Human pediatric and adult ventricular cardiomyocytes in culture: assessment of phenotypic changes with passaging

Ren-Ke Li, Donald A.G. Mickle *, Richard D. Weisel, Susan Carson, Sue A. Omar, Laura C. Tumiati, Gregory J. Wilson, William G. Williams

The Division of Cardiovascular Surgery and Department of Clinical Biochemistry and The Centre for Cardiovascular Research of the Toronto Hospital-General Division, the Division of Cardiovascular Surgery and the Department of Pathology of The Hospital For Sick Children, and the University of Toronto, Toronto, Ont., Canada

Received 21 September 1995; accepted 20 February 1996

Abstract

Objectives: The purpose of this study was to assess morphologically and biochemically the phenotypic changes which occur in vitro with passaging of human pediatric and adult ventricular cardiomyocytes. Methods: Human ventricular cardiomyocytes from 3 children (1 to 2 years of age) and an adult patient (65 years of age) undergoing open heart surgery and an adult heart transplant patient (55 years of age) were isolated, cultured, purified, and passaged. Growth curves and ³H-thymidine uptake studies were performed. Characterization of the cells was done by light microscopy, transmission electron microscopy, immunofluorescent staining for myoglobin, CK-MB, and cardiac-specific troponin I isoform, human ventricular myosin heavy chain (HVMHC) and light chain 1 (HVMLC1), Northern blot analysis of HVMHC, and CK-MB activity and mass measurements. Passage 3 cardiomyocyte and pediatric myocardial phospholipids were analysed by gas chromatography. Results: Pediatric cells were smaller (P < 0.01) and divided faster (P < 0.001, ANOVA) than adult cells. The cardiomyocytes showed phenotypic changes in primary culture with essentially complete loss of sarcomeres by 10 days and a gradual loss of myofilaments with passaging. The cells were identified as cardiomyocytes by immunohistochemistry for myoglobin, CK-MB, cardiac-specific troponin I isoform, HVMHC and HVMLC1, and by Northern blot analysis for the 3'-end of HVMHC mRNA. The composition of phospholipid fatty acids in the cultured pediatric cells was similar to that found in the pediatric myocardium. CK-MB activity and mass could be measured in the cardiomyocytes. The adult cardiomyocytes were more difficult to maintain than the pediatric cells which could be cultured for as long as 6 months. Conclusions: Primary cultures of human pediatric and adult partially differentiated ventricular cardiomyocytes can be passaged. Although rapid disorganization of the myofibrils occurs, the non-contractile cells can be identified as cardiomyocytes by morphological appearance, immunofluorescent staining. Northern blot analysis for HVMHC, and CK-MB activity.

Keywords: Human ventricular myocytes; Electron microscopy; Contractile proteins; Phospholipids

1. Introduction

Metabolic and molecular biological studies of the human myocardium have been limited to studies on tissue obtained from myocardial biopsies, myocardial resections and heart transplant patients. These restrictions have hindered human myocardial research. To facilitate our molecular biology research, we worked on a methodology to culture human cardiomyocytes.

The first long-term cultures of adult mammalian cardiomyocytes were established in the early 1980s. In the culture systems used [1-4], adult rat cardiomyocytes did not divide and underwent extensive morphological changes. Disorganization and re-establishment of myofibrils took place in the early stages of culture [1,5]. During the first 1 to 3 days in culture, 80 to 90% of the cardiomyocytes...
became rounded and lost their cylindrical rod-like shape. There was resorption of Z-lines and rapid disorganization of the contractile apparatus. In contrast to adult cardiomyocytes, fetal and neonatal cardiomyocytes disassembled and reorganized their myofibrils in the early stages of culture [6].

Although cardiomyocyte division in vivo in the mammalian heart is thought to stop at birth or shortly thereafter [7], the factors which control the capacity of the cardiomyocyte to divide are not well understood. It is generally assumed that adult cardiomyocytes, which do not divide in vivo, have also lost their capacity to divide in vitro. We have shown that cultured fetal rat cardiomyocytes, which can contract, proliferate and be passaged for over 3 months in vitro, lose only their capacity for in vivo hyperplasia shortly after being transplanted into the subcutaneous tissue of an adult rat [8]. These findings are consistent with non-cardiomyocyte-derived extracellular factors preventing cardiomyocytes from dividing in vivo. The factors present in the mammalian adult are unlikely to be present in fetal serum.

