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Gene Product Expression of Cyclin D_2 and p16 During the Transition from Cardiac Myocyte Hyperplasia to Hypertrophy

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Abstract: The current study was to investigate mRNA expression of cyclin D_2 and p16 during the transition from cardiac myocyte hyperplasia to hypertrophy. Cultured cardiac myocytes (CM) and fibroblasts (FC) obtained from 1-day-old Sparague-Dawley rats were used in this study. We have determined (1) hyperplasia by cell growth curve and fluorescence activated cell sorting (FACS); and (2) ultrastructure by electron microscope observation; and (3) expressions of cyclin D_2 mRNA and p16 mRNA by using in situ hybridization and image analysis. The results were shown (1) Results of cell growth curve and FACS analysis showed CM could proliferate in the first 3 cultured days (4 days in postnatal development). But the ability decreased quickly, concomitant with the differentiation. (2) The ultrastructure of CM showed the large amount of myofilaments and mitochondrion and FC showed moderate amount of rough endoplasmic reticulum. (3) The expression of cyclin D_2 mRNA in 3-, 4-, 5-day CM group was 0.89 times ($p<0.05$), 0.80 times ($p<0.05$) and 0.56 times ($p<0.01$) of that in 1-day group respectively. P16 mRNA in 2-, 3-, 4-, 5-day CM group were 1.63 times ($p<0.01$), 1.72 times ($p<0.01$), 1.99 times ($p<0.01$) and 2.84 times ($p<0.01$) of that in 1-day group respectively. It can be concluded that cultured neonatal rat cardiac myocytes could proliferate during the first 3 cultured days, but the ability of proliferation decreased, from the fourth day, concomitant with differentiation. Cyclin D_2 and p16 have the key roles during the transition from myocyte hyperplasia to hypertrophy.

Key words: cyclins; gene p16; hyperplasia; cardiac myocytes; rat

CLC number: R 361.3

Original view was introduced that adult cardiac myocytes are terminally differentiated cells and, therefore, cannot be recalled into the cell cycle. The ability of the mammalian cardiac myocytes to divide and proliferate is lost at, or just after, birth and in the rat the switch from cardiac myocytes hyperplasia (cell division) to hypertrophy (an increase in cell size) occurs between days 3 and 4 of postnatal development[1]. This cessation of proliferation has been attributed to a block in the cell cycle and cell-cycle machinery may be involved in[2,3].

The ordered progression of a cell through the cell cycle was known to be regulated both positively through the formation of cyclin and cyclin-dependent kinases (CDKs) complexes, and negatively by CDK inhibitors (CDKIs)[4]. Currently, the mechanisms that regulate the transition from cardiac myocytes hyperplasia to hypertrophy remain unknown. Cyclin D_2 is one kind of cyclin D, which can bind CDK4/CDK6. p16 is an inhibitor of the CDK4/CDK6, which is high expression in quiescent cell lines and can inhibit cell cycle progression[5]. In an attempt to understand better the functional role that cell cycle regulatory molecules may play in the transition from myocytes hyperplasia to hypertrophy, we have examined the proliferation

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by cell growth curve. In parallel, cell cycle profiles were detected by fluorescence-activated cell sorting (FACS) analysis. Ultra-thin sections were made to observe the ultrastructure of CMs and FCs under transmission electron microscope (TEM). In addition, we have measured changes in mRNA expressions of cyclin D_2 and p16 by using in situ hybridization and image analysis in an effort to compare those molecules involved in hyperplasia and those involved in hypertrophy.

1 Materials and Methods

1.1 Materials

Dulbecco ' s Modified Eagle ' s medium (DMEM) with Glutamax, fetal calf serum (FCS), newborn calf serum (NCS) and trypsin were obtained from Gibco, Paisley, Scotland. Propidium iodide (PI) was purchased from Sigma. Ribonuclease A (RNase A) and Triton X-100 were kind gifts from MSc. Jiang. Anti-Vimentin rabbit polyclonal antibody and anti-sarcomeric actin mouse monoclonal antibody were purchased from Dako, Denmark. UltraSensitive™ S-P kit was purchased from Maxim Biotech, Inc. Proteinase K was purchased from Sigma. Formamide, tRNA, DEPC, SDS, Tris were obtained from Serva. DNA labeling and detection kit nonradioactive was obtained from Boehringer Mannheim. Alkaline phosphatase streptavidin was purchased from Santa Cruz.

