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A Simple Isolation Method and Primary Culture of Neonatal Rat Cardiac Myocytes

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ABSTRACT **For long-term maintenance of functional cardiac myocytes in primary culture, we developed a simple method for isolation of cells from neonatal Wistar rat ventricles. The highest viable cell yield was obtained when using 13 to 20 ventricles for one isolation and employing collagenase as the tissue-digesting enzyme at 200 U/ml. Examination of the seeding density revealed that, at a density of 1.5×10^6 cells/35-mm dish, the cells were arranged into long, thick, fibrous masses exhibiting synchronous contraction with a constant rate for up to 20 days in culture, indicating that this density was appropriate for maintaining functional cardiac myocytes in culture. The cells at Day 7 of culture were stimulated by addition of 1×10^{-8} M isoproterenol, leading to an increase in the beating rate, but the beating rate of the cells at Day 14 decreased somewhat by the addition of isoproterenol. Electron microscopic examinations of the myocytes at Day 3 clarified the reconstitution of the myofibrils with the formation of Z-lines, accompanied by the formation of intercalated discs. The formation of these contractile structures was maintained throughout the 20 days in primary culture. The intermediate filaments, desmin and vimentin, were detected in the cultured myocytes by the immunofluorescence method, showing a cross-striated pattern. It is likely that both intermediate filaments were involved in the reconstitution and maintenance of the striated structures of the myofibrils. These results suggest that the neonatal rat cardiac myocytes isolated and cultured by the present method are useful for an in vitro experimental system of the heart.**

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Key Words: Neonatal rat cardiac myocytes, Cultured myocytes, Seeding density, Myofilaments, Intermediate filaments

1. Introduction

Primary culture of neonatal rat myocardial cells is an attractive experimental heart model. It has been used for the studies on cardiac development[1-3], cardiac metabolism[4], the research for adrenergic receptors[5-7] and the examinations of cardiac ischemia, inflammatory responses and myocytic hypertrophy[8-11]. Several proteolytic enzymes have been reported to be useful for digestion of neonatal rat ventricular tissues[1-16]. The first purpose of the present study is to determine which of the enzymes, trypsin and collagenase, and how many concentrations of the enzymes might be suitable for obtaining intact cardiac myocytes and therefore might be beneficial for the longevity of the isolated cells in primary culture. We also examined electron microscopically whether or not structual differences could be

induced by the different seeding density. The third purpose is to investigate to see whether the primary culture of the cells isolated and seeded under the appropriate conditions could be useful for an in vitro experimental system of the heart. It is shown that the myocytes continued spontaneously a synchronous contraction and that the cells maintained the fine structures characteristic of myocytes through 20 days in primary culture. The cells cultured for 7 days are stimulated by isoproterenol, resulting in an increase in the beating rate.

2. Materials and Methods

Materials

Dulbecco's modified Eagle's minimum essential medium (DMEM), collagenase, trypsin and fetal bovine serum (FBS) were obtained from Dainihon Seiyaku Co. (Ohsaka, Japan), Wako Chemical Co. (Tokyo, Japan), Sigma Chemical Co. (St. Louis, USA) and Flow Laboratories (Island, UK), respectively. All antibodies and chemicals for the immunocytochemistries, e. g., mouse monoclonal IgG to swine desmin and vimentin, biotinated $F(ab')_2$ fragment of rabbit anti-mouse immunogloblin, FITC-conjugated streptoavidin, were from DAKOPATTS a/s (Glostrup, Denmark). l-Isoproterenol bitartrate (ISO) was obtained from Sigma.

