STEM CELLS®
Original Article

In Vitro Differentiation of Size-Sieved Stem Cells into Electrically Active Neural Cells

SHIH-CHIEH HUNG,[a,b,g] HENRICH CHENG,[c,g] CHIEN-YUAN PAN,[d,e] MAY J. TSAI,[c] LUNG-SEN KAO,[f] HSIAO-LI MA[a,g]

[a]Department of Orthopaedics and Traumatology, [b]Medical Research and Education and [c]Neural Regeneration Laboratory, Neurological Institute, Veterans General Hospital-Taipei, Taipei, Taiwan; [d]Department of Medical Research, Chi-Mei Medical Center, Yungkang City, Tainan Hsien, Tainan, Taiwan; [e]Department of Zoology, National Taiwan University, Taipei, Taiwan; [f]Department of Life Sciences, School of Life Sciences, and [g]Department of Surgery, School of Medicine, National Yang-Ming University, Taipei, Taiwan

Key Words. *Bone marrow stromal cells · Mesenchymal stem cells · Plastic-adherent cells · Neural differentiation*

ABSTRACT

Size-sieved stem (SS) cells isolated from human bone marrow and propagated in vitro are a population of cells with consistent marker typing, and can form bone, fat, and cartilage. In this experiment, we demonstrated that SS cells could be induced to differentiate into neural cells under experimental cell culture conditions. Five hours after exposure to antioxidant agents (β-mercaptoethanol ± retinoic acid) in serum-free conditions, SS cells expressed the protein for nestin, neuron-specific enolase (NSE), neuron-specific nuclear protein (NeuN), and neuron-specific tubulin-1 (TuJ-1), and the mRNA for NSE and Tau. Immunofluorescence showed that almost all the cells (>98%) expressed NeuN and TuJ-1. After 5 days of β-mercaptoethanol treatment, the SS cells expressed neurofilament high protein but not mitogen-activated protein-2, glial filament acidic protein, and galactocerebroside. For such long-term-treated cells, voltage-sensitive ionic current could be detected by electrophysiological recording, and the intracellular calcium ion, Ca^{2+}, concentration can be elevated by high potassium (K^+) buffer and glutamate. These findings suggest that SS cells may be an alternative source of undifferentiated cells for cell therapy and gene therapy in neural dysfunction. ***Stem Cells*** *2002;20:522-529*

INTRODUCTION

Disorders of neural function, including cognitive dysfunction, psychiatric disorders, stroke, defects arising from injury, and neurodegenerative diseases, place an incalculable emotional and financial burden on our society. The discovery of new therapies that could alleviate neurodegeneration or facilitate neural regeneration has become a high priority in medical research. Neural progenitors isolated from the central nervous system (CNS) [1] and embryonic stem cells [2] derived from the inner cell mass of preimplantation embryos are candidate stem cells for cell therapy in neural dysfunction. Transplants of stem cells in vivo have clearly demonstrated that they can differentiate into neurons, astrocytes, and oligodendrocytes [3-8].

Correspondence: Shih-Chieh Hung, M.D, Ph.D, Department of Orthopaedics and Traumatology, Veterans General Hospital-Taipei, 201, Sec. 2, Shih-Pai Road, Taipei, Taiwan. Telephone: 886-2-28757557, ext 118; Fax: 886-2-28265164; e-mail: hungsc@vghtpe.gov.tw *Received March 25, 2002; accepted for publication July 11, 2002.*

Another source of undifferentiated cells is bone marrow stem cells, which have greater functional plasticity than previously suspected. After bone marrow transplantation, donor-derived stem cells have been found in such diverse nonhematopoietic tissues as bone [9, 10], cartilage [9, 10], skeletal muscle [11], lung [9, 10, 12], spleen [12], thymus [12], liver [13], cardiac muscle [14], neuron, and brain [15-17]. The stem cell compartment in human bone marrow is highly complex, comprising both $CD34^+$ and $CD34^-$ hematopoietic stem cells, mesenchymal progenitors, and perhaps other cell types whose activities remain to be defined. Among these, a stem cell may be identified to have potential similar to or equal to that of embryonic stem cells. This could simplify an approach to cell therapy in the nervous system by eliminating the need for harvesting autologous human neural stem cells, an admittedly difficult procedure. It is currently believed that heterogeneous cells may be used for the CNS, given the immune tolerance of the brain [5].

In the study reported here, we tested the novel size-sieved stem (SS) cells of $CD34^-$ stem cells [18], which were selected on the basis of cell size and adherent capacity via the use of a culture device—a plastic culture dish comprising a plate with 3-μm pores, for the capacity to form neurons in in vitro culture. SS cells isolated from human bone marrow and propagated in vitro are able to form bone, fat, and cartilage. SS cells have been well characterized previously with the use of flow cytometry over 30 more markers, for each marker cell expressed as a single peak in the histogram. SS cells used in this study were negative for surface markers associated with hematopoietic cells (e.g., CD14, CD34, and CD45), but positive for SH2, SH3, CD44, CD90, and CD105, consistent with previous reports [19]. In this report, SS cells were more thoroughly characterized by reverse transcription-polymerase chain reaction (RT-PCR), antibody staining, electrophysiological recording, and intracellular calcium ion concentration ($[Ca^{2+}]i$) measurement studies in order to better define the differentiation potential of these cells in in vitro culture.