In this study, we describe both morphological and biochemical characteristics of human pediatric and adult ventricular cardiomyocytes in our fetal bovine serum culture system for up to 24 passages and a duration of 6 months. We have found that cultured cardiomyocytes can be passaged with a purity greater than 95% for as long as 6 months. Although sarcomeres can sometimes be seen in passage 0, the cardiomyocytes undergo rapid phenotypic changes in primary culture with essentially complete loss of sarcomeres on ultrastructural analysis. The myofibrillar changes of our passaged human partially differentiated cardiomyocytes are similar to those reported above for unpassaged adult rat and monkey cardiomyocytes [1-4]. Nevertheless, these cells can be clearly identified as cardiomyocytes by detection of cardiac-specific contractile proteins, such as cardiac myosin heavy chain.

2. Methods

2.1. Cell culture

Myocardial ventricular resection specimens were obtained from 3 pediatric tetralogy-of-Fallot (TOF) patients (1 to 2 years of age) undergoing corrective cardiac surgery and an adult patient (55 years of age) immediately prior to heart transplantation because of multiple coronary artery occlusions. A transmural ventricular needle biopsy was also taken from a 65 year-old patient having aorto-coronary bypass surgery. Saphenous vein segments were acquired from an aorto-coronary bypass patient (65 years of age). This investigation conforms with the principles outlined in the Declaration of Helsinki. Permission was obtained from the hospitals' Human Experimental Committees. Cardiomyocytes were isolated, purified, cultured and subcultured [9,10]. In summary, 5 to 20 mg of ventricular tissue were taken under sterile conditions in the operating room and immediately immersed in culture medium (Iscove's modified Dulbecco's medium (Canada Life Technologies Inc., Burlington, Ontario), 10% fetal bovine serum, 0.1 mmol/1 1-mercaptoethanol, 100 U/ml penicillin, and 100 mg/ml streptomycin). The biopsy was washed with phosphate-buffered saline (PBS) (NaCl 136.9 mM, KCl 2.7 mM, Na2HPO4 8.1 mM, KH2PO4 1.5 mM, pH 7.3). The connective tissue was removed and the myocardium minced into small pieces. The minced tissue was digested in 0.2% trypsin and 0.1% collagenase for 5 min at 37°C. The supernatant was collected in medium and the tissue was processed twice more. The supernatants were combined and centrifuged at 580 x g for 5 min. The pellet was resuspended in culture medium and the cell number determined by an electronic counter. The cells were seeded at a density of 50 to 100 cells per 100 mm diameter dish and cultured at 37°C in 5% CO2. When the cells were seeded at a low density, the viable cells formed individual colonies after approximately 2 weeks in culture (the cloning dilution technique). If any fibroblasts were adjacent to a cardiomyocyte colony of interest, necrosis of the fibroblasts was caused through damage from a sterile needle. At this time cardiomyocyte colonies could be picked up using a sterile Pasteur pipette and transferred to new culture dishes. Saphenous vein endothelial cells and myocardial fibroblasts were cultured as described by Mickel et al. [11].

For all studies performed, individual clones were transferred into separate culture dishes. Throughout this paper, we denote n as the number of individual clones used from each patient and N as the number of patients from whom the colonies were derived.

2.2. Immunofluorescent staining

To identify cultured pediatric and adult cardiomyocytes, passage 3 cells (n = 4, N = 2 pediatric and 2 adult patients) were immunofluorescently stained for CK-MB, myoglobin, cardiac-specific troponin I isoform, human ventricular myosin light chain (HVMLC1) and human ventricular myosin heavy chain (HVMHC). To characterize the effect of culture time on a myocardial contractile protein, pediatric cardiomyocytes (n = 4, N = 2) cultured to passage 1 (2 weeks) and passage 12 and 24 (3 and 6 months, respectively) were immunofluorescently stained for HVMLC1. Briefly, the cultured cells were washed 3 times with PBS and fixed with 2 ml 100% cold methanol at -20°C for 15 min. After washing 3 times with PBS and drying by draining, the cells were exposed to monoclonal antibodies against CK-MB, myoglobin, troponin I, and HVMLC1 (Spectral Diagnostics, Toronto, Canada) and HVMHC (Rougier Bio-Tech, Montreal, Canada), at a concentration of 1:20 dilution with saline, for 45 min at 37°C. The control group cells were incubated with PBS under the same conditions. The cells were washed 3 times with PBS.
for 15 min at room temperature with gentle shaking. The secondary antibody, rabbit anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) at a concentration of 1:32 dilution with PBS, was added. The cells were incubated with the second antibody under dark and humid conditions for 45 min at 37°C. After washing with PBS, the cells in the test and control groups were visualized under ultraviolet light using an epi-microscope with a blue filter.

