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Neural and Cholinergic Differentiation of Mesenchymal Stem Cells Derived from the Human Umbilical Cord Blood

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인간 제대혈액에서 유래된 중간엽 줄기세포의 신경 및 콜린성 분화

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ABSTRACT : Human umbilical cord blood(HUCB) contains a rich source of hematopoietic stem cells, mesenchymal stem cells and endothelial cell precursors. Mesenchymal stem cells(MSCs) in HUCB are multipotent stem cells, differ from hematopoietic stem cells and can be differentiated into neural cells. We studied on transdifferentiation-promoting conditions in neural cells and cholinergic neuron induction of HUCB-derived MSCs. Neural differentiation was induced by adding-dimethyl sulphoxide(DMSO) and butylated hydroxyanisole(BHA) in Dulbeco's Modified Essential Medium(DMEM) and fetal bovine serum(FBS). Differentiation of MSCs to cholinergic neurons was induced by combined treatment with basic fibroblast growth factor(bFGF), retinoic acid(RA) and sonic hedgehog(Shh). MSCs treated with DMSO and BHA rapidly assumed the morphology of multipolar neurons. Both immunocytochemistry and RT-PCR analysis indicated that the expression of a number of neural markers including β-tubulin Ⅲ, GFAP and MBP, was markedly elevated during this acute differentiation. The differentiation rate was about 32.3±2.9% for β-tubulin Ⅲ-positive cells, 11.0±0.9% for GFAP, and 9.4±1.0% for Gal-C. HUCB-MSCs treated combinatorially with bFGF, RA and Shh were differentiated into cholinergic neurons. After cholinergic neuronal differentiation, the β-tubulin Ⅲ-positive cell population of total cells was 31.3±3.2% and of differentiated neuronal population, 70.0±7.8% was ChAT-positive showing 3 folds higher in cholinergic population than neural induction. Conclusively, HUCB-derived MSCs can be differentiated into neural and cholinergic neurons and these findings suggest that HUCB are alternative cell source of treatment for neurodegenerative diseases such as Alzheimer's disease.

Key words : Human umbilical cord blood, Mesenchymal stem cells, Cholinergic neuron, Basic fibroblast growth factor, Retinoic acid, Sonic hedgehog.

요 약 : 인간 제대혈 세포는 조혈모세포, 중간엽 줄기세포와내피전구세포를 풍부하게 포함하고 있다. 인간 제대혈 속의 중간엽 줄기세포는 조혈모세포와는 달리 다능성 줄기세포이며 신경세포로 분화할 수 있는 잠재성을 가지고 있다. 본 연구에서는 세포배양을 통해 제대혈의 중간엽 줄기세포를 신경세포와 콜린성 신경세포로 분화를 유도하였다. 중간엽 줄기세포를 신경세포로 분화시키기 위해 배양액에 dimethyl sulphoxide(DMSO)와 butylated hydroxyanisole(BHA)를 첨가하여 유도하였으며 basic fibroblast growth factor(bFGF), retinoic acid(RA), sonic hedgehog(Shh)를 처리하여 콜린성 신경세포로 분화시켰다. DMSO와 BHA에 처리된 중

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간엽 줄기세포가 빠르게 신경세포 모양으로 분화하는 것을 관찰하였으며, 이것은 면역조직학적 염색에서 신경세포 특이 표지인 β-tubulin Ⅲ, 별아교세포에 대한 특이 표지인 GFAP, 희돌기아교세포에 대한 특이 표지인 Gal-C에 대해 양성반응을 나타내었고, 그 비율은 각각 32.3±2.9%, 11.0±0.9%, 9.4±1.0%였다. RT-PCR 분석에서 배양 단계에 따라 신경세포에 특이적인 표지 인자가 발현됨을 통해, 중간엽 줄기세포가 신경세포로 분화됨을 확인하였다. 또한, 중간엽 줄기세포에 bFGF, RA, Shh를 처리하여 콜린성 신경세포로 분화시켰을 때, 전체 중간엽 세포 중 31.3±3.2%가 신경세포 특이 표지인 β-tubulin Ⅲ에 양성반응을 보였으며 이들 세포 중 70.0± 7.8%가 콜린성 신경 특이 표지인 ChAT에 양성반응을 보였고, 이것은 Woodbury 방법에 의한 신경분화의 경우보다 3배 가량 높은 비율로 콜린성 신경의 분화를 유도한 것이다. 이러한 실험 결과들은 인간 제대혈의 중간엽 줄기세포가 콜린성 신경세포로 분화가 가능하고 이러한 잠재성을 가진 제대혈 중간엽 줄기세포는 퇴행성 신경질환에 대한 세포 치료제로서 가능성을 제시한다.