Materials and Methods

Chemicals

The antibodies αnestin (polyclonal, AB5922), αNSE (polyclonal, AB951), αNeuN (monoclonal, MAB377), αNF-H (monoclonal, MAB1623), αGFAP (monoclonal, MAB360), αGal-C (monoclonal, MAB342), αrabbit IgG (AP132P), and αmouse IgG (AP124P) were purchased from Chemicon (Temecula, CA; http://www.chemicon.com). αMAP-2 (monoclonal, 5F9) was purchased from Upstate Biotechnology (Waltham, MA; http://www.upstate.com). αTuJ-1 (polyclonal, PRB-435p) was purchased from COVANCE (Princeton, NJ). Human brain lysate was from Chemicon. Fura-2 AM was purchased from Molecular Probes (Eugene, OR; http://www.probes.com).

Cell Culture

SS cells were isolated from human bone marrow as previously reported [18]. In brief, bone marrow aspirates, washed twice with phosphate-buffered saline (PBS), suspended in Dulbecco's modified Eagle's medium-low glucose (DMEM-LG) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.25 μg/ml amphotericin B, were plated in a 10-cm plastic culture dish comprising a plate with 3-μm pores at a density of 10^6 mononuclear cells/cm^2. SS cells that adhered to the pore-containing plate were recovered at 7 days after initial plating, plated at about 6,000 cells/cm^2, and subcultured at a ratio of 1:3 when cells reached more than 80% confluence.

Differentiation Protocol

Undifferentiated SS cells were cultured in DMEM-LG supplemented with 10% FBS. To induce differentiation, cells were seeded at a density of 4,000 cells/cm^2, pretreated with DMEM-LG supplemented with 10% FBS, 10% fetal calf serum (FCS), 10^{-3} M β-mercaptoethanol (β-ME) (Sigma; St. Louis, MO; http://www.sigmaaldrich.com) ± 5×10^{-7} M all-*trans*-retinoic acid (RA) (Sigma) for 24 hours, and then treated with serum depletion for 5 hours to 5 days.

Western Blot Analysis

Protein was extracted from subconfluent differentiated (β-ME ± RA) and undifferentiated cell cultures. The cells (5×10^5) were rinsed with PBS and lysed with 0.5 ml of ice-cold radio-immunoprecipitation buffer containing 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, and 0.1% SDS for 15 minutes on ice. After being heated for 5 minutes at 95°C, equal aliquots of the cell lysate were run on a 12% or 5% SDS-polyacrylamide gel. Proteins were transferred to PVDF membrane filters, probed with antibodies, and detected with the enhanced chemiluminescence system (PerkinElmer Instruments, Inc.; http://www.instruments.perkinelmer.com/index.asp).

Immunofluorescence

At 5 hours or 5 days postdifferentiation by serum depletion, cells were rinsed briefly in PBS, fixed with 2% paraformaldehyde (in PBS) for 10 minutes, permeabilized with 0.1% Triton X-100 in PBS for 10 minutes, and treated with 5% FCS in PBS for 30 minutes, all at room temperature. They then were incubated with the first antibody (αnestin 1:100, αNeuN 1:250, αTuJ-1 1:250, αNF-H 1:100, αMAP-2 1:100, αGFAP 1:100, and αGal-C 1:100) for 1 hour

at room temperature, rinsed three times with PBS, and incubated for a further hour with a secondary antibody (fluoroscein isothiocyanate- or rhodamine-conjugated αmouse or αrabbit IgG 1:100). Control staining without primary antibody was used as a negative control. For quantification, cells were further stained with Hoechst dye 33258 (Molecular Probes). Cells exhibiting strong fluorescence for each antibody were counted, compared with total cell counts, and the mean and standard deviation (SD) were computed.

RT-PCR

Total RNA was prepared from cells using the RNeasy purification system as described by the manufacturer (Qiagen; Valencia, CA; http://www.qiagen.com). Total RNA (1 μg) was reverse transcribed with Moloney murine leukemia virus reverse transcriptase at 42°C in the presence of oligo-dT primer. PCR was performed using specific primers designed from the published sequence of each cDNA as follows: NSE sense: 5′-CATCGA CAAGGCTGGCTACACG-3′, antisense: 5′-GACAGTTG CAGGCCTTTTCTTC-3′; Tau sense: 5′-GTAAAAG CAAAGACGGGACTGG-3′, antisense: 5′-ATGATG GATGTTGCCTAATGAG-3′; and β-actin sense: 5′-GCACTCTTCCAGCCTTCCTTCC-3′, antisense: 65′-TCACCTTCACCGTTCCAGTTTTT-3′. The thermal profile was 1 minute at 95°C, 1 minute at 60°C, and 2 minutes at 72°C. The amplification cycles of NSE, Tau, and β-actin were all 35. The expected sizes of NSE, Tau, and β-actin were 329 bp, 512/612 bp, and 515 bp, respectively. To exclude possible contamination of genomic DNA, PCR was also applied to reactions without RT. The amplified complementary DNA was electrophoresed through a 1% agarose gel, stained, and photographed under ultraviolet light.