The probe of Biotin-labeled synthesized oligonucleotides encoding rat p16 antigen[6] was described: 5′ Bio-GC TAG TCT ATC TGC AGA GGA CTC CAT G 3′.

The probe of Biotin-labeled synthesized oligonucleotides encoding rat cyclin D_2 antigen[7] was described: 5′ Bio-GTC CAC CTC GCA GCA CAG CAG CTC CAT AG 3′.

Oligonucleotides were synthesized by Sangon, purified by PAGE, and using TE (pH 8.0) for dilution, −20℃ stored.

1.2 Methods

1.2.1 Isolation and culture of cardiac myocytes

Primary culture of neonatal rat cardiac myocytes was prepared by the method originally described by Simpson[8] with minor modifications. Briefly, the ventricle from 1-day old single litter Sprague-Dawley rat were minced and dissociated with 0.1% trypsin. After dispersed cells were incubated on culture dishes for 2 h at 37℃ in a CO_2 incubator, nonattached viable cells were collected and seek into dishes (4×10^8 cells/L). Twenty-four hours later, at the end of 1 day, the cultures were washed with phosphate-buffered saline (PBS) (pH 7.2) containing 5.5 mm glucose. The culture medium (DMEM with 15% FCS, penicillin 10×10^4 U/L, streptomycin 100 mg/L, bovine insulin 230 U/L, pH 7.2) was renewed at this time and every 3 day thereafter. Cardiac myocytes were identified by immunocytochemistry study with anti-sarcomeric actin antibody.

1.2.2 Isolation and culture of fibroblasts

Primary culture of neonatal rat heart fibroblasts were prepared by the method described by Yao[9]. Briefly, the heart were dissociated with 0.1% trypsin and cell were incubated on culture dishes for 1 h at 37℃ in a CO_2 incubator, nonattached cells were discarded. Attached cell were mostly fibroblasts and maintained in DMEM containing 10% NCS in a humidified atmosphere containing 5% CO_2, 95% air at 37℃. Fibroblasts were identified by immunocytochemistry study with anti-Vimentin antibody.

1.2.3 Electron microscopy observation

Cultures were rinsed with PBS, fixed for 30 min at 24℃ in 2.5% glutaraldehyde, postfixed for 30 min at 24 ℃ in 1% OsO4, and dehydrated through graded alcohols, then Epon 812 containing. Thin sections were cut enface using a LKB-5 ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a H-600 electron microscope.

1.2.4 Cell growth curve observation

Cardiomyocytes and fibroblasts were plated into 24-wells plastic dishes at a density of 2×10^7 cells/L respectively and were grown at 37 ℃ in humidified air with sufficient CO_2 (about 5%) to maintain pH 7.3 for 7 d. The culture medium was changed (0.5 mL/well) every 2 d. Cell numbers were counted everyday to make cell grow curve.

1.2.5 FACS analysis

CMs and FCs cultured of 1, 2, 3, 4, 5 day were prepared. Cells were washed twice in PBS and then were fixed in ice-cold 80% ethanol and stored at 4℃ prior to analysis. After centrifugation, the ethanol was removed and PBS washed twice, 1 mL of staining solution (PI 100 mg/mL, RNase 100 mg/mL, 0.1% Triton X-100, 0.01% EDTA) was added and cells were stained at 4 ℃ in dark for 30 min. Labeled nuclei were analyzed for PI incorporation using a FACS analyzer (Becton Dickinson) and Cell Quest software.

