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Creation of mouse embryonic stem cell-derived cardiac cell sheets

Katsuhisa Matsuura [a,b,1], Shinako Masuda [a,1], Yuji Haraguchi [a], Noriko Yasuda [a], Tatsuya Shimizu [a], Nobuhisa Hagiwara [b], Peter W. Zandstra [c], Teruo Okano [a,*]

[a] Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku, Tokyo 162-8666, Japan
[b] Department of Cardiology, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku, Tokyo 162-8666, Japan
[c] Institute of Biomaterials and Biomedical Engineering, Donnelly Center for Cellular and Biomolecular Research, University of Toronto, 160 College Street, Room 1116, Toronto, Ontario M5S 3E1, Canada

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ABSTRACT

Research on heart tissue engineering is an exciting and promising area. Although we previously developed bioengineered myocardium using cell sheet-based tissue engineering technologies, the issue of appropriate cell sources remained unresolved. In the present study, we created cell sheets of mouse embryonic stem (ES) cell-derived cardiomyocytes after expansion in three-dimensional stirred suspension cultures. Serial treatment of the suspension cultures with noggin and granulocyte colony-stimulating factor significantly increased the number of cardiomyocytes by more than fourfold compared with untreated cultures. After drug selection for ES cells expressing the neomycin-resistance gene under the control of the α-myosin heavy chain promoter, almost all of the cells showed spontaneous beating and expressed several cardiac contractive proteins in a fine striated pattern. When ES-derived cardiomyocytes alone were seeded onto temperature-responsive culture dishes, cell sheets were not created, whereas cocultures with cardiac fibroblasts promoted cell sheet formation. The cardiomyocytes in the cell sheets beat spontaneously and synchronously, and expressed connexin 43 at the edge of adjacent cardiomyocytes. Furthermore, when the extracellular action potential was recorded, unidirectional action potential propagation was observed. The present findings suggest that stirred suspension cultures with appropriate growth factors are capable of producing cardiomyocytes effectively and easily, and that ES-derived cardiac cell sheets may be a promising tool for the development of bioengineered myocardium.

1. Introduction

Since heart failure is still a major cause of mortality in many developed countries, myocardial regeneration is considered to be a promising therapy for severe heart failure. Despite the principal concept of cardiac regeneration that transplanted stem cells differentiate into cardiomyocytes and replace the injured myocardium with newly cardiomyocytes, several recent studies have shown that paracrine effects mediated by growth factors secreted from the transplanted cells and the host tissue are the major mechanisms for adult stem cell transplantation-mediated improvement of cardiac function [1,2]. Since the adult human heart contains approximately 4×10^9 cardiomyocytes in the left ventricle [3], which suggests that it is an appropriate cell source for collecting huge amounts of cardiomyocytes, cell delivery methods and cell organization may be prerequisites for heart tissue reconstruction. Recently bioengineered myocardium has been proposed as one of the solutions for creating heart tissue [4]. We previously developed an original scaffold-free tissue engineering technology, designated "cell sheet-based tissue engineering", using temperature-responsive culture dishes covalently grafted to the temperature-responsive polymer poly(*N*-isopropylacrylamide) (PIPAAm) [5]. Lowering the culture temperature promotes a rapid surface transition from hydrophobic to hydrophilic, which enables us to collect a viable monolayer cell sheet with full preservation of the cell–cell contacts and extracellular matrices [6]. We also developed a cell sheet-based bioengineered myocardial tissue [7–9] using neonatal rat cardiomyocytes. However, the development of methods for the use of stem cell-based cardiomyocytes may be requisite for creating cell sheets and bioengineered heart tissue, since recent induced pluripotent stem (iPS) cell technologies have enabled the creation of autologous cardiomyocytes for clinical translation [10,11]. Furthermore, the following uncertainties

* Corresponding author. Tel.: +81 3 5367 9945; fax: +81 3 3359 6046.
E-mail address: tokano@abmes.twmu.ac.jp (T. Okano).
[1] These authors contributed equally to this work.

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remain for the cardiac cell sheets: (1) the ability to obtain a suitable number of stem cell-derived cardiomyocytes; (2) the necessity of using non-cardiomyocytes; and (3) the actual electrophysiological functions of cell sheets of stem cell-derived cardiomyocytes.

Many studies have reported that embryonic stem (ES) cells can differentiate into cardiomyocytes through embryoid body (EB) formation [12,13], and these studies have usually used hanging-drop methods. However, such methods may not be suitable for large-scale cultures, and three-dimensional stirred suspension cultures have therefore been used to create EBs and induce cardiac differentiation of ES cells [14,15]. Although suspension cultures are useful in terms of scaling up, it may be difficult to control the EB size, which may hinder cardiac differentiation [16]. On the other hand, robust studies have reported that several growth factors are related to heart development [17] and that certain growth factors such as noggin and granulocyte colony-stimulating factor (GCSF) promote cardiac differentiation of ES cells [18] and induce cardiomyocyte proliferation [19]. Accordingly, suspension cultures with the use of appropriate growth factors may promote cardiac differentiation, thereby enabling the collection of more cardiomyocytes.

The aims of this study were to establish easy and effective methods for collecting cardiomyocytes from mouse ES cells and creating cardiac cell sheets, and to elucidate the electrophysiological functions of ES-derived cardiac cell sheets.

2. Materials and methods

2.1. Animals and reagents

Wild-type C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). All the experimental protocols were approved by the Institutional Animal Care and Use Committee of Tokyo Women's Medical University. The following antibodies were used for immunocytochemistry: mouse monoclonal anti-sarcomeric α-actinin (Sigma-Aldrich, St. Louis, MO); mouse monoclonal anti-β-myosin heavy chain and mouse monoclonal anti-NG2 (Millipore, Temecula, CA); mouse monoclonal anti-cardiac troponin T (cTnT) (Thermo Scientific, Rockford, IL); guinea pig monoclonal anti-vimentin (Progen, Heidelberg, Germany); rabbit polyclonal anti-connexin 43 (Zymed Laboratories, South San Francisco, CA); and rabbit polyclonal anti-von Willebrand factor (Dako, Tokyo, Japan). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Unless otherwise specified, all reagents were purchased from Sigma-Aldrich.

2.2. Mouse ES cell cultures

R1 ES cells ubiquitously expressing EYFP and the neomycin phosphotransferase gene under the control of the α-myosin heavy chain promoter, and a phosphoglycerate kinase gene in front of a hygromycin-resistance gene were maintained as described previously [20].

For cardiac differentiation, trypsinized ES cells were seeded at 5×10^4 cells/mL (total, 125 mL/flask) into spinner flasks (Integra Biosciences, Zizers, Switzerland) and cultured as described previously [20] with a few modifications. Briefly, ES cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (FBS) without leukemia inhibitory factor until day 10. On day 6, the volume of medium was scaled up to 250 mL/flask. On day 10, 400 μg/mL G418 was added until day 18. The differentiation and selection media were changed every day. For noggin treatment, the cells were cultured with noggin (150 ng/mL) from 3 days before to 1 day after starting the suspension culture. For GCSF treatment, the cells were cultured with GCSF (1 ng/mL) from day 6 to day 10 of the spinner flask culture. The cell number at each time point was measured after EB dissociation with 0.25% trypsin/EDTA.

2.3. Cell isolation

Cardiomyocytes and fibroblasts were obtained from the hearts of neonatal mice (1–2 days of age) as described previously [21]. Cardiac fibroblasts from passage 4 were used for the experiments. Immunocytochemical analyses revealed that >99% of the cardiac fibroblasts expressed vimentin, and did not express von Willebrand factor (endothelial cell marker; data not shown) or NG2 (pericyte marker; data not shown).

2.4. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde and subjected to immunostaining as described previously [2]. Images of the stained samples were obtained by laser confocal microscopy (Carl Zeiss, Jena, Germany) or fluorescence microscopy (Nikon, Tokyo, Japan) with NIS-Elements software (Nikon).

2.5. Cell sheet preparation

Before seeding the cells, the surface of the temperature-responsive dishes (UpCell; CellSeed, Tokyo, Japan) was coated with FBS for 2 h. A mixed cell suspension of ES cell-derived cardiomyocytes and cardiac fibroblasts isolated from neonatal mouse hearts was plated onto each UpCell dish at 3.2×10^5 cells/cm^2, and the cells were cultured in DMEM supplemented with 15% FBS at 37 °C. After 4 days of culture, the cells were incubated at 20 °C. After 2 h, the cells spontaneously detached from the culture dish and floated in the medium as a monolayer cell sheet.

2.6. Electrophysiological analysis

The electrical activities of the cardiomyocyte sheets were obtained from the extracellular potentials measured by a multi-electrode array (MED) system (Alpha MED Sciences, Osaka, Japan) as described previously [9]. For monolayered cell sheet, 8 × 8 array prove (MED-515A: array size; 1.05 × 1.05 mm, distance between electrodes, 150 μm) and for partially overlaid cell sheets, cardiac sheet prove (MED-P5A15: electrode size; 50 μm, minimum distance between electrodes; 450 μm) were used.

2.7. Statistical analysis

Data are presented as means ± SD. The differences between groups were evaluated by analysis of variance followed by Bonferroni's correction. Values of $P < 0.05$ were considered to be statistically significant.

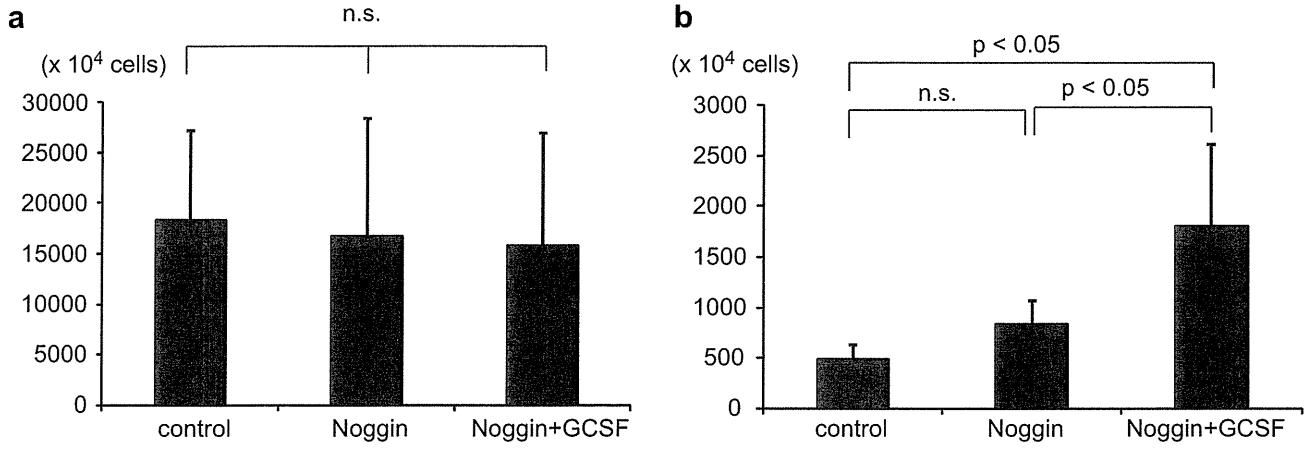

Fig. 1. Serial treatment with noggin and GCSF increases the number of cardiomyocytes in suspension cultures. The numbers of cells at 10 days (a) and 18 days (b) in suspension cultures ($n = 3$) are shown. The data are means ± SD.

Table 1
The total number of cells at day 0, 10 and 18 in each condition (n=3). The data are means ± SD.

