1989

Hypertrophic response in primary single-cell culture of adult rat myocardial cells

Phillip Chung-Ming Yang
Yale University

HYPERTROPHIC RESPONSE IN PRIMARY SINGLE-CELL CULTURE OF ADULT RAT MYOCARDIAL CELLS

PHILLIP CHUNG-MING YANG

1969

HYPERTROPHIC RESPONSE IN PRIMARY SINGLE-CELL CULTURE OF ADULT RAT MYOCARDIAL CELLS

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

Phillip Chung-Ming Yang

1989

ACKNOWLEDGEMENT

I would like to express my utmost gratitude to Dr. Paul C. Simpson of University of California, San Francisco for his endless support and generosity throughout the course of this research. I also extend my appreciation to Dr. Son Le of University of California, San Francisco for his kind supervision.

This thesis, however, could not have reached its final form without the sincere concern demonstrated by my thesis advisor, Dr. Lawrence S. Cohen of Yale University School of Medicine.

And of course, to my parents, words would not do any justice. "Thank You."

TABLE OF CONTENTS

ABSTRACT

Hypertrophic Response in Primary Single-Cell Culture of Adult Rat Myocardial Cells

Phillip Chung-Ming Yang

1989

We have devised a method to reliably isolate and culture adult rat heart cells. The average yield of 10 million total cells and approximately 70% viability have been consistently reproduced. (Rod-shaped criterion has been used to determine the viability.) The viable cells are cultured for 8 days at which time they undergo morphological changes into flat astrocyte-like configuration with multiple pseudopods. Primary culture of approximately 80% adult rat heart myocardial cells (MC) maintained in single layer and low density is obtained. We asked whether hypertrophy could be induced in the MC through the addition of the following agents: 1.) Norepinephrine (NE), 2.) NE and Propranolol (PRO), 3.) Phorbol ester (TPA:tumor promoting agent), and 4.) 5% Calf-serum (CS). The cell cultures were maintained in minimum essential media plus 1% CS for 6 days and minimum essential media plus transferrin and insulin (CS-free) for the remaining 2 days. By the end of incubation period, the myocardial cells had well differentiated and showed no sign of

proliferation. At this time, we brought the culture medium to 0.1uCi/ml with (u-14C) phenylalanine and added the following agents: 1.) Diluent (control), 2.) NE (0.2uM), 3.) NE (0.2uM) and PRO (0.2uM), 4.) TPA (0.1uM), and 5.) 5% CS. Following the incubaton period of 48 hours, we determined the extent of hypertrophy by measuring the increase in cell protein content using the radioisotopic method (scintillation count).

The results were the following: 1.) NE increased cell size by 1.15 fold, 2.) NE + PRO increased by 1.24 fold, 3.) TPA increased by 1.2 fold, and 4.) CS increased by 1.5 fold ($p<0.01$). While the level of response is not as large as in neonatal rats as previous studies have shown, this finding indicates that the adult rat MC possess the similar capability to undergo hypertrophy through alphal-adrenergic receptors. Furthermore, the addition of a beta adrenergic receptor antagonist, propranolol, has enhanced the hypertrophic effects of NE ($p<0.05$).

INTRODUCTION

Primary cultures of adult rat myocardial cells (MC) have been an extremely important model to enhance our understanding of cardiac cell structure and function (1). Many investigators have reported various techniques to improve the methods of dissociating adult rat MC and of preparing single-cell primary cultures (1,2,3,4,5,6). A definitive method, however, to reliably isolate and culture adult rat MC has been difficult to develop (1,2).

We have developed an inexpensive and simple method that reliably isolates and cultures adult rat MC. Our model consistently isolates 10 million total cells with 70% viability (rod-shaped criterion) and provides a cell culture which at the end of the incubation period (8 days) shows all of the following: MC predominance (>80%) in a single cell layer and low density, a well differentiated cellular morphology (flat astrocyte-like configuration with multiple pseudopods), and lack of MC proliferation. Such primary cell culture system is critical in order to isolate and study a single physiological phenomenon. The model allows the control of all the variables by eliminating numerous stimuli that are present in vivo. It allows the testing of a single factor in a systematic fashion. Limiatations, however, also exist. The experimental environment is artificial. Important factors such as 1.) cell-cell

interactions are altered since each cell is isolated in a low density culture, 2.) cellular morphology changes as each cell adapts to the culture substrate, and 3.) culture medium is different from tissue fluid. Nevertheless, a single cell-culture system allows the investigation of a single physiological phenomenon at a fundamental cellular level.

One interesting area where single cell culture system could be used effectively is in the study of myocardial hypertrophy. Myocardial hypertrophy has long been recognized both as an adaptive and a pathological response to cardiovascular needs (7). Hypertrophy plays a critical role in normal development and in response to exercise (7). Yet, hypertrophy has also been associated with congestive heart failure, hypertrophic cardiomyopathy, valvular diseases, and hypertension (8,9,10). So, is physiologic hypertrophy different from pathologic hypertrophy? If so, is hypertrophy an adaptive response to or an etiology of cardiovascular pathologies? In order to address these critcally important issues, a fundamental understanding of the mechanism of myocardial hypertrophy needs to be elucidated first.

The heart undergoes hyperplasia throughout the fetal life of both the rats and human beings. It continues to grow in a similar fashion during the first four weeks of a neo-natal rat and during the first three months of a newborn human baby (11,12). After this developmental period, cardiac growth becomes entirely dependent on hypertrophy as it reaches the normal size of an adult heart (8). Physiologic hypertrophy is this process of

increase in cardiac muscle mass (ventricular diameter and wall thickness) resulting from the addition of contractile proteins while the population of myocardial cells remains constant (13). It is physiologic since the increase in cardiac muscle mass translates to elevated contractile function necessary for normal development (14). The primary stimuli for this physiologic hypertrophy appears to be the hemodynamic variables (8). However, studies have suggested that the critical link between hemodynamic stresses and cardiac hypertrophy may be hormonal (15). The collected data have indicated that norepinephrine may play a significant role in induction of myocardial growth (15). In another study, chronic administration of subhypertensive dose of norepinephrine led to significant ventricular hypertrophy (16). While the hemodynamic stresses and the subsequent hormonal release appear to induce hypertrophy, more questions remain: 1.) what is the exact cellular mechanism leading to hypertrophy and 2.) are there other agents besides norepinephrine that induce hypertrophy?

There have been three major approaches to answer the questions. First of all, protein synthesis has been studied. One of the requirements for cardiac hypertrophy is that the rate of protein synthesis must exceed the rate of degradation (17). Data have been collected which suggest that the increased aortic pressure and the attendant stretch of the heart muscle are closely associated with the faster rates of protein synthesis(17). As to how the mechanical parameters translate to

increased protein synthesis is not clearly known. One postulate has involved the regulation of the synthesis of myosin iso-enzymes (18). There are three myosin iso-enzymes: V1 myosin- homodimer of 2 alpha heavy chains, V2 myosin - heterodimer of 1 alpha and 1 beta heavy chains, V3 myosin - homodimer of 2 beta heavy chains (18). Studies have correlated well-documented phenomenon of thyroid hormone-induced myocardial hypertrophy with the accumulation of alpha heavy chain mRNA and concomitant loss of beta heavy chain mRNA (12). Furthermore, the relative synthetic rates of alpha and beta heavy chains have been reported to reflect the proportion of their respective mRNA's present (12). On a molecular level, this implies that the regulation of protein synthesis may precede the translation of the message (12). Finally, a different study has provided a similar set of data that shows a rapid shift from one isoform of myosin mRNA to another isoform following aortic stenosis (19). Thus, an intricate regulation of protein synthesis at pre-translational level appear to respond to trophic stimuli inducing hypertrophy.

The second approach has involved the renin-angiotensin (R-A) system. A study has shown that the R-A system along with catecholamines play a role in cardiac hypertrophy (20). Furthermore in the same study, activation of the *sis* proto-oncogene has been detected in myocardial cells undergoing hypertrophy (20). This raises a possible similarity between the pathogenesis of myocardial hypertrophy and benign neoplasia (20,34).

Finally, our approach in the laboratory has been to study the role of alpha1-adrenergic receptor and its response to norepinephrine stimulation. Studies with the primary culture of neonatal rat myocardial cells (MC) have shown that when the MC are stimulated by norepinephrine, they undergo 1.5 to 2.0 fold increase in cell size and produce spontaneous contraction in essentially all of the cells (21). Hypertrophy and contractile activity have been found to be mediated through alpha1-adrenergic receptor (22). Nevertheless, the alpha1-adrenergic receptor mediated responses differ significantly in two major respects. First of all, hypertrophy only requires alpha1 stimulation while contractile activity requires both alpha1 and beta activations (23). This means that myocardial cells stimulated through alpha1 receptor can undergo hypertrophy without contractile activity. A similar observation of hypertrophy without contractile activity has been made when neonatal rat MC were stimulated with phorbol esters or with norepinephrine in the presence of calcium-channel antagonists (24,25).

