Biotube Technology for a Novel Tissue-Engineered Blood Vessels
Ishibashi-Ueda H and Nakayama Y 95

Clinical Application of Tissue-Engineered Blood Vessels
Matsumura G and Shin'oka T 105

Chapter 4: NEW ASPECTS OF ANGIOGENESIS

Vascular Regeneration and Remodeling by Circulating Progenitor Cells
Sata M and Nagai R 117

Gene Therapy with Hepatocyte Growth Factor for Angiogenesis in Severe Pulmonary Vascular Disease
Ono M, Sawa Y, and Matsuda H 129

Basic Fibroblast Growth Factor and Angiogenesis
Marui A, Doi K, Tambara K, Sakakibara Y, Ueyama K, Iwakura A, Yamamoto M, Ikeda T, Tabata Y, Komeda M 145

Gene Therapy for Angiogenesis under a Ventricular Assist System
Takewa Y, Shirakawa Y, Taenaka Y, Tatsumi E, Sawa Y, Matsuda H, Kitamura S, Takano H 157

The Role of Vascular Endothelial Growth Factor (VEGF) on Therapeutic Angiogenesis Using Bone Marrow Cells
Maeda Y and Ikeda U 173

Chapter 5: CLINICAL RESULTS OF THERAPEUTIC ANGIOGENESIS AND VASCULOGENESIS

Clinical Survey of Cell Therapy in Japan
Katsuda Y, Takeshita Y, Arima K, Saitoh Y, Imaizumi T, Asahara T, Nakatani T, Okano T, Kishida A, Ueda H, Shin'oka T, Nagai R, Sawa Y, Komeda M, Takewa Y, Matsuda H, Mori H 183

A Novel Micro-Angiography Detecting Angiogenesis, Application for Autologous Bone Marrow Mononuclear Cells Transplantation in the Patients with Critical Limb Ischemia
Nishigami K, Nakatani T, Chiku M, Mori H 191

Angiogenesis Induced by Intramyocardial Implantation of Autologous Bone Marrow Mononuclear Cells for the Treatment of Ischemic Heart Disease
Li T, Matsuzaki M, and Hamano K 201

Effect of Bone Marrow Transplantation in Patients with Critical Limb Ischemia
Katsuda Y, Takeshita Y, Arima K, Saitoh Y, Sasaki K, Shintani S, Murohara T, Imaizumi T 213

Therapeutic Angiogenesis for a Patient with Arteriosclerosis Obliterans by Autologous Transplantation of Bone Marrow Mononuclear Cells
Fujimoto K, Miyagi H, Miyao Y, Kajiwara I, Oe Y, Kawano F, Hidaka M 221

Autologous Bone Marrow Implantation for Burger's Disease
Ohtani M, Soma T, and Taguchi A 227

Closing Remarks 235

Index 237

CHAPTER 8

Regeneration of Cardiomyocytes from Bone Marrow Stem Cells and Application to Cell Transplantation Therapy

Keiichi Fukuda

Introduction

Although heart transplantation is the ultimate therapy for the treatment of severe heart failure, it has not been widely examined, because it requires donor hearts, and the inadequate supply of donor hearts is often a major problem everywhere in the world. As a result, the current challenge in cardiology is how to reserve pump failure by cell transplantation or regenerative medicine. Recent studies have shown that transplanted fetal cardiomyocytes can survive in heart scar tissue and that the transplanted cells limit scar expansion and prevent post-infarction heart failure. Transplantation of cultured cardiomyocytes into damaged myocardium has been proposed as a future method of treating heart failure,[1,2] but this revolutionary concept remains unfeasible in clinical settings because of the difficulty of obtaining donor fetal hearts. A cardiomyogenic cell line has long been awaited, and such a line might be capable of substituting for fetal cardiomyocytes in this therapy.

A number of studies have demonstrated that cardiomyocytes can differentiate from various multipotent stem cells, including embryonic stem (ES) cells[3] and embryonic carcinoma (EC) cells.[4] ES cells are an attractive cell source in regenerative medicine, but because the transplanted ES cells are allogeneic, the recipients must take immunosuppressant drugs throughout their lives. Use of these reagents impairs the quality of life of the recipients, and transplantation of undifferentiated ES cells often causes teratocarcinoma. In addition, the establishment of human ES cells involves ethical problems and is not allowed in every country. Because of these circumstances, the regeneration of cardiomyocytes from adult autologous stem cells has long been awaited.

Recent reports have demonstrated the existence of pluripotent stem cells in adult tissues. Roy et al reported the existence of neural stem cells in the brain that can differentiate into neurons, oligodendrocytes, and astrocytes in vitro.[5] Marrow stromal cells have been shown to possess many characteristics of mesenchymal stem cells,[6] and pluripotent progenitor marrow stromal cells can differentiate into various cell types, including osteoblasts,[7,8] myocytes,[9] adipocytes, tenocytes, and chondroblasts.[10] We recently reported the differentiation of mesenchymal stem cells into cardiomyocytes after exposure to 5-azacytidine and the establishment of cell line CMG (cardiomyogenic) that differentiates into cardiomyocytes in vitro.[11] CMG cells exhibit spontaneous beating and express atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), and they may provide a useful and powerful tool for cardiomyocyte transplantation after further characterization of their cardio-myocyte phenotype.

This chapter describes the characteristics of bone marrow-derived regenerated cardiomyocytes and discusses the possibility of using them for cardiovascular tissue engineering. The expression and function of α_1, β_1, and β_2-adrenergic receptors and muscarinic M_1 and M_2 receptors in CMG cells is also described, because these receptors play a critical role in modulating cardiac function.[12]

Does the Heart Have its Own Stem Cell Compartment?

It is well known that skeletal muscle cells contain stem cells, called "satellite cells". Satellite cells can both proliferate by cell division and differentiate into skeletal muscle cells, and the differentiated skeletal muscle cells can fuse to form myotubes. By contrast, fetal cardiomyocytes can proliferate by cell division, but they undergo terminal differentiation and stop dividing after birth. A number of studies have reported that cardiomyocytes increase in size by cell hypertrophy, not by cell hyperplasia. To our knowledge there have been no reports of the presence of satellite-cell-like cardiac stem cells in the heart. Beitrani et al recently reported that human cardiomyocytes express Ki67, a marker of cell division, and the M phase of the nucleus of the cardiomyocytes was observed in the border zone area of recent myocardial infarction in autopsied hearts.[13] These findings suggested that only a very few adult cardiomyocytes can divide after the terminal differentiation. Although their findings were very interesting, these cells were insufficient to improve cardiac function, since the population of these cells was very small.

Mesenchymal Marrow Stem Cells as a Possible Source of Cardiomyocytes: The Cardiomyogenic (CMG) Cell?

Figure 1 shows the classification of the stem cell system of adults.[14] Bone marrow stromal cells were previously used as a feeder layer to culture hematopoietic stem cells, and are known to be of mesodermal origin and produce various cytokines and

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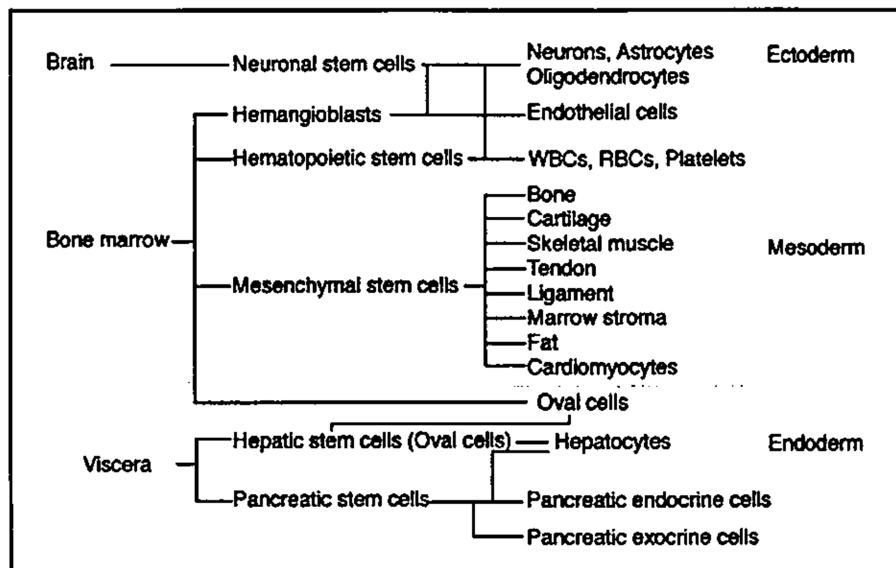

Figure 1. Classification of pluripotent stem cells in adult tissues. Bone marrow contains several kinds of stem cells. Mesenchymal stem cells may differentiate into various mesoderm-derived cells, such as osteoblasts, chondroblasts, adipocytes, skeletal muscle cells and cardiomyocytes.

growth factors. In the late 1990s, a number of papers reported that bone marrow stromal cells contain multipotent stem cells for non-hematopoietic tissues, called "marrow mesenchymal stem cells", that could differentiate into osteoblasts, chondroblasts, and adipocytes. All of these cells were known to be of mesodermal origin. If marrow mesenchymal stem cells are multipotent, we hypothesized that they might have the ability to differentiate into cardiomyocytes and instituted this study. We also recognized that bone marrow cells could be obtained from patients themselves and that autologous cells would not be rejected after cell transplantation.

Method of Establishing Bone-Marrow Derived Cardiomyocytes

Female C3H/He mice were anesthetized with ether, their femora were excised, and bone marrow cells were collected. The procedures were performed in accordance with the guidelines for animal experimentation of Keio University. Primary culture of the marrow cells was performed according to Dexter's method. Cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% fetal bovine serum and penicillin (100 μg/ml)/streptomycin (250 ng/ml)/ amphotericin B at 33°C in humid air containing 5% CO2. After a series of passages, the attached marrow stromal cells became homogeneous and were devoid of hematopoietic cells. The marrow stromal cells basically did not require co-culture with blood stem cells. Immortalized cells were obtained by frequent subculture for more than 4 months. Cell lines from different dishes were subcloned by limiting dilution. To induce cell differentiation, cells were treated with 3 μmol/L of 5-azacytidine for 24 hours. Subclones that included spontaneously beating cells were screened by microscopic observation (first screening), and cells surrounding spontaneous beating cells were subcloned with cloning syringes. Subcloned cells were maintained, exposed to 5-azacytidine again for 24 hours, and clones that showed spontaneous beating most frequently were screened (second screening). The clonal cell line thus obtained was named the CMG cell.