Electrophysiology

For whole-cell current recording, the cell was whole-cell patched as previously described [20]. The extracellular solution (loading buffer [LB Buffer]) contained 150 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM $MgCl_2$, 2.2 mM $CaCl_2$, and 10 mM Hepes, pH 7.3. The pipette solution contained: 120 mM aspartic acid, 5 mM $MgCl_2$, 0.5 mM EGTA, 40 mM Hepes, 2 mM ATP, and 0.3 mM GTP, pH 7.3 with KOH. Micropipettes were pulled from 1.5-mm O.D. capillary glass (1B150F4; World Precision Instruments; New Haven, CT) on a Brown-Flaming puller (model P-97; Sutter Instruments; Novato, CA) and had a resistance of about 8 MΩ with the above solutions used. Whole-cell current was recorded using an Axoclamp 200B amplifier (Axon Instruments, Inc.; Union City, CA) and pClamp software. Data acquisition was initiated when the whole-cell patch was formed and stable; the holding potential was set at -70 mV and depolarized to different potential for 20 milliseconds to evoke channel opening.

$[Ca^{2+}]i$ Measurement

The $[Ca^{2+}]i$ change was recorded as previously described, with some modifications [21]. Cells were loaded with Fura-2 AM (5 μM in loading buffer) under room temperature for 1 hour. After the washout of fura-2 AM, the coverslip was mounted on an Olympus IX-70 inverted microscope and observed with a 40× objective. The 340 nm and 380 nm excitation lights were provided by T.I.L.L Polychrome IV spectrophotometer (T.I.L.L. Photonics GMBH; Germany), and the emission light was recorded by Princeton charged-coupled device camera (CCD-130; Roper Scientific; Trenton, NJ). The excitation switch and emission acquisition were controlled by Axon Workbench software. The $[Ca^{2+}]i$ was represented as a ratio ($R_{340/380}$) between emission intensities induced by 340 and 380 nm. For stimulation, LB buffer contained glutamate (25 μM), or high K^+ (the NaCl was replaced with equilmolar KCl) was puffed from a glass micropipette placed right beside the cell. The opening of the micropipette was approximately 1.5 μm. The puff pressure was controlled by Eppendorf Microinjector 5242 (Eppendorf GMBH; Germany) with a P2 setting of 200 hPa.

Results

Neural Differentiation

To induce neural differentiation, SS cells were incubated in a serum-containing medium supplemented with 10^{-3} M β-ME for 24 hours, and then treated with serum depletion. After exposure to a serum-free condition, changes in morphology were observed over the first 5 hours (Fig. 1). Initially, cytoplasm in some of the flat SS cells retracted toward the nucleus and began to form a rounded cell body (1 hour). The percentage of responsive cells increased progressively and almost all the cells had the same morphologic changes (>95% at 2-3 hours). Neurite outgrowth was then observed, displaying primary and secondary branches (2-3 hours). Over the subsequent 2 hours, cells began to adopt their earlier morphology; rounded cell body and neurite outgrowth gradually disappeared (4-5 hours). At this time, cells exhibited greater expression of the neural markers (data shown below).

Western Blot Analysis (Fig. 2) (Table 1)

Western blot analysis demonstrated that all cell cultures had the same expression of actin, an internal control. Undifferentiated cell cultures had a faint expression of nestin, a marker for neural precursors, and several early neuron markers, such as NSE, NeuN, and TuJ-1. The differentiated cells, however, had a stronger expression of NSE and NeuN at 5 hours under conditions treated by the addition of

