MODULATION OF CARDIAC MYOCYTE BEATING RATE AND HYPERTROPHY BY CARDIAC FIBROBLASTS ISOLATED FROM NEONATAL RAT VENTRICLE

HIROYUKI ORITA, M.D., MANABU FUKASAWA, M.D.
SHIGEKI HIROOKA, M.D., HIDEAKI UCHINO, M.D.
KANA FUKUI, M.D. AND MASAHICO WASHIO, M.D.

We evaluated the in vitro modulation of cardiac myocyte beating rate and growth hypertrophy in the presence or absence of cardiac fibroblasts. Cardiac myocytes and fibroblasts were isolated from neonatal rat ventricles, and cultures of either myocytes alone or of myocytes in co-culture with fibroblasts were established and observed for 21 days. The beating rate in control myocytes increased rapidly, reached peak values on day 5 (266.2 ± 58.2 beat/min), and then decreased by day 21. The beating rate of myocytes which were co-cultured with fibroblasts in group 2-1 (2-1 ratio of myocytes to fibroblasts, 6.0×10^5 total cells/well) was significantly lower than that in control myocytes, and showed a decreased peak value on day 7 (135.1 ± 46.4). The beating rate in group 1-1 was significantly less than that of group 2-1, with a peak value on day 7 (85.9 ± 24.9). Group 1-2 myocytes did not show a beating rate increase.

Myocyte hypertrophy was measured after three days of culture. The surface area of myocytes which had been co-cultured with fibroblasts was significantly increased, as compared to that of control myocytes. In addition, myocyte surface area increased with increasing numbers of fibroblasts (Mean myocyte surface areas in group 2-1, 1-1 and 1-2 were 143 ± 21 , 163 ± 33 , $206 \pm 38\%$ of control, respectively).

In conclusion, suppression of the myocyte beating rate and acceleration of myocyte hypertrophy are characteristic expressions of myocyte differentiation and growth during the neonatal period. The present results suggest that fibroblasts play an important role in controlling myocyte development.

(*Jpn Circ J* 1993; 57: 912–920)

CARDIAC myocytes, non-myocytes, such as fibroblasts, and connective tissue matrices develop rapidly and dynamically during the neonatal period^{1,2}. Therefore, cardiac myocytes have various potential capacities, including cell proliferation, growth,

and differentiation. In addition, cardiac myocytes interact with, and are modified by, the presence of cardiac fibroblasts. Cardiac fibroblasts, which are present as a major cellular constituent in the neonatal rat ventricle, play an important role in producing necessary extracellular matrix components in the late fetal and early neonate stages^{3,4}. Recently, several investigators have indicated the localization of fibroblast growth factors

Key words:

Cardiac myocyte
Cardiac fibroblast
Myocyte hypertrophy
Myocyte differentiation

(Received September 12, 1992; accepted January 28, 1993)

The Second Department of Surgery, Yamagata University School of Medicine

This work was supported by a grant from the National Education Ministry of Japan

Mailing address: Hiroyuki Orita, M.D., The 2nd Department of Surgery, Yamagata University School of Medicine, Iida-nishi, Yamagata City 990-23, Japan

in cardiac myocytes, and suggested that these growth factors stimulate the process of cytodifferentiation and morphogenesis of the myocardium⁵⁻⁷. However, there are very few reports regarding the cell-cell interactions of cardiac myocytes and fibroblasts.

The purpose of the present study was to evaluate the in vitro modulation of cardiac myocyte beating rate and growth hypertrophy in the presence or absence of cardiac fibroblasts isolated from neonatal rat ventricles. The results indicate that cardiac fibroblasts significantly suppress myocyte beating rate and accelerate myocyte hypertrophy.