1.2.6 In situ hybridization

Cells were plated on glass coverslips. After 1, 2, 3, 4, 5 day, the cells were fixed with 4% PFA for 30 min at room temperature and washed twice with PBS, then dehydrated through graded alcohols, and the method *in situ* hybridization was performed with described. Briefly, the cells were incubated by 0.2 mol/L HCL 20 min and 1 mg/L Proteinase K 20 min at 37 ℃, then postfixed for 30 min at 24℃ in 4% PFA. Prehybridization solution contained: 50% formamide, 5 × SSC, 5 × Denhardt's, 10 g/L SDS, 100 mg/L DHA, 125 mg/L tRNA, every slip was added 20 μL prehybridization solution and kept for 1 h at 42 ℃. Replace the solution with hybridization solution containing 5 g/L probe, mounted on silicidized coverslips and kept for 24-36 h at 42 ℃. The slips washed in 2×SSC and 0.1% SDS for 5 min at 25 ℃ twice, then washed in 0.2×SSC and 0.1% SDS 15 min at 42 ℃ twice.

Alkaline phosphatase streptavidin was added and kept for 30 min at 37 ℃. Later NBT/BCIP was performed for stain, and dehydrated through graded alcohols and mounted on coverslips. Negative control contained two groups, one is probe negative, and the other is alkaline phosphatase Streptavidin negative. Positive stain was blue granulations in cell. Using image analyzer, 100 cells were counted to determine the expression of cyclin D_2 or p16 messenger RNA.

1.2.7 Statistical analysis

SAS software was used for analysis. Data were presented as mean ±S.E. and were determined by using one-way analysis of variance.

2 Results

2.1 General Features of CMs and FCs in Culture

The dissociation yields an average 1.4×10^6 cells per heart. At the end of the 6-hour plating period, many of the cells were round. The cells spread over the first day, developed their characteristic morphology by 3 or 4 d, and changed little over the next week. They were arranged in loose and interlacing networks of single cells. CMs were distinguished by a coarse, granular cytoplasm and the nucleus was small, round, dense and usually contained only one nucleolus. All morphological CMs beat spontaneously at 40-120 per minute (37 ℃). The ultrastructure of CM showed large amount of myofilaments and mitochondrion (Fig. 1). CMs showed as anti-sarcomeric actin positive cell (Fig. 2).

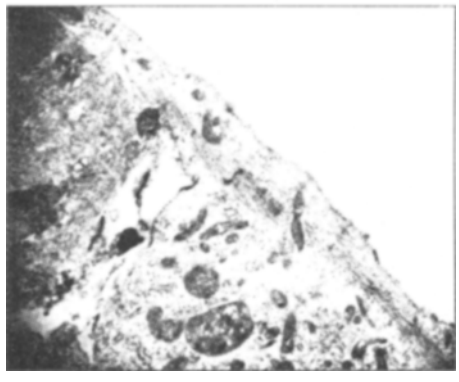

Fig. 1 The ultrastructure of CM cultured 3 d showed large amount of myofilaments and mitochondrion. (TEM ×10 000)

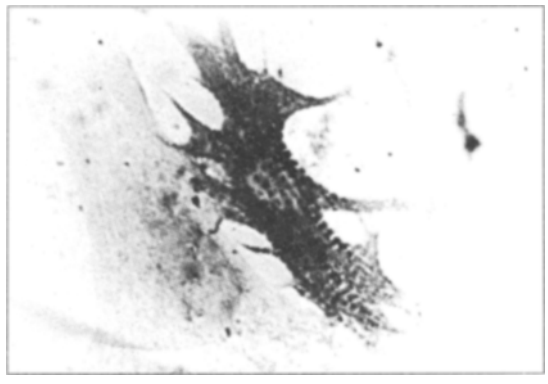

Fig. 2 Immunocytochemistry of CM cultured 5 d with monoclonal antibody against sarcomeric actin. Brown granulations could be observed in cytoplasm of CM. (S-P ×400)

The FCs could be readily distinguished. They were irregularly shaped with a thin, phase-lucent cytoplasm with inclusions usually clustered near the nucleus. The nucleus was distinctively larger, less dense, and after had two or more nucleoli. They could not beat, but their populations can increase. The ultrastructure of FC showed moderate amount of rough endoplasmic reticulum and small amount of mitochondrion. FC was anti-Vimentin positive cell.

