STUDIES ON ENGINEERED MICROENVIRONMENT FOR MANIPULATING FUNCTIONAL CELLS IN TISSUE REGENERATION

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Studies on engineered microenvironment for manipulating functional cells in tissue regeneration

(組織再生工学における細胞機能操作のための微小環境制御に関する研究)

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Preface

The work of this thesis has been carried out under the guidances of Professor Hiroshi Uyama at Department of Applied Chemistry, Graduate School of Engineering, Osaka University and Dr. Tetsuji Yamaoka, Director of Department of Biomedical Engineering, Advanced Medical Engineering Center, National Cardiovascular Center Research Institute, Japan.

The objectives of this thesis are to manipulate functional cells using well controlled *in vitro* microenvironments composed of (1) substrates on which cells are adhering, (2) chemical substances leading to cell differentiation, growth, or preservation, (3) cellular geometry such as monolayer, suspension, or spheroid, (4) culture medium (aeration, pH, nutrients, flow-condition and so on). This thesis focused on producing beating cardiac cells from MSC and on preserving isolated hepatocytes in bioartificial liver assist system.

The originalities of this thesis are the achievements in obtaining the beating cardiomyocytes from autogenic bone marrow massencymal stem cell, and the preservation of hepatocytes in bioartificial liver assist system at room temperature by control *in vitro* microenvironment.

The author wishes that the fundamental practicable results obtained in this work will provide useful information and suggestion for further development in tissue engineering and regenerative medicine.

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Contents

General Introduction

Tissue engineering and regenerative medicine

Tissue engineering and regenerative medicine aim to repair damaged tissue by replacing them with healthy and functional ones. Various somatic stem cells, embryonic stem cells, and specific functional cells were attracting great attention as their cell sources. Three types of potential cell sources were proposed; 1) allogeneic sources 2) xenogenic sources and, 3) autogeneic sources [1].

The microenvironment for the cells, which is called "niche", has been found to be sophisticated system and plays important roles in cell proliferation, differentiation, and functioning *in vivo*. Hence, how to design the artificial niche *in vitro* is one of the biggest challenges in tissue engineering. Scientists have been preparing various scaffolds with well designed interfaces between cells and scaffolds using synthetic polymer or natural substance to find out the most appropriate materials for cell growth and function. A variety of growth factors such as basic fibroblast growth factor (bFGF) or insulin-like growth factor (IGF) is very useful for to cells growth *in vitro* [2], and various kinds of cell-adhesion molecules, such as RGD and REDV, are known to improve cell-scaffold interactions [3].

Recently, many researchers have switched their attention into the effect of microenvironment on cell signaling with regard to cell differentiation [4]. Chemical methods to differentiate mesenchymal stem cells (MSC) into osteogenic cells or chondrogenic cells were established. However, myocardial differentiation of MSC was still under debate [5-6].

Cell preservation is also a big challenge in tissue engineering. Cryopreservation is a general technique for the cell preservation. A chemical substance such as dimethyl sulfoxide (DMSO), glycerol, and propylene glycol was used as cryoprotectant to protect cell from freezing damage. However, some functional cells like hepatocytes lost their function during the cryopreservation. Cho, H.R. et al. reported that the viability of hepatocytes decreased to lower than 60% when preserved at low temperature in University of Wisconsin (UW) and Euro-Collins (EC) solutions for 48 hours [7]. Not only the hepatocytes but also the other valuable cells generally lost their viability with preservation. For example, the viability of peripheral blood stem cell and hemopoietic progenitor cell decreased to less than 50% when preserved for 3 days at room temperature [8].

The objective of present study is to manipulate functional cells using well controlled *in vitro* microenvironments composed of (1) substrates on which stem cells are adhering, (2) chemical substances leading to cell differentiation, growth, or preservation, (3) cellular geometry such as monolayer, suspension, or spheroid, (4) culture medium (aeration, pH, nutrients, flow-condition and so on). Present study focused on producing beating cardiac cells from MSC and on preserving isolated hepatocytes in bioartificial liver assist system.

