TRANSFORMING GROWTH FACTOR-beta$_1$ AND DIFFERENTIATION OF CARDIAC FIBROBLASTS

HYPOTHESIS and OBJECTIVES: Cardiac myocytes are contractile units of the heart muscle. In postnatal periods, their mitotic division comes to an end and binucleation proceeds (1,2). Limited regenerative capacity of cardiac myocytes is the main cause of scar formation in cases of hemodynamic stress and myocardial injury due to ischemia and/or normal aging, hence deterioration of cardiac function and various cardiac diseases that are the leading cause of death in the United States. It is, therefore, highly desirable to compensate for the lack of regenerative capacity of cardiac myocytes. To date, little is known about cardiac myogenesis. Stem cells have not been identified and no cell line has been found to terminally differentiate into cardiac myocyte upon specific stimuli. Transforming growth factor-beta (TGF-β_1), is a multi-functional peptide hormone which is expressed in the myocardium (3,4) and involved in regulation of cell growth and differentiation. We discovered that TGF-β_1 induces cardiac fibroblasts transformation into a phenotype with certain characteristics of cardiac myocytes (5). The determination that modulation of cardiac fibroblasts phenotype occurs was based on four key items of evidence: 1) appearance of morphological features, characteristic to cardiac myocytes, in TGF-β_1-treated cells; 2) induction of sarcomeric actin mRNA and sarcomeric actin filaments in cardiac fibroblasts following treatment with TGF-β_1; 3) disappearance of intermediate filament vimentin, as evidenced by immunofluorescent light microscopy; and 4) continued expression of muscle-specific morphological features and sarcomeric actin filaments in the second generation of cells, stemmed from TGF-β_1-treated cells, even in the absence of TGF-β_1. Our hypothesis is that transformation of cardiac fibroblasts into cardiac muscle phenotype is possible and that TGF-β_1 or its combination with other factors could be exploited to induce a terminal differentiation of cardiac fibroblasts into beating cardiac myocytes. To test this hypothesis, our immediate objective is to elucidate the mechanisms of phenotypic modulation of cardiac fibroblasts by TGF-β_1. To this end, we will use cultured cardiac fibroblasts from rabbit heart to establish:

A. The antagonistic or cooperative effects of other growth factors, neurotransmitters or hormones.
B. The Role of the organization and components of extracellular matrix on TGF-β_1-induced transformation.
C. The type of TGF-β_1 receptor involved in phenotypic modulation of cardiac fibroblasts.
D. The autocrine role of TGF-β_1 on cardiac fibroblast transformation.

SIGNIFICANCE: The results of these studies are necessary for elucidation of regulatory steps that may prove critical for the terminal differentiation of cardiac fibroblasts into cardiac myocytes. Ultimately, these results may aid in developing new therapeutic strategies to compensate for the lack of regenerative capacity of cardiac myocytes. Those strategies can be used for the revitalization of the heart muscle in patients with cardiomyopathy and heart failure.

BACKGROUND: The non-myocyte populations of cardiac cells consist of cardiac fibroblasts, endothelial cells, smooth muscle cells, and nerve cells. By the use of monospecific antibodies to each cell type and immunofluorescent light microscopy of freshly isolated non myocyte heart cells we have established that greater than 90% of those cells are cardiac fibroblasts (6). Using in situ hybridization and Northern analysis combined with immunofluorescent light microscopy, we showed that cardiac fibroblasts synthesize types I, III and IV collagen, laminine and fibronectin (7,8). Cultured cardiac fibroblasts express mRNAs for cytoskeletal actin, TGF-β_1 (3) and the cardiac-specific gap junction protein, connexin-43 (9).

TGF-β_1 and Cell Function: TGF-β_1 is a growth factor peptide with molecular weight of 25 kd that controls a vast array of biological functions such as differentiation, adhesion, migration, proliferation, and production of extracellular matrix in different cell types (10). In vitro, TGF-β_1 has been also shown to have different effects depending on the cell type. In skeletal muscle myoblasts it inhibits myogenic differentiation (11,12,13). The keratinocyte proliferation is inhibited by TGF-β_1 and suppression of c-myc gene has been implicated in the inhibitory effect of TGF-β_1 in those cells (14). Endothelial cell differentiation and proliferation in culture is differentially affected by TGF-β_1 depending on the assay models and different stages of angiogenesis (15). The diversity of the effects of TGF-β_1 points to the multiple mechanisms by which this growth factor exerts its regulatory impact. The cellular mechanisms of actions of TGF-β_1 are mediated through cell surface receptor molecules (16). Three types of receptors have been identified: types I, II, and III. These receptors are the products of distinct genes. It has been postulated that the type III receptor, with a short cytoplasmic domain, is not involved in biological effects of TGF-β_1(17). However, the type I and II receptors are necessary for all TGF-β_1-induced signaling (18,19,20). These receptors have serine/ threonine kinase activities and TGF-β_1-induced signaling involves protein phosphorylation. However, specific substrates for the action of these receptors are not yet fully identified.