	Day 0	Day 10	Day 18
Control ($\times 10^4$ cells)	625	18350 ± 8888	491 ± 143
Noggin ($\times 10^4$ cells)	625	16725 ± 11707	829 ± 243
Noggin + GCSF($\times 10^4$ cells)	625	15838 ± 11124	1800 ± 813

3. Results

3.1. Stirred suspension cultures for collecting ES-derived cardiomyocytes

Suspension cultures of mouse ES cells are considered to be promising methods for cell expansion and differentiation. When ES cells were seeded at 5 × 10^4 cells/mL in spinner flasks (6.25 × 10^6 cells/flask), we observed an approximately 30-fold increase in the cells forming EBs after 10 days in culture (Fig. 1a, Table 1). When the cells were treated with neomycin from day 10 to day 18, approximately 5 × 10^6 cells remained at day 18 (Fig. 1b,

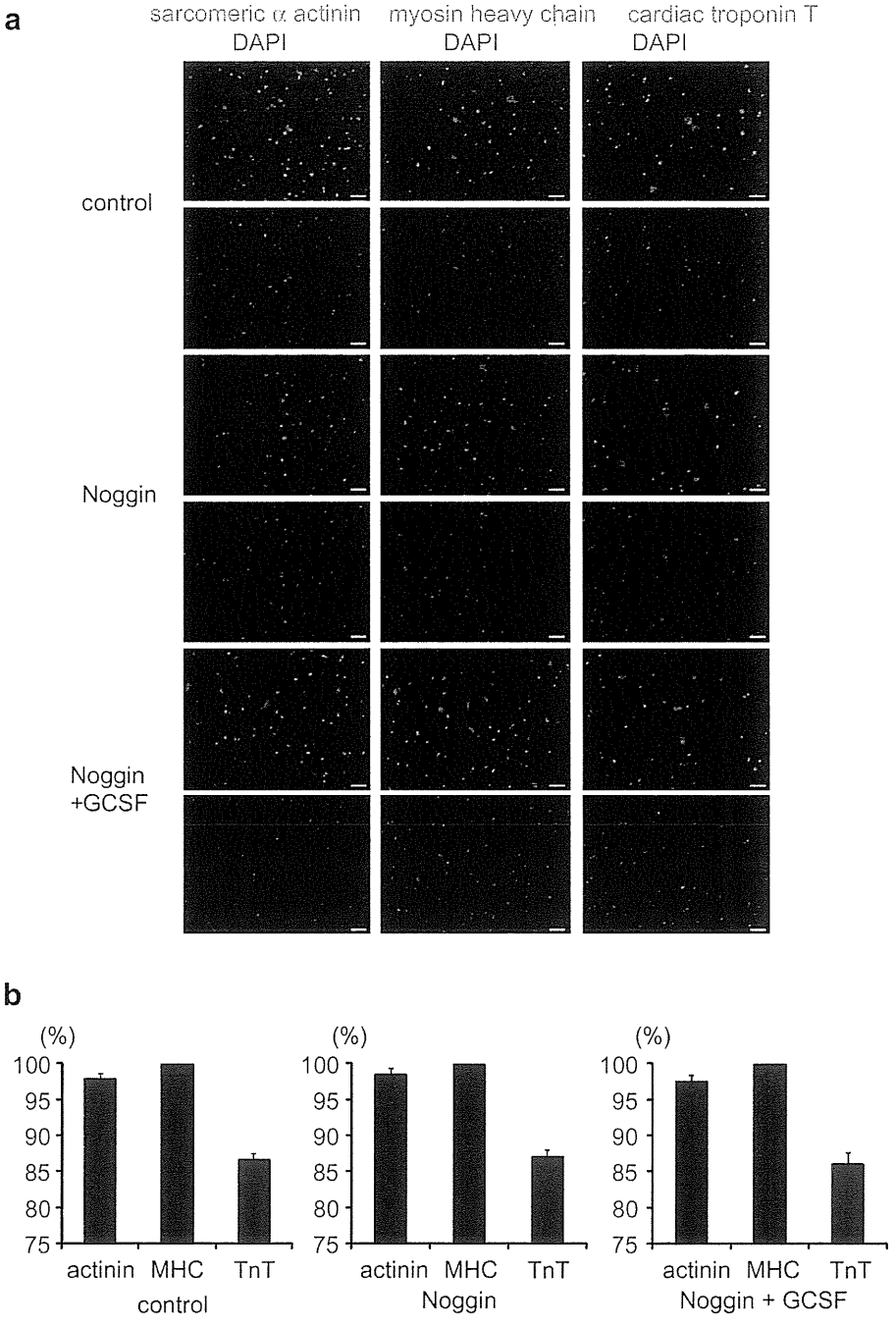

Fig. 2. Purity of cardiomyocytes after neomycin selection. (a) At 18 days after starting the culture in spinner flasks (at 8 days of culture with neomycin), the cells were dissociated and seeded onto 1% gelatin-coated 3.5-cm dishes. At 12 h after seeding, the cells were fixed and immunostained for sarcomeric-α actinin (left), myosin heavy chain (middle) and cTnT (right). The upper panels show images of the contractive proteins and the lower panels show images of DAPI nuclear staining. Scale bars, 100 μm. (b) The percentages of α-actinin (+), myosin heavy chain (+) and cTnT (+) cells were calculated and are shown in a graph (n = 3). The data are means ± SD.

Table 1). After dissociation with trypsin/EDTA, the cells were seeded onto gelatin-coated dishes. At 12 h after seeding, spontaneous beating was observed in almost all of the attached cells (Supplementary video 1). The purity of the cardiomyocytes in the remaining cells was examined by immunocytochemistry. As shown in Fig. 2, more than 99% of the cells were positive for myosin heavy chain, about 98% of the cells were positive for sarcomeric α-actinin and about 85% of the cells were positive for cTnT. These findings indicate that almost all of the remaining cells after neomycin selection in the suspension cultures might be cardiomyocytes, although the percentage of each cardiac contractive protein expression may vary probably according to the levels of maturation. Confocal microscopy observations revealed that the differentiated cardiac cells showed a fine striated pattern and that many cells had a single nucleus while some cells had double and triple nuclei (Fig. 3). These findings suggest that an 18-day culture period may promote not only cardiomyocyte differentiation but also cardiomyocyte maturation.

Supplementary video related to this article can be found at doi: 10.1016/j.biomaterials.2011.05.042.

Although we were able to collect many cardiomyocytes to a certain extent using the suspension culture system, better culture conditions should further increase the number of cardiomyocytes. Therefore, we tried to increase the number of cardiomyocytes in our suspension cultures by treatment with noggin [18] and/or GCSF [19]. The ES cells were treated with noggin from 3 days before to 1 day after starting the suspension cultures, and with GCSF from day 6 to day 10 in the suspension cultures. Even when ES cell cultures were treated with noggin and/or GCSF, the number of cells at day 10 in the suspension cultures did not differ compared with untreated cell cultures (Fig. 1a). However, at 8 days after neomycin selection (18 days after starting the culture), a significant increase in the number of remaining cells was observed in the cultures treated with noggin and GCSF (Fig. 1b, Table 1). Moreover, the remaining cells showed a similar expression pattern of cardiac contractive proteins to the untreated cells (Fig. 2), indicating that noggin and GCSF treatment may increase the number of cardiomyocytes. Conversely, noggin (Fig. 1b, Table 1) or GCSF (data not shown) alone did not significantly increase the number of cells after neomycin selection. Since it has been reported that transient early treatment with noggin (from 3 days before to 1 day after EB formation) induces the mesoendoderm [18] and that GCSF induces the proliferation of fetal cardiomyocytes and ES-derived cardiomyocytes [19], the sequential treatment with noggin and GCSF may strongly increase the number of cardiomyocytes in large-scale suspension cultures.

3.2. Cell sheet creation using ES cell-derived cardiomyocytes and cardiac fibroblasts

Next, we tried to create cardiac cell sheets using ES-derived cardiomyocytes and UpCell temperature-responsive culture dishes. According to our previous cell sheet experiments using neonatal rat cardiomyocytes [22], ES-derived cardiomyocytes were

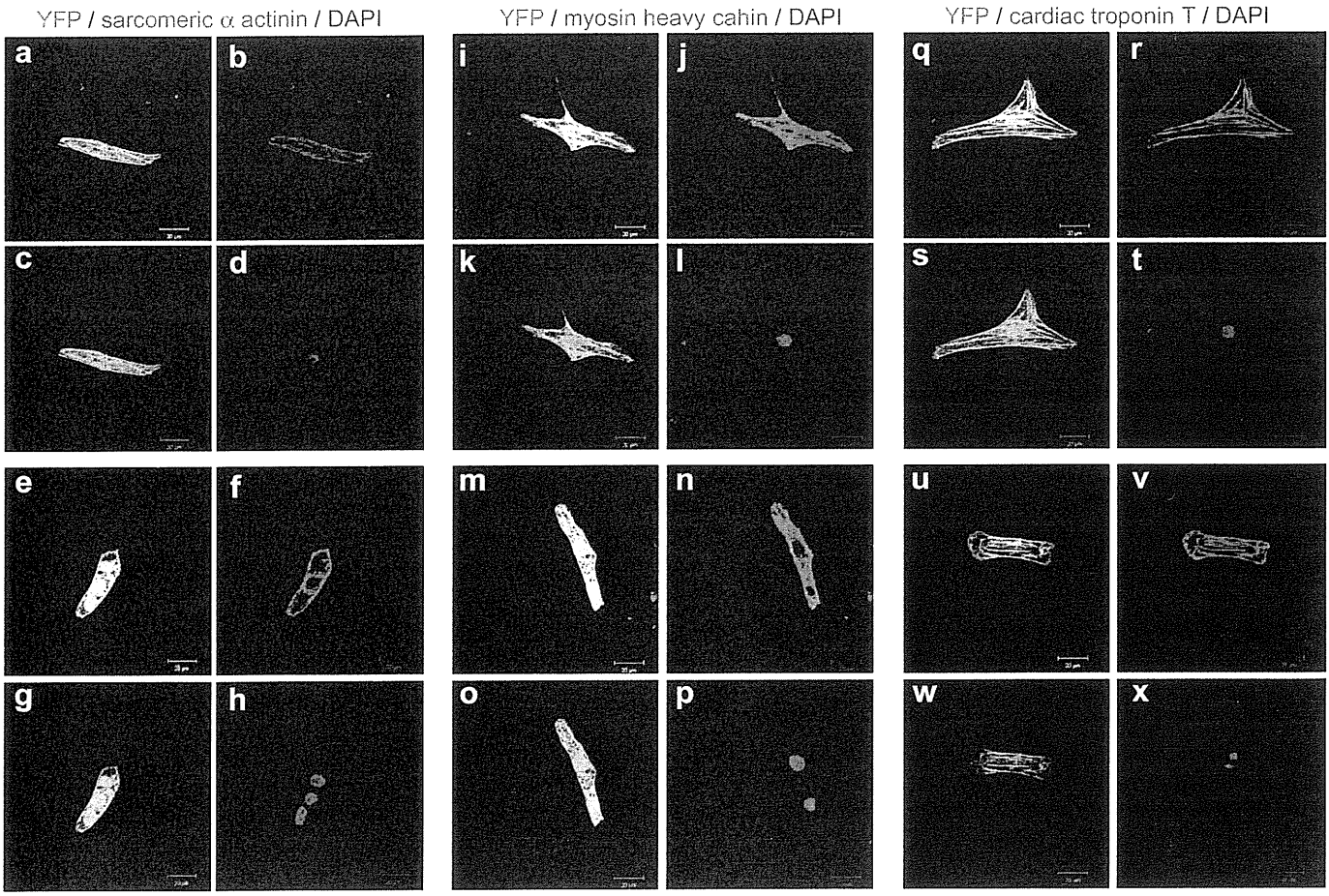

Fig. 3. Confocal microscopy images of the expression of cardiac contractive proteins in ES-derived cardiomyocytes after neomycin selection. Left panels: sarcomeric α-actinin; middle panels: myosin heavy chain; right panels: cTnT. Panels a–d, e–h, i–l, m–p, q–t and u–x show the same images, respectively. Panels a, e, i, q, m and u show merged images. Panels b, f, j, n, r and v show the expression of the contractive proteins (red). Panels c, g, k, o, s and w show YFP expression (green). Nuclei were stained with DAPI (blue in panels d, h, l, p, t and x). Scale bars, 25 μm.

