5-phenyl-2-oxazolyl]-benzene, 2,5-diphenyloxazole, sodium bicarbonate, sodium chloride, sodium dihydrogen phosphate, sodium hydroxide, toluene, trichloroacetic acid, concentrated hydrochloric acid, ethanol and ether were purchased from SISCO Research Laboratories, India.

III.1.3. Radiochemicals

[^{3}H]-Thymidine (17,200 mCi/mmol) and [^{3}H]-Proline (7500 mCi/mmol) were obtained from Board of Radioactivity and Isotope Technology, India.

III.1.4. ELISA Kits

Endothelin-1 enzyme immunoassay kit was purchased from Cayman Chemical, MI, USA. Angiotensin II enzyme immunoassay kit was procured from SPI BIO, France and Transforming Growth Factor-β1 ELISA kit was from Biosource International, Belgium.

III.1.5. Cell culture ware

35 mm and 100 mm cell culture dishes, T25 cell culture flasks, 4 well plates and thermanox cover slips were purchased from Nunc, Denmark. Cell culture filter ware was from Millipore, USA.

III.1.6. Equipments used

ELISA reader (Bio-Tek instruments, USA), Liquid scintillation counter (Wallac 1409), UV-visible spectrophotometer (Shimadzu, Japan), High speed refrigerated centrifuge (Hitachi, Japan), weighing balance (Sartorius, Germany), water bath (LKB,

Sweden), ice-machine (Hoshizaki, Japan), pH meter (Labindia, India), CO_2 incubator (Nuaire, USA), phase-contrast microscope (Nikon, Japan), confocal microscope (Carl Zeiss, Germany), laminar flow hood (CLAS, India), Magnetic stirrer (Schott, Germany), EASY pure UV/UF compact reagent grade water system (Barnstead, USA), Electrophoresis unit (Biorad laboratories, USA), Mini Blot (Biorad laboratories, USA), Transilluminator (Bangalore Genei, India), Submarine electrophoresis unit (Bangalore Genei, India) and Programmable Thermal Cycler (MJ Research Inc, USA).

III.2. COMPOSITION OF MEDIA, REAGENTS AND BUFFERS

III.2.1. Acrylamide 30%

29% (w/v) acrylamide and 1% (w/v) N,N'-methylene bisacrylamide in deionised water

III.2.2. Agarose Gel for DNA (1%)

200 mg agarose in 20 ml 0.5X TBE

III.2.3. Blocking Solution

5% (w/v) skim milk in TBST

III.2.4. Complete Growth Medium for Endocardial Endothelial Cells

Medium 199 HEPES modification with Earle's salts containing 20% FBS, 1% Endothelial cell growth factor, 6 mM L-Glutamine, 50 U/ml heparin, 50 U/ml benzyl penicillin and 50 µg/ml streptomycin

III.2.5. Complete Growth Medium for Cardiac Fibroblasts

Medium 199 with Earle's salts without HEPES containing 10% FBS, 50 U/ml benzyl penicillin and 50 μg/ml streptomycin

III.2.6. Diamino benzidine

6 mg diamino benzidine in 10 ml Tris (pH 7.6) containing 10 μl of 30% H_2O_2

III.2.7. Diethyl Pyrocarbonate (DEPC) Treated Water

0.1% DEPC in deionised water

III.2.8. Dissociation medium (pH 7.4) for Cardiac Fibroblasts

Sodium chloride 116.4 mM; HEPES 20 mM; Sodium dihydrogen phosphate 1.15 mM; Glucose 5.55 mM; Potassium chloride 5.37 mM; Magnesium sulfate 0.81 mM

III.2.9. DNA/RNA Gel Loading Dye

0.25% bromophenol blue; 0.25% xylene cyanol FF; EDTA 1 mM; 50% glycerol in water

III.2.10. Electrode Buffer 8X (pH 8.3) for Polyacrylamide gel electrophoresis

12 g Tris base, 57.6 g Glycine in 500 ml water

III.2.11. Ethidium Bromide

10 mg in 1 ml water

III.2.12. Ethylene Diamine Tetra Acetic Acid (EDTA) 0.5M (pH 8)

930 mg EDTA in 5 ml DEPC treated water

III.2.13. Hank's Balanced Salt Solution without calcium and magnesium (HBSS) (pH 7.4)

Potassium chloride 5.4 mM; Potassium dihydrogen phosphate 0.44 mM; Sodium chloride 137 mM; Disodium hydrogen phosphate 0.63 mM; D-Glucose 5.55 mM; Sodium bicarbonate 4.17 mM; Phenol Red 10 mg/ml

III.2.14. Lysis Buffer

Sodium hydroxide 0.1 M containing 0.1% Sodium dodecyl sulfate

III.2.15. MOPS Electrophoresis buffer 10X

MOPS (3-[N-morpholino] propanesulfonic acid) (pH 7.0) 0.2 M, Sodium acetate (pH 5.0) 3 M, EDTA (pH 8.0) 0.5 M in 1000 ml DEPC treated water

III.2.16. Phosphate Buffered Saline (PBS) (pH 7.4)

Sodium chloride 137 mM; Potassium chloride 2.7 mM; Disodium hydrogen phosphate 10.14 mM; Potassium dihydrogen phosphate 1.76 mM

III.2.17. Resolving Gel (10%) for Protein Electrophoresis

Water 7.9 ml, 30% acrylamide 6.7 ml, 1.5 M Tris (pH 8.8) 5 ml, 10% SDS 0.2 ml, 10% ammonium persulfate 0.2 ml, TEMED 0.008 ml

III.2.18. Scintillation cocktail

0.6% PPO and 0.02% POPOP in 100 ml Toluene

III.2.19. SDS Gel-loading Buffer 1X

0.067 M Tris base (pH 6.8), 2% (w/v) SDS, 0.03% bromophenol blue, 0.3% β-mercaptoethanol, 10% (v/v) glycerol

III.2.20. Sodium Acetate 3M (pH 5-6)

1.23 g Sodium acetate in 5 ml DEPC treated water

III.2.21. Stacking Gel for Protein Electrophoresis

Water 4.1 ml, 30% acrylamide 1.0 ml, 1 M Tris (pH 6.8) 0.75 ml, 10% SDS 0.06 ml, 10% ammonium persulfate 0.06 ml, TEMED 0.006 ml

III.2.22. Substrate Solution for Alkaline Phosphatase

1.0 mg/ml Fast Red TR (4-Chloro-2-methylbenzenediazonium), 0.4 mg/ml Naphthol AS-MX (3-Hydroxy-2-naphthoic acid 2,4-dimethylanilide phosphate), 0.15 mg/ml levamisol prepared in 0.1 M Tris buffer, pH 7.4

III.2.23. Towbin's Buffer

3g Tris base, 14.4 g Glycine, Methanol 200 ml in 1000 ml water

III.2.24. Tris Borate EDTA Buffer (TBE) 5X (pH 8.3)

54 g Tris base; 27.5 g boric acid; 20 ml 0.5 M EDTA (pH 8.0) per liter

III.2.25. Tris Buffered Saline 10X (pH 7.6)

24.2 g Tris base, 80 g Sodium chloride in 1000 ml water

III.2.26. Tris Buffered Saline with Tween-20 (TBST) 1X

1X TBS containing 0.2% Tween-20

III.2.27. Tris-$CaCl_2$ Buffer 4X (pH 7.4)

200 mM Tris, 200 mM $CaCl_2$

III.2.28. Trypsin/EDTA solution

PBS, pH 7.4 containing 0.25 mg/ml trypsin and 0.2 mg/ml EDTA

III.3. ISOLATION, CULTURE AND CHARACTERIZATION OF PORCINE VENTRICULAR ENDOCARDIAL ENDOTHELIAL CELLS

III.3.1. Isolation of endocardial endothelial cells

All experiments in the study had the approval of the Institute Animal Ethics Committee.

Endocardial endothelial cells from freshly collected pig hearts were isolated by the method described by Smith *et al.* (1993).

The animals were those used for short-term evaluation of carotid artery stents. Porcine hearts were collected and transported in sterile ice-cold HBSS (Ref. III. 2. 13) containing 100 U/ml heparin and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin). Atria, valves and chordae tendinae were removed and ventricles flushed with HBSS. The ventricles were filled with 0.1% collagenase (Type IA) in medium E199 and incubated for 45 minutes at 37^0C. The inside of the ventricles was rubbed gently with a cell scraper to remove loosely attached cells. The cell suspension was collected and centrifuged at 1000 rpm for 10 minutes. The pellet was resuspended in complete medium (Ref. III. 2. 4), seeded in 2% gelatin coated 35 mm culture dishes and incubated in a CO_2 incubator (37^0C in 95% air-5% CO_2). The cells were washed with medium at 6 hours to remove the debris and unattached cells. Fresh medium was supplied to the cells. Following day, cells were washed in medium to remove any floating cells and incubated with fresh complete medium. The cells reached confluence in 7-8 days.

III.3.2. Sub-culture of endocardial endothelial cells

At confluence, the cells were split at 1:3 ratio. Cells were washed twice with PBS and trypsinized using trypsin/EDTA solution (Ref. III. 2. 28) by incubation at 37^0C for 1 minute. Trypsinization was stopped by adding 10% FBS containing Medium E199 and the detached cells collected immediately. Fresh medium was added to the culture dish and pippetted repeatedly without foaming, to dislodge remaining cells from the dish. The suspension was centrifuged, pellet was resuspended in complete medium and cells seeded in fresh culture dishes coated with gelatin.

III.3.3. Characterization of endocardial endothelial cells

Endocardial endothelial cells were characterized by their morphology, immunocytochemistry or immunofluorescence and growth kinetics.

III.3.3.1. Morphology

The cells were routinely viewed under an inverted phase contrast microscope to analyze their morphology and growth pattern.

III.3.3.2. Immunocytochemistry

Cells grown on gelatin-coated cover slips to 60-70% confluence were washed thrice with PBS and fixed in 100% ice-cold methanol for 10 minutes. After two washings in PBS (Ref. III. 2. 16), endogenous peroxidase was quenched with two drops of 3% hydrogen peroxide for 5 minutes. Cells were again washed with PBS. Nonspecific binding was blocked with 2% BSA in PBS for 10 minutes. Two drops of primary antibody diluted in PBS containing 1% BSA was added and incubated for 60 minutes. The cells were washed thrice with PBS and incubated for 30 minutes with

diluted secondary antibody conjugated with either HRP or alkaline phosphatase. Cells were washed thrice with PBS and incubated with either the substrate reagent containing 3-amino, 9- ethyl-carbazole or the substrate solution for alkaline phosphatase (Ref. III.2.22) for up to 10 minutes and observed under a microscope. After sufficient color development, the cells were washed in deionized water for 5 minutes. The cells were counterstained with hematoxylin, mounted in glycerol and observed under a microscope.

Monoclonal anti-vimentin, monoclonal mouse anti-human anti-cytokeratin, anti-mouse IgM and anti-mouse IgG antibodies were diluted 1:50; anti-human von Willebrand factor was diluted 1:800. Immunostaining for desmin was done using a commercially available kit, in which the all reagents were provided ready-to-use.

III.3.3.3. Uptake of DiI-Acetylated LDL by endocardial endothelial cells

Cells grown on gelatin coated thermanox cover slips to near confluent stage were used for testing the scavenger pathway of lipoprotein metabolism. The procedure followed was that of Voyta *et al* (1984). Cells were incubated with 10μg/ml DiI-Ac-LDL at 37°C, in growth medium containing 20% FBS, for 4 h. The media was then removed and the cells were washed once with probe-free media for 10 minutes, rinsed with PBS, and fixed with paraformaldehyde for 5 min. Cover slips were inverted over a drop of 10% PBS in glycerol prior to viewing under a fluorescent microscope or confocal microscope.

III.3.3.4. Growth kinetics of endocardial endothelial cells

Growth kinetics of endocardial endothelial cells was studied following the method of Kovacs and Fleishmajer (1974).

Cells from passage 3 were plated in 35 mm cell culture dishes at a density of 1 x 10^5 cells/ml. Cell count was determined using a Neubauer chamber at 0, 24, 48, 72 and 96 hours after seeding. A graph was plotted with cell number on the y-axis and time on the x-axis. The best fitting curve was constructed by connecting the mean points. A time interval was selected in the middle of the exponential phase where the relationship between time and log density is linear. The beginning of the time interval was marked as t_1 and the end as t_2. The time interval, t_2- t_1 was taken as Δt. The cell densities at t_1 and t_2 were taken as N_1 and N_2 respectively. The growth constant, k, was calculated using the formula:

$$k = [2.3 \log (N_2/ N_1)] / \Delta t \text{ hours}^{-1}.$$

The doubling time, T_d, was determined using the formula:

$$T_d = 0.693/k \text{ hours}$$

III.4. IMMORTALIZATION AND CHARACTERIZATION OF PORCINE VENTRICULAR ENDOCARDIAL ENDOTHELIAL CELLS

III.4.1. Preparation of telomerized endocardial endothelial cells

Endocardial endothelial cells were transfected with the mammalian expression vector LZRS-hTERT-IRES-GFP containing the full-length human telomerase reverse

transcriptase (hTERT) carrying puromycin resistance gene (Kindly provided by Erik Hooijberg, Department of Immunology, The Netherlands Cancer Institute).

One day prior to transfection, EECs in early passages (1 or 2) were seeded at 6 x 10^4 cells/well into 4 well plates and allowed to grow up to 80-90% confluence. The cells were transfected with 2 µg of Plasmid DNA using lipofectamine reagents. 100 µl OptiMEM medium, 5 µl plasmid and 16 µl Plus reagent was mixed and incubated for 15 minutes. In another tube, 100 µl OptiMEM medium and 8 µl Lipofectamine 2000 were mixed. The above two were mixed to form the transfection complex and incubated for 15 minutes. The cells were washed twice with OptiMEM and the medium replaced with 200 µl OptiMEM without antibiotics and serum. 25 µl of transfection complex was added to the medium. Six hours later, the transfection complex was replaced with complete medium (medium E199 supplemented with 20% FBS, 100 U/ml benzyl penicillin and 100 µg/ml streptomycin). Forty-eight hours after transfection, the cells were incubated with complete medium containing 0.5 µg/ml of puromycin for the selection of transfected population. The selected clones of cells were expanded and maintained separately. Cells were frozen by slow freezing at different passages and stored at -80^{0}C in freezing mixture containing 90% FBS and 10% DMSO. Cells were revived whenever required by fast thawing in a 37^{0}C water bath and resuspension in complete medium followed by a wash to remove DMSO. The pellet was resuspended in complete medium and cells seeded to 35 mm cell culture dishes.

III.4.2. Characterization of transfected cells

III.4.2.1. Morphology

The cells were viewed under an inverted phase contrast microscope to observe the morphology.

III.4.2.2. Immunocytochemistry

The cells were fixed with 100% ice-cold methanol for 10 minutes and washed with PBS. The proteins were blocked by incubation in 2% BSA. The cells were then incubated with the primary antibody to vimentin (1:50), cytokeratin (1:50) or desmin for 1 hour, biotinylated secondary antibody (1:200) for 30 minutes and streptavidin-HRP or alkaline phosphatase for 15 minutes. After each incubation step, the cells were washed with PBS. The color was developed using the chromogen, 3-amino-9-ethylcarbazole or the substrate solution (Ref. III. 2. 22) and cells viewed under the microscope.

III.4.2.3. Immunofluorescence

Cells grown on gelatin-coated cover slips to 60-70% confluence were washed thrice with PBS and fixed in 100% ice-cold methanol for 10 minutes. After two washings in PBS, endogenous peroxidase was quenched with two drops of 3% hydrogen peroxide for 5 minutes. Cells were again washed with PBS. Nonspecific binding was blocked with 2% BSA in PBS for 10 minutes. Two drops of the primary antibody, anti-human von Willebrand Factor raised in rabbit, diluted 1:500 in PBS containing 1% BSA was added and incubated for 60 minutes. The cells were washed thrice with PBS and incubated for 30 minutes with FITC- conjugated anti-rabbit

secondary antibody diluted 1:100. The cells were washed with PBS and viewed under a confocal microscope.

III.4.2.4. Uptake of DiI-acetylated LDL

The ability of hTERT-transfected EECs to scavenge modified LDL was determined as described for primary EECs in Section III. 3. 3. 3. The cover slips were inverted over a drop of PBS in glycerol prior to viewing. The cells were observed under a confocal microscope.

III.4.2.5. Growth kinetics

The cells were seeded at an initial density of 1 x 10^5 cells/ml in medium E199 containing 5% FBS or 10% FBS. The cells were harvested at intervals of 24 hours for 6 days and counted using a Neubauer chamber. The doubling time was calculated using the formula Td = 0.693/k hours where k is the growth constant.

III.4.2.6. Proliferation assay

[^{3}H]-Thymidine uptake by hTERT-transfected EECs was measured as an index of DNA synthesis. The protocol is same as that followed for cardiac fibroblast [^{3}H]-Thymidine uptake as described in Section III. 6. 2.

III.4.2.7. RNA isolation

Total RNA was isolated from primary EECs and hTERT-transfected cells grown in 100 mm culture dishes, by an improvement of the single-step method reported by Chomczynski and Sacchi (1987) using TRI reagent as per the instructions of the manufacturer. The purity of RNA was determined from the 260/280 ratio. The yield of RNA was calculated from the formula

$$\text{RNA in } \mu g/\mu l = \frac{OD_{260} \times 40 \times DF}{1000}$$

Where, OD_{260} is the OD at 260 nm

DF is the dilution factor and

40 is included assuming that OD of 1 ≡ 40 µg/µl of RNA

III.4.2.7.1. RNA gel

To determine whether the isolated RNA is intact, the 3 µl of RNA samples were run on 1% agarose gel (Ref. III. 2. 2) containing ethidium bromide (Ref. III. 2. 11), by submarine electrophoresis using 1X MOPS buffer (Ref. III. 2. 15). The sample run was tracked by adding 3 µl of the loading dye (Ref. III. 2. 9) along with the sample. The gel was run at 65 mA for 15 minutes.

