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Paracrine Factors from Cultured Cardiac Cells Promote Differentiation of Embryonic Stem Cells into Cardiac Myocytes

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Abstract: Embryonic stem (ES) cell-derived cardiac myocytes could be valid source for cell transplantation therapy. The primary goal of the present study is to explore the efficient method to obtain cardiac cells derived from ES cells. We examined the effects of conditioned medium from cultured cardiac cells of neonatal mouse on the differentiation of ES cells toward cardiac myocytes. When Nkx2.5-GFP knock-in ES cells were incubated in the medium extracted from cultured neonatal mouse ventricular myocytes, they showed more efficient differentiation to cardiac myocytes, compared with incubation in the control medium (6.8±1.6 vs 8.6±1.9%, n=4, $p<0.05$). Neonatal cultured myocytes may produce some paracrine factors which promotes ES cell differentiation to cardiac cells.

Key words: embryonic stem cell, fluorescence-activated cell sorting, conditioned medium

Introduction

Myocardinal reconstruction with cardiac myocytes derived from embryonic stem (ES) cells is expected to a potential therapeutic option for heart diseases refractory to conventional treatments. To make this regenerative therapy feasible, it is required to establish efficient methods to obtain a large number of ES cell-derived cardiac myocytes (ESCMs). A recent study has demonstrated that human endothelial progenitor cells (EPCs) can transdifferentiate to cardiac myocytes when co-cultured with cardiac cells (Badorff C 2003). A transdifferentiation of murine skeletal myoblasts co-cultured with rat cardiomyocytes has also been reported (Iijima Y 2003).

In the present study, we investigated the effects of the medium of neonatal mouse cultured myocytes. We used ES cells in which GFP coding sequence is knocked in Nkx2.5, a cardio-specific homeobox gene, to recognize ESCMs.

Materials and methods

Nkx2.5-GFP knock-in ES Cells (hCGP7)

A cell line of Nkx2.5-GFP knock-in ES cells (hCGP7) was established as described elsewhere . Briefly, one of the murine Nkx2.5 loci was knocked-in with a promoter-less EGFP gene (Clontech, Palo Alto, USA) and a puromycin resistance gene driven by the Pgk1 promoter. The parental ES strain, ht7, was provided by Dr. Hitoshi Niwa, Osaka University.

Cell culture

Undifferentiated ES cells of ht7 or hCGP7 were digested with trypsin-EDTA and suspended in "ES cell differentiation medium" containing Glasgow minimum essential medium (GMEM: Sigma) supplemented with 10% FBS (Gibco), 2mM L-glutamine, 50U/μl penicillin, 50μg/ml streptomycin, 100μM 2-mercapto ethanol. The ES cells were then cultured in small drops hanged from the lid of culture dish ("hanging-drop") to aggregate and form embryo-like cells called embryoid bodies (EBs) for 3 days (Hidaka K 2003). Each drop (20 μl) contained 1600 cells (Miwa K 2002). EBs were then transferred to tissue culture dishes (Falcon, USA) and further cultivated for 7 days with the reference medium or a conditioned medium. Medium was changed every third day.

Conditioned medium

Primary culture of cardiac cells was prepared from ventricles of 1-day-old ICR mouse using Neonatal Rat Cardiomyocyte Isolation System (Worthington, USA). Cardiomyocytes were plated at a field density of 2×10^5 cells/cm^2 on 6-well tissue culture plate (Falcon, USA) and cultured in medium 199 (M199 GIBCO, USA) supplemented with 10% fetal bovine serum (FBS) and 50μg/ml gentamicin sulfate. On the next day, the medium was replaced by the ES cell differentiation medium.

FACS analysis

After 10 days of culture in differentiation medium (with

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or without conditioned medium), EBs (day3+7) were dispersed with trypsin-EDTA (0.25%). Flow cytometric analyses were performed using a dual laser FACS Calibur (Becton Dickinson, USA). Cells were excited with 488 nm Argon laser and a 530 nm bandpass filter was used to detect GFP positive cells. Data were recorded using the CellQuest acquisition software (Becton Dickinson, USA) (Muller M 2000). ES cells derived from ht7 were used as control.

Statistics

Data were expressed mean±SE. Statistical analysis was performed using Student's paired t test. Differences are considered significant at $p<0.05$

Results and Discussion

Spontaneously beating cells appeared in each EBs with 5 or 6 days of plated culture. In FACS analysis, ESCMs were recognized as GFP-positive cells. Figure 1 shows representative scatter plots of flow cytometric analyses. Abscissas and ordinates indicate intensities of intensities of GFP and PI (propidium iodide), respectively. The fraction of high PI intensity (>20) indicates dead cells. Viable ESCMs should be included in the GFP-positive and low PI intensity zone (R2 area). In non-labeled cells (ht7-derived EBs), only a small fraction of the GFP-positive cells were detected in R2 (0.1±0.05%, n=4) (Fig. 1A). In cells from hCGP derived EBs cultured with the reference medium, a certain population of GFP-positive variable cells were detected (6.8±1.6%, n=4) (Fig. 1B). In cells from hCGP-derived EBs cultured with conditioned medium, a higher population of GFP-positive viable cells were obtained (8.6±1.9%) (Fig. 1C).