As a result of repeated rounds of limiting dilution, we succeeded in isolating 192 single clones, several of which differentiated into cardiomyocytes and showed spontaneous beating. The experiments were reproducible, but the percentage of cells that differentiated into cardiomyocyte differentiation was specific to each clone. Phase-contrast photography and/or immunostaining with anti-sarcomeric myosin antibodies were used to identify the morphological changes in the CMG cells. CMG cells showed a fibroblast-like morphology before 5-azacytidine treatment (0 week), and this phenotype was retained through repeated subculturing under non-stimulating conditions. After 5-azacytidine treatment, however, the morphology of the cells gradually changed (Fig. 2). Approximately 30% of the CMG cells gradually increased in size at 1 week, and they formed a ball-like appearance, or had lengthened in one direction to exhibit a stick-like morphology. They connected with adjoining cells after 2 weeks and had formed myotube-like structures at 3 weeks. The differentiated CMG myotubes maintained the cardiomyocyte phenotype and beat vigorously for at least 8 weeks after the final 5-azacytidine treatment and did not de-differentiate.[11] Most of the other non-myocytes had an adipocyte-like appearance.

Regenerated Cardiomyocytes Display a Fetal Ventricular Phenotype

Various cardiac contractile protein isoforms are differentially expressed in cardiomyocytes at different developmental stages and in different chambers. At around the time of birth there is a developmental switch in the ventricular muscle of small mammals from expression of β-myosin heavy chain (MHC), which is the predominant fetal form, to expression of α-MHC. There is also a developmental switch from expression of α-skeletal actin, which is the predominant fetal and neonatal form, to that of α-cardiac actin, the predominant adult form. We investigated the contractile protein isoforms of bone marrow-derived CMG cells to characterize their phenotype as cardiomyocytes. Table 1 summarizes the results. Fetal, neonatal, and adult ventricle and atrium were used as controls.[14] Expression of both α- and β-*MHC* was detected in differentiated CMG cells by RT-PCR, but β-*MHC* expression was overwhelmingly greater than that of α-*MHC*. CMG cells expressed both α-*cardiac* and α-*skeletal actin*, but the

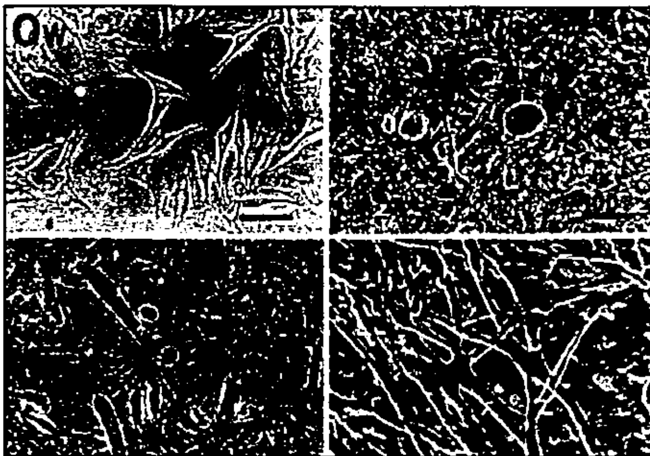

Figure 2. Phase-contrast photographs of marrow-derived cardiomyogenic (CMG) cells before and after 5-azacytidine treatment. Upper left: Marrow mesenchymal cells have a fibroblast-like morphology before 5-azacytidine treatment (0 week). Upper right: One week after treatment, some cells gradually increased in size, and developed a ball-like or stick-like appearance. These cells began spontaneous beating thereafter. Lower left: Two weeks after treatment, the ball-like or stick-like cells connected to adjoining cells, and began to form myotube-like structures. Lower right: Three weeks after treatment. Bars indicate 100 μm.

α–skeletal actin gene was expressed at markedly higher levels than the α-cardiac actin gene. Interestingly, CMG cells expressed the myosin light chain (MLC)-2v gene, but not the MLC-2a gene. MLC-2v is specifically expressed in ventricular cells, while MLC-2a is specifically expressed in atrial cells. Skeletal muscle cells do not express either α-MHC or MLC-2v. These results indicated that differentiated CMG cells possess the specific phenotype of the fetal ventricular cardiomyocytes.[11]

CMG Cells Have a Cardiomyocyte-Like Ultrastructure

Representative transmission electron micrographs are shown in Figure 3. A longitudinal section of the differentiated CMG myotubes clearly revealed the typical striation and pale-staining pattern of the sarcomeres.[11] CMG myotube nuclei were positioned in the center of the cell, not beneath the sarcolemma. The most conspicuous feature of the differentiated CMG myotubes was the presence of membrane-bound dense secretory granules measuring 70-130 nm in diameter. They were thought to be atrial granules, and were especially concentrated in the juxtanuclear cytoplasm, but some were also located near the sarcolemma. These findings indicated that the CMG cells possessed cardiomyocyte-like rather than skeletal muscle ultrastructure.

Developmental Stage of Undifferentiated and Differentiated CMG Cells

Various cardiac specific transcription factors have been cloned, and their genes are serially expressed in the developing heart during myogenesis and morphogenesis. Figure 4 shows the time course of the expression of cardiomyocyte-specific transcription factors in fetal developing heart and CMG cells. The genes coding Nkx2.5[15] (homeobox type transcription factor specifically expressed beginning in the early developing heart), GATA4[16] (GATA-motif-binding Zinc finger type transcription factor expressed beginning in the early stage developing heart), HAND1/2 (basic helix-loop-helix type transcription factor expressed in the heart and autonomic nervous system), and MEF2-B/C[17] (muscle enhancement factor: a MADS box family transcription factor expressed in the myocytes) were expressed in the early stage of heart development, and MEF2A and MEF2-D in the middle stage. The CMG cells already expressed GATA4, TEF-1[18](transcription enhancement factor 2), Nkx2.5, HAND, and MEF2-C before exposure to 5-azacytidine, and they expressed MEF2-A and MEF2-D after exposure to 5-azacytidine. This pattern of gene expression in CMG cells was similar to that of developing cardiomyocytes in vivo,[11] and indicated that the developmental stage of the undifferentiated CMG cells is close to that of cardiomyoblasts or the early stages of heart development. We estimated that the stage of differentiation of the CMG cells lies between the cardiomyocyte-progenitor stage and the differentiated cardiomyocyte stage.

Serial Changes in Action Potential Shape in CMG Cells Simulate Those of Fetal Ventricular Cardiomyocytes in Vivo

CMG cells exhibit at least two types of distinguishable morphological action potentials: sinus-node-like potentials (Fig. 5A) and ventricular myocyte-like potentials (Fig. 5B).[11] The cardiomyocyte-like action potential recorded from these spontaneous beating cells is characterized by: (1) a relatively long action potential duration or plateau; (2) a relatively shallow resting membrane potential; and (3) a pacemaker-like late diastolic slow depolarization. Peak-and-dome-like morphology was observed in ventricular-myocyte-like cells. Figure 5C shows the time course of the percentages of the sinus node-like and ventricular-myocyte-like action potentials. All action potentials recorded from CMG cells until 3 weeks were sinus-node-like action potential. The ventricular-myocyte-like action potentials were first recorded after 4 weeks, and their percentage gradually increased thereafter.

Table 1. Isoforms of the contractile proteins in differentiated CMG cells

	Atrium		Ventricle			
Developmental Stage	**Fetus**	**Adult**	**Fetus**	**Neonate**	**Adult**	**CMG**
α-actin	skeletal	cardiac	skeletal>cardiac	skeletal	cardiac	skeletal>cardiac
myosin heavy chain	α>β	α	β>α	α>β	α	β>α
myosin light chain	2a	2a	2v	2v	2v	2v

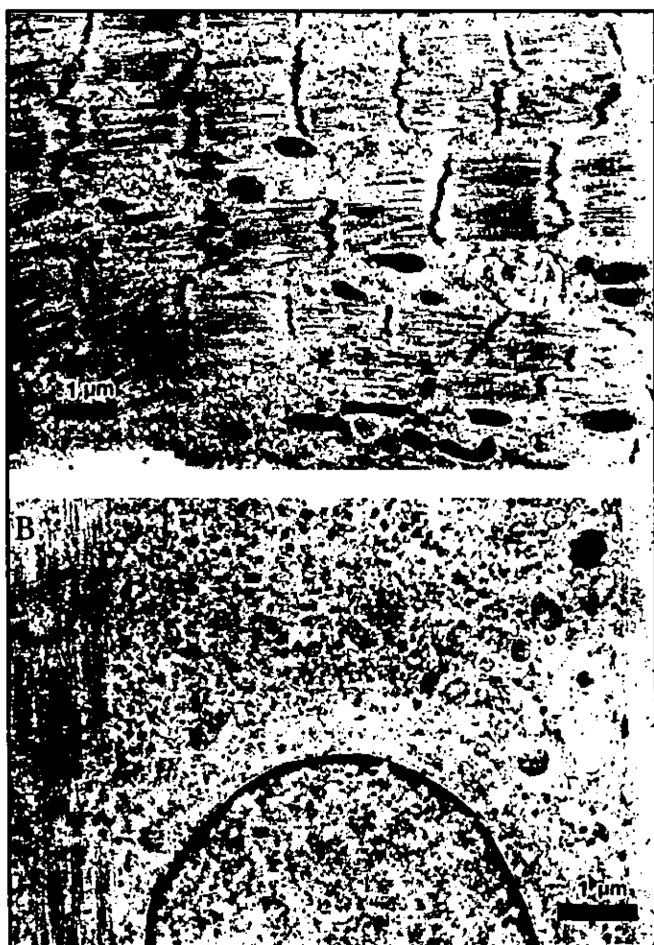

Figure 3. Transmission electron micrograph of CMG cells. A) Differentiated CMG cells had well-organized sarcomeres. Abundant glycogen granules and a number of mitochondria were observed. B) Ultrastructural analysis revealed atrial granules measuring 70-130 nm in diameter in the sarcoplasm that were especially concentrated in the juxtanuclear cytoplasm. Bar indicates 1 μm.

The observation of several distinct patterns of action potential in CMG cells may reflect different developmental stages. Yasui et al studied action potentials and the occurrence of one of the pacemaker currents, I(f), by the whole-cell voltage and current-clamp technique at the stage when a regular heartbeat is first established (9.5 days post coitum) and at 1 day before birth.[19] They showed a prominent I(f) in mouse embryonic ventricles in the early stage, and that it decreased by 82% before birth in tandem with the loss of regular spontaneous activity by the ventricular cells. They concluded that the I(f) current of the sinus node type is present in early embryonic mouse ventricular cells. Loss of the I(f) current during the second half of embryonic development is associated with a tendency for the ventricle to lose pacemaker potency. Our findings in CMG cells may reflect the developmental changes in the action potentials that occur in embryonic ventricular cardiomyocytes.

