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GLIAL DIFFERENTIATION OF HUMAN ADIPOSE-DERIVED STEM CELLS: IMPLICATIONS FOR CELL-BASED TRANSPLANTATION THERAPY

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Abstract—Increasing evidence has shown that adipose-derived stem cells (ASCs) could transdifferentiate into Schwann cell (SC)-like cells to enhance nerve regeneration, suggesting potential new cell-based transplantation therapy for peripheral nerve injuries and neurodegenerative disorders. For the implementation of these results to the clinical setting, it is of great importance to establish the differentiation of human ASCs (hASCs) into a SC phenotype. In this study, we studied hASCs obtained from subcutaneous fat tissue of healthy donors. By a mixture of glial growth factors we differentiated them into Schwann cell-like cells (dhASCs). We then assessed their ability to act as Schwann cells *in vitro* and *in vivo* and also compared them with primary human Schwann cells (hSCs). Enzyme-linked immunosorbent assay showed that dhASCs secreted brain-derived neurotrophic factor (BDNF)/nerve growth factor (NGF) at a comparable level, and glial cell-derived neurotrophic factor (GDNF) at a level even higher than hSCs, whereas undifferentiated hASCs (uhASCs) secreted low levels of these neurotrophic factors. In co-culture with NG108-15 neuronal cells we found that both dhASCs and hSCs significantly increased the percentage of cells with neurites, the neurite length, and the number of neurites per neuron, whereas uhASCs increased only the percentage of cells with neurites. Finally, we transplanted green fluorescent protein (GFP)-labeled hASCs into the crushed tibial nerve of athymic nude rats. The transplanted hASCs showed a close association with PGP9.5-positive axons and myelin basic protein (MBP)-positive myelin at 8 weeks after transplantation. Quantitative analysis revealed that dhASCs transplantation resulted in significantly improved survival and myelin formation rates (a 7-fold and a 10-fold increase, respectively) as compared with uhASCs transplantation. These findings suggest that hASCs took part in supporting and myelinating regenerating axons, and thus have achieved full glial differentiation *in vivo*. In conclusion, hASCs can differentiate into SC-like cells that possess a potent capacity to secrete neurotrophic factors as well as to form myelin *in vivo*. These findings make hASCs an interesting prospect for cell-based transplantation therapy for various peripheral nerve disorders.

Key words: human adipose-derived stem cell, glial differentiation, peripheral nerve, Schwann cell, neurotrophic factor, myelination.

INTRODUCTION

Injured axons in the peripheral nervous system have a capacity to regenerate as opposed to those in the central nervous system. However, the clinical outcomes are often suboptimal especially in cases with a long nerve gap or in more proximal injuries such as brachial plexus injury. In those cases, the axons have to regrow over a long distance to reach their targets. While the native Schwann cells (SCs) in the distal nerve stump are initially supportive of the regenerating axons, it has been shown that a prolonged loss of axonal contact results in atrophy of SCs (Gordon et al., 2003, 2011; Hoke, 2006). This further translates into a loss of support with neurotrophic factors (Funakoshi et al., 1993; Terenghi, 1999) and loss of the basal lamina tube which serves as a scaffold for the regenerating axons (Son and Thompson, 1995; Stoll and Muller, 1999). In such situations, supplementing the resident atrophic SCs with exogenously activated SCs could be a promising therapeutic approach (Walsh et al., 2010; Tomita et al., 2012). However, autologous SCs have limited clinical application since a need of nerve biopsy often results in neuropathic pain at the harvest site, and the *in vitro* cell expansion is time consuming. Therefore, alternative sources of easily accessible and rapidly expandable cells would be needed.

Over the past decade, increasing evidence has established that adult bone marrow-derived mesenchymal stem cells (MSCs) can transdifferentiate into non-mesenchymal cells, including SCs (Sanchez-Ramos et al., 2000; Dezawa et al., 2001; Caddick et al., 2006; Keilhoff et al., 2006; Ladak et al., 2011). More

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Abbreviations: ASCs, adipose-derived stem cells; BDNF, brain-derived neurotrophic factor; b-FGF, basic fibroblast growth factor; BSA, bovine serum albumin; dhASCs, differentiated human adipose-derived stem cells; FBS, fetal bovine serum; GDNF, glial cell-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; hASCs, human adipose-derived stem cells; HBSS, Hank's balanced salt solution; hSCs, human Schwann cells; MBP, myelin basic protein; MSCs, mesenchymal stem cells; NGF, nerve growth factor; PBS, phosphate-buffered saline; PE, phycoerythrin; PFA, paraformaldehyde; SC, Schwann cell; T-PER, Tissue Protein Extraction Reagent; uhASCs, undifferentiated human adipose-derived stem cells.

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recently, human adipose-derived stem cells (ASCs) have been identified as a source of multipotent cells (Zuk et al., 2002; Gimble and Guilak, 2003; Strem et al., 2005), which can be easily harvested from the abundant subcutaneous fat tissue by small skin surgeries or conventional liposuction procedures. Importantly, a recent work has revealed that the rat ASCs could also transdifferentiate into SC-like cells (dASCs) under specific conditions (Kingham et al., 2007). The rat dASCs then could take an active part in the axonal regeneration, enhancing myelination and functional recovery after peripheral nerve injury (di Summa et al., 2010, 2011; Tomita et al., 2012).