2.2 Cell Growth Curve in Culture

The data in Fig. 3 showed that the number of fibroblasts increased in a time-dependent way, which indicated that fibroblasts can proliferate in these stable conditions. The number of CM also increased slowly in 3 days (4 days after birth), but 3 days later, CM number was not change, which indicated that CM could not proliferate.

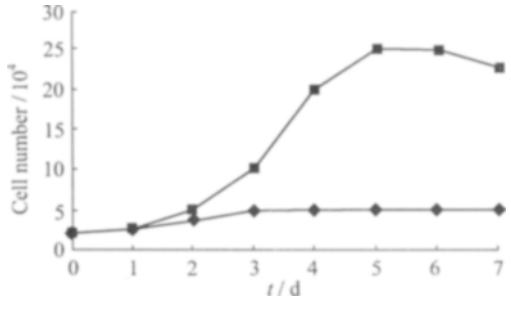

Fig. 3 Cell number of CMs and FC

—◆— CM —■— FC

2.3 FACS Analysis

The data in Fig. 4 showed that the percentage in S phases of CMs decreased significantly [(13±2.1)%, (7.3±1.5)%, (6.0±0.1)%, (5.2±0.6)% and (4.8±0.2)%] in 1, 2, 3, 4, 5 days, respectively, $p < 0.01$), concomitant with a significant increased in the percentage of G_0/G_1 phase cells [(76.8±1.1)%, (86.3±0.7)%, (89.9±0.5)%, (90.2±0.3)% and (90.7±0.2)%] in 1, 2, 3, 4, 5 days, respectively, $p < 0.05$). In parallel, the percentage of G_2/M phase cells was also decreased obviously. Cell cycle changes were not observed among fibroblasts groups (Fig. 5).

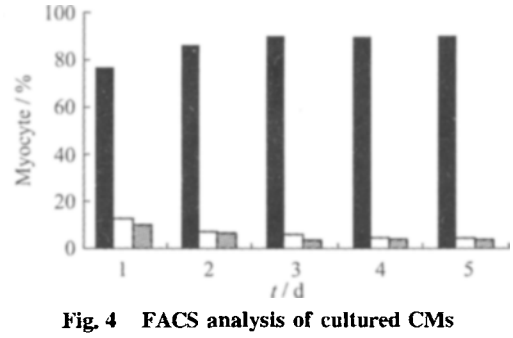

Fig. 4 FACS analysis of cultured CMs

■ G0/G1 □ S ▒ G2/M

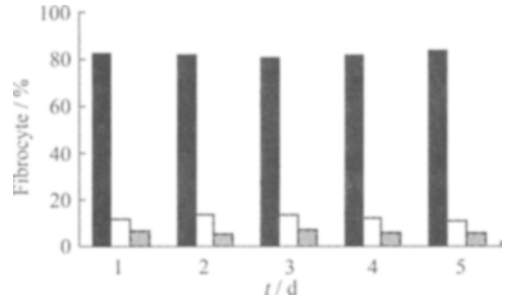

Fig. 5 FACS analysis of cultured FCs

■ G0/G1 □ S ▒ G2/M

2.4 Cyclin D_2 Messenger RNA Analysis

Expressions of cyclin D_2 messenger RNA were viewed in nucleus of CMs and FCs as blue purple granulations (Fig. 6). The data in Table 1 showed that the expression amount of cyclin D_2 mRNA of CM in 3, 4, 5 days were 0.89