MATERIALS AND METHODS

Isolation of Cardiac Myocytes and Fibroblasts

Cardiac myocytes were prepared from neonatal rat ventricles by a modification of Klein's method^{8,9}. Hearts were removed from 1-2 day-old neonatal male Wistar rats after decapitation. The ventricles from 10 hearts were minced into fine fragments with scissors in 0.025 M Hepes buffered minimum salt solution (MSS, Gibco, Grand Island, NY). The fragments were rinsed twice with MSS to remove contaminating red blood cells, and placed in a 50 ml flask containing 10 ml of 0.1% collagenase (Wako Chemical, Tokyo) in MSS. The flask was gently agitated for 60 min at 37°C. The enzyme digest was centrifuged at 1000×g for 2 min and the resulting pellet was washed twice with MSS. The resuspended cells and small aggregates were gently passed through a wire-mesh screen (90 μm porosity) to remove large aggregates and debris. The cellular filtrate was suspended in MCDB 107 medium (Kyokuto Pharmaceutical, Tokyo) containing 5% fetal calf serum (FCS) (Flow Labs, Rockville, MD) and placed in a 75 cm² tissue culture flask which was incubated for 60 min at 37°C in a humidified atmosphere of 5% CO₂: 95% air to separate the pure myocytes from fibroblasts by a differential adherence protocol⁹⁻¹². After incubation, unattached cells were decanted, centrifuged, and rinsed twice with MSS. Thereafter, the cell pellet was resuspended in MCDB 107 containing 2% FCS, transferin (10 μg/ml, Sigma, St. Louis, MO), and insulin (10 μg/ml, Sigma)⁹, and then gently passed

through a 28 μm wire-mesh screen (>95% myocytes). The attached cells remaining in the 75 cm² flasks were washed twice with MSS and treated with 0.25% trypsin-0.025 M EDTA to facilitate resuspension of the fibroblasts. The cells were washed twice with MSS, resuspended in MCDB 107-2% FCS, and then passed through a 28 μm wire-mesh screen (>90% fibroblasts)⁹⁻¹². Myocytes were identified by their beating activity, which began on the 1st day of culture. Fibroblasts were determined by immunohistochemical staining of a fibroblast-specific intracellular component (Wako Chemical, Tokyo) 6 h after the cells were plated⁹.

The attached and unattached cells consisted primarily of fibroblasts and myocytes, respectively, and were contaminated by non-fibroblasts or non-myocytes at less than 10 or 5 percent of the respective total cell component. In addition, non-fibroblasts and non-myocytes consisted primarily of myocytes and fibroblasts, respectively. Accordingly, in the present study, these contaminations were thought to be a minor concern.

The investigation was performed in accordance with the Guidance on the Operation of Animals (Scientific Procedures) Act 1986, published by Her Majesty's Stationary Office, London.

Cardiac Myocyte Contraction Rate With and Without Co-culture With Fibroblasts

Cardiac myocytes were cultured at various concentrations in 35 mm multiwell culture plates which had been previously treated with fibronectin (Wako Chemical, Tokyo)⁹. Fibronectin treatment was performed by adding 1 ml of 10 μg/ml fibronectin in phosphate-buffer-saline (PBS), pH 7.4, to each culture well and incubating at room temperature for 1 h just prior to the addition of myocytes and fibroblasts. The unbound fibronectin was decanted before the addition of myocytes and fibroblasts. Thereafter, cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂: 95% air and the media was changed daily. The cultured myocytes began to beat on the first day and the contraction rate of each well was observed for 21 days. The mean contraction or beating rate of 10 myocytes/culture well was determined microscopically.

Initially, myocytes were cultured without

Fig.1. Cardiac myocyte beating rate associated with various cell concentrations. Each data point represents the geometric mean of three experiments.

added fibroblasts to optimize the myocyte beating rate response relative to cell density. Three ml of various myocyte cell concentrations ($0.3, 0.5, 0.7, 1.0, 1.5, 2.0, 2.5, 2.5$ and 3.0×10^5 cells/ml) were added to triplicate wells. This experiment was performed a total of three separate times. Next, myocytes were cultured in the presence of fibroblasts and the beating rate was observed for 21 days. For these experiments, a myocyte and fibroblast concentration of 2.0×10^5 cells/ml was chosen and the proportion of fibroblasts varied from 0 to 67% (ml myocyte: ml fibroblast) as follows: 3:0 (group C), 2:1 (group 2-1), 1.5:1.5 (group 1-1), 1:2 (group 1-2). Thus, the total number of cells/well was 6.0×10^5 . Triplicate wells were evaluated for each group and the experiment was repeated an additional 5 times.