The second major difference between the two alpha1 mediated responses is that hypertrophy requires RNA and protein synthesis while contractile activity does not (26). Alpha1 mediated hypertrophic response was blocked by inhibition of protein synthesis with cyclohexamide or by inhibition of RNA synthesis with actinomycin D (26). The contractile activity, however, persisted despite the addtion of cyclohexamide or actinomycin D (26). These observations suggest dual functions of alpha1-

adrenergic receptor and a possibility of two seperate intracelluar pathways mediated by alpha1-adrenergic receptor.

The above findings regarding the role of alpha1-adrenergic receptors have been made using the neonatal rat primary culture system. In this study, we asked whether norepinephrine could similarly induce hypertrophy in adult rat myocardial cells. The investigation was divided into three major stages. The first stage comprised of isolating viable myocardial cells. We devised a simple and reliable method which consistently produced a yield of 10 million cells with approximately 70% viability. The second stage involved the maintenance of adult rat MC in long-term primary culture. At the end of 8 day incubation period, we were able to develop single-layer, low density culture of approximately 80% MC predominance, a well-differentiated cellular morphology, and lack of MC predominance. The third stage entailed the testing of the trophic response of adult rat MC to the following agents: 1.) Diluent (control), 2.) Norepinephrine (NE), 3.) NE + Propranolol (PRO), 4.) Phorbol Ester (TPA:tumor promoting agent), and 5.) 5% CS. Our data indicate that a significant hypertrophic response ($p<0.01$) was seen with the addition of all four agents. The hypertrophic response to NE was further enhanced when the beta-receptor was blocked with the addition of PRO. This finding suggests that the alpha1-receptor plays a key role in inducing hypertrophy while the beta-receptor acts to inhibit hypertrophy.

MATERIALS AND METHODS

The experiment has been divided into three major stages:

I. Isolation of single adult rat myocardial cells (MC)

II. Maintenance of MC in long-term primary culture

III. Stimulation of the MC to test hypertrophic response with the following agents: 1.) Diluent (control), 2.)Norepinephrine (NE), 3.) NE + Propranolol (PRO), 4.) Phorbol Ester (TPA:tumor promoting agent), and 5.) 5% Calf-serum (CS).

Material

Male Sprague-Dawley rats weighing 250-300g. and approximately 3-4 months old were used. Reagents used in this experiment were obtained from the following sources: collagenase CLS II, 143 U/mg, lot #45M7903 (Cooper Biomedical); bovine serum albumin, lot#73F-9305 (Sigma); calf-serum (Sterile System); Minimum Essential Media Eagle with Hanks BSS with glutamine, with 0.35 GRA/LT NaHCO3 #671 (UCSF Cell Culture Facility); (u-14C) phenylalanine, SWO #225316 (ICN); heparin sodium (Invenex); arabinoside cytosine #C-6645 (Sigma); percoll #P-1644 (Sigma); aquasol (New England Nuclear); and all of the following came from Sigma: ether, trichloroacetic acid, sodium dodecyl sulfate, phosphate-buffered solution, streptomycin sulfate, transferrin, insulin, norepinephrine, propranolol, and phorbol ester.

Perfusion Solution and Culture Media

The following solutions were prepared:

Hepes buffer solution: NaCl, 115mM; KCl, 5.36mM; MgSO4+7H2O, 0.81mM; dextrose, 27.5mM; KH2PO4+7H2O, 0.34mM; Na2HPO4+7H2O, 0.34mM; Hepes, 20mM, (pH=7.4); lactate, 1.5mM; pyruvate, 2.0mM; penicillin, 50U/ml; and streptomycin, 50ug/ml. The solution was adjusted to pH=7.3 and sterilized by filtration through a Millipore Filter (pore size=0.45um).

Enzyme solution: collagenase, 1.5mg/ml; bovine serum albumin, 1 mg/ml; and buffer solution, 80ml.

Perfusion solution: heparin sodium, 1ml; and buffer solution, 200ml.

Dissociation solution: calf-serum, 2%; and buffer solution, 100ml.

Percoll solution: Isotonic percoll solution, 40% volume in isotonic Hepes-buffer solution.

Growth medium: Minimum essential media; CS, 10%; penicillin, 50U/ml; and Vitamin B12, 1.5uM.

Serum-free medium: Minimum essential media; transferrin, 10ug/ml; insulin, 10ug/ml; penicillin, 50U/ml; and Vitamin B12, 1.5uM.

Laminin solution: Minimum essential media; laminin, 10ug/ml; penicillin, 50u/ml; and Vitamin B12, 1.5uM.

Stage I: Isolation of Single Adult Rat Myocardial Cells (MC)

Anaesthesia of the rat: Three anaesthetic agents were tested.

Methoxyflurane induced severe hypoxemia, hypercarbia, and acidosis based on ABG obtained directly from the heart at the time of sedation suggesting significant ventilatory suppression. Pentobarbital produced prominent myocardial infarcts. Ether was the most effective agent producing minimal respiratory and cardiac suppression. Ether is now routinely used.

Sex of the animal: When the cell yields and viability are corrected for body weight, we found that there was no significant difference between male and female rats. The mean cell yields for female rats were 33.5±9.3 million cells/kg body weight and those for males were 36.3±10.4 million cells/kg body weight. The mean percentage of viable rod shape MC for both sexes was 70±6%.

Cell isolation technique: The rats were injected with heparin sodium, 0.75ml, at least 20 minutes prior to the excision of the heart. The heart was then quickly removed from the rat and immersed in a calcium-free isotonic perfusion solution maintained at 4 degrees celcius. The heart was then immediately mounted to a large 13-gauge Eppendorf tip attached to a Langendorf perfusion apparatus. The mounting is a critical step (<3 minutes) since the Eppendorf tip could be easily advanced too far into the left ventricle preventing the proper perfusion of the coronary circulation or damaging the aortic valve. Langendorf perfusion was then performed using the perfusion solution (calcium-free and contains 1000U heparin) during the first four minutes. The perfusion was performed using gravity from the height of 81.6cm (equivalent to 60mmHg). During this time the heart was

essentiallly washed to remove blood and extracellular calcium. This was then followed by enzyme (collagenase) solution perfusion at a slow, constant flow rate of 1.5ml/min maintained by a peristaltic pump for 40-50 minutes. During the enzyme solution perfusion, the heart was completely immersed in a perfusion solution maintained at 37 degrees celcius. In addition, 85% oxygen and 15% nitrogen mixture gas was being constantly bubbled into both enzyme and perfusion solutions. The heart should remain uniformly pink throughout the procedure and should not show any spontaneous contraction.

Following the enzyme solution perfusion period, the heart was placed into a 37 degrees celcius perfusion solution that also contains 0.5mM CaCl2. The intact heart was then able to be gently pulled apart using a pair of tweezers and a scalpel. The heart pieces were transferred into 8ml of enzyme solution which also contains 0.5mM CaCl2. This suspension was gently shaken for 10 minutes in water bath maintained at 37 degrees celcius to dissociate the heart into single MC. The supernatant which contains the single MC was decanted to be saved and additional 8ml of enzyme solution was added to the intact heart pieces. This dissociation procedure was repeated for a total of three times. After letting the supernatant settle by gravity for twenty minutes following the last dissociation, a pellet of MC formed at the bottom of the tube. The supernatant was decanted and the pellet of MC was resuspended in growth medium and allowed again to settle by gravity for 20 minutes. Following this period, the

supernatant was decanted and the MC pellet was added to a tube containing percoll solution. The percoll solution containing the MC pellet was centrifuged at 29g for 5 minutes. Viable MC settle at the bottom of the tube while the dead, rounded MC and other non-MC formed a distinct layer on the top which was easily decanted. This percoll seperation step was repeated and the final MC pellet was resuspended in growth medium. Cell count was then performed on an eosinophil counting chamber using the rod-shape criterion to count the viable MC.

Stage II: Maintenance of MC in Long-term Primary Culture

For long-term culture of adult cardiac myocytes, care must be taken to minimize contamination during the isolation procedure. After isolation of the cells, all tubings needed to be rinsed with 10% bleach, then with sterile distilled water and again soaked in distilled water overnight. They were then air dried and kept under UV light until use. Collagenase solution should be filtered with a 0.22um filter. All buffer solutions contain penicillin and streptomycin. The growth and serum-free media contain penicillin.

Cell Culture

The 35mm Corning dishes were used to plate the cells. Each dish was coated with 1ml of laminin solution for at least 12 hours prior to plating the cells. Laminin promotes initial adherence of the MC to culture dishes at an impressive number and facilitates the cellular transformation into a flat, star-like

configuration with long-term survival in culture (41,42). At the end of the laminin coating, the solution was removed from the dishes.