2.3. Cardiomyocyte purity

Passage 3 cells in a culture dish were immunofluorescently stained for myosin heavy chain using a monoclonal antibody to HVMHC. The percentage of the cells staining positively in 10 random light microscopic fields (200 × ) was a measure of the purity of the cardiomyocyte culture. This process was repeated using cells purified from 8 clones of 2 pediatric patients and from 6 clones of 2 adult patients.

2.4. Cell size

The sizes of passage 3 cardiomyocytes (n = 4, N = 2 pediatric and 2 adult patients), fibroblasts and vascular endothelial cells were determined using planimetry on micrographs at 200 × magnification. Eight cells of each cell type were measured.

2.5. Northern blot analysis of cardiac myosin heavy chain

Total RNA from passages 0, 1, 3 (1 month), passage 5 (2 months), and passage 10 (3 months) pediatric cardiomyocytes (n = 6, N = 2), adult vascular endothelial cells (n = 6, N = 1), and pediatric myocardial fibroblasts (n = 6, N = 2) were isolated according to the method of Chomczynski and Sacchi [15].

Total RNA, 4 μg, from each cell group was fractionated through 1% formaldehyde/agarose gel [16]. The RNA was transferred onto a nitrocellulose membrane over-night with 10 × SSC. RNA was fixed to nitrocellulose by baking in a vacuum oven for 45 min at 80°C.

Northern blot analysis [1] was carried out using a 3' cDNA myosin heavy chain probe derived from the human β-cardiac myosin heavy chain cDNA clone (pHMC3) [18] (gift from Dr. C.C. Liew, The Centre for Cardiovascular Research, The Toronto Hospital).

2.6. Creatine kinase activity and mass

Passage 1 (2 weeks) to 9 (8 weeks) pediatric (n = 8, N = 2) cardiomyocytes and passage 1 (1 week) and passage 9 (6 weeks) adult (n = 8, N = 1) endothelial cells were washed 3 times with PBS, harvested with a rubber policeman and collected in microcentrifuge tubes. After centrifugation for 1 min, the cell pellets were lysed with 0.4 ml (1:6) PBS/distilled water on ice for 20 min. The samples were centrifuged for 1 min. The supernatants were assayed for creatine kinase (CK) and CK-MB activities with a COBAS-FARA analyser and for CK-MB mass with ROCHE Isomune-CK kit [9]. Protein concentrations were measured by the Bio-Rad protein kit (Bio-Rad, Richmond, CA) with bovine serum albumin as the standard.

2.7. Transmission electron microscopy

Passage 0 (1 week), passage 1 (2 weeks), and passage 5 (5 weeks) pediatric cardiomyocytes cultured from 4 clones of 2 patients and passage 0 (3 weeks) and passage 8 (12 weeks) cardiomyocytes cultured from 4 clones of 2 adult patients were used for transmission electron microscopy. After washing 3 times with PBS, cardiomyocytes were fixed with 1% glutaraldehyde in 0.1 M phosphate buffer (0.1 M NaH2PO4, 0.1 M Na2HPO3) for at least 24 h. The cells were rinsed again with phosphate buffer before post-fixing with 1% osmium tetroxide in 0.1M phosphate buffer for 1 h. The samples were then dehydrated in graded alcohols (50–70–90–100% ethanol for 10 min each), and embedded in Epon-Araldite epoxy resin. EM blocks were positioned over single preselected areas in each tissue culture dish. Thin sections were cut from the EM blocks en face, in order to view the whole monolayer, and mounted on copper grids. Following uranyl acetate and lead citrate staining, sections were viewed and photographed using a Philips 201 electron microscope at 60 kV.

2.8. Fatty acids levels

Passage 3 to 4 pediatric cardiomyocytes (n = 10, N = 2) cultured at a pO2 of 40 mmHg and myocardial resections obtained from 10 pediatric patients were immediately frozen with liquid nitrogen and freeze-dried for 12 h at −50°C. The cells were removed with a cell scraper in 2 ml methanol, collected in a glass homogenizer, and homogenized with 15 nmol each of heptadecanoic and 2,3-dihydroxypropyl-sn-glycerol-1-phosphorylcholine (internal standards) following the method of Shaikh and Downar [13]. The freeze-dried myocardial biopsies were homogenized in 2 ml methanol containing the internal standards. The phospholipid lipids were separated by passing through a silice acid column and the fatty acids were analysed by gas chromatography. Cellular DNA was quantified using the colorimetric method of Burton [14]. The fatty acid concentrations were expressed as nmol fatty acid per μg cellular DNA.