Cardiac Myocyte Hypertrophy

Three days after the initiation of the co-culture, at which time most of the myocytes were beating synchronously and steadily, the extent of myocyte hypertrophy was evaluated by measuring the surface area of myocytes in each group. The areas were determined by using an image-analyzing system (EM-II, Rise Inc., Sendai) with a personal computer (PC-9801, NEC Inc., Tokyo). Fifty myocytes from each well were evaluated. Myocytes in group C were used as a control and myocyte hypertrophy in fibroblast co-

culture groups was expressed as a percent of control myocytes.

Statistical analysis

Data were initially evaluated by rankit analysis to determine the distribution, and then by Kruskal-Wallis one-way analysis of variance to examine the differences within each group followed by the Mann-Whitney U test. Results are presented as the mean $\pm$ standard deviation and differences were considered significant if the p value was less than 0.05.

RESULTS

Myocyte Beating Rate Associated With Various Cell Concentrations

All myocytes started to beat on the first culture day although the plating density appeared to significantly affect the early beating rate (Fig. 1). The frequencies of myocyte beating for cell concentrations of 1.0×10^5 cells/ml (3.0×10^5 cells/well) or greater showed similar profiles, with initial rapid increase, peak values reached by day 5 (ranging from 254.8 ± 56.8 to 291 ± 61.3 beats/min), and gradual decreases until day 21. The highest cell concentration tested (3.0×10^5 cells/ml) maintained a maximum beating rate for about 14 days, which was twice as long as that of any other cell density. The beating frequency in the preparation

Fig.2. Cardiac myocyte beating rate with and without co-cultured fibroblasts. A myocyte and fibroblast concentration of 2.0×10^5 cells/ml was used and the proportion of fibroblasts varied from 0 to 67% (ml myocyte: ml fibroblasts) as follows; 3 : 0 (group C), 2 : 1 (group 2-1), 1.5 : 1.5 (group 1-1), 1 : 2 (group 1-2). The total number of cells/well was 6.0×10^5 . Each data point represents the mean $\pm$ standard deviation (SD) of 6 experiments.

Fig.3. Cardiac myocyte beating rate of group 2-1 compared to that of group C. Each data point represents the mean $\pm$ SD of 6 experiments. **: $p < 0.025$ and ***: $p < 0.005$.

containing 0.7×10^5 myocytes/ml (2.1×10^5 cells/well) increased on day 5 (123.7 ± 42.5 beat/min.), peaked on day 12 (476.7 ± 30.7 beat/min.), and then gradually decreased until day 21. The beating in the preparation containing 0.5×10^5 myocytes/ml (1.5×10^5 cells/well) did not increase until after day 10, peaked on day 12 (150.2 ± 28.6 beat/min.), and then decreased. These latter two cell

concentrations showed delayed onset of beating rate increases and had peak rate levels significantly lower than those of the other concentrations tested, with the exception of the lowest concentration tested (0.3×10^5 cells/ml) which showed no rate increase.

Cardiac Myocyte Beating Co-cultured With

Fig. 4. Cardiac myocyte beating rate of group 1-1 compared to that of group 2-1. Each data point represents the mean $\pm$ SD of 6 experiments. *: $p < 0.05$

Fig. 5. Cardiac myocyte beating rate of group 1-2 compared to that of group 1-1. Each data point represents the mean $\pm$ SD of 6 experiments. ***: $p < 0.005$

Fibroblasts

The beating rate in control group C (6.0×10^5 myocytes only) increased rapidly, reached a peak value on day 5 (266.2 ± 58.2 beat/min.), and then decreased gradually by day 21. The beating rate in group 2-1 (2.0×10^5 fibroblasts/well) was significantly lower than that in group C with a delayed beating rate increase and a peak value on day 7 (135.1 ± 46.4 beat/min.) (Fig. 2 and 3). The beating rate in group 1-1 (3.0×10^5

fibroblasts/well) was significantly less than that of group 2-1, with a peak value on day 7 (85.9 ± 24.9 beat/min.) (Fig. 2 and 4). The beating rate of group 1-2 (4.0×10^5 fibroblasts/well) did not increase and had no peak value throughout the 21 day interval (Fig. 2 and 5). When compared to the control myocytes without co-cultured fibroblasts, the beating rate of 1.0×10^5 cells/ml was similar to that of group C. However, the myocyte peak beating rates in groups 2-