Two ml of the uniform suspension of MC were plated on laminin-coated 35mm Corning dishes and incubated for 1 hour. The plating density of the MC was set at 200 cells/mm2. At the end of the incubation period, the growth medium was aspirated to remove the unattached cells and newly made growth medium with 10 uM cytosine arabinoside and 10uM gentamycin was added. On culture day 3, the growth medium was changed with freshly prepared growth medium containing 10uM cytosine arabinoside. On culture day 6, the growth medium was replaced with serum-free medium for 2 days.

In order to measure the hypertrophic effects of the various trophic agents, 0.1 uCi of (u-14C) phenylalanine was added to newly made serum-free medium to prepare a radiolabeling solution (21,43). On culture day 8, the 2 day old serum-free medium was replaced by the radioisotopic medium. Several hours later, the following agents were added for 48 hours to the cell-culture dishes of the respective group: 1.) diluents (control), 2.) 0.2uM Norepinephrine (NE), 3.) 0.2uM NE + 0.2uM Propranolol (PRO), 4.) 0.1uM Phorbol ester (TPA: tumor promoting agent), and 5.) 5% Calf-serum (CS). The protein content of each cell culture was determined 48 hours later by scintillation count method (23). The protein degradation of NE was prevented by the addition of 100uM Vitamin C to the NE solution and also by the maintenance of the medium strictly at pH7.3.

Cell Count

The cells were counted in 17 randomly selected fields throughout the entire dish (44). Each field was equivalent to 0.96mm2 surface area. We, therefore, counted 2% of the total 800mm2 surface area of the dish. In order to measure the number of cells per dish, the following calculation was performed:
# of cells/dish=(cells/field)x(1.006xfield/mm2)x(800mm2/dish).
The number of MC was counted for each dish.

Stage III: Stimulation of the MC to Test Hypertrophic Response

Cell size was measured by quanitifying the total cell protein concentration. Since the cells do not divide, the increase in dish protein indicated the increase in cell size or hypertrophy. The total protein content was measured by using asymptotic labelling with a radioactive amino acid (23). Using (u-14C) phenylalanine, the culture medium was brought up to 0.1uCi/ml. Phenylalanine ws used since the amino acid is not metabolized by myocardial tissue (23). After incubating the cell culture with radioisotopic medium and with the trophic agents for 48 hours: 1.) Diluent (control), 2.) NE, 3.) NE+PRO, 4.) TPA, and 5.) 5% CS, 14C-phenylalanine incorporation was measured by scintillation count. The scintillation count for each dish was divided by the number of MC per dish to measure the protein concentration per cell.

The cell protein defined as material that was trichloroacetic acid (TCA)-insoluble and sodium dodecyl sulfate

(SDS)-soluble was quantified by liquid scintillation counting (23). On culture day 10, the radioisotopic labelling medium was removed, the attached MC was rinsed with phosphate buffered saline (PBS) and treated with 10% TCA at 0 degree celcius for at least 1 hour. Then the MC was rinsed with TCA three times and dissolved in 1ml of 1% SDS overnight. The entire SDS solution was transferred into plastic vial with 5ml of Aquasol and was counted.

Statistical Analysis

All data were presented as mean $\pm$ SE. For comparison, Student's t-test for unpaired and paired observations was used. A p-value<0.05 was considered a statistically significant difference between means.

RESULTS

General Characteristics of Adult Rat Heart Single-Cell Cultures

The dissociation method yielded an average of 10 million cells per heart with approximately 70% viable rod-shaped myocardial cells (MC). The initial attachment of the cells to the culture dishes consisted predominantly of MC (>80%). The morphology of MC was initially marked by the characteristic rod-shape, cross-striated cytoarchitecture, and contractility (Fig. 1). On culture day 2, the cells became round-shaped. By culture day 3, the cross-striations disappeared and the MC started to flatten out at each end of the cell. By day 6, the initial morphology completely changed into a flat cellular appearance with translucent cytoplasm and developed multiple, slender pseudopods growing in all directions (Fig.2). Throughout the course of cellular development, the MC did not proliferate. Instead, the cell culture showed attrition in the number of MC.

Hypertrophic Response

Hypertrophy was quantified through an increase in the cell protein content using the 14C-phenylalanine incorporation technique measured by the scintillation counting method. HCl- and DMSO-treated (diluent for NE, PRO, and TPA) MC served as a control (Fig.3). The difference in the hypertrophic response between the testing agents-treated (NE, NE+PRO, TPA, & CS) and

the control-treated MC was described as % of the control. Control was set at 100%. All the data were obtained from 8 experiments with each experiment consisting of 30 culture dishes with 6 dishes in each respective group: 1.) Control (diluent), 2.) NE, 3.) NE+PRO, 4.) TPA, and 5.) 5% CS.

NE-treated MC showed a significant increase in the cell protein content. The increase of 14C-phenylalanine incorporation of NE-treated MC averaged 115%$\pm$4.9 ($p<0.01$) of the control (Fig.4 & 8).

NE+PRO-treated MC also showed a significant increase in the cell protein content. The increase of 14C-phenylalanine incorporation of NE+PRO-treated MC averaged 124%$\pm$4.3 ($p<0.01$) of the control (Fig.5 & 8).

TPA-treated MC similarly showed a significant increase in the cell protein content. The increase of 14C-phenylalanine incorporation of TPA-treated MC averaged 120%$\pm$4.9 ($p<0.01$) of the control (Fig.6 & 8).

Five percent CS-treated MC showed the largest increasae in the cell protein content. The increase of 14C-phenylalanine incorporation of 5% CS-treated MC averaged 159%$\pm$6.7 ($p<0.01$) of the control (Fig.7 & 8).

In addition, the NE+PRO-treated MC showed significant increase in the cell protein content when compared to the NE-treated MC. The 14C-phenylalanine incorporation of NE+PRO-treated MC averaged 108%$\pm$6.3 ($p<0.05$) of the NE-treated MC (Fig.9).

DISCUSSION

Methods

We succeeded in devising a simple and reliable method to prepare single-cell culture of adult rat heart. We consistently produced a yield of 10 million cells with approximately 70% viability. The time required for preparation was 6-7 hours and the amount of viable myocardial cells (MC) was quantitatively sufficient to conduct our experiment. We believe that our model provides an acceptable system to study the cell biology of the adult rat heart (1,6,8,30).

Our method improved several important areas of adult rat heart single-cell preparation. First of all, we examined two critical parameters of the dissociation (Stage I) of adult rat heart into single MC: enzyme perfusion rate and maintenance of the temperature of the heart. The first variable involved the enzyme perfusion rate. The normal diastolic blood pressure of the rat is between 58-145mmHg. In theory, a good retrograde perfusion requires a physiological pressure of at least 60mmHg. However, a 60mmHg pressure delivers a high flow rate of approximately 14$\pm$4ml/min which would require large volume of the collagenase solution or necessitate the enzyme to be recirculated. The large volume of the collagenase solution would be economically prohibitive and the recirculation of the solution would introduce many intracellular substances that may damage the viable cells. Furthermore, the perfusion pressure set at 60mmHg may induce

vasoconstriction of the coronary vessels interfering with the perfusion process. We, therefore, opted to select a constant flow rather than constant pressure technique to perfuse the heart. Different flow rates have been carefully examined. Under identical conditions, the most effective flow was the one that will consistently offer the highest cell yields with the greatest fraction of viable cells. We have found that a flow rate of 1.5ml/min (maintained constant by peristaltic pump) repeatedly achieved this objective. This slow flow rate had many advantages:

1.) It allowed maximal time of exposure to the collagenase without any additional damage done to the MC due to the perfusion pressure.

2.) Gas analysis indicated that the oxygen tension and saturation of the enzyme solution obtained after perfusion is sufficiently adequate to oxygenate the MC.

3.) Air embolism, when occured, could not cause any damage to the heart at such slow flow rate. The embolus is slowly pushed out of the coronary circulation and no infarct occurs. This phenomenon has been observed numerous times.

4.) The slow flow rate allows a prolonged perfusion with a small volume of collagenase without requiring its recirculation.

Secondly, we devised a method to maintain the ambient temperature of the heart during the enzyme solution perfusion at 37 degrees celcius. This was accomplished by totally immersing the heart into a double-jacket chamber that contains the Hepes-

buffer solution and maintained at 37 degrees celcius. This chamber is drained at the same rate as the enzyme perfusion flow rate. The enzyme solution was also maintained at 37 degrees celcius.

We, furthermore, examined two parameters to improve the technique of maintaining the long-term culture of single MC (Stage II). Two modifications were critical in order to sustain a viable long-term primary culture: elimination of non-myocardial cells and reduction of cell death after its attachment to the dish. First of all, when establishing a long-term culture of MC in the presence of calf-serum, proliferation of non-MC such as fibroblasts, endothelial cells, smooth muscle cells, neurons, macrophages, lymphocytes, and other blood-born cells presents a major problem. We eliminated this unnnecessary growth of non-MC by two steps:

1.) Density sedimentation using Percoll solution before plating seperated the isolated, small size non-myocytes.