Expression of α_1- and β-Adrenergic Receptor mRNA in CMG Cells

In the heart in vivo, α and β adrenergic receptors play a key role in modulating cardiac hypertrophy and cardiac function, such as heart rate, contractility, and conduction velocity. CMG cells expressed all the α_1 receptor subtypes (α_{1A}, α_{1B}, and α_{1D}) before 5-azacytidine exposure (Fig. 6A),[12] and their expression in undifferentiated CMG cells may be explained by their ubiquitous or wide expression in vivo.[20] A low level of expression of α_{1A} was observed before 5-azacytidine exposure, and it increased markedly after exposure. Expression of α_{1B} was unaffected by 5-azacytidine. A high level of expression of α_{1D} was detected before 5-azacytidine exposure, but it decreased considerably after exposure. This transcriptional switch may be attributable to the CMG cells having acquired the cardiomyocyte phenotype. The ventricular cardiomyocytes in vivo mainly expressed α_{1A} and α_{1B}, and expressed a low level of α_{1D} receptor. The temporal changes in expression of α_1-adrenergic receptor subtypes in CMG cells are very similar to the postnatal changes observed in neonatal rat heart.[21,22]

The cardiomyocytes of the mammalian hearts express both β_1 and β_2-adrenergic receptors, the β_1 receptor being the predomi-

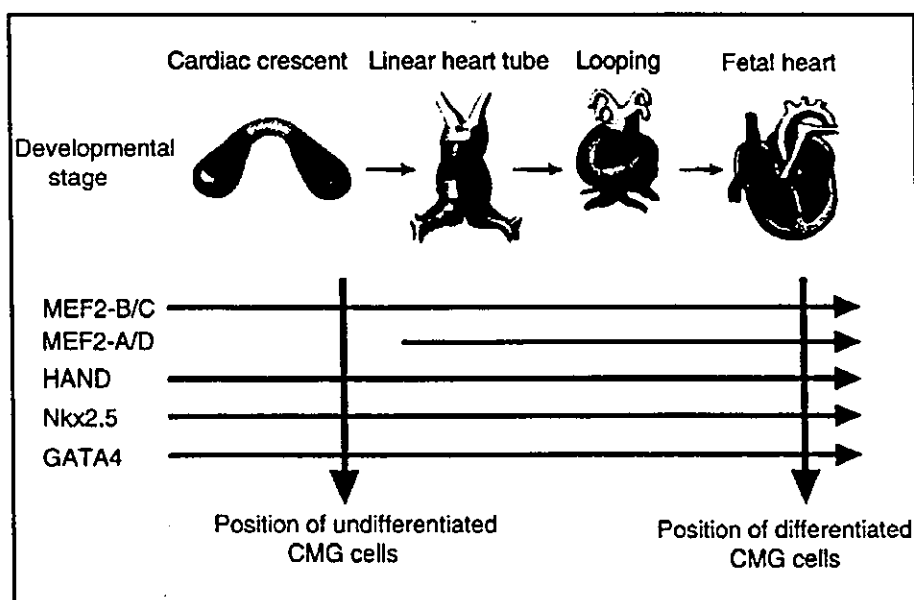

Figure 4. Expression of cardiac-specific transcription factors in the developing heart and in CMG cells. The horizontal arrows indicate the time course of the expression of cardiac-specific transcription factors in the developing fetal heart. The dotted vertical arrows indicate the expression of these factors in undifferentiated and differentiated CMG cells. CMG cells expressed MEF2-A and MEF2-D after 5-azacytidine exposure, when they acquired a cardiomyocyte phenotype.

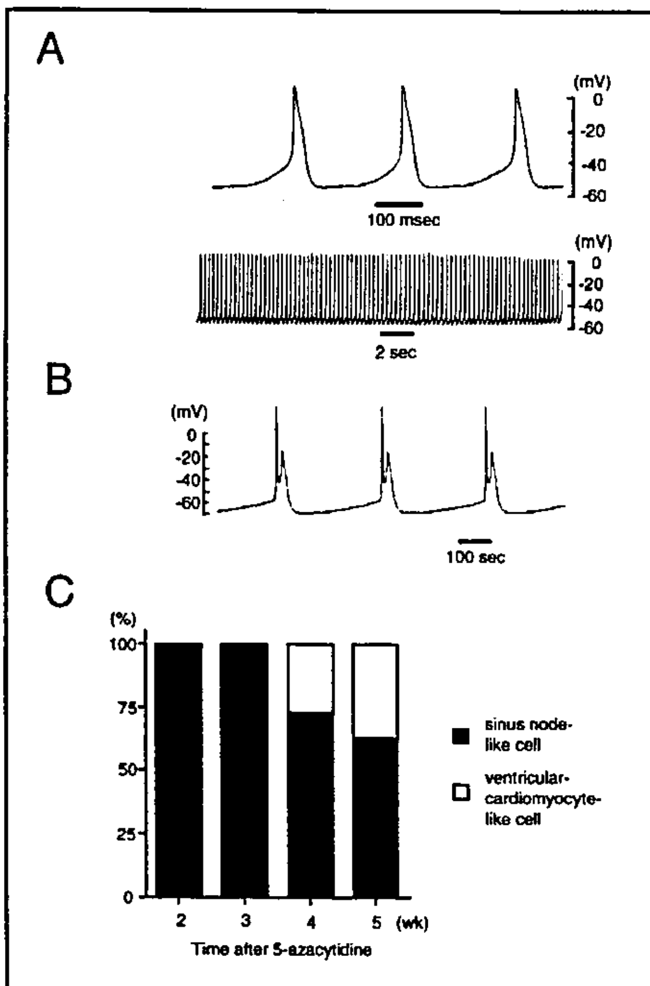

Figure 5. Representative tracing of the action potentials of CMG myotubes. Action potential recordings from spontaneous-beating cells were obtained with a conventional microelectrode at day 28 after 5-azacytidine exposure. The action potentials were classified into two groups: A) sinus-node-like action potentials and B) ventricular-cardiomyocyte-like action potentials. C) Percentages of CMG cells exhibiting sinus-node-like and ventricular-cardiomyocyte-like action potentials after 5-azacytidine exposure. A ventricular cardiomyocyte-like action potential was first recorded 4 weeks after 5-azacytidine exposure, and it rapidly became more prevalent thereafter.

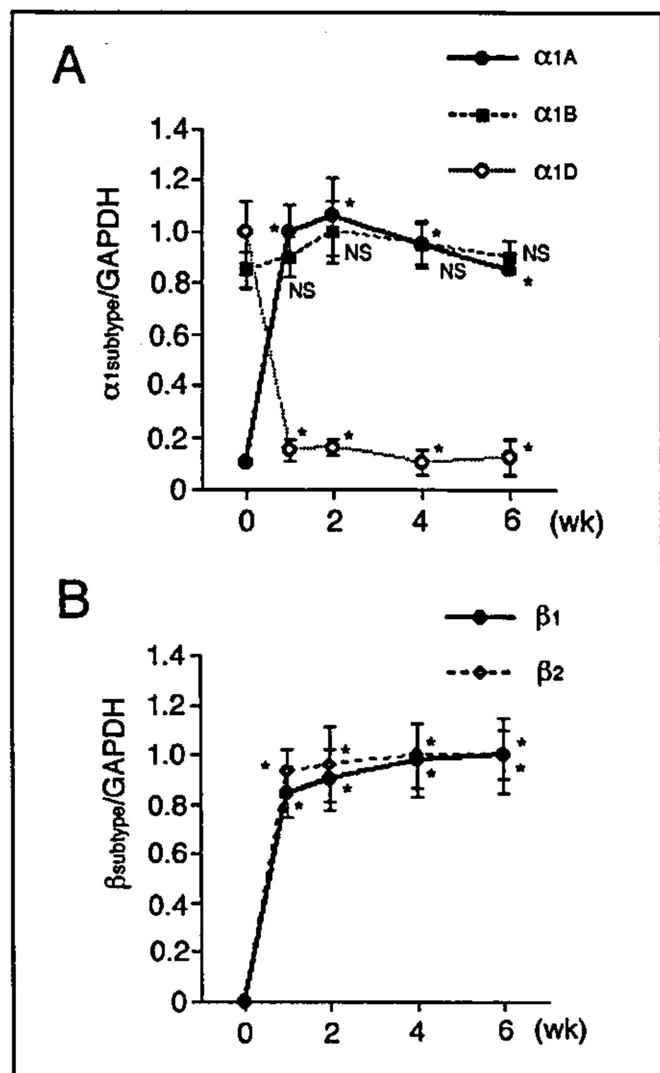

Figure 6. Temporal expression of α_1- and β-adrenergic receptor subtype messenger ribonucleic acid (mRNA) in CMG cells. A) Densitometric analysis was performed, and the ratio of the reverse transcriptase polymerase chain reaction (RT-PCR) product of α_1 subtype (α_{1A}, α_{1B}, α_{1D}) receptors to that of glyceraldehyde-3-phosphate dehyrogenase (GAPDH) is shown. Data were obtained from 5 separate experiments and are shown in arbitrary units compared to the controls. Values are mean ± SE. *=p<0.01 vs. controls (before 5-azacytidine exposure). NS= not significant. B) Densitometric analysis was performed, and the ratio of the RT-PCR product of β subtype (β_1 and β_2) receptors to that of GAPDH is shown.

nant subtype (approximately 75–80% of total β receptors).[23] CMG cells did not express β_1 and β_2 receptor transcripts before 5-azacytidine exposure, but RT-PCR showed expression of their mRNAs forward day 1 after exposure onward, and exposure, and expression was stable after 1 week (Fig. 6B).[12] CMG cells expressed β_1 and β_2 mRNA after acquiring the cardiomyocyte phenotype. The temporal pattern of expression of these receptors differed from that of α_1.

