Establishment of Mouse Embryonic Stem Cell and Effects of Herbal Medicine on Induction of Cardiomyocyte Differentiation

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Abstract - This study was conducted to investigate the effects of Woohwangcheungsimsweun (ox bezoar), deer antlers, and wild ginseng on induction of cardiomyocyte differentiation using the established mouse embryonic stem (ES) cells. The expression of atrial natriuretic peptide (ANP) was highest in Woohwangcheungsimsweun treatment group. The expression of rabbit anti-GATA-4(GATA-4) and troponin (TnI) were highest in wild ginseng and Woohwangcheungsimsweun treatment groups, respectively. Fluorescence activated cell sorting (FACS) analysis showed that the expression of ANP was highest in Dimethyl sulfoxide(DMSO) and Woohwangcheungsimsweun treatment groups. The expression of GATA-4 was relatively high in wild ginseng treatment group. The expression of TnI was highest in Woohwangcheungsimsweun treatment group. In the gene expression analysis, DMSO greatly inhibited GATA-4 expression to 25% of control. Woohwangcheungsimsweun treatment caused to increase cTnI and cardiac ANP expression significantly. Wild ginseng extract upregulated GATA-4 gene expression. In conclusion, DMSO widely used as cardiomyocyte differentiation inducer did not show significant effects on the expression of ANP, GATA-4 and TnI in this study. Woohwangcheungsimsweun showed upregulation of ANP and TnI expression. Wild ginseng extract showed greater effects than DMSO on GATA-4 expression. These results might suggest that the combination of Woohwangcheungsimsweun and wild ginseng extract treatment can be expected to increase expressions of all three genes.

Key words - Mouse ES cell, Cardiomyocyte, DMSO, Woohwangcheungsimsweun, Deer antlers, Wild ginseng

Introduction

Since Evans and Kaufman (1981) first isolated an embryonic stem (ES) cell from the blastocyst embryo of a mouse and *in vitro* culture of ES cells succeeded (Thomson et al., 1995; Bongso, 1994), induction of differentiation to a specific cell from the ES cell (Hirashima al., 1999; Kawasaki et al., 2000; Kramer et al., 2000; Lee et al., 2000; Nakayama et al., 2000; Soria et al., 2000; Yamane al., 1999) were studied by many researchers.

The induction of differentiation into a specific cells has recently been extensively studied. As Reubinoff and others (2000) were successful in differentiating a neural progenitor cells from hES cells by applying the culturing process of the mouse ES cell, it was possible to utilize the ES cell as a tool for treatment of human diseases. However, more studies for the separation and cultivation, characterization, mass production

and induction of ES cell differentiation are required.

Woohwangcheungsimsweun, deer antlers and wild ginseng have been applied to prevent and cure the heart diseases and their clinical efficacy has been highly estimated in clinical aspects. These oriental medicines may contain functional components to stimulate heart function.

Therefore, as part of basic research to develop functional substance related to the differentiation of the ES cell into the cells of specific tissues, we have isolated mouse embryonic stem cells and then examined the effect of medical herb (Woohwangcheungsimsweun, deer antlers and wild ginseng extract) on the induction of ES cell differentiation into the cardiomyocytes.

Materials and Methods

Isolation and culturing of ES cell

Female BDF1 mice (C₅₇BL x DBA, 6~10 weeks old) were hyperstimulated ant then mated with males in individual cage.

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Mice were killed by cervical dislocation, ovaries removed and zygotes of 1-cell stage collected. All procedures used in this study were performed in accordance with the Guide for the Care and Use of Laboratory animals and the work was approved by the Ethics Committee of the Faculty. Culture medium was Modified Basal XI media (Modified Cleavage medium, Sage Inc.) with 10% Serum Substitute Supplement (SSS, Irvine Sci. 92705) under paraffin oil for further culture. Embryos were cultured when they had developed into expanded blastocysts or early hatching stage in an incubator at 37°C in a humidified atmosphere of 5.0% CO₂ for 5-6 days.

The mouse embryonic feeder fibroblasts (MEF) cells were prepared from ICR mouse embryos at 13.5-16.5 days post-coitum. The culture medium was Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, 11995). The cells were cultured in 60-mm culture dish (Flacon) at 37°C, 5.0% CO₂ incubator until confluency, and then resuspended in 1 ml freezing medium. Freezing medium contained 10% dimethyl sulfoxide (DMSO) and 0.5~1x10⁷ cell/ml. The frozen feeder aliquots were thawed and cultured until confluency and treated with 10 µg/ml mitomycin C (Sigma) in MEF culture medium with 20% FBS for 2 hours in 37°C, washed three times with phosphate-buffered saline (PBS), and plated onto a coated dish (4 well dish; Nunc) without gelatin treatment. The MEF cells were obtained at passage 3~5 and were passed four more times before freezing in liquid nitrogen. Frozen vials containing ES cells were thawed and passed three more times before differentiation.

To characterize ES cells alkaline phosphatase activity, Immunocytochemical test (SSEA-1 and Oct4-ES cell marker sample kit, Chemicon), teratoma formation using nude mouse

(nu/nu B/C, 36~42 days old, non-obese diabetic-severe combined immunodeficiency) and general histomorphological test were examined.