2.) The adult rat MC do not undergo mitosis while the non-MC do profliferate rapidly in the presence of the calf-serum. The use of potent anti-mitotic agent such as cytosine arabinoside at 10uM effectively reduced the percentage of non-MC to <10%.

Secondly, the rate of cell death following their attachment to the culture dishes and during the 6 days of culture needed to be reduced. The viability of the MC during the long-term culture was improved by the following measures:

1.) Supplementation of the culture medium with 1% calf-serum

significantly improved the attrition rate of viable MC during the 6 days of culture. The unnecessary proliferation of non-MC as mentioned above was eliminated by the addition of 10uM cytosine arabinoside.

2.) On culture day 3 and 6, the culture dishes were rinsed to remove the non-attached round cells presumed to be dead and the growth medium was also replaced. This left behind a highly purified culture of predominantly viable MC.

Hypertrophic Response

Using this system, we studied the possible hypertrophic effect of different agents on adult rat MC. Previous studies have shown that hypertrophy of neonatal rat heart cells is stimulated by NE through alphal adrenergic receptors (21,43). We similarly have determined that NE induce hypertrophy in adult rat MC. However, the hypertrophic response appears markedly reduced in adult rat heart MC. While the exact etiology is not known, this phenomenon may be possibly correlated with a well-established peculiarity of adult rat MC which is known to have shortened action potential (18). Another postulate may be that the alphal-mediated hypertrophy might be an early developmental response that is only fully re-expressed in the adult by an alternative stimulus such as the thyroid hormone (12). Furthermore, the adult rat MC essentially are matured MC (6). The matured MC may no longer have any physiological need to increase its size. Thus, the alphal receptors may have developed to mediate other cellular

mechanisms that have become increasingly important during growth such as contractile activity (26). Finally, it is also possible that the alpha1 adrenergic receptors may have been down-regulated and the intracellular mechanism involved in hypertrophy may have lost its efficacy. These hypotheses, however, require further investigations.

The enhanced hypertrophic response seen when propranolol was added to norepinephrine suggests a possible inhibitory effect of beta-adrenergic receptors in hypertrophy. Although the mechanism of the beta receptors' involvement in hypertrophy is hardly known, based purely on our experimental observation, it is possible to propose a certain antagonistic effects of beta-mediated response to hypertrophy. One hypothesis may be related to the phenomenon in which contractile activity requires both alpha1- and beta-receptors (26). It is possible that the hypertrophic and contractile responses share a similar intracellular pathway. The simultaneous stimulation of alpha1- and beta-receptors by NE may preferentially stimulate the contractile activity and thus, reducing the efficacy of hypertrophy which is purely an alpha1-mediated response (22). By adding propranolol, a beta-anatagonist, the inhibitory effect of beta-receptor to hypertrophy may have been reduced.

In the case of TPA-treated MC, it has been suggested that the TPA-induced hypertrophy might serve as model for pathological hypertrophy (31). Studies have shown that the TPA-treated MC that undergo hypertrophy have reduced contractile protein content when

compared to the NE-treated counterpart (31). While the mechanism of hypertrophy is unclear, the effect of TPA to increase overall cell size without an increase in contractile protein provide a possible explanation for pathological hypertrophy (31). In vivo, cells from pathologically hypertrophied hearts (decompensated aortic stenosis) show a selective reduction in the volume fraction of myofibrils (32).

Finally, the cells that were treated with 5% CS showed the highest hypertrophic response. This result attests to the enormous complexity of hypertrophy which responds best to the multiple growth factors that are present in the calf-serum. It also reinforces the difficulty of isolating one dominant humoral agent that might be responsible for hypertrophic response. It appears most likely that numerous trophic agents work singly or in conjunction with other agents to induce hypertrophy in response to different physiological circumstances such as normal growth, exercise, hypertension, cardiomyopathy, etc. All these conditions place unique hemodynamic demands to the heart probably triggering different factors in each circumstance.

While the data from our experiment provide a new insight to the phenomenon of hypertrophy in adult rat MC, the subsequent discussion and explanation for the results are primarily hypothetical. In order to better understand the phenomenon of alphal-stimulated hypertrophy of adult rat MC, it is critical to elucidate the intracellular mechanism. Although the intracellular pathway of adult rat MC is currently investigated, some studies

have already shown the mechanism involved in the alpha1-mediated hypertrophy of neonatal rat MC.

Intracellular Mechanism

There have been two approaches to study the intracellular mechanisms involved in alpha1-stimulated hypertrophy of neonatal rat MC: 1.) genetic expression in alpha1-stimulated hypertrophy and 2.) intracellular pathway leading to the induction of gene expression. First of all, studies have looked at the mRNA and protein expression of c-myc proto-oncogene, actin, and myosin. It has been shown that alpha1 stimulation induces transient increase in c-myc proto-oncogene mRNA (34). Recent studies have also associated the c-myc mRNA with cell proliferation (34). This may imply common intracellular mechanism between cell proliferation and hypertrophy. Another area of investigation looked at the expression of actin and myosin mRNA isoforms during MC hypertrophy. It has been shown that the alpha1-mediated hypertrophy induces an increase in the expression of actin and myosin mRNA isoforms that are prevalent in immature, growing heart (35). The studies suggest alpha1-stimulated hypertrophy may be associated with expression of growth and developmental genes (35). Furthermore, another study has shown that during pressure-load hypertrophy of the adult rat heart in vivo, there is also an increased expression of a similar immature isoform of actin mRNA (36). These studies suggest the possibility of an increased expression of the developmental mRNA isofrom in both alpha1-

mediated and pathological hypertrophy.

The second approach to elucidate the mechanism of alpha1-mediated hypertrophy involved the intracellular pathways that are activated by the alpha1 receptors to induce gene expression. The primary intracellular event following the activation of alpha1-adrenergic receptor is a hydrolysis of phosphatidylinositol-4,5-biphosphate (PI) in the plasma membrane to produce two intracellular messengers: myo-inositol-1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG) (37). IP3 activates the release of the calcium from endoplasmic reticulum which then binds to the regulatory protein, calmodulin (37). DAG activates protein kinase C which subsequently phosphorylates and alters the cellular proteins (37). Studies have been made to determine the level of PI hydrolysis by measuring IP3 and PKC in neonatal rat MC following alpha1-stimulation (38,39). The levels of both IP3 and PKC productions have been seen to increase following alpha1 stimulation (38,39). These findings suggest a possible role of PI, IP3, and PKC in mediating the alpha1-stimulated hypertrophy.

Conclusion

We were able to provide new information regarding the alpha1-mediated hypertrophy in adult rat MC. We found that the adult rat MC respond to lesser magnitude than the neonatal rat MC to the trophic factors. Some type of physiologic adaptation of the alpha1 mechanism must exist from neonatal to adult rat to reduce the adult rat MC's potential to hypertrophy (40). In order

to explain such phenomenon, the intracellular pathway of hypertrophy of adult rat MC need to be studied to answer the following questions: 1.) do the intracellular events change as the rat ages and 2.) do the alpa1 receptors themselves undergo morphologic changes? Finally, the over-riding concern is how do all these cellular and molecular biology ultimately translate to in vivo in human beings. While the answers can not be obtained immediately, it is of utmost importance to at least comprehend the phenomenon of cardiac hypertrophy at the most fundamental levels before any medical application is achieved.