Beating cardiac cells

Cardiomyocyte transplantation for patients with ischemic heart disease or dilated cardiomyopathies is great potential therapeutic option to enhance the contractile function of the failing heart. However, the best cell sources for clinical cardiomyocyte transplantation are still under debate. Allogenic source including human embryonic

stem cells or fatal allogenic cardiomyocytes have been proposed, but there still remains an ethical issue. Genetically engineered animal cardiomyocytes have been studied for reducing the rejection reaction *in vivo*, which takes still long period time to secure the safety. Most promising cell source is autogeneic. Isolating cardiomiocytes from the patient hearts is unrealistic at present, and autologous skeletal muscle precursors, fibroblast, or mesenchymal stem cells have been studied so far [9]. However, since beating cardiomyocytes are more promising [10], in the present study focusing on to differentiate bone marrow mesenchymal stem cells (BMSCs) into "beating" cardiomyocytes.

Producing autologous beating cardiomyocytes is then attractive issue for cell-based therapy. The most crucial part is how to differentiate them to cardiomyocytes *in vitro* and how to maintain the beating feature. The effect of extracellular matrix (ECM) proteins such as collagen type I, collagen type IV, gelatin, laminin, fibronectin, Matrigel™ (mixture of laminin, collagen type IV, heparan sulfate proteoglycans and entactin), and Cardiogel™ (mixture of collagen type I and III, glycoproteins, laminin, fibronectin and proteoglycans) on cell viability, proliferation rate, and cardiomyocyte gene expression were reported [11-12]. However, the effects of ECM on cardiomyocytes beating and differentiation behaviors were not fully discussed.

There is no certain induction method for BMSCs differentiation into beating cardiomyocytes. Many researchers observed cardiac gene expression in MSCs treated with various inducers [13-15] or passage number [16], but they do not beat spontaneously. Wakitani, S. et al and Makino, S. et al. reported that murine BMSCs were differentiated to beating cardiomyocytes-like cells *in vitro* by exposing to DNA-demethylating agent 5-azacytidine [13-14]. This is in contrast with a report stated

that the functional cardiac cells and gene expression were not obtained after treated with 5-azacytidine [5].

Therefore, in part I, effects of microenvironment on various behaviors of cardiac cells were studied. In chapter 1, neonatal primary cardiomyocytes was cultured on various extracellular matrix (ECM) proteins (gelatin, collagen type-I, and fibronectin). The effect on beating duration and intracellular cardiac gene expression (troponin T type-2 and troponin C type-1) and cardiac differentiation marker gene (troponin C type-2) were evaluated. The beating period and the expression of troponin T type-2, troponin C type-1 of cardiomyocytes cultured on gelatin-coated dish were longer and higher than the others. The effect of ECM on beating duration will be further discussed in this chapter.

In chapter 2, the differentiation efficiency of murine embryonal carcinoma (EC) stem cells (P19.CL6) on various ECM proteins (gelatin, collagen type-I, and fibronectin) was studied. The beating colonies and intracellular cardiac gene expression and cardiac differentiation marker gene were evaluated. For cardiac differentiation of P19.CL6 cells, troponin T type-2 expression on gelatin- or fibronectin-coated dish was 5 times higher than that on collagen type I-coated dish or polystyrene dish 11days after induction. The effect of ECM proteins on differentiation behavior was discussed in this chapter. The fundamental information from chapter 1 and chapter 2 would be important for cardiac differentiation of various stem cells including autologous BMSCs.

In chapter 3, effect of chemical inducer, substrates, and cell geometry (suspension and monolayer induction) on differentiation of bone marrow MSCs to beating cardiomyocytes were studied. The number of myotube-like cells and the expression of troponin T type-2, troponin C type-1, and troponin C type-2 were

evaluated. In this chapter, the differentiation of BMSCs to beating cardiomyocytes was successfully achieved.

Preservation of isolated hepatocytes in bioartificial liver asssit system

Hepatocyte-based treatments for fulminant or chronic hepatitis have been attracting great attention so far. At present, injection of hepatocytes isolated from unused donor livers and bioartificial liver assist systems (BAL) is used for treating acute liver failures and liver-based metabolic defects [17-20]. A large number of hepatocytes must be prepared for emergency care and for repeated treatment [21]. However, because the isolation and reconstruction into 3D structure of the hepatocytes would take long time with complex steps, the metabolic activity rapidly decreased during the preparation process. However, to maintain the specific functions of the hepatocytes by conventional cryoperservation is difficult [22-27].