The present study has shown that ESCM differentiation is promoted by a medium conditioned by cultured cardiac myocytes. Conditioned medium may include a variety of hu-

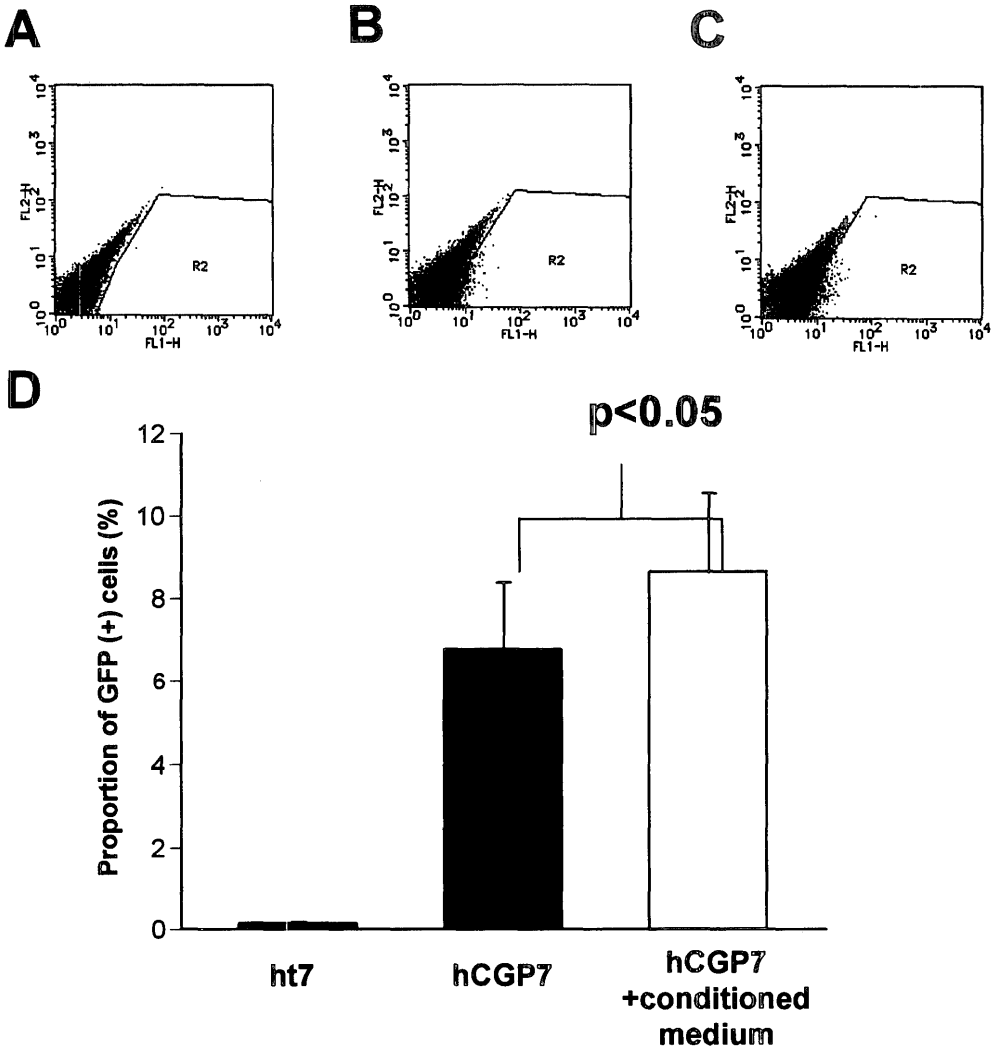

Fig. 1 FACS analysis of single cell preparation from embryoid bodies. EBs were enzymatically digested at the stage of day 3+7. (A) Control (ht7 cultured with ES cells differentiation medium), (B) hCGP7, (C) hCGP7 cultured with conditioned medium from mouse cardic cells. (D) Summary of GFP positive fraction (GFP-positive and PI negative cells, n=4).

moral factors such as angiotensin II, atrial natriuretic polypeptide or growth factors. EPCs and skeletal myoblasts have been shown to require direct cell-to-cell contact and contraction of neighboring cardiomyocytes for the transdifferentiation into cardiomyocytes (Badorff C 2003) (Iijima Y 2003). The discrepancy between ES cells and these progenitor cells of endothelium and skeletal muscle could be attributed, at least in part, to pluripotency of ES cells. ES cells can differentiate to cardiac cells directly, whereas dedeferentiation is needed for EPCs and skeletal myoblasts to transdifferentiation to cardiac myocytes. Thus, paracrine factors may not be sufficient to cause the transdifferention. It is also conceivable that conditioned medium may affect non-cardiac cells in EBs and then autocrine factors would affect the differentiation of ES cells to cardiac myocytes.

In the future, there may be increasing demands for ESCMs as a source for cellular therapy of myocardial reconstruction. It is of great importance to specify the factors affecting the promotion of differentiation of ES cells to cardiac myocytes. Further experimental studies will be required to elucidate the point.

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References

Badorff C, Brandes RP, Popp R, et al. Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes. Circulation 2003; 107: 1024–1032.

Hidaka K, Lee JK, Kim HS, et al. Chamber-specific differentiation of Nkx2.5-positive cardiac precursor cells from murine embryonic stem cells. FASEB J 2003; 17: 740–742.

Iijima Y, Nagai T, Mizukami M, et al. Beating is necessary for transdifferentiation of skeletal muscle-derived cells into cardiomyocytes. FASEB J 2003; 17: 1361–1363.

Miwa K, Lee JK, Hidaka K, et al. Optimal population of embryonic stem cells in "hanging drop" culture for in-vitro differentiation to cardiac myocytes. Environ Med 2002; 46: 92–94.

Muller M, Fleischmann B.K, Selbert S, et al. Selection of ventricular-like cardiomyocytes from ES cells in vitro. FASEB J 2000; 14: 2540–2548.

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