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)
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 95% air-5% CO₂ environment of the 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.

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 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, x211. D: original magnification, x98.

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 crossstriations (Figure 1A) and 70-80% trypan blue negative cells. This results in an average of 1.04x10^6 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...
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.
FIGURE 3. Myosin isoform expression in cultures of adult myocytes. $V_1$ and $V_3$ band identity had previously been established by comigration with known standards. The pattern of expression in 3–4-week cultures did not differ significantly from what is seen in this figure though it could take 25 days to reach the 1:1 ratio of $V_1$:$V_3$.

**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 $V_1$ 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 $V_3$ have been observed as early as 7 days after plating. With increasing time in culture, however, both $V_2$ and $V_3$ appear until a 1:1 ratio of $V_1$:$V_3$ 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 $V_1$ 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. 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-
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it has been suggested that attachment to the sub-
strate is sufficient to produce an external load and
thus maintain isolated myocytes in a differentia-
ted state. Regardless of whether the isolated myocyte
is loaded, it still maintains many of the adult myo-
cyte characteristics during this period of morpho-
logical change. That cytoskeletal rearrangement
is an important adaptive strategy for the myocytes is
further suggested by the fact that such rearrange-
ment 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 V2 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 appear-
ance of the V3 isoform with aging of the in vivo
heart, with hypertrophy due to adaptation to an
altered work load, 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). Further-
more, 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 ap-
parent that we can extend the in vivo myosin pheno-
type (V1 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 rearrange-
ment are in general agreement with the immuno-
fluorescent 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
more diffuse staining towards the periphery similar
to that seen in Figure 5A appear to coexist in the
same culture. It is not clear which of these types of
myofibrillar reorganization precedes the other. In
both cases, the original cell appears to form a
nucleus for new myofibrillogenesis. Another diffe-
rence in our results is that we appear to have more
complete myofibrillogenesis since packing in our
cells appears denser than in the previous study.
Either of these differences may result from our use
of laminin, which promotes the initial cell attach-

| TABLE 2. Comparison of Cultured Embryonic/Neonatal and Adult Cardiac Myocytes |

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Embryonic/neonatal</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial striated structure</td>
<td>Immediately lost</td>
<td>Lost with time</td>
</tr>
<tr>
<td>Striated structure regained</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Morphology</td>
<td>Unique</td>
<td>Unique</td>
</tr>
<tr>
<td>T-tubule system</td>
<td>Rudimentary</td>
<td>Well-developed</td>
</tr>
<tr>
<td>Beating rate</td>
<td>70–100 ms</td>
<td>175–220 ms</td>
</tr>
<tr>
<td>Mass beating</td>
<td>Synchronous</td>
<td>Asynchronous initially; becomes synchronous in time</td>
</tr>
<tr>
<td>Isozymes</td>
<td>V1:V3 1:1</td>
<td>V1 Only (during 1st week)</td>
</tr>
<tr>
<td>PAS positive</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Cell division</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>DNA synthesis</td>
<td>Yes16,17</td>
<td>Yes16,17</td>
</tr>
<tr>
<td>Epinephrine responsive</td>
<td>Yes18</td>
<td>Yes16,17</td>
</tr>
<tr>
<td>Resting potential</td>
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<td>-76.3 mV20</td>
</tr>
</tbody>
</table>

The previously mentioned characteristics, as well as several others are compared between embryonic or neonatal cells and adult cells in Table 2. Upon examination of this table, it quickly becomes apparent that adult myocytes represent a distinct cell population that differs significantly from myocytes in culture obtained during the perinatal developmental period. That is, they do not "dedifferentiate" to resemble embryonic or neonatal cells; in fact, they remain highly differentiated. We think that the dramatic morphological changes which we, as well as others, have observed most likely represent an adaptation to a new environment. When adult 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
Acknowledgments

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References

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