Cell isolation method

Neonatal Wistar rats (2-5 days old) were employed for isolation of cardiac myocytes. Ten to 20 neonatal rats were decapitated. Their ventricles were aseptically separated and placed in Hanks' balanced salt solution (HBSS) containing 40 U/m*l* penicillin G and 40 μg/m*l* streptomycin sulfate. The ventricles were cut into two fragments and treated with 200 U/m*l* collagenase in 30 m*l* of Ca^{++} and Mg^{++}-free HBSS buffered with 10 mM N-2-hydroxyethylpiperazine-N'-ethansulfonic acid (HEPES), pH 7.4 [medium A]. Medium A was gassed with 5% CO_2+95% O_2 by shaking in a water-bath incubator at 37℃ for 30 min. During this incubation period, most blood cells were washed out from the ventricle fragments. The fragments were then minced with fine scissors and digested in the fresh medium A gassed with 5% CO_2+95% O_2 at 37℃ for 15 min. The solution containing dissociated cells was filtrated through a mesh screen with a pore size of 150 μm, and the residual tissues were treated with the fresh medium A again. This digestion process was repeated three times. Each filtrate was centrifuged at 50×g for 5 min. The sedimented cells were collected and suspended in 15 m*l* of DMEM containing 10% heat-inactivated (56℃ for 30 min) FBS, 5 mM HEPES, 40 U/m*l* penicillin-G and 40 μg/m*l* streptomycin sulfate [medium B]. The cells were seeded on 60×15 mm tissue culture dishes (Corning Glass Works, Corning, NY) and placed in a CO_2-incubator for 30 min. During this pre-plating period, nonmyocardial cells have been reported to attach rapidly[6,13-16]. After 30 min, the dishes were swirled gently and unattached cells (enriched with myocardial cells) were collected and suspended in 30 m*l* of medium B. The number and viability of the dissociated cells were determined by a trypan blue exclusion test. The isolated cells were seeded on 35×15 mm tissue culture dishes (Corning) coated with collagen type I (rat tail collagen) at the seeding densities of 0.5×10^6, 1.5×10^6 or 2.5×10^6 cells/1.5 m*l*/dish, followed by maintaining in a CO_2-incubator. The cardiac myocytes have been reported to attach to culture substrata within 24 hours[13,14]. The medium was therefore aspirated off at Day 1 of culture and 1.5 m*l*/dish of the fresh medium B was added gently. The cultured cells were then fed the fresh medium B every 2 to 3 days.

Measurement of beating rate and the effect of isoproterenol

Measurement of beating rate of the myocytes seeded at a density of 1.5×10^6 cells/dish was done using the phase contrast microscope equipped with a box-type incubator maintained at 37℃. In this incubator, the chamber gassed with 5% CO_2+95% O_2 was placed for maintaining the cells in a culture

dish. After the incubation of the cultured myocytes in serum-free DMEM (pH 7.4) for 15 min, the number of beating/30 sec of the cells was counted.

Preliminary studies revealed that the spontaneous and continual contraction of the cultured myocytes occurred in the medium adjusted pH in the range of 7.4 to 7.5. Isoproterenol (ISO) was dissolved in serum-free DMEM at 1×10^{-8} M and adjusted pH to 7.4. The counting of the beating rate started at 1 min after addition of the ISO-containing medium.

Morphological examinations

Fine structures of the isolated or cultured myocytes were examined electron microscopically by the previously reported method [17]. Development of the intermediate filaments, desmin and vimentin, was also examined by the immunofluorescent staining method according to that of Furukawa, K. *et al.* [18].

3. Results

Yield and Viability

The highest yield and viability of the isolated cells per one ventricle was obtained when 13-20 ventricles were used for one isolation (Table 1). Collagenase at 200 U/m*l* had a similar effect as collagenase at 300 U/m*l* for the isolation of viable cardiac myocytes, but the viability was higher at 200 U/m*l* than at 300 U/m*l* (Table 2).