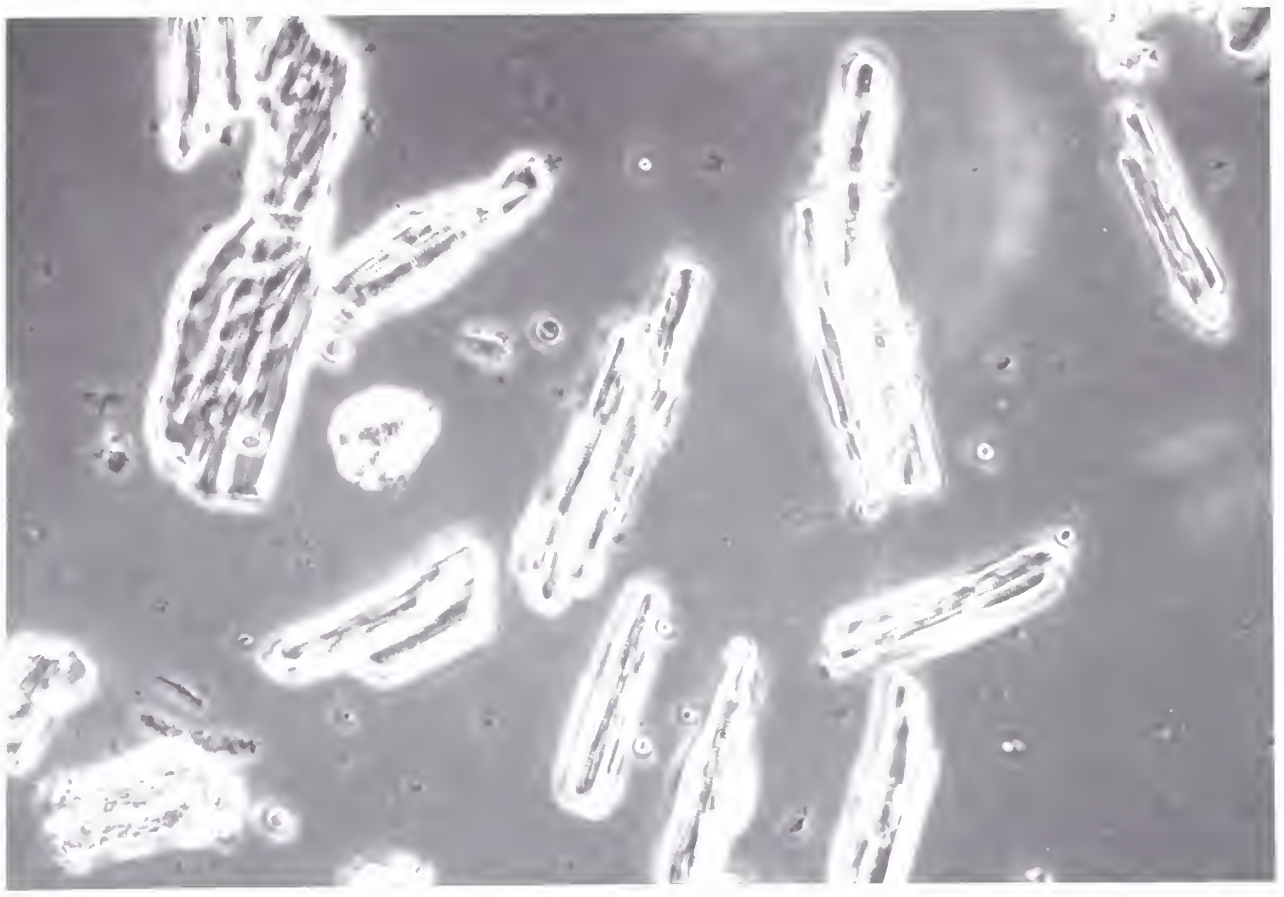

Figure 1. Photograph of adult rat MC immediately following the disssociation. The morphology of MC is marked by the characteristic rod-shape, cross-striated cytoarchitecture, and contractility.

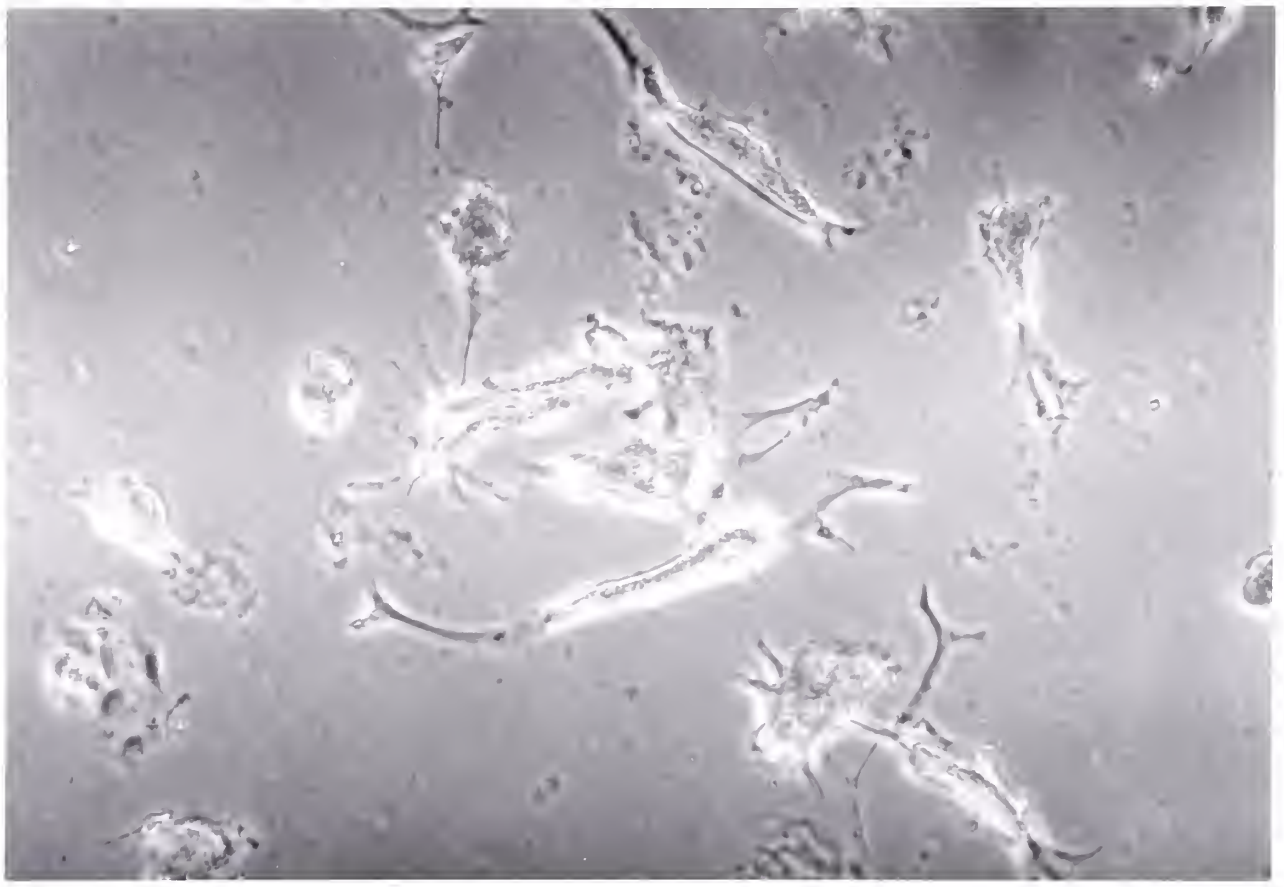

Figure 2. The photograph of adult rat MC on culture day 6. The morphology of the MC is marked by its flat cellular appearance with translucent cytoplasm and well-developed multiple, slender pseudopods growing in all directions.

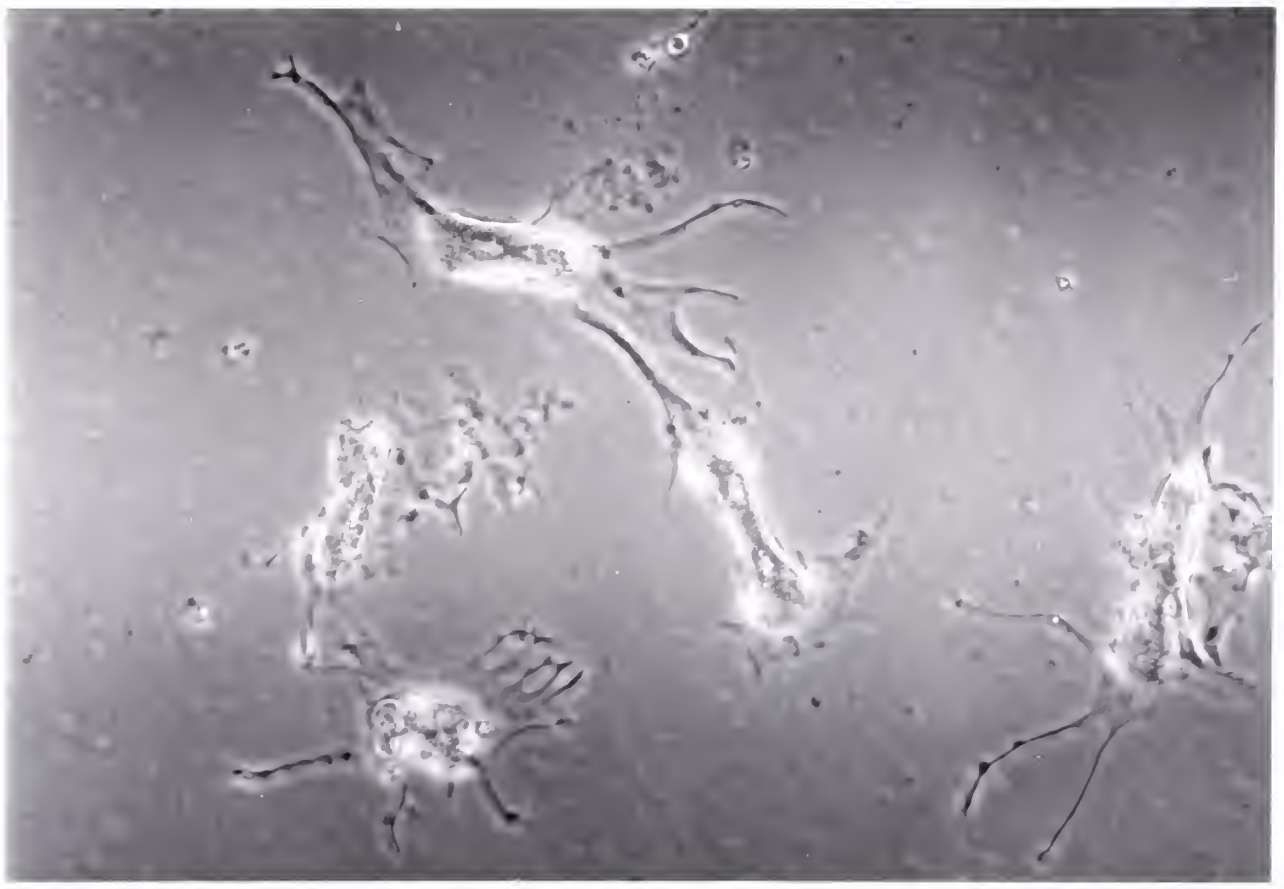

Figure 3. The photograph of adult rat MC on culture day 10 following 2 days of diluent-treatment (control).

