Load Regulation of the Properties of Adult Feline Cardiocytes

The Role of Substrate Adhesion

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SUMMARY. We have recently described rapid and reversible changes in cardiac structure, function, and composition in response to surgical load alteration in vivo. In the present study, we used a simple, well-defined in vitro experimental model system, consisting of terminally differentiated quiescent adult cat ventricular cardiocytes maintained in serum-free culture medium, to assess more definitively the role of loading conditions in regulating these same biological properties of heart muscle. Cardiocytes considered to be externally loaded were adherent throughout their length to a protein substrate, such that the tendency for the ends of the cells to retract was prevented. Cardiocytes considered to be unloaded were not adherent to a substrate and, thus, were free to assume a spherical shape. Cardiocyte structure and surface area were assessed in initially identified cells, both by serial light microscopy and by terminal electron microscopy. Cardiocyte function was assessed in terms of the ability to exclude trypan blue, to remain quiescent with relaxed sarcomeres containing I-bands, and to shorten in response to electrical stimulation. Cardiocyte composition was first assessed by quantitative gel electrophoresis of proteins and then by microfluorimetric measurement of ribonucleic acid, protein, and deoxyribonucleic acid. In addition, cardiocyte incorporation of [3H]thymidine into deoxyribonucleic acid and [14C]hydridine into ribonucleic acid were measured. Loading via substrate adhesion was found to be very effective in terms of each of these measurements in retaining the differentiated features of adult cardiocytes for up to 2 weeks in culture; unattached and thus unloaded cardiocytes quickly dedifferentiated. Conditions thought to stimulate cardiac growth, including catecholamine stimulation, were found to be ineffective. These experiments demonstrate that external load has a primary role in the maintenance of the basic differentiated properties of adult mammalian cardiocytes. (Circ Res 58: 692-705, 1986)

IT has become clear, during our more recent studies of cardiac hypertrophy (Cooper et al., 1981; Cooper and Marino, 1984; Marino et al., 1983, 1985), that, while greater precision is being achieved in the description of pathophysiologically relevant models of this entity, little insight into the basic mechanisms responsible for the initiation of cardiac hypertrophy in particular and the maintenance of normal cardiac properties in general has been forthcoming. Therefore, in a parallel series of studies (Tomanek and Cooper, 1981; Cooper and Tomanek, 1982; Thompson et al., 1984; Kent et al., 1985; Cooper et al., 1985), we have begun to investigate the basic regulation of the structure, composition, and function of adult mammalian myocardium. These studies have considered the entire potential spectrum of cardiac loads, ranging from underloading through normal loading to overloading. Based on these experiments, employing surgical and pharmacological animal models, we have concluded that the regulation of myocardial properties in the setting of either a normal or an abnormal hemodynamic environment represents a primary load response intrinsic to the heart, and that other potential trophic factors have a largely supportive or modulatory role.

The major limitations of these studies of cardiac load regulation are 2-fold. First, it is extremely difficult in the intact animal to isolate and unequivocally evaluate the role of any single factor in controlling the biology of heart muscle. Second, cardiac tissue consists of striated muscle cells, or cardiocytes, embedded in a complex of neural, vascular, and interstitial cells; the interaction of these various tissue components with the cardiocytes in response to a varying hemodynamic input is impossible to characterize completely. In addition, there are recent data which suggest that the primary stimulus for cardiac hypertrophy may be through factors other than load, such as α1-adrenergic stimulation (Simpson, 1983). Therefore, in order to test our hypothesis better that hemodynamic load is the primary determinant of the properties of the heart and to begin our study of how load alterations in the adult are translated
into biochemical events, we have turned to a far simpler experimental system: the cultured, terminally differentiated, quiescent feline cardiocyte. The present study uses this system to examine that part of the cardiac loading spectrum extending from underloading to normal loading.

In addition to the several rather straightforward advantages with respect to simplicity of experimental design and control which this system provides (Marsh, 1983), four other seemingly disparate pieces of information made it seem to us that adult cultured cardiocytes might provide an optimal tool with which to probe the role of either load or a variety of other putative factors in regulating myocardial biology. First, theoretical considerations suggest that the form of both cells and tissue is a net function of the minimum free energy achieved by the balance between mechanical strain resulting from deformation and the opposing adhesive force, such that deformable biological materials not influenced by external forces assume a spherical shape (Mittenthal and Mazo, 1983). Second, on the cellular level, fibroblast morphology during the transition from a rounded to an elongated form in culture is largely determined by the traction forces which these cells exert (Kreis and Birchmeier, 1980) during substrate adhesion (Harris et al., 1980). Third, on the tissue level, as well, morphogenesis may be determined by traction forces (Lewis, 1984). Fourth, and of particular relevance to the present study, experimental data derived from cultured endothelial cells demonstrate two important findings: cellular spreading is enhanced when substrate adhesion is increased, and the dispersed shape of the adherent cell as opposed to the compact shape of the loosely attached cell is coupled closely both to increased DNA synthesis and to cellular growth (Folkan and Moscona, 1978). Indeed, the potential importance of adhesion to extracellular components for the long-term maintenance of adult cardiocytes in culture is now beginning to be recognized (Piper et al., 1982; Borg et al., 1984: Lundgren et al., 1984; Eckel et al., 1985).

Since most of the resting stiffness of isolated cardiocytes resides in the myofilaments, with a lesser amount residing in the cytoskeletal intermediate filaments (Brady, 1984; Fish et al., 1984), and since these organelles are not static structures but are instead quite labile, being comprised largely of proteins which are in a dynamic equilibrium defined by readily variable synthesis and degradation rates (Kira et al., 1984), we have developed for the present study an experimental model in which the role of external load in determining the structure and function of cultured adult mammalian cardiocytes can be evaluated by controlling the adhesion of these cells to a substrate. The experimental construct is as follows.