Investigation under the phase contrast microscope

The freshly isolated cells were round in shape and they did not show any beating (Fig. 1-a). Within 18 to 24 hours of culture, many of the cells became flatten and attached ot the dish. As shown in Fig. 1-b, three cell types were found in the culture; cardiac myocytes, fibroblast-like cells and epithelioid cells. When seeded at 1.5×10^6 cells/dish, over 80% of the cells at Day 3 were beating cardiac myocytes. These cells were arranged into long, thick, fiber-like masses as culture aged (Fig. 1-c) and showd synchronized beating. At the seeding density of 0.5×10^6 cells/dish, the majority of attached

Table 1 *Changes in the yield and viability of the isolated cells : Effects of the number of ventricles employed for one isolation procedure.*

The number of ventricles	n	Yield (viable cells/ventricles)	Viability (%)
5-10	6	$1.417\pm0.120\times10^6$	71.3 ± 2.16
13-20	6	$2.260\pm0.060\times10^{6a}$	86.5 ± 0.98^b

Collagenase was applied to the tissue digestion at 200 U/m*l*. n; number of examinations. The values are represented as the means±S. E. (a; $p<0.01$, b; $p<0.05$. The values indicated are significantly different from those of the experiments used 5 to 10 ventricles for one isolation.).

Table 2 *Changes in the yield and viability of the isolated cells : Effect of trypsin and collagenase used as the tissue digestion enzyme.*

Enzyme	n	Yield (viable cells/ventricle)	Viability (%)
Trypsin (0.015%)	4	$1.076\pm0.362\times10^6$	61.5 ± 4.21
Trypsin (0.030%)	6	$1.465\pm0.248\times10^6$	74.3 ± 3.19
Collagenase (200U/m*l*)	6	$2.260\pm0.060\times10^6$	86.5 ± 0.98^a
Collagenase (300U/m*l*)	4	$2.520\pm0.141\times10^6$	69.0 ± 1.62

The number of ventricles employed in each examination was 13 to 20. n; the number of examinations. The values are represented as the means±S. E. (a; significantly different from the values of the experiments used collagenase at 300 U/m*l*, $p<0.01$).

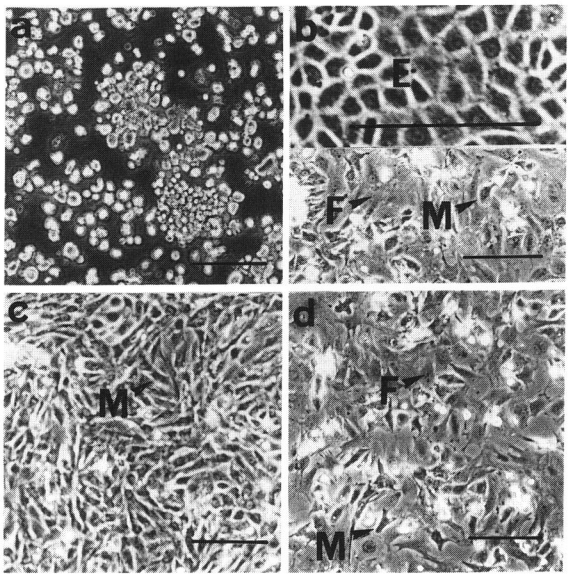

Fig. 1 The phase contrast microscopic photographs of the cultured cells. a, Rounded cells attached to collagen-coated substratum, 2.5 hours after seeding. b, Three types of the cells observed in primary culture at Day 7. M ; myocytes, F ; fibroblats-like cells, E ; epithelioid cells. c, The cardiac myocytes at Day 3 in primary culture. The seeding density is 1.5×10^6 cells/dish. d, The cardiac myocytes at Day 3 in primary culture. The seeding density is 0.5×10^6 cells/dish. The bars represent 150 μm.