INTRODUCTION

Stem cells are primal undifferentiated cells which possess the ability to differentiate into other cell types. Stem cells are also categorized, according to their source, as either adult or embryonic(Fortier, 2005). Adult stem cells exist in most tissues, including hematopoietic, neural, gastrointestinal, epidermal, hepatic and mesenchymal stem cells(MSCs)(Tuan et al., 2003). Compared with embryonic stem(ES) cells, adult stem cells have less self-renewal ability although they differentiate into multiple lineages(Jiang et al., 2002). It has been thought that tissue-specific stem cells could only differentiate into cells of the origin tissue. However, there are increasing body of evidences suggesting that adult stem cells can differentiate into lineages other than the origin and certain adult stem cell types are pluripotent(Dazzi & Horwood, 2007; Mays et al., 2007).

Human umbilical cord blood(HUCB) has been recognized recently as one of useful sources of stem cells, which might be preferable to other available source. HUCB is rich in hematopoietic progenitor cells and has been considered an efficient source of stem cells for clinical reconstitution of bone marrow and blood cell lineages(de Wynter & Testa, 2001). HUCB is collected from the placental umbilical cord stump after delivery and the collection is much easier than bone marrow. Another advantage of using HUCB is its reactive immune immaturity like fetal cells thus, the risk for rejection is low and long-term immunosuppressive treatment may not be required(Rogers & Casper, 2004). In addition, HUCB contains adult stem cells that display characteristics similar to those of MSCs found in bone marrow. The cells also are capable of differentiating into cells of different connective tissue lineages such as hematopoietic, epithelial, endothelial and neural tissues(Harris et al., 2007).

MSCs are one of the well-studied adult stem cells. In recent years, great interest has been generated in MSCs, due to their ability to self-renew, proliferate, and differentiate into a variety of cell types. They can be reprogrammed by extracellular cues to alter their character in ways that could have important implications for cell therapy and other forms of regenerative treatment. Recently, a number of studies have shown that MSCs could differentiate into neuron and glial cells *in vitro* and *in vivo*. Ischemic rats that received human MSCs exhibited significantly improved functional performance and histological analysis revealed that transplanted human MSCs expressed markers for astrocytes, oligodendrocytes, and neurons(Zhao et al., 2002). When MSCs were injected into the Parkinson's disease model mice, the transplantation resulted in the significant improvement on the rotarod test and some of these cells showed immunoreactivity for tyrosine hydroxylase(TH) (Lindvall et al., 2004). A number of studies have shown that bone marrow-derived stromal cell/MSC could differentiate into neuron or glial cells *in vitro*. Adult rat bone marrow cultured *in vitro* with epidermal growth factor (EGF) and basic fibroblast growth factor(bFGF) gave rise to cellular spheres which differentiated into neurons and glia(Kabos et al., 2002). Human bone marrow stromal cells also differentiated into a neuronal phenotype, under a sim-

ple culture condition(Woodbury et al., 2000) or by co-culture with rat fetal mesencephalic or striatal cells(Sanchez-Ramos et al., 2001). These observations indicate that MSCs can differentiate into neural cells. Therefore MSCs could restore the damaged brain function and thus might be suitable for cell therapy.

Central cholinergic neurons, mainly located in basal forebrain, send their projections to different structures including the cortex. The cholinergic system is implicated in functional, behavioral and pathological states. Degeneration of the cholinergic system of the basal forebrain occurs in many neurodegenerative diseases including Alzheimer's disease(Arendt et al., 1983) and Parkinson's disease(Dubois et al., 1983). Cholinergic neuron differentiation involves several steps. ES cells generate early neuroectodermal cells, which are organized into rosettes and express neuroectodermal transcription factor Pax6 but not Sox1, and then late neuroectodermal cells, which form neural tube-like structures and express both Pax6 and Sox1. Only the early, but not the late, neuroectodermal cells are efficiently posteriorized by retinoic acid(RA) and in the presence of sonic hedgehog(Shh), differentiate into cholinergic neurons. Current studies reported that ES cells differentiate specific cholinergic neuron *in vitro*. Human ES cells differentiate cholinergic neurons by treatment with bFGF, RA, and Shh(Li et al., 2005; Shin et al., 2005). Specific cholinergic neuron differentiation from mouse ES cells were induced by co-culturing with murine bone marrow-derived stromal feeder cell lines(Barberi et al., 2003). However, few studies on cholinergic differentiation of adult stem cells have done *in vitro*.