If external load, defined as experimentally imposed cardiocyte adhesion opposing retraction, is important to the maintenance of differentiated cellular properties, one would expect nonadherent cells gradually to round up and differentiate, whereas adherent cells would remain differentiated. If this is not the case, one would expect the properties of adherent and nonadherent cardiocytes to remain similar as culture duration increases. In addition, if α1-adrenoceptor activation has a trophic effect, one would expect adherent cardiocytes exposed to norepinephrine to exhibit growth.

Whereas no trophic response to norepinephrine was identified, the data do support the hypothesis that load plays a critical role in maintaining the differentiated structural, functional, and biochemical properties of adult cardiac muscle cells.

**Methods**

**Cardiocyte Isolation**

The techniques for cardiocyte isolation, usually from rodents, have been reviewed in detail (Dow et al., 1981; Farmer et al., 1983; Bkaily et al., 1984). We have described a technique which generates a large number (≈3 x 10^6 cells/g wet weight) and percentage (~60%) of viable, calcium and temperature tolerant cardiocytes from the cat heart (Silver et al., 1983). The cat was chosen, first, to eliminate interspecies variations when correlating our present work on feline cardiocytes with our extensive previous work in the cat on cardiac load regulation and, second, because calcium control systems (Langer et al., 1975) and basic biochemical responses to load alterations (Cooper and Tomanek, 1983; Wisenbaugh et al., 1984) are substantially different in rodent as opposed to cat and human hearts.

**Experimental Procedure**

The heart and great vessels were removed under sterile conditions from adult cats of either sex (1.8-4.0 kg) after anesthesia with ketamine hydrochloride (50 mg/kg, im) and acepromazine maleate (5 mg/kg, im). The aorta was cannulated, and the coronary arteries were perfused at 37°C with a nonrecirculating, nominally Ca++-free (8-10 mM) Krebs-Henseleit buffer of the following composition (mM): NaCl, 130.0; KCl, 4.8; MgSO₄, 1.2; NaH₂PO₄, 1.2; NaHCO₃, 25.0; and glucose, 12.5, with 0.12% type II collagenase. The osmolality of this buffer was 290-310 mosm, and the pH was maintained at 7.4 to 7.5 by equilibration with 95% O₂-5% CO₂. The perfusion was terminated when the heart was flaccid.

The atria, great vessels, and anulus fibrosus were removed from the heart, and the remaining cardiac tissue was minced for 10 minutes in buffer with collagenase until most of the cardiac tissue was dissociated; it then was filtered through 300-μm nylon mesh and rinsed thoroughly with collagenase-free buffer. The filtrate was centrifuged at 100 g for 1 minute, and the cardiocytes then were resuspended in collagenase-free buffer. After the cardiocytes had settled out of suspension, they were resuspended in buffer containing 1 mM Ca++ and 1% bovine serum albumin.

**Cardiocyte Culture**

Isolated cat cardiocytes were maintained in a serum-free, chemically defined medium. When cardiocyte adhe-
sion was desired, a laminin substrate was employed; this basement membrane glycoprotein was found to induce firm substrate attachment along the entire length of these rod-shaped cells.

Experimental Procedure

The freshly isolated cardiocytes were allowed to settle out of the final isolation buffer and then resuspended, at a density of ~5 x 10^5 cells/ml, in the standard culture medium used in this study: M-199 medium supplemented with 0.2% bovine serum albumin, 100 U/ml penicillin, 1 x 10^{-5} M streptomycin, and 10^{-5} M insulin. In order to separate cardiocytes from other myocardial cells, the cardiocyte suspension was incubated for 3 hours in 10-ml aliquots in 100-mm polystyrene dishes kept in a 37°C incubator in which the atmosphere was humidified and equilibrated with 5% CO₂. Only noncardiocyte cells attached to these dishes.

After this 3-hour incubation, the cardiocytes were removed and maintained in the incubator under one of the five types of culture conditions described below. Minimal (<1%) fibroblast or endothelial cell contamination was noted under any of these experimental conditions; the culture medium was changed every other day.

Group 1: Adherent Cardiocytes. A 1.5-ml aliquot of the cardiocyte suspension, containing ~8 x 10^4 cells, was placed in each of multiple 2.5 cm² wells mounted on glass tissue culture slides. These slides had been coated previously with 0.5 ml of a 1.0% solution of laminin and allowed to air-dry. By the next day, considered day 1 of culture, the cardiocytes had attached to the laminin substrate along their entire resting length and remained attached in the same location through subsequent changes of culture medium. These cardiocytes were maintained in the standard culture medium for as long as 4 weeks.

Group 2: Nonadherent Cardiocytes. A 40-ml aliquot of the cardiocyte suspension, at a density of ~5 x 10^5 cells/ml, was placed in each of multiple 80 cm² flat-bottomed polystyrene tissue culture flasks, to which the cells did not adhere. They settled in a dispersed manner on the bottom of these flasks, but could be collected in a corne by tilting the flasks to facilitate changes of culture medium. These cardiocytes were maintained in the standard culture medium for up to 2 weeks.

Group 3: Adherent Cardiocytes in Low [Kᵢ⁺]. The conditions here were duplicative of those for Group 1, with the single exception that the potassium concentration in the M-199 culture medium was reduced to 0.67 mm on the second day of cell culture and maintained at that level for the subsequent 2 weeks. This was done in order to cause these ordinarily completely quiescent cardiocytes to hyperpolarize and contract spontaneously because of the deviation from linearity at low [Kᵢ⁺], in the relationship between log [Kᵢ⁺] and Eₘ (Murphy et al., 1983; Kim et al., 1984).