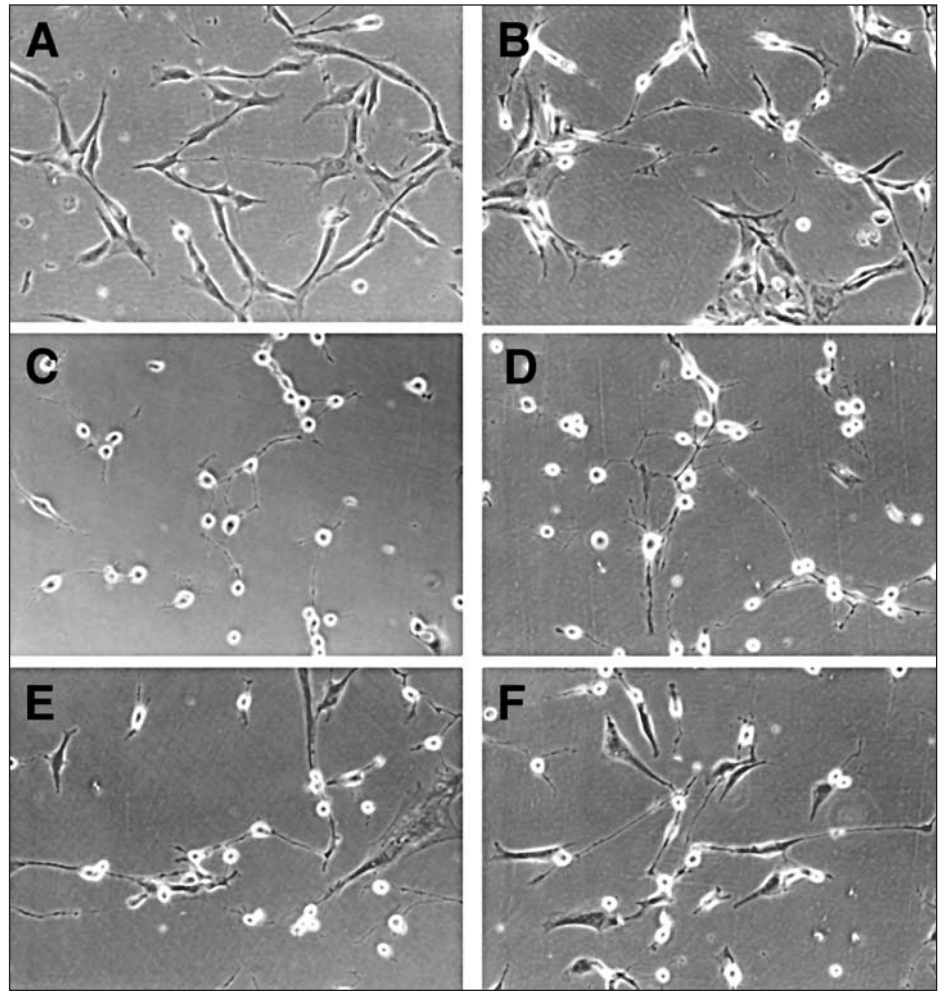

Figure 1. Induction of neural morphology in SS cells treated with β-ME and serum depletion. *SS cells were treated with β-ME for 24 hours and then were further treated with serum depletion for 0 (A), 1 (B), 2 (C), 3 (D), 4 (E), and 5 (F) hours. Original magnification 150×.*

antioxidants (β-ME ± RA) and serum depletion. The addition of RA to the medium did not significantly increase the level of neural protein expression. The expression of NF-H, a marker for mature neurons, was only observed at 5 days after exposure to serum-free conditions. A decrease in the expression of nestin and NSE was also found at that time. MAP-2, a marker for mature neurons, GFAP, and Gal-C, markers for mature astrocytes and oligodendrocytes, were negative in these culture conditions.

Immunofluorescence on Differentiated SS Cells

Subsequently, a more detailed analysis of the expression of nestin, NeuN, TuJ-1, and NF-H was performed using immunofluorescence. Control staining without primary antibody did not detect any autofluorescence in these cells (Fig. 3A). β-ME-treated SS cells, exposed to serum-free conditions for 5 hours expressed nestin. Nestin was chiefly preserved in the cytoplasm with little maintained in the nucleus (Fig. 3B). Up to 98% of the cell population was stained with NeuN (98% ± 2%) and TuJ-1 (>98%) antibodies. The NeuN and TuJ-1 proteins were dispersed in a dependable manner both in the nucleus and in the cytoplasm of the cells (Fig. 3C and Fig. 3D). After exposure to serum-free conditions for 5 days, cells also expressed NF-H, which was located primarily in the cytoplasm (Fig. 3E). However, these cells did not express GFAP (Fig. 3F), Gal-C (Fig. 3G), and MAP-2 (Fig. 3H), before, and 5 hours or 5 days after the depletion of serum from the medium, as shown in the figures.

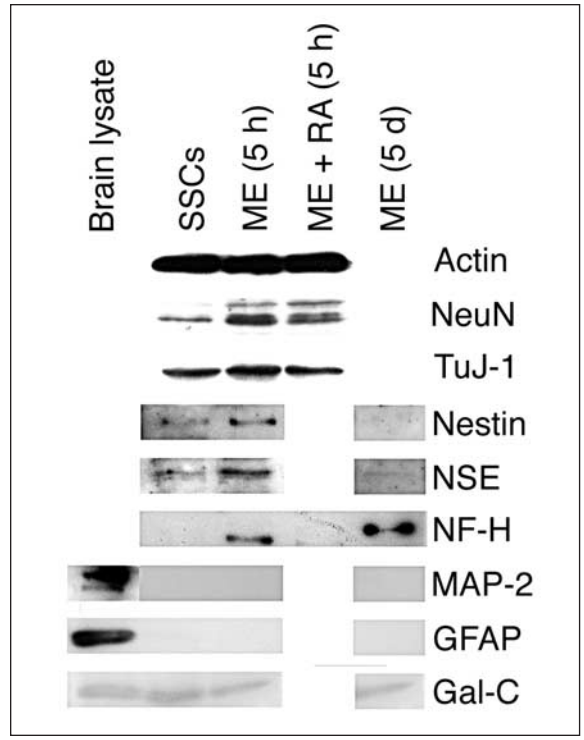

Figure 2. Western blot assay of the expression of actin, NeuN, TuJ-1, nestin, NSE, NF-H, MAP-2, GFAP, and Gal-C in brain lysate and SS cells. *SSCs, untreated SS cells. ME (5 h), SS cells treated with β-ME and serum depletion for 5 hours. ME + RA (5 h), SS cells treated with β-ME/retinoic acid and serum depletion for 5 hours. ME (5 d), SS cells treated with β-ME and serum depletion for 5 days.*

Table 1. Comparisons of protein expression on neural differentiation of SS cells

Conditions	Undifferentiated cells	β-ME (serum free for 5 hours)	β-ME + RA (serum free for 5 hours)	β-ME (serum free for 5 days)
Actin	++	++	++	
Nestin	±	+		–
NSE	+	++		±
TuJ-1	++	++	++	
NeuN	+	++	++	
NF-H	–	–	–	+
MAP-2	–	–	–	–
GFAP	–	–	–	–
Gal-C	–	–	–	–