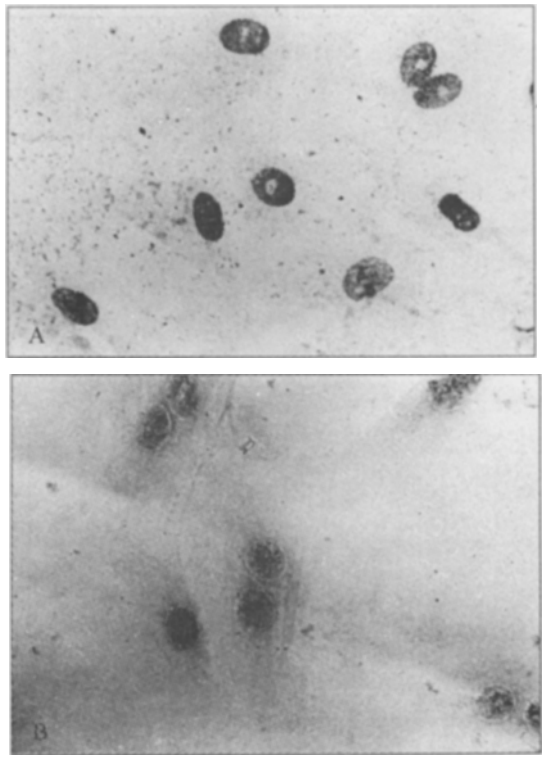

Fig. 6 Cyclin D_2 messenger RNA of CM observed in situ hybridization. (ISH ×400)

A. In 1 d, cyclin D_2 mRNA was expressed in nucleus of CM and stained deep blue. B. In 5 d, compared with A, the stain of CM was weak.

times ($p<0.05$), 0.80 times ($p<0.05$) and 0.56 times ($p<0.01$) of that in 1 day group respectively, and the difference between 1 day and 2 day was not observed. The expression amount of cyclin D_2 mRNA in FC groups was stable during five days culture (Table 2).

2.5 P16 messenger RNA analysis

The positive blue materials were observed on the nucleus membrane and in nucleus of cardiac myocytes (Fig. 7). The data in Table 1 showed that the expression amount of p16 mRNA in CMs was progress increased during the cultured process; 2, 3, 4, 5 days group were 1.63 times ($p<0.01$), 1.72 times ($p<0.01$), 1.99 times ($p<0.01$) and 2.84 times ($p<0.01$) of that in 1-day group, respectively. There were no statistic differences on the expression amount of p16 mRNA among FCs groups cultured (Table 2).

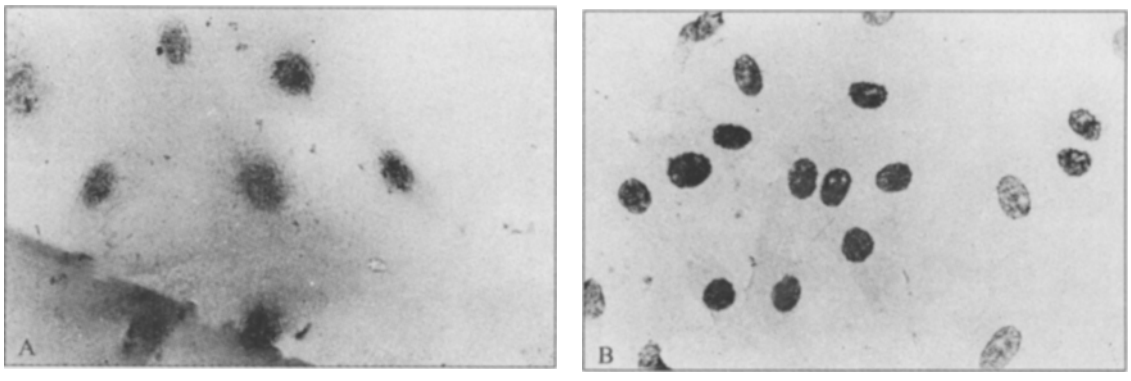

Fig. 7 P16 messenger RNA of CM observed in situ hybridization. (ISH ×400)

A. In 1 d, the positive blue purple materials were observed in part of CMs on the nucleus membrane and in nucleus; B. In 5 d, the blue purple stain in nucleus was deeper than A

Table 1 Messenger RNA expression of cyclin D_2 and p16 in CMs($\bar{x}\pm s$, mean ± S. E.)