Group 4: Adherent Cardiocytes Exposed to Norepinephrine. In assaying the effect of norepinephrine on adherent cardiocytes, the initial conditions were the same as those described for the Group 1 cardiocytes. On the third day of culture, the following concentrations of norepinephrine were added to six wells in each case: 0.01, 0.10, or 1.00 x 10^{-5} M. To inhibit catecholamine oxidation, 10^{-4} M ascorbic acid was added to each well. Norepinephrine was made up fresh from powdered stock for each change of the medium during the 2 weeks that these cardiocytes were maintained in culture. Using methods which we have recently described in detail (Cooper et al., 1985), we analyzed the norepinephrine content of the medium both initially and at 24 and 48 hours by liquid chromatography coupled with electrochemical detection (Felice et al., 1978).

For each concentration used, the norepinephrine content had declined to 44.8 ± 0.3% of the initial value at 24 hours and to 18.4 ± 0.9% of the initial value at 48 hours. This decline in norepinephrine concentration may well have been due largely to the fact that ascorbic acid in the concentration range which we used declines to ~20% of the initial value after 12 hours in a similar culture medium (Peterkovsky, 1972).

Group 5: Adherent Cardiocytes Exposed to Ascorbic Acid. Because the concentration of ascorbic acid used in group 4 for the inhibition of norepinephrine oxidation has been shown to have an independent stimulatory effect on the protein synthesis of cultured vascular smooth muscle cells (Libby and O'Brien, 1983), we used an additional control to which 10^{-4} M ascorbic acid but no norepinephrine was added during 2 weeks of culture.

Cardiocyte Evaluation

Morphology

The cultures were examined daily and photographed twice weekly using an inverted light microscope. In all but the second set of experiments, wherein the cells were nonadherent, ~10 cardiocytes of each group in each of six culture wells were followed sequentially as individuals identified by initially recorded spatial coordinates.

For transmission electron microscopy of the adherent cardiocytes, the cells were fixed for 1 hour in situ in the tissue culture wells in 5% glutaraldehyde in 0.10 M sodium cacodylate buffer adjusted to ~300 mosm with 5% sucrose. After osmication, the cells were dehydrated in graded alcohols, cleared in propylene oxide, and embedded in epon. Thin, 60–90 nm sections were cut, placed on a Formvar-coated copper grid, and stained with uranyl acetate and lead citrate.

For electron microscopy in the second set of experiments, the nonadherent cardiocytes were allowed to settle out of suspension in a centrifuge tube before they were fixed and embedded. The face of the block containing the cells was cut at 60–90 nm, and these thin sections were stained and examined as described above.

Cardiocyte size was determined for the group 1 adherent cells after 1 day in culture and for all groups of adherent cells after 2 weeks in culture. For this purpose, the tissue culture slides were placed on the stage of an inverted microscope having optics which allowed the cursor of a digitizer to be projected onto the image of a cell. Since cardiocyte thickness is much less than cardiocyte length or width, the area just determined was doubled to give an estimate of cellular surface area (Simpson et al., 1982). The outlines of 100 randomly selected cells on at least two different tissue culture slides were digitized for each measurement.

Function

On day 1 of culture, a general assessment of the initial functional integrity of the cultured cardiocytes was made in terms of the following characteristics, each of which depends on an intact sarcolemma: the ability to exclude trypan blue, to retain a rod-shaped morphology, and to
remain quiescent. In addition, at the time of terminal study, the presence of actin and myosin filaments in the array characteristic of relaxed striated muscle was taken as evidence of long-term functional viability.

A more specific longitudinal assessment of cardiocyte functional integrity was made in the first set of experiments in terms of the contractile response of these cells to 0.5-Hz electrical stimulation at 37°C, since this depends not only on sarcoplasmic integrity, but also on the integrity of a broad range of other cardiocyte properties. The contractile response quantitated was percent shortening, expressed as % Δ rest length. Field stimuli of alternating polarity were employed to minimize electrolytic contamination. After a brief positive shortening treppe, photographs were taken just before stimulation, and then at the time of peak cardiocyte shortening. For the nonadherent cells on day 0 and day 7, the maximum length of the cells on the sylastic and diastolic frames was measured. For the adherent cells on day 1 and day 14, the distance between two obvious intracellular densities was measured on the sylastic and diastolic frames. We made 10 such measurements of % Δ rest length on a single cell at each of these intervals in order to estimate the error of this measurement; the measurement then was repeated on 10 cardiocytes in that well.

Biochemistry

Three biochemical techniques were used. First, the response of an entire population was assessed by gel electrophoresis of the pooled contents of a number of tissue culture wells in which the cardiocytes had been exposed to a particular set of experimental conditions. This approach provided a survey of the protein composition of a heterogeneous population. Second, the response of individual members of a population to the same sets of experimental conditions was assessed by the microfluorimetric quantification of the amount of DNA, RNA, and protein in particular cardiocytes. This approach allowed the selection of only viable cardiocytes from this heterogeneous population, which included both cardiocytes and contaminating fibroblasts and endothelial cells, for the biochemical determinations. Third, autoradiography was used to estimate the DNA and RNA synthesis of defined members of cardiocyte populations.

Gel Electrophoresis of Cardiocyte Proteins. Polyacrylamide gel electrophoresis of the cultured cardiocytes was performed after dissolution of the cells with sodium dodecyl sulfate. The amounts of selected proteins were compared to that of the cardiocyte-specific protein, myoglobin. This technique has been applied to the isolation of proteins from cardiac tissue (Kent et al., 1985) and from cultured cardiac cells (Lemanski et al., 1976). The samples from each of five tissue culture wells exposed to any particular set of experimental conditions were electrophoresed on 6–15% gradient polyacrylamide slab gels containing 0.15% sodium dodecyl sulfate and stained with Coomassie blue. Each gel then was scanned at 595 nm by a densitometer, and the area under the optical density tracing for each protein of interest, including the standards, was calculated using a programmable digitizer.