Various strategies to maintain cell functions have been proposed. Watts, P. et al. and Mckay, G.C. et al. reported that monolayer culture is superior to suspension culture in preserving hepatocytes [28-29]. Kakinoki, R. et al have succeeded in storing peripheral nerves for over a month using green tea polyphenol [30], and Hyon, S.H. et al. reported that green tea polyphenol also preserved the rat pancreatic islet for over 2 months [31].

In the part II, the microenvironment for preserving hepatocyte functions were studied. Before preserving the isolated hepatocytes in 3D bioreactor, the improvement of hepatocytes functions in radial-flow bioreactor for BAL was studied in chapter 4. BAL for treating acute liver failure has been studied for 50 years. Majority of the research has been conducted using hollow fiber perfusion-type bioreactor. However, the

efficiency in supplying oxygen and nutrients is not satisfactory. In this chapter, radial-flow bioreactor was developed for prolonged hepatocyte functions. This radial flow allows the medium culture to flow in the bioreactor entirely. In 1985, it is reported that spheroid structure of the hepatocytes are important for maintaining their functions [32]. In this chapter, bioreactor in which hepatocytes are cultured in spheroid-like structure was established by using hydrophilic non-woven matrices. The non-woven sheet was polytetrafluoroethlyne (PTFE) non-woven fabric coated with copolymer of poly (γ-methyl-L-glutamate) (PMLG) and the polyurethane (PAU-coated PTFE). The hepatocyte functions were kept at high value for 1 week and will be described further in this chapter.

In chapter 5, new technique to preserve the cultured hepatocytes in BAL bioreactor in the presence of epigallocatechin-3-gallate (EGCG) was studied. In this chapter, the preservation experiment in 3D BAL system was conducted after the effect of EGCG on hepatocyte preservation at room temperature was evaluated by monolayer culture. The effect of EGCG concentration, monolayer and spheroid-culture, and culture medium on hepatocyte functions were studied and will be discussed in this chapter.

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Part I : Manipulating cardiomyocytes

Chapter 1

Beating behavior of neonatal cardiomyocytes in different ECM matrices

Chapter 2

The effect of ECM matrices on differentiation of P19.CL6 carcinoma stem cells

Chapter 3

A novel system for myocardial differentiation of rat mesenchymal stem cells on various ECM proteins

Part I: Manipulating cardiomyocytes

Chapter 1

Beating behavior of neonatal cardiomyocytes in different ECM matrices

1.1 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. However, the best cell sources for clinical cardiomyocyte transplantation are still under debate.

In this decade, fetal or neonatal rat cardiomyocytes were reported to form mature cardiac tissue in syngeneic heart, acutely injured myocardium, and granulation tissue in the heart [1]. Rather neonatal cardiomyocytes will be used for the clinical transplantations or not, the most crucial part is how to maintain the beating duration of neonatal cardiomyocytes *in vitro*. As we know, the substrate microenvironment or niche proves to play an important role in providing essential signals to influence major intracellular pathways such as proliferation, differentiation and cell metabolism *in vitro*. 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 (mixture of collagen type I and III, glycoproteins, laminin, fibronectin and proteoglycans) on cell viability, proliferation rate, and cardiomyocyte gene expression were reported [2-3]. However, the effect of ECM on duration of isolated cardiomyocytes beating behavior and BMSCs

differentiation to cardiomyocytes has not fully been discussed.

Therefore, the aim of this chapter was to investigate the beating duration or beating rate of cardiomyocytes on different ECM proteins (gelatin, fibronectin, and collagen type I) with respect to intracellular cardiac marker genes (troponin T type-2 and troponin C type-1) [4] and cardiac differentiation marker gene (troponin C type-2) [5].