Cultured/d	Messenger RNA expression of CMs(A)		
	Cell number	Cyclin D_2	p16
1	100	0.189 8±0.078 8	0.096 1±0.013 1
2	100	0.187 0±0.017 1	0.156 6±0.011 7**
3	100	0.170 8±0.033 9*	0.165 7±0.032 9**
4	100	0.158 7±0.033 6*	0.191 3±0.055 7**
5	100	0.132 2±0.028 8**	0.273 1±0.047 4**

* $p<0.05$ and ** $p<0.01$ compared with 1 d group culture.

Table 2 Messenger RNA expression of cyclin D_2 and p16 in FCs($\bar{x}\pm s$, mean ± SE)

Cultured time/d	Messenger RNA expression of FCs(A)		
	Cell number	cyclin D_2	p16
1	100	0.174 4±0.009 4	0.087 7±0.002 6
2	100	0.175 0±0.008 7	0.087 4±0.002 0
3	100	0.176 0±0.012 9	0.087 0±0.003 0
4	100	0.175 4±0.005 5	0.088 2±0.003 9
5	100	0.175 6±0.001 6	0.084 0±0.002 2

3 Discussion

Cell growth curve and FACS analysis were usually used for investigation cell proliferation. From cell growth curve, we could investigate that cultured cardiac myocytes could proliferate in first cultured three days, but it became stable in the later days soon. These indicated that the number of S phase cardiac myocytes was decreased, consistent with the outcome of FACS. Furthermore, FACS analysis showed that the cessation of proliferation has been attributed to a block in the cell cycle with myocytes locked either in the G_0 or G_1 phases of cell cycle. In our study, fibroblasts were used as comparison, and were observed have stable proliferation ability. From these we can conclude that the block factor which made cardiac myocytes quiescent in cell-cycle was

not from excellular but from cardiac myocytes itself.

Cell cycle progression in mammalian cells can be divided into 4 phases, those were G_1, S, G_2 and M phase. Mitogenic responses in undifferentiated cells were known to be regulated by cell cycle regulators, such as cyclins, CDKs and CDKIs. Cyclins were family of proteins that have oscillating levels of expression during the cell cycle. Cyclins associated with CDKs and acted as a regulatory subunit of CDKs, thus activating them in a cell cycle-specific manner. Activity of CDKs was regulated not only by binding of cyclins but also by phosphorylation of threonine and tyrosine residues and by binding of CDKIs, such as p16. The activated cyclin-CDK complex was thought to phosphorylate various cellular substrates relevant to cell cycle progression. The most intensely studied among CDK substrate was pRb. In the G_1 phase, pRb was hyperphosphorylated by the cyclin D-CDK4 complex, which caused release and activation of the E2F/DP transcription factor complex, which was otherwise sequestered and inhibited by pRb. E2F/DP in turn activated expression of genes required for S-phase progression [10]. Cyclin D_2 was one kind of cyclin D, which can bind to and activate CDK2, CDK4, CDK5, CDK6, inducing cell into S phase [11]. p16 was an inhibitor of CDK 4/CDK6 and can inhibit cell cycle progression[12]. Our study showed that during the cultured process, the expression amount of cyclin D_2 messenger RNA was decreased, otherwise the p16 messenger RNA, along with the loss of proliferation ability of cardiac myocytes. These results suggested cyclin D_2 and p16 played a key role in cardiac myocytes postnatal development. Downregulation of cyclin D_2, upregulation of p16 may induce cardiac myocytes differentiation. These were in accordance with Flink[13]. In this study, we found the first time that p16 gene also has important role on ventricular myocytes terminal differentiation during development. At present, we do not know why changes of cyclin D_2 and p16 exist in cardiac myocytes. Further studies are necessary to elucidate the role of them in terminal differentiation of cardiac myocytes and cardiac hypertrophic responses while growth factors stimulating.

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