III.4.2.8. cDNA synthesis

cDNA reaction mix (30 µl)

5X RT buffer	6.0 µl
dNTPs	2.5 µl
Oligo dT	3.0 µl
RNAse inhibitor	0.5 µl
RT	2.0 µl
RNA	10.0 µl
DEPC water	6.0 µl

The cDNA mix was incubated in a 37^{0}C water bath for 1 hour. The cDNA was stored at -20^{0}C till PCR reaction was performed.

III.4.2.9. RT- PCR analysis for telomerase transcripts

The PCR primers for amplification of hTERT and porcine TERT were designed using the Oligos software.

The primer sequences were:

hTERT

Forward primer: 5'-GCC CCC GAG GAG GAG GAC-3'

Reverse primer: 5'- CGC ACA CGC AGC ACG AAG G-3'

Porcine TERT

Forward primer: 5'-GCG TCT CAC GGG GCA GGT C-3'

Reverse primer: 5'-GCA GGA AGT CGT CCA CCA AGC-3'

PCR mix (50 µl)

10X Taq buffer	5.0 µl
$MgCl_2$	3.0 µl
dNTPs	3.0 µl
Forward primer	1.0 µl
Reverse primer	1.0 µl
Taq	0.5 µl
cDNA	3.0 µl
Sterile water	33.5 µl

PCR was done in a MJ thermal cycler and the cycling conditions followed were: denaturation at 95^0C for 5 min, annealing at 55^0C for 1 min for hTERT and 62^0C for

porcine TERT, extension at 72^0C for 7 minutes. The cycle was repeated 34 times. PCR products were analyzed by electrophoresis (Ref. III. 2. 24) on a 1% agarose gel stained with ethidium bromide.

III.4.2.10. Western blot analysis for telomerase catalytic subunit

Western analysis of telomerase catalytic subunit was done using mouse monoclonal antibody to human telomerase reverse transcriptase (catalytic subunit). Transfected cells were grown on 100 mm dishes and the lysate for Western blot analysis was prepared when the cells reached confluence. The medium was discarded and cell layer was washed twice with ice-cold PBS. The cells were scraped using a cell scraper, transferred to a micro centrifuge tube and centrifuged at 10,000g for 10 minutes at 4^0C. After washing and removing all the residual PBS, 150 µl of 1X SDS gel-loading buffer (Ref. III. 2. 19) was added to the pellet. The samples were placed in a boiling water bath for 10 minutes to denature the proteins and centrifuged at 10,000g for 10 minutes at room temperature. The supernatant was transferred to a fresh tube and pellet discarded.

Aliquots of 35 µl of sample were electrophoretically fractionated on 10% SDS-PAGE minigels (Refs. III. 2. 17. and III. 2. 21) using electrode buffer (Ref. III. 2. 10) and were electroblotted onto a nitrocellulose filter cut to the exact size of the gel. The bottom electrode (anode) was laid with the graphite side up, over which 3 sheets of Whatman paper strips, nitrocellulose filter, gel, 3 sheets of Whatman paper strips and the upper electrode (cathode) graphite side down were placed in that order and properly aligned after removal of any trapped air bubbles. The transfer was done

for 2 hours in transfer buffer (Ref. III. 2. 23). The filter was stained with Ponceau S to visualize the molecular weight markers and to confirm that electrophoretic transfer of proteins has occurred. The filter was washed with several changes of deionised water to proceed to immunological probing.

To reduce background of nonspecific staining, potential blocking sites were blocked for 45 minutes with skim milk (Ref. III. 2. 3). The filter was incubated overnight in a sealed plastic bag at 4^0C with the primary antibody- mouse monoclonal antibody to human telomerase reverse transcriptase (catalytic subunit) at 1:100 dilution. The filter was washed thrice with TBST (Ref. III. 2. 26). After incubation for 1 hour with HRP conjugated anti-mouse secondary antibody diluted 1:1000, the filter was washed with TBST and incubated with the chromogen, diamino benzidine (Ref. III. 2. 6) till brown colored bands were clearly visible. The filter was washed twice in TBST, dried and scanned. Lysate from the cancer cell line HCT-116 was used as a positive control for telomerase expression.

III.4.2.11. Estimation of nitrite in culture supernatants

The stable metabolite of NO synthesized by the cells, nitrite was assayed by Griess reaction by the method of Marletta *et al.* (1988). The cells were cultured in 25 cm^2 tissue culture flasks in M199 without phenol red until confluence. The cells were then incubated for 24 hrs in 0.4% serum containing medium and subsequently treated with 10 ng/ml TNF-α or 1 μg/ml LPS. After 24 hr incubation, the medium was collected and centrifuged at 1500g for 15 minutes at 4^0C to remove cellular debris. The culture supernatant was added to a 1:1 (v/v) Griess reagent. The absorbance at

550 nm was measured and nitrite concentration determined using a calibration curve with sodium nitrite standards.

The assay was done at passages 7 and 82 to determine whether repeated passaging of cells altered their functional characteristics.

III.5. ISOLATION, CULTURE AND CHARACTERIZATION OF CARDIAC FIBROBLASTS

III.5.1. Isolation of cardiac fibroblasts

Cardiac fibroblasts were isolated from neonatal rats following the method of Nair and Gupta (1988) with some modifications.

Newborn rat pups (3-4 days old) were anaesthetized with ether. The thoracic cavity was exposed and heart excised. Hearts were collected in PBS containing antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin) and amphotericin (2.5 µg/ml) and washed in PBS. The ventricular tissue was separated, minced into bits of approximately 1 mm^3 size and transferred to a conical flask containing a 3 mm x 5 mm teflon coated magnetic bar. The tissue bits were subjected to a series of 8 digestions, each of 10 minutes duration, in dissociation medium (Ref. III. 2. 8) containing collagenase Type IA (0.3 mg/ml), trypsin (0.3 mg/ml), deoxyribonuclease (5.5 µg/ml), BSA (1 mg/ml), 1 mM $CaCl_2$, antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin) and amphotericin (2.5 µg/ml). Digestion was aided by stirring using a magnetic stirrer maintained at 37°C. Supernatants from second to eighth digestions were collected in centrifuge tubes containing equal volume of M199 with 10% FBS. Cell suspension was centrifuged at 1200 rpm for 6 minutes. The cells from

all seven digestions were pooled and resuspended in complete growth medium (Ref. III. 2. 5). The cells were seeded to 35 mm cell culture dishes and incubated in a CO_2 incubator at 37^0C in 95% air-5% CO_2.

III.5.2. Pre-plating of cardiac fibroblasts

Cardiac fibroblasts were separated from other contaminating cell types such as myocytes and endothelial cells by a pre-plating method. The cells seeded on the culture dishes were incubated for 90 minutes. After discarding the non-adherent cells, the attached fibroblasts were washed and supplied fresh growth medium. The cells were again given a medium change the following day. The cells reached confluence in 48 hours.

The cells were subcultured by the standard trypsinization protocol described earlier for EECs.

III.5.3. Characterization of cardiac fibroblasts

Cardiac fibroblasts were characterized by their morphology and immunostaining properties.

III.5.3.1. Morphology

The cells were examined under an inverted phase contrast microscope for their morphological analysis.

III.5.3.2. Immunostaining

Immunocytochemical analysis of cardiac fibroblasts was done by staining the cells for vimentin and von Willebrand factor by the procedure described earlier for EECs.

III.6. EFFECT OF ENDOCARDIAL ENDOTHELIAL CELL CONDITIONED MEDIUM ON CARDIAC FIBROBLASTS

III.6.1. Preparation of conditioned medium

The experiments were performed on EECs in the 3rd - 4th passages. Cells were seeded at a density of $2x10^5$ cells/ml in 35mm culture dishes. After confluence was reached (2-3 days) the cells were made quiescent by reducing the serum content of the medium to 0.4% for 24 hours. On the day of the experiment, these cells were washed with PBS and maintained in 0.4% serum containing medium. At the end of the incubation period of 24 hours, the medium was collected, centrifuged at 10,000 rpm for 10 minutes to remove cellular debris and stored at -80^0C for further assay. DMEM was used in place of M199 for generating conditioned medium to assay collagen synthesis in cardiac fibroblasts.

III.6.2. Measurement of DNA synthesis

DNA synthesis was measured in terms of [^{3}H]-Thymidine incorporation into TCA-insoluble material, as described by Shivakumar *et al.* (1992), with some modifications.

Experiments were performed on cardiac fibroblasts exclusively in their 2nd passage. The cells were seeded at a density of $1x10^5$ cells/ml and incubated for 24 hours. The experiments were conducted on cultures that have been incubated with 0.4% serum containing medium for the past 24 hours. These cells were treated with 0.4% serum containing medium or medium conditioned by EECs, with [^{3}H]-Thymidine at a final concentration of 1 μCi/ml. At 24 hours, medium was discarded;

cell layer was washed with phosphate buffered saline, lysed in lysis buffer (Ref. III. 2. 14) and precipitated with 10% ice-cold TCA. The TCA-precipitated material was filtered onto a Whatman no.3 filter paper using a vacuum filtration unit and radioactivity determined (Ref. III. 2. 18) using a scintillation counter.

III.6.3. Measurement of collagen synthesis

Cardiac fibroblast collagen synthesis was measured according to a modification of the method described by Diegelmann and Peterkofsky (1972). After a 24 hour incubation period in DMEM supplemented with 0.4% FBS, the cells were washed and incubated for another 24 hours with 2μCi/ml [^{3}H]-Proline and 50 μg/ml ascorbic acid in DMEM containing 0.4% FBS or EEC conditioned medium. Thereafter, the supernatant was collected and cells were lysed using 1% Triton-X 100 containing 5 mM N-ethylmaleimide. The cell lysate and medium were pooled and the mixture divided into two aliquots. Proteins in one aliquot were precipitated with 10% ice-cold TCA. The second aliquot was digested with 30 μg/ml collagenase Type VII in Tris-$CaCl_2$ buffer (pH 7.4) (Ref. III. 2. 27) for 5 hours at 37^{0}C. At the end of collagenase digestion, the proteins were precipitated with 10% ice-cold TCA. The TCA-precipitated material in the two aliquots was filtered separately onto Whatman no.3 filter paper. Radioactivity (cpm) was determined using a scintillation counter. Collagen synthesis was calculated from the counts obtained for the two aliquots as follows

$$\text{Collagen (\% of total protein)} = \frac{\text{Collagenase released cpm} \times 100}{(\text{Non-collagen cpm} \times f) + \text{collagenase released cpm}}$$

A correction factor of f = 5.4 for non-collagen protein was used to adjust for the relative abundance of proline and hydroxyproline in collagen containing proteins.

III.6.4. Delineation of signal transduction pathway involved in endocardial endothelial cell induced proliferation of cardiac fibroblasts

III.6.4.1. Assay of endocardial endothelial cell derived factors in the conditioned medium

The levels of EEC derived factors, Endothelin-1, Angiotensin-II and Transforming growth factor-β1 in the conditioned medium from cells treated with TNF-α and LPS were assayed by sandwich ELISA using commercially available kits.

III.6.4.2. Effect of anti-TGF-β antibody and ET-1 receptor antagonists on endocardial endothelial cell induced proliferation of cardiac fibroblasts

Quiescent cultures of cardiac fibroblasts were pretreated for one hour with 10 μg/ml neutralizing anti-TGF-β-antibody (recognizes porcine TGF-β1.2 and TGFβ2), 1 μmol/l BQ123 (selective ET_A receptor antagonist) or 1 μmol/l PD 142893 (non selective ET_A/ET_B receptor antagonist), following which cells were incubated for 24 hours with or without the EEC conditioned medium containing 1μCi/ml [^{3}H]-Thymidine. The inhibitors were present throughout the entire incubation period. The direct effect of the TGF-β-antibody and ET receptor antagonists on cardiac fibroblast proliferation was studied by incubating the cells with these inhibitors in 0.4% FBS containing medium with 1μCi/ml [^{3}H]-Thymidine for 24 hours. The cells were processed for measurement of DNA synthesis as described in Section III. 6. 2. Radioactivity was measured using a liquid scintillation counter.

III.6.4.3. Effect of Protein Kinase C and Mitogen Activated Protein Kinase antagonists on endocardial endothelial cell induced proliferation of cardiac fibroblasts

Cardiac fibroblasts made quiescent by incubation in medium containing 0.4% medium for 24 hours were pretreated for one hour with 1 μmol/l Bis-indolylmaleimide (inhibitor of PKC isozymes α, β1, δ, ε and ζ) (Martiny-Baron *et al.* 1993) or 10 μmol/l PD 098059 (MEK inhibitor) following which cells were incubated for 24 hours with or without the endocardial endothelial cell conditioned medium containing 1 μCi/ml [^{3}H]-Thymidine. The inhibitor was present throughout the entire incubation period. The direct effect of BIM and PD 098059 alone on proliferation of cardiac fibroblasts, in the absence of conditioned medium was studied by incubating the cells with these inhibitors in 0.4% FBS containing medium with 1 μCi/ml [^{3}H]-Thymidine for 24 hours. The cells were processed for measurement of DNA synthesis and radioactivity measured in the scintillation counter.

III.6.4.4. Western Blot analysis to confirm whether MAPK activation is PKC dependent

To confirm whether the EEC conditioned medium-mediated growth of cardiac fibroblasts occurred via PKC activation of ERK1/2, the effect of BIM on ERK1/2 phosphorylation was examined. Cardiac fibroblasts from neonatal rats were cultured in 100 mm dishes. The cells were made quiescent by incubation in 0.4% FBS containing medium for 24 hours, after which the cells were preincubated for 1 hour with 1 μmol/l BIM and then treated with the EEC conditioned medium for 30 minutes in presence of the inhibitor (Cheng *et al.* 2003). Cardiac fibroblasts grown in 0.4%

M199 and in presence of EEC conditioned medium were also analyzed for ERK1/2 phosphorylation. At the end of the incubation period, the medium was discarded and cells washed twice with ice-cold PBS. The protocol followed was as described in Section III. 4. 2. 10. The primary antibody used was either rabbit anti-phospho-ERK1/2 at a dilution of 1:1000 or mouse anti-non-phospho-p42 ERK2 (MAPK2) at 1:600 dilution, to serve as control. The filter was washed thrice with TBST (Ref. III. 2. 26). After incubation for 1 hour with HRP conjugated anti-rabbit or anti-mouse secondary antibody respectively, both diluted 1:1000, the filter was washed with TBST and incubated with the chromogen, diamino benzidine (Ref. III. 2. 6) till brown colored bands were clearly visible. The filter was washed twice in TBST, dried and scanned.

III.6.5. Effect of pro-inflammatory cytokines on endocardial endothelial cell - cardiac fibroblast interaction

The experiments were performed on EECs in the 3^{rd} - 4^{th} passages. Cells were seeded at a density of $2x10^5$ cells/ml in 35mm culture dishes. After 2-3 days, when confluence was reached, the cells were made quiescent by incubation in M199 containing 0.4% FBS for 24 hours. These cells were washed with PBS and treated with 10 ng/ml TNF-α or 1 μg/ml bacterial LPS. Cells incubated in medium containing 0.4% FBS served as controls. Conditioned medium was collected after 24 hours, centrifuged at 10,000 rpm for 10 minutes to remove cellular debris and stored at – 80^0C for further assay. DMEM was used in place of M199 for generating conditioned medium to assay collagen synthesis in cardiac fibroblasts.

Proliferation of cardiac fibroblasts was assayed by [^{3}H]-Thymidine incorporation and rate of collagen synthesis by [^{3}H]-Proline uptake as described in Sections III. 6. 2. and III. 6. 3. respectively.

Direct effect of the inhibitors- BQ123, PD 142893, anti- TGF-β, PD 098059 and BIM on cardiac fibroblast proliferation was studied by first pre-treating fibroblast cultures with the inhibitors for one hour and then in fresh 0.4% FBS containing medium with 1 μCi/ml [^{3}H]-Thymidine for 24 hours in presence of the inhibitors. The cells were processed and proliferation assayed as described in Section III. 6. 2.

To ascertain whether TNF-α has a proliferative effect on cardiac fibroblasts, the cells were incubated with 10 ng/ml TNF-α and 1 μCi/ml [^{3}H]-Thymidine for 24 hours. Proliferation was assayed as described in Section III. 6. 2.

III.6.5.1. Cell viability test

The viability of the cells after treatment with the pro-inflammatory agents TNF-α and LPS was tested by trypan blue exclusion test. After incubating the cells for 24 hours with TNF-α or LPS, the cells were trypsinized and suspended in 10% FBS containing medium to inactivate trypsin. The cells were centrifuged, washed with PBS and resuspended in PBS. To the cell suspension, trypan blue solution in PBS was added to a final concentration of 0.04% and incubated for 4 minutes. The cells were counted in a Neubauer chamber. Cells which incorporated the dye and turned blue were counted as non-viable. The percentage of non-viable cells was calculated considering the total number of cells in the suspension. The total

percentage of non-viable cells in the TNF-α or LPS treated dishes was compared to non-treated controls.

III.6.5.2. Estimation of EEC derived factors in culture supernatants from TNF-α and LPS treated cells

Nitrite levels in the culture supernatants were assayed by Griess reaction. The assay was done as described in Section III. 4. 2. 11. The levels of ET-1 and TGF-β were assayed by ELISA.

III.7. STATISTICAL ANALYSIS

For experiments with more than two variables, ANOVA was employed for the comparisons. In case of a significant difference, Bonferroni t-test was carried out for comparison of pairs of variables. The values are expressed as mean ± SD. $p<0.05$ was considered statistically significant.

IV. RESULTS

IV.1. CHARACTERIZATION OF ENDOCARDIAL ENDOTHELIAL CELLS

IV.1.1. Morphological analysis

Endocardial endothelial cells attached to the cell culture dishes by 6 hours of incubation at 37^0C in a CO_2 incubator. Except for a few single cells, mostly the cells remained attached in clumps (Figure 2). The cells had well defined nuclei and numerous cytoplasmic granules. The cells were found to spread out starting from the periphery of the clumps, gradually making space for the cells in the centre to spread out, thus forming several colonies of cells in the dish. The cells reached confluence by the 7th or 8th day and exhibited the typical cobblestone morphology (Figure 3).