seeded onto FBS-coated UpCell dishes at 3.2×10^5 cells/cm^2. When we checked the cell distribution on the dishes by fluorescence microscopy after 4 days in culture, many spontaneously beating YFP(+) cell aggregates were observed (Supplementary video 2). However, synchronous beating among the aggregates was not observed. When the cells were cultured in a 20 °C incubator, the aggregates detached from the culture dishes and no cell sheet formation was observed (Fig. 4a). Furthermore, even when the number of seeded cells was increased to 14.8×10^5 cells/cm^2 (four times the initial number of seeded cells) and the dishes were precoated with gelatin, fibronectin or laminin, the cell aggregates still detached from the culture dishes (data not shown), suggesting that the cell–cell interactions between cardiomyocytes may be insufficient to create cell sheets. To examine the cell components in the cell sheets, we created cell sheets using neonatal mouse cardiomyocytes seeded at 3.2×10^5 cells/cm^2. At 4 days after cell seeding, cell sheets were created upon culture in a 20 °C incubator. Immunocytochemical analyses showed the coexistence of cardiomyocytes and vimentin-positive cells in the cell sheets (Fig. 4b), suggesting that a certain level of non-cardiomyocytes that produce extracellular matrix may be necessary to create cell sheets. Therefore, we tried to create cell sheets by coculturing ES-derived cardiomyocytes and cardiac fibroblasts isolated from neonatal mouse hearts. To examine the appropriate ratio for the cell numbers of cardiomyocytes and fibroblasts, we cocultured ES-derived cardiomyocytes and fibroblasts at ratios of 9:1, 8:2, 7:3, 6:4 and 5:5. When cardiomyocytes and fibroblasts were cocultured at the ratio of 9:1, cell aggregates that may have been larger than those formed by cardiomyocytes alone were detached from the culture dishes after culture at 20 °C (Fig. 4a). On the other hand, when cardiomyocytes were cocultured with fibroblasts at the ratio of 8:2, YFP(+) cells were evenly distributed and beat synchronously with one another (Supplementary video 3), and cell sheets were created at 4 days after culture at 20 °C (Fig. 4a). Furthermore, even when the ratio of fibroblasts was increased up to 50%, cell sheets were created (Fig. 4a), suggesting that the existence of fibroblasts was important for creating cardiac cell sheets using ES-derived cardiomyocytes.

Supplementary video related to this article can be found at doi: 10.1016/j.biomaterials.2011.05.042.

3.3. Electrophysiological evaluation of ES-derived cardiac cell sheets

Finally, we examined the electrophysiological functions of the ES-derived cardiac cell sheets created by the mixture of 80% cardiomyocytes and 20% fibroblasts. Confocal microscopy observations revealed vimentin(+) cells surrounding cTnT(+) cells in horizontal and vertical directions in the cell sheets. Consistent with the observation that the cardiomyocytes in the cell sheets beat spontaneously and synchronously, connexin 43 was expressed at the edge of adjacent cardiomyocytes (Fig. 5a). To confirm the electrophysiological connections among the cardiomyocytes in the cell sheets, the extracellular action potentials were measured using the

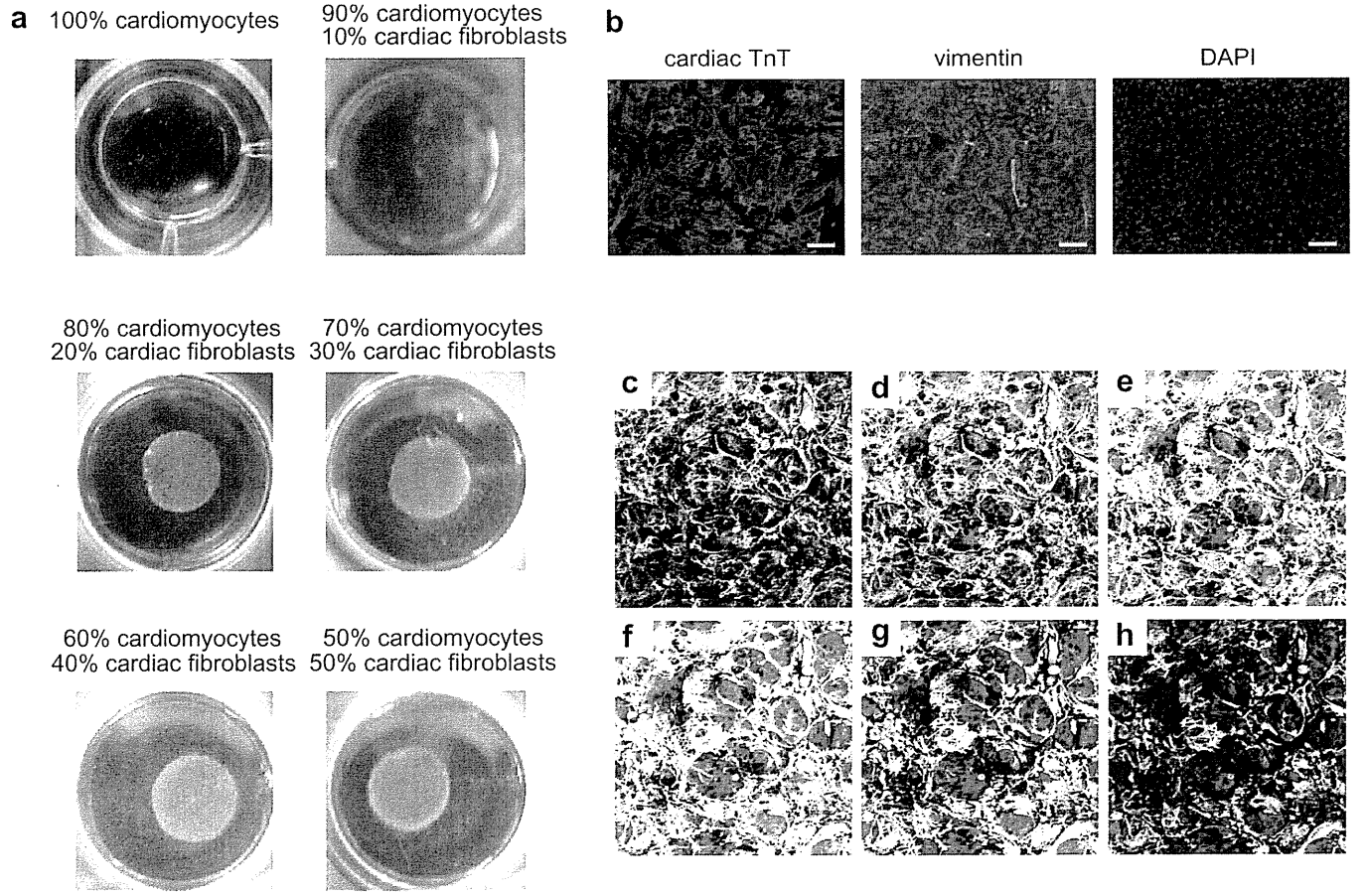

Fig. 4. Cell sheet creation using ES-derived cardiomyocytes and cardiac fibroblasts. (a) Representative images of cell aggregates and cell sheets in the well of a 24-well plate after 2 h of incubation at 20 °C. (b) Distribution of cardiomyocytes and fibroblasts in a neonatal mouse cardiac cell sheet. Left: cTnT (red); middle: vimentin (green); right, DAPI (blue). Scale bars, 50 μm (c–h) Distribution of cardiomyocytes and fibroblasts in ES-derived cardiac cell sheets. The cell sheets were stained for cTnT (red) and vimentin (white). Images were taken at 1-μm intervals in the vertical direction. Scale bar, 50 μm.

MED system [9]. As shown in Fig. 5b, unidirectional action potential propagation was observed. To clarify the electrophysiological connections between cell sheets, a cell sheet was seeded onto one side of the MED system. After 30 min, another cell sheet was seeded onto the other side of the MED system. Under the conditions used, the two cell sheets were partially overlaid and synchronous beating in the two cell sheets was observed (Fig. 5c). These findings indicate that electrical communication was established between the two cardiac cell sheets.

4. Discussion

Many reports have suggested that ES cells differentiate into cardiomyocytes via EB formation [12,13]. Since the classical methods to create EBs using hanging-drop procedure might not be useful for obtaining enough amounts of cardiomyocytes for fabricating bioengineered myocardium, various methods to overcome its limitation have been developed including multi-well plates [16,23], microwell substrates [24,25] and stirred suspension culture [14]. Consistent with previous studies [14,15], many EBs were created and cardiac differentiation was observed in the present study. Stirred suspension cultures have been thought to be unsuitable for differentiation because it is difficult to control the size of the EBs. In the present study, the number of cardiomyocytes was around 4.9×10^6 cells after neomycin selection (~78.1% of the initial ES cell number and ~2.7% of the cell number at day 10). It has been reported that ~1.5% of cells were cardiomyocytes on day 10 when mouse ES cells were cultured on a size-controlled cell pattern surface [26], suggesting that size control of EBs may not affect cardiac differentiation as obviously as previously thought. Furthermore, we applied serial treatment with noggin and GCSF to the stirred suspension cultures. The serial treatment significantly increased the number of cardiomyocytes to 1.8×10^7 cells (3.7-fold the number of untreated cells), whereas noggin treatment alone tended to increase the number of cardiomyocytes. Zandstra et al. reported that around 9.0×10^6 cardiomyocytes were obtained when mES cells were cultured in stirred suspension culture after neomycin selection, and retinoic acid treatment enhanced cardiomyocytes production doubly [14]. Although the number of cardiomyocytes without growth factor treatments in our study was smaller than that in their report, time-dependent growth factor stimulation with noggin and GCSF might produced more cardiomyocytes effectively with synergistic promotions of cardiac differentiation and proliferation in stirred suspension cultures.

