Beating behavior of primary neonatal cardiomyocytes and cardiac-differentiated P19.CL6 cells on different extracellular matrix components

Abstract  Stem cell-based therapy in cardiac tissue engineering is an emerging field that shows great potential for treating heart diseases. However, even preliminary issues, such as the ideal niche for cardiomyocytes, have not been clarified yet. In the present study, the effects of extracellular matrix (ECM) components on the beating duration of neonatal rat cardiomyocytes (RCMs) and on the cardiac differentiation of P19.CL6 carcinoma stem cells were studied. RCMs were cultured on gelatin-, fibronectin-, and collagen type I-coated dishes and on noncoated polystyrene dishes, and their beating rate, beating duration, and cardiac gene expression were evaluated. The beating period and the expression of troponin T type-2 (TNNT2) and troponin C type-1 (TNNC1) of cardiomyocytes cultured on gelatin-coated dishes were longer and higher than for those on dishes with other coatings. For the cardiac differentiation of P19.CL6 cells, troponin T type-2 expression on gelatin- and fibronectin-coated dishes was five times that on collagen type I-coated dishes or polystyrene dishes 11 days after induction. These results indicate that a gelatin-coated surface has a high ability not only to maintain the cardiac phenotype but also to enhance cardiac differentiation.

Key words  Extracellular matrix · Cardiomyocyte · Beating · Differentiation

Introduction

Cardiac tissue engineering, such as cardiomyocyte transplantation for patients with ischemic heart disease or dilated cardiomyopathies, is of great potential therapeutic value to enhance the contractile function of the failing heart. Recently, fetal or neonatal rat cardiomyocytes were reported to form mature cardiac tissue in syngeneic hearts, acutely injured myocardium, and granulation tissue in the heart. However, the best cell sources for clinical cardiomyocyte transplantation are still under debate. In general, three types of potential cell sources have been proposed. One is the allogeneic source, including human embryonic stem cells or fetal allogeneic cardiomyocytes, but there still remain ethical issues. Another is the transgenic source. Genetically engineered animal cardiomyocytes have been studied in an attempt to reduce the rejection reaction in vivo, which is still a long-term problem in recipients.

The most promising cell source is the autogeneic one. Isolating cardiomyocytes from patients' hearts is unrealistic at present, and autologous skeletal muscle precursors, fibroblasts, or mesenchymal stem cells have been studied so far. However, since beating cardiomyocytes are more promising, we have been trying to differentiate bone marrow mesenchymal stem cells (BMSCs) into “beating” cardiomyocytes. There is no certain induction method for BMSC differentiation into beating cardiomyocytes. Many researchers have observed cardiac gene expression in MSCs treated with various inducers or passage numbers, but they do not beat spontaneously. Waki et al. and Makino et al. reported that murine BMSCs were differentiated to beating cardiomyocyte-like cells in vitro by exposing them to DNA-demethylating agent 5-azacytidine. This is in contrast with a report stating that functional cardiac cells and gene expression were not obtained by treatment with 5-azacytidine.

Producing autologous beating cardiomyocytes is thus an attractive goal for cell-based therapy. The crucial part is how to differentiate cells to cardiomyocytes in vitro and how to maintain the beating. Various microenvironments surrounding the cells (niches) play important roles not only...
in cell proliferation but also in cell differentiation. The effect of extracellular matrix (ECM) proteins such as collagen type I, collagen type IV, gelatin, laminin, fibronectin, Matrigel (a mixture of laminin, collagen type IV, heparan sulfate proteoglycans, and entactin), and Cardiogel (a mixture of collagen types I and III, glycoproteins, laminin, fibronectin, and proteoglycans) on cell viability, proliferation rate, and cardiomyocyte gene expression have been reported. However, the cardiomyocyte beating behavior has not fully been discussed.

