Differentiation of Adult Rat Cardiac Myocytes in Cell Culture

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Cardiac myocytes isolated from adult rat hearts were grown on laminin coated culture dishes for more than a month. During this time, the cells underwent a morphological transformation which has also been referred to by others as cell remodeling (Guo J-X, Jacobson SL, Brown DL: *Cell Mot Cytoskeleton* 1986;6:291–304). This results in a change in myocyte morphology from its typical in vivo cylindrical shape to one which is more pleiomorphic. Despite the long-term change in morphology, myocytes expressed for differing lengths of time several aspects of the adult phenotype as evidenced by the following: 1) maintenance of cylindrical shape and/or evident cross-striations for the first 24–48 hours in culture, 2) reappearance of cross-striations during the second week in culture, 3) little or no spontaneous contractility for the first 4 days in culture, 4) expression of only the V, isoform of myosin for at least 7 days, and 5) altered myosin isoform expression in response to changes in environmental conditions. These factors taken together suggest that in culture the adult cardiac myocyte remains a highly differentiated cell (as opposed to possible dedifferentiation) and maintains many of its previous in vivo characteristics. Such highly differentiated adult cells should be suitable as an in vitro system for studying the direct cellular effects of factors which regulate growth and differentiation of the in vivo heart. (*Circulation Research* 1989;64:493–500)

The adult cardiac myocyte of the rat is a binucleated, nondividing cell which participates in organ growth solely by cellular hypertrophy. While this cell type constitutes only about 25% of the total cellular population in the heart, it represents approximately 75% of the organ's total mass and thus forms the major functional and structural unit of the heart. Little is known about those factors that at the cellular level directly regulate growth, differentiation, and the response to physiological stress in this cell type. To investigate these processes, it is necessary to have a well-characterized cell culture system in which the properties of the cardiac myocyte can be studied devoid of systemic influences. Investigators began to approach this problem more than 20 years ago using cells cultured from embryonic or neonatal hearts. Unlike myocytes from the adult rat heart, however, myocytes from embryonic and neonatal hearts undergo cell division during growth. Thus, cells from embryonic/neonatal hearts studied in culture may use different strategies from adult cells to respond to growth stimuli. Since these characteristics (cellular division versus cellular hypertrophy) as well as others unique to embryonic and neonatal cells may be carried over into cell culture, it may be inappropriate to extrapolate results obtained with immature cells in an in vitro system to describe the response of the adult myocyte.

Adult cardiac myocytes in small quantities have been successfully cultured for several years, but only recently have techniques become available for the long-term in vitro growth of sufficient numbers of cells for biochemical analyses. Previous studies were limited to a description of the morphological and ultrastructural changes that occurred in those few cells that survived the enzymatic dissociation and attached to the surface of the culture dish. Briefly, this transformation involved an initial loss of the myocyte's characteristic cylindrical shape (approximate dimensions, 100 μm x 15 μm) and cross-striations resulting in a more pleiomorphic cell, which investigators interpreted to be structurally dedifferentiated.

A major morphological characteristic of the cardiac myocyte is its evident cross-striations, which
are the result of the interaction of several contractile proteins, particularly actin and myosin. Myosin can be observed as three different isoforms of the native molecule (V₁, V₂, and V₃), which differ not only in their relative electrophoretic mobility on nondenaturing gels but also in ATPase activity. Changes in the relative proportions of myosin isoform composition occur both during normal development and induced growth of the heart and correlate with altered contractility. Furthermore, changes in the isoforms can also be modulated by thyroxine, both in vivo and in neonatal cardiac myocytes in primary culture. In this report, we use myosin isoform profiles in conjunction with the pattern of immunofluorescent staining of myofibrils to examine the state of differentiation of isolated adult myocytes in long-term culture. Despite previous descriptions of myocytes in long-term cultures as “dedifferentiated cells,” we demonstrate that many of the aspects of the adult myocyte phenotype continue to be expressed in tissue culture. Based on these observations, it is proposed that adult myocytes in culture remain highly differentiated and distinct from neonatal or embryonic myocytes. Furthermore, we suggest that the previously observed dramatic morphological alterations, as well as other biochemical changes that occur, most likely represent an adaptation to the new environment of tissue culture.

