Potential Characteristics of Stem Cells from Human Exfoliated Deciduous Teeth Compared with Bone Marrow–derived Mesenchymal Stem Cells for Mineralized Tissue-forming Cell Biology

Kenji Hara, DDS,* Yoichi Yamada, DDS, PhD,* Sayaka Nakamura, DDS, PhD,* Eri Umemura, DDS,* Kenji Ito, DDS, PhD,† and Minoru Ueda, DDS, PhD*

Abstract

Introduction: Tissue engineering and regenerative medicine using stem cell biology has been a promising field for the treatment of local and systemic intractable diseases. Recently, stem cells from human exfoliated deciduous teeth (SHED) have been identified as a novel population of stem cells. This study focused on the characterization of SHED as compared with bone marrow–derived mesenchymal stem cells (BMMSCs). Methods: We investigated potential characteristics of SHED by using DNA microarray, real-time reverse transcriptase polymerase chain reaction, and immunofluorescence analysis. Results: Multiple gene expression profiles indicated that the expression of 2753 genes in SHED had changed by $\geq 2.0$-fold as compared with that in BMMSCs. One of the most significant pathways that accelerated in SHED was that of bone morphogenetic protein (BMP) receptor signaling, which contains several cascades such as PKA, JNK, and ASK1. When the BMP signaling pathway was stimulated by BMP-2, the expression of BMP-2, BMP-4, Runx2, and DSPP was up-regulated significantly in SHED than that in BMMSCs. Furthermore, the BMP-4 protein was expressed much higher in SHED but not in BMMSCs, as confirmed by immunofluorescence. Conclusions: By using the gene expression profiles, this study indicates that SHED is involved in the BMP signaling pathway and suggests that BMP-4 might play a crucial role in this. These results might be useful for effective cell-based tissue regeneration, including that of bone, pulp, and dentin, by applying the characteristics of SHED. (J Endod 2011;37:1647–1652)

Key Words
Bone marrow-derived mesenchymal stem cells, bone morphogenetic protein, microarray analysis, regenerative medicine, stem cells from human exfoliated deciduous teeth

The concept of tissue engineering and regenerative medicine (TERM), which applies stem cells, biocompatible scaffolds, and growth factors, has attracted much attention and is a growing field that has been well-implemented in medicine (1). Stem cell biology in TERM provides an important key to the treatment of local and systemic intractable illnesses. Currently, stem cells such as bone marrow–derived mesenchymal stem cells (BMMSCs) are considered the most important elements for TERM. The BMMSCs have been well-characterized and used for clinical applications, including bone augmentation in the field of dentistry (2–4). Postnatal MSC-like populations have been isolated from various dental tissues such as dental pulp, periodontal ligament, dental papilla, and dental follicle (5–14). Dental pulp stem cells have been reported to be especially useful in the repair of bone and dental pulp (5, 15). Stem cells from human exfoliated deciduous teeth (SHED) have been identified as a novel population of stem cells that has the capacity of self-renewal and of multilineage differentiation (6). A previous study reported that SHED was able to differentiate into multiple cell types, including osteoblastic and odontoblastic cells, and to generate bone or dentin-pulp complex tissue (6–8). Some of the greatest advantages of using SHED are that a cell population might be obtained from deciduous teeth of each individual patient, with very low morbidity, without any ethical concern, and which are highly proliferative (9, 10). SHED also might provide a useful cell source as an alternative to BMMSCs for mineralized tissue formation, but their specific characteristics and differentiation mechanisms are still unknown. On the other hand, TERM is the most advanced in the field of dentistry, and growth factors such as basic fibroblast growth factor (FGF) and bone morphogenetic proteins (BMPs) have been applied for tissue regeneration (1–4, 16). BMPs have been identified as protein regulators of bone and dentin formation and have been involved in embryogenesis and morphogenesis of various organs and tissues (17–21). BMPs also stimulate differentiation of osteoblasts and odontoblasts and of mineralization via the BMP signaling pathway. Among them, it is known that BMP-2 acts in a paracrine fashion in concert with other BMPs and induces to stimulate bone or osteodentin formation (20–23). BMP-4 also accelerates cell differentiation in similar fashion to BMP-2 (17, 24, 25). However, the precise cellular and molecular mechanisms behind this remain unknown. In this study, we investigated the characteristics of SHED that could provide a basis for effective clinical applications in the future for mineralized tissue regeneration for TERM by comparing SHED and BMMSCs.
Materials and Methods

Subjects and Cell Cultures

Human exfoliated deciduous teeth and bone marrow aspirates of healthy subjects were obtained with informed consent. The ethics committee of Nagoya University approved all experimental protocols. SHED and BM-MSCs were isolated and cultured as previously described (4, 6). To examine the effects of BMP-2, 150 ng/ml human recombinant BMP-2 (Invitrogen, Camarillo, CA) was added to the medium.