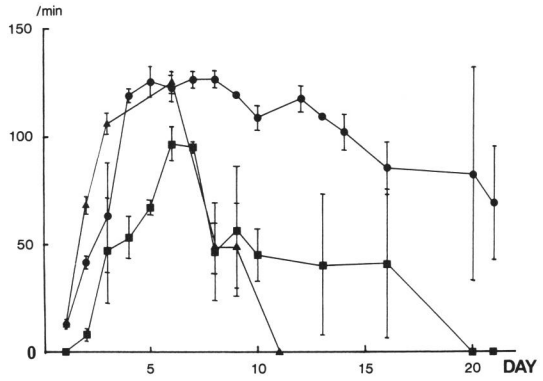

Fig. 2 Changes with culture age in the beating rate of the cardiac myocytes seeded at the densities of 0.5×10^6 cells/dish (■), 1.5×10^6 cells/dish (●), 2.5×10^6 cells/dish (▲).

cells were nonmyocardial cells, mainly fibroblasts-like cells (Fig. 1-d). Although a few beating cells were found in the low density cultures, their beating was not synchronized.

Changes in the beating rate

The beating cardiac myocytes were observed already 18 hours after seeding. When seeded at 1.5×10^6 cells/dish, the beating rate increased with culture days (Fig. 2), and at Days 4 to 6 of culture, it reached at a relatively constant rate of 90-130/min. At the density of 2.5×10^6 cells/dish, the beating rate of the myocytes decreased after 7 days of culture and many of the cells were detached at Days 10 to 15 of culture. At 0.5×10^6 cells/dish, a few beating myocytes survived up to 20 days in culture (Fig. 2), without showing synchronization of the contraction. The cardiac myocytes seeded at the density of 1.5×10^6 cells/dish continued beating constantly up to 20 days in culture (Fig. 2).

Electron microscopic examinations

The isolated cardiac myocytes showed myofilaments run at random in direction (Fig. 3-a). With culture aged, the rearrangement of myofilaments to constitute myofibril structures became apparent in the cultured myocytes seeded at the density of 1.5×10^6 cells/dish (Fig. 3-b, c, d). At Day 3 of culture, the reconstituted myofibrils were detected in the myocytes, accompanied by the formation of a striated pattern, namely Z-lines. Moreover, a part of the myofibrils in the adjoining myocytes were found to connect with the intercalated disc structures which were formed coopereatively by the adjacent plasma membranes (Fig. 3-b). In the myocytes seeded at 0.5×10^6 cells/dish, the reconstitution of myofibrils was not induced so well as compared with the cells seeded at 1.5×10^6 cells/dish, and even Day 7 of

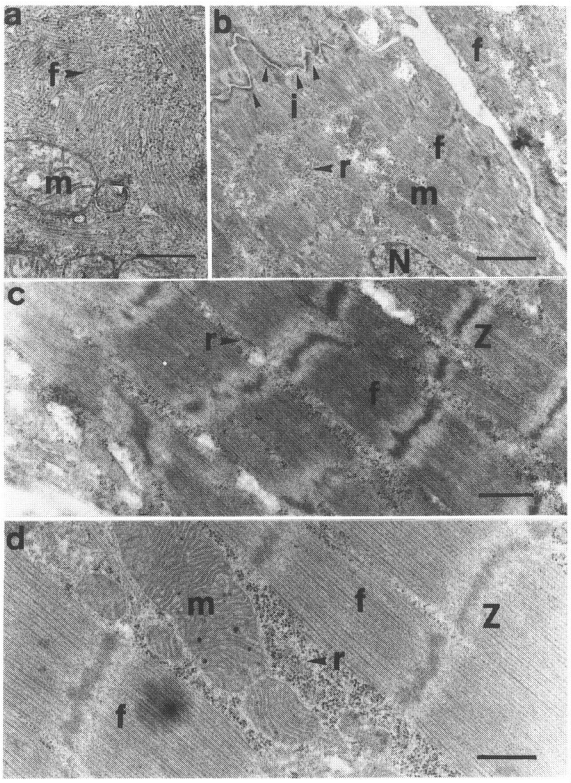

Fig. 3 Electron photomicrographs of the cardiac myocytes in primary culture, seeded at 1.5×10^6 cells/dish. N ; nucleus, m ; mitochondria, r ; ribosomes, f ; myofilaments, Z ; Z-lines, i ; intercalated discs. a, Day 0. Myofilaments are shown to run at random in direction in the cell. b, Day 3, c, Day 7, d, Day 14. The bars represent 2 μm in a and b, 500 nm in c and d. Re-arrangement of myofilaments leading to the formation of typical myofibrils in shown.