Fig. 6. Photographs ($\times 50$) of cardiac myocytes three days after the initiation of culture with and without co-culture with fibroblasts. Top photograph: group C, bottom photograph: group 2-1

Fig. 7. Photographs ($\times 50$) of cardiac myocytes three days after the initiation of culture with co-cultured fibroblasts. Top photograph: group 1-1, bottom photograph: group 1-2

1, 1-1, and 1-2 (135.1 ± 46.4 vs 254.8 ± 56.8 , 85.9 ± 24.9 vs 254.8 ± 56.8 , and 46.1 ± 14.6 vs 176.7 ± 33.6 beat/min, respectively) were significantly suppressed compared to the matching control myocyte study groups.

Cardiac Myocyte Hypertrophy Co-cultured With Fibroblasts

After three days in culture, the surface areas of the myocytes co-cultured with fibroblasts were significantly increased, as compared to those of control myocytes (group C), and were also shown to increase with increasing numbers of fibroblasts (Mean myocyte surface areas in groups 2-1, 1-1 and 1-2 were 151.4 ± 27.3 , 193.0 ± 39.7 , and 210.8 ± 51.8 percent of control, respectively). In the corresponding myocyte-only cultures, myocyte hypertrophy increased with decreasing cell number, but was not significantly different from control group C (4.0×10^5

myocytes/well: 105.3 ± 9.2 percent of control, 3.0×10^5 cells/well: 112.6 ± 13.9 , 2.0×10^5 cells/well: 132.1 ± 21.2). In addition, each group which had been co-cultured with fibroblasts showed significantly greater hypertrophy than the myocyte-only controls (vs group 2-1, $p < 0.005$; vs group 1-1, $p < 0.001$; vs group 1-2, $p < 0.01$). Among the co-culture groups, the myocyte area in group 1-2 was significantly greater than that in group 2-1 ($p < 0.05$) (Fig. 6, 7 and 8). Accordingly, the presence of fibroblasts induced significantly greater myocyte hypertrophy. Myocytes cultured in the presence of fibroblast-conditioned media only were unaffected and behaved similarly to cells in control group C (data not shown).

DISCUSSION

In this series of experiments, cell compo-

Fig.8. Cardiac myocyte hypertrophy with co-cultured fibroblasts three days after the initiation of culture. The surface areas of myocytes were determined from photographs of each group using a digitizer. Myocytes in group C were used as a control and hypertrophy was expressed as a percentage of control. Each data point represents the mean $\pm$ SD of 6 experiments.

nents were isolated from neonatal rat ventricles, and myocytes, either alone or in co-culture with fibroblasts, were evaluated for beating rate changes and growth hypertrophy. Initially, myocyte-only studies showed that myocytes formed confluent monolayers by the fourth culture day, and at that time almost all of the myocytes beat synchronously and steadily at a constant frequency. These observations were consistent for cell concentrations equal to or greater than 1.0×10^5 cells/ml (3.0×10^5 cells/well). However, at 0.7×10^5 cells/ml (2.1×10^5 cells/well) or less, the increase in the myocyte beating rate was either markedly delayed or not induced at all. When cardiac fibroblasts were co-cultured with myocytes, the myocyte beating rate was significantly suppressed and this suppression increased with an increasing proportion of fibroblast cells. In co-culture group 1-1, which contained 3.0×10^5 cells/well of both myocytes and fibroblasts, the beating was suppressed strongly, as compared to the corresponding control myocyte group. Moreover, no beating rate increase was observed in group 1-2, which contained 2.0×10^5 myocytes/well, although the control group exhibited a moderate increase in the beating frequency.