IV.1.2. Immunostaining

The cells grown on cover slips were tested for immunoreactivity to anti-Factor VIII related antigen, anti-desmin, anti-vimentin and anti-cytokeratin. The cells stained positive for anti- Factor VIII-related antigen (Figure 4), with the typical perinuclear distribution pattern and for anti-vimentin antibody. Smooth muscle cell contamination in the culture was <2% as indicated by desmin positivity in the cells (Figure 5). The cells stained negative for cytokeratin (Figure 6) excluding the possibility of epithelial cells in the culture. Considering these immunostaining results, it was concluded that the cultures contained >98% endothelial cells.

IV.1.3. Uptake of DiI-Acetylated LDL by endocardial endothelial cells

Primary EECs exhibited the ability for uptake of DiI-Ac-LDL. Fluorescence was predominantly punctuate with a perinuclear distribution (Figure 7). Individualized nuclei with no nuclear overlap were seen, indicating that the cells were organized in a monolayer and exhibited contact inhibition. Since the LDL uptake is done using live cells, their ability to incorporate DiI-Ac-LDL also confirms that the cells are functionally active.

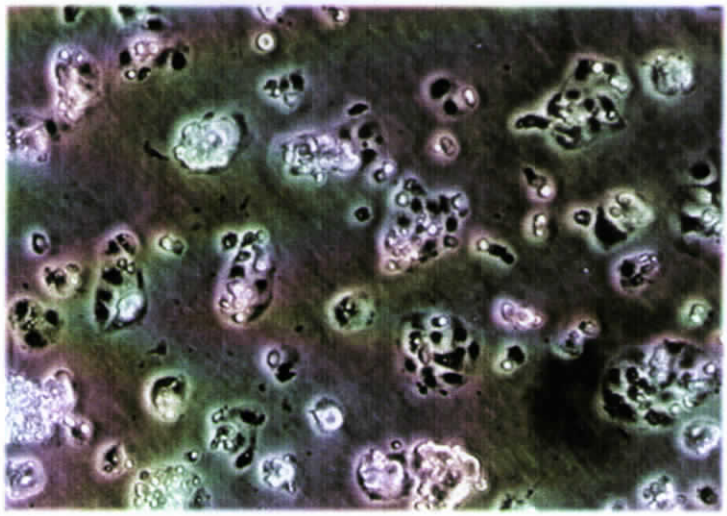

Figure 2: Photomicrograph of endocardial endothelial cells 6 hours after seeding (100 X)

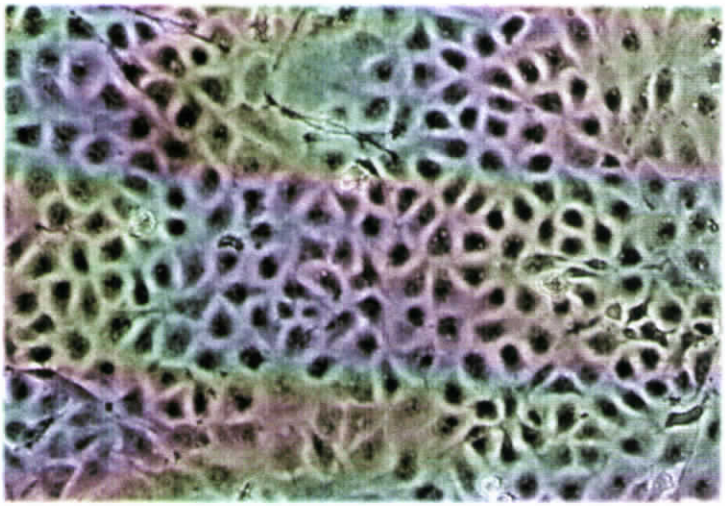

Figure 3: Photomicrograph of endocardial endothelial cells at confluence (100 X)

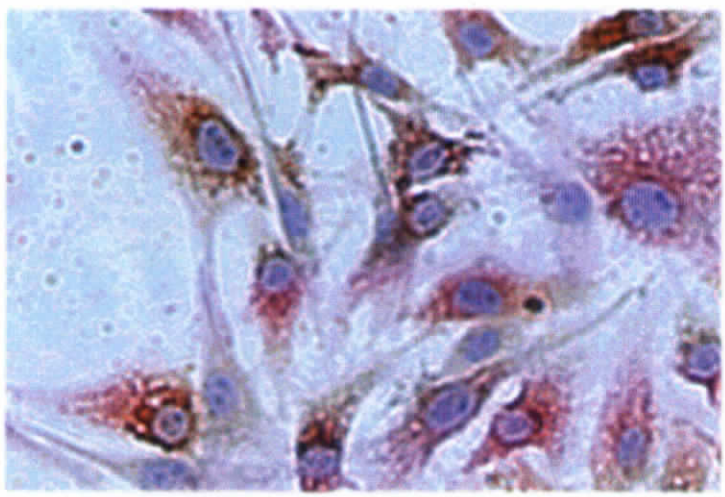

Figure 4: Photomicrograph of primary endocardial endothelial cells stained positive for factor -VIII related antigen (200 X)

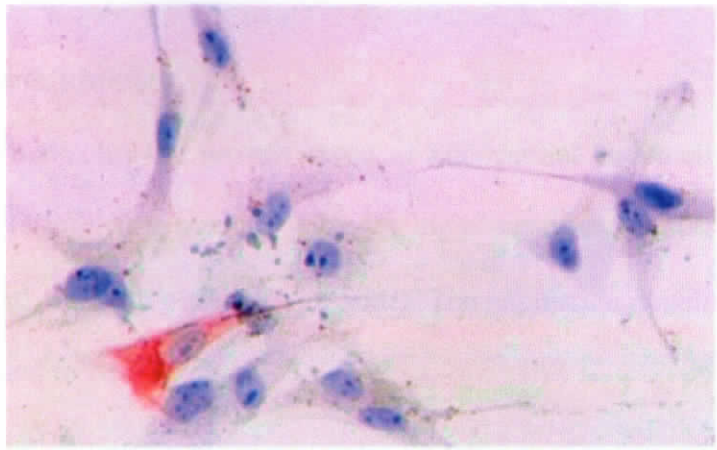

Figure 5: Photomicrograph of primary endocardial endothelial cells stained negative for desmin (200 X). There is one smooth muscle cell stained positive for desmin, the population of which constituted <2% of the total number of cells in the culture

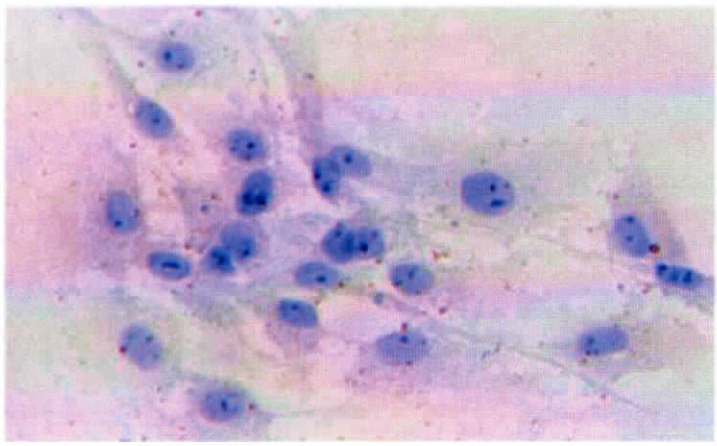

Figure 6: Photomicrograph of primary endocardial endothelial cells stained negative for cytokeratin (200 X)

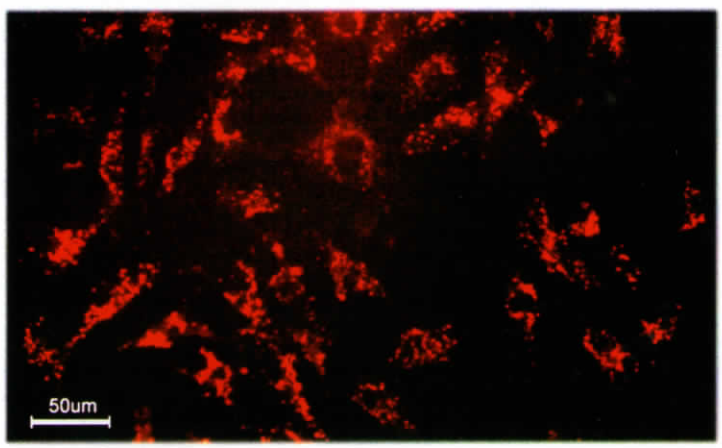

Figure 7: Fluorescence image of DiI-Acetylated-LDL uptake by primary endocardial endothelial cells (100 X)

IV.1.4. Growth kinetics

Figure 8 indicates the growth curve of endocardial endothelial cells for a period of 96 hours following seeding. After the log phase of growth till 72 hours, the cells demonstrated a growth plateau by 96 hours. The population doubling time of the cells was calculated to be 30 hours.

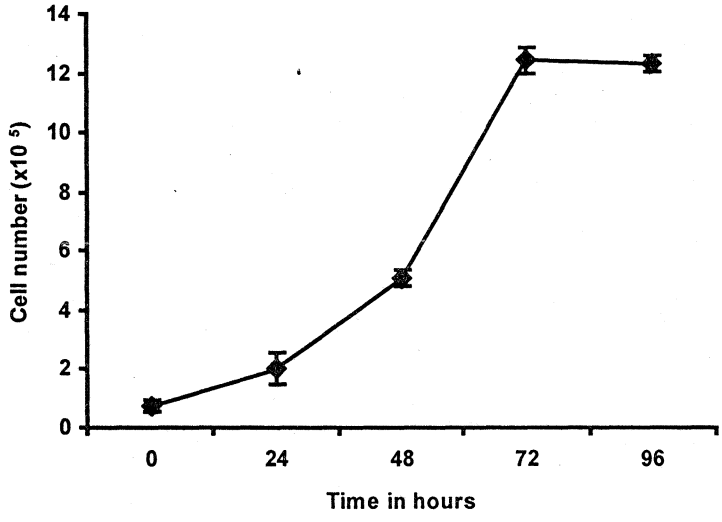

Figure 8: Growth curve of primary endocardial endothelial cells in culture

IV.2. IMMORTALIZATION OF ENDOCARDIAL ENDOTHELIAL CELLS

IV.2.1. Preparation of telomerized endocardial endothelial cells

Incubation of primary EECs with the transfection mix containing the mammalian expression vector LZRS-hTERT-IRES-GFP carrying full-length hTERT as well as the puromycin resistance gene, for six hours caused damage to many cells resulting in their detachment from the dish surface. Remaining cells attached to the culture dish regained their morphology and survived when incubated in complete growth medium. On incubation in the selection medium containing puromycin, only the cells which had taken up the plasmid survived. The selected population of cells formed colonies on further incubation and were passaged at confluence.

IV.2.2. Characterization of transfected cells

IV.2.2.1. Morphology

The morphological characteristics of puromycin-resistant cells were similar to the normal primary EECs. Polygonal cells at confluence organized in a homologous cobblestone pattern were seen under the phase contrast microscope (Figure 9).

IV.2.2.2. Immunocytochemistry .

The endothelial nature of the cells was demonstrated by positive staining for Factor VIII- related antigen by immunofluorescence. By confocal microscopy, rod shaped Wiebel-Palade bodies, which are the storage granules for Factor-VIII related

antigen was clearly evident in the perinuclear region of the FITC-stained cells (Figure 10).

Immuonocytochemical analysis demonstrated that the cells also stain positive for vimentin. The cells were negative for desmin (Figure 11) and cytokeratin (Figure 12), indicating that smooth muscle cells and epithelial cells are absent in the culture.

IV.2.2.3. Uptake of DiI-acetylated LDL

The transfected cells exhibited the ability for uptake of DiI-AC- LDL. When observed under a confocal microscope, the cells exhibited fluorescence which was predominantly punctuated with a perinuclear distribution, similar to primary EECs (Figure 13). Individualized nuclei with no nuclear overlap were seen, indicating the monolayer organization as well as contact inhibition exhibited by the cells.

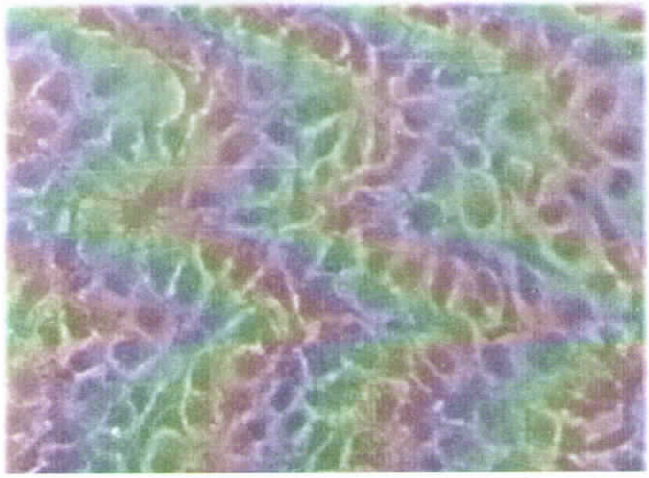

Figure 9 : Photomicrograph o f transfected endocardial endothelial cells at confluence demonstrating cobblestone morphology (100 X)

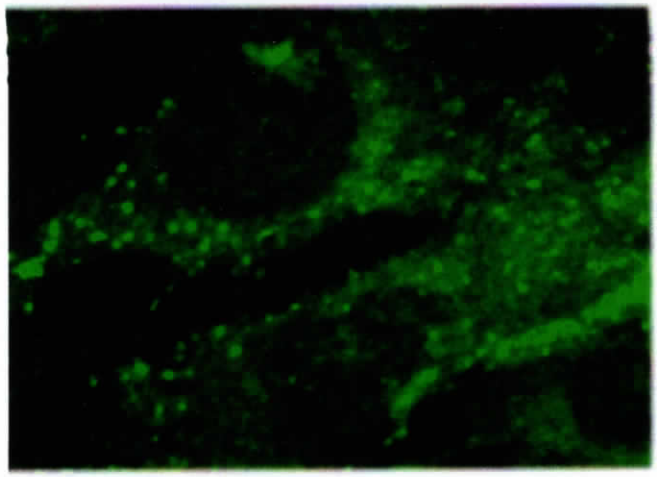

Figure 10 : Confoca l image o f transfected endocardial endothelial cells stained positive for factor -VIII related antigen (400 X)

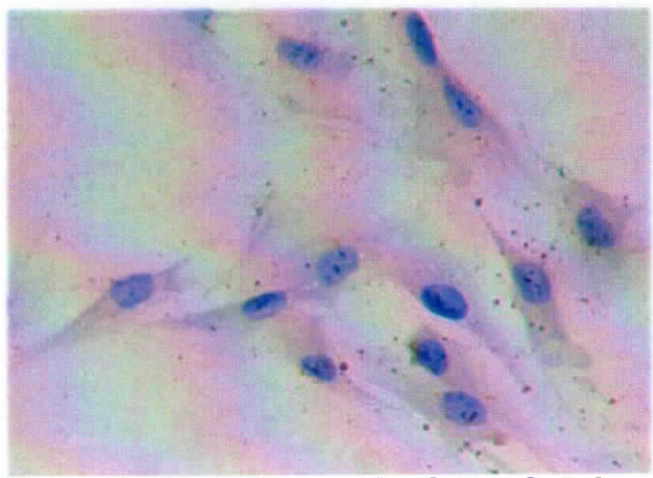

Figure 11: Photomicrograph of transfected endocardial endothelial cells stained negative for desmin (200 X)

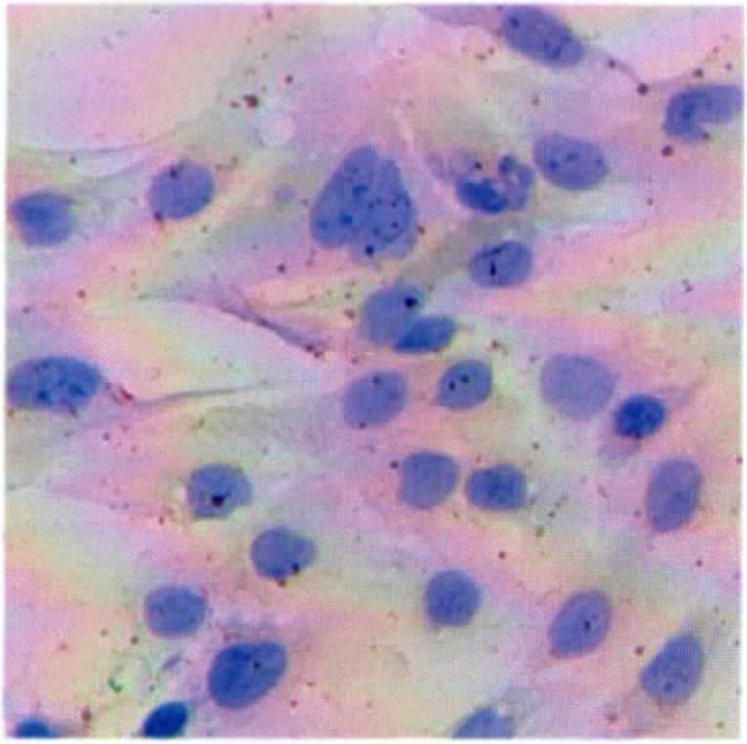

Figure 12: Photomicrograph of transfected endocardial endothelial cells stained negative for cytokeratin (200 X)

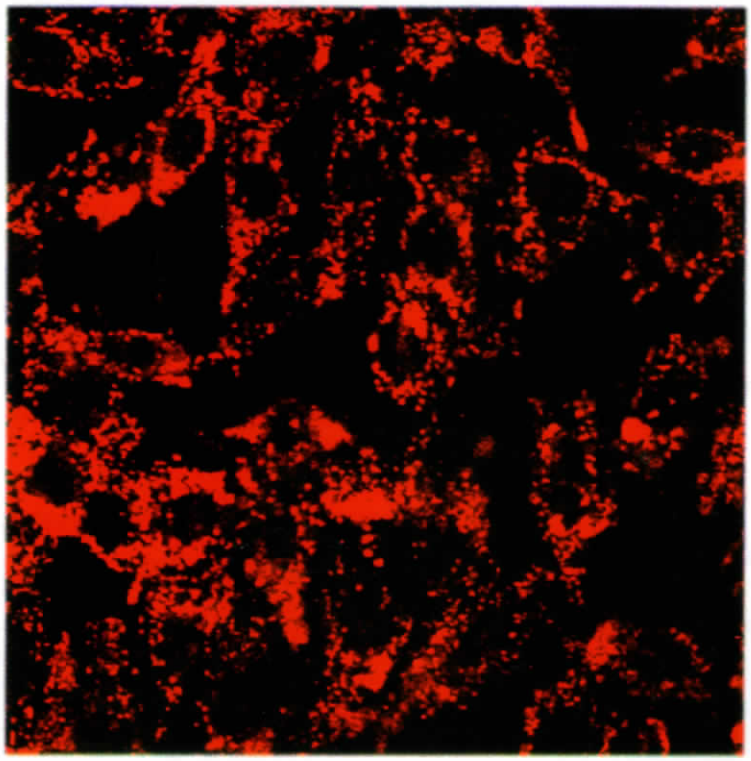

Figure 13: Confocal image of DiI-Acetylated-LDL uptake by transfected endocardial endothelial cells (200 X). Fluorescence is seen localized around the nucleus.