In the present study, differentiation to beating cardiomyocytes and the beating duration of the cardiomyocytes were studied using two types of model cells. Murine embryonal carcinoma (EC) stem cells (P19.CL6), which are widely used for investigating cardiac differentiation, were treated with differentiation medium containing 1% dimethyl sulfoxide (DMSO) on various ECM proteins (collagen, gelatin, laminin, fibronectin, and proteoglycans) to evaluate cell viability, proliferation, and cardiomyocyte gene expression. Differentiation to beating cardiomyocytes was also investigated, along with intracellular cardiac marker genes (troponin T type-2 (TNNT2) and troponin C type-1 (TNNC1)) and skeletal muscle marker gene (troponin C type-2 (TNNC2)), which is reported to be expressed in the early developing heart. Any fundamental information obtained would be important for the cardiac differentiation of various stem cells, including autologous BMSCs.

**Materials and methods**

Cardiomyocytes

Cardiomyocytes were isolated from neonatal Sprague–Dawley rat heart (1 to 2 days old) by the collagenase digestion method with modifications. Institutional guidelines for the care and use of laboratory animals were observed. The hearts were removed and carefully minced with a scalpel blade into fragments and were rinsed several times with Hanks’ balanced salt solution (Sigma-Aldrich, St. Louis, MO, USA) to remove blood and cellular debris. The minced hearts were gently stirred in 50 ml collagenase solution (0.15 M Sodium Chloride (NaCl), 5.63 mM Potassium Chloride (KCl), 0.02 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.02 M Sodium Hydrogen Carbonate (NaHCO₃), 3.74 mM Calcium Chloride Dihydrate (CaCl₂·2H₂O), and 6.5 × 10⁻³ U collagenase (Wako, Osaka, Japan, Lot no: 06032W) at 37°C for 30 min. The resulting cell suspension was filtered through a nylon mesh (220 μm) and centrifuged at 78 g for 3 min.

Isolated cardiomyocytes (1.0 × 10⁶) were cultured in minimum essential medium alpha medium (α-MEM, Gibco, Invitrogen, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, MP Biomedicals, Eschwege, Germany, lot no: 7297H), and 100 IU/l penicillin–streptomycin (Wako, Osaka, Japan) on 60-mm gelatin-dihydrate (CaCl₂·2H₂O), and 6.5 × 10⁻³ U collagenase (α-MEM supplemented with 10% (v/v) FBS containing 1% DMSO (Wako). As a control experiment, P19.CL6 cells were cultured with α-MEM supplemented with 10% (v/v) FBS without 1% DMSO. The medium was changed every 2 days.

Measurement of action potential

Cultured plates on which beating colonies appeared were placed on the stage of an inverted phase-contrast optical microscope (ZEISS, Axiovert 135, Munich, Germany) and action potentials were measured immediately by a conventional microelectrode. The measurements were conducted after 1, 2, and 3 weeks of cultivation. Silicon-coated Ag wire (A-M System, Carlsborg, WA, USA, 250 μm bare, 330 μm coated) was used as the microelectrode. The microelectrode was set in a micromanipulator system (MONT-202D, Nikon Narishige, Tokyo, Japan) and connected to a bioelectric amplifier (AB-621G, Nihon Kohde, Osaka, Japan). The sensitivity and time constant of the bioelectric amplifier were set at 0.1 mV/div and 0.003 s. For the measurements, the microelectrode was advanced using the micromanipulator until it was attached to the membrane of beating cells. The voltage difference was amplified by the bioelectric amplifier and was displayed and recorded using Chart 5 software (AD Instrument, Bella Vista, Australia).

Total RNA isolation and reverse transcription

Total RNAs of cardiomyocytes and DMSO-treated P19.CLC6 cells cultured on various dishes were extracted by QuickGene RNA cultured cell kit S (Fujifilm Life Science, Tokyo, Japan) 4 weeks after culture and 11 days after culture, respectively.

First-strand cDNAs were synthesized using a mixture of oligo(dT)ₙ primer. Total cellular RNAs (200 ng) were incubated with 2.5 μM oligo(dT)₁₆ primer at 70°C for 10 min to denature the RNA secondary structure and then incubated at 4°C to let the primer anneal to the RNA. A given amount of 5X RT buffer (Toyobo, Osaka, Japan) and 2.5 mM Deoxynucleotide Triphosphate (dNTP) mixture (Takara Bio, Shiga, Japan) (4 μl) were added and incubated at 37°C for 5 min. The reverse transcriptase (100 Units, Toyobo) was added into the mixture and the reverse transcriptase (RT) reaction was extended at 37°C for 1 h. Then, the reac-