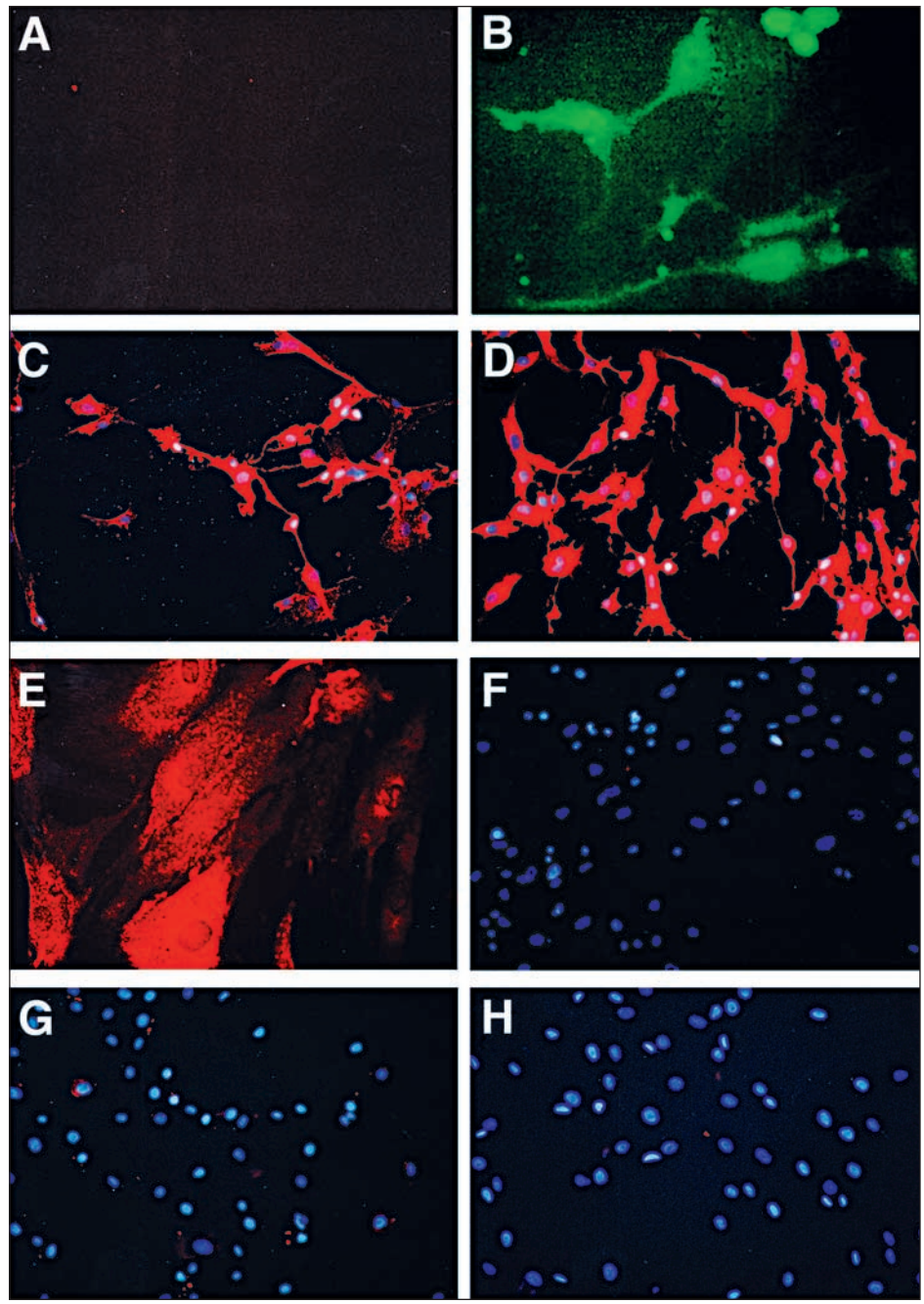

Figure 3. Immunofluorescence for expression of neural protein in SS cells treated with β-ME and serum depletion for 5 hours (A-D) and 5 days (E-H). *These cells were fixed, then stained without primary antibody (A) or with an antibody against nestin (B), NeuN (C), TuJ-1 (D), NF-H (E), GFAP (F), Gal-C (G), or MAP-2 (H), and finally stained with a secondary antibody. Cells were also stained with Hoechst dye 33258 (C, D, F-H). Original magnification 200× (A, C, D, F-H), 300× (B), and 400× (E).*

Expression of Neural Genes in Differentiated SS Cells (Fig. 4)

In order to identify neural differentiation after treatment (β-ME ± RA), we examined the expression of neural genes in SS cells before and after differentiation. Total RNA isolated from undifferentiated and from differentiated cells, which had been maintained in serum-free conditions for 5 hours, was analyzed by RT-PCR. Expression of β-actin, which served as an internal control, was the same in undifferentiated and differentiated cells. In contrast, NSE and Tau were detected only in the differentiated SS cells. Tau had an alternative splicing and expressed two bands, 512 bp and 612 bp. There was no significant difference in the expression of the neural genes between the two protocols for differentiation.