In an additional experiment, an attempt was made to identify by Western blot analysis a specific protein band on the gels of the cultured cardiocytes which became more intense as the duration of cell culture increased, but which did not appear to be a standard striated muscle protein (Syrovoy, 1984). Because the protein band in question had an electrophoretic mobility comparable to that of both adenosine monophosphate (AMP) deaminase from the cardiocytes and bovine serum albumin from the culture medium, antibodies to these two proteins were employed. For AMP deaminase, a rabbit polyclonal antibody to human skeletal muscle (Fischbein et al., 1983) was employed. For bovine serum albumin, a commercially available rabbit anti-bovine serum albumin was employed.

Microfluorimetry of Individual Cardiocytes. Computer-operated microfluorimetry was used to determine the relative amounts of DNA, RNA, and protein in individual cardiocytes after staining the cells with chemically reactive dyes and quantifying the relative fluorescence intensities at the wavelengths appropriate to each of these substances. Additional discrimination in these measurements was provided by the selection of cardiocyte sampling sites for the measurements of RNA and protein, the sampling area was confined to the central cytoplasm; for the measurement of DNA, the sampling area was confined to the nucleus. Cardiocyte morphology is very well delineated by these stains, so that it was possible to exclude from analysis both noncardiocyte cells and those few cardiocytes in which pyknotic nuclei suggested questionable viability at fixation.

Acridine orange was used to quantify DNA and RNA in 80 cardiocytes exposed to each set of experimental conditions. When intercalated with double-stranded nucleic acid, this metachromatic dye fluoresces green with an emission peak at 530 nm; when intercalated with single-stranded nucleic acid, it fluoresces red with an emission peak at 640 nm. The intensity of the fluorescence is proportional to the quantity of that nucleic acid in the cell (Darzynkiewicz et al., 1975, 1979). Green fluorescence for DNA was determined with a barrier filter which passes light with wavelengths in the range of 520–560 nm, and red fluorescence for RNA was determined with a barrier filter which passes light at wavelengths >600 nm (Mercer et al., 1984).

Propidium iodide and fluorescein isothiocyanate were used to quantify protein and DNA in 80 cardiocytes exposed to each set of experimental conditions (Crisman and Steinkamp, 1973). In the excited state, propidium iodide-stained DNA fluoresces reddish-orange with an emission peak at 590 nm, and the fluorescein-isothiocyanate stained proteins fluoresce greenish-yellow with an emission peak at 530 nm. Fluorescence intensity for DNA was determined with a barrier filter which passes light at wavelengths >570 nm, and fluorescence intensity for protein was determined with a barrier filter which passes light with wavelengths in the 520–560 nm range.

Autoradiography of Individual Cardiocytes. The techniques for the estimation of DNA and RNA synthesis by autoradiography are standard in these laboratories (Beserga and Malamud, 1969) and have been described in detail in our recent publications (Mercer et al., 1982, 1984). Briefly, DNA synthesis was estimated in cardiocytes labeled for 24 hours with 0.5 μCi/ml of [3H]thymidine (6 Ci/mmol). RNA synthesis was estimated in cardiocytes pulse-labeled for 2 hours with 5.0 μCi/ml of [3H]uridine (25 Ci/mmol). To estimate DNA synthesis, grain density over the nuclei of 250 cardiocytes in each experimental group was assessed; a nucleus was considered to be labeled if the grain density in the area overlying the nucleus was significantly greater than that over a background area of comparable size which contained no cells. To estimate nuclear RNA synthesis, grain density in excess of that over a background area was enumerated over the nuclei.
of 250 cardiocytes. Both of these measurements were confined to isolated binucleate cardiocytes, which comprised the majority (>90%) of these cells.

Statistical Analysis

Each value is presented as a mean ± SE. Differences either among or within the cardiocyte treatment groups were sought with a one-way analysis of variance. If a difference was found, this was followed either by a Newman-Keuls test to identify the differences among these groups or by a Dunnett's test to identify the differences within a group with respect to a single, sequentially measured variable (Winer, 1971). A significant difference was said to exist if P was less than 0.05.

Results

Morphology

Cardiocyte Ultrastructure

The major new morphological finding of this study is that long-term retention of adult cardiocyte differentiation is critically dependent on cellular loading through substrate adhesion. Cardiocytes from group 1, attached to the substrate along the entire length of the cell, retained much of their initial differentiated morphology, as shown in Figure 1, panels A–F. This was also true for the other adherent cardiocytes in groups 3–5. The internal structure of these cardiocytes on the first day of culture appears to be very similar to the ultrastructural level to that of cardiocytes in intact cat myocardium (Thompson et al., 1984: Fig. 3A), and there were only rather minor changes during the first 2 weeks of culture. In contrast, those cardiocytes from group 2, which were not loaded by substrate attachment, rounded up and lost some of the internal structure and most of the internal organization characteristic of differentiated striated muscle, as shown in Figure 1, panels G and H. The morphology of these unloaded cardiocytes is strikingly similar, both to that of the cardiocyte in adult myocardium unloaded in situ (Kent et al., 1985, Fig. 5), and to that of cardiocytes in poorly differentiated neonatal myocardium (Kent et al., 1985, Fig. 9D).

Day 1 of Culture. While the dissociation procedure separated the group 1 cardiocytes at the intercalated discs, it is still possible to identify a disc region in these cultured cells in Figure 1, A and B. The myofibrils are aligned with the long axis of the cardiocyte, the Z-lines are in register throughout the length of the cell, and mitochondrial morphology appears normal.

Day 7 of Culture. During the first week of culture, the single consistent change in the adherent cardiocytes is that at the ends of these cells, where intercalated disc sites had been located previously, the angular appearance seen in Figure 1A is no longer present. The ends of the cardiocytes have become rounded, as shown in Figure 1C, and in these regions, the myofibrils have started to lose the usual alignment and registration.