2.9. Growth curve

The purified cardiomyocytes from 2 pediatric patients (n = 8) and 1 55-year-old adult patient (n = 8) were trypsinized and then cultured at concentrations of 5.0 ± 2.27 × 104 and 5.7 ± 1.95 × 104 cells/60 mm diameter
Fig. 1. Representative photomicrographs of pure cultures of passage 3 pediatric (A, 1 year of age) and adult cardiomyocytes (B, 55 years of age) (200×). The adult cells (1231 ± 2103 μm²; n = 2, N = 2) were larger (P < 0.01) than the pediatric cells (4395 ± 436 μm²; n = 4, N = 2). Cell size is expressed as mean ± 1 s.e. In this and subsequent figures, n is the number of individual clones used from each patient and N is the number of patients from whom the colonies were derived. Eight cells of each type were measured.

dish, respectively. For 10 successive days, 8 plates of cells of pediatric cardiomyocytes or adult cardiomyocytes were washed 3 times with PBS and trypsinized with 1 ml of 0.05% trypsin in PBS for 5 min. After adding 1 ml cell culture medium, 200 μl of cell suspension were mixed with 9.8 ml of saline and the cell number measured 8 times by an electronic cell counter.

2.10. ³H-Thymidine uptake

As part of the growth curve study, for 10 successive days 1 plate per clone (n = 4, N = 2 pediatric patients and n = 4, N = 1 55-year-old patient) were cultured with 1 μCi/ml ³H-thymidine-supplemented medium for 12 h [12]. After discarding the medium, the cells were washed 3

Fig. 2. Representative photomicrographs of pure cultures of adult vascular endothelial cells (A) and pediatric myocardial fibroblasts (B) (40×). The oval-shaped vascular endothelial cells (927 ± 64 μm²; n = 4, N = 1) differed from the spindle-shaped myocardial fibroblasts (468 ± 82 μm²; n = 4, N = 1).
times with PBS, detached with 0.05% trypsin and 0.02% glucose in PBS and collected in a Whatman glass micropore filter. The filter was washed 3 times with PBS, 3 times with 10% TCA in distilled water and and once with 100% ethanol. The filter was then dried under a heat lamp, transferred into a scintillation vial containing 10 ml Aquasol and counted in a beta counter for 3 min.

2.11. Percentage of binucleated pediatric cardiomyocytes

The percentage of binucleated cardiomyocytes were counted in 8 randomly selected fields of passage 3 cells \( n = 4, N = 1 \) pediatric patient immediately before trypsinization and then 6 h, 24 h, and days 4, 6 and 10 after trypsinization. The cells were confluent on day 10.

Fig. 3. Representative photomicrographs of passage 3 pediatric (2 years of age, pictures A,C,E,G,I) and adult (65 years of age, pictures B,D,F,H,J) cardiomyocytes immunofluorescently stained for human CK-MB (A,B), myoglobin (C,D), cardiac-specific troponin I isoform (E,F), myosin heavy chain (G,H), and myosin light chain 1 (I,J) (200×). Control cardiomyocytes, vascular endothelial cells and myocardial fibroblasts did not stain (results not shown).
2.12. Statistical analysis

Data was expressed as mean ± 1 s.d. except that cell size data was expressed as mean ± 1 s.e. The growth rates of pediatric and adult cardiomyocytes were assessed by Analysis of Covariance (ANCOVA). Student’s t-test was used to determine the difference between groups.

3. Results

When the human ventricular cells isolated from pediatric and adult myocardium were seeded at low densities, pure cardiomyocyte and fibroblast colonies formed. Individual colonies were readily transferred using a sterile Pasteur pipette to a new culture dish and cultured. The purities of pediatric and adult cardiomyocyte cultures were 94.1 ± 0.52% (n = 8, N = 2) and 93.6 ± 2.9% (n = 6, N = 2), respectively.

The pediatric and adult cardiomyocytes were identified by morphological appearance (Fig. 1). The cells were polygonal in shape. The pediatric cardiomyocytes were approximately 4395 ± 436 µm² (n = 4, N = 2). The cell size became slightly smaller with confluence. The size of the adult cardiomyocytes was approximately 12315 ± 2103 µm² (n = 4, N = 2) and was significantly (P < 0.01) larger than that of the pediatric cardiomyocytes. The morphological appearance of the cardiomyocytes in culture was distinctly different from vascular endothelial cells (Fig. 2A) and myocardial fibroblasts (Fig. 2B). The pediatric myocardial fibroblasts were spindle-shaped and 468 ± 82 µm² (n = 4, N = 2) in size while the adult vascular endothelial cells were oval in shape and 927 ± 64 µm² (n = 4, N = 1) in size.