Induction of differentiation into cardiomyocytes

For induction of differentiation, embryoid bodies (EBs) were formed using hanging drops (25 µL/drop, 10³ cells/drop) and cultured in the medium containing 1% DMSO, 1% Woohwangcheungsimweun (K. co. Korea), 1% deer antlers, 1% wild ginseng extract and 0.1 mg/ml wild ginseng powder on coated 4 well-dish without gelatin treatment (4~5 EBs/ well) for 5 days until formation of beating cell cluster. For preparation of natural products, 500 g of deer antlers and 500 g of wild ginseng were extracted with MeOH 3 times for 5 hours each in cooling water reflux cisten, and then concentrated under decompression. Final yield was approximately 120 mg.

Immunocytochemical analysis of cardiomyocyte was carried out on day 9 using rabbit anti-atrial natriuretic peptide (ANP, 1:500, Chemicon), and rabbit anti-GATA-4 (GATA-4, 1:100, chemicon, AB4132) polyclonal antibody and mouse anti-human cardiac Troponin I monoclonal antibody (cTnI, 1:1000, Chemicon). Fluorescence-activated cell sorting (FACS) analysis, beating rate and reverse transcription-polymerase chain reaction (RT-PCR) analysis were also performed for analysis of cardiomyocyte differentiation.

Beating ratio of EB (number of beating EB/5 EBs plated) of each treatment group was measured at day 5 after plating of 5 EBs per well. The number of beating EB was measured 8 times and mean values were calculated.

Total RNA was extracted from ES cells and EBs using TRI reagent (Sigma) according to the manufacturer's instructions.

Table 1. The primers used in RT-PCR, the nucleotide position and the estimated size of the amplified fragments

Gene	Primers	Position	Expect size (Known gene)
TnI	Forward GATGGAACGAGAGGCAGAAG	183-202	450bp
	Reverse CCCTCAAACCTTTTCTTGCG	632-613	(Tnni3)
ANP	Forward AACCTGCTAGACCACCTGGA	121-140	325bp
	Reverse GGAAGCTGTTGCAGCCTAGT	445-426	(Nppa)
GATA-4	Forward CCGTCTACGTGCCACTCC	106-123	624bp
	Reverse GTGATAGAGGCCACAGGCAT	729-710	(ATA4)
β-actin	Forward TGTTACCAACTGGGACGACA	225-244	573bp
	Reverse AAGGAAGGCTGGAAAAGAGC	797-778	(Actb)

First strand cDNA was synthesized from 5 mg/ml total RNA using M-MLV Reverse transcriptase kit (Promega). Complementary DNAs were subjected to PCR amplification with primers specific for mouse cardiac genes (Table 1). PCR was performed in a reaction volume of 30 μ L with 2.5 mM dNTP mixture Ex-Taq polymerase (2.5 units, TaKaRa), 0.2 μ M forward and reverse primers, and 1 μ L cDNA template. The profile for PCR reaction was 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57~60°C, depending on the melting temperature of the primer sequence, for 30 sec, and extension at 72°C for 45sec, then 10 min at 72°C for the final extension. The PCR products were size fractionated by electrophoresis on a 1.5% agarose gel containing 0.1 mg/ml ethidium bromide and photographed with BioRad Gel Imaging System using a GeneRuler DNA ladder mix as a size marker (Fermentas). Statistical analysis was performed by students' t-test (SPSS 13.0). The probability at $p < 0.05$ was considered significant.

Results and Discussion

Identification of the isolated and cultured mouse embryonic stem cell

For characterization of the established mouse embryonic stem cell, alkaline phosphatase activity, immunocytochemical analysis (SSEA-1 and Oct-4 cell markers), teratoma formation using nude mouse and histochemical test were conducted. Alkaline phosphatase activity about 100% of cell colony were positive for alkaline phosphatase. Immunocytochemical analysis demonstrated that most of colonies were positive for SSEA-1 and Oct-4 (Fig. 1).

Established ES cells were injected to a nude mouse and teratoma formation was observed (Fig. 2). Histological examination of teratoma demonstrated the presence of endoderm, mesoderm and ectoderm, suggesting pluripotency of this ES cell line.

Induction of differentiation into cardiomyocytes

We investigated the effect of functional natural products on the induction of ES cell differentiation into cardiomyocytes.

As shown in Fig. 3 EB, Beating was observed in EB outgrowth suggesting ES cells were induced to differentiate

into cardiomyocytes.

To examine protein expression, immunohistochemical analysis was performed using ANP, GATA-4 and TnI antibodies.

Expression of these proteins were confirmed by this method (Figs. 4, 5 and 6). Immunocytochemical test for ANP

Fig. 1. Immunocytochemistry test of SSEA-1 and Oct-4 in ES cells. Scale bar = 100 μ m.

Fig. 2. Formation of teratoma on 35 days after injection on ES cell in the nude mouse.