Day 14 of Culture. After 2 weeks of culture, there were modest further changes in the group 1 cardiocytes. As seen in Figure 1F, the striations are not as clearly evident in these cells by light microscopy, and at the ultrastructural level, as seen in Figure 1F, the relative amount of the myofibrils appears to be less than was the case for cardiocytes from younger cultures. However, the myofibrils continue to line up with the long axis of the cell, and mitochondria remain interposed between them.

In several additional studies, it was found that after 4 weeks, the adherent cardiocytes showed some further loss of myofibril density and lysosome accumulation. The remaining myofibrils were well organized and aligned with the cellular long axis.

In contrast, the elongated shape of the non-adherent group 2 cardiocytes was lost during 2 weeks of culture and, as seen in Figure 1G, the cells rounded up and no longer show striations. These changes are very similar to those seen in non-adherent adult rat cardiocytes (Claycomb and Palazzo, 1980). At the ultrastructural level in Figure 1H, the most striking features of these cells are the loss of myofibrils and the circumferential orientation of the residual myofibrils within these round cardiocytes. It is important to note that these cells appear to be viable, in that they have normal mitochondria, an intact sarcolemma, and a nucleus containing a prominent nucleolus.

Sequential Studies. Since cultured adult rat cardiocytes may initially round up and then later spread out (Schwarzfeld and Jacobson, 1981), we followed initially identified cardiocytes sequentially by daily microscopy in order to be sure that discontinuous changes in cellular morphology were not overlooked. No group 1 cell was ever observed to round up and then spread out; the fully adherent cells remained attached and uniformly exhibited the sequence of morphological changes shown in Figure 1, panels A–F.

Cardiocyte Dimensions

Cardiocyte surface area was determined for the group 1 adherent cardiocytes after 1 day in culture and then for each of the four groups of adherent cardiocytes after 2 weeks in culture under the experimental conditions defined for that group. The results are shown in Table 1. Because the non-adherent cardiocytes of group 2 were rounded rather than flattened, this type of analysis would have been inappropriate for these cells and, therefore, was not done.

The group 1 cardiocytes did not enlarge during 2 weeks in culture. Exposure of the cardiocytes either to ascorbic acid or to low [K+]o caused a significant increase in cardiocyte surface area, more marked in the latter instance. In addition, exposure to graded concentrations of norepinephrine caused significant, graded increases in cardiocyte surface area. Neither the control cardiocytes nor those exposed to ascorbic...
FIGURE 1. Modulation contrast (Hoffman, 1977) light micrographs and transmission electron micrographs of feline cardiocytes maintained in tissue culture for 1 day after isolation (panels A and B), or for 7 days (panels C and D), or for 14 days (panels E–H). The cardiocytes in panels A–F are adherent to a laminin substrate. The cardiocytes in panels G and H are not adherent to a substrate. The 10-μm bars in panels A and B apply to the light and electron micrographs, respectively.
Table 1
Adherent Cardiocyte Dimensions

| Day   | Group 1 Control | Day 14 Group 1 Control | Group 3 Control | Group 4 Low [K+] | Group 5 Ascorbic acid 0.01 µM | Group 5 Ascorbic acid 0.10 µM |
|-------|-----------------|------------------------|-----------------|------------------|-------------------------------|-------------------------------|
|       | 2.12 ± 0.08     | 2.08 ± 0.05            | 2.08 ± 0.05     | 4.58 ± 0.18*     | 4.10 ± 0.23*                  | 3.39 ± 0.14*                  |

Each result is expressed as a mean ± 5%. For each measurement, the surface areas of a minimum of 100 randomly selected cells from the four wells on two cell culture slides were digitized. Note that each of the three subgroups exposed to norepinephrine was also exposed to 0.10 µM ascorbic acid.

*Significant difference from the control values at both day 1 and day 14 when examined by one-way analysis of variance, followed by a Newman-Keuls test.

acid contracted spontaneously, so that the increased surface area of the cardiocytes exposed to ascorbic acid probably can be ascribed to a primary effect of this agent. Low [K+] caused virtually all of the cardiocytes to contract spontaneously, albeit at a rather slow initial rate which decreased further with time. In the absence of any putative or known trophic effect of low [K+], itself, the increased surface area of the cardiocytes exposed to this treatment probably can be ascribed to some effect of the ongoing spontaneous contractions, such as repetitive intracellular strain, which this treatment induces. Exposure to norepinephrine, as might be expected from earlier observations (Jacobsen, 1977), caused spontaneous cardiocyte contractions at each concentration employed, with contraction frequency varying directly with drug concentration. In view of the increased cardiocyte surface area observed in response both to ascorbic acid in quiescent cells and to low [K+], in contracting cells, the increased cardiocyte surface area observed here with norepinephrine cannot be ascribed solely to the activated activity of this drug, both because of the spontaneous contractions which it caused and because of the concomitant use of ascorbic acid. As a cautionary note, it should be clear that this technique cannot differentiate an increase in cardiocyte surface area caused by cellular spreading from one caused by actual cellular growth.

Function
At the time that the freshly isolated cardiocytes first were suspended in the culture medium, just before they were divided into the various experimental groups, ~75% of the cells were found to be quiescent rods of the type seen in Figure 1A. These cells, without exception, excluded trypan blue. The remaining damaged cells rapidly rounded up, took up trypan blue, and were spontaneously contractile. These damaged cardiocytes quickly degenerated. The adherent, group 1 cells continued to exclude trypan blue throughout the period of culture and did not exhibit spontaneous contractions. The non-adherent group 2 cells remained quiescent, but began to round up by day 2 and were spherical by day 7. At this time and thereafter, there was obvious bleeding of the sarcolemma in ~50% of these cells, which was associated consistently with trypan blue uptake. The remaining spherical cells with smooth sarcolemmas, of the type seen in Figure 1G, continued to exclude trypan blue.