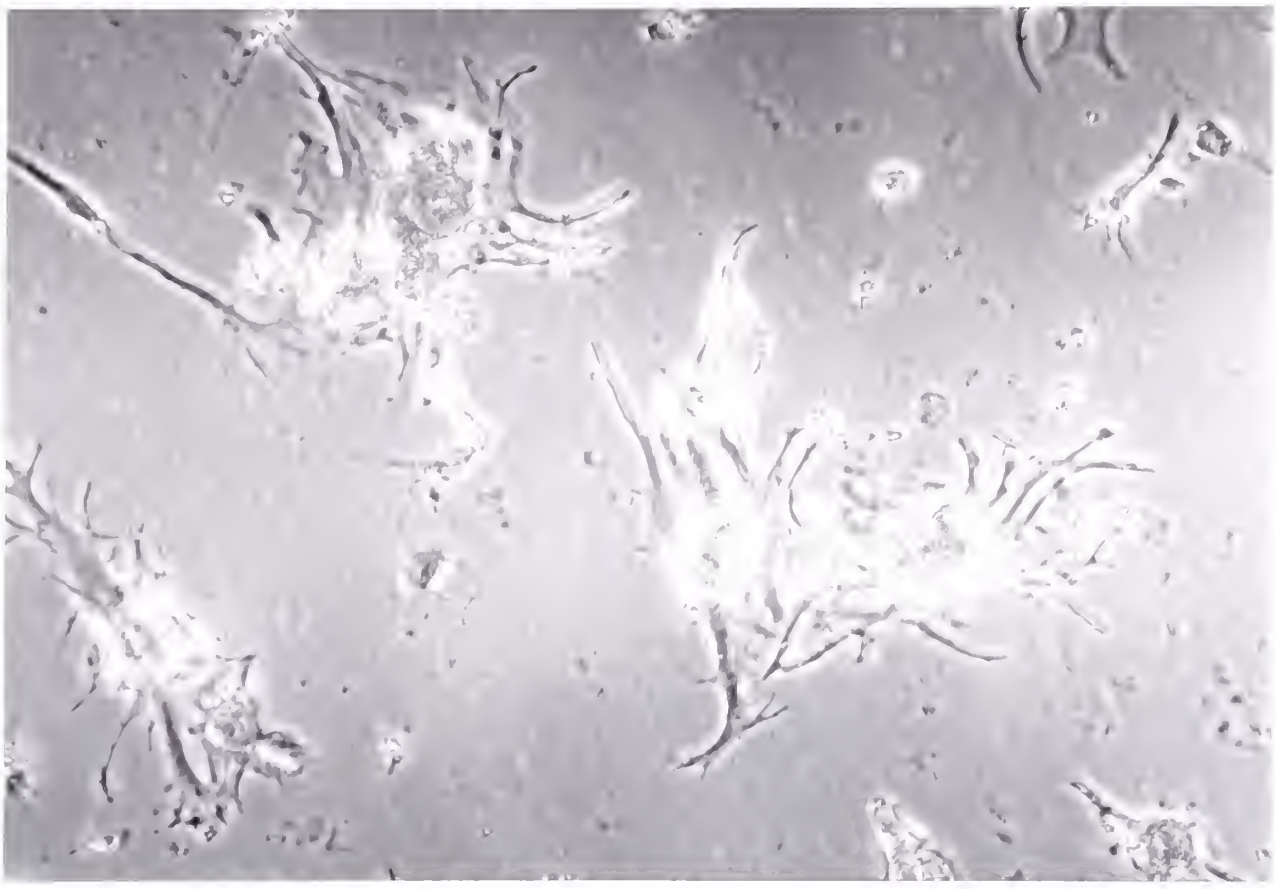

Figure 4. The photograph of adult rat MC on culture day 10 following 2 days of NE-treatment. The increased cell size is readily apparent in comparison to the control MC.

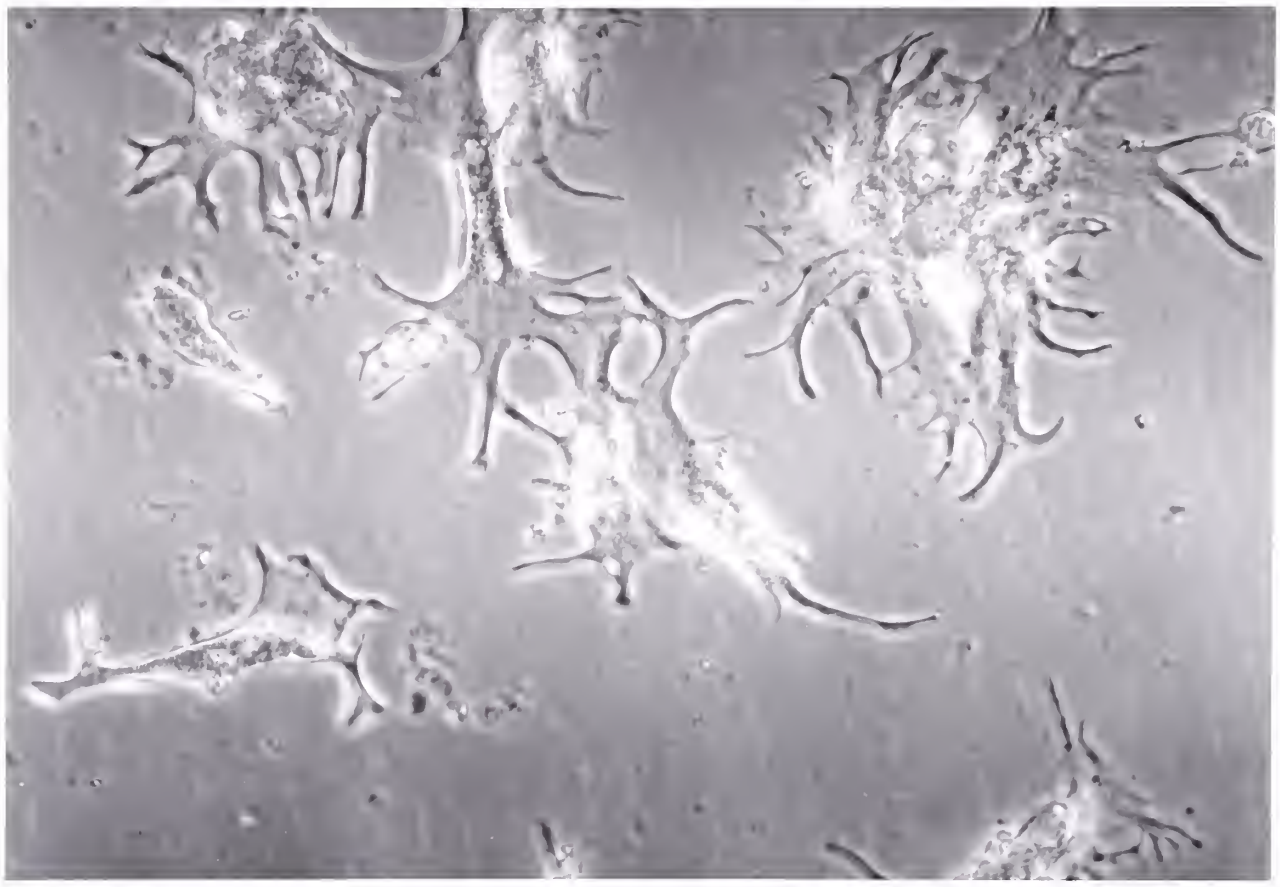

Figure 5. The photograph of adult rat MC on culture day 10 following 2 days of NE+PRO-treatment. The increase in cell size is readily apparent when compared to control MC but not obvious when compared to NE-treatment.