Cell sheet technologies have been used as promising cell delivery tools for regenerative medicine in both animal experiments and clinical settings [2,27–29]. Although transplantation of cardiac cell sheets formed from neonatal rat cardiomyocytes was reported to improve the cardiac function in a rat infarcted heart model [30] and serial subcutaneous transplantation of cardiac sheets enabled the creation of thickened heart-like tissue of >1 mm [7], methods for creating cell sheets and the function of cell sheets formed from ES

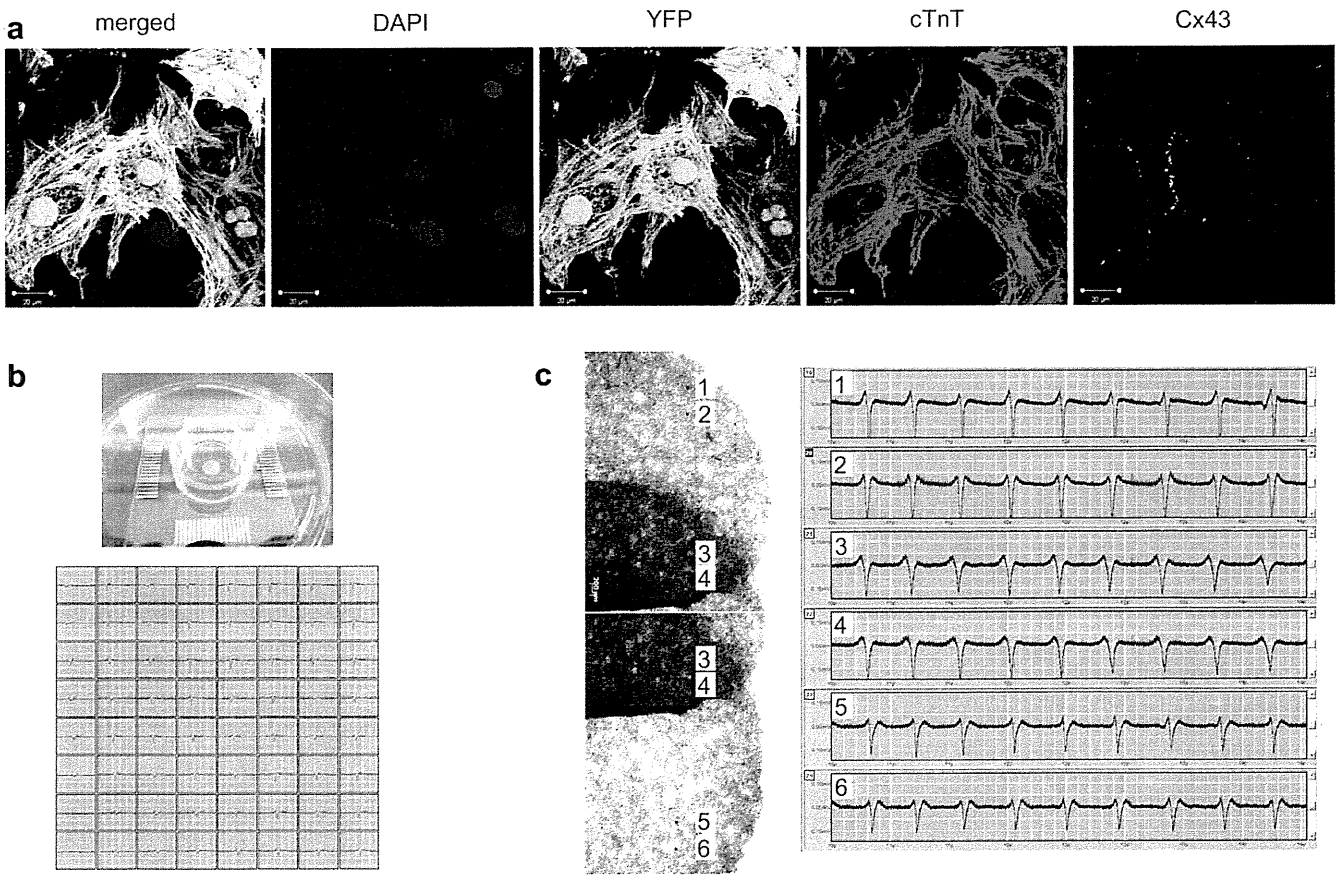

Fig. 5. Electrophysiological functions of ES-derived cardiac cell sheets. (a) Confocal microscopic images of cell sheets. Connexin 43 (white) is expressed at the edge of adjacent cardiomyocytes (YFP, green; cTnT, red). Nuclei were stained with DAPI (blue). Scale bars, 20 μm. (b) Extracellular action potential evaluations using an MED system. The upper panel shows a macroscopic view of a cell sheet on the MED system. Unidirectional action potential propagation is observed in the monolayer cardiac cell sheet (lower). (c) Electrophysiological connection between two cell sheets. The two cell sheets were partially overlaid on the MED system. The left panels show microscopic view of the electrode positions in cell sheets. Right panel shows the extracellular action potential at each electrode position. The number in each action potential shows the position of electrode under the cell sheets and No. 3 and 4 are under the overlaid area. Scale bars, 200 μm.

cell-derived cardiomyocytes have remained elusive. In the present study, ES-derived cardiomyocytes seeded onto temperature-responsive culture dishes did not form cell sheets, even when the number of seeded cells was increased. On the other hand, when we cocultured ES-derived cardiomyocytes with cardiac fibroblasts on the temperature-responsive culture dishes, cell sheets were created. These findings indicate that the existence of non-cardiomyocytes potentially expressing extracellular matrix components may be important for forming cardiac cell sheets. We further found that the cardiomyocytes in these cell sheets expressed connexin 43 at the edge of adjacent cardiomyocytes and beat spontaneously and synergistically with neighboring cardiomyocytes. Furthermore, the MED system revealed that unidirectional action potential propagation was present in the cell sheets, suggesting that ES-derived cardiac cells sheets might have the electrical functional gap junctions. Consistent with previous report indicating that the excitation of a cardiac cell sheet of neonatal rat cardiomyocytes was conducted and spread to other cell sheet via newly formed gap junction [9], the action potential of a ES-derived cardiac cell sheet could be propagated to another cell sheet. These findings suggest that ES-derived cardiac cell sheet might have functions as bioengineered myocardium in term of synchronous electrical coupling as similar to cardiac cell sheets of neonatal rat cardiomyocytes [9,22] and might provide us the helpful models for evaluating genetic disordered myocardium if patient-specific iPS cells are available [31,32]. Vascularization of cell sheets is considered to be important for fabricating thickened myocardium [7], recently we have developed the pre-vascularized cell sheets in vitro [33]. Since endothelial cells have been already reported to be isolated from ES-derived EBs [34], to create the multilayered ES-derived cardiac cell sheets with vascularization using ES-derived vascular endothelial cells is our next challenge.

5. Conclusions

In the present study, we have demonstrated the usefulness of combined treatment with noggin and GCSF of stirred suspension cultures for cardiac differentiation and the function of cardiac cell sheets using ES-derived cardiomyocytes. Recently, iPS cell technologies have enabled the creation of autologous cardiomyocytes [10]. Further understanding of the mechanisms of the expansion and differentiation in stirred suspension cultures may provide new insights for the development of human ES/iPS cell cardiomyocyte culture systems and human heart tissue engineering using cell sheet technologies.

Competing interests statement

T.S. is a consultant for CellSeed Inc. T.O. is an investor in CellSeed Inc.

Acknowledgments

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ORIGINAL ARTICLE
Preventive Medicine

Depression and Outcomes in Hospitalized Japanese Patients With Cardiovascular Disease

– Prospective Single-Center Observational Study –

Tsuyoshi Suzuki, MD; Tsuyoshi Shiga, MD; Kazue Kuwahara, MD; Sayaka Kobayashi; Shinichi Suzuki, PhD; Katsuji Nishimura, MD; Atsushi Suzuki, MD; Hisako Omori, MD; Fumiaki Mori, MD; Jun Ishigooka, MD; Hiroshi Kasanuki, MD; Nobuhisa Hagiwara, MD

Background: Several studies have suggested that depression poses a risk in cardiovascular patients. The aim of the present study was to evaluate the prevalence of depression and its effect on cardiovascular events and mortality in Japanese inpatients with cardiovascular disease.

Methods and Results: A total of 505 patients hospitalized with cardiovascular disease (28% female; mean age, 61±14 years; 31% ischemic heart disease; 47% New York Heart Association [NYHA] class II–IV; 25% implantation of pacing devices) were enrolled in the present prospective observational study. The Zung Self-Rating Depression Scale (SDS) was used to screen for depression. The primary outcome was the time to death or cardiovascular event, and the secondary outcome was death. In total, 109 patients (22%) were diagnosed with depression (Zung SDS index score ≥60). NYHA class III/IV, defibrillator implantation, and being unmarried were independently associated with depression. During an average follow-up period of 38±15 months, 92 patients (18%) reached the primary outcome. There was a higher incidence of the primary outcome in patients with depression than in those who were not depressed (P<0.01). Depressed patients had a significantly higher rate of mortality than non-depressed patients (P<0.01). Depression was an independent predictor of the primary outcome (hazard ratio, 2.25; 95% confidence interval: 1.30–3.92, P<0.01).

Conclusions: Depression was not uncommon in Japanese inpatients with cardiovascular disease and was associated with cardiovascular outcomes. (*Circ J* 2011; **75:** 2465–2473)

Key Words: Cardiovascular disease; Depression; Inpatient; Mortality; Outcome

Several studies have suggested that depression is a possible risk factor for adverse outcomes in patients with coronary artery disease or heart failure.[1–7] While cardiac events may cause and prolong depression in patients with cardiac disease,[8–10] the prevalence of depression is reported to be approximately 20% in outpatients with coronary artery disease and 30–40% in outpatients with heart failure.[6,11–14] In patients hospitalized for acute myocardial infarction, 16–45% are depressed,[6,8,11] and the presence of depressive symptoms is a significant risk factor for subsequent cardiac events in elderly myocardial infarction patients.[15] In hospitalized heart failure patients, depression is also common and is independently associated with poor outcomes.[2,3,16,17] Understanding these issues could help cardiologists identify inpatients with depression and deliver the most appropriate care.

Cultural and ethnic differences influence depressive symptoms and the interpretation of depression as an illness.[18–20] In Japan, there have been few reports about the prevalence of depression and its effect on patients with cardiovascular disease.[14,15,21] To date, there have been no reports concerning the prevalence of depression in hospitalized patients with cardiovascular disease in Japan.

The aim of the present study was to evaluate the prevalence of depression and the effect of depression on subsequent cardiovascular events and mortality in Japanese patients hospitalized with cardiovascular disease.

Methods

We conducted a prospective observational study in patients who

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Department of Cardiology (T. Suzuki, T. Shiga, K.K., A.S., H.O., F.M., N.H.), Department of Psychiatry (K.K., S.K., K.N., J.I.), Tokyo Women's Medical University, Tokyo; Faculty of Science and Engineering, Waseda University, Tokyo (H.K.); and Faculty of Human Sciences, Waseda University, Tokorozawa (S.S.), Japan

Mailing address: Tsuyoshi Shiga, MD, Department of Cardiology, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan. E-mail: mshiga@hij.twmu.ac.jp

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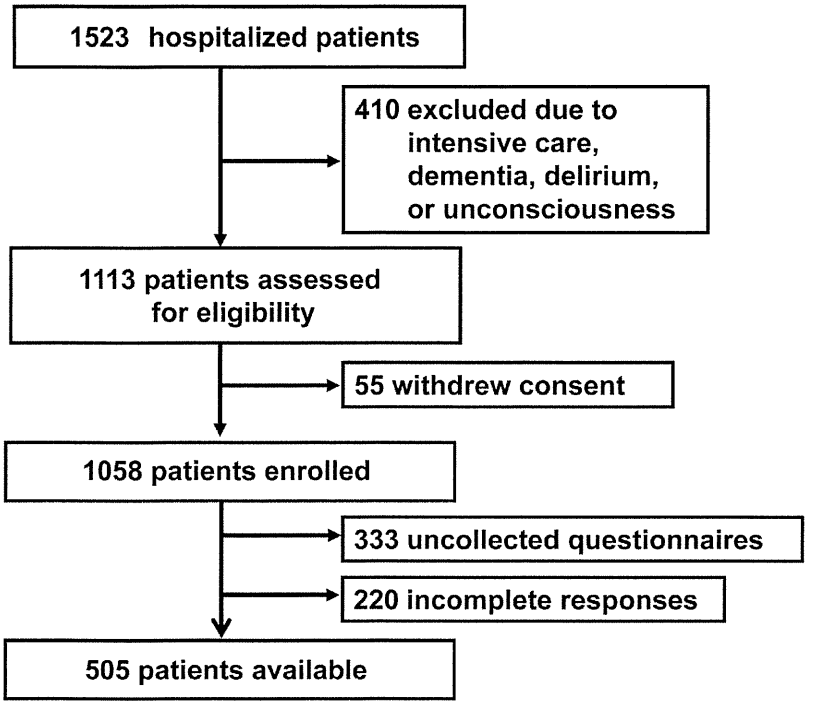

Figure 1. Flow diagram of the study subjects.

were admitted to the cardiology department of Tokyo Women's Medical University Hospital between June 2006 and April 2008. Patients with dementia, delirium, or other conditions that make it difficult to complete a self-reported written questionnaire (eg, unconsciousness, in intensive care, end-stage of another life-threatening disease) were excluded. The protocol was approved by the institutional review board of Tokyo Women's Medical University. All patients gave written informed consent.