The contractile response to 0.5 Hz electrical stimulation, expressed as % Δ rest length, was measured in fresh cardiocytes immediately after the initial suspension in the culture medium and at days 1 and 14 in culture. The coefficient of variation for this measurement in the group 1 cells was 5.47% initially and 4.16% and 5.73% on day 1 and day 14 respectively. The extent of shortening was 6.57 ± 0.72% Δ rest length on day 0, 6.62 ± 0.33% on day 1, and 6.59 ± 0.38% on day 14. The group 2 cells did not contract measurably in response to electrical stimulation at one week in culture or thereafter. Thus, the adherent cardiocytes retained an unimpaired ability to carry out the complex, integrated processes required for contraction and relaxation, whereas the non-adherent cardiocytes lost this ability, as might be expected from the extensive structural changes seen in Figure 1H.

Biochemistry

Gel Electrophoresis

Electrophoretograms, and the quantitative results of the densitometric scans of these gels, are shown for sequentially sampled group 1 cardiocytes in Figure 2A and Table 2 respectively. Lane 1 in Figure 2A identifies the positions in each gel lane of the specific proteins of interest. Lanes 2–5 show an apparent decrease with time in the staining intensity of the bands representing the major contractile proteins, actin and myosin, as well as that of a structural Z-line protein, α-actinin. Western blot analysis identified the band showing a progressive increase in staining intensity as bovine serum albumin, presumably adsorbed onto the cardiocytes from the culture medium or taken up by these cells through pinocytosis.

Based on our previous experience with unloaded and then reloaded cardiac tissue (Kent et al., 1985), we did not expect major changes in the myoglobin content of these cultured cardiocytes. The data in the first column of Table 2 bear out this expectation. The contents of each of the other three proteins then were related to that of the myoglobin in the same sample, both to provide an internal reference and to
avoid the effects of any contaminating cell types, such as fibroblasts or endothelial cells, in which myoglobin would not be expected to occur. As might be anticipated from Figure 1, which shows a gradual reduction in myofilament density in the group 1 adherent cardiocytes, Table 2 shows for these cells a moderate, progressive decrement with time in each of the two major contractile proteins, actin and myosin. There is a more marked decrement in α-actin, an organizing center and directionality controller for the actin filaments (Lazarides and Burridge, 1975; Suzuki et al., 1976), which we have considered to be an early and sensitive marker for sarcomere integrity and sarcomereogenesis. The electrophoretograms in Figure 2B, which were obtained separately from those in Figure 2A, suggest a modest effect of low [K+]o, norepinephrine, and ascorbic acid in retaining the loss of contractile proteins from adherent cardiocytes.

Microfluorimetry

The results of these measurements are presented in two formats. Figure 3 shows examples of the frequency distributions for the measurements of RNA, protein, and DNA when obtained in 80 group 1 cardiocytes at day 1 and day 14 of culture. Table 3 is a summary of these measurements of RNA, protein, and DNA in 80 cells from each group.

The frequency histograms shown in panels A and B of Figure 3 demonstrate a substantial reduction in the cytoplasmic RNA content of these adherent cardiocytes from group 1 during 2 weeks in culture. Panels C and D of Figure 3 demonstrate that the cytoplasmic protein content of these group 1 cardiocytes is similar on day 1 and day 14. Panels E and F of Figure 3 demonstrate that the nucleoplasmic DNA content of the group 1 cardiocytes does not change during 2 weeks in culture.

The full data for these measurements, shown in Table 3, allow two conclusions. First, cytoplasmic RNA content in all groups of adult feline cardiocytes is at a fairly low level (11–17% of the day 1 value) after 2 weeks of culture. Together with the cardiocyte surface area data shown in Table 1, this strongly suggests that the increased cell size seen in response to certain experimental conditions is a result of cell spreading rather than the increased protein synthesis attendant on cell growth. Second, the protein content is reduced after 2 weeks only in the nonadherent cells of group 2. Together with the micrographs in Figure 1, this shows that cardiocyte loading through substrate adhesion has a major effect in terms of maintaining the structural integrity of adherent cells. Nevertheless, even for the adherent group 1 cardiocytes, the decrease in specific contractile protein concentrations shown in Table 2, and the cytoplasmic total protein content shown in Table 3, suggest that some dedifferentiation may be occurring.

Autoradiography

Cardiocytes exposed to [3H]thymidine did not have a nucleoplasmic grain density significantly greater than that of background areas under any of the experimental conditions or culture intervals em-

### Table 2

| Protein          | Day 0 | Day 1 | Day 3 | Day 7 | Day 14 |
|------------------|-------|-------|-------|-------|--------|
| Myoglobin (µg/mg total protein) | 8.5   | 8.0   | 8.2   | 8.4   | 7.8    |
| Myosin/myoglobin (% day 0 value) | 91.2  | 94.3  | 84.7  | 79.9  | 73.7   |
| Actin/myoglobin (% day 0 value)   | 94.3  | 83.7  | 74.3  | 61.1  | 35.6   |
| α-Actin/myoglobin (% day 0 value) | 90.9  | 69.5  | 64.8  |       |        |

Each datum is the average of two values obtained from densitometric scans of a pair of electrophoretograms such as that shown in Figure 2A. Myoglobin content is expressed as the ratio of the quantity of this protein to that of the total protein in that gel lane. The contents of the other three proteins were first related to that of myoglobin in that gel lane and then, as a percentage, to the value for this ratio obtained in the fresh, day 0 cardiocytes.
Figure 3. Amounts of cytoplasmic RNA and protein and of nucleoplasmic DNA in 80 adherent group 1 cardiocytes on day 1 (panels A, C, and E) and day 14 (panels B, D, and F) of culture. The amount of each substance, expressed in arbitrary units as percent fluorescence at the appropriate wavelength, was determined by computer-operated microfluorimetry. Panels A and B quantify RNA in terms of cytoplasmic fluorescence at >600 nm in cardiocytes stained with acridine orange. Panels C and D quantify protein in terms of cytoplasmic fluorescence at 520-560 nm in cardiocytes stained with propidium iodide and fluorescein isothiocyanate. Panels E and F quantify DNA in terms of nucleoplasmic fluorescence at 520-560 nm in cardiocytes stained with acridine orange.
employed. The occasional contaminating fibroblasts in cultures exposed to norepinephrine showed substantial nucleoplasmic labeling, so that the lack of cardiocyte nuclear labeling was real rather than artifactual.