In the present study, we tried to induce neural cells from HUCB-MSCs and further differentiation to cholinergic neurons *in vitro*.

MATERIALS AND METHODS

1. Collection, Isolation and Propagation of HUCB Derived MSCs *in vitro*

HUCB was collected after delivery of the placenta by cannulation of the umbilical vein into an umbilical cord blood collection bag(Green Cross Company, Korea) containing heparin. Mononuclear cells were separated from HUCB using Ficoll-Hypaque Plus(Amersham biosciences, Piscataway, NJ) as previously described(Buzanska et al., 2002). The cells that bound to the immunomagnetic beads coated with the anti-CD34 antibody were eliminated by immunomagnetic sorting system(Dynal CD34 progenitor cell collection system, Dynal Biotech A.S.A., Oslo, Norway). After washing, the remaining cells were resuspended in Iscove's modified Dulbecco's medium(IMDM; Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum(FBS; Gibco) at a final concentration of 1×10^6 cells/mL. The plastic-adherent cells were cultured for 3 weeks in IMDM plus 10% FBS at 37℃ in a fully humidified atmosphere containing 5% CO_2. Before the cells reached confluence, the cells were trypsinized and recultured in similar conditions for the next 3 weeks.

2. Neural Induction of MSCs from HUCB and Cholinergic Neuronal Differentiation

HUCB-MSCs were induced to differentiate into neural cells using a modification of the method used by Woodbury et al.(Woodbury et al., 2000). For dedifferentiation, the cells were cultivated on poly-L-lysine-coated tissue culture plates in DMEM supplemented with 20% FBS, 0.1% β-mercaptoethanol(Sigma, St Louis, MO), 1×nonessential amino acid(Gibco) and 2 mM L-glutamine(Sigma). The cells are cultured for 2~3 days. Twenty four hours prior to neural induction, medium were substituted with pre-induction medium consisting of N2 medium(Gibco), 1× N2 supplement(Gibco), 20 ng/mL EGF(Sigma) and 20 ng/mL bFGF(Sigma). Neural differentiation was induced with 2% dimethylsulfoxide(DMSO; Sigma) and 200 μM butylated hydroxyanisole(BHA; Sigma) in N2 medium plus 1 ×N2 supplement(Gibco). After 5 hr, the medium was modified for long-term induction by adding 25 mM KCl, 2 mM valproic acid(Sigma) and 1 μM hydrocortisone(Sig-

ma) and after incubation for 1.5 days, the cells were subjected to immunohistochemistry.

Cholinergic neuronal differentiation was induced by modified method of Barberi et al.(Barberi et al., 2003). The HUCB-MSCs were cultured on cover slips coated poly-D-lysine and ornithine in tissue culture plate for 2~3 days for dedifferentiation. Then, the cells were cultured in the preinduction medium for overnight. At day 4 of culture, 500 ng/mL Shh(Sigma) and 1 μM RA(Sigma) were added to N2 medium and then the cultured was transferred to N2 medium supplemented with 10 ng/mL bFGF and 500 ng/mL Shh from day 8 to day 11. Differentiation was induced at day 11 in N2 medium supplemented with 200 μM ascorbic acid(AA; Sigma) and 20 ng/mL brain derived neurotrophic factor(BDNF; Gibco).

3. Immunocytochemistry

Immunocytochemistry was done by using immunofluorescence staining. For immunofluorescence, cells were washed with PBS and fixed by treating with 4% paraformaldehyde at room temperature for 30 min. This was followed by washing with three changes of PBS and PBS plus 0.2% Triton X-100. Slides were blocked with 10% normal goat serum and normal rabbit serum(derived from the same species as the secondary antibody) in PBS for 1 hr, β-tubulin Ⅲ(1:400, Sigma) for 24 hr at 4℃ or for 3 hr at RT. The slides were then washed with PBS, and incubated with antibody for TH or choline acetyltransferase(ChAT)(1:100, Vector Lab., Burlingame, CA) and antibodies for three times with PBS and incubated with a Cy3-conjugated rabbit anti-goat IgG(1:500, Chemicon, Temecula, CA) and FITC-conjugated goat anti-rabbit IgG(1:128, Sigma) secondary antibody for 1 hr at 4℃. Mounted slides were observed with a fluorescence microscope(Carl Zeiss Axioskop2+, Germany) to visualize the cellular colocalization of ChAT (or TH) and β-tubulin Ⅲ in the same cells. The number of ChAT- or TH-positive cells per slides was counted to obtain the percentage of ChAT- or TH-positive cell population in β-tubulin Ⅲ positive cells.