Material and Methods

Myocyte Isolation and Culture

Calcium tolerant myocytes were obtained using a combination of the perfusion technique of Claycomb and Palazzo and the attachment procedure of Borg et al. Adult female Sprague-Dawley rats weighing 175–225 g were injected intraperitoneally with heparin (100 units/100 g body wt) 30 minutes before death. Rats were killed by either a blow to the head or by injection of Ketamine-Rompun anesthesia, and the hearts were aseptically removed into the head or by injection of Ketamine-Rompun anesthesia, and the hearts were aseptically removed into a 25-ml Erlenmeyer flask. The ventricles were cut into small pieces and then shaken at 37 °C on a rotary shaker for 5 minutes. Five milliliters of Joklik’s medium containing 5% calf serum (Hyclone Lab, Logan, Utah) were added and released cells removed. Fresh collagenase solution was added and the process repeated two or three times. After the last incubation, the remaining tissue was broken up by trituration through a 10-ml pipette. Released cells were allowed to settle (10–15 minutes) without centrifugation and washed twice with Joklik’s medium containing 5% calf serum. After the last wash, cells were suspended in basal medium Eagle’s (BME) containing 5% calf serum and antibiotics.

Cells from one heart were usually split among 10–15 6-cm dishes that had been precoated for at least 2 hours at 37 °C with 15 μg of laminin (Bethesda Research Laboratories, Gaithersburg, Maryland). Attachment of many cells could be observed to begin almost immediately, but to ensure a good yield of attached cells, they were left undisturbed for 2–3 hours at 37 °C. After this time, the dishes were washed with BME+5% calf serum, and no further media changes occurred for at least 2 days. After 2–4 days in culture, 0.01 mM cytosine arabinoside was added for 2–3 days to prevent fibroblast overgrowth. Media was then routinely changed every other day. Cells to be grown in the serum-free medium PC-1 (Ventrex Labs, Portland, Maine) were allowed to initially attach in BME plus 5% calf serum and then switched to the serum-free medium after 1–5 days.

Cell counts per plate were obtained by counting 10 random fields of known area on several dishes. This average value was then extrapolated to the area of the entire dish.

Beating Rates

Beating rates were obtained on cultures maintained at 37 °C in an incubator-enclosed stage of a Nikon Diaphot Microscope. Only 10 cells per dish were counted to minimize the time they were out of the 95% air-5% CO₂ environment of the tissue culture incubator. Prolonged time out of the incubator results in a change in medium pH and may have an effect on the overall beating rate. Three dishes per culture and at least five different cultures were counted for a given timepoint, which resulted in a minimum of 150 cells per day being used to obtain information for Figure 2.

Myosin Extraction

Cells from three to six culture dishes (6 cm; Falcon) were combined for myosin isoform analysis. The cells were washed three times with phosphate-buffered saline and then scraped into preweighed tubes and centrifuged for 15–30 seconds in an Eppendorf centrifuge. After centrifugation, the weight of the pellet was determined and 6 volumes of ice-cold Guba-Straub solution containing 5 mM ATP was added. Cells were extracted 90–120 minutes on ice with occasional agitation and pelleted. Six volumes of running buffer (30 mM sodium pyrophosphate [pH 8.6], 1.0 mM EDTA, 5.0 mM cysteine, 0.0004% Bromphenol Blue, and...
FIGURE 1. Morphological transformation of adult cardiac myocytes in long-term culture. A–E are cells that have been maintained in basal medium Eagle + 5% calf serum as follows: A) Day 0+3 hours B and C) Day 5 D) Day 10 E) Day 15 F) 15 days in culture, as in E but the last 10 days were in PC-1. B and C, both from 5-day cultures, illustrates the heterogeneity present in terms of the degree of transformation on the same day. Arrow in B points out a fibroblast in the culture. Newly expressed sarcoplasm is shown in C (arrow) and sarcoplasm that appears to be less firmly attached is shown in F. A–C, E–F: Original magnification, ×211. D: original magnification, ×98.

50% glycerol) were added to the supernatant, and it was stored at −20°C until analysis.

Native Gel Electrophoresis
Polyacrylamide gels (3.88%) were prepared similar to Clark et al12 and myosin extracts loaded onto the gels. Electrophoresis was conducted for 20–24 hours at a voltage gradient of 11 V/cm and a temperature between 2° and 4° C. Gels were stained with Coomassie Brilliant Blue R-250 and electrically destained.