Cardiovascular Disease

In the present study, structural heat disease consisted of the following disorders: left ventricular (LV) systolic dysfunction and/or marked LV dilatation (unless secondary to severe valve regurgitation), LV diastolic dysfunction associated with congestive heart failure, coronary heart disease, right heart disease with at least moderate right ventricular dilation, moderate or severe tricuspid regurgitation, pulmonary hypertension, LV hypertrophy, left-sided valvular disease, and congenital heart disease. Coronary artery disease was defined as positive stress test findings, coronary angiography demonstrating at least 75% of stenosis or coronary spastic angina as documented on an acetylcholine provocation test, a history of prior myocardial infarction, or a history of revascularization procedures. Valvular and congenital heart diseases were diagnosed on angiographic, hemodynamic or echocardiographic findings or a history of valvular or congenital cardiac surgery. Aortic and mitral regurgitation were defined as valvular disease with at least moderate regurgitation on color-flow Doppler echocardiography. Non-ischemic cardiomyopathies were defined as ventricular myocardial abnormalities in the absence of coronary artery disease, or valvular, pericardial or congenital heart disease. Pulmonary artery hypertension was defined as an increase in mean pulmonary arterial pressure of ≥25 mmHg with a pulmonary wedge pressure of ≤15 mmHg at rest, as estimated on right heart catheterization. Aortic disease, peripheral artery disease and other vascular diseases were diagnosed on angiographic or echocardiographic findings, or a history of vascular surgery or intervention. Arrhythmias and conduction disorders without structural heart disease included atrial, supraventricular and ventricular arrhythmias, sick sinus syndrome and atrioventricular block in the absence of structural heart disease. Hypertension was defined as a systolic blood pressure ≥140 mmHg, a diastolic blood pressure ≥90 mmHg, or a history of treatment for hypertension. LV ejection fraction (LVEF) was calculated using left ventriculography, echocardiography or radionuclide angiography.

Assessment of Depression

Most patients received psychological questionnaires within a few days after hospital admission. For patients who initially required intensive treatment, these questionnaires were given after their transfer to the general cardiology wards. The Zung Self-Rating Depression Scale (SDS) has been used to screen for depression and to measure the severity of depression in numerous settings.[22–26] The Zung SDS is a self-reporting, 20-question instrument that assesses the psychological and somatic symptoms of depression. It has good internal consistency and validity, encompassing most DSM-IV criteria for major depression.[26–32] The Zung SDS has been found to be the primary discriminating variable for distinguishing depressed from non-depressed people.[33] It has shown a positive likelihood ratio for major depression of 3.3 (95% confidence interval [CI]: 1.3–8.1), and negative likelihood ratio of 0.35 (95%CI: 0.2–0.8).[24] The Zung SDS has also been used in clinical studies to assess depression in cardiovascular disease.[15,34–37] Ten questions are positively worded, and 10 are negatively worded. Each question is scored on the following 4-point scale: 1, a little of the time; 2, some of the time; 3, good part of the time; and 4, most of the time. To obtain a total score, the positive items are reversed, and then the items are summed. This raw score is converted to a 100-point scale (SDS index). Zung SDS index scores range from 25 to 100 and are interpreted as follows: within the nor-

Table 1. **Patient Characteristics**

	Total (n=505)	Depression (n=109)	No depression (n=396)	P value
Age (years)	61±14	61±13	59±15	0.45
Female	143 (28)	36 (33)	107 (27)	0.26
Cardiovascular disease				0.24
Coronary artery disease	159 (31)	24 (22)	135 (34)	
Non-ischemic cardiomyopathy	114 (23)	30 (28)	84 (21)	
Valvular heart disease	65 (13)	15 (14)	50 (13)	
Arrhythmia without structural heart disease	143 (28)	32 (29)	111 (28)	
Pulmonary artery hypertension	3 (1)	1 (1)	2 (1)	
Congenital heart disease	6 (1)	2 (1)	4 (1)	
Others	15 (3)	5 (5)	10 (3)	
Plasma BNP on admission (pg/ml)	251 (4–4,335)	378 (5–4,335)	215 (4–3,400)	<0.01
NYHA functional class on admission (I/II/III/IV)	269/191/30/15	41/45/16/7	228/146/14/8	<0.01
NYHA functional class at discharge (I/II/III/IV)	275/206/23/1	41/46/21/1	234/160/2/0	<0.01
LVEF (%)	48±15	49±15	46±16	0.11
eGFR (ml·min^{-1}·1.73 m^{-2})	61±14	61±14	61±14	0.73
Current smoker	70 (14)	14 (12)	56 (14)	0.72
History of atrial fibrillation	85 (17)	16 (15)	69 (17)	0.49
Medical comorbidities				
Hypertension	166 (32)	29 (27)	137 (35)	0.11
Diabetes	86 (17)	16 (15)	70 (18)	0.46
Dyslipidemia	141 (28)	23 (21)	118 (30)	0.06
Hemodialysis	32 (6)	10 (9)	22 (6)	0.18
Cerebrovascular disease	8 (1.5)	2 (2)	6 (2)	0.81
Major depression	8 (1.5)	5 (5)	3 (1)	0.01
Implanted pacing devices before admission				
Pacemaker/CRT-P	54 (11)	13 (12)	41 (10)	0.64
ICD/CRT-D	73 (14)	26 (24)	47 (12)	0.02
Implanted pacing devices at discharge				
Pacemaker/CRT-P	64 (13)	13 (12)	51 (13)	0.79
ICD/CRT-D	95 (19)	29 (27)	66 (17)	0.01
Medications at the time of questionnaire				
β-blockers	248 (49)	52 (48)	196 (49)	0.74
ACE inhibitors/ARBs	278 (55)	60 (55)	218 (55)	0.99
Spironolactone/eplerenone	120 (24)	37 (34)	83 (21)	0.68
Calcium channel blockers	284 (56)	54 (50)	230 (58)	0.11
Aspirin	172 (34)	29 (27)	143 (36)	0.06
Warfarin/heparin	142 (28)	34 (32)	108 (27)	0.64
Amiodarone/nifekalant	60 (12)	22 (20)	40 (10)	<0.01
Intravenous inotropics	3 (1)	2 (2)	1 (0.3)	<0.01
Intravenous vasodilator	5 (1)	4 (4)	1 (0.3)	<0.01
Antidepressants	8 (2)	5 (5)	3 (1)	0.01
Medications at discharge				
β-blockers	259 (51)	57 (52)	202 (51)	0.81
ACE inhibitors/ARBs	308 (61)	72 (66)	236 (59)	0.21
Spironolactone/eplerenone	136 (27)	40 (37)	96 (24)	0.01
Calcium channel blockers	289 (57)	55 (50)	234 (59)	0.10
Aspirin	186 (37)	33 (30)	153 (39)	0.10
Warfarin	160 (32)	44 (40)	116 (29)	0.03
Amiodarone	68 (13)	25 (23)	43 (11)	0.05
Antidepressants	8 (2)	5 (5)	3 (1)	0.01
Education				0.33
High school	314 (62)	74 (68)	240 (61)	
College	124 (25)	24 (22)	100 (25)	
Others	67 (13)	11 (10)	56 (14)	
Marital status				<0.01
Unmarried	35 (7)	13 (12)	22 (6)	
Married	448 (89)	83 (76)	365 (92)	
Widowed	22 (4)	13 (12)	9 (2)	
Work status				0.02
Employed	205 (41)	32 (29)	173 (44)	
Housewife	89 (18)	26 (24)	63 (16)	
Unemployed/retired	211 (42)	51 (47)	160 (40)	

Data given as n (%) or mean±SD or median (range).
BNP, B-type natriuretic peptide; NYHA, New York Heart Association; LVEF, left ventricular ejection fraction; eGFR, estimated glomerular filtration rate; CRT, cardiac resynchronization therapy; CRT-P, CRT with a pacemaker; ICD, implantable cardioverter defibrillator; CRT-D, CRT with a defibrillator; ACE, angiotensin-converting enzyme; ARB, angiotensin II receptor blocker.

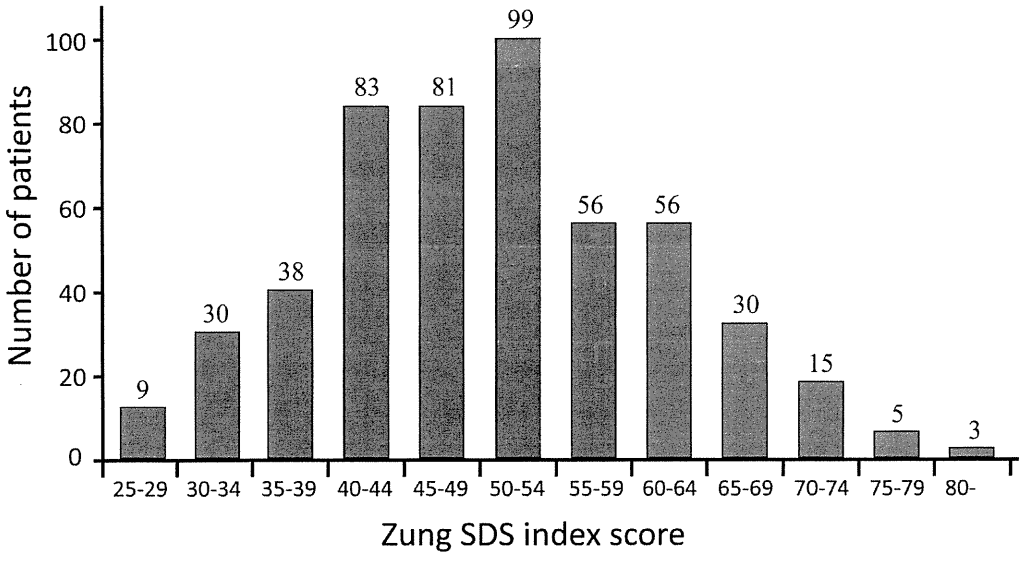

Figure 2. Zung Self-Rating Depression Scale (SDS) index score in 505 hospitalized patients with cardiovascular disease. Red, depression defined by a Zung SDS index score ≥60.

mal range, <50; mildly depressed, 50–59; moderately depressed, 60–69; and severely depressed, ≥70. Because the psychological and physical symptoms of depression may overlap with those of cardiovascular disease, there is a possibility that cardiovascular symptoms may be attributed to depression. Previous studies with cardiovascular disease have often used a cut-off index score of 50 (raw score 40) as a definition of depression.[15,34–37] Higher depression scores (eg, SDS score index ≥60) are associated with increased morbidity and mortality in patients with coronary artery disease.[37,38] A cut-off index score of 60 has been shown to detect clinical depression while avoiding an abundance of false-positive results in patients with cardiovascular or other disease.[10,39–41] In the present study, depression was defined as a Zung SDS index score ≥60.

Follow-up

After discharge, patients were seen as outpatients or at their general practitioner's clinic at 1–3-month intervals up to October 2010. Patients receiving pacing device therapy, including pacemakers, cardiac resynchronization therapy (CRT) and implantable cardioverter defibrillators (ICD), were also followed every 3–6 months at the pacemaker/ICD clinic. The occurrence of ventricular tachyarrhythmias requiring ICD therapy, including shock and anti-tachycardia pacing, was obtained by reviewing event details and electrograms stored on the ICD disks. Only episodes of ventricular tachycardia or fibrillation requiring ICD therapy for termination were included in the analysis. Information about deceased subjects was obtained from medical records, family members, their general practitioners and the admitting hospital.