Flow Cytometry Analysis

Cultured SHED and BM-MSCs were trypsinized and incubated with mouse antibodies against human CD14, CD29, CD54, CD44, CD73, or CD146 (BD Pharmingen, San Diego, CA). Nonlabeled cells were used as negative controls. Fluorescence-activated cell sorter analysis (FACS) was performed as previously described (8).

Complementary DNA Microarray Analysis and Pathway Analysis

Total RNA was prepared from SHED and BM-MSCs obtained from the same donor by using the RNeasy Mini Kit (Qiagen, Valencia, CA). Microarray experiments were performed as previously described (10). Data collections were imported to GeneSpring GX 10.0 software (Agilent Technologies, Santa Clara, CA) and Ingenuity Pathways Analysis (Ingenuity Systems, Inc, Redwood City, CA) for further analysis. A fold-change cutoff of 2 for gene up-regulation and a P-value cutoff of .05 were set to identify the genes to be analyzed in pathway analysis.

Quantitative Real-time Reverse Transcriptase Polymerase Chain Reaction

On days 0, 3, 7, 14, and 21 after BMP-2 induction, total RNA was extracted from SHED and BM-MSCs. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis was performed as previously described (11, 12, 26). Specific primers and probes were as follows: BMP-2, forward, 5'-TGTCGCACTTCACG-3', reverse, 5'-CTC GGGTTTGGTCAC-3', and probe, 5'-TGAAGAATCTTGGGAAGACT ACCAGAAACG-3'; BMP-4, forward, 5'-CTGCTCTTGAAGTCCTCAAG-3', reverse, 5'-AAGGAAAGAAGCAAAGCA-3', probe, 5'-ACTGTCCTCGGG ATGTTCC-3', MAPK10: forward, 5'-GAGGTTGCTCAAGATGTGTTA-3', reverse, 5'-TATTTCTACCATGCATTCTA-3', and probe, 5'-ATGGCGA TCTCACTGTTCACT-3'; PRKAR2B: forward, 5'-ACAC ATCTTCACAACATCATA-3', reverse, 5'-TGTTCACTTTGGCCATGG-3', and probe, 5'-CC ACTCAGTCACACCTTACGC-3'; osteocalcin (OCN): forward, 5'-AG TGTCGCAAGGGTTCAG-3', reverse, 5'-CCAGCAGTACAGTACCC GC-3', and probe, 5'-CTCCACTATTGTCCTGGCTGT-3'; dentin sialophosphoprotein (DSP): forward, 5'-GCACTCAGCTATGTTGCAAC-3', reverse, 5'-CAGCGAAGAAGGCCACAT-3', and probe, 5'-TGTGTTGC ACTGAGATATCTTACTCC-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer and probe (Taqman GAPDH detection reagents) were purchased from Perkin-Elmer and Applied Biosystems (Foster City, CA). Relative expression levels were normalized to GAPDH expression. Statistical analyses were performed with a Student's t test.

Immunofluorescence for BMP-4

After 7 days of cultivating with or without BMP-2, immunofluorescence analysis of BMP-4 was performed according to the manufacturer's instructions. The primary antibody was mouse anti-human BMP-4 monoclonal antibody (1:50; Millipore, Billerica, MA), and a secondary goat anti-mouse immunoglobulin G antibody was conjugated to Alexa Fluor 488 (1:500; Invitrogen) and mounted by using Vectashield with DAPI (Vector Laboratories Inc, Burlingame, CA).

Results

Expression of Stem Cell Markers in SHED and BM-MSCs

FACS analyses revealed that both SHEDs and BM-MSCs showed more than 90% positivity for all mesenchymal lineage markers (CD29, CD44, CD73, and CD146) (Fig. 1A). On the other hand, SHED and BM-MSCs were negative for the monocytic marker CD14 and the hematopoietic lineage marker CD34. These findings indicated that SHED, similar to BM-MSCs, possessed the phenotype of MSCs.