The time-dependent distribution of nucleoplasmic labeling for cardiocytes exposed to [3H]uridine is shown in Figure 4. The adherent group 1 cells, pulse-labeled for 2 hours on the indicated day, showed an initially high level of nuclear RNA labeling, followed by a decline to a relatively stable level of nuclear RNA labeling over the next 2 weeks. In contrast, the nonadherent group 2 cells showed no detectable nuclear RNA labeling at the earliest time studied, despite the fact that, as seen in Figure 1H, they appear to be fully viable. Note that both group 1 and group 2 cardiocytes were exposed on day 0 to [3H]uridine beginning at the same time after

| TABLE 3 Cardiocyte Biochemistry |
|--------------------------------|
|                                |
| RNA fluorescence (%) maximum intensity | Protein fluorescence (%) maximum intensity | DNA fluorescence (%) maximum intensity |
| Day 1                          |
| Control                        | 42.06 ± 2.63 | 19.20 ± 1.12 | 27.00 ± 1.36 |
| Group 1                        |
| Control                        | 5.56 ± 0.20* | 22.69 ± 1.43 | 28.73 ± 1.17 |
| Group 2                        |
| Nonadherent                    | 5.47 ± 0.24* | 15.77 ± 0.38*| 25.85 ± 1.00 |
| Group 3                        |
| Low [K+]                      | 6.63 ± 0.32* | 19.36 ± 1.05 | 24.98 ± 1.61 |
| Group 4 (norepinephrine)       |
| 0.01 µM                        | 7.21 ± 0.32* | 20.73 ± 0.78 | 25.67 ± 1.05 |
| 0.10 µM                        | 7.16 ± 0.35* | 20.55 ± 1.10 | 29.39 ± 1.25 |
| 1.00 µM                        | 6.91 ± 0.24* | 22.63 ± 0.99 | 29.19 ± 1.28 |
| Group 5 (ascorbic acid)        |
| 0.10 µM                        | 4.57 ± 0.25* | 23.34 ± 0.74 | 26.50 ± 1.01 |

Each result is expressed as a mean ± SE. For the measurement of each substance, the fluorescence intensity relative to a reference standard was determined at a wavelength appropriate to that substance in 80 morphologically viable cardiocytes. Each of the three subgroups exposed to norepinephrine was also exposed to 0.10 µM ascorbic acid. When examined by one-way analysis of variance followed by a Newman-Keuls test, each asterisk (*) indicates a significant difference from the control value in that column at day 1, and the dagger (!) indicates a significant difference from all other values in that column.

![Figure 4: Distribution of grain density over the nucleoplasm of group 1 adherent cardiocytes and group 2 nonadherent cardiocytes pulse-labeled for 2 hours with [3H]uridine at each of the four specified culture intervals. Only binucleate cardiocytes were examined, and the total counts over both nuclei of 250 cells were enumerated. No attempt was made to count individual grains when the labeling density was >50 grains/nucleus. As expected for this brief RNA-labeling interval, only nuclear RNA were labeled; grain density over the cytoplasm did not differ from that over acellular background areas.](image-url)
isolation from a single heart, but only the group 1 cardiocytes were exposed to a laminin substrate. Finally, the nucleoplasmic labeling for the adherent cardiocytes of groups 3–5 was indistinguishable from that of the group 1 cells. That is, low [K⁺], in group 3, 1.0 μm norepinephrine in group 4, and ascorbic acid in group 5 did not result in labeling different from that of group 1 over a 2-week period.

Discussion

This study, employing a simple and well-defined experimental system, directly supports the hypothesis that external load is an important regulator of the differentiated properties of the adult mammalian cardiac muscle cell. Two major lines of evidence support this statement. First, cardiocyte loading through substrate adhesion results in a substantial retention of the normal structure, function, and composition of these cells in long-term culture. Second, while other experimental interventions which might plausibly be thought to have either a direct or an indirect effect on the biological properties of these cells appear to cause an increase in cardiocyte surface area, they do not cause cardiocyte growth.

Load Regulation

It is clear that cell shape plays a fundamental role in the control of cell growth for a variety of normal mammalian cell types (Folkman and Moscona, 1978). It is equally clear that cell shape and growth are the combined result of forces actively exerted by the cells themselves (Harris et al., 1980) and of external forces to which the cells are subjected (Nakatsui and Johnson, 1984). The way in which these factors may operate on cardiac muscle is suggested by three observations. First, adult cardiocytes in culture which are not confluent adherent to a substrate appear to round up rather quickly (Schwarzfeld and Jacobson, 1981). Second, juvenile cultured Purkinje cells show structural evidence of myofilament formation in cytoplasmic extensions (Canale et al., 1983), wherein a stress axis is presumably present. Third, myofibril formation, with the incorporation of α-actinin into nascent Z-lines, is localized to the growing pseudopods and the spreading borders of embryonic cardiocytes (Sanger et al., 1984).