Phenylephrine Induces Activation of ERK1/2 and Hypertrophy in CMG Cells Via α_1 Receptors

ERK1/2 was activated by phenylephrine, an α_1 stimulant, within as little as 5 minutes, and the activation peaked at 10 minutes (Fig. 7A,B). The phenylephrine-induced phosphorylation was completely inhibited by prazosin (Fig. 7C), and phenylephrine increased the cell area and perimeter of the CMG cardiomyocytes (Fig. 7D,E). These findings indicated that CMG cells express functionally active α_1-adrenergic receptors.[12]

Isoproterenol Increases the cAMP Content, Spontaneous Beating Rate, and Contractility of CMG Cells

Isoproterenol, a β stimulant, increased the cAMP content of CMG cells, and propranolol completely inhibited the isoproterenol-induced cAMP accumulation (Fig. 8A,B). Isoproterenol was applied to the cells to determine whether it would increase the spontaneous beating rate (Table 2), and the results showed

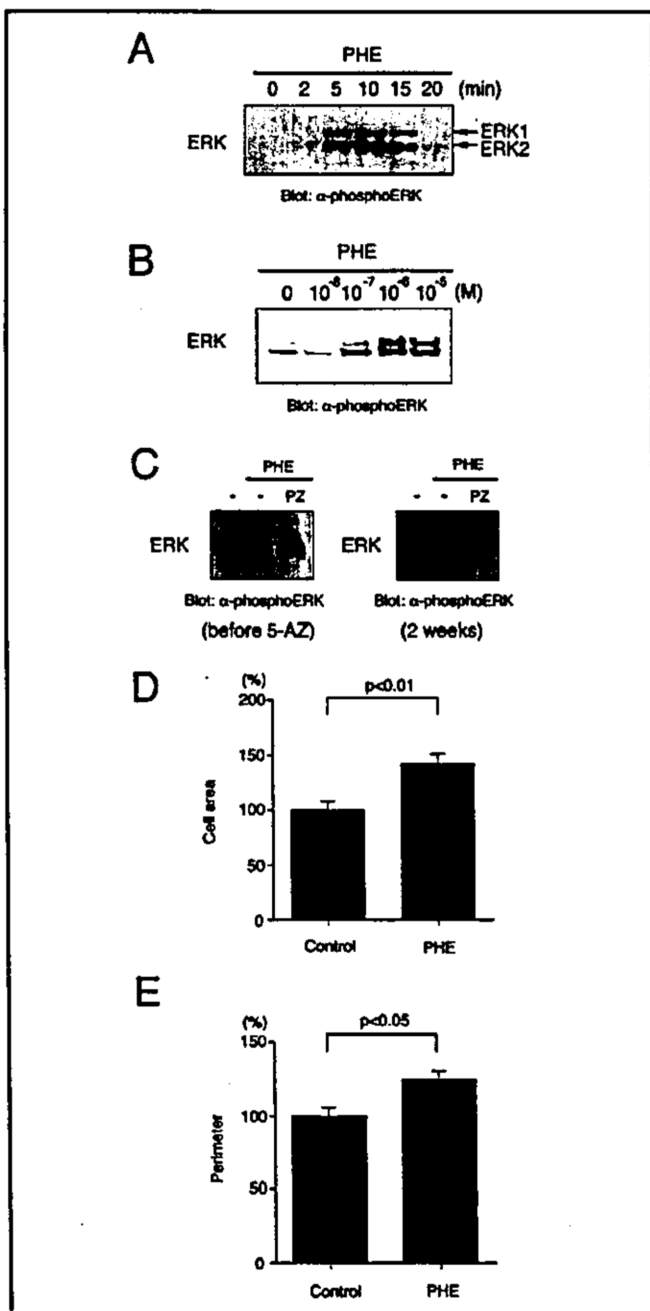

Figure 7. Effect of phenylephrine on phosphorylation of ERK1/2 and cell size in CMG cells. A-C. A) Cells at 2 weeks after 5-azacytidine exposure were stimulated with phenylephrine (PHE) (10^{-4} mol/L), and Western blot analysis was performed to detect phosphorylation of ERK1/2. B) Cells were stimulated with phenylephrine (10^{-7}-10^{-5} mol/L) for 10 minutes, and phosphorylation of ERK was detected. C) Prazosin (10^{-6} mol/L) was added to cells 20 minutes before stimulation with phenylephrine (10^{-6} mol/L). PHE= phenylephrine; PZ= prazosin. D-E. Cells were serum depleted for 24 h, stimulated with phenylephrine for 24 h, and stained with anti-sarcomeric myosin antibody. Cell area (D) and perimeter (E) were quantitated with NIH Image software. (n= 100) *= p<0.01 vs. control.

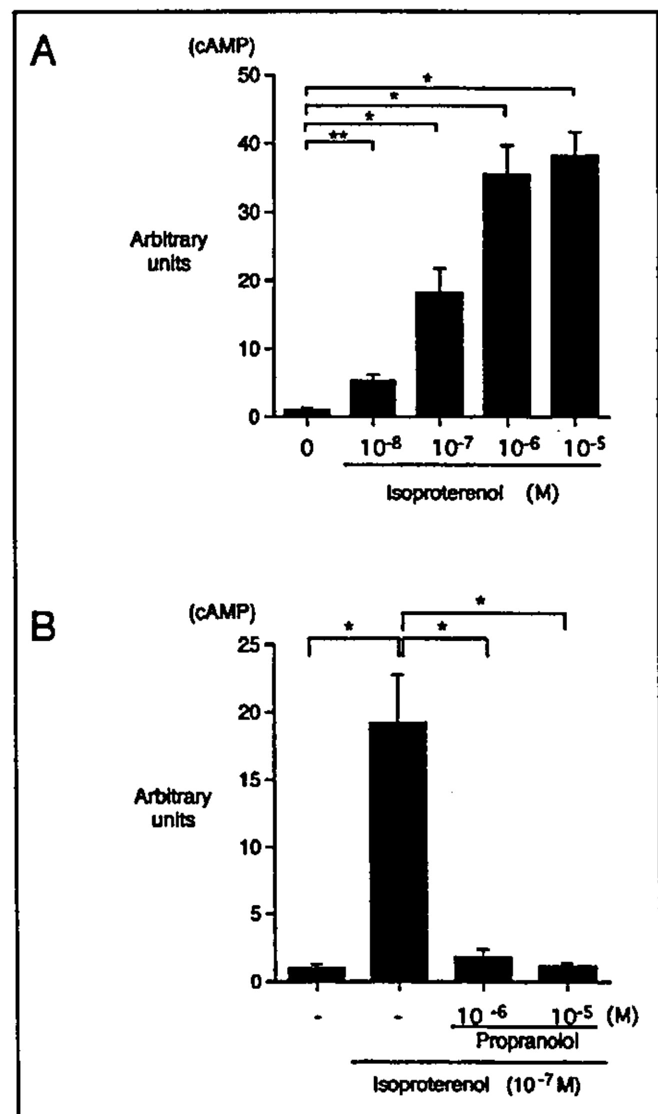

Figure 8. β receptor-mediated cyclic 3'5' adenosine monophosphate (cAMP) accumulation in CMG cells. A) Effect of isoproterenol on cAMP accumulation in CMG cells at 2 weeks after 5-azacytidine exposure. B) Cells were preincubated with propranolol (10^{-6} or 10^{-5} mol/L) for 20 minutes and stimulated with isoproterenol (10^{-7} mol/L) for 10 minutes. Data were obtained from 5 separate experiments and are shown as arbitrary units compared with the controls. *= p<0.01, **= p<0.05 vs. controls.

that it increased it significantly to 48% over the rate in the control cells.[12] Preincubation with propranolol (non-selective β blocker), or CGP20712A (β_1-selective blocker) strongly reduced the isoproterenol-induced increase in beating rate, and preincubation with ICI118551 (β_2-selective blocker) only slightly decreased the beating rate. The increase in beating rate was similar to that of adult murine cardiomyocytes and ES cell-derived cardiomyocytes.

We also investigated the effect of isoproterenol on the contractile function of CMG cells and found that it increased cell motion distance, % shortening, and contractile velocity. The isoproterenol-induced increase in contractility was almost completely inhibited by both propranolol and CGP20712A. Collectively, these results indicated that the β_1 and β_2-adenergic receptors expressed in CMG cells are functional, and that the

Table 2. Isoproterenol increased the spontaneous beating rate and contractility of CMG cells, mainly via β_1 receptors

	Control	Isoproterenol (10^{-7} mol/L)			
		Vehicle	Propranolol (10^{-7} mol/L)	CGP20712A (10^{-7} mol/L)	ICI118551 (10^{-7} mol/L)
% increase in beating rate	-	47.6±8.4*	10.0±1.9†	13.8±2.4†	37.6±1.9‡
cell motion (μm)	5.0±0.3	6.8±0.7*	5.6±0.8‡	5.3±0.6‡	ND
% shortening (%)	6.9±0.5	8.5±1.2*	7.2±0.8‡	5.6±0.6‡	ND
contractile velocity (μm/s)	71.1±5.2	100.9±11.0*	71.3±8.8‡	70.6±6.6‡	ND

CMG cells at 4 weeks after 5-azacytidine exposure were initially exposed to prazosin (10^{-6} mol/L) for 30 minutes to block α_1-adrenergic receptors. Cells were then preincubated for 20 minutes with vehicle (PBS), propranolol, CGP20712A, or ICI118551, and then stimulated with isoproterenol. The beating rate was counted 3 minutes after stimulation. Contractile parameters were analyzed 90 seconds after stimulation. Each contractile parameter value was calculated as the mean of 3 randomly selected beats in one cell. PBS was added to the control. Values are means ± SE (n= 100, each). *= $p<0.05$ vs. control; †= $p<0.01$ vs. vehicle (isoproterenol only); ‡= $p<0.05$ vs. vehicle; ND= not determined.

isoproterenol-induced increase in spontaneous beating rate and contractility is mainly mediated by β_1 receptors. The β_1 receptor was the predominant subtype that mediated changes in the beating rate in CMG cells, and the beating rate and the contractility were significantly increased by isoproterenol, and completely inhibited by propranolol and CGP20712A. β_1-Receptors played a critical role in mediating the isoproterenol-induced signaling in differentiated CMG cells. This expression pattern was consistent with that of cardiomyocytes in vivo.

Phenylephrine and Isoproterenol Induce Atrial Natriuretic Peptide (ANP) and Brain Natriuretic Peptide (BNP) mRNA Expression

Hypertrophic stimuli are well known to induce reprogramming of gene expression in cardiomyocytes. Phenylephrine and isoproterenol significantly induced expression of the ANP (24 hour) gene, and they also induced the BNP (1 hour) gene (Fig. 9). These findings demonstrated that α and β adrenergic signal transduction systems in CMG cells are linked to the gene expression that induces cardiac hypertrophy.