The pediatric and adult cultured cardiomyocytes, but not the endothelial cells or fibroblasts, stained positively for CK-MB, myoglobin, cardiac-specific troponin I isoform, HVMHC, and HVMLC1 (Fig. 3). Northern blot
analysis of the 3' end of the myosin heavy chain mRNA showed expression in the cardiomyocytes, but not in the myocardial fibroblasts and the vascular endothelial cells (Fig. 4). Cellular enzymatic studies showed that the cardiomyocytes, but not the vascular endothelial cells, had CK activity and CK-MB mass and activity (Fig. 5).

In culture, cardiomyocytes divided and underwent phenotypic changes. Although sarcomeres can sometimes be found in passage 0 cardiomyocytes (Fig. 6), an essentially complete loss of sarcomeres on ultrastructural analysis occurs by 10 days in culture. Numerous myofibrils with dense bands were found in passage 1 and 5 pediatric cardiomyocytes (n = 4, N = 2) (Fig. 7A,B) and passage 0 and 8 adult cardiomyocytes (n = 4, N = 2) (Fig. 7C,D). A progressive decrease and greater disorganization of the myofibrils was apparent with subsequent passages. Consistent with the loss of myofibrils with passaging, the intensity of the immunofluorescent staining for HVMLC1 decreased (Fig. 8). Using the 3' human cardiac myosin heavy chain cDNA as a hybridization probe, Northern blot analysis showed a similar decrease in myosin heavy chain mRNA levels with time of culture in pediatric cardiomyocytes (Fig. 4). In contrast to the changes in immunofluorescent staining for the contractile proteins, CK, CK-MB activities and mass did not change significantly (Fig. 5).

Total phospholipid fatty acids per μg DNA in the cultured pediatric cardiomyocytes were similar to the levels in the pediatric myocardial biopsies (Table 1). The major fatty acids in the phospholipid fractions for both cardiomyocytes and myocardium were 16:0, 18:0, 18:1(n-9), 18:2(n-6) and 20:4(n-6).

The rate of growth of the pediatric cardiomyocytes was significantly (P < 0.0001, ANOCOVA) faster in culture than that of the adult cardiomyocytes (Fig. 9). The pretrypsinization percentage (n = 4, N = 1) of pediatric binucleated cardiomyocytes was 4.3 ± 2.0%. After trypsinization the percentages were 3.4 ± 1.6% (6 h), 1.9 ± 2.1% (day 1), 1.1 ± 1.1 (day 4), 1.1 ± 1.0% (day 6), and 5.1 ± 3.6% (day 10). Trypsinization decreased (P < 0.01) the percentage of binucleated cells by day 1. Between days 1 and 6 the percentage of binucleated cells remained unchanged. By day 10 when the cells were confluent, there was an increase (P < 0.002) in binucleated cardiomyocytes. The percentages of binucleated cardiomyocytes on day 10 and immediately before trypsinization were not significantly different. When the pediatric cardiomyocytes reached confluence, they demonstrated contact inhibition. The incorporation of [3H]-thymidine in the pediatric cardiomyocytes reflected their rate of growth. The pediatric cardiomyocytes could be passaged up to 24 times and for as long as 6 months. By 6 months of culture, the pediatric cardiomyocytes became larger and star-shaped and had lost their capacity to proliferate. In contrast, adult cardiomyocytes proliferated at a very slow rate until the plate was approximately 50% confluent. The cell number remained constant at this stage and the cells hypertrophied.

4. Discussion

Since longitudinally arranged myofibrils divided into sarcomeres extend the length of fully differentiated cardiomyocytes in vivo, it would be unlikely that these cells
could divide either in vivo or in vitro without myofibrillar breakdown. In long-term culture the pediatric and adult cardiomyocytes became less differentiated. The cells lost their cylindrical shape. There was rapid disorganization and loss of the contractile apparatus with resorption of the Z-lines, resulting in loss of the capacity of the cardiomyocyte to contract.