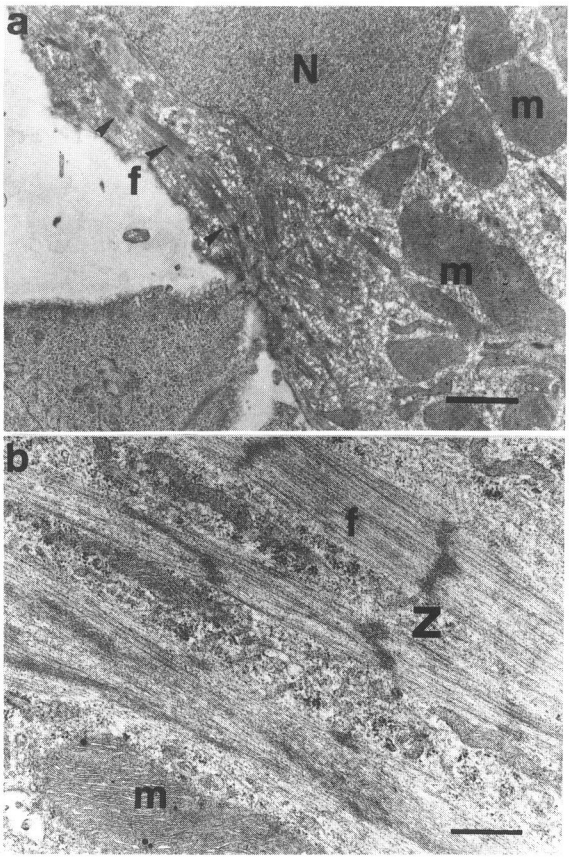

Fig. 4 Electron photomicrographs of the cardiac myocytes in primary culture, seeded 0.5×10^6 cells/dish. N ; nucleus, m ; mitochondria, f ; myofilaments. a, Day 3, b, Day 7. Poorly developed Z-lines (Z) are seen in the cell at Day 7. The bar represent 2 μm in a and 500 nm in b.

culture, only poorly developed myofibrils were observed in the cells of the low density cultures (Fig. 4-a, b).

Effect of isoproterenol

The responses of the myocytes to ISO were clearly detected at Day 7 of culture as a positive chronotropic effect (Fig. 5). This effect of ISO was not found in the myocytes at Day 14 of culture (Fig. 5), in spite of having well-developed myofilaments (Fig. 3-d).

Expression of the intermediate filaments

At Day 1 of culture, filamentous structures positive in desmin or vimentin were not detected (in the myocytes) by the immunofluorescent method (Fig. 6-a, c). The cardiac myocytes at Day 5 of culture exhibited the cross striated filamentous structures positive in desmin and vimentin (Fig. 6-b, d). The fibroblasts-like cells showed vimentin-positive filaments around their nuclei (Fig. 6-b), but no filaments positive in desmin were found.

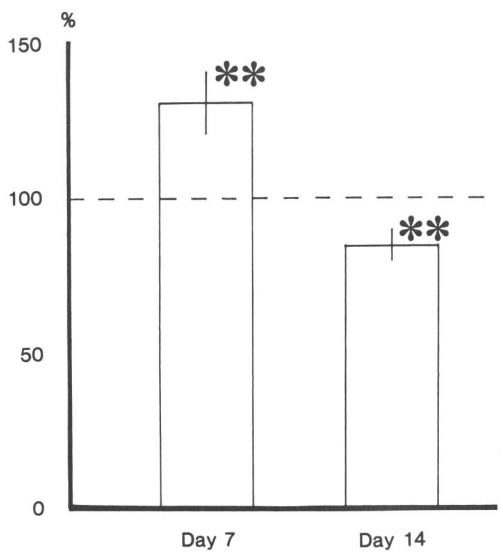

Fig. 5 Effect of 1×10^{-8} M isoproterenol on the beating rate of cardiac myocytes at Days 7 and 14 of primary culture. The percentage indicates the relative value of the beating rate of the cells exposed to isoproterenol versus that of the corresponding unexposed cells. Asterisks mean significant differences, $p<0.01$.