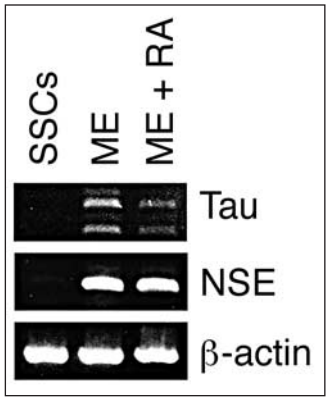

Figure 4. RT-PCR analysis of untreated SS cells (SSCs), 5 hour β-ME-treated SS cells (ME), and 5 hour β-ME- and RA-treated SS cells (ME + RA). *β-actin served as an internal control (515 bp). Tau has an alternative splicing (512 bp and 612 bp). NSE is neuron-specific enolase (329 bp).*

Electrical Properties of SS Cells

To confirm that SS cells have the potential to differentiate into functional neuronal cells, the membrane currents and $[Ca^{2+}]i$ were measured. Figure 5A is a representative whole-cell current trace from a voltage-clamped SS cell in β-ME-supplemented serum-free culture. The current shows an apparent inward Na^+ current, which inactivated very quickly, and an outward current, which was likely to be K^+ current.

Since differentiated cells can have voltage-sensitive ion channels, we used high K^+ or glutamate to stimulate the cell and record the $[Ca^{2+}]i$ change. Figure 5B shows a representative $R_{340/380}$ ratio recorded from cells stimulated with glutamate. Though glutamate could induce the elevation of $[Ca^{2+}]i$ in almost all of the SS cells tested, the response was slowly developed. This suggests that the treated SS cells may express glutamate receptors. Similarly, using high K^+ buffer to stimulate the differentiated cell also elevated the $[Ca^{2+}]i$, but also in a slow mode. This suggests that voltage-sensitive Ca^{2+} channels may appear on differentiated SS cells. The averaged results (Fig. 5C) show that cells without treatment did not have a significant $[Ca^{2+}]i$ change when stimulated; however, differentiated cells had a significant elevation in $[Ca^{2+}]i$ either stimulated with glutamate or high K^+ buffer. This suggests that long-term β-ME-treated SS cells may develop some basic neuronal membrane properties.

Discussion

Replating stem cells from embryos on an adhesive substrate can lead to the formation of multiple cell types, including skeletal muscle-, cardiac-, neuron-, and hematopoietic-like cells (mesoderm, endoderm, and ectoderm) [22]. Until recently, the fate of adult cells had been thought to be restricted to their tissues of origin. Cells are well known to be capable of repairing damage in tissues in which they reside, such as blood, muscle, liver, and skin. Our previous