The partially differentiated ventricular cardiomyocytes grown in our cell culture system were identified by microscopic appearance, immunohistochemistry for myocardial proteins, Northern blot analysis for HVMHC, CK-MB activity and mass measurements, and ultrastructural analysis. The cardiomyocytes, endothelial cells, and fibroblasts could be distinguished by morphological appearance. The adult cardiomyocytes were 4 times larger than the pediatric. Compared to the cardiomyocytes, the oval-shaped endothelial cells and spindle-shaped fibroblasts were much smaller in size. Only the cardiomyocytes, not the endothelial cells and fibroblasts, stained positively for the myocardial proteins CK-MB, myoglobin, troponin I, human ventricular myosin heavy chain, and human ventricular myosin light chain I. The antibodies to troponin I were specific for cardiac troponin I isoform and did not cross-react with the other two isoforms related to skeletal muscle. The remaining antibodies used in the immunofluorescent staining reacted with CK-MB, myoglobin, myosin heavy chain and

![Fig. 7. Representative electron micrographs of the myofibrils of cultured pediatric cardiomyocytes from a 2-year-old patient (passage 1 (A, 20,500×) and passage 5 (B, 16,400×)] and of cultured adult cardiomyocytes [passage 0 (C, 25,600×), 65-year-old patient; and passage 8 (D, 35,040×), 55-year-old patient]. Note the loss and disorganization of the myofibrils with irregular or "smeared" Z-lines with passaging and age of culture.\]
myosin light chain present in the human myocardium and skeletal muscle. Northern blot analysis for the 3' end of mRNA for HVMHC was positive. Pericytes, which contain smooth muscle actin, non-muscle actin and tropomyosin, would not have stained positively for the myocardial contractile proteins reported in this study. The cardiomyocytes contained creatine kinase MB fraction activity and mass. Ultrastructural analysis revealed a dramatic loss of sarcomeres in primary culture. The loss of sarcomeres could be attributed to cell division with the sarcomeres breaking up in the mitotic process and not being reformed in the daughter cells, most probably because these cells were not contracting in the cell culture environment. Myofibrils with dense bands were seen by electron microscopy on primary culture and subsequent passages, but it required the identification of myosin by immunohistochemistry and Northern blot analysis to definitively identify these cells as cardiomyocytes. Although it was possible to grow the pediatric cardiomyocytes in culture for up to 24 passages over a duration of 6 months, a gradual loss in contractile proteins occurred. The adult cardiomyocytes could not be maintained as long as the pediatric cardiomyocytes.

The ventricular cardiomyocytes obtained from adult patients were more difficult to culture than pediatric cells. Similar age-related results have been reported by Smith et al. [19] who cultured human atrial cardiomyocytes. The explanation for the effect of donor age on the rate of cell
division is unknown, but is a well-known phenomenon in primary cell culture. The pediatric cardiomyocytes increased in number with a doubling time of approximately 2 days until confluence was reached on day 8. When confluent, the cells exhibited contact inhibition and the cell number did not change. The adult cells grew more slowly with a doubling time of 4 days until the culture dish was approximately 50% confluent by day 9. At this stage the cell number did not change. The changes in cell numbers were reflected by 3H-thymidine uptake studies. Between days 0 and 3, the small number of pediatric cells increased from 50,000 to 150,000 cells per dish which was due to cell division and to binucleated cardiomyocytes becoming mononucleated within the first 24 h after trypsinization. The increase was mirrored by the small change in the 3H-thymidine uptake from 0 to 10,000 dpm per dish. The increase occurred despite the use of trypsin to suspend the cells to initially seed the culture dishes. Proteolytic enzymes, such as trypsin, have been shown in non-malignant cultured cells to induce growth promotion and to reduce contact inhibition [20]. The limited initial growth was likely due to trypsin-derived internal organelle disorganization, in a fashion similar to that which occurs in neonatal cardiomyocytes [21,22]. Cardiomyocyte recovery from the trypsin injury can take as long as 36 h. We attributed the dramatic increase in thymidine uptake between days 3 and 4 to the non-confluent cells, after recovering from the trypsin-derived injury, starting to synthesize DNA. It should be noted that since the cells were labelled for 12 h with 3H-thymidine, the increase in thymidine uptake cannot be attributed to synchronous growth. A short labelling time relative to the cell cycle time is needed to determine whether there is synchronization of the cardiomyocytes with respect to the mitotic cycle. The steady increase in cell count between days 4 and 8 despite a plateauing of the 3H-thymidine uptake could be explained by a constant number of cells dividing while increasing numbers of cells were losing the capacity to divide because of contact inhibition. By day 8 the culture dish was confluent and cell numbers had stabilized. The decrease in 3H-thymidine uptake probably reflected normal turnover of the cultured cells and nuclear duplication.