CMG Cells Express Muscarinic Receptor mRNA after 5-Azacytidine Exposure

Heart rate, conduction velocity, and contractility were negatively regulated by the parasympathetic nervous system in cardiomyocytes, and muscarinic (cholinergic) receptors play an important role in mediating this function. To date, 5 subtypes (M_1-M_5) of muscarinic receptors have been cloned. The expression of the muscarinic receptors is tissue-specific, and cardiomyocytes mainly express M_2 receptors in the mouse and human.[22] The M_1 receptor subtype is also expressed in murine neonatal and adult cardiomyocytes. Figure 10A shows the temporal expression pattern of M_1 and M_2 receptor mRNA. Neither receptor was detected prior to 5-azacytidine exposure. CMG cells began to express these receptors when they acquired the cardiomyocyte phenotype.

M_1 receptors coupled to Gq/G_{11} and activated phospholipase Cβ via Gqα, leading to inositol triphosphate (IP_3) production, and M_2 receptors coupled to $Gi/G_0/Gz$ and activated phospholipase Cβ via Giβγ, leading to IP_3 production.[25,26] Carbachol, an acetylcholine homologue, increased the content of a second messenger, IP_3 (inositol triphosphate), in CMG cells (Fig. 10B), and preincubation with atropine (non-selective muscarinic blocker) and AFDX116 (M_2-selective blocker) inhibited the carbachol-induced IP_3 production (Fig. 10C). These findings indicated that muscarinic receptors can transduce their signals, and that M_2 receptors play a critical role in this carbachol-induced IP_3 production in CMG cells. This expression pattern is similar to that of cardiomyocytes in vivo.

Significance of Expression of Adrenergic and Muscarinic Receptors in CMG Cells

Cardiomyocytes in vivo respond to stimulation by both sympathetic and parasympathetic nerves, and such stimulation alters the heart rate, conduction velocity, and contractility, enabling the cells to adapt to rapid changes in systemic oxygen demand. To date, and to our knowledge, ES cells and mesenchymal-stem-cell-derived CMG cells are the only possible candidates for regeneration of cardiomyocytes. We have already transplanted these cells into normal adult mouse hearts, and have observed that transplanted cells survived in recipient hearts for at least several weeks. Regenerated cardiomyocytes must express functional adrenergic and muscarinic receptors to be useful for transplantation, and although we did not investigate all signaling pathways and their functions, CMG cells are potential candidates for cardiomyocyte cell transplantation, because they possess such receptors.

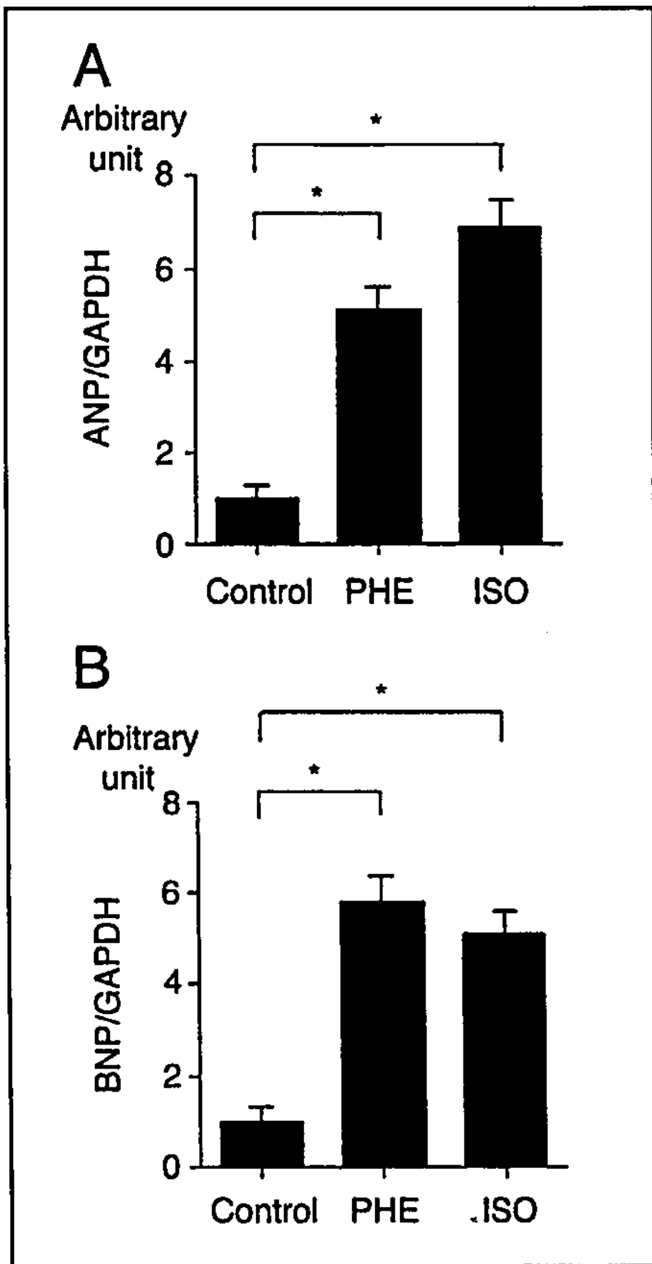

Figure 9. Both α and β stimulation induced mRNA expression of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) genes. CMG cells were serum depleted for 24 hours and pretreated with propranolol and stimulated with phenylephrine (PHE) (50 μM) or isoproterenol (ISO) (100 μM). RNA was extracted for 1 hour (BNP) and 24 hours (ANP), and reverse transcriptase polymerase chain reaction (RT-PCR) was examined. All values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *= p<0.01 vs. control.

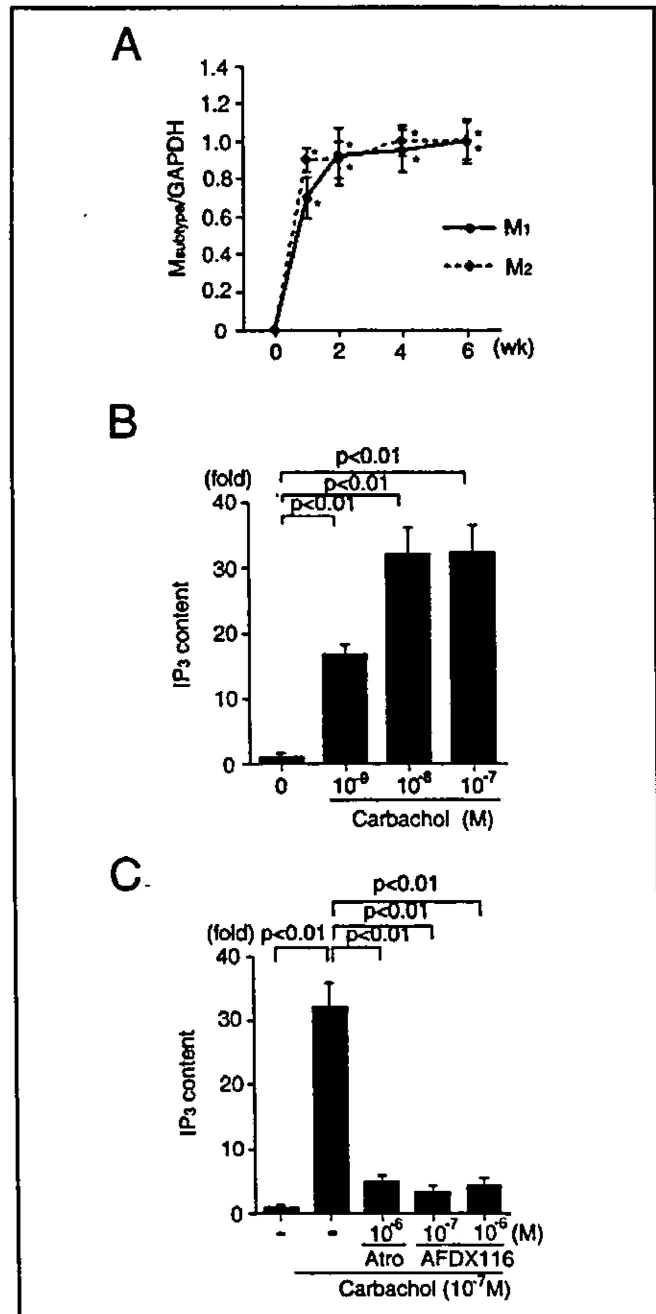

Figure 10. Expression and function of M_1- and M_2-muscarinic receptors in CMG cells. A) The ratio of the reverse transcriptase polymerase chain reaction (RT-PCR) product of muscarinic subtype to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown. Data were obtained from 5 separate experiments and are shown as arbitrary units over controls. *= p<0.01 vs. controls. B) Effect of carbachol on inositol triphosphate (IP_3) production in CMG cells at 2 weeks after 5-azacytidine exposure. C) Effect of atropine (10^{-6} mol/L) and AFDX116 (M_2-selective blocker) (10^{-7} or 10^{-6} mol/L) on carbachol-induced IP_3 production. Data were obtained from 5 separate experiments and are shown as arbitrary units compared with the controls. *=p<0.01 vs. controls. Atro= atropine.

Cell Transplantation Therapy for the Treatment of Heart Failure

We have already transplanted CMG cells into normal adult mouse hearts, and observed that the transplanted cells could survive in the recipient heart for at least several months. Fibroblasts, smooth muscle cells, and skeletal muscle cells were the first cells used for transplantation into scar tissue secondary to experimental myocardial infarction in the heart in vivo. While transplantation

of these cells into scar tissue might improve cardiac remodeling or diastolic function, it is unlikely to improve systolic function. Transplantation of cardiomyocytes, however, might rescue systolic function. The only potential sources of regenerated cardiomyocytes available to date are embryonic stem (ES) cells and mesenchymal stem cells. ES cells differentiate into cardiomyocytes in vitro and have both advantages and disadvantages for cardiomyocyte regeneration. Transplanted ES cells may form teratomas if some undifferentiated totipotent cells are still present, and recipients must take immunosuppressants, because ES cells are allogeneic. By contrast, since mesenchymal stem cells do not carry any inherent risks of tumor formation and are syngeneic, it is reasonable to use autologous mesenchymal stem cells to treat heart disease. Nevertheless, there is a need to improve both the current methods for identification and culture of mesenchymal stem cells, and for induction of CMG cell differentiation, which are still inefficient and slow. Identification of specific growth factors, cytokines, or extracellular matrix factors that regulate cardiomyocyte differentiation may help to accelerate this process faster and make it more efficient.

In Vivo Evidence that Marrow Cells Can Generate Functional Cardiac Tissues

Recent studies have revealed that bone-marrow-derived cells differentiate into various types of cells in vivo. Shimizu et al reported that smooth-muscle-like cells (SMCs) in graft-vs-host arterial lesions could arise from circulating bone-marrow-derived precursors. They used murine aortic transplants to formally identify the source of SMCs in lesions in grafted arteries.[27] Allografts in beta-galactosidase transgenic recipients showed that intimal SMCs arose almost exclusively from host cells, and bone-marrow transplantation of beta-galactosidase-expressing cells into aortic allograft recipients demonstrated that the intimal cells included those of marrow origin.