4. RNA Isolation and RT-PCR

Cells were harvested and washed once in PBS. Total RNA was extracted from cultured cells using Tri-reagent (Sigma), and then 5 μg RNA per condition was reverse-transcribed into first-strand cDNA using oligo-dT primer (Promega). Reverse transcription was carried out in the presence of M-MLV reverse transcriptase(Promega) for 1 hr at 42℃, followed by inactivation at 99℃ for 5 min and cooling to 4℃. The cDNA was amplified by 35 cycles of PCR using Taq polymerase(Promega). The amplified cDNAs were separated on 1% agarose gels, and the bands were visualized by ethidium bromide staining. Primer sequences and product sizes are summarized in Table 1.

RESULTS

1. Characterization of HUCB-MSCs

Upon serial passage of adherent HUCB-MSCs, a homogenous population of bipolar fibroblast-like cells progressively arose(Fig. 1). In order to verify the nature of these adherent HUCB-MSCs, they were labeled for MSC-related surface markers or intracellular antigens such as ICAM-1, TRA-1-60, vimentin, type I collagen and fibronectin. In those analyses, these cells turned out to be positive for all those markers(data not shown)

2. Induction of Neural Cells from HUCB-MSCs

HUCB-MSCs that had been grown to near confluence were treated with bFGF overnight and then N2 medium plus BHA and DMSO, according to the neural-inducing protocol described by Woodbury et al.(Woodbury et al., 2000). This treatment caused HUCB-MSCs to undergo profound changes in morphology(Fig. 2A, B, C), with some cells developing multiple neurite extensions from cell body (Fig. 2C). Immunocytochemical staining for neural markers was performed to confirm the neural markers expressing cells in cultured HUCB-MSCs. The immunoreactive cells for β-tubulin Ⅲ, a neuron-specific marker, GFAP, an astrocyte marker, or Gal-C, an oligodendrocyte marker, were

Table 1. Primer sets used for PCRs

Gene	Forward(5'-3')	Reverse(5'-3')	Size(bp)
STEM CELL MARKER			
SCF	ttacaagggcagttgaaaat	ttccaactgaatcatcc	263
C-kit	gttgaggcaactgcttatgg	gcttctgcatgatcttcctg	289
Stat-3	tctcctacttctgctatctttgag	atgggtctcagagaacacatc	117
NEURAL PROGENITOR CELL MARKER			
Nestin	ccagaaactcaagcaccac	ttttccactccagccatcc	398
Glypican	tgggaaaggcaaaagcag	cagaagacagtgaggaggtag	582
ECTODERM CELL MARKER			
Wnt-1	atggggctctgggcgctgttgc	cccactcacgctgtgcaggatc	252
Pax-6	agattcagatgaggctcaaa	aattggttggtagacactgg	313
NEURAL CELL MARKER			
β-tubulin Ⅲ	cctgacaatttcatctttgg	cagtaggtttcatccgtgtt	344
NeuroD1	agtccgccttacggtaccatg	gacagtcactgtaagcacag	448
GFAP	tcatcgctcaggaggtcctt	ctgttgccagagatggaggtt	383
MBP	acacgggcatccttgactccatcgg	tccggaaccaggtggttttcagcg	263
INTERNAL CONTROL			
GAPDH	acaactgtatcgtggaa	aaattcgttgtcataccagg	456