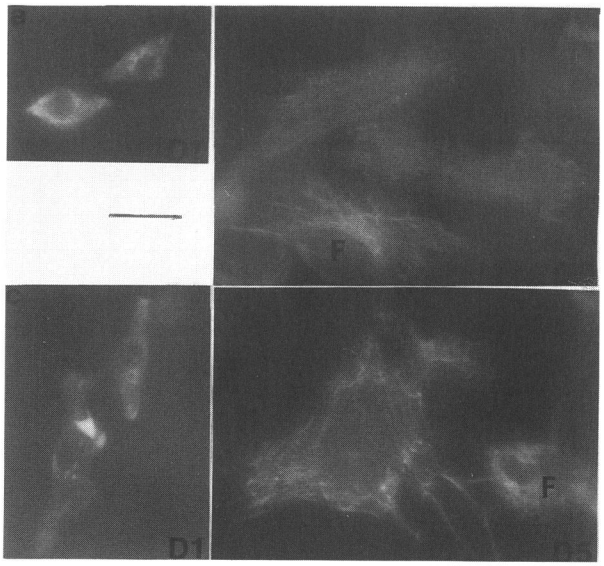

Fig. 6 Immunofluorescence photomicrographs of the cardiac myocytes exposed to the anti-vimentin antibody (a, b) and anti-desmin antibody (c, d). a and c; Day 1. b and d; Day 5. F; fibroblast-like cells. The bars represent 10 μm.

4. Discussion

To establish a long-term maintenance of primary culture system of the cardiac myocytes of neonatal rat ventricle, several conditions affecting yield and viability of the cells were examined. It is shown that the highest yield and viability per one ventricle was obtained when 13 to 20 ventricles were used for one isolation and collagenase was applied at 200 U/m*l* to the tissue dissociation. Trypsin was found to be not so effective for the dissociation, even when used at the concentrations which were reported previously to be optimal[6,7,13-16]. It seems likely that the combined use of collagenase and trypsin[1,19] may not necessarily provide higher yield and viability than the single use of collagenase.

An interference of non-myocardial cells to the maintenance of cardiac myocytes in primary culture have not been a disregarded problem[2,6,7,13-15] and therefore, various attempts to eliminate non-myocytic cells have been reported[13-16,20]. We also employed the pre-plating method as reported previously[15] and succeeded to eliminate a large number of non-myocardial cells. However, complete exclusion of these cells from the cultures was not accomplished. In fact, at the low seeding density of 0.5×10^6 cells/dish, fibroblasts-like cells became dominant as culture aged and the rearrangement of myofilaments to myofibrils of myocytes was not accomplished. On the other hand, a mass of the beating myocytes was observed dominantly in the cultures seeded at density of 1.5×10^6 cells/dish. These functional myocytes maintained well-developed myofibrils, accompanied by the formation of Z-lines and intercalated discs.

It is apparent that the cultures seeded at the density of 1.5×10^6 cells/dish is of great advantage to maintain functional cardiac myocytes and to decrease effectively the possibility of interference of non-myocardial cells to the development of function and structure of the myocytes in the primary culture.

The growth of non-myocardial cells may be inhibited at the seeding density of 1.5×10^6 cells/dish by a mechanism of the contact inhibition as reported previously[2]. Additionally, the seeding density of 2.5×10^6 cells/dish is considered to be a over-density leading to the detachment of the myocytes and therefore not available for the present primary culture system of cardiac myocytes.