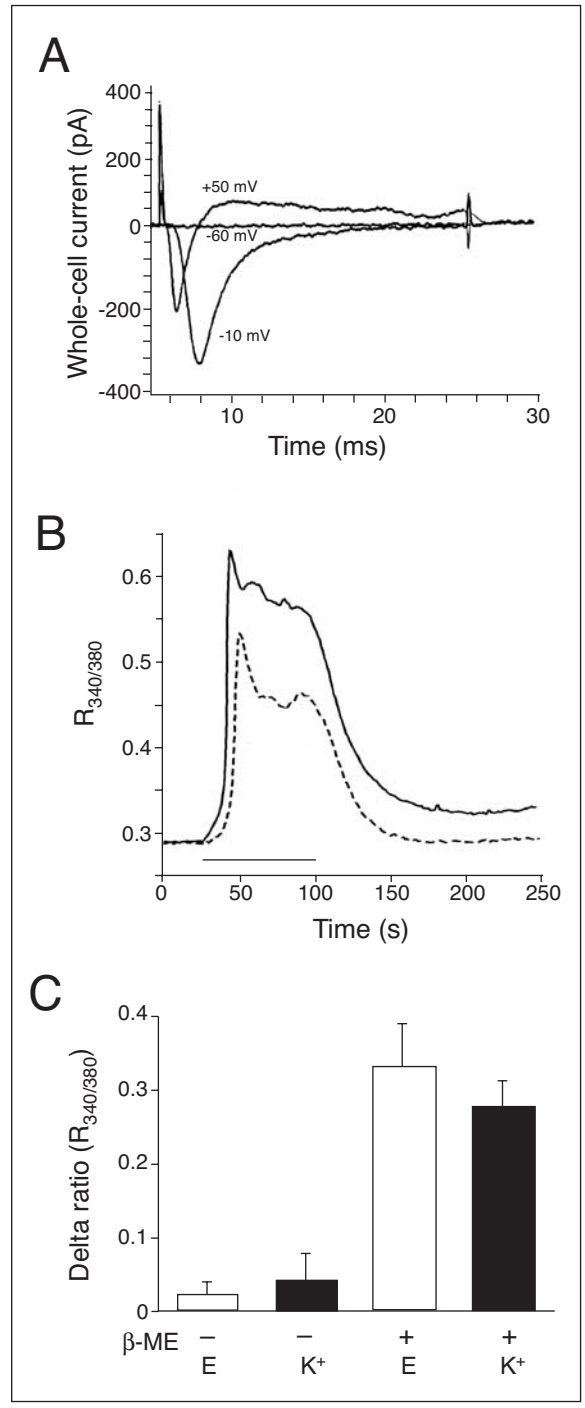

Figure 5. The membrane properties of long-term treated SS cells. *SS cells were treated with β-ME in serum-free conditions for 5 days and the membrane currents and $[Ca^{2+}]i$ were measured. A) Long-term treated cell has voltage-sensitive ion currents. The current traces were recorded from a treated SS cell. The cell was whole-cell patch-clamped and depolarized to -60, -10, and +50 mV for 20 milliseconds (ms) from a holding potential of -70 mV as indicated. B) $[Ca^{2+}]i$ could be elevated by high K^+ buffer and glutamate. Cells were puffed with high K^+ buffer (dashed line) or 25 μM glutamate (solid line) for 75 seconds as indicated by the bar. $[Ca^{2+}]i$ was measured and represented as $R_{340/380}$. C) The averaged results of $[Ca^{2+}]i$ response induced by high K^+ buffer (K^+) and glutamate (E) from cells with or without β-ME treatment. The differences between the basal (before stimulation) and maximum (during stimulation) $R_{340/380}$ from each cell were averaged. The treatment of each group of cells is indicated under the figure.*

data indicated that SS cells from human bone marrow are a population of cells with consistent marker typing and can form bone, fat, and cartilage [18]. The current observations indicate that SS cells are capable of differentiating into cells that express several neural proteins, including the neuron precursor marker (nestin), immature neuron markers (NeuN, TuJ-1), and more mature neuron markers (NF-H). This documents the presence of a population of potent stem cells in adult bone marrow that can form both nonmesenchymal and mesenchymal derivatives.

The results were similar, but not identical to, the observations recently reported by *Woodbury et al*. [23], *Sanchez-Ramos et al*. [24], and *Deng et al*. [25] using different culture conditions. Similar morphological changes were seen with all these studies, but the number and percentage of neural-like cells varied widely. The percentage varied from 0.4%-50%, and the addition of basic fibroblast growth factor to the medium increased the ratio of neural-like cells from 50% to 80% in β-ME or dimethylsulfoxide + butylated hydroxyanisole-treated cells [23]. Possible factors that may influence the results include the culture conditions used and the cells made to differentiate into neural-like cells. β-ME in serum-free conditions seemed to be the most effective method for neural differentiation, as seen in *Woodbury et al.'s* results and also in ours. Our study [18] was the only one that emphasized the characterization of the cells applied for neural differentiation. The previous studies left two fundamental questions. The first is whether the differentiated cells are more mature neuron-like cells that possess functional and electrophysiological characteristics of neurons. The second is how to identify and characterize the subfraction of marrow stem cells that gives rise to the neuron-like cells. SS cells are a relatively homogeneous population as evidenced by a consistent profile on 30 more surface epitopes. Previous studies demonstrated that they can form bone, fat, and cartilage [18]. In this report, following a treatment that included antioxidants and serum-free conditions, about 100% of SS cells assumed a neuron-like morphology. The morphological change coincided with an increase in the expression of both nestin and early neuronal markers (NSE, NeuN, and TuJ-1) at 5 hours after exposure to a differentiation medium. The expression of mature neuronal markers (NF-H), the decreased expression of nestin and NSE, and a feedback in the functional assay, namely the membrane currents and calcium channels for putative neurons at 5 days, are evidence of a more mature differentiation. To our knowledge, this investigation is the first to have more mature and electrically active neurons differentiated from adult stem cells. The failure to express MAP-2, GFAP, and Gal-C in these results does not conclusively indicate that the cells we tested here lost their potential to form MAP-2-expressed neurons, astrocytes, or oligodendrocytes. These need different or more specialized culture conditions.

β-ME has been shown to protect neurons from cell death in serum-free conditions, and the survival rates of cells were 200-fold higher than those in control cultures for neurons [26]. The ability of neurons to extend neurites was drastically enhanced by the addition of β-ME in a serum-free medium. β-ME itself had trophic activity for primary cultures of neurons. In the present investigation, we clearly demonstrated that β-ME also has effects on neural differentiation of adult stem cells. The functional mechanisms of the effects of β-ME on neural differentiation were speculated as the synthesis of glutathione [27] and the release of cells from oxygen stress [28]. RA also has been reported to promote the replication and survival of multipotential neural crest precursor cells in culture and lead to a greater number of differentiated neurons [29]. That there was no elevation in the expression of neuron-specific proteins after the addition of RA into the medium in our experiment might indicate that the mechanisms or pathways undertaken by β-ME or RA to initiate neural differentiation are the same.

The elevation in $[Ca^{2+}]i$ induced by glutamate or high K^+ buffer strongly suggests that SS cells have the potential to differentiate into neuron-like cells. The delayed response of differentiated SS cells to these stimuli suggests that the mechanisms underneath the response are not well coupled. However, with appropriate induction factors, it is possible to strengthen the coupling between stimuli and responses. More importantly, it is possible for SS cells to differentiate into the specific type of neuronal cells.