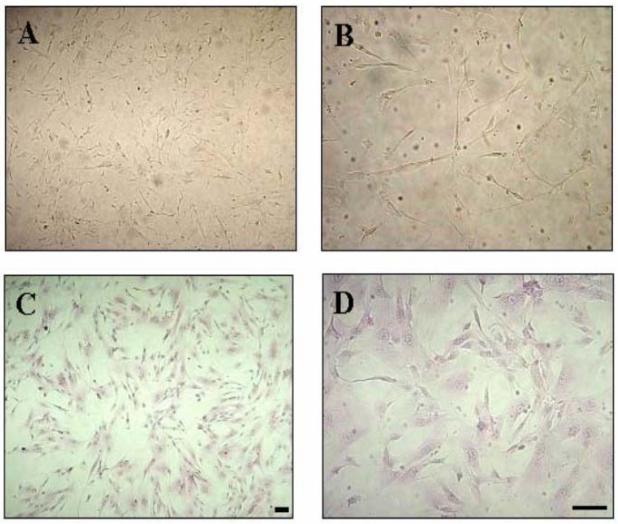

Fig. 1. The morphology of the homogeneous population of mesenchymal-like cells derived from HUCB. The cells shows bipolar fibroblast like feature. C and D were hematoxylin and eosin-stained. A and C, ×40; B and D, ×100. Scale bars=100 μm.

found. The differentiation rate was about 32.3±2.9% for β-tubulin Ⅲ-positive cells, 11.0±0.9% for GFAP, and 9.4±1.0% for Gal-C(Fig. 3). RT-PCR analysis confirmed that

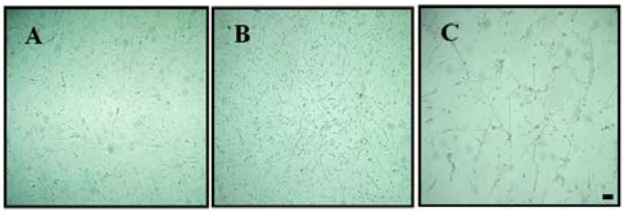

Fig. 2. Cellular morphology of HUCB-MSCs during neural differentiation. A, HUCB-MSCs before addition of differentiation medium. Most cells were fibroblast like. B, the cells were exposed to 20 ng/mL bFGF and EGF in preinduction medium for 24 hr. Cell morphology became thin. C, the cells were exposed to neural induction medium containing DMSO and BHA for 1.5 days. Extensive outgrowth of cellular processes was noted. Scale bar=100 μm.

HUCB-MSCs differentiated into neural cell. Nestin expression is restricted to neuroepithelial precursor cells in the neural tube and thus has been used as a cell type-specific marker for neural stem and precursor cells. While the stem cell markers such as SCF, C-kit, and Stat-3 were not expressed after preinduction medium culture, expression of NuroD1, GFAP, and MBP were induced during neural differentiation(Fig. 4).

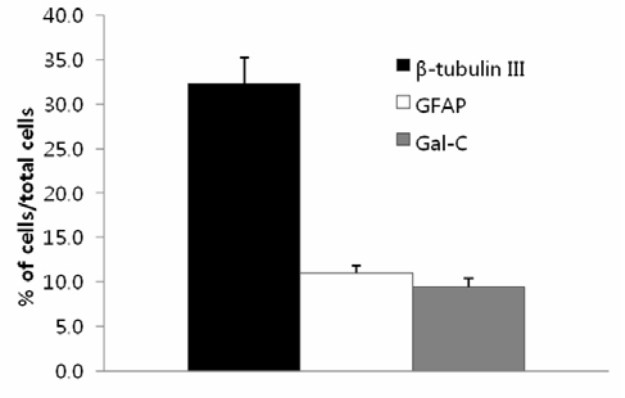

Fig. 3. Subpopulation of neural differentiation from HUCB-MSCs. After culture in neural induction medium for 1.5 days, immunohistochemistry was done with antibodies for β-tubulin Ⅲ, a neuronal marker, GFAP, an astrocyte marker and Gal-C, an oligodendrocyte maker. The proportions of subpopulation of the differentiated cells were calculated as differentiated cell's percentage of the total cells and expressed as the mean±SE(n=3).