W. A. Clark *et al.*[21] have reported that the reappearance of striated myofibrils in cultured adult feline cardiac myocytes results in the spontaneous initiation of beating. The synchronous beating but not yet accompanied by a constant rate was observed in the present study even for the cells 18 hours after seeding, while the reconstitution of the myofibrils showing a striated pattern was detected apparently in the myocytes at Day 3 of culture. Therefore, in the case of neonatal rat cardiac myocytes, development of myofibrils in primary culture may be preceded by the initiation of synchronous beating.

The response of the cardiac myocytes to ISO was clearly detected at Day 7, but not at Day 14 of culture. Nevertheless, the myocytes at Day 14 were comparable to those at Day 7 in having a well-developed myofibrils and showing synchronous beating with a constant rate. It is possible that the differences in the function of adrenergic receptors and in the membrane fluidity or viscosity may exist between the cells at Days 7 and 14 of cultures. Further studies are required.

The intermediate filaments (IFs) are classified on the basis of their amino acid sequences into four broad categories[22]. Vimentin and desmin are those of Type II IFs. Vimentin is widely distributed in the cells of mesenchymal origin such as fibroblasts, and has been expressed often in cultured cells[22]. Desmin has been found characteristically in striated and smooth muscle cells or cardiac myocytes[23-26]. Vimentin as well as desmin has also been detected in developing cardiac muscles[23] and in embryonic cardiac muscle cells in culture[24]. In our primary culture system, the cardiac myocytes at Day 5, but not at Day 1 showed a striated distribution of desmin and vimentin, suggesting a close association of these IFs with the formation of contractile apparatus including Z-lines. A. C. Nag *et al*[24]. have reported that vimentin and desmin gradually increased in concentration as the cardiac myocytes matured in culture. Interestingly, B. L. Granger and E. Lazarides[25] have revealed coexistence of these IFs at the periphery of the Z-lines of skeletal muscle cells. In the neonatal rat cardiac myocytes in the culture, these two types of IFs may be synthesized by the manner similar to that of the cells *in vivo* and may have an essential role on the reconstitution and long-term maintenance of Z-line and myofibril structures.

In conclusion, we established in the present study the method of isolation of cardiac myocytes from neonatal rat ventricles, and our primary culture system of the cells is suggested to be a useful tool for investigating physiological and pharmacological states of the heart *in vitro*.

5. Summary

(1) The isolation method of cardiac myocytes from neonatal rat ventricles is established.

(2) A long-term maintenace of the functional myocytes in primary culture is demonstrated.

(3) Histological change in the cultured cardiac myocytes are investigated with the electron microscopic and immunofluorescent methods.

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新生仔ラット心筋細胞の分離・培養について

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培養心筋細胞を長期間維持するために，我々はWistar系新生仔ラットの心室から，より簡単な方法を用い心筋細胞の分離をおこなった．一度の操作では，13から20個の心室を200 U/mlのcollagenaseにより分離した際，最も高い収量・収率を得ることができた．また心筋細胞は，その播種密度を1.5×10^6 cells/dishとしたとき自動拍動を20日間も続ける長く太い線維状の塊を形成し，このことから，この播種密度は今回報告する系において最適のものと考えられた．培養第7日において，1×10^{-8} MのIsoproterenolにより心筋細胞の拍動数は増加したが，培養第14日では拍動数は減少した．電子顕微鏡による観察では，培養第3日においてZ-line形成を伴った筋線維の再構成が認められ，intercalated discsの形成も認められた．これら収縮構造は培養第20日でも確認された．中間径フィラメントであるdesminやvimentinは蛍光抗体法により格子状に認められた．これは，両方の中間径フィラメントが，格子状の筋線維の再構成と維持に役割をはたしていることを示していると考えられた．以上の結果より，我々の方法により分離培養された心筋細胞は心臓のin vitroの実験系として役立つものと考えられた．