Kocher et al showed that bone marrow from adult humans contains endothelial precursors with phenotypic and functional characteristics of embryonic hemangioblasts and that they can be used to directly induce new blood vessel formation in the infarct-bed (vasculogenesis) and proliferation of preexisting vasculature (angiogenesis) after experimental myocardial infarction.[28] The neoangiogenesis resulted in decreased apoptosis of hypertrophied myocytes in the peri-infarct region, long-term salvage and survival of viable myocardium, reduction in collagen deposition, and sustained improvement in cardiac function.

We also observed that transplanted bone marrow cells differentiated into cardiomyocytes in the recipient heart in vivo (unpublished observation). These findings provided direct evidence that bone marrow cells can regenerate various types of cells in cardiac tissue. We expect cardiac tissues damaged by myocardial infarction or other diseases to be repaired by bone-marrow-derived stem cells in the near future, and the precise mechanism should be investigated to achieve this goal.

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第１章 心臓病診療の課題と展望(トピックス)

1.　再生医学による心臓病治療

1.1 はじめに

ここ 10 年の心臓病の治療を振り返る時、カテーテル治療や植え込み型除細動器の開発、アンジオテンシンⅡ受容体遮断薬の一般臨床への普及、マルチスライス CT による画像診断の進歩など、その目覚ましい発展には驚かされるものが多い。しかし、この 10 年間で著しい進歩がみられなかったものに、難治性重症心不全の治療が挙げられよう。この疾患に対してはこれまで心臓移植が根本治療とされてきたが、ドナーの不足は如何ともしがたく、補助人工心臓による一時的な延命がはかられるのみであった。これに対し、細胞生物学、遺伝子工学の発達は新たな展開をもたらそうとしている。未分化幹細胞を心筋細胞に分化誘導し、これを移植治療の材料として使うという、いわゆる再生医学である。本稿では再生医学による心臓病治療というテーマをいただいたが、そのなかでも特に心筋細胞に分化可能な多能性幹細胞に焦点をあて、現状を解説することとした。

1.2 心筋細胞に分化が可能な多能性幹細胞

近年の精力的な研究により、さまざまな幹細胞が明らかにされている。このうち心筋細胞に分化可能なことが報告され、将来的に臨床応用可能と考える細胞は胚性幹細胞、骨髄間葉系幹細胞、心筋内組織幹細胞であろう。それぞれに利点・欠点を有しており、現時点ではどの細胞が有望であるかについて結論は出ていない。以下、各々の細胞の特徴と現状につき説明する。

1.2.1 胚性幹細胞

胚性幹細胞（ES 細胞）は受精早期の胚盤胞という時期の内部細胞塊という将来胎児になる部分より得られた細胞（図 1.1.1）で、身体のすべての細胞に分化し得るという意味で万能幹細胞とも呼ばれる。しかし、*in vitro* で実際に分化することができるのは、胎生早期の段階で分化が達成される細胞である。心筋細胞は胎児期の早期に分化する細胞であり、胚性幹細胞からは比較的得られやすい。マウスの場合には培養液中に LIF（白血病阻止因子）を入れておくことにより、未分化機能が維持されることが知られている。多くの細胞株では feeder layer として MEF（マウス胎児線維芽細胞）の上に胚性幹細胞を重層して培養することが多いが、近年は技術の向上により feeder layer を必要としない培養も可能になった。ヒトの胚性幹細胞もすで

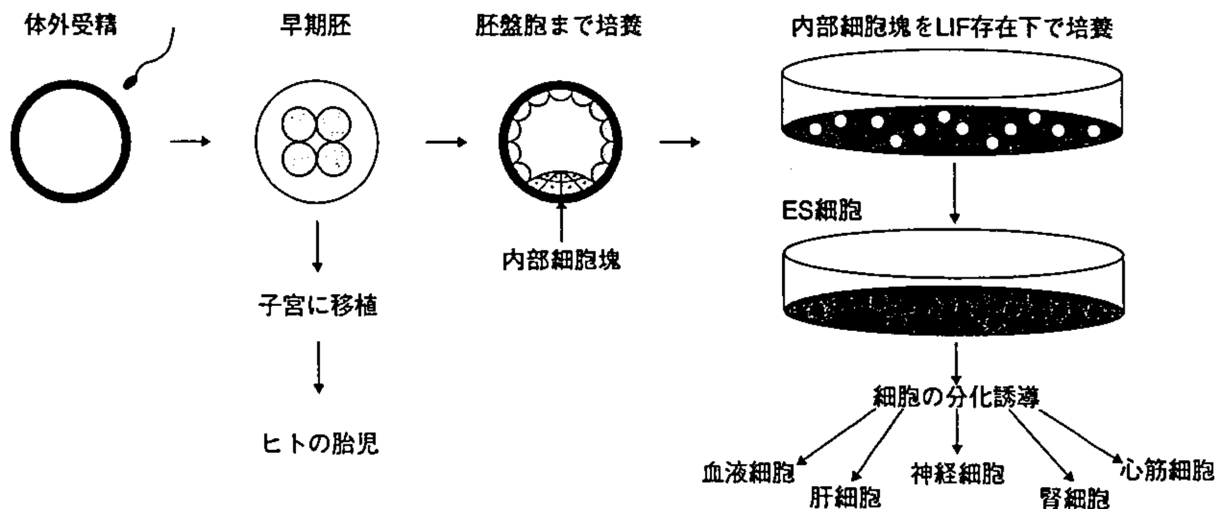

図 1.1.1 胚性幹細胞の樹立と利用
受精早期の胚盤胞より内部細胞塊を取り出し、胚性幹細胞を作成する。

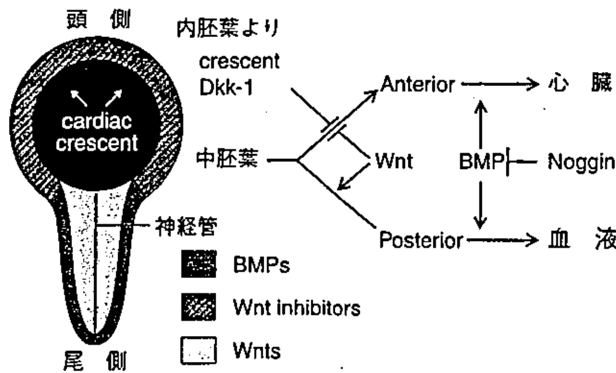

図1.1.2 中胚葉より心筋細胞への分化経路(文献3より改変)

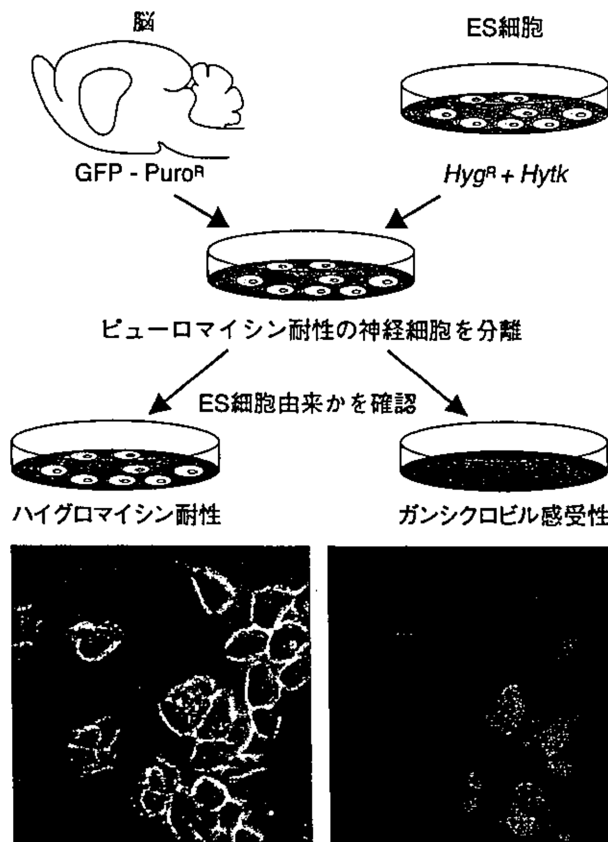

図1.1.3 細胞融合の概念と証明（口絵1参照）
神経細胞と胚性幹細胞の細胞融合を証明した実験の概要を示した。

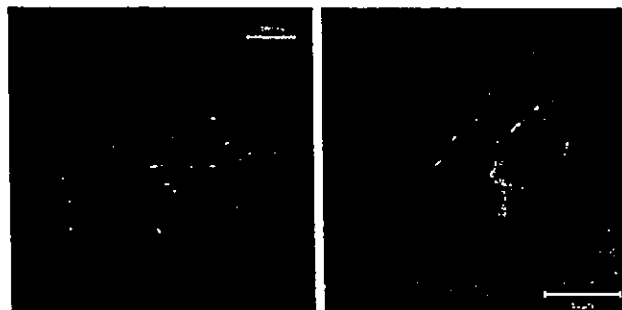

図1.1.4 骨髄移植モデルマウスを用いた再生心筋(口絵2参照)
GFP過剰発現マウスより得た骨髄を同系統マウスに骨髄移植し、心筋梗塞を作成した。骨髄由来細胞はGFP陽性細胞として観察される。右図にはGFP陽性、アクチニン陽性の心筋細胞が少数観察された。

に多数樹立され、本邦においても京都大学の中辻先生により3株ほど胚性幹細胞が樹立されている[1]。ヒトの胚性幹細胞はLIFにより未分化機構が維持されることはなく、MEFとの共培養が必須であるとされている。また、マウスと同様にヒトの胚性幹細胞も心筋細胞に分化することがすでに報告されている[2]。胚性幹細胞から心筋細胞への分化には図1.1.2のようにBMP2やWntといったシグナル伝達経路が重要であるとされているが、その詳細は明らかではない[3]。胚性幹細胞の臨床応用上の問題点は、①未分化機構維持のため動物細胞（MEF）と共培養が必要である点、②胚性幹細胞から心筋細胞への選択的分化誘導法の確立、③未分化細胞の混入による奇形腫形成の抑制、④同種移植であるため免疫拒絶が避けられない点、などが挙げられる。患者本人の体細胞の核を卵細胞に移植することによるクローン胚の形成は、動物レベルでは可能となった。近年、韓国よりヒトクローン胚の成功が報じられた。しかし、その場合でも100個以上の卵を用いて1個成功したとされている。先進諸国のほとんどでクローン人間の作成は禁止されているが、臓器作成を目的としたクローン胚性幹細胞は一部の国で行われる可能性はある。この点に関しては国民的な議論がなされるべきである。技術革新により、卵細胞を用いずに胚性幹細胞レベルでのクローン化ができれば、倫理的にはハードルが低くなって応用される可能性もあり、さらなる研究が望まれる。