Immunofluorescent Studies
For immunofluorescent studies, cells were grown on laminin coated coverslips. They were washed three times with phosphate buffered saline (PBS) before fixation and were then fixed with ice-cold 50% methanol and permeabilized with 1:1 methanol-acetone. Coverslips were then washed for 30 minutes with successive solutions of PBS, anti-myosin antibody (CCM #52),12 PBS, fluorescein-conjugated goat immunoglobulin G (Cooper Biomedical, Malvern, Pennsylvania), and PBS. After the last wash, they were mounted in PBS-glycerol and observed with either a Zeiss or Nikon Epifluorescent microscope with an FITC filter block.

Results
Adaptation to Tissue Culture Conditions
The initial percentage of cylindrical cells following perfusion appears to be highly dependent on the batch of collagenase used for perfusion. In early experiments we could often obtain as many as 80–90% cylindrical cells in the starting cultures. Recently, however, our cultures on the average begin with 50–60% cylindrical cells containing cross-striations (Figure 1A) and 70–80% trypan blue negative cells. This results in an average of 1.04×10⁶ total attached cells from each heart. During the first three days in culture the percentage of cylindrical cells decreases from 55.0±3.3% to 12±4.0% while the percentage of trypan blue negative cells remains virtually constant (81.5±2.8% vs. 81.0±3.8%) (Table
TABLE 1. Changes in Morphology and Trypan Blue Uptake During Adaptation to Culture

<table>
<thead>
<tr>
<th>% Cylindrical cells</th>
<th>% Trypan blue negative cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>Day 1</td>
</tr>
<tr>
<td>55.0±3.3</td>
<td>44.5±6.6</td>
</tr>
<tr>
<td>(6)</td>
<td>(6)</td>
</tr>
<tr>
<td>81.5±2.8</td>
<td>72.8±3.2</td>
</tr>
<tr>
<td>(6)</td>
<td>(6)</td>
</tr>
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Values are mean±SEM. Number in parentheses is number of animals.

1). This data illustrates that some noncylindrical myocytes appear to establish themselves in culture and that trypan blue uptake and the percentage of cylindrical myocytes in a culture does not necessarily correlate with each other. The majority (80% on average) of the initial population of cylindrical cells remain that way and still possess cross-striations following 24 hours in culture. With increasing time in culture the myocytes express cytoplasmic pseudopodia, lose their typical cylindrical shape (Figures 1B and 1C), and eventually become fully spread out on the tissue culture dish (Figures 1D and 1E). This process appears to be initially accompanied by myofibrillar disorganization visible at the light microscope level, which is then followed by reorganization. The series of morphological changes we observed are similar to those reported by other investigators, though the time course may differ somewhat, with many but not all cells losing their cylindrical shape before becoming fully spread out during the first week. Thereafter, almost all cells have this spread out appearance by 10 days in culture. Interestingly, there is a marked morphological heterogeneity during the transformation period with both spread out cells and nearly cylindrical cells apparent in the same culture at early time-points (4–7 days in culture) (Figure 1C). The use of Hoffman Modulation Contrast optics allows one to readily distinguish the heterogeneity in these cell populations not only in terms of gross morphology but also in terms of the myocyte's relation to the attachment substratum. It can easily be seen that parts of some cells are very close to the dishes' surface while other cells are not (Figure 1C, arrow). During this period, cells in close proximity to each other will extend their sarcoplasm (Figures 1C and 1D) in an almost tactic response until they appear as a single cell that can contain several nuclei. We also observe a considerable increase in myocyte surface area in these cultures.

After initial plating, a few cells (less than 10%) demonstrate sporadic beating; however, the vast majority are quiescent. After several hours, virtually no cells are beating, and remain so for several days. This contrasts with embryonic and neonatal cells, which show significant spontaneous contractility within 24 hours of being placed in culture. While some cells (less than 5%) have been observed to beat spontaneously as early as 2 days in culture, it is not until 4–6 days that large numbers (greater than 50%) of the isolated adult myocytes demonstrate intrinsic beating rates greater than 150 beats/min. Because these are individual cells that do not make contact with each other there is not synchronous beating during this period. While cells that make contact with one another do not initially beat synchronously, they do, however, eventually establish synchronous beating rates. With increasing time in culture, the average rate approaches and surpasses 200 beats/min, with individual cells beating as fast as 250 beats/min (Figure 2). After 2 weeks, the average beating rate returns to about 180 beats/min. The contiguous sarcoplasm resulting from three apparently single cells, as seen in Figure 1C, does not initially result in a synchronously beating mass of cells. In fact, we have frequently observed what appeared to be a single cell containing contiguous sarcoplasm and several nuclei demonstrating three distinct rhythms. With increasing time in culture, however, these multinucleated masses of cells eventually demonstrate a uniform contraction.