1.2 Materials and methods

1.2.1 Cardiomyoctes

Cardiomyocytes were isolated from neonatal Sprague-Dawley (SD) rat heart (1 to 2-day-old) by the collagenase digestion method with modifications [6-7]. The hearts were removed and carefully minced with a scalpel blade into fragment and were rinsed several times with Hanks' balanced salt solution (Sigma-Adrich Inc, St. Louis, MO) 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-piperazineethanesulfunic acid (HEPES), 0.02 M Sodium Hydrogen Carbonate ($NaHCO_3$), 3.74 mM Calcium Chloride Dihydrate ($CaCl_2{\cdot}2H_2O$), and 6.5 x 10^4 U collagenase (Wako, Ltd., Osaka, Japan, Lot no: 06032W)) at 37 °C for 30 minutes. The resulting cell suspension was filtered through a nylon cell strainer (BD Falcon, BD Biosciences, Bedford) with a 40 μm pore size and centrifuged at 1000 rpm (78g) for 3 minutes.

Isolated cardiomyocytes (1.0 x 10^5) were cultured in minimum essential medium alpha medium (α-MEM, Gibco, Invitrogen Co., Grand Island, NY) supplemented with 10 % (v/v) fetal bovine serum (FBS, MP Biomedicals Inc.,

Eschwege, Germany, Lot no: 7297H), and 100 IU/L penicillin-streptomycin (Wako, Ltd., Osaka, Japan) on 60 mm gelatin- (IWAKI, Asahi Glass Co.,LTD., Tokyo, Japan), fibronectin- (BD Falcon™, BD BioCoat, New Jersey), collagen type I-coated dish and non-coated polystyrene dish (IWAKI, Asahi Glass Co.,LTD., Tokyo, Japan).

1.2.2 Measurement of action potential

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

1.2.3 Total RNA isolation and reverse transcription

Total RNAs of cardiomyocytes cultured on various dishes were extracted by QuickGene RNA cultured cell kit S (Fujifilm Life Science, Tokyo, Japan) 4 weeks after culture, respectively. First-strand cDNAs were synthesized using a mixture of

oligo(dT)$_{18}$ primer. Total cellular RNAs (200 ng) were incubated with 2.5 μM oligo(dT)$_{18}$ primer at 70 °C for 10 minutes to denature RNA secondary structure and then incubated at 4 °C to let the primer anneal to the RNA. A givent amount of 5X RT buffer (TOYOBO Co., Ltd., Osaka, Japan) and 2.5 mM deoxynucleotide trisphosphate (dNTP) mixture (Takara Bio Inc., Shiga, Japan) (4 μl) were added and incubated at 37 °C for 5 minutes. The reverse transcriptase (100 Units, TOYOBO Co., Ltd., Osaka, Japan) were added into the mixture and reverse transcriptase (RT) reaction was extended at 37 °C for 1 hour. Then, the reaction mixture was heated at 94 °C for 5 minutes to inactivate the enzyme and cooled at 4 °C for 15 minutes. The RNase (DNase-free, 0.5 μg, Roche Diagnostics GmbH, Mannheim, Germany) was added into the mixture and incubated at 37 °C to remove the template of RNA.

1.2.4 Real-time quantitative polymerase chain reaction (PCR)

Real-time quantitative polymerase chain reaction (PCR) was conducted with SYBR Green. Primers for PCR analysis for troponin T type-2, troponin C type-1, and troponin C type-2 were designed using Primer Express software (Perkin-Elmer Applied Biosystems, Cheshire, UK). Primer sequences are shown in Tab. 1-1. Reaction mixtures contained 23.74 μl distilled water, 25 μl SYBR Green Realtime PCR master mix (TOYOBO Co., Ltd, Osaka, Japan), 100 nM of each primer, and 0.26 μl cDNA. The thermal profile for PCR was 50 °C for 2 min, followed by 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Distilled water 0.26 μl was used as a negative control PCR reaction to ensure the absence of template contamination in PCR reagents. The average threshold cycle (Ct) values of triplicate measurements were used for all subsequent calculations on the basis of the delta Ct method.

Tab. 1-1. Polymerase chain reaction primers used in this study.