1.2.2 骨髄間葉系幹細胞

骨髄は造血の主たる場であり、骨髄中の細胞のほとんどは造血幹細胞に由来する血液細胞である。しかし、骨髄中には間質細胞と呼ばれる接着系の細胞（培養皿に付着する細胞）が存在し、造血幹細胞の機能維持に働いている。そして、間質細胞の一部に多分化能を有する間葉系幹細胞と呼ばれる細胞が存在し、*in vitro*、*in vivo*でさまざまな細胞に分化することが知られている[4]。骨芽細胞、軟骨芽細胞、脂肪細胞、骨格筋細胞、心筋細胞などの中胚葉系の細胞に主として分化するが、一部神経細胞などの外胚葉系の細胞にも分化することが報告されている[5,6]。一時期造血幹細胞にも多分化能があることが報告され、造血幹細胞の多分化能が誇張された。これは心臓移植後の剖検心蔵を用いた研究より始まった。すなわち、女性ドナーから提供された心臓が男性レシピエントに移植され、一定時間後に別の理由で死亡し剖検したところ、心臓内にY染色体陽性の心筋細胞が観察されたと報告された[7]。この現象は男性骨髄から多能性幹細胞が心臓に移動

し、心筋に分化したのであろうとされた。しかしその後、細胞融合という現象が報告され（図 1.1.3）、造血幹細胞のみかけの多能性に関しては細胞融合が原因ではないかという否定的な見解が多く示された[8]。

われわれの GFP トランスジェニックマウスを用いた骨髄移植モデルによる検討でも、造血幹細胞の多能性に関しては否定的な所見が得られている（図 1.1.4）。しかし、間葉系幹細胞に関しては *in vitro* の実験同様、*in vivo* でも心筋細胞に分化する所見が得られており、骨髄由来細胞の多能性は間葉系幹細胞に起因するものと考えられる。

最近２年ぐらいのトピックは骨髄内の MAPC 細胞（mulitipotent adult progenitor cell）であろう[9]。MAPC 細胞はミネソタ大学の Verfeillie 教授らのグループが報告した多分化能を有する細胞で、あらゆる種類の細胞に分化誘導可能な細胞であると報告されている[10]。MAPC を別のマウスの受精卵の胚盤胞に注射してキメラマウスを形成させると、MAPC は胚性幹細胞と同様にほぼすべての細胞に分化することが報告された。しかし、MAPC の培養法は極めて細胞密度の薄い条件で培養しないと細胞が分化するとされ、再現性も乏しいことから、細胞を *in vitro* で長期間培養することによるアーチファクトではないかとする声もある。MAPC に関しては今後の研究をみた上で慎重に判断されるべきであろう。

1.2.3 心筋内の幹細胞

昨年後半に米国で相次いで成体心臓内に存在する多能性幹細胞の存在が報告された。Anversa らは心筋細胞中の SCF（stem cell factor）受容体である c-kit 陽性の小型の細胞を単離し培養すると、心筋、平滑筋、内皮細胞に分化すると報告した。この c-kit 陽性細胞はクローナルに増殖し、心筋梗塞時には梗塞巣で心筋細胞に分化し、梗塞巣の修復に関与しているのではないかとしている[11]。また、この細胞は心尖部と心房内に散在して存在し、心筋梗塞時などには梗塞巣に移動するとしている。これに対し Schneider らは成体心組織に Sca-1（stem cell antigen-1）陽性の細胞が存在し、この細胞は多分化能をもつわけではないが DNA 脱メチル化剤の 5-azacytidine を用いることにより心筋細胞に分化が可能であるとし、心筋細胞の progenitor（前駆細胞）ではないかとしている[12]。この Sca-1 陽性細胞は尾静脈より注射すると心筋梗塞部に homing するという。さらに Hierlihy らは心臓内に存在する SP（side population）細胞を採取してくると一部の細胞が心筋細胞に分化すると報告した[13]（図 1.1.5）。SP 細胞は Mulligan らが骨髄の造血幹細胞を濃縮する方法として開発したもので、DNA 結合色素の Hoechst33342 の細胞外への汲み出し能力を指標として幹細胞を分離する方法である。Hoechst33342 を細胞外に汲み出すポンプは ABC トランスポーターである MRD1 が関与しているとされ、この蛋白を発現する細胞に幹細胞として

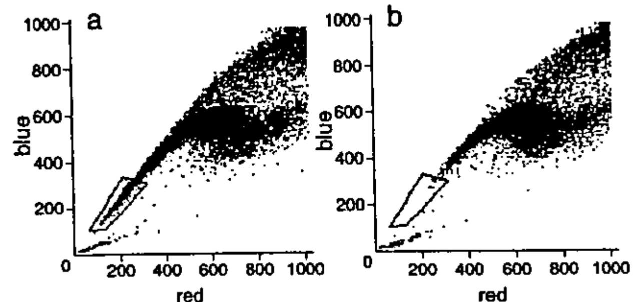

図 1.1.5 心臓由来の SP 細胞分画
a : reserpine（−）　b : reserpine（+）

	c-kit	MRD1	Sca-1
構　造	免疫グロブリン様ドメイン チロシンキナーゼドメイン	細胞外領域 細胞内領域 ATP	PtdIns anchor
分　布	メラノサイト、マスト細胞、生殖細胞、幹細胞	肝細胞、胆管細胞、brush border cells 腎尿細管細胞、癌細胞、脳血管内皮細胞、幹細胞	血管壁、腎皮質細胞、胸腺、脾臓、T リンパ球、幹細胞
機　能	増殖、遊走、分化、分泌	膜に存在する排出ポンプ、アポトーシスの抑制	細胞接着、シグナル伝達、T 細胞活性化

図 1.1.6 c-kit、MDR-1、Sca-1 の各種細胞における構造、機能と分布

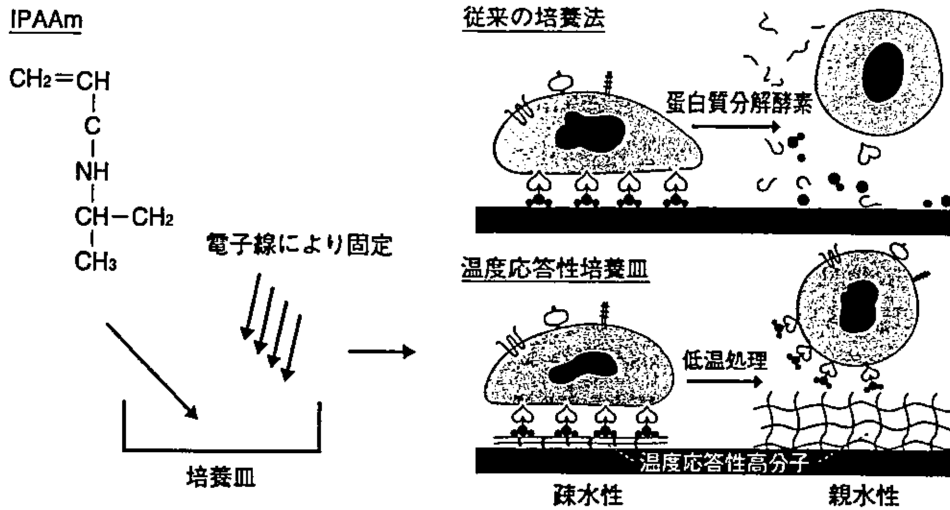

図 1.1.7 東京女子医科大学岡野らが開発した温度感受性培養皿の構造と機能

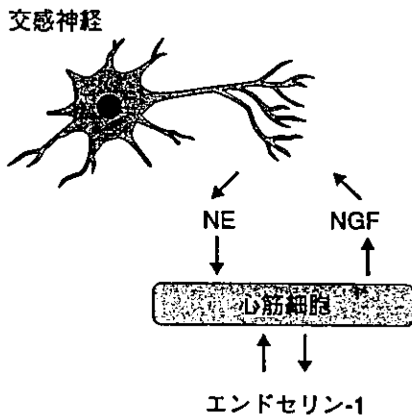

図 1.1.8 心臓と交感神経の関係
心筋細胞自身が分泌したエンドセリン-1 が autocrine に心筋細胞に作用し、NGF を分泌する。このNGF が交感神経を呼び込むことにより心臓の交感神経が発達する。

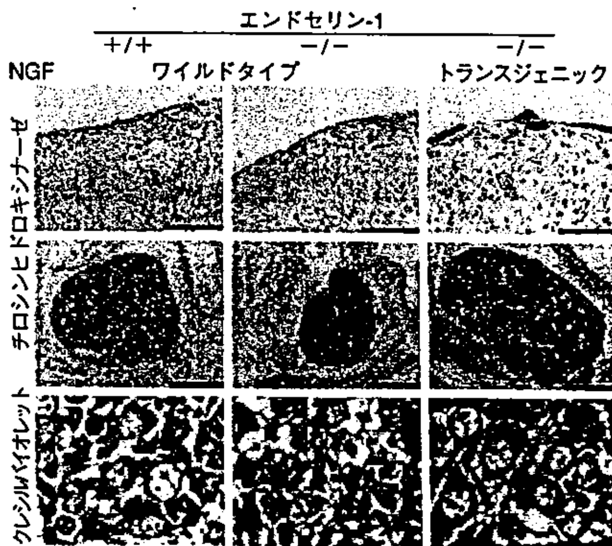

図1.1.9 エンドセリン-1欠損マウスの心臓における交感神経(口絵3参照)
エンドセリン-1 欠損マウスでは心臓の交感神経が欠損するとともに星状神経節のアポトーシスが観察される。この表現型は心臓特異的に NGF を過剰発現することにより回復する。

の能力が高いとされる。c-kit、Sca-1、MRD1 自体の構造、機能、発現する細胞をまとめたものを図1.1.6 に示した。これらの c-kit 陽性細胞、Sca-1 細胞、MRD1 陽性の SP 細胞は一部重複する性質をもつが、基本的には異なる細胞であると思われ、その存在頻度、生体での機能と存在意義は今後の解決すべき問題であろう。