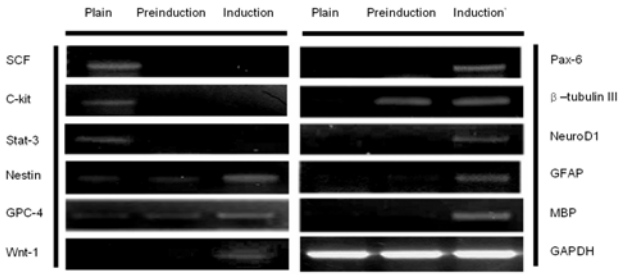

Fig. 4. Expression of various specific maker genes in HUCB-MSCs during differentiation. RT-PCR analyses showed that SCF, C-kit and Stat-3(stem cell markers) were only expressed by plain medium, and an increasing tendency in levels of Nestin and GPC-4(neural stem cell markers) after culture in preinduction medium. Expressions of Wnt-1 and Pax-6 (ectoderm markers), β-tubulin Ⅲ and NeuroD1(neuronal markers), GFAP(astrocyte marker) and MBP(oligodendrocyte marker) were induced by neural induction medium. Reaction condition and cycle number for PCR was optimized for each gene.

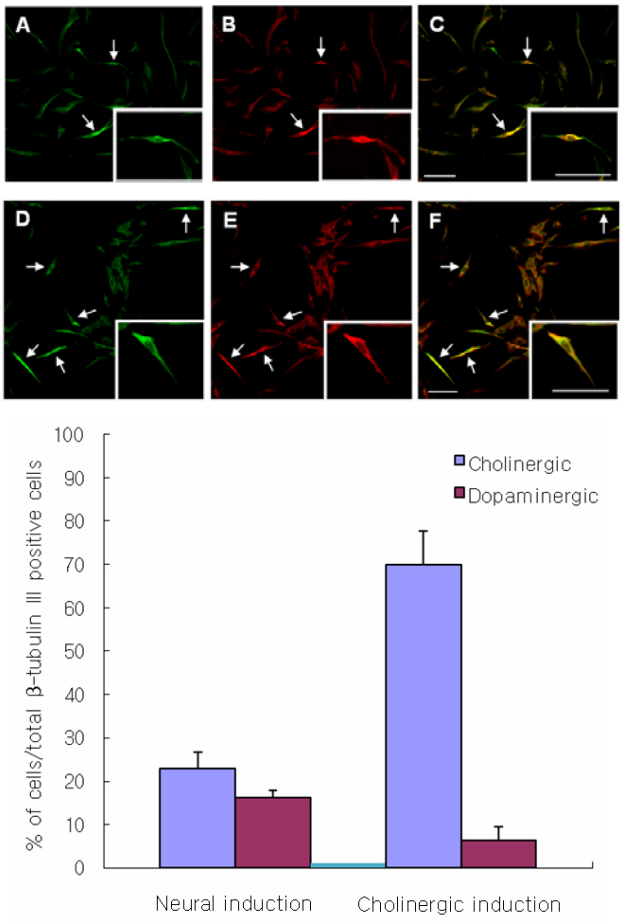

Fig. 5. Cholinergic differentiation of HUCB-MSCs. Cell-type specific immunostaining for cholinergic neurons were performed after neural induction according to Woodbury's method (A, B, C) and cholinergic induction(D, E, F). Green fluoresce indicates β-tubulin Ⅲ-immunoreactivity(A, D). Red fluoresce indicates ChAT-immunoreactivity(B, E). C and F represent the merged images of the previous two figures. The lower panel shows comparison of cholinergic neuronal proportions after neural induction(Woodbury's methods) and cholinergic induction. The proportions were calculated as a percentage of cell number of each type over total β-tubulin Ⅲ-positive cell number. The bars are expressed as the mean±SE(n=3). Scale bars= 100 μm.

3. Cholinergic Neuron Differentiation

Immunocytochemistry for the TH and ChAT has performed to identify dopaminergic or cholinergic cell population respectively after neural induction for 6 days or cholinergic neuronal induction(Fig. 5). After cholinergic neuronal differentiation, the β-tubulin III-positive cell population of total cells was 31.3±3.2% which is similar to neuronal proportion after neural induction. While ChAT- and TH-posi-

tive cell populations of the differentiated neuronal population were 23.0±3.8 and 16.3±1.5% respectively after neural induction, ChAT- positive cells were 70.0±7.8% and TH-positive cells 6.4±3.0% after cholinergic neuronal induction showing 3 folds in cholinergic population(Fig. 4).

DISCUSSION

In the present study, we isolated MSCs population derived from the HUCB. Based on morphological characteristics and immunophenotypes, adherent fibroblast-like cells isolated from HUCB-derived MSCs were identified to be similar to BM-derived MSCs. Immunophenotyping confirmed that these cells expressed MSC-related antigens, ICAM-1, TRA-1-60, vimentin, type I collagen and fibronectin.