Phenotypic modulation of Cardiac Fibroblasts by TGF-β_1: Our previous studies that are the basis for this proposal showed that rabbit cardiac fibroblasts, when exposed to TGF-β_1, undergo phenotypic modulation. As described

above, the determination that modulation of cardiac fibroblasts phenotype occurs was based on four key items of evidence: 1) appearance of morphological features, characteristic to cardiac myocytes, in TGF-$ß_1$-treated cells; 2) induction of sarcomeric actin mRNA and sarcomeric actin filaments in cardiac fibroblasts following treatment with TGF-$ß_1$; 3) disappearance of intermediate filament, vimentin, following treatment with TGF-$ß_1$ as evidenced by immunofluorescent light microscopy; and 4) continued expression of muscle-specific morphological features and sarcomeric actin filaments in the second generation of cells, stemmed from TGF-$ß_1$-treated cells, even in the absence of TGF-$ß_1$. While expression of sarcomeric actin mRNA and appearance of sarcomeric actin filaments in cardiac fibroblasts could be the result of the effects of TGF-$ß_1$ on actin gene expression, loss of vimentin filaments in TGF-$ß_1$-treated cells is an indication of phenotypic modulation of cardiac fibroblasts by TGF-$ß_1$, because terminal differentiation of myoblasts is associated with reduced expression of vimentin in differentiated cells. The transforming effect of TGF-$ß_1$ seems to be specific for cardiac fibroblasts because treatment of skin fibroblasts and NIH 3T3 cells did not lead to the induction of muscle-specific features in those cells. On the other hand, other regulatory factors such as angiotensin II, interleukin-1, norepinephrine, thyroid hormones failed to induce muscle-specific features in cardiac fibroblasts (5). Together, these data suggest that modulation of cardiac fibroblasts phenotype by TGF-$ß_1$ is caused by the specific effect of this growth factor that cannot be mimicked by other factors to which cardiac fibroblasts are naturally exposed. In the same study we showed, by immunofluorescent light microscopy, that the second generation of cells, stemmed from TGF-$ß_1$-treated cardiac fibroblasts, expressed sarcomeric actin filaments even in the absence of TGF-$ß_1$. In addition, those cells had a diminished rate of proliferation compared with control cells. Therefore, modulation of cardiac fibroblasts phenotype by TGF-$ß_1$ was proved to be a non-transient effect.

EXPERIMENTAL PLANS: The main goal of this proposal is to understand how to exploit TGF-$ß_1$ or its combination with other factors to induce a terminal differentiation of cardiac fibroblasts into beating cardiac myocytes. To accomplish this goal we need to know those mechanisms that potentiate and those that inhibit or reverse the effects of TGF-$ß_1$ on cardiac fibroblasts. To determine the differentiation of cardiac fibroblasts we will use **molecular, structural** and **functional** criteria. The molecular differentiation will be determined by the expression (mRNA and protein by Northern and Western blot analyses) of proteins of contractile apparatus (sarcomeric actin, α- and ß-myosin heavy chains), contractile enzyme (creatine kinase) and proteins of conduction system (cardiac Na, K-ATPase isoforms) and intermediate-sized filament, vimentin. Structural differentiation will be examined by transmission electron microscopy and laser scanning confocal microscopy to identify the contractile apparatus, namely z band structure and the conduction system (21). Functional differentiation of cardiac fibroblasts will be determined by their diminished or loss of proliferative capacity (measurement of DNA synthesis by ^{3}H-thymidine incorporation and doubling time by cell counting) and their acquiring the ability to beat in culture (the beating of cells will be monitored under the inverted microscope) (22). For these studies we will use rabbit cardiac fibroblasts which we have previously shown to have a predisposition to convert into muscle phenotype by TGF-$ß_1$ (5). As a positive control for cardiac myocyte-specific features, we will use cultured cardiac myocytes.