Genes	*Sens*	*Anti-sens*
Troponin T type-2	5'-GAAACAGGATCAACGACAACCA-3'	5'-CGCCCGGTGACTTTGG-3'
Troponin C type-1	5'-GATCTCTTCCGCATGTTTGACA-3'	5'-TGGCCTGCAGCATCATCTT-3'
Troponin C type-2	5'-AGATCGAATCCCTGATGAAGGA-3'	5'-CATCTTCAGAAACTCGTCGAAGTC-3'
GAPDH	5'-CTACCCCCAATGTATCCGTTGT-3'	5'-TAGCCCAGGATGCCCTTTAGT-3'

1.3 Results:

1.3.1 Beating behavior of isolated cardiomyocytes

One week after culture, the action potential of cardiomyocytes on gelatin-coated dishes was higher than that for other conditions (Fig. 1-1), and the beating duration was also longer than that for other conditions. The action potential and beating rates on each matrix are summarized in Tab. 1-2. After 7 days of culture, the action potential was around 6.7 ± 0.49 mV, for cardiomyocytes cultured on gelatin-coated dishes, 1.1 ± 0.97 mV on fibronectin-coated dishes, 2.0 ± 0.35 mV on collagen type I-coated dishes, and 2.0 ± 0.75 mV on noncoated polystyrene dishes. These results indicate that the action potential rate on fibronectin-, collagen type I-coated dishes and noncoated polystyrene dishes were 84%, 61%, and 70% lower than gelatin-coated dishes after 1 week of cultivation.

After 14 days of culture, the action potential became 6.6 ± 1.26 mV on gelatin-coated dishes, 6.9 ± 1.15 mV on fibronectin-coated dishes, and 1.7 ± 0.03 mV on collagen type I-coated dishes. These mean the action potential rate on fibronectin-, and collagen type I-coated dishes were 5% higher, and 74% lower than gelatin-coated

dishes. No action potential was detected on polystyrene dishes after 2 weeks of cultivation.