Fig. 3. Beating cell clusters (arrow) of cardiomyocyte on 5 and 6 days after hanging drop culture.

as shown in Fig 4. Showed green fluorescence in all treatment groups, indicating that ES cells were differentiated into cardiomyocytes. However, expression was different among treatment groups. The intensity of green fluorescence radiation between control group and deer antlers treatment group was similar. The intensity of control and deer treatment groups were weak, compared with those in DMSO treatment group, Woohwangcheungsimsweun treatment group, wild ginseng extract treatment group and wild ginseng powder treatment groups. The intensity was greatest in Woohwangcheungsimsweun treatment group.

Expression of GATA4 are shown in Fig. 5. Intensity of green fluorescence was lowest in DMSO treatment group among all the treatment groups. Intensity in deer antlers

Fig. 4. Immunocytochemistry test of ANP in the differentiation cell.

Fig. 5. Immunocytochemistry test of GATA-4 in the differentiation cell.

Fig. 6. Immunocytochemistry test of cTnI in the differentiation cell.

Fig. 7. FACS analysis of ANP concentration in the differentiated cardiomyocyte.

treatment group, Woohwangcheungsimweun treatment group and wild ginseng powder treatment group were similar with that in control group. The intensity were higher in wild ginseng extract treatment group than any other treatment groups.

Expression of TnI are shown in Fig. 6. Intensity of green fluorescence radiation were highest in Woohwangcheungsimweun treatment group. Intensity of DMSO treatment group and wild ginseng extract treatment group were greater than those in control group. Intensity in wild ginseng powder treatment group were less than that in control group.

To further examine differentiation into cardiomyocyte quantitatively, fluorescence activated cell sorting (FACS)

analysis was performed for ANP, GATA-4 and TnI. FACS analysis for ANP (Fig. 7) demonstrated slight increase of cells with high expression 1×10^2 (FLI-H) (M3) in Woohwangcheungsimweun and deer antlers treated groups. High expression was also observed in Woohwangcheungsimweun treatment in RT-PCR. FACS analysis for GATA-4 (Fig. 8) demonstrated increase of cells with intensity of higher than 4×10^2 (FLI-H), consistent with RT-PCR result. FACS analysis for TnI (Fig. 9) demonstrated no obvious difference in all treatments.

The ratio of beating EB at day 5 after plating onto medium containing each natural products is shown in Table 2. The ratio of beating EB ranged from 72% to 85%. The ratios of

Fig. 8. FACS analysis of GATA-4 concentration in the differentiated cardiomyocyte.

Fig. 9. FACS analysis of cTnI concentration in the differentiated cardiomyocyte.

Table 2. The ratio of beating embryoid bodies on day 5 after plating onto medium containing each natural products

Treatment	Ratio (%) of beating EB*
Control	72.28±19.86 [†]
DMSO	77.34±15.27 [†]
Woohwangcheungsimsweun	85.14±17.38 [†]
Deer antlers	81.65±10.21 [†]
Wild ginseng extract	79.36±14.24 [†]
Wild ginseng dry powder	82.08±12.75 [†]

[†]Not significantly different (P<0.05).

*Counting test of beating EB was performed 8 times for the ratio of beating embryoid bodies NO./ 5 embryoid bodies.

beating EB in Woohwangcheungsimsweun treatment group, deer antlers treatment group and wild ginseng powder treatment group appeared to be higher than in control group, but there was no significant difference.

The results of RT-PCR analysis for ANP, GATA-4 and TnI in the differentiated cardiomyocytes are shown in Fig. 10. TnI and ANP gene expressions were higher in Woohwangcheungsimsweun treated group compared to other treatment groups. GATA-4 gene expression was the highest in wild

ginseng extract treatment group and the lowest in DMSO treatment group. Relative expression of ANP, GATA-4 and TnI normalized by β -actin as shown in Figs. 11, 12 and 13, DMSO had no effect on the expression of cardiac ANP and GATA-4 genes, which are indicator genes for cardiac differentiation. DMSO, however, suppressed the GATA-4 gene expression. Woohwangcheungsimsweun treatment induced

Fig. 10. RT-PCR analysis for specific gene expressions.

Fig. 11. The ratio of ANP/ β -actin in RT-PCR gene expression. (★:P<0.05)

Fig. 12. The ratio of GATA-4/ β -actin in RT-PCR gene expression. (★:P<0.05)

Fig. 13. The ratio of TnI/ β -actin in RT-PCR gene expression. (★:P<0.05)

TnI and cardiac ANP expressions significantly. GATA-4 gene expression was increased in wild ginseng extract treatment.

These results demonstrate that DMSO, currently widely used as cardiomyocyte differentiation inducer, does not have significant effect on ANP, GATA-4 and TnI gene expressions. On the other hand, Woohwangcheungsimsweun or wild ginseng extract as herb medicines have greater effects than DMSO. Effect of induction of ANP and TnI by Woohwangcheungsimsweun, and GATA-4 by wild ginseng extract were also observed by FACS analysis and showed good agreement. Combination of Woohwangcheungsimsweun and wild ginseng extract treatment need to be investigated further.

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