IV.2.2.4. Growth kinetics

Unlike primary EECs, the transfected cells could be passaged continuously and did not enter replicative senescence. These cells continued to proliferate and did not exhibit the morphological changes described in senescent cells. The cells are frozen and stored in their 98th passage. The transfection-positive cells had a greater doubling rate in 5% FBS containing medium compared to the 10% FBS containing medium. During the growth phase, doubling time of the cells in 5% FBS (43 hours) was shorter than those in 10% FBS (53 hours) (Figure 14).

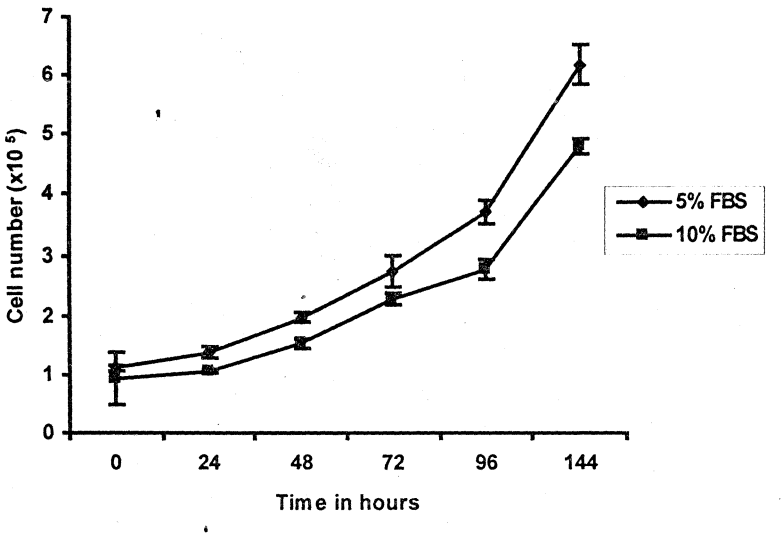

Figure 14: Growth kinetics of hTERT-transfected EECs at passage number 33 in medium 199 containing 5% fetal bovine serum (diamonds) and 10% fetal bovine serum (squares); (n=6)

IV.2.2.5. Proliferation assay

[^{3}H]-Thymidine uptake by the transfected cells reached the maximum at 24 hours and remained at a steady or slightly lower rate by 48 hours (n=4; $p<0.01$) and exhibited the same pattern of greater incorporation of [^{3}H]-Thymidine and increased DNA synthesis in 5% FBS compared to 10% FBS (Figure 15).

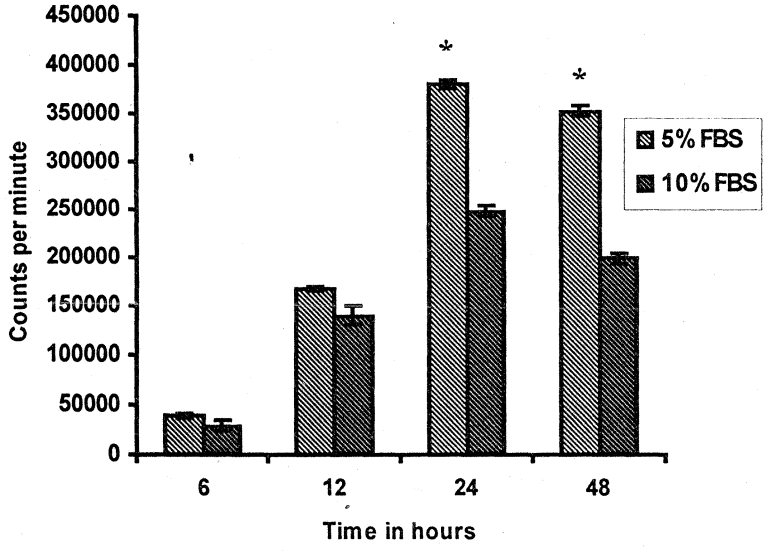

Figure 15: [^{3}H]-Thymidine incorporation by hTERT-transfected cells cultured in medium 199 containing 5% fetal bovine serum and 10% fetal bovine serum. The values are mean ± SD (n = 4; ANOVA; *$p<0.01$)

IV.2.2.6. RT-PCR for telomerase transcripts

The RNA yield from the cells was in the range of 35-60 μg/100 mm dish. The 260/280 ratio was ≥ 1.7 and the RNA was confirmed to be of good quality. The RNA samples gave clear and distinct bands on the gel indicating that the isolated RNA is intact, not degraded or sheared and are without protein or DNA contamination.

To ascertain that the extended life span of the cells was due to the expression of hTERT and not due to porcine TERT, the transfected cells were screened for hTERT and porcine TERT RNA, by RT-PCR. The cells expressed hTERT but lacked porcine TERT implying that the increased life span of the cells is due to the ectopic expression of hTERT and not due to the sustained expression of porcine TERT (Figure 16).

IV.2.2.7. Western blot analysis for telomerase catalytic subunit

To verify whether the cells with extended life span expressed hTERT protein, Western blot analysis was performed. The specific band of telomerase catalytic subunit was detected at 100 kDa (Figure 17).

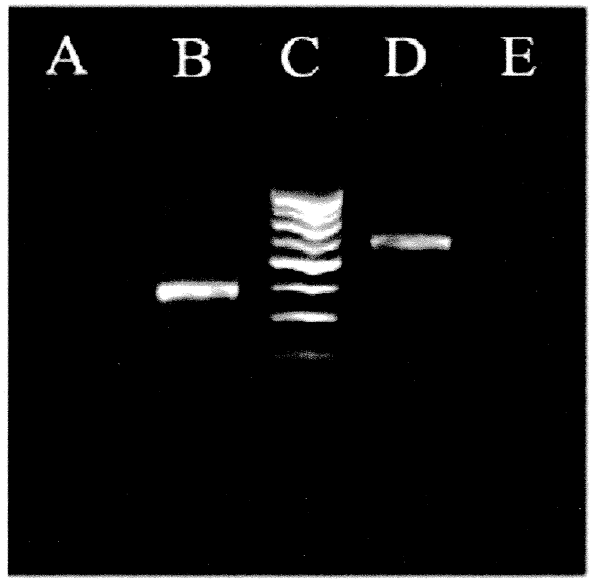

Figure 16: RT-PCR analysis of hTERT mRNA expression.

Lane A – absence of hTERT expression in primary EECs; Lane B – hTERT expression in transfected cells in 88th passage; Lane C – DNA ladder (100bp); Lane D – porcine TERT expression in primary EECs; Lane E - absence of porcine TERT in transfected cells in 88th passage.

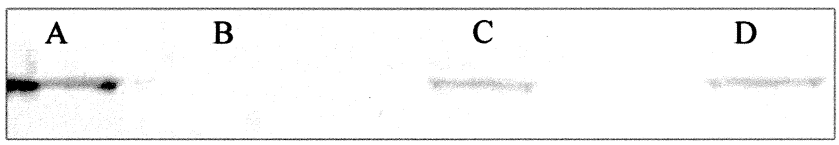

Figure 17: Western Blot analysis for the expression of hTERT.

Lane A - High levels of telomerase expression in cancer cell line HCT-116; Lane B - absence of telomerase in primary endocardial endothelial cells ; Lane C and D - Telomerase expression in hTERT-transfected endocardial endothelial cells in 92nd and 80th passage respectively.

IV.2.2.8. Transfected cells release comparable levels of nitrite as primary EECs when stimulated by pro-inflammatory agents

Normal primary EECs constitutively synthesize NO and release increased levels of NO in response to treatment by the pro-inflammatory cytokine TNF-α or bacterial LPS. The transfected cells retained the ability to release levels of nitrite comparable to that of the primary EECs both constitutively and when treated with the inflammatory agents. Nitrite levels in primary cells were 1.57 ± 0.33 μM. Tumor necrosis factor -α and LPS caused respectively 64% and 155% increase in the nitrite levels. Transfected cells in their 7th passage released 1.62 ± 0.16 μM nitrite. The pro-inflammatory agents increased the levels by 76% and 229% respectively. In the 82nd passage, the levels of nitrite released were 1.67 ± 0.2 μM. TNF-α and LPS induced 62% and 108% increase in nitrite release from the cells (Figure 18 & Figure 19).

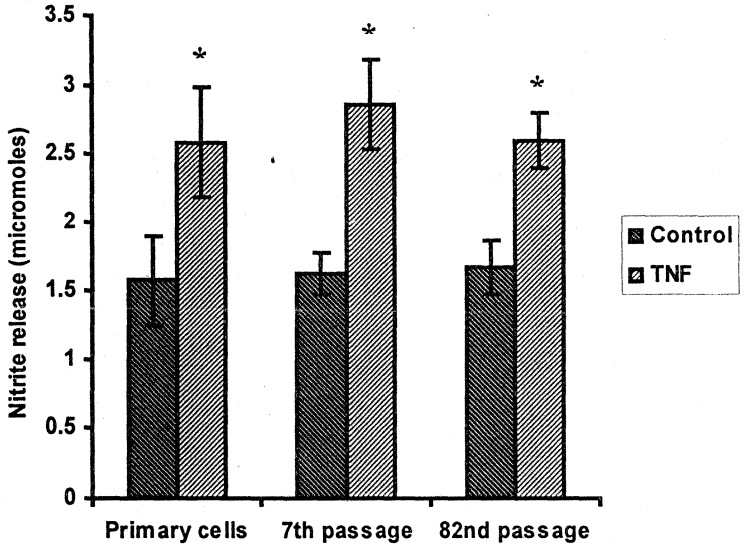

Figure 18: Nitrite release by primary endocardial endothelial cells (EECs), hTERT-transfected EECs in 7th passage and hTERT-transfected EECs in 82nd passage in response to 10 ng/ml TNF-α. The values are mean ± SD (n=6 in EECs, n = 3 in hTERT-transfected EECs; ANOVA; *$p<0.01$)

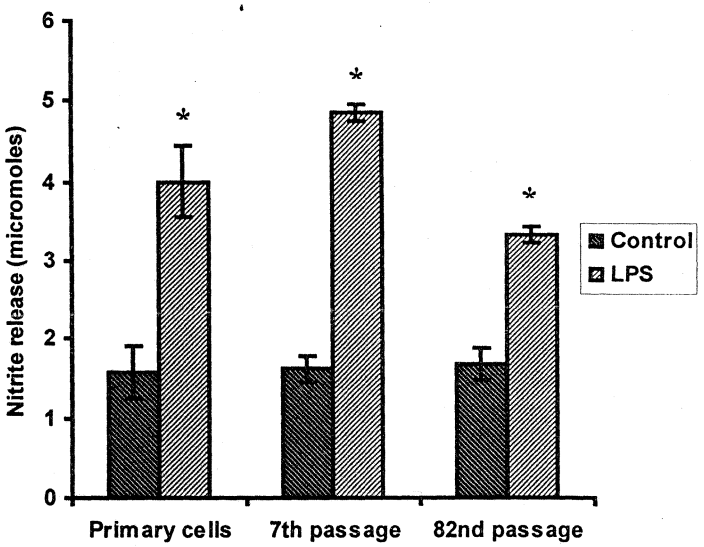

Figure 19: Nitrite release by primary endocardial endothelial cells (EECs), hTERT-transfected EECs in 7th passage and hTERT-transfected EECs in 82nd passage in response to 1μg/ml bacterial LPS. Control is the basal release of nitrite from the cells without any treatment. The values are mean ± SD; (n=6 in EECs, n = 3 in hTERT-transfected EECs; ANOVA; *$p<0.01$).

IV.3. CHARACTERIZATION OF CARDIAC FIBROBLASTS

IV.3.1. Morphological analysis

Cardiac fibroblasts from neonatal rats attached to the cell culture dishes by 90 minutes of incubation at 37^0C in a CO_2 incubator. The other cell types present in the cell suspension, such as myocytes and endothelial cells require longer time period to attach and therefore were eliminated from the culture by this pre-plating method. The cells attach and spread out within 90 minutes of incubation (Figure 20). The cells reached confluence in 48 hours (Figure 21) and were ready for passaging.

IV.3.2. Immunostaining

The cells grown on cover slips were tested for immunoreactivity to anti-vimentin and anti-Factor VIII related antigen. It was found that $\geq$ 99% of the cells stained positive for anti-vimentin (Figure 22) but negative for anti- Factor VIII-related antigen. Considering these results, it was confirmed that the cultures contained $\geq$ 99% cardiac fibroblasts.

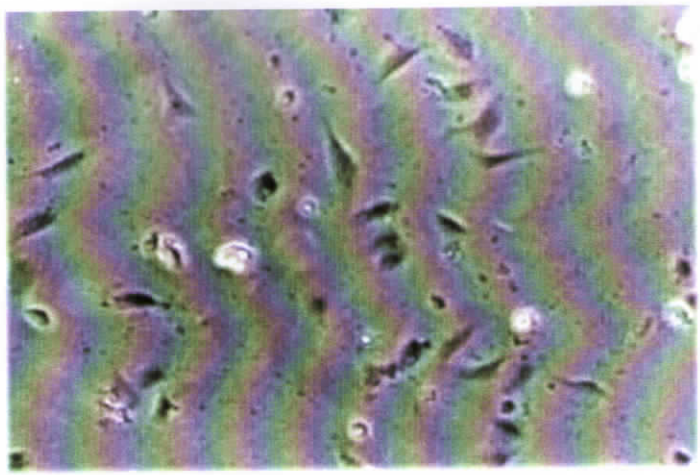

Figure 20: Photomicrograph of neonatal rat cardiac fibroblasts 90 minutes after seeding (100 X)

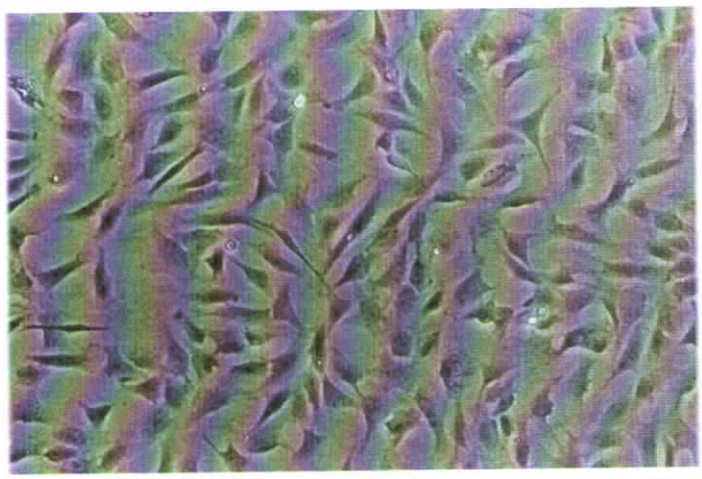

Figure 21: Photomicrograph of neonatal rat cardiac fibroblasts at confluence (100 X)

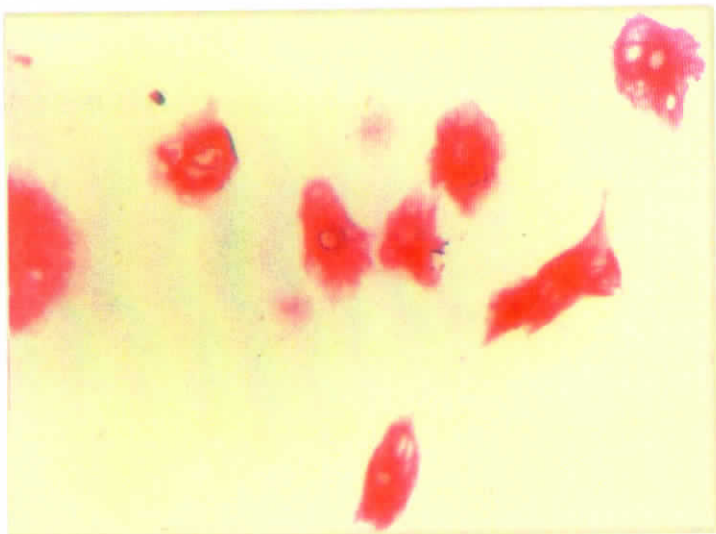

Figure 22: Photomicrograph of neonatal rat cardiac fibroblasts stained positive for vimentin (100 X)

IV.4. EFFECT OF ENDOCARDIAL ENDOTHELIAL CELL CONDITIONED MEDIUM ON CARDIAC FIBROBLASTS

IV.4.1. Endocardial endothelial cell conditioned medium increases [^{3}H]-Thymidine uptake by cardiac fibroblasts

To determine whether EECs exert a proliferative effect on cardiac fibroblasts, quiescent cultures of cardiac fibroblasts were incubated in the EEC conditioned medium for 24 hours. When compared to fibroblasts grown in the absence of conditioned medium, the incorporation of [^{3}H]-Thymidine into fibroblast DNA was increased by 53% ($n=15$; $p<0.05$) in cells incubated with the EEC conditioned medium (Figure 23).