The number of patients reported in this study is small. In the last 8 years we have cultured ventricular cardiomyocytes from over 100 tetralogy-of-Fallot patients aged 6 months to 14 years and from 5 adult patients over 55 years of age having aorto-coronary bypass surgery. Over 10 clones per patient are usually cultured. No inter-clone variability has been detected in our metabolic and molecular biology studies except that the cells obtained from the younger patients grew more rapidly than cells obtained from older patients. Otherwise, we have not detected any

Table 1
Fatty acid composition in cultured cardiomyocytes and the myocardium

<table>
<thead>
<tr>
<th>Composition</th>
<th>Phospholipid fatty acids</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fatty acid (nmol/g DNA)</td>
<td>Cardiomyocyte</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Percentage</td>
</tr>
<tr>
<td>14:0</td>
<td>5.85 ± 1.05</td>
<td>0.91 ± 0.78</td>
</tr>
<tr>
<td>16:0 DMA</td>
<td>2.56 ± 0.24</td>
<td>3.04 ± 0.69</td>
</tr>
<tr>
<td>16:0</td>
<td>34.25 ± 5.00</td>
<td>22.78 ± 4.18</td>
</tr>
<tr>
<td>18:0 DMA</td>
<td>1.85 ± 0.36</td>
<td>2.55 ± 0.42</td>
</tr>
<tr>
<td>18:0</td>
<td>24.00 ± 3.73</td>
<td>23.92 ± 3.83</td>
</tr>
<tr>
<td>20:0</td>
<td>trace</td>
<td>0.47 ± 0.07</td>
</tr>
<tr>
<td>22:0</td>
<td>trace</td>
<td>1.00 ± 0.28</td>
</tr>
<tr>
<td>24:0</td>
<td>trace</td>
<td>0.81 ± 0.40</td>
</tr>
<tr>
<td>16:1 (n-7 and -9)</td>
<td>1.48 ± 0.71</td>
<td>0.98 ± 0.29</td>
</tr>
<tr>
<td>18:1 DMA</td>
<td>0.14 ± 0.05</td>
<td>1.10 ± 0.31</td>
</tr>
<tr>
<td>18:1 (n-9)</td>
<td>20.46 ± 4.23</td>
<td>16.70 ± 1.71</td>
</tr>
<tr>
<td>20:1 (n-9)</td>
<td>trace</td>
<td>0.17 ± 0.04</td>
</tr>
<tr>
<td>22:1</td>
<td>trace</td>
<td>trace</td>
</tr>
<tr>
<td>24:1</td>
<td>trace</td>
<td>0.99 ± 0.59</td>
</tr>
<tr>
<td>18:2 (n-6)</td>
<td>5.90 ± 0.98</td>
<td>22.66 ± 4.44</td>
</tr>
<tr>
<td>18:3 (n-3)</td>
<td>5.39 ± 0.91</td>
<td>0.22 ± 0.08</td>
</tr>
<tr>
<td>20:4 (n-6)</td>
<td>8.85 ± 1.18</td>
<td>29.95 ± 4.93</td>
</tr>
<tr>
<td>22:6</td>
<td>5.24 ± 0.95</td>
<td>3.51 ± 0.81</td>
</tr>
<tr>
<td>Saturated</td>
<td>68.51 ± 8.08</td>
<td>55.44 ± 8.48</td>
</tr>
<tr>
<td>Unsaturated</td>
<td>56.46 ± 7.71</td>
<td>76.79 ± 9.74</td>
</tr>
<tr>
<td>Total</td>
<td>124.97 ± 15.35</td>
<td>131.73 ± 17.02</td>
</tr>
</tbody>
</table>

Data are the means ± s.d. Lipids were extracted from passage 3 and 4 cardiomyocytes cultured from 10 clones of 2 patients as well as from myocardial biopsies obtained from 10 pediatric patients (N = 10). The phospholipid fractions were isolated, and fatty acid content was determined as described in 'Methods'. Fatty acids are shown as carbon number: no. of unsaturated bonds and n-3 and n-6 represent the carbon number of the first double bond from the methyl-end of the fatty acid chain. DMA (= dimethylacetals) represents alkenyl moieties.
Our only experience in culturing cardiomyocytes from patients with familial myocardial disorders has been with approximately twenty 30–65-year-old patients with hypertrophic cardiomyopathy. These cells always divided faster than pediatric cells [23]. The hypertrophic cells showed abnormal contact inhibition at confluence and continued to proliferate.