Cardiac myocyte hypertrophy, on the other hand, was significantly accelerated in the presence of fibroblasts. Increased myocyte surface area correlated positively with an increasing percentage of fibroblasts, such that in group 1-2, the hypertrophy was twice as great as that in control myocytes. Thus, cardiac fibroblasts from neonatal rat ventricles suppressed the cardiac myocyte beating rate and accelerated myocyte hypertrophy.

While the mechanisms by which these phenomena occur are not well known, three explanations may be postulated. First, these effects may be induced via myocyte-fibroblast connections, or by gap junctions between myocytes and fibroblasts. Cardiac myocyte gap junctions have a well-defined role as low-resistance pathways for propagation of the action potentials and current transfer of small molecular size substances. In addition, conduction velocity is directly related to junction conductance, which affects both the upstroke velocity and amplitude of the action potential¹³⁻¹⁵. Therefore, fibroblasts may interfere with myocyte-myocyte gap junctions by a dilutional or mass action effect, thus accounting for the reduced or delayed myocyte beating rate increase. Second, these effects may be

induced by extracellular matrix (ECM) components produced by the fibroblasts. The synthesis of ECM components by specific cell types in the heart is well documented, and several reports indicate that fibroblasts are major producers of interstitial collagens in the late fetal and neonate stages.^{3,4} Collagens and other matrix components have been shown to be critical in the regulation of developmental processes, including differentiation and cell growth.^{16,17} Third, the effects may be induced by substances secreted from the fibroblasts. However, preliminary studies in our laboratory showed that myocyte beating rate and hypertrophy were unaffected when cultured with fibroblast-conditioned media from the initial stages of culture. Therefore, the actual presence of the fibroblasts themselves is apparently required to induce the observed changes (unpublished data). However, further experiments will be needed to elucidate the precise mechanisms by which fibroblasts regulate myocyte growth and beating, because our data could not exclude the possibility that the fibroblasts may secrete unstable and active substances in the culture media.

Taken together, these observations support a process which has been called physiological hypertrophy,¹⁸ in which the neonatal heart undergoes rapid growth in response to several physiological stimuli, which is then translated into an increase in both pressure (contraction) and volume (hypertrophy). In addition, protein synthesis in myocardium increases rapidly during the neonatal period in response to the increased work load.¹⁹ Moreover, it has been reported that beating and hypertrophy in neonatal rat myocytes are independently regulated in vitro.²⁰ Accordingly, we believe that the suppression of beating may reflect an increase in myocyte contractility and that acceleration of myocyte hypertrophy may stimulate growth. In contrast, several other investigators have suggested that myocyte and nonmyocyte cells grow independently of each other.^{2,21-23} Thus, further studies will be necessary to elucidate the mechanisms by which myocytes and fibroblasts interrelate during this crucial perinatal period of development.

In conclusion, the suppression of myocyte beating rate and the acceleration of hyper-

trophy may be characteristic expressions of myocyte differentiation and growth in an in vitro culture system, which suggests that fibroblasts play an important role in modulating cardiac myocyte development during the neonatal period.