Here we suggest that HUCB-derived MSCs retain the capacity to differentiate into nonmesenchymal derivatives, specifically neural cells. HUCB-MSCs showed an response to antioxidants such as DMSO and BHA(Jeong et al., 2004). This antioxidant-dependent neural differentiation occurred with a remarkable speed so that we could observe major changes in cellular and molecular levels within 24 hr. This acute neural differentiation occurs 5~10 times faster than other differentiation events. Cells with neuronal and glial phenotypes were induced, and several cell type-specific markers for neurons and glial cells(Buzanska et al., 2002; Jang et al., 2004) were identified in these cells by RT-PCR and immunocytochemistry. DMSO- and BHA-treated cells expressed mRNA transcripts for β-tubulin III, a neuron-specific marker, GFAP, an astrocyte marker or MBP, an oligodendrocyte marker, determined by RT-PCR. DMSO- and BHA-treated cells also showed β-tubulin Ⅲ-, GFAP- or Gal-C-immunoreactivity, as demonstrated by immunocytochemistry.

Basic Helix-Loop-Helix(bHLH) transcription factors are known to regulate neuronal differentiation of embryonic stem cell and neural stem cells and appear in early stages of neural development(Bertrand et al., 2002). The bHLH transcription factors included Pax6, Wnt1 and NeuroD1. Pax6, transcription factor crucial during the early phase of neurogenesis and Wnt1 which controls the initial phase of neural plate formation were not detected in control medium but expression was induced by neural induction medium. Also NeuroD1, which is reported to be expressed in subsets of CNS neurons during terminal differentiation(Lee et al., 1995), was expressed after cultivation in induction medium, indicating that NeuroD1 participates closely in trans-differentiation of HUCB-MSCs into neurons. Therefore, it might be possible to induce neuronal and glial cell types from HUCB-MSCs through controlling expression of neurogenic bHLH factor genes.

We tried to differentiate HUCB-MSCs into further specific cholinergic neuron. The cholinergic neurons in the human basal forebrain play a key role in neurodegenerative disorders such as Alzheimer's disease. In the present study, the HUCB-MSCs were differentiated into cholinergic neurons in N2 medium containing Shh, RA, bFGF, BDNF and AA combinatorially(Barberi et al., 2003). Some cells of the differentiated HUCB-MSCs were shown to express ChAT, a cholinergic neuronal marker by immunocytochemistry. RA and Shh are well-known molecules involved in cholinergic neuron differentiation. When mouse ES cells were induced to neural fate and exposed to Shh and RA, the differentiating population was preferentially directed to cholinergic neuron fate(Islet1-, HB9-, and Tuj1-positive) (Wichterle et al., 2002; Wu et al., 2002). In addition, bFGF alone induced long-term cultured fetal human neural stem cells to form cholinergic neurons(Weis et al., 2001), suggesting that bFGF may also be an effective growth factor for *in vitro* differentiation to human cholinergic neurons. A recent study reported that combined treatment with Shh, RA, BDNF and AA had additive and synergistic effects on cholinergic neuron differentiation from human embryonic stem cells(Lee et al., 2007). We found that the combined treatment with such factors could differentiate HUCB-MSCs to cholinergic neuron phenotypes very efficiently.

In conclusion, HUCB-derived MSCs retain potential mesenchyme-related multipotency and differentiate into cells

that express neuronal and glial to differentiate into cells that express neuronal and glial markers *in vitro*. Also HUCB-MSCs differentiated into cholinergic neuron *in vitro*. The findings here implicate that HUCB-derived MSCs may be alternative cell source of treatment for neurodegenerative diseases such as Alzheimer's disease.

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REFERENCES

Arendt T, Bigl V, Arendt A, Tennstedt A (1983) Loss of neurons in the nucleus basalis of Meynert in Alzheimer's disease, paralysis agitans and Korsakoff's Disease. Acta Neuropathol 61:101-108.

Barberi T, Klivenyi P, Calingasan NY, Lee H, Kawamata H, Loonam K, Perrier AL, Bruses J, Rubio ME, Topf N, Tabar V, Harrison NL, Beal MF, Moore MA, Studer L (2003) Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice. Nat Biotechnol 21:1200-1207.

Bertrand N, Castro DS, Guillemot F (2002) Proneural genes and the specification of neural cell types. Nat Rev Neurosci 3:517-530.