The reason why our culture technique permitted partially differentiated cardiomyocytes to divide for as long as 6 months is unknown. Ng and Cheng [4] reported adult rat cardiomyocyte mitosis and cell division only during the first week of culturing. Their culture medium consisted of 90% minimum essential medium and 10% horse serum, which is similar to that of other researchers [1,24]. We tested Dulbecco’s modified Eagle’s medium, medium 199, minimum essential medium, α-medium, and Iscove’s modified Dulbecco’s medium all with 10% fetal bovine serum. Iscove’s modified Dulbecco’s medium was the best with fetal bovine serum. With the other media, the cultured cardiomyocytes underwent marked phenotypic changes and were spider-shaped by 3 to 4 days of culture. Since fetal bovine serum should have more growth factors than horse serum, it could account in part for the success in passaging the cells. Cardiomyocyte division did not occur in the absence of fetal bovine serum and was facilitated by β-mercaptoethanol. The action of β-mercaptoethanol is unexplained. The addition of cytosine arabinoside and bromodeoxyuridine to culture medium has been used by investigators [24–27] to inhibit fibroblast proliferation. From our experience these chemicals also inhibited cardiomyocyte proliferation.

To obtain cardiomyocyte culture purities greater than 95%, we used a clonal dilution technique, rather than density sedimentation, selective plating, and the addition of cytosine arabinoside. The freshly isolated cells were seeded at a low density (e.g., 50 to 100 cells per 100-mm-diameter culture dish) to permit cardiomyocyte and fibroblast colonies to grow separately. A single pure colony of cardiomyocytes or fibroblasts was then transferred using a Pasteur pipette to another culture dish. If a fibroblast were adjacent to a cardiomyocyte colony of interest, the fibroblast was necrosed by slicing it with a sterile needle before transferring the colony. This technique is superior to density sedimentation and selective plating and avoids the use of chemicals, such as chemotherapeutic agents, to inhibit

---

Fig. 8. Representative photomicrograph of pediatric cardiomyocytes (from a 2-year-old patient) cultured for one half (A, 200 ×), 3 (B, 200 ×) and 6 (C, 200 ×) months immunofluorescently stained for human ventricular myosin light chain 1. The progressive loss of this contractile protein with passaging and age of the culture is consistent with the Northern blot analysis for myosin heavy chain mRNA (Fig. 4) and the loss of myofibrils (Fig. 7).

Fig. 9. Growth curves and H thymidine uptake studies using cardiomyocytes cultured from 2 pediatric (n = 8) and from 1 adult patient (n = 4). The growth curve and H-thymidine results (mean ± 1 s.d.) are expressed as the number of cell and disintegrations per minute per 60 mm diameter culture dish, respectively. The rate of growth of the pediatric cells was significantly greater (P < 0.0001, ANOVA) than the adult cells.
fibroblast proliferation. A potential disadvantage with the clonal dilution technique is that it selects the fastest growing cardiomyocytes. If these cells have upregulated their 'growth genes', they may not be representative of the general cardiomyocyte population. This difference could account for the failure of other research groups using a non-clonal isolation technique to culture cardiomyocytes.

Our long-term goals are to transplant cultured cardiomyocytes into a patient's myocardium. We hope to reconstruct some forms of congenital disease and to 're-muscularize' the post-infarcted ventricle to prevent the onset of heart failure. Using the same culture method described in this paper, we [28] have successfully transplanted fetal rat cardiomyocytes into experimentally-derived myocardial scar tissue in the adult rat heart. Angiogenesis occurred, contractile myocardial tissue formed, and the development of heart failure was prevented. When a technique is developed to reform sarcomeres in the adult cardiomyocyte, transplantation of the patient's own cells becomes possible.

In summary, primary cultures of pediatric and adult partially differentiated ventricular cardiomyocyte can be established and passaged. Although there is rapid disorganization of the myofilaments, the cultured cells can be identified by their morphological appearance and by the presence of cardiac-specific contractile proteins.

Acknowledgements

We thank Dr. D.B. Cowan for help in Northern blot analysis; Julia Hwang for assistance with electron microscopy; Dr C.C. Liew for human β-cardiac myosin heavy chain cDNA clone (pHMC3); and Dr G. Jackowski (Spectral Diagnostics) for the monoclonal antibodies to identify human CK-MB, myoglobin, troponin I, and ventricular myosin light chain 1. This work was supported by the Medical Research Council of Canada (MT-10392) and Heart and Stroke Foundation of Ontario (A2604).

References