REFERENCES

1. BORG TK: Development of the connective tissue network in the neonatal hamster heart. *Am J Anat* 1982; **165**: 435-444
2. WEBER KT, BRILLA CG: Pathological hypertrophy and cardiac interstitium. Fibrosis and renin-angiotensin-aldosterone system. *Circulation* 1991; **83**: 1849-1865
3. BORG TK, JOHNSON LD, LILL PH: Specific attachment of collagen to cardiac myocytes. *Dev Biol* 1983; **97**: 417-423
4. EGHBALI M, CZAJA MJ, ZEYDEL M, WEINER FR, ZERN MA, SEIFTER S, BLUMENFELD OO: Collagen chains mRNAs in isolated heart cells from young and adult rats. *J Mol Cell Cardiol* 1988; **20**: 267-276
5. KARDAMI E, FANDRICH RR: Basic fibroblast growth factor in atria and ventricles of the vertebrate heart. *J Cell Biol* 1989; **109**: 1865-1875
6. SASAKI H, HOSHI H, HONG YM, SUZUKI T, KATO T, SAITO M, YOKI H, KARUBE K, KONNO S, ONODERA M, SAITO T, AOYAGI S: Purification of acidic fibroblast growth factor from bovine heart and its localization in the cardiac myocytes. *J Biol Chem* 1989; **264**: 17606-17612
7. SPIRITO P, FU YM, YU ZX, EPSTEIN SE, CASCCELLS W: Immunohistochemical localization of basic and acidic fibroblasts growth factors in the developing rat heart. *Circulation* 1991; **84**: 322-332
8. KLEIN I, DAOOD M: Regulation of the growth of nonmuscle heart cells in culture. *In Vitro Cell Dev Biol* 1985; **21**: 693-696
9. SUZUKI T, OHTA M, HOSHI H: Serum-free, chemically defined medium to evaluate the direct effects of growth factors and inhibitors on proliferation and function of neonatal rat cardiac muscle cells in culture. *In Vitro Cell Dev Biol* 1989; **25**: 601-606
10. BLONDEL B, ROIJEN I, CHENEVAL JP: Heart cells in culture: a simple method for increasing the population of myoblasts. *Experientia* 1971; **27**: 356-358
11. POLINGER IS: Separation of cell types in embryonic heart cell culture. *Exp Cell Res* 1970; **63**: 78-82
12. YAGEV S, HELLER M, PINSON A: Change in cytoplasmic and lysosomal enzyme activities in cultured rat heart cells: the relationship to cell differentiation and cell population in culture. *In Vitro Cell Dev Biol* 1984; **20**: 893-898
13. PAGE E, MANJUNATH CK: Communicating junctions between cardiac cells. In: FOZZARD HA, HABER E, JENNINGS RB, KATZ AM, MORGAN HE (eds): *The Heart and Cardiovascular System*. New York, Raven Press, 1986,

- 537–600
14. SEVERS NJ, SHOVEL KS, SLADE AM, POWELL T, TWIST VW, GREEN CR: Fate of gap junctions in isolated adult mammalian cardiomyocytes. *Circ Res* 1989; **65**: 22–42
15. LUKE RA, BEYER EC, HOYT RH, SAFFITZ JE: Quantitative analysis of intracellular connections by immunohistochemistry of the cardiac gap junction protein connexin-43. *Circ Res* 1989; **65**: 1450–1457
16. BLUMENFELD OO, SEIFTER S: Biochemistry of connective tissue with special emphasis on the heart, in Cardiac myocyte connective tissue interactions in health and disease. ROBINSON TF, KINNE RKH (eds): Basel, Karger, 1990, 5–36
17. BORG TK, TERRACIO L: Interaction of the extracellular matrix with cardiac myocytes during development and disease, *In*: ROBINSON TF, KINNE RKH (eds): Cardiac myocyte connective tissue interactions in health and disease. Basel, Karger, 1990, 113–129
18. WILHMAN-COFFELT J, PARMLY WW, MASON DT: The cardiac hypertrophy process. Analysis of factors determining pathological vs physiological development. *Circ Res* 1979; **45**: 697–708
19. PETERSON CJ, WHITMAN V, WATSON PA, SCHULER HG, MORGAN HE: Mechanisms of differential growth of heart ventricles in newborn pigs. *Circ Res* 1989; **64**: 360–369
20. SIMPSON PC, LONG CS, WASPE LE, HENRICH CJ, ORDAHL CP: Transcription of early developmental isogenes in cardiac myocyte hypertrophy. *J Mol Cell Cardiol* 1989; **21**: 79–89
21. LUND DD, TWIETMEYER AT, SCHMID PG, TOMANEK RJ: Independent changes in cardiac muscle fibers and connective tissue in rats with spontaneous hypertension, aortic constriction and hypoxia. *Cardiovasc Res* 1979; **13**: 39–44
22. WEBER KT, CLARK WA, JANICKI JS, SHROFF SG: Physiologic versus pathologic hypertrophy and the pressure-overload myocardium. *J Cardiovasc Pharmacol* 1987; **10**: S37–S49
23. RUSKOAHO HJ, SAVOLAINEN ER: Effects of long-term verapamil treatment on blood pressure, cardiac hypertrophy and collagen metabolism in spontaneously hypertensive rats. *Cardiovasc Res* 1985; **19**: 355–362