IV.4.2. Endocardial endothelial cell conditioned medium enhances rate of collagen synthesis in cardiac fibroblasts

The effect of EEC conditioned medium on the rate of collagen synthesis by cardiac fibroblasts was examined by incorporation of [^{3}H]-Proline. Incubation of confluent cultures of fibroblasts in EEC conditioned medium for 24 hours caused a 65% increase ($p<0.001$) in their collagen synthesis (**Figure 24**) compared to the control cells grown in 0.4% FBS containing medium.

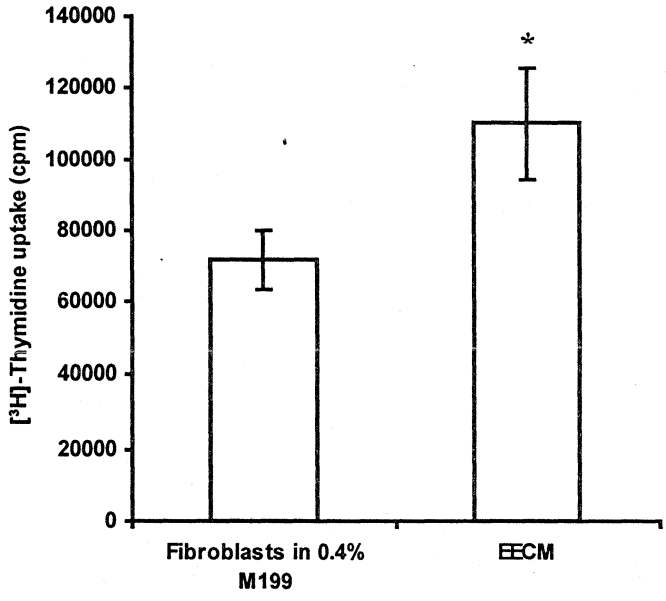

Figure 23: [^{3}H]-Thymidine uptake in cardiac fibroblasts cultured in 0.4% M199 and incubated with EEC conditioned medium (EECM) for 24 hours. The values are mean ± SD (n=15; *$p<0.05$)

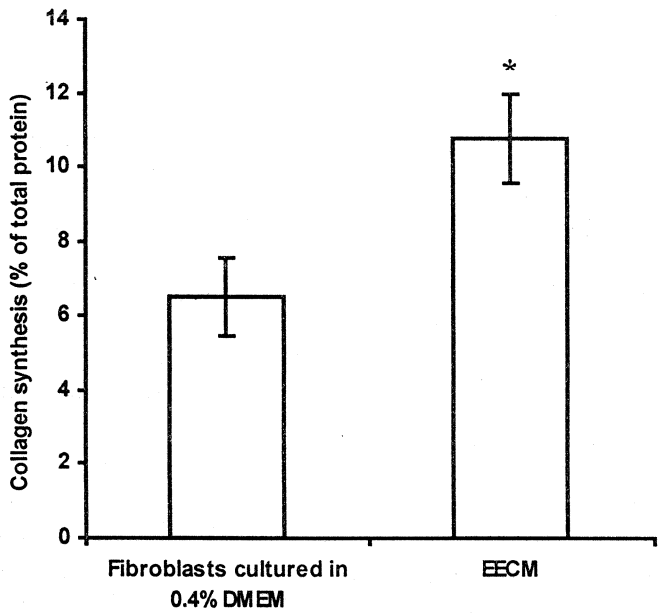

Figure 24: Collagen synthesis in cardiac fibroblasts cultured in 0.4% DMEM and incubated with EEC conditioned medium (EECM). The percentage of collagen represented is the relative proportion of [^{3}H]-Prollne incorporation into collagen vs. non-collagen protein. The values are mean ± SD (n=9. *p<0.001)

IV.4.3. Delineation of signal transduction pathway involved in endocardial endothelial cell induced proliferation of cardiac fibroblasts

IV.4.3.1. Endocardial endothelial cell derived factors in the conditioned medium

To determine the factors involved in the EEC induced effect on cardiac fibroblasts, the levels of EEC secreted factors such as ET-1, TGF-β and Ang-II in the conditioned medium was assayed using ELISA. Nitric oxide released into the medium was measured as nitrite by Griess reaction.

The level of ET-1 in the culture supernatants was 6.63 ± 0.0095 ng/ml (n=6; p<0.01).

The TGF-β level was 6.56 ± 0.1 ng/ml (n=6; $p<0.01$) and Ang II level was undetectable in the conditioned medium (n=6).

Nitrite level in the culture supernatant was 1.59 ± 0.25 μM (n=6; $p<0.01$).

IV.4.3.2. Selective inhibition of ET_A receptor of ET-1 using BQ123 depresses EEC induced proliferation of cardiac fibroblasts whereas nonselective inhibition of ET_A/ET_B receptors using PD 142893 enhances [^{3}H]-Thymidine incorporation

To examine the role of ET-1 in the EEC-induced proliferation of cardiac fibroblasts, ET-1 action through its ET_A and ET_B receptors were blocked using the selective ET_A receptor antagonist, BQ123 and nonselective ET_A/ET_B receptor antagonist PD 142893. Subsequently, the extent of [^{3}H]-Thymidine incorporation was measured.

Proliferation induced in cardiac fibroblasts by EEC conditioned medium was abolished on inhibition of ET_A receptor on the fibroblasts. Incubation of fibroblasts in conditioned medium, after pretreatment with BQ123 decreased the [^{3}H]-Thymidine uptake to basal values, similar to that of the fibroblasts which were grown in non-conditioned medium (n=6; $p<0.001$) (Figure 25).

Nonselective ET_A/ET_B receptor antagonist, PD 142893 decreased DNA synthesis levels by 34.8% (Figure 25) compared to fibroblasts grown in conditioned medium.

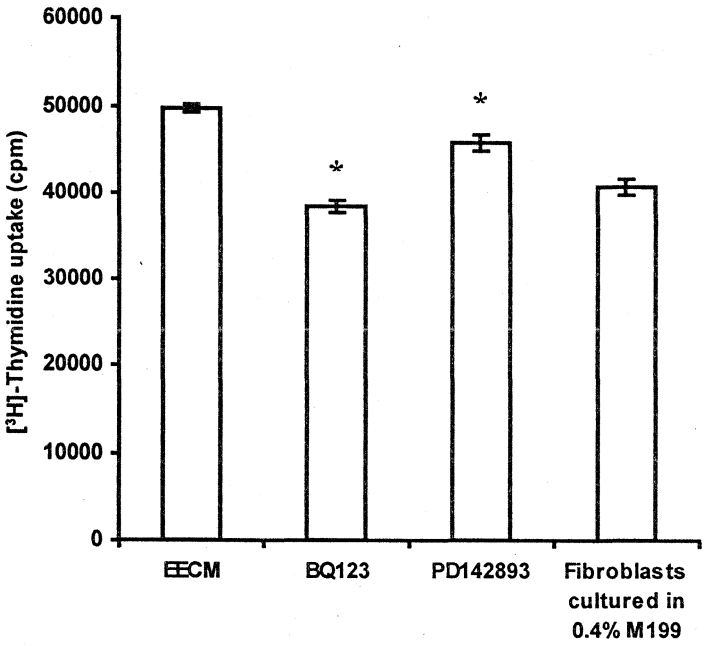

Figure 25: [^{3}H]-Thymidine uptake in cardiac fibroblasts incubated with EEC conditioned medium for 24 hours in the presence of ET_A antagonist BQ123 or ET_A/ET_B antagonist PD 142893 or in the absence of inhibitors (EECM) and in fibroblasts cultured in 0.4% M199. The values are mean ± SD (n=3; ANOVA; EECM vs. BQ123 and EECM vs. PD 142893, *$p<0.001$)

The direct effect of endothelin receptor antagonists on cardiac fibroblast proliferation was studied by incubating the cells with these inhibitors in 0.4% FBS containing medium for 24 hours. While incubation of cardiac fibroblasts with PD 142893 demonstrated a 40% ($n=3$; $p<0.01$) increase in [^{3}H]-Thymidine uptake, the inhibition of ET_A by BQ123 did not cause any variation in the DNA synthesis (Figure 26).

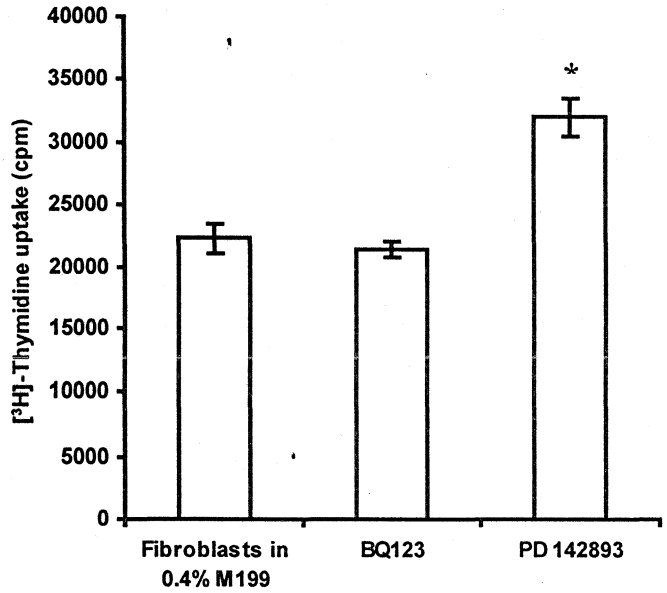

Figure 26: [^{3}H]-Thymidine uptake in cardiac fibroblasts incubated for 24 hours in the presence of ET_A antagonist BQ123, ET_A/ET_B antagonist PD 142893 or in the absence of inhibitors (Fibroblasts cultured in 0.4% M199 - control). The values are mean ± SD (n=3; ANOVA; Control vs. PD 142893, *$p<0.01$; Control vs. BQ123, non significant).

IV.4.3.3. Blocking of TGF-β augments proliferation of cardiac fibroblasts

To ascertain whether the significant levels of TGF-β in the conditioned medium contributed to the mitogenic effect of EEC conditioned medium on cardiac fibroblasts, [^{3}H]-Thymidine incorporation in fibroblasts was assayed after addition of anti-TGF-β antibody.

Cardiac fibroblasts incubated with EEC conditioned medium containing neutralizing anti- TGF-β antibody demonstrated an increase of 29.6% in DNA synthesis ($n=3$; $p<0.05$) compared to fibroblasts incubated without the antibody (Figure 27).

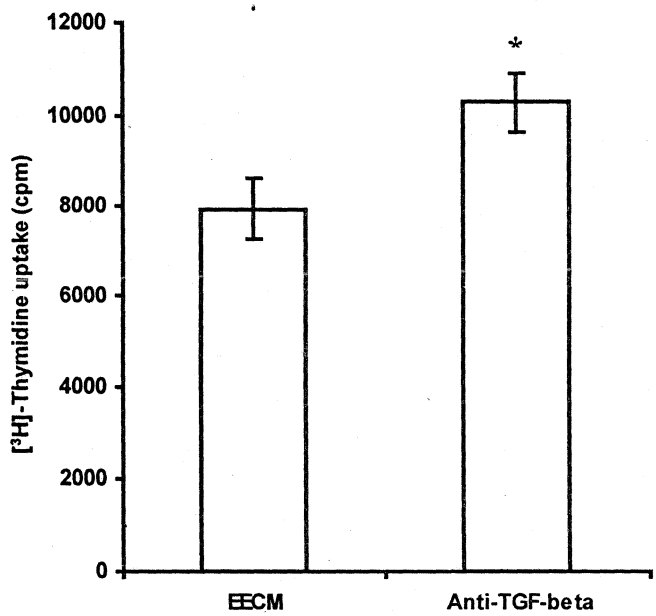

Figure 27: [^{3}H]-Thymidine uptake in cardiac fibroblasts incubated with EEC conditioned medium for 24 hours (EECM) and in the presence of neutralizing anti-TGF-β. The values are mean ± SD (n=3; *$p<0.05$)

Incubation of cardiac fibroblasts in 0.4% FBS containing medium with neutralizing anti-TGF-β demonstrated a 44% (n=3; $p<0.05$) increase in [^{3}H]-Thymidine uptake (Figure 28).

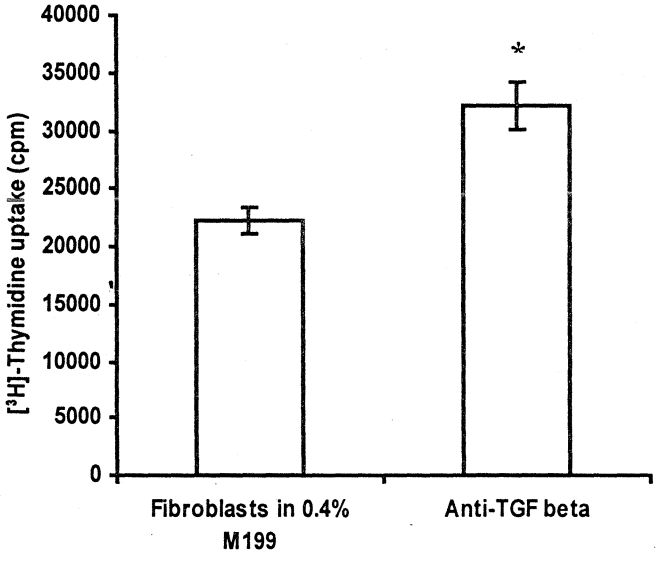

Figure 28: [^{3}H]-Thymidine uptake in cardiac fibroblasts incubated for 24 hours in the presence of anti-TGF-β or in the absence of the antibody (Fibroblasts cultured in 0.4% M199 - control). The values are mean ± SD (n=3; *$p<0.01$)

IV.4.3.4. Inhibition of PKC and MEK decreases EEC conditioned medium induced proliferation of cardiac fibroblasts

To delineate the signaling pathways involved in the mitogenic activity of EEC conditioned medium on cardiac fibroblasts, the key molecules, protein kinase C and mitogen activated protein kinase kinase were inhibited using the specific inhibitors, bis-indolylmaleimide and PD 098059. Inhibition of MAPK by pretreatment of fibroblasts with PD 098059 resulted in complete blockade of EEC induced proliferation of fibroblasts ($n=3$; $p<0.001$). PKC inhibitor BIM in the EEC conditioned medium also depressed the EEC induced DNA synthesis in cardiac fibroblasts by 48% ($n=3$; $p<0.001$) (Figure 29).

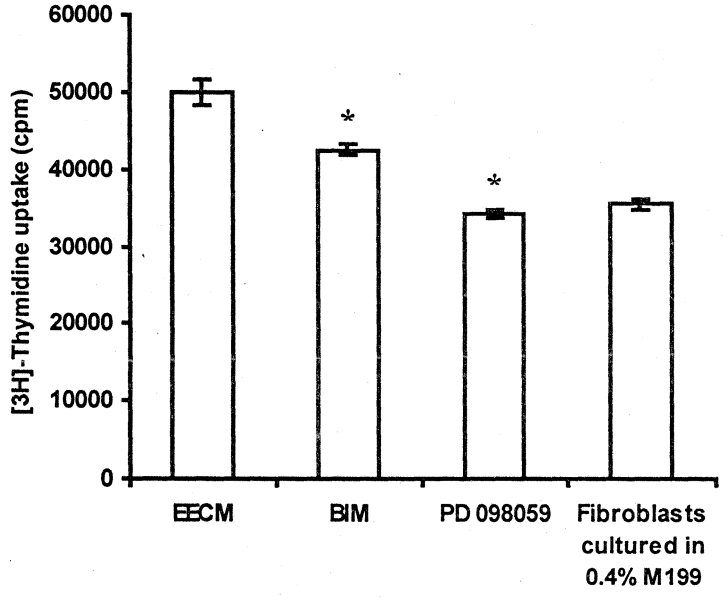

Figure 29: [^{3}H]-Thymidine uptake in cardiac fibroblasts incubated with EEC conditioned medium for 24 hours in the presence of PKC inhibitor bis-indolyl maleimide (BIM), MAPK inhibitor PD 098059, in the absence of inhibitors (EECM) or in fibroblasts cultured in 0.4% M199. The values are mean ± SD (n=3; ANOVA; *$p<0.001$)

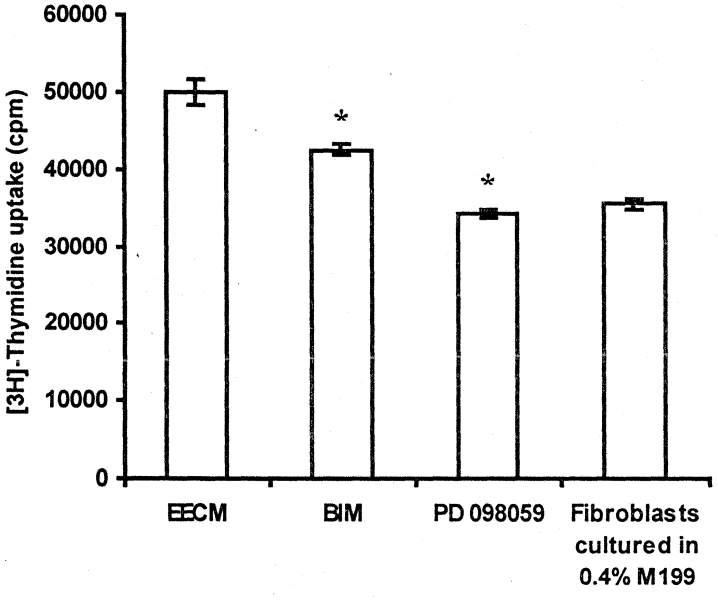

Figure 29: [^{3}H]-Thymidine uptake in cardiac fibroblasts incubated with EEC conditioned medium for 24 hours in the presence of PKC inhibitor bis-indolyl maleimide (BIM), MAPK inhibitor PD 098059, in the absence of inhibitors (EECM) or in fibroblasts cultured in 0.4% M199. The values are mean ± SD (n=3; ANOVA; *$p<0.001$)

In cardiac fibroblasts incubated without the conditioned medium, bis-indolyl maleimide reduced the [^{3}H]-Thymidine uptake by 11% (n=3; $p<0.01$) compared to the controls and the MEK inhibitor decreased the DNA synthesis levels by 29.5% (n=3; $p<0.005$) (Figure 30).