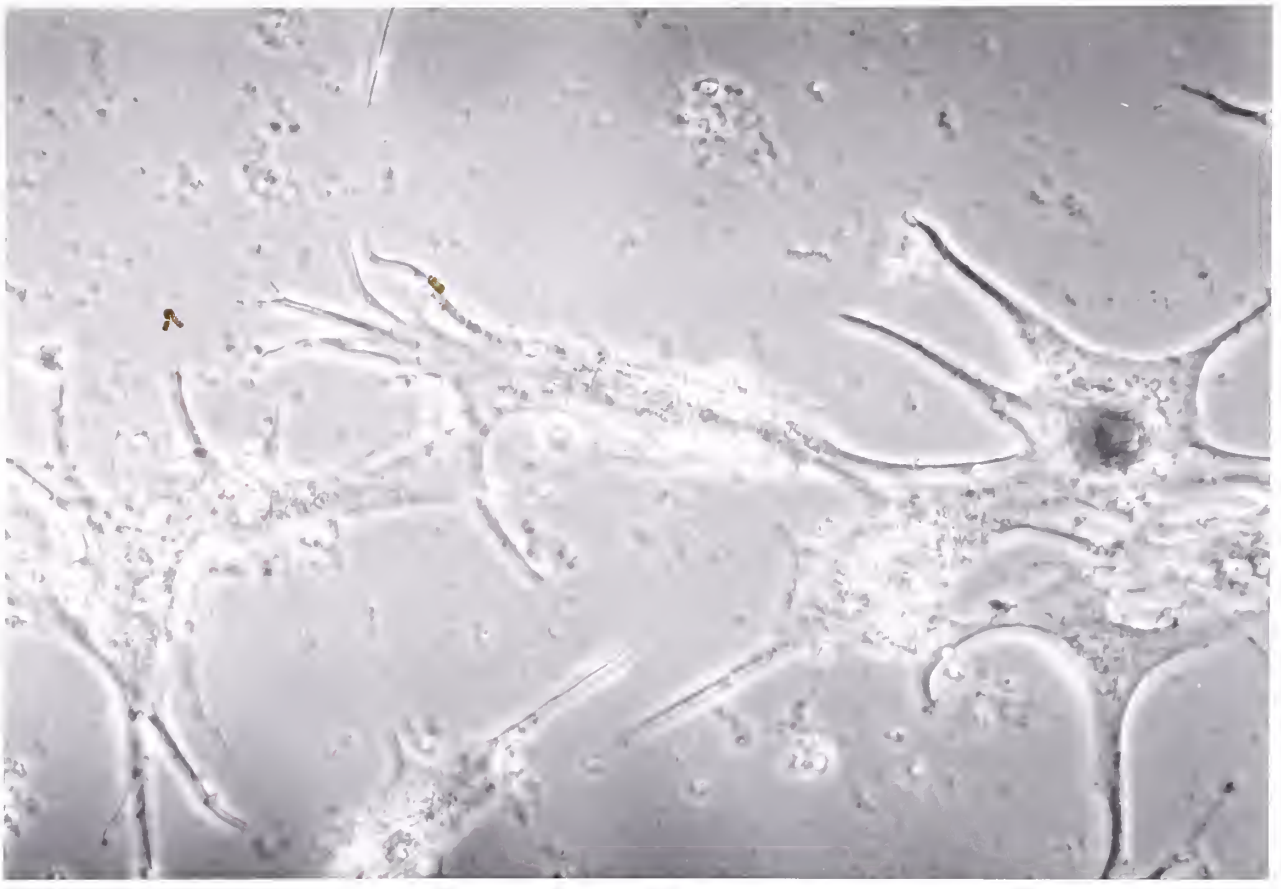

Figure 6. The photograph of adult rat MC on culture day 10 following 2 days of TPA-treatment. The increase in cell size is readily apparent when compared to the control MC. A close observation shows increased granularity within the cytoplasm which may possibly be indicative of its possible pathological state of hypertrophy.

Figure 7. The photograph of adult rat MC on culture day 10 following 2 days of 5% CS-treatment. The increased cell size is readily apparent when compared to all the other MC. In addition, the CS-treated MC appear to have increased number of pseudopods growing in all directions.

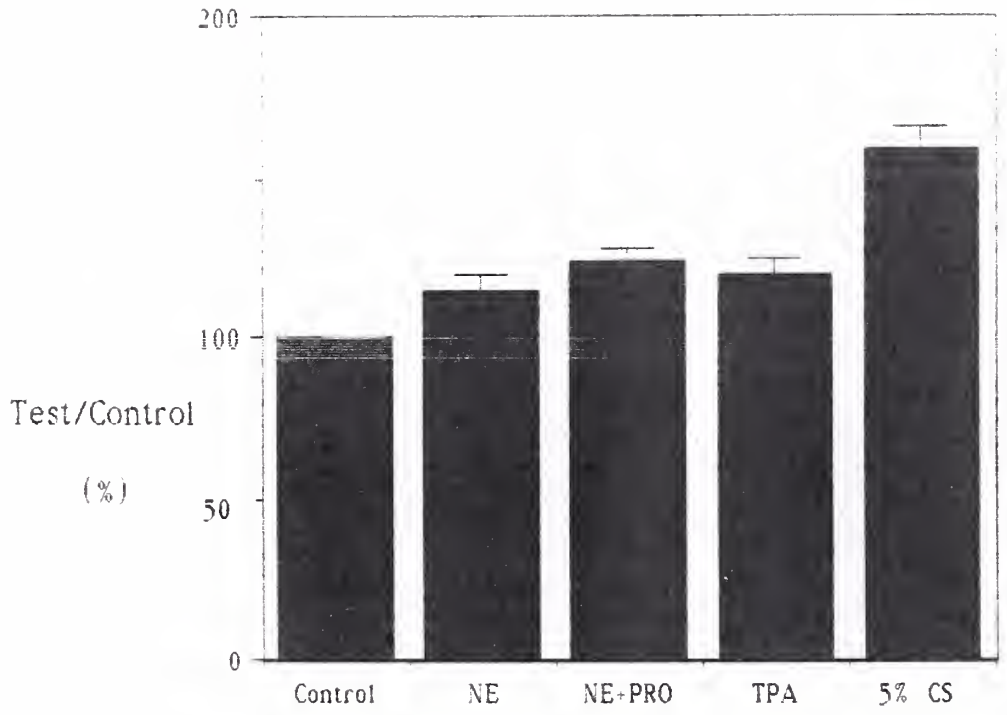

Figure 8 Hypertrophy Measured Through 14C-Phenylalanine Incorporation by MC.

The control was set at 100%. The NE, NE+PRO, TPA, and 5% CS treated MC were comparedto the control MC. The differences were described as % of control. All test agents significantly induced hypertrophy ($p<0.01$). The data were obtained from 8 experiments with each experiment consisting of 30 culture dishes with 6 dishes in each condition.

Control: 100%. NE: 115+4.9 NE+PRO: 124+4.0
TPA: 120+4.9 5% CS: 159+6.7

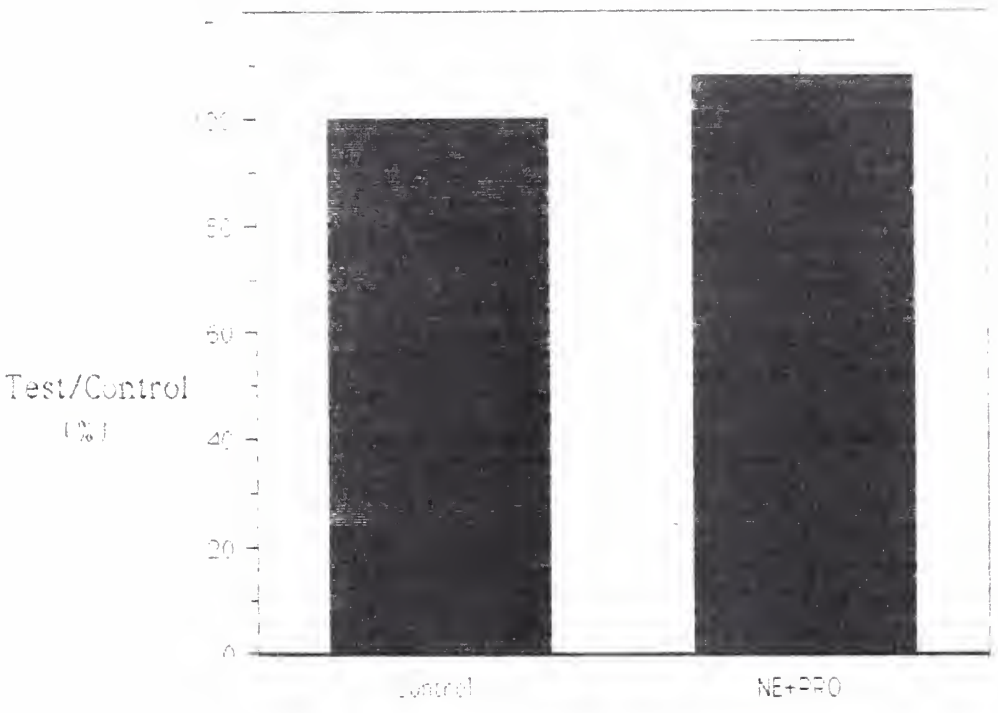

Figure 9 Comparison of Hypertrophy Induced by NE vs. NE+PRO

Hypertrophy was measured through 14 C-phenylalanine incorporation by MC. NE was set as control at 100%. The effect of NE+PRO was described as % of control. NE-PRO induced significant increasae in hypertrophy when compared to NE alone ($p<0.05$). The data are from 6 experiments with each experiment consisting of 6 dishes in each condition.
NE: 100% NE+PRO: 108%-6.3