Clinical Outcomes

The primary outcome was a composite of death from any cause or cardiovascular events from the time of enrollment to the first event. Cardiovascular death was defined as death due to myocardial or cerebral infarction, other vascular causes, heart failure or documented sudden cardiac death. Cardiovascular events included non-fatal myocardial infarction, hospitalization for heart failure, unstable angina, revascularization, stroke, refractory arrhythmia, and ventricular tachyarrhythmia requiring ICD therapy. Unstable angina was defined according to the Braunwald criteria.[42] Revascularization included angioplasty, stenting and coronary artery bypass grafting. Heart failure was defined on the basis of symptoms and signs such as dyspnea, rales and ankle edema and the need for treatment with diuretics, vasodilators, positive inotropic drugs or an intra-aortic balloon pump. Stroke was defined as a new focal neurological deficit of vascular origin lasting >24 h. Stroke was further classified by etiology, including intracranial hemorrhage, ischemia (diagnosed on computed tomography or magnetic resonance imaging if available) or uncertain cause. Refractory arrhythmia was defined as supraventricular or ventricular tachyarrhythmia requiring external defibrillation or pacing, i.v. anti-arrhythmics such as amiodarone and nifekalant, catheter ablation, or implantation of an ICD, and bradyarrhythmia requiring implantation of a pacemaker. Other cardiovascular events included peripheral artery disease, dissecting aortic aneurysm, and rupture of an aortic aneurysm. The second outcome was death from any cause.

Statistical Analysis

The data are given as either mean±SD or numbers of patients. Baseline clinical data were compared between groups with and without depression using Student's t-test and the Mann–Whitney U-test. Categorical variables were subjected to chi-squares analysis. Multivariate analysis using the Cox proportional hazards model was performed to assess the relationship of the following baseline characteristics to depression: age ≥65 years, female gender, New York Heart Association (NYHA) functional class III/IV, LVEF≤35%, estimated glomerular filtration rate (eGFR) by the Modification of Diet in Renal Disease formula $<60\,ml\cdot min^{-1}\cdot 1.73\,m^{-2}$,[43] diabetes mellitus, hemodialysis, implantation of an ICD/CRT with a defibrillator (CRT-D), β-blocker use on admission, marital status and work status. Cumulative event-free rate was calculated using the Kaplan–Meier method. Differences in event-free rates were

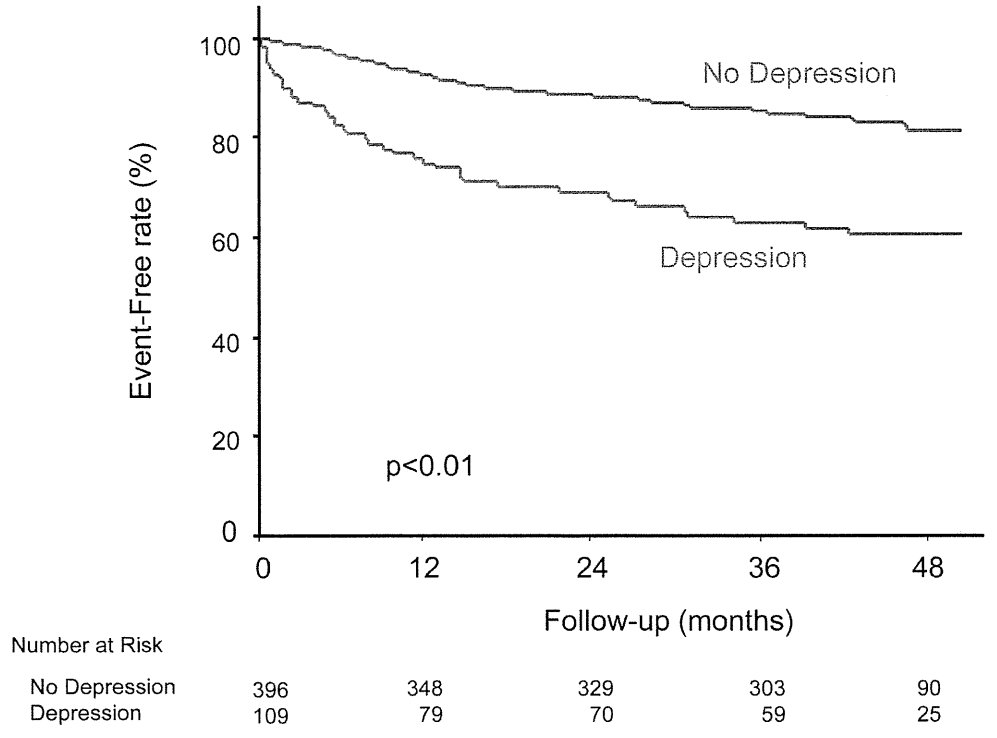

Figure 3. Kaplan–Meier curve for the primary outcome (death from any cause or cardiovascular events) in cardiovascular inpatients with or without depression.

compared using the log-rank test. Multivariate analysis using the Cox proportional hazards model was performed to assess the relationships between depression and the primary outcome, independent of the following confounders at discharge: age ≥65 years, female gender, NYHA functional class III/IV, LVEF≤35%, eGFR<60 ml·min^{-1}·1.73 m^{-2}, diabetes mellitus, hypertension and implantation of an ICD/CRT-D. P<0.05 was considered significant. SPSS version 11.01 (SPSS, Chicago, IL, USA) was used for analysis.

Results

Patients

Of the 1,523 consecutively hospitalized patients, 1,058 patients were enrolled in the present study. Seven hundred and twenty-five questionnaires were collected (collection rate of 68%). Of these, 505 questionnaires had valid responses (response rate of 48%), and these patients were available to participate in the study (Figure 1). The patient characteristics are shown in Table 1. The mean age on admission was 61±14 years, and 28% of the patients were female. A total of 159 patients (31%) had coronary artery disease, 236 (47%) were rated as being in NYHA functional class II–IV on admission, and 127 (25%) had implanted pacing devices on admission. Eight patients (2%) had been treated for major depressive disorder prior to admission. All 505 patients were discharged from hospital, and 230 (46%) were in NYHA functional class II–IV at discharge. At discharge, 159 (31%) had implanted pacing devices. Regarding concomitant medications at discharge, 259 patients (51%) were taking β-blockers, and 68 patients (13%) were taking amiodarone. Eight patients (2%) who were diagnosed with major depression by a psychiatrist were taking antidepressants. No patients were receiving non-pharmacological therapy such as cognitive behavior therapy.

Depression Prevalence

The Zung SDS index scores of all studied patients at baseline are shown in Figure 2. In total, 109 patients (22%) had depression. A comparison of patients' clinical characteristics according to the presence or absence of depression is shown in Table 1. There was no significant difference in age, gender, underlying cardiovascular disease, coexisting conditions or implanted devices between groups. The plasma B-type natriuretic peptide (BNP) level on admission and NYHA functional class on admission and at discharge were higher in patients with depression than in those who were not depressed. There was a higher rate of ICD/CRT-D implantation on admission in patients with depression. There were higher rates of amiodarone/nifekalant use, i.v. inotropic use, i.v. vasodilator use and antidepressant use at the time of the questionnaire in patients with depression. There was no significant difference, however, in the rate of β-blocker use between patients with (48%) and without depression (49%). There were higher rates of spironolactone/eplerenone use, warfarin use and antidepressant use at discharge in patients with depression. Compared with patients without depression, fewer depressed patients were married or employed. Multivariate analysis showed that ICD implantation (hazard ratio [HR], 1.92; 95%CI: 1.00–3.80, P=0.04), NYHA functional class III/IV (HR, 3.03; 95%CI: 1.38–6.67, P<0.01), and unmarried status (HR, 4.32; 95%CI: 2.31–8.09, P<0.01) were significantly associated with depression.

Depression and Clinical Outcomes

During an average follow-up period of 38±15 months, 92

Table 2. **Cause of Death and Rate of Cardiovascular Events**

	Depression (n=109)	No depression (n=396)	P value
Death from any cause	21	20	<0.01
Cardiovascular death	18	17	<0.01
Sudden death	1	8	0.42
Heart failure	17	5	<0.01
Myocardial infarction	0	2	0.45
Cerebral infarction	0	1	0.59
Peripheral artery disease	0	1	0.59
Non-cardiovascular death	3	3	0.08
Infection-related death	1	1	0.32
Surgery-related death	1	0	0.06
Hepatocellular carcinoma	0	1	0.59
Hepatic failure	1	0	0.06
Pulmonary hemorrhage	0	1	0.59
Hospitalization for heart failure	22	30	<0.01
Hospitalization for unstable angina	2	3	0.31
Hospitalization for revascularization	5	5	0.02
Hospitalization for stroke	0	1	0.59
Hospitalization for refractory arrhythmia	1	3	0.86
Ventricular tachyarrhythmia requiring ICD therapy	3	9	0.77
Hospitalization for other cardiovascular events	1	2	0.61

Abbreviation see in Table 1.

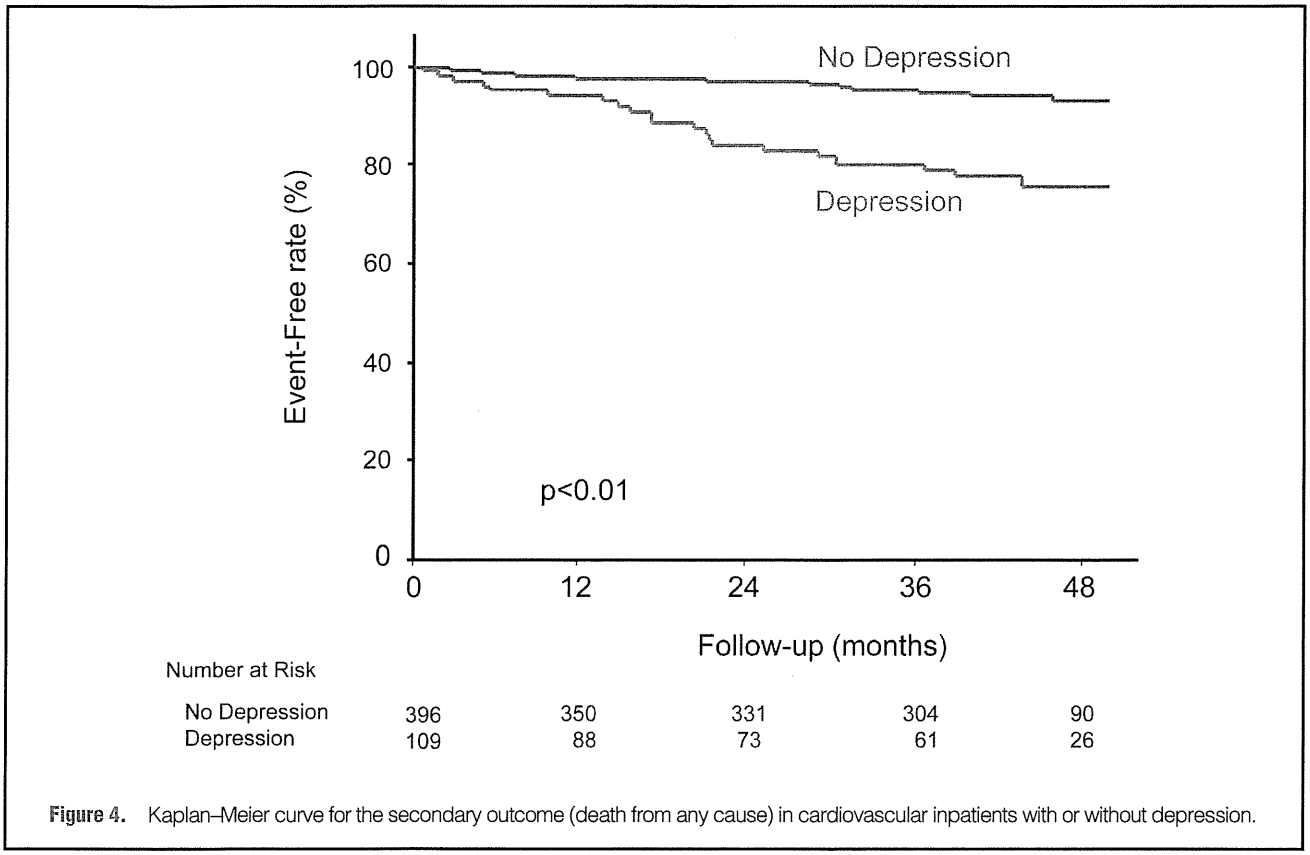

Figure 4. Kaplan–Meier curve for the secondary outcome (death from any cause) in cardiovascular inpatients with or without depression.

patients (18%) reached the primary outcome. Kaplan–Meier curves for the primary outcome are shown in Figure 3. There was a significantly higher incidence of the primary outcome in patients with depression than in those without depression. Causes of death and each cardiovascular event are listed in Table 2. Kaplan–Meier curves for death from any cause are shown in Figure 4. There was a significantly higher mortality in patients with depression than in those who were not depressed.