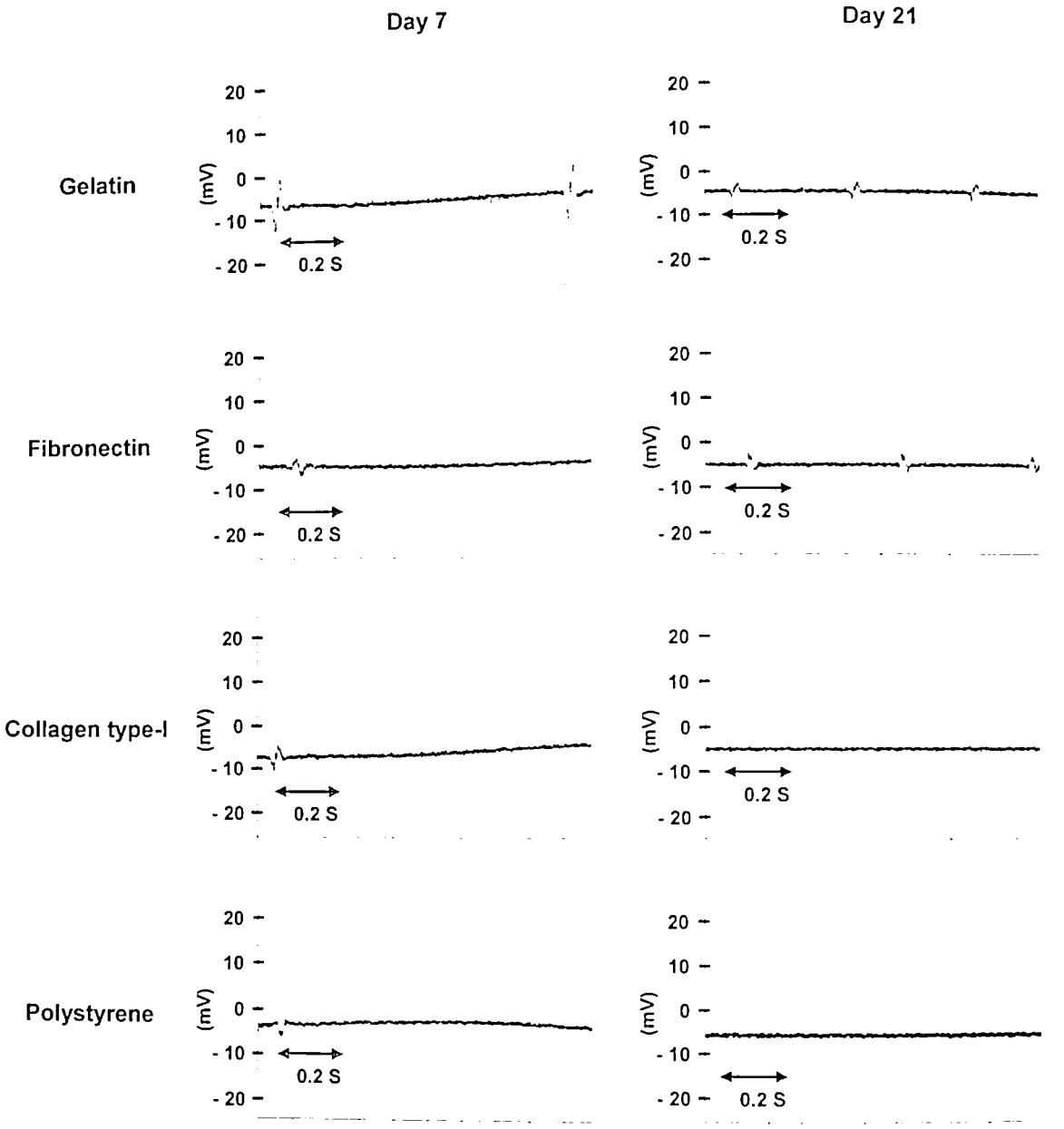

Fig. 1-1. Electrophysiological assessment of isolated cardiomyocytes after 7 and 21 days of cultivation on different substrates.

After 21 days of culture, the action potential was 3.1 ± 0.21 mV on gelatin-coated and 2.8 ± 0.11 mV on fibronectin-coated dishes, signifying that the action

potential on fibronectin-coated dishes was 10% lower than gelatin-coated dish. No action potential was detected on collagen type I-coated dishes and polystyrene dishes after 21 days of cultivation.

The beating rate of cardiomyocyte was also affected by the ECM proteins. After 7 days of culture, the beating rate was 1.2 ± 0.05 Hz for cardiomyocyte cultured on gelatin-coated dishes, 1.1 ± 0.3 Hz on fibronectin-coated dishes, 0.8 ± 0.02 Hz on collagen type I-coated dishes and 0.3 ± 0.04 Hz on noncoated polystyrene dishes. After 14 days of culture, the beating rate became 1.3 ± 0.01 Hz on gelatin-coated dishes, 1.3 ± 0.42 Hz on fibronectin-coated dishes, and 2.3 ± 0.05 Hz on collagen type I-coated dishes. After 21 days, the beating rate was 2.8 ± 0.03 Hz on gelatin-coated dishes and 2.0 ± 0.11 Hz on fibronectin-coated dishes, whereas, cardiomyocytes cultured on polystyrene dishes and collagen type I-coated dishes did not beat well and stopped at an early stage of cultivation. These results indicated that, gelatin could maintain the action potential of cardiomyocytes at high value for 2 weeks compared to fibronectin and collagen type-I.

Tab. 1-2. Summary of voltage potential in several types of ECM-coated dish.

Substrate	*Action potential (mV) [Beating rate (Hz)]*		
	Day 7	*Day 14*	*Day 21*
Gelatin	6.7 ± 0.49 [1.2 ± 0.05]	6.6 ± 1.26 [1.3 ± 0.01]	3.1 ± 0.21[2.8 ± 0.03]
Fibronectin	1.1 ± 0.97 [1.1 ± 0.30]	6.9 ± 1.15 [1.3 ± 0.42]	2.8 ± 0.11 [2.0 ± 0.11]
Collagen type-I	2.6 ± 0.35 [0.8 ± 0.02]	1.7 ± 0.03 [2.3 ± 0.05]	ND
Polystyrene	2.0 ± 0.75 [0.3 ± 0.04]	ND	ND