To obtain better control over the environment of the cell, we have also grown adult myocytes in PC-1 (Ventrex Labs, Portland, Maine), which is a serum-free medium containing insulin, transferrin, T3, fatty acids, and proprietary proteins, but no growth factors such as epidermal or platelet-derived growth factor. Myocytes grown in this medium undergo similar morphological changes and have beating rates comparable to cells grown in 5% calf serum (Figures 1E and 1F). With increasing time in culture, however, the cells appear to become somewhat less spread out, with the edges of the myocytes appearing to become somewhat less firmly attached to the dish. This is evident in Figure 1F as a much more marked delineation of the cell's edge.
Myosin Expression in Culture

Using native gel electrophoresis, we have examined the myosin isozyme profile of myocytes grown on the laminin coated dishes. Figure 3 demonstrates the myosin isoform pattern obtained from cells which have been in culture for 2 weeks. Despite the dramatic morphological changes taking place during the first week in culture, only the V, isoform which was initially expressed in vivo continues to be present. This is usually the case for at least 7–8 days in culture, though traces of V3 have been observed as early as 7 days after plating. With increasing time in culture, however, both V2 and V3 appear until a 1:1 ratio of V1:V3 is usually reached. The 1:1 ratio has been observed as early as 15 days in culture and as late as 35 days.

We have also studied myosin isoform expression during culturing in serum-free PC-1 medium. Cells were transferred to this medium after at least 24 hours in medium containing 5% calf serum or as late as after 5 days in serum. Growth of cells in the PC-1 can extend expression of solely the V1 isoform for at least an additional 10 days. Figure 4 shows the myosin isoform profile of cells after 15 days in culture and from cells which had been switched into PC-1 after an initial period of 5 days in 5% calf serum.

Concomitant with the morphological transformation there is a reorganization of myofibrils, which is demonstrated in Figure 5. The wide variety of staining patterns in this figure often can be observed in a single culture during the first and second week. This once again demonstrates the heterogeneity of myocytes in culture. The general pattern of changes can be described as starting with an initial bright apparently nonspecific staining similar to that reported by Nag et al.29 This takes place during massive internal reorganization in cells converting from a cylindrical to a round shape. This is followed by more diffuse indistinct staining as new sarcoplasm is expressed. It is not a necessary condition for a cell to become round to observe this type of staining since cylindrical cells can demonstrate similar fluorescent patterns. The next stage in the temporal progression to well organized myofibrils is not obvious since several different patterns become apparent in culture (Figures 5A, 5C, and 5D). In one case (Figures 5A and 5D), the original cell almost forms a nucleating structure, while in the second (Figure 5C), staining patterns somewhat reminiscent of stress fibers can be observed. Regardless, with increasing time in culture an extremely well-organized myofibrillar network reappears (Figures 5B, 5E, and 5F). In some cases (Figures 5E and 5F), the striations run parallel in fairly regular register, while in other cases (Figure 5D), the fibers organize from the center somewhat like the spokes of a wheel. Figure 5F shows what are probably two or three cells in close proximity. At the center of this mass (arrow) one can see overlapping myofibrils with differing orientation. As a rule, myocytes with well-reorganized myofibril staining have been observed as early as 4–5 days in culture, with large numbers of cells demonstrating such staining by 7–10 days in culture.