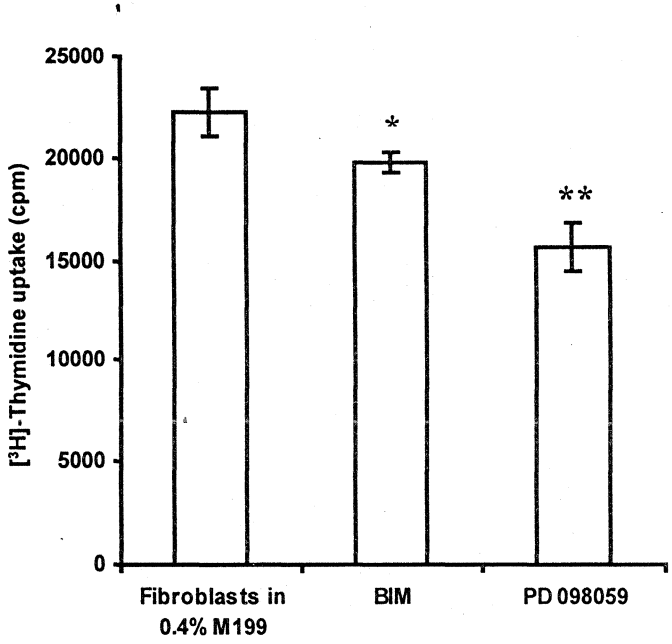

Figure 30: [^{3}H]-Thymidine uptake in cardiac fibroblasts incubated for 24 hours in the presence of PKC inhibitor BIM, MAPK inhibitor PD 098059 or in the absence of inhibitors (Fibroblasts cultured in 0.4% M199 - control). The values are mean ± SD (n=3; ANOVA; Control vs. BIM, *$p<0.05$. Control vs. PD 098059, **$p<0.005$).

IV.4.3.5. Endocardial endothelial cell induced proliferation of cardiac fibroblasts is through PKC dependent MAPK activation

To examine whether the activation of MAPK is through a PKC dependent signaling cascade, MAPK activation was analyzed by western blot, after inhibition of PKC with BIM.

Protein expression analysis in cardiac fibroblasts after incubation in EEC conditioned medium demonstrated that the conditioned medium activates ERK1/2 (p44 and p42) in cardiac fibroblasts. As shown in Figure 31, incubation in conditioned medium caused an increased phosphorylation of ERK1/2. The PKC inhibitor, BIM significantly inhibited the conditioned medium-induced phosphorylation of ERK1/2, particularly p44, in cardiac fibroblasts. This finding confirms the premise that MAPK activation occurs via PKC.

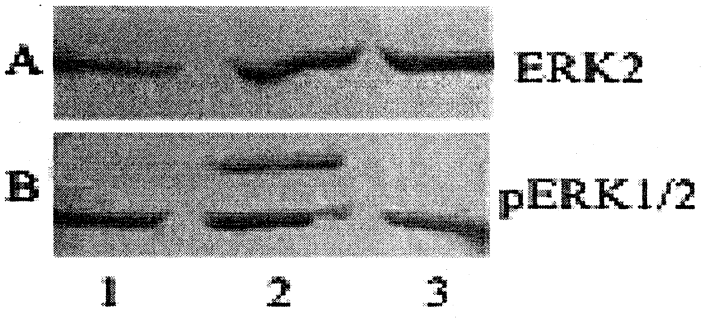

Figure 31: Western blot analysis to detect whether the activation of MAPK is through a PKC dependent signaling cascade. MAPK activation was analyzed by western blot, after inhibition of PKC with bis-indolyl maleimide. A: Non-phosphorylated ERK2 (p42). B: Phosphorylation of ERK1/2 (p44 and p42) detected by means of Western blotting with anti-phospho-ERK1/2 antibody.

Lane 1- Fibroblasts incubated in M199 containing 0.4% M199 for 30 minutes. Lane 2 - Fibroblasts incubated in EEC conditioned medium for 30 minutes. Lane 3 - Fibroblasts preincubated for 1 hour with BIM and incubated with the EEC conditioned medium for 30 minutes. Endocardial endothelial cell conditioned medium induced an increase in ERK1/2 phosphorylation and BIM significantly inhibited the conditioned medium induced ERK phosphorylation.

IV.4.4. The pro-inflammatory cytokine TNF-α and bacterial lipo polysaccharide attenuate the EEC-induced cardiac fibroblast proliferation

To determine whether pro-inflammatory agents, TNF-α and LPS alter the mitogenic effect of EEC conditioned medium on cardiac fibroblasts, the proliferation of fibroblasts was assayed after incubating in conditioned medium from EECs treated with TNF-α or LPS for 24 hours.

Both TNF-α and LPS minimized the proliferative effect that EEC conditioned medium had on cardiac fibroblasts. TNF-α attenuated DNA synthesis level by 40.08% (n=15; $p<0.01$) and LPS by 41.03% (n=15; $p<0.01$) of the value for EEC conditioned medium induced proliferation in cardiac fibroblasts (Figure 32).

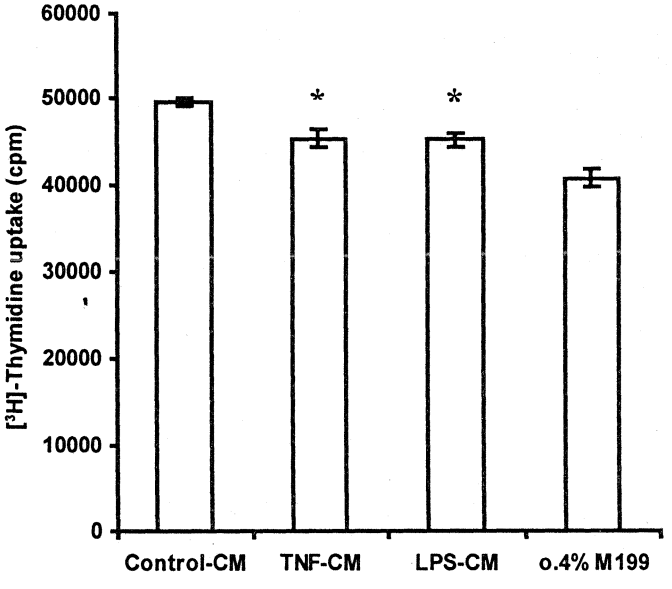

Figure 32: [^{3}H]-Thymidine uptake by cardiac fibroblasts incubated with conditioned medium from EECs treated with TNF-α and LPS. The values are mean ± SD (n=15; ANOVA; Control-CM vs. TNF-CM and Control-CM vs. LPS-CM, *$p<0.01$)

IV.4.4.1. TNF-α and LPS attenuate EEC-induced collagen synthesis in cardiac fibroblasts

When fibroblasts were incubated with conditioned medium from EEC treated with TNF-α or LPS, the rate of collagen synthesis in fibroblasts was attenuated to values below the basal synthesis rate. TNF-α decreased collagen synthesis (by 25.5% of that of the basal synthesis rate in 0.4% FBS containing medium and) by 54.25% of the synthesis rate in EEC conditioned medium. LPS reduced the value to (21.35% of the basal rate and) 51.69% of the synthesis rate in the EEC conditioned medium (Figure 33).

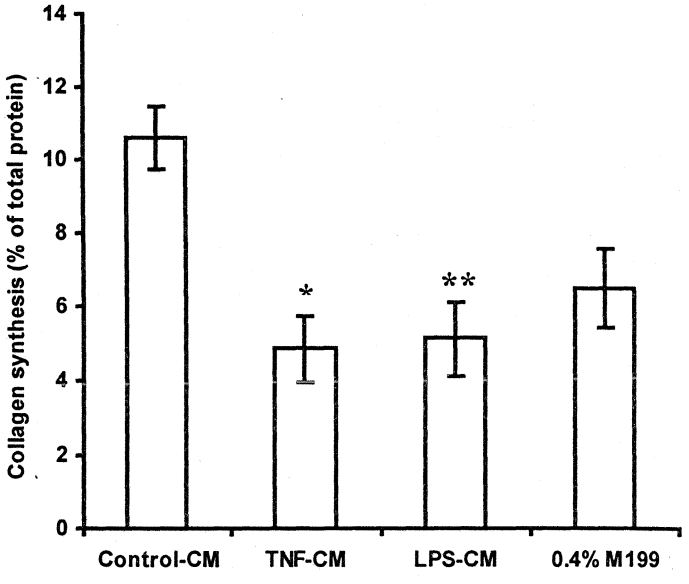

Figure 33: Collagen synthesis by cardiac fibroblasts in response to incubation with conditioned medium from EECs treated with TNF-α and LPS for 24 hours. The values are mean ± SD (n=6; ANOVA; Control-CM vs. TNF-CM and Control-CM vs. LPS-CM, *$p<0.05$)

IV.4.4.2. Cells treated with TNF-α and LPS are viable

To establish that the cells treated with the pro-inflammatory agents are still viable when their conditioned medium is analyzed for the various mediators, the viability of cells was determined by the trypan blue dye exclusion test. From the cell counts, it was calculated that only >2% of the cells are non-viable and the remaining cell population remained viable after 24 hours of treatment with these agents.

IV.4.4.3. Endocardial endothelial cells release increased levels of nitrite in response to pro-inflammatory stimuli

In order to determine the factors that could contribute to the attenuation of EEC mediated effects on cardiac fibroblasts, the levels of endocardium derived factors such as NO, TGF-β and ET-1 were measured.

The EECs were stimulated with the pro-inflammatory agents and NO release was measured after 24 hours. Treatment of EECs with TNF-α and LPS caused an increased release of nitrite from these cells into the culture supernatant. Stimulation of EECs with TNF-α increased endocardial cell nitrite release by 64% whereas stimulation with LPS caused 155% increase in the nitrite levels (Figure 34).

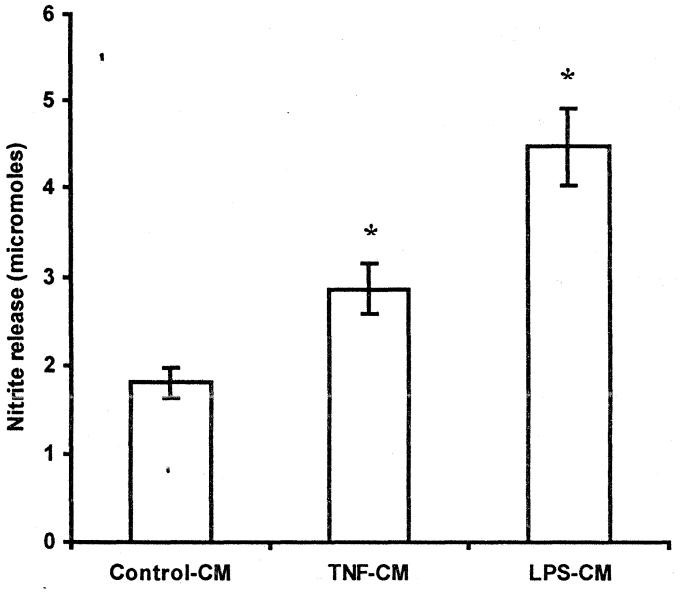

Figure 34: Nitrite release by endocardial endothelial cells in response to pro-inflammatory agents TNF-α and LPS. Values are mean ± SD (n=6; ANOVA; *$p<0.05$)

IV.4.4.4. TNF-α and LPS alters release of endocardial endothelial cell derived factors into the conditioned medium

Treatment of EECs with TNF-α and LPS altered the release of the major EEC derived mediators, ET-1 and TGF-β. While TNF-α depressed the release of ET-1 by 16.13%, it increased TGF-β release by 10%. However, LPS depressed the secretion of both ET-1 and TGF-β into the conditioned medium. ET-1 secretion was reduced by 13% and TGF-β secretion by EECs was decreased by 12.5% on treatment with LPS (Figure 35 and Figure 36).

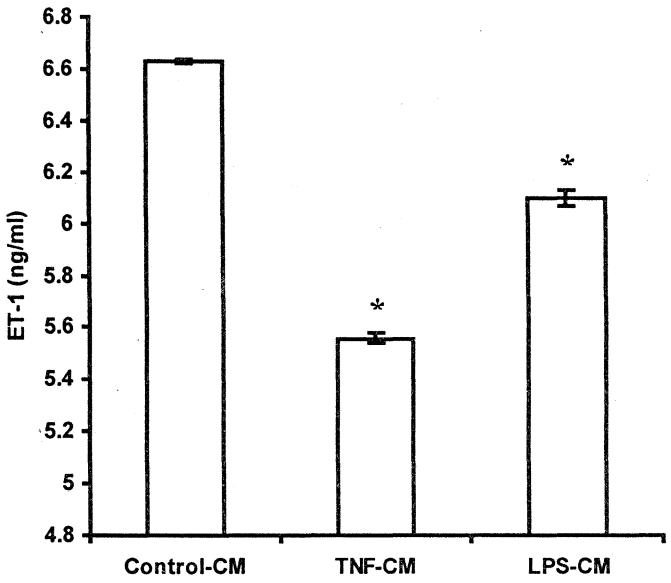

Figure 35: Effect of TNF-α and LPS on endothelin-1 release by endocardial endothelial cells. The values are mean ± SD (n=6; ANOVA; *p<0.01)

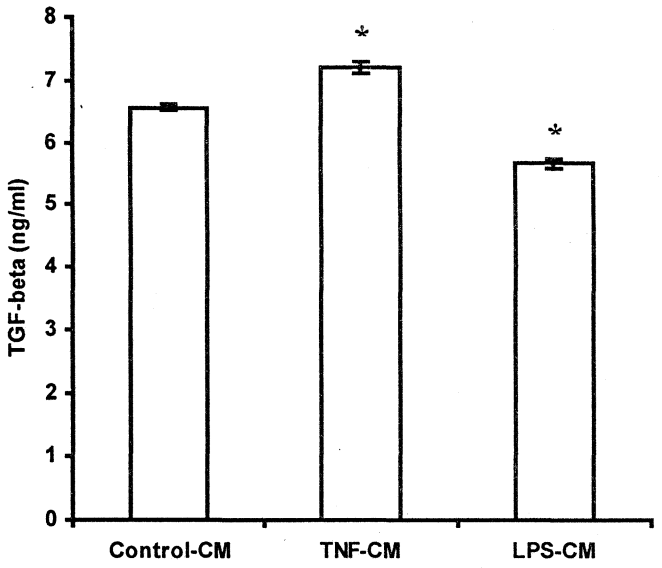

Figure 36: Effect of TNF-α and LPS on transforming growth factor-β release by endocardial endothelial cells. The values are mean ± SD (n=6; ANOVA; *p<0.01)

IV.4.4.5. Residual proliferation induced by EECs treated with TNF-α is not mediated through ET-1 or TGF-β

To determine whether the residual levels of proliferation occurring in response to incubation in conditioned medium from EECs treated with TNF-α, the fibroblasts were pretreated with ET-1 receptor inhibitors, BQ123 and PD 142893 or anti-TGF-β antibody and subsequently incubated in the conditioned medium. There was no significant difference in the [^{3}H]-Thymidine incorporation compared to controls in any of these treated groups (Figure 37 and Figure 38).

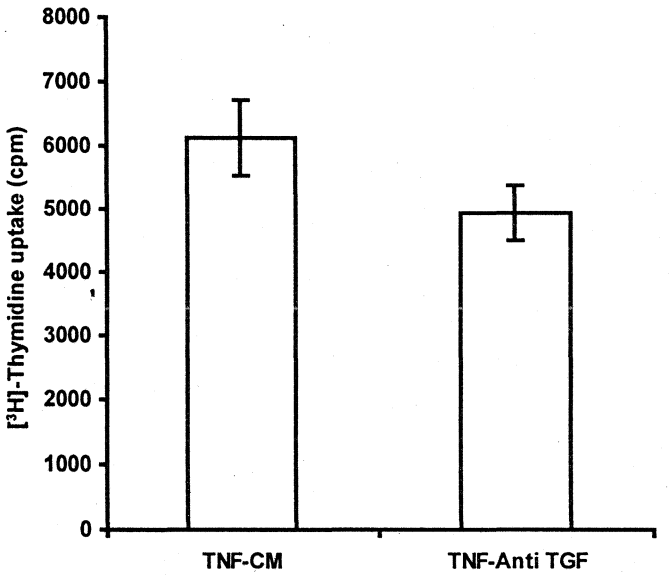

Figure 37: Effect of Anti-TGF-β antibody on TNF-α mediated [^{3}H]-Thymidine uptake in cardiac fibroblasts. The values are mean ± SD. (n=3; TNF-CM vs. TNF-Anti-TGF-β is non significant)

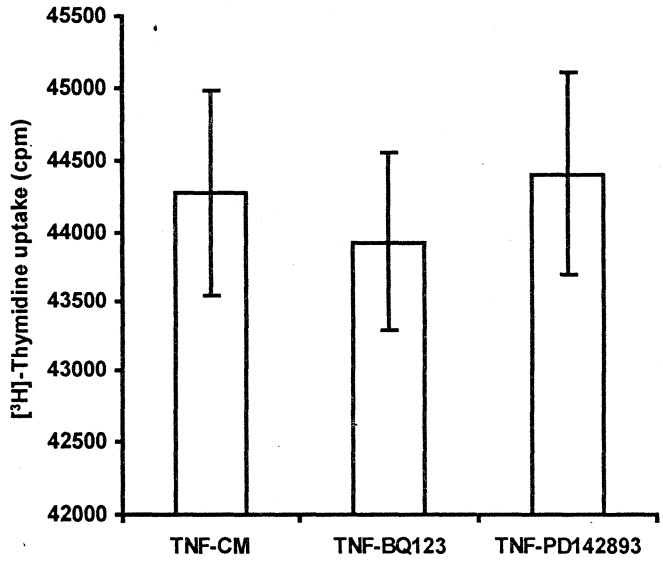

Figure 38: Effect of specific ET_A inhibitor, BQ123 and non-specific ET_A/ET_B inhibitor on [^{3}H]-Thymidine uptake by cardiac fibroblasts incubated with conditioned medium from EECs treated with TNF-α. The values are mean ± SD (n=6; ANOVA; TNF-CM vs. TNF-BQ123 and TNF-PD 142893 are non significant)

It was inferred from the above data that the residual proliferation seen in cardiac fibroblasts incubated in conditioned medium from EECs treated with TNF-α is possibly because of the direct effects of surplus TNF-α in the medium. To ascertain this possibility, [^{3}H]-Thymidine uptake in cardiac fibroblasts was assayed after incubation in TNF-α for 24 hours. TNF-α was found to be a potent mitogen, inducing 5.7 fold increase in [^{3}H]-Thymidine uptake in cardiac fibroblasts (Figure 39).