Multivariate analysis showed that patients with depression had an increased risk of the primary outcome: death from any

cause and cardiovascular events (HR, 1.98; 95%CI: 1.32–2.98, P<0.001; Table 3). This risk was independent of whether patients met the criteria of NYHA functional class III/IV, LVEF≤35% and eGFR<60 ml·min^{-1}·1.73 m^{-2}.

Table 3. **Multivariate Analysis for the Primary Outcome**

	HR (95%CI)	P value
NYHA class III/IV	2.07 (1.14–3.72)	0.01
Implantation of ICD/CRT-D	4.04 (2.15–7.06)	<0.01
eGFR <60 ml·min^{-1}·1.73 m^{-2}	3.26 (1.84–5.76)	<0.01
LVEF≤35%	2.06 (1.03–4.13)	0.04
Depression	2.25 (1.30–3.92)	<0.01
Female gender	1.02 (0.55–1.87)	0.94
Age≥65 years	0.83 (0.48–1.44)	0.83
Diabetes	1.47 (0.74–2.94)	0.26
Hypertension	0.97 (0.53–1.74)	0.91

HR, heart rate; CI, confidence interval. Other abbreviations see in Table 1.

Discussion

The present study has shown that the prevalence of depression was 22% in hospitalized patients with cardiovascular disease. ICD/CRT-D implantation, NYHA functional class III/IV at baseline, unmarried status, and unemployment were associated with depression. Furthermore, higher mortality and death from any cause and cardiovascular events were more prevalent in patients with depression than in those who were not depressed. Finally, depression was shown to be an independent factor for worsening clinical outcome.

Depression is often comorbid with chronic physical disease. The World Health Organization World Health Survey reported that an average of 9.3–23.0% of subjects with one or more physical diseases, such as angina, arthritis, asthma and diabetes, also suffer from depression.[44] A large study based on National Health Interview Survey data of 30,801 US adults reported that the 12-month prevalence of major depression was 9.3% in subjects with coronary artery disease, 9.3% in subjects with diabetes, 8.0% in subjects with hypertension and 7.9% in subjects with congestive heart failure, compared with 4.8% in those with no chronic medical disorder.[45] Recently, the American Heart Association recommended routine depression screening in patients with coronary artery disease using the 2- and 9-item tests from the Patient Health Questionnaires (PHQ-2 and PHQ-9).[46] Sowden et al reported that approximately 9% of 3,504 screened inpatients in cardiac care units had positive PHQ-2 scores (≥3). Of these patients, 74.1% had a PHQ-9 score ≥10, but the details of the patients' clinical backgrounds are unknown.[47] Previous studies have used several methods to measure depression, including the Beck Depression Inventory, SDS, the Hospital Anxiety and Depression Scale, and the Centre for Epidemiologic Studies Depression Scale (CES-D).[5,8] The Sowden et al PHQ-2 cut-off score was higher than that in general use (≥2)[48] to avoid false-negative results. The prevalence of patients with a PHQ-2 ≥2 was at least 15% in the Sowden et al study.[47] In the present study, 22% of all cardiovascular disease inpatients met the criteria for depression (Zung SDS index score ≥60).

The prevalence of depression in the present inpatients was comparable to the prevalence reported previously in Western countries, but the methods for measuring depression varied. In the present patients, ICD/CRT-D implantation and NYHA functional class III/IV as baseline were associated with depression. Previous studies have indicated that ICD implantation improves quality of life (QOL) in most ICD patients,[49,50] but an underlying disease or comorbidity, poor social support, or ICD-specific problems, such as frequent shocks and poor understanding of ICD therapy, increase depressive symptoms and reduce the QOL for ICD patients.[10,50–52] This is an important problem in clinical practice because the number of ICD implantations being carried out to prevent sudden cardiac death is increasing. A meta-analysis showed that depression is common among patients with heart failure, and substantially higher rates of clinically significant depression are present among patients with more severe heart failure.[53] In the present study, concomitant use of amiodarone/nifekalant, i.v. inotropics and i.v. vasodilators at the time of the questionnaire was higher in patients with depression. These findings might be due to a higher proportion of moderate to severe heart failure patients among patients with depression. More than half of the heart failure patients in Japan have non-ischemic etiologies, unlike in Western countries, where the majority of heart failure patient have ischemic etiologies.[54–56] From the present results, regardless of the etiology, severe heart failure, higher plasma BNP and higher NYHA functional class were associated with depression and are risk factors for cardiovascular events and mortality. The prevalence of heart failure increases with age, and depression will be expected to rise in coming years because of the growing elderly population.

Single or widow status was associated with depression. Regarding socioeconomic status, the employment rate was lower in patients with depression, although work status was not a statistically independent factor for depression. Education also was not related to depression. Using national survey data, Inaba et al reported that the depression score according to CES-D is higher in women, single people, and people with lower incomes in both Japan and the USA, but there is no association between education and depression in Japan; however, depression is inversely related to education in the USA.[57] The present findings that higher prevalences of single people and people with low employment status, but not level of education, were seen in patients with depression might be due to certain common features of Japanese patients with depression.

There are several mechanisms to consider concerning the relationships between depression and poor outcomes in patients with cardiovascular disease.[6] First, behavioral problems decrease patient compliance. Depressive symptoms have been associated with poor adherence to medications, diet, fluid restriction, and exercise as well as poor social support.[2,6,58,59] In the present subjects, poor social status, such as being unmarried or unemployed, was associated with depression. Poor social support also has been reported to be independently associated with worse cardiovascular outcome.[60] Second, biological mechanisms are involved in poor cardiovascular outcomes. Several events have been associated with these poor outcomes, including changes in cardiac autonomic tone, activation of the sympathetic nervous system, enhanced activity of the hypothalamic–pituitary–adrenal axis, and elevated inflammatory and pro-inflammatory processes.[1,2,6,61] Although depression is associated with poorer outcome in patients with cardiovascular disease, its pathophysiologic mechanisms are not completely understood. In the present study, death due to heart failure and hospitalization for heart failure were major adverse cardiovascular events, and the rates of these events were significantly different between patients with and without depression. There was significantly higher use of spironolactone/eplerenone and warfarin at discharge in patients with depression than in those who were not depressed. This difference might be related to a

higher rate of coexisting heart failure in patients with depression. Recently Zuluaga et al suggested that the association between depression and higher long-term mortality in patients hospitalized for heart failure is explained largely by the presence of comorbidities, physical inactivity, and disability.[62] Moreover, several reports concluded that therapy for depression improved depressive symptoms but not cardiovascular outcomes in patients.[6,63,64] In the present study, antidepressant use was higher in patients with depression, but the small rate of usage of these drugs did not contribute to patient outcomes. Depression may be merely a surrogate marker of poor prognosis but it may be an important marker, especially in patients with heart failure. The management of depression and cardiovascular disease, including proactive follow-up by nurses or care managers,[65] intervention with cognitive behavioral therapy, or social support,[60] is important for improving compliance and therapeutic outcomes in patients with cardiovascular disease and depression.

Study Limitations

There were some limitations in the present study. First, this was a single-center cohort study. The clinical characteristics of the present patients might not reflect those of general cardiovascular patients in Japan because the present institution is a university hospital. The prevalence of coronary heart disease was only 31%, and half of the patients were in NYHA functional class II–IV. In addition, there was a treatment bias. Therefore, the present results have limited generalizability in overall cardiac care. Second, the present patients were not consecutively enrolled, and many patients who received emergent or intensive care were not enrolled because it was not possible for them to complete the questionnaire. Moreover, there was an approximately 50% response rate for the Zung SDS questionnaire in the enrolled patients. This self-report 20-item written questionnaire was used as a convenient screening method but was limited by the document return rate from all subjects and the validity of the responses. From these limited data, we could not determine the contribution of depression to clinical condition in several patients with cardiovascular disease. Third, the questionnaire was not completed before discharge. The primary aim of the present study was to evaluate the prevalence and distribution of depression in hospitalized patients. Moreover, the length of hospital stay ranged from a few days to several months because cardiovascular diseases are heterogeneous. For long-term prognosis, an assessment immediately before discharge might be more appropriate. Previous research has demonstrated, however, that depression at the time of hospitalization, not only before discharge, is associated with poor prognosis in patients with cardiovascular disease.[66–69] Although this problem exists, the present results demonstrate the importance of assessment at an early stage of management of cardiovascular patients. Four, because the number of subjects in the present study was relatively small, subgroup analysis was not feasible. To clarify these issues, large multicenter clinical investigations that include several regions in Japan are needed.

Conclusion

The present results suggest that depression is not uncommon in Japanese cardiovascular inpatients, especially in those with heart failure or who are on ICD therapy. Depression is associated with subsequent cardiovascular outcomes or mortality and may be an important marker of poor prognosis.

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Disclosures

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ORIGINAL ARTICLE

Accelerated BMIPP uptake immediately after reperfused ischemia in the isolated rat heart model

Kenji Fukushima · Mitsuru Momose · Chisato Kondo · Nobuhisa Hagiwara · Shuji Sakai

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Abstract
Objective ^{123}I-beta-methyl iodophenyl pentadecanoic acid (BMIPP) can visualize myocardial fatty acid metabolism and has extensive potential for diagnosing cardiac diseases such as acute coronary syndrome in the clinical setting. Increased BMIPP uptake with decreased perfusion occasionally occurs under acute reperfusion ischemia and the kinetics of BMIPP remain unclear. The present study uses the isolated rat heart model to measure kinetic changes in BMIPP under acute reperfusion ischemia.
Methods Male Wistar rats were allotted to normal control (NG), mild (MG) and severe (SG) ischemia groups. The hearts were perfused according to the Langendorff method at a constant flow rate, and BMIPP wash-in and wash-out were studied. No-flow ischemia was applied for 15 and 30 min to the MG and SG groups, followed immediately by the wash-in and wash-out study. Whole heart radioactivity was determined using an external gamma detector throughout the experiment. Rates of myocardial uptake (K_1, mL/min) and clearance (k_2, min^{-1}) were generated using a compartmental model analysis. The same procedures and protocols were performed using ^{99m}Tc-sestamibi (MIBI) as a perfusion study.
Results Perfusion pressure significantly increased and mean heart rate significantly decreased in the severe ischemia group (heart rate: 244 ± 76, 304 ± 105 and 94 ± 140 bpm; perfusion pressure: 67 ± 13, 101 ± 31 and 160 ± 84 mmHg for NG, MG and SG, respectively). MIBI-K_1 significantly decreased, whereas BMIPP-K_1 increased in the MG and SG groups (MIBI-K_1: 3.45 ± 1.10, 1.95 ± 0.82, and 1.05 ± 0.13 mL/min; BMIPP-K_1: 3.06 ± 0.88, 3.91 ± 0.87, and 4.94 ± 1.51 mL/min for NG, MG and SG, respectively) with an inverse relationship to the severity of ischemia. MIBI-k_2 increased markedly in severe ischemia (NG vs. MG: $p < 0.05$), whereas BMIPP-k_2 did not change in the ischemic groups (MIBI-k_2: 0.00072 ± 0.0011, 0.00038 ± 0.00076 and 0.043 ± 0.033; BMIPP-k_2: 0.0056 ± 0.0028, 0.0029 ± 0.0010 and 0.0037 ± 0.0022 min^{-1} for NG, MG and SG, respectively).
Conclusion Myocardial BMIPP uptake increased immediately upon reperfusion after no-flow ischemia, and was inversely related to the severity of ischemia. The increased uptake was not due to reduced clearance, but to accelerated extraction.