これらの心臓内に存在する幹細胞が心筋細胞として存在するとして、これらを生体外に取り出して大量培養し、*in vitro* で心筋細胞に分化させて、再移植するには *in vitro* での大量培養法の確立と心筋分化誘導法が必要不可欠のものとなろう。むしろ、生体内で増殖・分化させる方が現実的である。今後のさらなる研究の発展が期待される。

1.3 心筋細胞移植の方法

これまでの心臓への(再生)心筋細胞移植は、*in vivo* の心臓に直接注射針で細胞を移植するという方法がとられた(図 1.1.7)。しかし、この方法では移植した細胞の生着率が低く、移植できる細胞の数も限られたものであった。これに対し、東京女子医科大学の Okano らは、培養皿の表面に温度感受性に分子形態が変化し、脂溶性・水溶性を転換させる poly-N-isopropylacryl-amide (PIPAAm) という化合物をコーティングする温度感受性培養皿を作成した[14]。この培養皿を用いると培養細胞をシート状に回収することができ、移植に直接用いることができる。Shimizu らはこの培養皿を用いて心筋細胞シートを作成し、ラット皮下に長期間生着し、拍動を続ける層状の心筋組織の開発に成功した[15]。Shimizu らの研究成果は心筋細胞の移植法に大きなインパクトを与えた。これまで細胞レベルの再生のみに注目が集められてきたが、細胞レ

ベルから組織レベルの再生を論じる素地を作るものとなったといえよう。組織レベルの再生にはこれ以外にも血管の構築や神経支配も重要な問題であり、今後の研究を待つことになろう。

1.4 心臓の交感神経支配の形成

心臓は諸臓器のなかでも唾液腺と並び交感神経の分布密度が高い臓器である。交感神経の刺激により、心臓は心拍数の上昇、心収縮力の増強、刺激伝導速度の増強が起こる。心臓を支配する交感神経は主として星状神経節の神経細胞の支配を受けるが、これまで心臓を支配する交感神経がいかなる機序で形成されるかは解明されてこなかった。われわれの近年の解析では、心臓への交感神経支配は胎生後期に心筋細胞から分泌される神経成長因子（NGF）を指標に星状神経節から軸索が伸長してくる。そしてこのときに心筋細胞がautocrineに分泌するエンドセリン-1が心筋細胞自身に作用してET-A受容体、Gi$\beta\gamma$を介して経路でNGFを分泌させることが明らかとなった（図1.1.8）[16]。そしてこのエンドセリン-1/NGF経路が存在しない場合には心筋細胞に交感神経がこないだけでなく、星状神経節の交感神経細胞体自身がアポトーシスを起こすことも明らかとなった（図1.1.9）。心臓移植あるいは再生心筋細胞移植した臓器・組織に再神経支配を行うためには、種々のさらに基礎レベルの研究が必要であろう。

1.5 再生医学の今後

われわれが研究を始めた10年前は、心臓の再生などはSFの世界ではないかと考えられていた。しかし、今では骨髄間葉系幹細胞や胚性幹細胞を用いた心筋細胞の再生が可能となり、再生心筋をシート状にした組織レベルでの再生も可能となってきた。もちろんこれだけでは現実の医療にすぐ結び付くものではない。血管構築や神経支配を伴う再生組織の作成にはまだ越えねばならないステップは山ほどあるであろう。過去100年間の医学の進歩を顧みるとき、これから先の100年後には必ず再生臓器は臨床応用されていると考えられる。子供の頃にみた鉄腕アトムが2足歩行するロボットとして現実味を帯びてきた今、再生臓器の具現化もそう遠い将来ではないと確信している。

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（福田恵一）

Weakly ionized plasma flash x-ray generator and its distinctive characteristics

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ABSTRACT

In the plasma flash x-ray generator, a high-voltage main condenser of approximately 200 nF is charged up to 50 kV by a power supply, and electric charges in the condenser are discharged to an x-ray tube after triggering the cathode electrode. The flash x-rays are then produced. The x-ray tube is a demountable triode that is connected to a turbo molecular pump with a pressure of approximately 1 mPa. As electron flows from the cathode electrode are roughly converged to a rod copper target of 3.0 mm in diameter by the electric field in the x-ray tube, weakly ionized linear plasma, which consists of copper ions and electrons, forms by target evaporation. At a charging voltage of 50 kV, the maximum tube voltage was almost equal to the charging voltage of the main condenser, and the peak current was about 15 kA. When the charging voltage was increased, the linear plasma formed, and the K-series characteristic x-ray intensities increased. The K-series lines were quite sharp and intense, and hardly any bremsstrahlung rays were detected. The x-ray pulse widths were approximately 700 ns, and the time-integrated x-ray intensity had a value of approximately 30 μC/kg at 1.0 m from the x-ray source with a charging voltage of 50 kV.

Keywords: Flash x-ray, weakly ionized linear plasma, K-series characteristic x-rays, monochromatic x-rays, x-ray divergence, rectilinear power

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1. INTRODUCTION

Flash x-rays have been produced by several different methods, and various generators have been developed corresponding to specific radiographic objectives.[1-3] Currently, the maximum photon energy has been increased to approximately 1 MeV using multiple-stage Marx pulse generators[1,2] in order to produce hard x-rays for military studies. In soft x-ray generators,[4-8] high-intensity single generators with large capacity condensers were originally developed. Subsequently, repetitive generators[9-12] have been developed, and the repetition rate has been increased to sub-kilohertz using a cold-cathode triode.

Recently, soft x-ray lasers have been produced by a gas-discharge capillary,[13-16] and the laser pulse energy substantially increased in proportion to the capillary length. These kinds of fast discharges can generate hot and dense plasma columns with aspect ratios approaching 1000:1. However, it is difficult to increase the laser photon energy to 10 keV or beyond. Because there are no x-ray resonators in the high photon energy region, new methods for increasing coherence will be desired in the future.

By forming weakly ionized linear plasma[17-21] using plate and rod targets, we confirmed irradiation of intense K-series characteristic x-rays from the plasma axial direction. In these experiments, because we employed a transmission-type x-ray spectrometer utilizing an x-ray film, it was difficult to determine the relative intensities of the characteristic x-rays. In former experiments, because we have succeeded in producing fairly intense and sharp K-series characteristic x-rays, monochromatic x-rays should be produced using a filter.

In this paper, we describe a plasma flash x-ray generator utilizing a rod-target radiation tube, used to perform a preliminary experiment for generating intense and sharp monochromatic x-rays by forming a linear copper plasma cloud around a fine target.

2. GENERATOR

2.1 High-voltage circuit

Figure 1 shows a block diagram of the high-intensity plasma flash x-ray generator. This generator consists of the following essential components: a high-voltage power supply, a high-voltage condenser with a capacity of approximately 200 nF, a turbo-molecular vacuum pump, a krytron pulse generator as a trigger device, and a flash x-ray tube. In this generator, a low-impedance transmission line is employed in order to increase maximum tube current. The high-voltage main condenser is charged to 50 kV by the power supply, and electric charges in the condenser are discharged to the tube after triggering the cathode electrode with the trigger device. The plasma flash x-rays are then produced.

2.2 X-ray tube

The x-ray tube is a demountable cold cathode triode that is connected to the turbo-molecular pump with a pressure of approximately 1 mPa (Fig. 2). This tube consists of the following major parts: a pipe-shaped carbon cathode with a bore diameter of 10.0 mm, a trigger electrode made from copper wire, a stainless steel vacuum chamber, a nylon insulator, a polyethylene terephthalate (Mylar) x-ray window 0.25 mm in thickness, and a rod-shaped copper target 3.0 mm in diameter with a tip angle of 60°. The distance between the target and cathode electrodes is approximately 20 mm, and the trigger electrode is set in the cathode electrode. As electron beams from the cathode electrode are roughly converged to the target by the electric field in the tube, evaporation leads to the formation of a weakly ionized linear plasma, consisting of copper ions and electrons, around the fine target.

2.3 Principle of characteristic x-ray irradiation

In the linear plasma, bremsstrahlung photons with energies higher than the K-absorption edge are effectively absorbed and are converted into fluorescent x-rays (Fig. 3). The plasma then transmits the fluorescent rays easily, and bremsstrahlung rays with energies lower than the K-edge are also absorbed by the plasma. In addition, because bremsstrahlung rays are not emitted in the direction opposite that of electron acceleration, intense characteristic x-rays are generated from the plasma-axial direction.

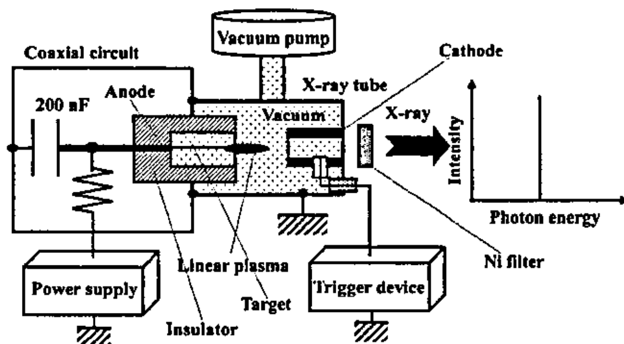

Figure 1: Block diagram of the high-intensity plasma flash x-ray generator.

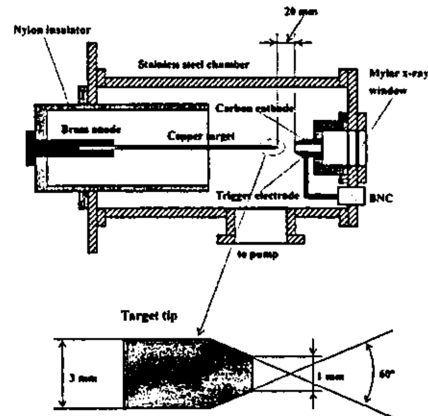

Figure 2: Schematic drawing of the flash x-ray tube with a rod target.

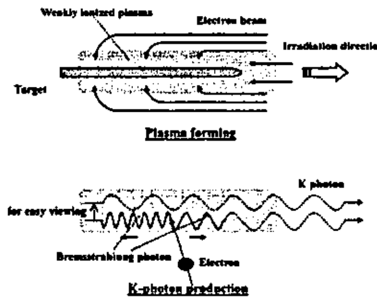

Figure 3: K-photon irradiation from the plasma.

3. CHARACTERISTICS

3.1 Tube voltage and current

Tube voltage and current were measured by a high-voltage divider with an input impedance of 1 GΩ and a current transformer, respectively. Figure 4 shows the time relation for the tube voltage and current. At the indicated charging voltages, they roughly displayed damped oscillations. When the charging voltage was increased, both the maximum tube voltage and current increased. At a charging voltage of 50 kV, the maximum tube voltage was almost equal to