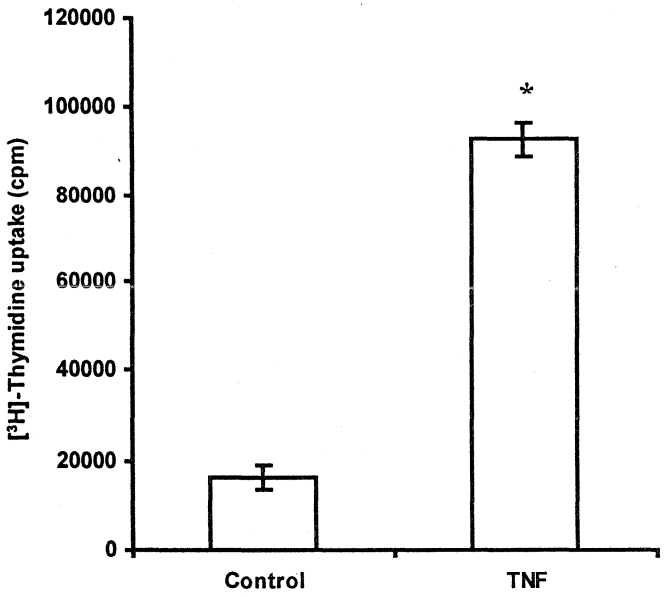

Figure 39: [^{3}H]-Thymidine uptake by cardiac fibroblasts in response to treatment with TNF-α for 24 hours. The values are mean ± SD. (n=6; *p<0.001)

IV.4.4.6. Residual proliferation induced by EECs treated with TNF-α involves signaling through PKC and MAPK

To verify whether the residual proliferation occurring with conditioned medium from TNF-α involved PKC and MAPK, the proliferation of cardiac fibroblasts was assayed after pretreating with BIM or PD 098059 and then with conditioned medium. Inhibition of PKC decreased [^{3}H]-Thymidine incorporation by 52.8% whereas inhibition of MAPK caused 39% decrease in proliferation rates (Figure 40).

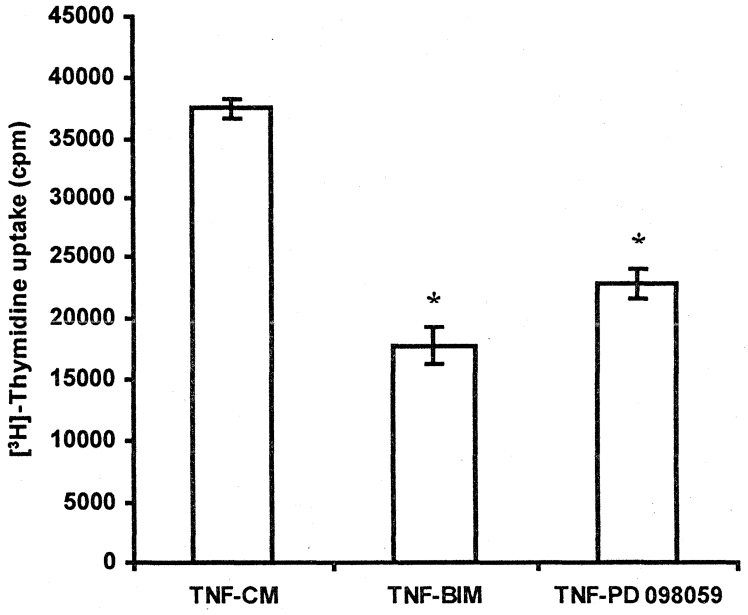

Figure 40: Effect of PKC inhibitor, bis-indolyl maleimide and MAPK inhibitor, PD 098059 on [^{3}H]-Thymidine uptake by cardiac fibroblasts incubated with conditioned medium from EECs treated with TNF-α. The values are mean ± SD (n=6; ANOVA; TNF-CM vs. TNF-BIM and TNF-CM vs. TNF-PD 098059, *$p<0.001$)

V. DISCUSSION

Endothelial cells and their subjacent ECM form a heterogeneous complex with specific physiological and morphological properties in different parts of the vasculature (Andries *et al.* 1995). The vascular endothelial cells have an established regulatory role on neighboring non-endothelial cells such as myocytes, fibroblasts, pericytes and SMCs (Gajdusek *et al.* 1980; Gajdusek and Schwartz 1982; Baird and Ling 1987; Villanueva *et al.* 1991; Guarda *et al.* 1993). Considering the regulatory role of endothelial cells on neighboring cells and the modulator role of EE on the subjacent myocardium the present *in vitro* study explored whether EE has a role in regulating cardiac interstitium, in normal as well as in conditions of elevated cytokine levels in the heart. The regulatory role of EECs on cardiac fibroblast proliferation and collagen synthesis was confirmed, after which an attempt was made to examine the possible mechanisms of regulation and delineate the signaling pathways involved.

V.1. ISOLATION, CHARACTERIZATION AND IMMORTALIZATION OF ENDOCARDIAL ENDOTHELIAL CELLS

V.1.1. Isolation and characterization of endocardial endothelial cells

The cells isolated from porcine ventricles were confirmed to be EECs as described by Smith *et al* (Smith *et al.* 1991). Myocytes were absent on observation under phase contrast microscopy. Negative immunostaining for desmin ruled out the possibility of the presence of both SMCs and myocytes in the culture. The cells at confluence formed a monolayer, without a 'hill and valley' pattern characteristic of VSMCs. Furthermore, the cells incorporated DiI-Ac-LDL, a feature characteristic of

endothelial cells and macrophages and absent in other cell types of the heart (Voyta *et al.* 1984). Individual nuclei were clearly evident, without any nuclear overlap, on uptake of DiI-Ac-LDL, indicating that the cells exhibit contact inhibition. Together with the fact that the cells demonstrate cobblestone pattern in culture and stain positive for Factor VIII-related antigen establishes their endothelial nature. It has been earlier shown that the strategy of filling the ventricular cavities with collagenase and removing the loosened cells ensures isolation of cells of endocardial origin, not contaminated by vascular endothelial cells (Smith *et al.* 1991; Mebazaa *et al.* 1993).

V.1.2. Immortalization of endocardial endothelial cells

In the present study it is demonstrated that porcine ventricular EECs can be immortalized by the ectopic expression of hTERT. Somatic cells have a limited life span *in vitro*. Shortening of the telomeres occurs upon each cell division eventually leading to cell cycle arrest. This shortening of telomere can be prevented by expression of telomerase reverse transcriptase, which elongates short telomeres, thereby preventing replicative senescence. Results of the present study imply the general applicability of using the ectopic expression of hTERT to bypass replicative senescence while maintaining endothelial cell morphogenetic and phenotypic characteristics *in vitro*.

Upon transfection of hTERT, telomerase protein expression was detectable in the EECs. To rule out the possibility of spontaneous transformation of EECs, mRNA expression of hTERT by RT-PCR was performed. This method is now regarded as an alternative assessment of telomerase function since hTERT mRNA expression is

closely associated with telomerase activity (Kyo *et al.* 1999). There was also evidence for the expression of telomerase catalytic subunit in the immortalized cells in comparison with normal primary EECs and cancer cell lines. This one-step immortalization of EECs by telomerase expression, to our knowledge has not been reported earlier and is thus significant. The data shows that it is possible to use hTERT to generate immortal EECs in a convenient, reliable and reproducible manner.

The transfected cells have been continuously passaged without evidence of altered morphology. To date, the hTERT expressing clones have achieved a passage number 5 to 6 times that of parental cells and therefore are considered immortal.

The cells exhibit the property of contact inhibition, ruling out malignant transformation. The notion that hTERT expression mediates life span extension but not transformation was emphasized by studies of ectopic hTERT expression in other human somatic cells that showed that these cells do not display characteristics of transformed cells (Jiang *et al.* 1999; Morales *et al.* 1999).

V.1.2.1. Telomerase activity confers survival advantage in hTERT transfected EECs

Normal EECs require 20% FBS for their optimum growth. The immortalized cells exhibit a distinct survival advantage as these cells have a higher growth rate in reduced serum percentage of 5%. The reduction in serum and growth factor requirement for the growth of these cells is beneficial in the culture of larger number of cells for various studies. The doubling time of transfected cells during their growth phase in 5% FBS (43 hours) was shorter than those in 10% FBS (53 hours). Greater incorporation of [^{3}H]-Thymidine and increased DNA synthesis was also observed in

5% FBS compared to 10% FBS. These findings imply that ectopic hTERT expression confers a level of survival advantage in these cells. Telomerase expression lowers the demand for higher serum percentage or other growth factors for the survival of these cells. Therefore telomerase could act as a survival enzyme.

From various studies it has been concluded that telomerase not only allows long-term unlimited growth but also improves cellular resistance against a wide variety of stressors and cytotoxic agents and more over, in many cases, this survival function appeared unrelated to maintenance of telomere length. It is established that maintenance of telomerase activity during differentiation of embryonic stem cells confers several advantages on the differentiated cells, such as enhanced proliferation, resistance to apoptosis and oxidative stress, and improved differentiation toward hematopoietic lineages by expansion of the progenitor population. (Armstrong *et al.* 2005). The results from a study using $CD4^+$ helper and regulatory helper T-cell clones indicate that ectopic hTERT expression extends the replicative capacity of human T cells upon activation and prolongs survival by protecting against oxidative stress–induced DNA damage and apoptosis (Luiten *et al.* 2003). The data from the present study is in line with the other available reports on the survival- promoting effects of telomerase.

V.1.2.2. Immortalized endocardial endothelial cells constitutively release nitric oxide

Matsushita *et al* (Matsushita *et al.* 2001) have earlier demonstrated that replicative aging result in decreased endothelial expression of eNOS and stable expression of hTERT results in a younger endothelial cell phenotype, exhibiting

greater amount of eNOS and NO activity. Our finding that stable levels of NO are released by the immortalized EECs corroborate their observation that transduction of hTERT into endothelial cells retain the constitutive NOS expression and prevent senescence in the cells.

V.1.2.3. Immortalized endocardial endothelial cells respond to pro-inflammatory agents by releasing increased levels of nitric oxide

Treatment of primary EECs with TNF-α and LPS caused an increased release of nitrite from the cells into the culture supernatant. Similarly, transfected cells release higher levels of nitrite in response to treatment by pro-inflammatory agents. It therefore appears that hTERT transfection has not affected the ability of these cells to respond to pro-inflammatory stimuli, which is an important functional capability of EECs. Even after repeated passaging, these cells retain the functional characteristics of the primary cells implying that they are suitable for experiments in a manner similar to that of primary EECs.

V.2. EFFECT OF ENDOCARDIAL ENDOTHELIAL CELL CONDITIONED MEDIUM ON CARDIAC FIBROBLASTS

The regulatory effect of EECs on cardiac fibroblasts was studied by assaying fibroblast proliferation and collagen synthesis in response to conditioned medium from EECs. The *in vitro* model was ideal for the study mainly because the results are not affected by the complex hemodynamic parameters and is purely because of the factors present in the conditioned medium. The fact that EECs are arranged as a single

layer of cells, both *in vivo* as well as in culture, implies that the responses of these cells *in vitro* may be comparable to that *in vivo*.

V.2.1. Endocardial endothelial cells stimulate proliferation and collagen synthesis of cardiac fibroblasts

The significant increase in [^{3}H]-Thymidine incorporation into cardiac fibroblast DNA when incubated in EEC conditioned medium connotes that EE has a mitogenic effect on cardiac fibroblasts. Endocardial endothelial cells also enhanced collagen synthesis in cardiac fibroblasts. These effects on fibroblasts are possibly mediated by substances released by EEC. Endocardial endothelial cells secrete pro-proliferative and pro-fibrotic factors, which have a paracrine effect on cardiac fibroblasts. The data supports the hypothesis that EE, besides its physiological control over myocyte mechanics, have a significant role in the regulation of fibroblast proliferation and collagen synthesis in the cardiac interstitium.

Earlier studies with vascular endothelial cells have indicated the trophic effect of vascular endothelial cells on fibroblasts. DiCorleto, in studies using BAECs and human umbilical vein endothelial cells demonstrated that multiple mitogens are actively secreted by cultured endothelial cells (DiCorleto 1984). In addition, Villanueva *et al* in studies using BAECs and human embryonic lung fibroblasts suggested that endothelial cells release a factor which stimulates protein production and thus plays a role in the remodeling of vascular connective tissue by stimulating collagen synthesis. Their results established that the protein formation in fibroblasts stimulated by endothelial cell-derived factor is mediated by a substance distinct from

either TGF-β or insulin-related peptides (Villanueva *et al.* 1991). Guarda and Weber (Guarda and Weber 1995) on examining the influence BAECs on rat cardiac fibroblast collagen synthesis under co-culture conditions reported that an endothelial-cell-derived-factor other than aldosterone and Ang II signals an increase in cardiac fibroblast collagen synthesis and augmentation of collagenase activity. Results of the present study supplement these observations and provide evidences that the modulator role of endothelial cells is not restricted to the vascular endothelium, but is present in the heart as well. The findings are in keeping with the complex interaction that exists between endothelial cells and neighboring non-endothelial cells. Such a cell-cell interaction, mediated by factors having paracrine properties on fibroblasts can alter ECM protein turnover.

In general, growth of the non-myocyte cells in the heart can lead to structural remodeling of the myocardium and vasculature. Pathological myocardial fibrosis which is a serious complication of chronic heart disease generally results from an imbalance between the synthesis and breakdown of collagen (Jugdutt 2004). Cardiac remodeling that occurs in response to myocardial infarction is often accompanied by a disproportionate increase in collagen deposition. Collagen accumulation has a dual effect on cardiac structure and function. Though increased collagen deposition is necessary for preventing dilatation of the infracted area, excessive growth of cardiac fibroblasts and accumulation of collagen can lead to diastolic and systolic dysfunction, disturbances in conduction and can also contribute to the development of heart failure (Cleutjens 2004; Kim and Iwao 2000). Inhibition of the development of

cardiac fibrosis may exert a favorable effect on cardiac integrity (Maki *et al.* 2002). The EE mediated effect on cardiac fibroblasts is likely to be of significance in the pathogenesis of perivascular fibrosis and endomyocardial fibrosis (Weber 1989; Weber and Brilla 1991; Kartha 1995).

V.3. SIGNALING PATHWAYS INVOLVED IN THE REGULATION OF CARDIAC FIBROBLAST PROLIFERATION BY ENDOCARDIAL ENDOTHELIAL CELLS

V.3.1. Endocardial endothelium induced proliferation of cardiac fibroblasts is not mediated through TGF-β and Ang II

Endothelium is known to produce several growth factors and substances such as ET, TGF-β and Ang II, (Shah *et al.* 1996; Shah 1996; Brutsaert *et al.* 1996) which are stimulatory for connective tissue cells. Among the secreted substances known to be mitogenic for cardiac fibroblasts, the levels of ET-1, TGF-β and Ang II were assayed. An attempt to identify the mitogenic factor in the conditioned medium was made by inhibiting the above factors and subsequently assaying the proliferation of fibroblasts.

These factors, though primarily secreted by endothelial cells, can be expressed by cardiac fibroblasts as well, under varying conditions. Eghbali *et al* have reported that TGF-β in the heart is also expressed by cardiac fibroblasts (Eghbali 1989; Zhao and Eghbali-Webb 2001). Endothelin-1 is endogenously induced in cardiac fibroblasts in response to Ang II (Clozel and Salloukh 2005; Gray *et al.* 1998) and in pathological states of the heart. In the present study, Ang II was not detected in the

EEC conditioned medium. Guarda and Weber had previously reported that neither Ang II nor aldosterone influences the response in cardiac fibroblast collagen metabolism to vascular endothelial cell conditioned medium (Guarda and Weber 1995). Findings of the present study using EECs validate their observations in vascular endothelial cells.

Inhibition of TGF- β in the EEC conditioned medium caused an increase in incorporation of [^{3}H]-Thymidine by cardiac fibroblasts. Increased proliferation levels were attained when TGF-β was inhibited in cultures of cardiac fibroblasts alone. This obviously suggests that TGF-β inhibits proliferation of cardiac fibroblasts, whether endogenously secreted by fibroblasts or secreted by the overlying EECs. These findings are consistent with the observations in previous studies on human and rabbit cardiac fibroblasts (Agocha *et al.* 1997-a; Agocha *et al.* 1997-b; Sigel *et al.* 1996).