Discussion

In this study, we have demonstrated that several characteristics of the adult cardiac myocyte phenotype are maintained for different lengths of time in
FIGURE 5. Myosin staining pattern of cultured adult myocytes. A and B are from 7-day cultures. C–F are from 12-day cultures. Arrow points out overlapping myofibrils which probably come from different cells.

culture: 1) The cylindrical shape and evident cross striations characteristic of cells in vivo are maintained by virtually all initially cylindrical myocytes during the first 1–2 days in culture. Some cylindrical cells can even be found up to 4–5 days in culture. 2) Quiescence of virtually all myocytes is maintained for the first 3 days in culture. Although contractility is a property of cardiac myocytes, with the exception of pacemaker cells, spontaneous contractility is not an in vivo property of cells in the intact heart. 3) Expression of the adult myosin isoform (V₁ isomyosin) continues for at least 7 days in culture when grown in 5% calf serum. The above characteristics differ significantly from embryonic/neonatal cul-
A load on these isolated myocytes is debatable since a strain is sufficient to produce an external load and placed on the in vivo heart. Whether there is in fact from an environment that required a significant dimensional relationships and/or 2) they are removed. Myocytes are placed in culture, they lose their cylindrical shape and cross-striations for two possible reasons: 1) they are released from strict in vivo three-dimensional relationships and/or 2) they are removed from an environment that required a significant amount of work because of the hemodynamic load placed on the in vivo heart. Whether there is in fact a load on these isolated myocytes is debatable since it has been suggested that attachment to the substrate is sufficient to produce an external load and thus maintain isolated myocytes in a differentiated state. Regardless of whether the isolated myocyte is loaded, it still maintains many of the adult myocyte characteristics during this period of morphological change. That cytoskeletal rearrangement is an important adaptive strategy for the myocytes is further suggested by the fact that such rearrangement has been observed in vivo in preparation for increased cell growth following imposition of a hypertrophic stress in hearts of both young and adult rats.

While the V1 isoform, which is a marker for the adult phenotype remains present for at least the first 7 days in culture, with increasing time the V1 and V3 myosin isoforms appear. One interpretation for the appearance of these isoforms may be that the cells are reverting to an embryonic phenotype in culture. There are, however, several alternate explanations. Reports in the literature demonstrate the appearance of the V3 isoform with aging of the in vivo heart, with hypertrophy due to adaptation to an altered workload and several other events (for summary, see Table 3 of Reference 25). An increase in the surface area (in at least two dimensions) suggestive of hypertrophy does appear to occur in the adult myocyte in culture (Figure 1E). Furthermore, Simpson et al have shown increases in protein accumulation for neonatal myocytes in long term culture despite an obvious lack of workload on the cell. A similar process may also occur for adult myocytes in culture. Regardless of the reason for the shift in myosin isoform composition, it is apparent that we can extend the in vivo myosin phenotype (V3 only) at least a week past the point when it usually changes. This is presumably due to the presence of triiodothyronine (0.5 nM) and lack of putative factors necessary to stimulate synthesis of the V3 myosin in the defined medium.

While our results concerning myofibril rearrangement are in general agreement with the immunofluorescent work of Guo et al, we differ in some specifics. During the first 2 weeks in culture, we also observe dramatic morphological changes or cell remodeling similar to their study as well as others. Our results suggest, however, that two alternate intermediate steps may concurrently occur during the remodeling process. Staining patterns obtained with a myosin antibody comparable to stress-fiberlike structures (Figures 5C and 5D) and also intense staining near the center of the cell with fluorescent work of Guo et al, we differ in some specifics. Our results suggest, however, that two alternate intermediate steps may concurrently occur during the remodeling process. Staining patterns obtained with a myosin antibody comparable to stress-fiberlike structures (Figures 5C and 5D) and also intense staining near the center of the cell with fluorescence.
ment in less than 15 minutes, or from differences in the cell culture medium. These differences aside, however, the basic morphological transformation is similar.

Based on this study, which demonstrates that the adult myocyte in culture retains many of the characteristics of its in vivo phenotype, as well as additional information in the literature, we feel that the isolated adult cell in culture represents a good in vitro system to study those factors which regulate cardiac muscle growth and differentiation. In this regard, we have already demonstrated in a preliminary report that cultured adult myocytes are sensitive to positive and negative adrenergic modulation. These studies as well as our recent report demonstrating the feasibility of using adult human cardiac myocytes lay the groundwork for future comparison of cells from normal and abnormal hearts. In this way, we should eventually be able to determine whether specific types of human heart malfunctions, such as cardiomyopathies, can be traced to intrinsic properties of the cardiac myocyte itself.

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References


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