SS cells, a relatively homogeneous population of adult stem cells that can form bone, fat, and cartilage, have been found in many cases to differentiate into electrically active neurons in vitro. SS cells offer significant advantages over other "stem cells," and may be useful in the treatment of a wide variety of neurological diseases. SS cells are readily isolated from marrow, thus overcoming the risks of obtaining neural stem cells from the brain, and providing a renewable population. Autologous transplantation overcomes the ethical and immunologic concerns associated with the use of fetal or embryonic tissue. Moreover, SS cells grow rapidly in culture, precluding the need for immortalization, and differentiate into neurons exclusively with the use of a simple protocol.

REFERENCES

1 Kalyani AJ, Rao MS. Cell lineage in the developing neural tube. Biochem Cell Biol 1998;76:1051-1068.

2 Gokhan S, Mehler MF. Basic and clinical neuroscience applications of embryonic stem cells. Anat Rec 2001;265:142-156.

3 Brustle O, Choudhary K, Karram K et al. Chimeric brains generated by intraventricular transplantation of fetal human brain cells into embryonic rats. Nat Biotechnol 1998;16:1040-1044.

4 Flax JD, Aurora S, Yang C et al. Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes. Nat Biotechnol 1998;16:1033-1039.

5 Ourednik V, Ourednik J, Flax JD et al. Segregation of human neural stem cells in the developing primate forebrain. Science 2001;293:1820-1824.

6 Brustle O, Jones KN, Learish RD et al. Embryonic stem cell-derived glial precursors: a source of myelinating transplants. Science 1999;285:754-756.

7 Zhang SC, Wernig M, Duncan ID et al. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol 2001:19:1129-1133.

8 Reubinoff BE, Itsykson P, Turetsky T et al. Neural progenitors from human embryonic stem cells. Nat Biotechnol 2001;19:1134-1140.

9 Pereira RF, Halford KW, O'Hara MD et al. Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc Natl Acad Sci USA 1995;92:4857-4861.

10 Pereira RF, O'Hara MD, Laptev AV et al. Marrow stromal cells as a source of progenitor cells for nonhematopoietic tissues in transgenic mice with a phenotype of osteogenesis imperfecta. Proc Natl Acad Sci USA 1998;95:1142-1147.

11 Ferrari G, Cusella-De Angelis G, Coletta M et al. Muscle regeneration by bone marrow-derived myogenic progenitors. Science 1998;279:1528-1530.

12 Sandhu JS, Clark BR, Boynton EL et al. Human hematopoiesis in SCID mice implanted with human adult cancellous bone. Blood 1996;88:1973-1982.

13 Theise ND, Nimmakayalu M, Gardner R et al. Liver from bone marrow in humans. Hepatology 2000;32:11-16.

14 Orlic D, Kajstura J, Chimenti S et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001;410:701-705.

15 Kopen GC, Prockop DJ, Phinney DG. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc Natl Acad Sci USA 1999;96:10711-10716.

16 Brazelton TR, Rossi FM, Keshet GI et al. From marrow to brain: expression of neuronal phenotypes in adult mice. Science 2000;290:1775-1779.

17 Mezey E, Chandross KJ, Harta G et al. Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. Science 2000;290:1779-1782.

18 Hung SC, Chen NJ, Hsieh SL et al. Isolation and characterization of size-sieved stem cells from human bone marrow. STEM CELLS 2002;20:249-258.

19 Pittenger MF, Mackay AM, Beck SC et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143-147.

20 Hamill OP, Marty A, Neher E et al. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch 1981;391:85-100.

21 Pan CY, Kao LS. Catecholamine secretion from bovine adrenal chromaffin cells: the role of the Na^+/Ca^{2+} exchanger and the intracellular Ca^{2+} pool. J Neurochem 1997;69:1085-1092.

22 Pera MF, Reubinoff B, Trounson A. Human embryonic stem cells. J Cell Sci 2000;113:5-10.

23 Woodbury D, Schwarz EJ, Prockop DJ et al. Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 2000;61:364-370.

24 Sanchez-Ramos J, Song S, Cardozo-Pelaez F et al. Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol 2000;164:247-256.

25 Deng W, Obrocka M, Fischer I et al. In vitro differentiation of human marrow stromal cells into early progenitors of neural cells by conditions that increase intracellular cyclic AMP. Biochem Biophys Res Commun 2001;282:148-152.

26 Ishii K, Katayama M, Hori K et al. Effects of 2-mercaptoethanol on survival and differentiation of fetal mouse brain neurons cultured in vitro. Neurosci Lett 1993;163:159-162.

27 Droge W, Eck HP, Gmunder H et al. Modulation of lymphocyte functions and immune responses by cysteine and cysteine derivatives. Am J Med 1991;91:140S-144S.

28 Kaplan FS, Brighton CT, Boytim MJ et al. Enhanced survival of rat neonatal cerebral cortical neurons at subatmospheric oxygen tensions in vitro. Brain Res 1986;384:199-203.

29 Henion PD, Weston JA. Retinoic acid selectively promotes the survival and proliferation of neurogenic precursors in cultured neural crest cell populations. Dev Biol 1994;161:243-250.

In Vitro Differentiation of Size-Sieved Stem Cells into Electrically Active Neural Cells

Shih-Chieh Hung, Henrich Cheng, Chien-Yuan Pan, May J. Tsai, Lung-Sen Kao and Hsiao-Li Ma

Stem Cells 2002;20;522-529
DOI: 10.1634/stemcells.20-6-522