V.3.2. Endothelin-1 mediates EEC conditioned medium induced proliferation of cardiac fibroblasts through its ET_A receptor

Endothelin has been shown to have a mitogenic effect on SMCs, bronchial epithelial cells and cardiac fibroblasts (Clozel and Salloukh 2005; Fujisaki *et al.* 1995). ET-1 is also identified to have profibrotic potential (Hafizi *et al.* 2004; Guarda *et al.* 1993). Since it is known that the effect of ET is mediated through the G protein coupled receptors - ET_A and ET_B on cardiac fibroblasts (Katwa *et al.* 1993; Piacentini *et al.* 2000), effect of blocking the ET receptors was studied. It was found that ET_A receptor blockade nullified the EEC induced proliferation of cardiac fibroblasts. The effect of ET_A selective receptor antagonist BQ123 was more pronounced than that of

the non-selective ET_A/ET_B inhibitor PD 142893. This differential effect is not surprising since the distinct effect of the two types of ET receptor antagonists have been demonstrated earlier with respect to blood vessels, where selective ET_A receptor blockade caused a greater increase in cardiac output and reduction in systemic vascular resistance than non-selective ET_A/ET_B receptor blockade (Leslie *et al.* 2005). Further, the ET_A receptor is reported to launch most of the excitatory and proliferative response, whereas ET_B mediates predominantly inhibitory and/or excitatory responses (Drimal *et al.* 1999). Thus the results of this study clearly suggest that the EEC mediated stimulation of cardiac fibroblasts is mediated by ET-1 through its ET_A receptor. This inference is also supported by observations on the direct effects of ET-receptor blockade on cardiac fibroblasts. While non-selective ET_A/ET_B receptor blockade, which removes the inhibitory effect of the predominant ET_B receptor in fibroblasts, increased the proliferation in these cells, blockade of ET_A receptor alone did not alter the proliferation rates. This finding imply that endogenous ET-1 from fibroblasts has no role in cardiac fibroblast proliferation and that ET-1 in the EE conditioned medium is responsible for the proliferation.

V.3.3. Proliferation of cardiac fibroblasts induced by EEC conditioned medium is through MAPK activated signaling cascade dependent on PKC

Divergent pathways are involved in the action of ET-1. In an attempt to trace the signaling pathways involved in the mitogenic activity of EECs on cardiac fibroblasts, the role of MAPK or ERKs was elucidated. The synthetic inhibitor, PD 098059 that selectively inhibits the MAPK-activating enzyme, MAPK/ERK kinase

(MEK) without significant inhibitory activity of MAPK itself was used in the study. Inhibition of MEK by PD 098059 prevents the activation of MAPK and subsequent phosphorylation of MAPK substrates (Dudley *et al.* 1995). MAPK blockade decreased the proliferation of cardiac fibroblasts even lower than the basal levels, demonstrating the involvement of MAPK in growth of cardiac fibroblasts. Pretreatment and continued incubation of fibroblasts in conditioned medium with PD 098059 completely abolished EEC induced response in cardiac fibroblasts. Gi and Gq mediated MAPK activation is distinguished by differences in dependence upon PKC (Hawes *et al.* 1995) and GPCRs might be able to utilize pathways bypassing the requirement for Ras activation. Incubation of cardiac fibroblasts in EEC conditioned medium containing the PKC inhibitor, bis-indolyl maleimide decreased the DNA synthesis significantly while inhibition of PKC in cardiac fibroblasts alone did not decrease the proliferation levels as much, indicating that EEC conditioned medium activates PKC.

Signaling cascade leading to activation of MAPK is subject to varied and complex regulation. It can occur either via PKC dependent or independent pathway. ET-1 has also been shown to activate ERK in cardiac myocytes (Clerk and Sugden 1999). Therefore to explore whether the signaling cascade involved in the definitive activation of MAPK is via activation of PKC, Western blot analysis was performed. The EEC conditioned medium activates ERK in cardiac fibroblasts and this effect is ameliorated by pretreatment with BIM. This significant reduction in ERK phosphorylation on inhibition of PKC confirms that the signaling system involved in the EEC induced proliferation of cardiac fibroblasts is through PKC dependent

MAPK activation. Other signaling pathways known to be activated by ET-1 include those involving protein kinase B, phosphatidylinositiol-3-kinase and non-receptor tyrosine kinase Src (Clerk and Sugden 1999). Besides PKC, the ET-1 stimulated proliferation of cardiac fibroblasts may possibly involve one or more of these kinases (Cheng *et al.* 2003).

Considering the data from the present study, it can be deduced that stimulation of fibroblasts with ET-1 present in the EEC conditioned medium stimulates MAPK activity through PKC, prompting the proliferation of fibroblasts and formation of collagen.

V.4. CARDIAC FIBROBLASTS RESPOND DIFFERENTLY TO CONDITIONED MEDIUM FROM EECs TREATED WITH INFLAMMATORY AGENTS

V.4.1. Pro-inflammatory agents attenuate EEC induced proliferation of cardiac fibroblasts

Heart function can be profoundly affected during periods of increased cytokine production such as in endotoxic shock, transplant rejection and ischemia/reperfusion (Palmer *et al.* 1995). A major source of cytokine and growth factor production in the area of wound healing post infarction are the non-cardiomyocytes such as myofibroblasts and endothelial cells. Cytokine and growth factors such as TNF-α, IL-1β, IL-6 and TGF-β can act via autocrine and paracrine pathways to influence fibroblast growth and collagen turnover.

When cardiac fibroblasts were grown in conditioned medium from EECs treated with TNF-α and LPS, the [^{3}H]-Thymidine uptakes as well as collagen syntheses by the cells were lowered, compared to the percentage of increase induced by EEC conditioned medium not treated with these agents. Since the viability of the cells treated with these agents was not affected to any greater extent compared to the controls, the attenuated response was not because the cells were non-viable and unable to respond in a normal physiological manner. This finding is supported by the earlier reports that 0.4% serum does not alter the viability of endothelial cells significantly, though these cells die in no-serum conditions (Myers and Tanner 1998).

Raised concentrations of the endotoxin, LPS have been demonstrated in patients with chronic heart failure. LPS contribute to and initiate a cascade of cellular events that lead to decreased contractile efficiency and left ventricular enlargement and dysfunction. Also, marked abnormalities in cardiovascular function accompany septic shock and LPS is believed to be one of the primary mediators of these abnormalities. It is an established fact that myocardial depression is a complication of human septic shock. Kumar *et al* demonstrated that the negative effects of LPS on myocardial function are mediated at least in part through TNF-α (Suffredini *et al.* 1989). LPS plays a role in cardiac dysfunction by causing an enhancement of cardiac-derived inflammatory mediator expression, with release of pro-inflammatory cytokines such as TNF-α and IL-1β and over production of NO (Chagnon *et al.* 2005; Kruger *et al.* 2006). Local myocardial levels of TNF-α increases after LPS challenge and contribute to the development of left ventricular dysfunction

(Knuefermann *et al.* 2002). Similar results were observed *in vitro* in studies using human coronary endothelial cells. Cells stimulated with the endotoxin, expressed higher levels of TNF mRNA and released increased levels of the cytokine (Chan *et al.* 2001). Since most of the effects of LPS are through TNF, it can be assumed that in cardiac fibroblasts, the endotoxin elicits responses similar to that induced by TNF-α.

TNF-α has been detected in myocardium during sepsis, viral myocarditis, heart failure and transplant rejection. Ischemia and bacterial LPS are two clinically relevant stimulus that induce TNF-α production in the heart (Kapadia *et al.* 1995). TNF-α has been proposed as an autocrine/paracrine mediator in myocardial remodeling (Yokoyama *et al.* 1997). In studies on the direct effect of TNF-α on cardiac fibroblasts, Siwik *et al* and Sano *et al* reported that the cytokine decreases total collagen synthesis (Siwik *et al.* 2000; Sano *et al.* 2001). TNF-α can favor either cell growth or death, depending on the cell type, the developmental stage and the association of other stimuli. In the present study, it was interesting to note that while TNF-α directly caused several fold increase in proliferation in neonatal cardiac fibroblasts, it diminished the mitogenic effect of EECs on the fibroblasts.

A notable action of TNF-α is its ability to induce NOS activity in different cell types, including cardiac myocytes (Balligand and Cannon 1997). NO in turn is an important modulator of TNF-α in the heart. Increased levels of TNF-α consequent to the increase in NO possibly enhances the innate immune response in the heart, promoting the recruitment of neutrophils, monocytes and natural killer cells to the affected areas of the myocardium. This action may therefore confer some beneficial

effects (Peng *et al.* 2003). In most cases, the expression of inducible NOS is enhanced by LPS. In the presence of a co-stimulus such as LPS, TNF-α can stimulate proliferation of cardiac myocytes, suggesting its potential mitogenic action, which may be relevant to issues such as development of embryonic heart or the remodeling of the pathological heart (Tantini *et al.* 2001).

In the present study, it was detected that EECs release significantly higher levels of nitrite in response to pro-inflammatory agents. In addition to being an immunomodulator and a potent inhibitor of platelet aggregation and cell migration, NO is also an antimitogen (Lau and Ma 1996; Rizvi and Myers 1997; Sarkar *et al.* 1995). Thus the attenuation of cardiac fibroblast proliferation by TNF-α activated EECs can be attributed to the higher levels of nitrite in the conditioned medium. The multiple actions of NO and its role in vascular wall diseases are consistent with an important role it plays in the metabolism of the ECM. In studies using endothelial cells and SMCs from coronary arteries, inhibition of NO caused increase in concentration of collagen types I and III. This data also supports an inhibitory role for NO on collagen synthesis and suggest that diseases impairing endogenous NO production may adversely affect an important regulatory mechanism underlying the control of vascular collagen metabolism (Myers and Tanner 1998). Extrapolating these findings to the present study, it can be suggested that increased release of NO from EECs on treatment with TNF-α and LPS, causes the attenuation of the proliferative response in cardiac fibroblasts.

When treated with TNF-α and LPS, EECs demonstrated a diverse pattern of release of endothelium-derived factors, such as NO, TGF-β and ET-1 into the conditioned medium, which might contribute to the altered response elicited in cardiac fibroblasts. Levels of ET-1 in the conditioned medium from EECs treated with TNF-∝ and LPS were found to be lowered when compared to that from untreated cells. This implies that in addition to the elevated levels of the anti-mitogenic NO, a decrease in the levels of ET-1 could be another factor contributing to the diminished proliferative response in fibroblasts.

Treatment of EECs with TNF-∝ also demonstrated an elevation of TGF-β in the conditioned medium. Dose-dependent increase in TGF-β release has been observed in microglial cells treated with TNF-∝ (Chao *et al.* 1995). It is probable that TNF-∝ may regulate TGF-β expression as a feed back mechanism to limit extra cellular degradation in response to injury. TGF-β being inhibitory to cardiac fibroblast proliferation, the increased levels of this peptide in the conditioned medium from TNF and LPS treated EECs obviously accentuates the attenuation of proliferation brought about by increased NO and decreased ET-1. The findings in this study using EECs and neonatal cardiac fibroblasts demonstrate that pro-inflammatory cytokines caused altered expression of paracrine factors in EECs and inhibit proliferation and lower collagen synthesis in fibroblasts. Taken together, the results suggest that pro-inflammatory cytokines are capable of creating a shift in ECM metabolism. Such mechanisms may operate in ventricular remodeling in dilated cardiomyopathy, given that cardiac endothelium is a major source of TNF-α production in human DCM

(Palmer *et al.* 1995). The responses observed in this study are also in accordance with the consequences of inflammatory cytokine activation observed in heart failure. Endocardial endothelial cells in times of pathologic stress may respond by altered production of factors that limits fibroblast proliferation. The data from the present study thus underscores the importance of EE in pathologic stress when these cells may act as negative modulators and limit fibroblast proliferation to prevent excessive scarring response.

V.4.2. Signaling pathway involved in TNF-α effect on proliferative action of EEC conditioned medium on cardiac fibroblasts

Compared to fibroblasts grown in 0.4% FBS containing medium, the conditioned medium from EECs treated with TNF-α induced proliferation in cardiac fibroblasts, though the effect was significantly less than that of EEC conditioned medium without TNF-α. It was explored whether this effect was mediated by ET-1 or TGF-β. Neither BQ123, the specifi ET_A receptor inhibitor nor PD 142893, the non-specific ET_A/ET_B receptor inhibitor altered the proliferative response of cardiac fibroblasts to the conditioned medium from EECs treated with TNF-α. This suggests the non-involvement of ET-1 in mediating TNF-induced response. Inhibition of TGF-β using anti-TGF-β also did not result in a significant decrease in the proliferative response. Given that TNF-α alone induced proliferation in cardiac fibroblasts grown in 0.4% FBS containing medium, these results imply that the proliferation induced by TNF-α is possibly because of the direct mitogenic effect of TNF-α on cardiac

fibroblasts and not through the endothelium-derived factors such as ET-1 or TGF-β from activated EECs.

V.4.3. Activity of TNF-∝ involves PKC and MAPK

TNF-α and LPS may activate multiple signaling pathways in the cells. Since phosphorylation cascades are implicated in cytokine signaling, the effect of PKC and MEK inhibition on TNF action was investigated. In a previous report using skin fibroblast cell line it has been suggested that MAPK proteins represent the most abundant targets of enhanced tyrosine kinase activity resulting from TNF action, which could be mediated by MEK (Vietor *et al.* 1993). Inhibition of PKC and MAPK inhibited the proliferation of cardiac fibroblasts significantly implying that both these signaling molecules are required in the residual mitogenic activity of TNF-α. The presence of unique elements in the TNF signal transduction cascade which are amenable to selective inhibition by reagents may prove to be useful targets in developing therapies for pathologies associated with cytokine elaboration (Marino *et al.* 1996).

Further investigations are warranted to determine whether the signaling events coupled to EEC mediated growth of cardiac fibroblasts is implicated in collagen synthesis and also to delineate the exact signaling cascade in the response to pro-inflammatory cytokines. Given that neonatal cardiac myocytes are distinct from adult cardiac myocytes it is probable that adult cardiac fibroblasts may respond to EECs in a manner different from what is observed in the present study conducted with neonatal cardiac fibroblasts.

VI. SUMMARY AND CONCLUSIONS

VI.1. ENDOCARDIAL ENDOTHELIAL CELL REGULATION OF CARDIAC FIBROBLAST FUNCTION

The endocardial endothelium that lines the inner cavity of the heart is distinct from the microvascular endothelial cells and it modulates cardiac muscle performance exactly as the vascular endothelium modulates vascular structure and vasomotor tone. One of the goals of the study was to establish protocols for the isolation, characterization and immortalization of EECs. Endocardial endothelial cells were isolated from porcine ventricles and characterized by their cobblestone morphology, positive staining for factor VIII-related antigen and uptake of DiI-acetylated LDL.

Endocardial endothelial cells were immortalized by ectopic expression of human telomerase reverse transcriptase gene. The transfection was confirmed by repeated passaging and demonstration of hTERT expression at the transcript and protein levels. The transfected cells were found to retain the phenotypic characteristics of EECs and released similar levels of nitrite as primary EECs, in response to pro-inflammatory stimuli. The transfected cells have typical endothelial morphology of cobblestone pattern at confluence, exhibit contact inhibition which rules out malignant transformation, express the endothelial cell marker Factor VIII-associated antigen, retain the capacity to scavenge acetylated LDL and respond to pro-inflammatory agents TNF-α and LPS.

The influence of EECs on cardiac fibroblasts is of considerable interest because cardiac fibroblasts, as the principal cellular constituent of the interstitium, play an important role in maintaining the structural and functional integrity of the

myocardium. An additional goal of the study was to examine the influence of endocardial endothelial cells on cardiac fibroblasts, using primary cultures of EECs and neonatal rat cardiac fibroblasts. Specifically, the effects of paracrine factors, such as ET-1 and TGF-β derived from EECs, on cardiac fibroblast proliferation and collagen synthesis were focused upon. EEC-conditioned medium caused a significant increase in fibroblast proliferation and collagen synthesis in cardiac fibroblasts by a mechanism involving protein kinase C-dependent activation of p42/p44 MAPK. Endothelin-1, acting via the ET_A receptor, was identified as the factor in the EEC-conditioned medium responsible for the stimulatory action. Interestingly, TNF-α and LPS attenuated the stimulatory action of EEC-conditioned medium on fibroblasts, implying a complex interplay between pro-inflammatory cytokines, EECs and cardiac fibroblasts, which could be relevant in pathological states that are associated with marked elevations in the levels of the cytokine. Together, the findings underscore the influence of the endocardial endothelium on cardiac interstitium that could contribute to the development of myocardial fibrosis and ventricular remodeling.

VI.2. FUTURE DIRECTIONS

The present study established the regulatory role of endocardial endothelium on cardiac fibroblasts, mediated by ET-1 through its ET_A receptor. It would be further interesting to explore

1) The signaling pathways involved in the EEC-induced stimulation of collagen synthesis in cardiac fibroblasts

2) Whether EECs induce transformation of fibroblasts to myofibroblasts in conditions with elevated cytokines

3) Whether EEC induced effects on cardiac fibroblasts are different depending on the developmental stage of the organism

4) The significance of EEC-cardiac fibroblast interaction in ventricular remodeling in cardiac diseases

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VIII. LIST OF PUBLICATIONS

1. **Kuruvilla L**, Kartha CC. Molecular mechanisms in endothelial regulation of cardiac function. *Mol Cell Biochem* 2003;**253**:113-123

2. **Kuruvilla L,** Santhosh Kumar TR, Kartha CC. Immortalization and characterization of porcine ventricular endocardial endothelial cells. *Endothelium* 2007;**14**:1-9

3. **Kuruvilla L**, Nair RR, Umashankar PR, Lal AV, Kartha CC. Endocardial endothelial cells stimulate proliferation and collagen synthesis of cardiac fibroblasts. *Cell Biochem Biophys* 2006;**47** (In press)

4. **Kuruvilla L,** Kartha CC. TNF-α and bacterial lipopolysaccharide attenuate endocardial endothelial cell mediated stimulation of cardiac fibroblasts (Communicated)