A. Since in the heart cardiac fibroblasts are exposed to an array of cytokines, growth factors, neurotransmitters and hormones, we will determine the potential interplay between TGF-$ß_1$ and those factors. We will start these studies with candidate factors, bFGF, TNF-α, IL-1, thyroid hormones and angiotensin II. These factors are chosen as candidates for two reasons: **first,** our preliminary results showed that although none of them induces molecular features of phenotype modulation in cardiac fibroblasts, those cells respond to them by changes in gene expression for functional and structural proteins such as different types of collagen, collagenase, cytoskeletal actin, and transcriptional regulators as well as changes in DNA synthesis. Therefore, these factors are capable of triggering intracellular mechanisms in cardiac fibroblasts that may or may not help the transformation of cardiac fibroblasts by TGF-$ß_1$; **second,** the in vivo exposure of cardiac fibroblasts to these factors is certain because they are either synthesized in the myocardium or gain access to it by circulating blood (23, 24).

B. The autocrine role of TGF-ß molecules in biological processes in diverse cell types has been previously documented. For example, transformation of embryonic cardiac endothelial cells into epithelial-mesenchymal cells is dependent on the expression of autocrine TGF-$ß_3$ (26). TGF-$ß_1$ is produced by cardiac fibroblasts (3). In our studies, we will determine the parameters of TGF-$ß_1$-induced differentiation in TGF-$ß_1$-depleted cardiac fibroblasts. To achieve this, we will block the translation of TGF-$ß_1$ mRNA in cardiac fibroblasts by using an anti-sense oligonucleotide (propyne derivative of phosphorothioate linkage) to TGF-$ß_1$ mRNA. To test the blocking of TGF-$ß_1$ synthesis, we will determine TGF-$ß_1$ activity in the conditioned medium of cardiac fibroblasts by measuring inhibition of mink lung epithelial cell proliferation against the activity of standard solutions of TGF-$ß_1$ (R&D System). As a negative control, we will use cells treated with the sense oligonucleotides (for our preliminary results on the use of antisense oligonucleotides to TGF-$ß_1$ mRNA please see the enclosed abstracts, ref# 25,26).

C. In the heart there is an extensive and highly organized extracellular matrix that is necessary for both structural

and functional integrity of the myocardial function. The impact of the extracellular matrix on cell differentiation and function is now established in a variety of cell culture systems such as mammary cells, keratinocytes, hepatocytes, myoblasts, myocytes and endothelial cells (27). The effects of TGF-$ß_1$ on endothelial cell differentiation has been shown to be dependent on the composition and organization of the extracellular matrix (15). It is, therefore, our goal to study the impact of extracellular matrix organization and its components on the transformation of cardiac fibroblasts by TGF-$ß_1$. To this end, we will determine the effect of matrix organization by culturing cardiac fibroblasts on a three-dimensional collagen type I gel. We will then prepare sections of the gel for both immunofluorescent light microscopy and transmission electron microscopy for the observation of differentiation parameters (28). The effect of matrix components will be determined by culturing cardiac fibroblasts on culture dishes that are coated with individual matrix components (fibronectin, laminin, collagen type I and basement membrane-specific collagen type IV).

D. Since cellular signaling pathways that are involved in TGF-$ß_1$-induced transformation of cardiac fibroblasts are not known, we need to know whether type I or II receptors or their complex are involved in the mediation of cell transformation in cardiac fibroblasts. To achieve this goal, we will **first,** identify the type of TGF-$ß_1$ receptor on cardiac fibroblasts in culture. For these studies we will use cross linking studies, using chemical crosslinker disuccinimidyl suberate, and iodinated TGF-$ß_1$ to label the receptor molecules(16). The resulting labelled receptors will then be resolved by SDS gel electrophoresis of membrane proteins to identify the TGF-$ß_1$ receptor based on their molecular weight as previously described (16,18); and **second,** selectively block the expression of either type or both receptors by the use of anti-sense oligonucleotides to the translation initiation site of mRNAs for TGF-$ß_1$ receptor types I and II. The sequence of these oligonucleotides will be deduced from the sequences of a full length cDNA to type I receptor (19) and a cloned cDNA to type II receptor (18). We will test the blocking of the expression of receptors by cross-linking experiments as described above. TGF-$ß_1$-induced transformation of the cells will then be determined by the criteria of differentiation as outlined above.

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