These observations, together with our own observations of hypertrophy in response to increased loads and atrophy in response to decreased loads, led to a comparison in the present study between adult cardiocytes cultured under two basically different conditions; that is, either as nonadherent unloaded cells or as substrate-adherent loaded cells. When the data from this study are considered as a whole, the fact that they were gathered from quiescent, terminally differentiated cells is important. The group 1 cardiocytes show during 2 weeks a slight loss of myofibrillar density in Figure 1 and a trend toward decreasing myofibrillar protein concentra-

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tion in the electrophoretic data of Figure 2 and Table 2, which was not apparent in Figure 3 and Table 3 when total protein content in the central region of the cytoplasm was measured by microfluorimetry. At the same time, cytoplasmic RNA, largely rRNA, content decreased ~8-fold (Fig. 3; Table 3), and nucleoplasmic RNA, at least partly mRNA, labeling decreased ~2-fold (Fig. 4). Given that these cells are not growing or dividing, and even show a tendency toward a decrease in structural proteins, the marked drop in cytoplasmic RNA content and the gradual drop in nucleoplasmic RNA labeling are to be expected. Further, given that the half-lives of RNA species are typically on the order of minutes, while those of myofibrillar proteins are on the order of days, the more gradual decrease of protein content is also to be expected. It must be recognized that mRNA is typically 1% or less of cellular RNA, and that this study provides no direct measure of the rate of RNA transcription, or even of whether labeled nucleoplasmic RNA is actually leaving the nucleus. Indeed, these data provide no insight into the critical question of whether muscle-specific mRNAs, as opposed to common mRNAs, are being transcribed in these isolated cells, and there are data which show that for hepatocytes, the transcription of organ-specific mRNAs is critically dependent on the normal structural organization of these cells within the liver parenchyma (Clayton et al., 1985a, 1985b). However, the more rapid differentiation of the nonadherent as opposed to adherent cardiocytes, and the retention of more nearly normal structure and function in the latter cells, would suggest the possibility that at least some muscle-specific mRNAs are being transcribed in the latter group. The two most important points in these data are that none of the experimental treatments of the adherent cardiocytes caused a significant increase in any of the markers of cellular growth, whereas, in the absence of substrate adhesion, the decrease in cardiocyte synthetic activity was so much more rapid and pronounced than that in the adherent cells that the greater loss of differentiated cellular properties is apparent both visually in Figure 1 and biochemically in terms of both RNA labeling in Figure 4 and protein content in Table 3. Thus, although growth during culture is not induced in these cells at their normal diastolic rest length in a simple culture medium, a variety of differentiated features, and at least some of the synthetic activity to support them, are well maintained in adherent cells while they are being rapidly lost in nonadherent cells.

On the levels of structure, function, and composition, there are striking parallelisms between our previous findings in unloaded cardiac tissue and our present findings in unloaded cardiac cells. Structurally, unloaded cardiocytes in tissue (Kent et al., 1985; Fig. 5) are virtually indistinguishable from unloaded cardiocytes in culture, such as those shown in Figure 1H. Functionally, the rapid loss of effective contrac-
tion in response to electrical stimulation observed before for unloaded myocardium (Cooper and Tomanek, 1982; Figs. 2 and 3) is the same as that observed here for unloaded cardiocytes. In terms of composition, the protein loss seen before in unloaded myocardium (Kent et al., 1985; Fig. 3) is reflected in the protein loss shown in Table 3 for unloaded cardiocytes.

Other Regulators

Our previous in vivo study showed that cardiac hypertrophy following a hemodynamic overload in the adult cat is a direct response to the load increase rather than being an indirect response mediated by either circulating or neural factors (Cooper et al., 1985). The present study demonstrates that, although the retention of cellular differentiation is load dependent, neither hyperplasia nor hypertrophy of adult feline cardiocytes occurs in response either to adrenergic stimulation or to other interventions which cause a modest amount of spontaneous contractile activity. Given that these terminally differentiated adult cardiocytes have exited the cell cycle, the failure to find DNA synthesis either with or without karyokinesis or cytokinesis under any of the experimental conditions employed in this study was expected. In addition, the observed decline in RNA synthesis and content of adherent cardiocytes under each experimental condition shows that none of these conditions caused cardiac growth.

The results of the present study of adult feline cardiocytes are in distinct contrast to the results of several recent studies of neonatal rat cardiocytes (Simpson and Savion, 1982; Simpson et al., 1982, 1983, 1985; Bishopric et al., 1985). In these latter studies, hypertrophy was said to be induced by an α₂-specific effect of catecholamines on ventricular cardiocytes isolated from 1-day-old rats. There were definite α₂ effects on these cardiocytes, which were documented by the use of specific adrenergic agonists and antagonists in terms both of cell size and of cell protein and RNA content. However, these effects do not appear to be directly applicable, in the context of the present data, to an understanding of the control of growth in adult mammalian cardiocytes. That is, neither hyperplastic nor hypertrophic growth in response to norepinephrine was seen in the present study of adult cardiocytes, despite the use of assays which should have detected either.

These contrasting experimental results may be based both on fairly discrete and obvious differences in experimental methodology as well as on more general differences in the basic biological properties of the cardiocytes which were studied. There is one very basic distinction between the present study of cat cardiocytes and the previous studies of rat cardiocytes: in the present study, terminally differentiated adult cardiocytes (Zak, 1974) were employed; in the previous studies, neonatal cardiocytes which were traversing the cell cycle (Clubb and Bishop, 1984) were employed. In view of this very basic difference between neonatal and adult cardiocytes with respect to growth control in the cell cycle, it is not surprising that, in the present study, growth control in adult cardiocytes was found to differ from that reported for neonatal cardiocytes. Nonetheless, definite conclusions about the role of any given factor in adult cardiocyte growth control must await the demonstration of cellular growth in vitro in response to that factor.

Conclusion

External leading through substrate adhesion allows the differentiated properties of adult feline quiescent cardiocytes to be maintained for relatively long periods of time in culture. Other factors employed in this study, including adrenergic stimulation, do not modify this response. Two further questions will be of considerable interest: first, does increased load, imposed in this simple experimental system, cause cardiac growth; second, what is the mechanism by which mechanical load exerts a primary effect on cardiocyte biology.

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