Keywords Fatty acid metabolism · I-123 BMIPP · Isolated rat heart · Myocardial ischemia · SPECT

K. Fukushima · M. Momose (✉) · C. Kondo · S. Sakai
Department of Diagnostic Imaging and Nuclear Medicine, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan
e-mail: mmomose@rad.twmu.ac.jp

N. Hagiwara
Department of Cardiology, Tokyo Women's Medical University, Tokyo, Japan

Introduction

Myocardial fatty acid metabolism constitutes the major portion of the energy pathway that supplies the ATP required for healthy cardiac contraction. Free fatty acids account for 50–70% of the major energy source under normal conditions [1, 2]. Because ATP production via fatty acid metabolism requires abundant oxygen, metabolism can shift to glucose when the myocardium is compromised by ischemic or hypoxic insult. Therefore, to detect dysfunctional fatty acid metabolism is considered to be quite important.

Since the 1990s, ^{123}I-beta-methyl iodophenyl pentadecanoic (BMIPP) has been commercially available in Japan, where it has been extensively applied to the scintigraphic evaluation of various cardiac diseases [3]. BMIPP is a branched-chain fatty acid analog of *p*-iodophenyl pentadecanoic acid in which a methyl group added to the β-3 position inhibits mitochondrial β-oxidation. The rate of fatty acid metabolism is usually rapid and difficult to track, but BMIPP enables prolonged retention in the myocardium, a feature that is suitable for SPECT image acquisition [4].

BMIPP is clinically useful for detecting ischemia in patients with coronary artery disease and non-ischemic myocardial disease such as hypertrophic or dilated cardiomyopathy. Abnormal findings in BMIPP images might predict future cardiac events in patients with cardiomyopathy [5].

Cardiac imaging using BMIPP can sensitively detect myocardial ischemia and damage. Therefore, ischemic heart diseases are frequently visualized by dual isotope imaging with a myocardial perfusion tracer such as ^{201}thallium, or ^{99m}Tc-sestamibi (MIBI) [6]. Fatty acid metabolism is considered to decrease more rapidly than perfusion tracers when ischemia has occurred. Perfusion–metabolism mismatches are quite frequent in patients with ischemic heart disease [7, 8].

While many studies have examined the features and applications of BMIPP, some clinical reports have uncovered a controversial phenomenon [9]. Higuchi and Noriyasu et al. found in an animal experiment that the myocardial uptake of BMIPP is transiently accelerated during the very early phase of acute reperfusion ischemia compared with that of a perfusion tracer [10, 11]. These studies have visualized increased BMIPP uptake under acute reperfusion ischemia, but changes in the kinetics during this phenomenon remain unclear. Furthermore, to measure the precise kinetics of the tracer is difficult because of systemic effects (i.e. radioactivity in adjoining organs, systemic tracer recirculation, and hemodynamic influences). The present study aimed to elucidate the myocardial extraction and wash-out kinetics of BMIPP immediately after reperfusion ischemia in the isolated rat heart model.

Materials and methods

Preparation of radiopharmaceuticals

Nihon Medi-Physics Co. Ltd. (Kobe, Japan) supplied BMIPP (1 mg) in a kit and Fujifilm RI Pharma Co. Ltd. (Chiba, Japan) supplied MIBI (1 mg) in a lyophilized kit.

Experimental animals

Thirty-four male Wistar rats (body weight, 250–350 g; age, 6 weeks; Saitama Experimental Animals Co. Ltd, Saitama, Japan) were assigned to normal control (NG, $n = 6$), mildly ischemic (MG, $n = 5$) or severely ischemic (SG, $n = 5$) groups. The group assignment was similar for the MIBI kinetic study groups (NG, $n = 6$; MG, $n = 6$; SG, $n = 6$).

The heart was quickly isolated under deep anesthesia induced with an intraperitoneal injection of pentobarbiturate (25 mg/g), and the aorta was cannulated to initiate retrograde perfusion according to the Langendorff method (LE 05.200®; Panlab, Barcelona, Spain). A thermometer was inserted into the left ventricle of the isolated heart and connected with a thermostat (LE 13206 Thermostat Letica®, Barcelona, Spain) to measure and maintain the temperature of the chamber at 37°C. Circulation pressure and ECG were continuously monitored using an amplifier (Bio Amp®; Power Lab System AD Instruments, Barcelona, Spain). The isolated heart was perfused with Krebs–Henseleit bicarbonate buffer [g/L: D-glucose (2.0), magnesium sulfate (0.141), potassium phosphate monobasic (0.16), potassium chloride (0.35), and sodium chloride (6.9); SIGMA Chemical Co., St. Louis, MO, USA] containing $NaHCO_3$ 2.1 mg/L and $CaCl_2$ 0.175 mg/L. The buffer was continuously bubbled with 95% O_2/5% CO_2 throughout the study. The isolated heart was wrapped with Parafilm® to maintain the moisture content throughout the study and perfused with the buffer using a pump system (Miniplus3 Peristaltic Pump® and STH Pump Controller®; Gilson Inc., Middletown, WI, USA) at a constant flow rate of 10 mL/min. A Parafilm®-covered, external gamma probe (ALOKA Co., Ltd., Tokyo, Japan) connected to an analyzer (Gamma-Chaser®, ALOKA) was applied to whole hearts to determine radioactivity levels on a count-per-sec basis throughout the study.

Figure 1 shows the study protocol. Stabilization of the heart for 15 min achieved by perfusion with BMIPP or MIBI in the buffer at the above constant rate for 20 min was followed by 25 min of tracer-free wash-out. In the ischemia groups, global myocardial ischemia was induced by stopping pump infusion with perfusion medium (15 min for mild ischemia, and 30 min for severe ischemia) prior to tracer wash-in and out study. The same protocols were performed for both BMIPP and MIBI. The radioactivity in the buffer was determined using a gamma-well counter (Well Counter®, ALOKA). The calibration factor for the radioactivity between the buffer and heart was determined as described [12].

Sampling energy windows were set to 140–180 keV. Sampling data were recorded every second in the Excel format on a personal computer, and time–activity curves were generated using Excel®. All curves were corrected for

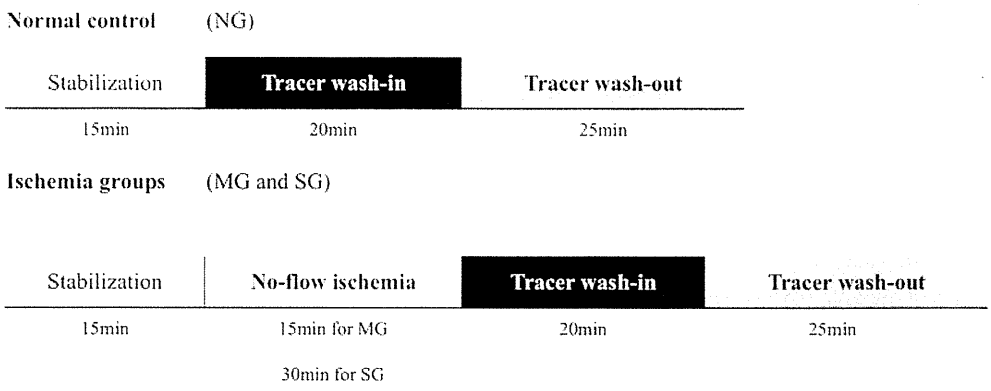

Fig. 1 Study protocols. After 15 min of stabilization, tracer (BMIPP or MIBI) wash-in of the rat heart proceeded for 20 min, followed by wash-out for 25 min. No-flow ischemia was implemented before these two studies. NG, MG and SG: control, mild and severe ischemia groups

radioactive decay, and we adopted a single-compartmental tracer kinetic model in which K_1 is described as flow velocity into myocardium, and k_2 as diffusion from myocardial cell and extra cellular space, namely myocardial wash out. Since flow in vessel should be constant due to continuous pump infusion (10 ml/min), K_1 was obviously defined as an extraction rate into myocardium. The principles and equation have been previously described [12].

Biochemical concentration

Effluent buffer from the rat heart was sampled at 10, 15 and 20 min during constant perfusion, and 10 ml of each sample was collected using EDTA tubes before measurements. Lactate was measured using lactate oxidase enzyme and visible spectrophotometry according to the Japanese Society of Laboratory Medicine. Mean values were calculated from sampled data in each group.

Statistical analysis

Values are expressed as mean values ± SD and were compared with nonparametric data by the Kruskal–Wallis and Mann–Whitney tests using StatView® (version 5.0, SAS Institute, NC) for Windows. A value of $p < 0.05$ was considered statistically significant.

Results

Hemodynamics during retrograde perfusion

The temperature of the left ventricle did not change significantly (36.6 ± 0.4°C; $n = 27$) in any of the groups, and oxygen saturation was at least 90% in all rats throughout the study. Table 1 shows significantly higher circulation pressure and a significantly lower heart rate in SG compared with NG and MG ($p < 0.05$, respectively).

Tracer kinetics

Figure 2 showed the K_1 value from the results. MIBI-K_1 was significantly decreased in the MG and SG groups compared with NG and MG groups, respectively, and SG also significantly differed among the groups (3.45 ± 1.10, 1.95 ± 0.82 and 1.05 ± 0.13 ml/min for NG, MG and SG, respectively; $p < 0.05$ for each). On the other hand, BMIPP-K_1 was significantly higher in the ischemic groups than in NG, and BMIPP-K_1 was significantly higher in SG than in MG (3.06 ± 0.88, 3.91 ± 0.87 and 4.94 ± 1.51 ml/min for NG, MG and SG, respectively; $p < 0.05$ for each).

An inverse relationship was observed between MIBI- and BMIPP-K_1 in ischemia groups, and a significantly higher ratio of metabolism-perfusion uptake (BMIPP-K_1/MIBI-K_1) was found according to ischemic severity (0.89 ± 0.25, 2.0 ± 0.44 and 4.70 ± 1.43 for NG, MG and SG, respectively; $p < 0.05$ for each comparison).

From the wash-out kinetics, MIBI-k_2 was markedly higher for SG than for MG and NG (0.00072 ± 0.0011, 0.00038 ± 0.00076 and 0.043 ± 0.033 for NG, MG and SG; $p < 0.05$ for SG vs. NG and MG), whereas BMIPP-k_2 did not significantly differ between any of the groups (0.0056 ± 0.0028, 0.0029 ± 0.0010 and 0.0037 ± 0.0022 for NG, MG and SG, respectively). As shown in Fig. 3, MIBI-k_2 showed a lower value compared to BMIPP, but was further accelerated in SG than in those of MIBI ($p < 0.05$).

Biochemical profile

Lactate was significantly increased in MG compared with NG (2.22 ± 0.62 and 0.53 ± 0.15 mg/dl, respectively; $p < 0.0001$) and tended to increase in SG (5.15 ± 3.12 mg/dl) compared with MG but the difference did not reach statistical significance.