Buzanska L, Machaj EK, Zablocka B, Pojda Z, Domanska-Janik K (2002) Human cord blood-derived cells attain neuronal and glial features *in vitro*. J Cell Sci 115:2131-2138.

Dazzi F, Horwood NJ (2007) Potential of mesenchymal stem cell therapy. Curr Opin Oncol 19:650-655.

de Wynter EA, Testa NG (2001) Interest of cord blood stem cells. Biomed Pharmacother 55:195-200.

Dubois B, Ruberg M, Javoy-Agid F, Ploska A, Agid Y (1983) A subcortico-cortical cholinergic system is affected in Parkinson's disease. Brain Res 288:213-218.

Fortier LA (2005) Stem cells: classifications, controversies, and clinical applications. Vet Surg 34:415-423.

Harris DT, Badowski M, Ahmad N, Gaballa MA (2007) The potential of cord blood stem cells for use in regenerative medicine. Expert Opin Biol Ther 7:1311-1322.

Jang YK, Park JJ, Lee MC, Yoon BH, Yang YS, Yang SE, Kim SU (2004) Retinoic acid-mediated induction of neurons and glial cells from human umbilical cord-derived hematopoietic stem cells. J Neurosci Res 75:573-584.

Jeong JA, Gang EJ, Hong SH, Hwang SH, Kim SW, Yang IH, Ahn C, Han H, Kim H (2004) Rapid neural differentiation of human cord blood-derived mesenchymal stem cells. Neuroreport 15:1731-1734.

Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418:41-49.

Kabos P, Ehtesham M, Kabosova A, Black KL, Yu JS (2002) Generation of neural progenitor cells from whole adult bone marrow. Exp Neurol 178:288-293.

Lee H, Shamy GA, Elkabetz Y, Schofield CM, Harrsion NL, Panagiotakos G, Socci ND, Tabar V, Studer L (2007) Directed differentiation and transplantation of human embryonic stem cell-derived motoneurons. Stem Cells 25:1931-1939.

Lee JE, Hollenberg SM, Snider L, Turner DL, Lipnick N, Weintraub H (1995) Conversion of Xenopus ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. Science 268:836-844.

Li XJ, Du ZW, Zarnowska ED, Pankratz M, Hansen LO, Pearce RA, Zhang SC (2005) Specification of motoneurons from human embryonic stem cells. Nat Biotechnol 23: 215-221.

Lindvall O, Kokaia Z, Martinez-Serrano A (2004) Stem cell therapy for human neurodegenerative disorders-how to make it work. Nat Med 10 Suppl:S42-50.

Mays RW, van't Hof W, Ting AE, Perry R, Deans R

(2007) Development of adult pluripotent stem cell therapies for ischemic injury and disease. Expert Opin Biol Ther 7:173-184.

Rogers I, Casper RF (2004) Umbilical cord blood stem cells. Best Pract Res Clin Obstet Gynaecol 18:893-908.

Sanchez-Ramos JR, Song S, Kamath SG, Zigova T, Willing A, Cardozo-Pelaez F, Stedeford T, Chopp M, Sanberg PR (2001) Expression of neural markers in human umbilical cord blood. Exp Neurol 171:109-115.

Shin S, Dalton S, Stice SL (2005) Human motor neuron differentiation from human embryonic stem cells. Stem Cells Dev 14:266-269.

Tuan RS, Boland G, Tuli R (2003) Adult mesenchymal stem cells and cell-based tissue engineering. Arthritis Res Ther 5:32-45.

Weis C, Marksteiner J, Humpel C (2001) Nerve growth factor and glial cell line-derived neurotrophic factor restore the cholinergic neuronal phenotype in organotypic brain slices of the basal nucleus of Meynert. Neuroscience 102:129-138.

Wichterle H, Lieberam I, Porter JA, Jessell TM (2002) Directed differentiation of embryonic stem cells into motor neurons. Cell 110:385-397.

Woodbury D, Schwarz EJ, Prockop DJ, Black IB (2000) Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 61:364-370.

Wu P, Tarasenko YI, Gu Y, Huang LY, Coggeshall RE, Yu Y (2002) Region-specific generation of cholinergic neurons from fetal human neural stem cells grafted in adult rat. Nat Neurosci 5:1271-1278.

Zhao LR, Duan WM, Reyes M, Keene CD, Verfaillie CM, Low WC (2002) Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. Exp Neurol 174:11-20.