REFERENCES

1. Farmer BB, Mancina M, Williams ES, Watanabe AM: Isolation of calcium tolerant myocytes from adult rat hearts: review of the literature and description of a method. Life Sci 33: 1-18,1983
2. Nag AC, Zak R: Dissociation of adult mammalian heart into single cell suspension: an ultrastructural study. J Anat 129: 541-559, 1979
3. Kao RL, Christman EW, Luh SL, Krauhs JM, Tyers GF, Williams EL: The effects of insulin and anoxia on the metabolism of isolated mature rat cardiac myocytes. Arch Biochem Biophys 203: 587-599, 1980
4. Onorato JJ, Rudolph SA: Regulation of protein phosphorylation by inotropic agents in isolated rat myocardial cells. J Bio Chen 256: 10697-10703, 1981
5. Jacobson SL, Banfalvi M, Schwarzfeld TA: Long-term primary cultures of adult human and rat cardiomyocytes. Basic Res Cardiol 80 Suppl 1: 79-82, 1985
6. Wollenberger A: Isolated heart cells as a model of the myocardium. Basic Res Cardiol 80 Suppl 2: 9-13, 1985
7. Badeer HS: Biological significance of cardiac hypertrophy. Am J Cardiol 14: 133, 1964
8. Grossman W: Cardiac hypertrophy: useful adaptation or pathological process? Amer J Med 69: 576-584, 1980
9. Witzke DJ, Kaye MP: Hypertrophic cardiomyopathy induced by

administration of nerve growth factor. Circ 53/54 Suppl 2: II-83, 1976

10. Dodge HT, Baxley WA: Left ventricular volume and mass and their significance in heart disease. Am J Cardio 23: 528, 1969
11. Zak R: Development and proliferative capacity of cardiac muscle cells. Circ Res 35: II-17,1974
12. Brown AJ Jr: Morphological factors in cardiac hypertrophy. In Alpert NR, Ed. Cardiac Hypertrophy. New York: Academic Press, 1971
13. Zak R: Cell proliferation during cardiac growth. Am J Cardiol 31:211, 1973
14. Wikman-Coffelt J, Parmley WW, Mason DT: The cardiac hypertrophy process. Analysis of factors determining pathological vs. physiological development. Circ Res 45: 697-710, 1979
15. Laks MM: Norepinephrine: the myocardial hypertrophy hormone? Am Heart J 91: 674, 1976
16. Laks MM, Morady F, Swan HJC: Myocardial hypertrophy produced by chronic subhypertensive doses of norepinephrine in the dog. Chest 64: 75, 1973
17. Morgan HE: Mechanical factors affecting protein turnover in isolated rat hearts. Fed Proc 45: 2563-2567, 1986
18. Everett AW: Expression of myosin heavy chains during thyroid-hormone-induced cardiac growth. Fed Proc 45: 2568-2572, 1986
19. Rappaport L: Physiological adaptation of the heart to

pathological overloading. Fed Proc 45: 2573-2579, 1986

20. Re RN: Cellular mechansisms of growth in cardiovascular tissue. Am J Cardiol 60: 104I-109I, 1987.

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

22. Simpson P: Norepinephrine-stimulated hypertrophy of cultured rat myocardial cells is an alpha1-adrenergic response. J Clin Invest 72: 732-738, 1983

23. Simpson P: Stimulation of hypertrophy of cultured neonatal rat heart cells through an alpha1-adrenergic receptor and induction of beating through an alpha1- and beta-adrenergic receptor interaction: evidence for independent regulation of growth and beating. Circ Res 56:884-894, 1985

24. Simpson P, Karliner JS: Regulation of cardiac myocyte hypertrophy by a tumor-promoting phorbol ester. Clin Res 33: 229A, 1985

25. Simpson P: Calcium entry blockers inhibit catecholamine-induced beating but not catecholamine-stimulated hypertrophy of cultured rat heart cells. Clin Res 33: 90A, 1984

26. Simpson P, Bishopric N, Coughlin S, Karliner J, Ordahl C, Starksen N, Tsao T, White N, Williams L: Dual trophic effects of the alpha1-adrenergic receptor in cultured neonatal rat heart muscle cells. J Mol Cell Cardiol 18 (Suppl 5): 45-58, 1986

27. Simpson P: Norepinephrine-stimulated hypertrophy of cultured

rat heart cells requires continuous alpha1-adrenergic receptor occupancy. Circulation 70: II-198, 1984

28. Powell T: Isolated adult heart cells. The development of a new experimental model. Basic Res Cardiol 80 suppl 1: 93-96, 1985
29. Dow JW: Isolated cardiac myocytes II: functional aspects of mature cells. Cardiovasc Res 15: 549-579, 1981
30. Farmer BB, Harris BA, Jolly WW, Hathaway DR, Katzberg A, Watanabe AM, Whitlow AL, Besch HR, JR: Isolation and characterization of adult rat heart cells. Arch Biochem Biophys 179: 545-558, 1977
31. White N, Tsao T, Simpson P: Contractile protein content is increased in hypertrophy stimulated by norepinephrine but not by a phorbol ester: An example of pathological hypertrophy in cultured neonatal rat heart muscle cells. J Amer Coll Cardiol 7: 122A, 1986
32. Schwarz F, Schaper J, Kittstein D, Flameng W, Walter P, Schaper W: Reduced volume fraction of myofibrils in myocardium of patients with decompensated pressure overload. Circ 63: 1299-1304, 1981
33. Morgan HE: Biochemical mechanisms of cardiac hypertrophy. Ann Rev Physiol 49: 533-543, 1987
34. Starksen NF, Simpson P, Bishopric N, Coughlin SR, Lee WMF, Escobedo JA, Williams LT: Cardiac myocyte hypertrophy is associated with c-myc proto-oncogene expression. Proc Natl Acad Sci USA 83: 8348-8350, 1986

35. Bishopric NH, Simpson P, Ordahl CP: Induction of the skeletal alphal adrenoceptor-mediated hypertrophy of rat cardiac myocytes. J Clin Invest 80(4): 1194-1199, 1987

36. Schwartz K, de la Bastie D, Bouvert P, Oliveriro P, Alonso S, Buckingham M: Alpha-skeletal muscle actin mRNAs accumulate in hypertrophied adult rat hearts. Circ Res 59: 551-555, 1986

37. Exton JH: Mechanisms involved in alpha-adrenergic phenomena. Amer J Physiol 248:E633-E647, 1985

38. Henrich CJ, Simpson P: Activation of protein kinase C in hypertrophy of cultured rat heart myocytes. J Mol & Cell Card (in press), 1988

39. Karliner JS, Simpson PC, Braun L, Honbo N, Woloszyn W: Alphal-adrenoceptor regulation of phosphoinositide turn-over in hypertrophied myocardial cells. Circ 72: III-182, 1985

40. Jacobson SL: Cell cultures of adult cardiomyocytes as models of the myocardium. J Mol Cell Cardiol 18: 661-678, 1986

41. Lundgren E, Terracio L, Borg TK: Adhesion of cardiac myocytes to extracellular matrix components. Basic Res Cardiol 80 Suppl 1: 69-74

42. Piper HM, Spahr R, Probst I, Spieckerman PG: Substrate for the attachment of adult cardiac myocyte in culture. Basic Res Cardiol 80 suppl 2: 175-180, 1985

43. Simpson P: Norepinephrine-stimulated hypertrophy of cultured rat myocardial cell is an alphal adrenergic response. J Clin Invest 31: 81A, 1983

44. Simpson P, Savion S: Differentiation of rat myocytes in single cell cultures with and without proliferating non-myocardial cells. Circ Res 50: 101-116, 1982

YALE MEDICAL LIBRARY

Manuscript Theses

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Yale Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by has been used by the following persons, whose signatures attest their acceptance of the above restrictions.

NAME AND ADDRESS	DATE