For implementation of these encouraging results to the clinical transplantation therapy, it is clearly of great importance to establish the differentiation of human adipose tissue-derived ASCs (hASCs) into a SC phenotype. In this study, we first identified the morphological and phenotypic characteristics of hASCs after differentiation into SC-like cells (dhASCs) *in vitro*. Secondly, we assessed their ability to produce neurotrophic factors *in vitro*, and we also looked at their ability to promote neurite outgrowth by examining their interaction with NG108-15 neuronal cells. Finally, we transplanted hASCs into an injured peripheral nerve of athymic nude rats to study their interactions with regenerating axons *in vivo*.

EXPERIMENTAL PROCEDURES

Isolation and culture of hASCs

Samples of human subcutaneous adipose tissue were taken from three healthy donors during reconstructive surgery after obtaining informed consent. Those patients were designated as follows: P1; female aged 56 years, P2; female aged 33 years, and P3; male aged 63 years. The anatomical sites where subcutaneous adipose tissue was harvested were abdomen for P1, buttock for P2, and thigh for P3. All procedures were approved by the Local Ethics Committee for Clinical Research in the Osaka University (No. 11147).hASCs were isolated as previously described (Kingham et al., 2007). Briefly, subcutaneous human fat was enzymatically dissociated for 60 min at 37 °C using 0.15% collagenase type I (Invitrogen). The solution was passed through a 70-μm filter to remove undissociated tissue, neutralized by the addition of stem cell growth medium [MEM (Minimum Essential Medium) containing 10% fetal bovine serum (FBS)] and centrifuged for 5 min. The stromal cell pellet was resuspended in growth medium. Cultures were maintained at subconfluent levels in a 37 °C-incubator with 5% CO_2.

Flow cytometry analysis for surface antigens of hASCs

Surface antigens of the undifferentiated hASC (uhASCs) were identified by flow cytometry using anti-human CD29 (1:20, Biolegend, 303003), CD90 (1:10, AbD Serotec, MCA90PET), CD44 (1:10, AbD Serotec, MCA89PET), and CD45 (1:20, Biolegend, 304007) antibodies. After subconfluent uhASC cultures were detached, the cells were resuspended in buffer containing phosphate-buffered saline (PBS) plus 1% bovine serum albumin (BSA) to achieve a concentration of 1×10^7 cells/ml, and incubated with phycoerythrin (PE)-conjugated antibodies for 30 min on ice. Flow cytometry analysis was performed on the FACS Vantage SE (BD Biosciences, San Jose, USA) and the data were analyzed with CellQuest Pro software.

Multilineage differentiation of hASCs

For osteogenic differentiation, uhASCs at passage 2 were plated at 4×10^3 cells/cm^2 and cultured for 2 weeks in osteogenic induction medium [MEM containing 10% FBS, 100 μg/ml ascorbate, 0.1 μM dexamethasone and 10 mM β-glycerophosphate (All from Sigma)]. Cells were then fixed with 4% paraformaldehyde (PFA) for 30 min, washed with Hank's balanced salt solution (HBSS) containing 1% BSA, and then incubated with 1 mg/ml Alizarin Red (Sigma) solution to stain for calcium deposition.

For adipogenic differentiation, subconfluent passage 2 cultures were incubated for 2 weeks in adipogenic induction medium [high glucose-DMEM (Dulbecco's Modified Eagle Medium) containing 10% FBS, 1 μM dexamethasone, 0.5 mM methyl-isobutylxanthine, 10 μg/ml insulin, and 100 μM indomethacin (All from Sigma)]. Cells were fixed with 4% PFA for 60 min, washed with PBS, and then incubated 0.3% Oil Red O (Sigma) solution in 60% isopropanol for 20 min.

Differentiation of hASCs into SC-like cells (dhASCs)

uhASCs were differentiated into dhASCs as previously described (Kingham et al., 2007) using Her-β instead of glial growth factor 2. Briefly, subconfluent ASCs at passage 2 were cultured in a growth medium containing 1 mM 2-mercaptoethanol (Sigma) for 24 h. The cells were then washed and fresh medium supplemented with 35 ng/ml all-trans-retinoic acid (Sigma). After 72 h, the cells were washed and the medium was replaced with differentiation medium: growth medium supplemented with 5 ng/ml platelet-derived growth factor (PDGF; PeproTech Ltd., USA), 10 ng/ml basic fibroblast growth factor (b-FGF; PeproTech), 5.7 μg/ml forskolin (Sigma) and 200 ng/ml recombinant human heregulin-β1 (HRG-β1; R&D Systems, USA). The cells were incubated for 14 days to achieve full differentiation while the medium was changed every 3 days.

Isolation and culture of human SCs

Samples of human peripheral nerve were taken from two patients during reconstructive surgery after obtaining informed consent. The patients were designated as follows: P1; female aged 36 years, and P2; male aged 65 years. After removing the epineurium, the nerves were cut to 1-mm segments. The segments were cultured in a Petri dish with SC growth medium (DMEM containing 10% FBS, 4.1 μg/ml forskolin, and 100 ng/ml HRG-β1) at 37 °C with 5% CO_2. Two weeks later, the medium was aspirated, and 0.0625% collagenase type IV and 0.585 U dispase were added to the dish. After 24-h incubation, the cell suspension was filtered through a 70-μm cell strainer, followed by centrifugation for 5 min. Finally, the cell pellet was resuspended in SC growth medium and plated in 25-cm^2 flask coated with laminin (BD). The cultures were maintained at subconfluent levels in a 37 °C-incubator with 5% CO_2.

Immunostaining

For immunocytochemistry, the cells in culture were fixed in 4% PFA for 20 min, followed by permeabilization using 0.2% Triton X for 30 min. Background staining was blocked using normal animal serum for 1 h at room temperature and the cells were then incubated at 4 °C overnight with the primary antibodies. The cells were washed three times, and the secondary