Gene Expression Profiles of SHED and BM-MSCs

Complementary DNA microarray technology was used to compare multiple gene expression profiles representative of SHED and BM-MSCs. These results indicated that out of 41,078 genes, the expression of 2,753 in SHED changed by $\geq 2.0$-fold as compared with that in BM-MSCs. The expression of 1,309 genes increased, whereas that of 1,444 decreased in SHED, with a change of $\geq 2.0$-fold.

Pathway Analysis

Pathway analysis revealed that higher expression in SHED was observed for genes in several pathways including c-kit, the BMP receptor signaling pathway, and the basic mechanisms of action of peroxisome proliferator-activated receptor (PPAR)α and PPARγ, and the effects on gene expression (Table 1). Among these pathways, we focused on the BMP receptor signaling pathway, because it might have a crucial role in the differentiation of SHED into osteogenic/odontogenic cells. The genes of the BMP receptor signaling pathway whose expression was up-regulated in SHED as compared with that in BM-MSCs are shown in Table 2, and the network is shown in Figure 1B. The expression profiles (ratio) of up-regulated genes in this pathway are as follows: BMP-2, 4.64; BMP-4, 1.62; MAP3K5 (ASK1), 1.48; PRKAR2B (PKA), 4.39; PRKACB (PKA), 1.06; MAP2K6 (MKK6), 2.22; MAPK10 (JNK), 1.66; CAMK4, 1.29; MEF2C, 1.07; ELK4, 1.70; and ETS2, 1.01.

Real-time RT-PCR Analysis

To confirm the significance of the relative gene expression between SHED and BM-MSCs, the expression patterns of genes related to the BMP signaling pathway were validated (Fig. 1C). The expressions of BMP-2, BMP-4, MAPK10, and PRKAR2B were significantly up-regulated in SHED as compared with those in BM-MSCs ($P < .01$). These results are consistent with those from the microarray results.

Effect of BMP-2 Signaling on the Differentiation of SHED and BM-MSCs

The BMP-2 signaling pathway provides one of the strongest signals for stimulating biomineralization. To compare the effects of this pathway, the expression of osteogenic/odontogenic-related genes, including BMP-2, BMP-4, Runx2, OCN, and DSP, was examined by using real-time RT-PCR analysis. BMP-2 increased the expression of BMP-2, Runx2, OCN, and DSP in both SHED and BM-MSCs (Fig. 2, C–E). Exposure to BMP-2 led to a strong expression of BMP-4 in SHED but only to a weak expression in BM-MSCs (Fig. 2B). The expression levels of BMP-2, BMP-4, and DSP in SHED were significantly higher than those in BM-MSCs at all time points. The expression levels of Runx2 at days 0, 3, 7, and 14 were higher in SHED as compared with those in BM-MSCs. In contrast, there were no significant differences observed in OCN expression between SHED and BM-MSCs.
Figure 1. (A) Typical flow cytometric analysis diagrams for expression of mesenchymal stem cell markers (CD29, CD44, CD73, and CD146) and the monocytic marker CD14 and hematopoietic marker CD34 antigens on SHED and BMMSCs. (B) Schema of intracellular signaling related to the BMP receptor signaling pathway. Genes in red showed a highly increased expression of 2.0-fold or more in SHED than in BMMSCs in this study. (C) Real-time RT-PCR analysis was performed to validate the expression levels of genes involved in the BMP receptor signaling pathway. BMP-2, BMP-4, MAPK10, and PRKAR2B showed a significantly higher expression in SHED than in BMMSCs (**P < .01, Student’s t test).
Immunofluorescence for BMP-4

To confirm the up-regulated BMP-4 protein expression induced by BMP-2, immunofluorescence staining for BMP-4 was performed. The BMP-4 expression level was strongly increased in SHED but only mildly in BMMSCs, compared with BMP-2 exposure (Fig. 2F).

Discussion

Regenerative medicine is progressing toward clinical application. We have previously reported the application of BMMSCs in clinical trials for the effective treatment of osseous defects, on the basis of translational research rather than autogenous bone grafting, and favorable results have been obtained (2–4). On the other hand, SHED have been reported to possess similar characteristics as BMMSCs, namely that of self-renewal, multilineage differentiation, and expression of mesenchymal stem cell markers such as CD29, CD44, CD73, and CD146 (6–8). SHED can differentiate into osteogenic/odontogenic lineage cells (6–8), although a difference between stem cells from dental pulp and BMMSCs has been reported (11, 13, 27). SHED, which are specific for bone/dentin (17, 24, 25), was specifically expressed in SHED. BMP-2 is known to enhance the expression of differentiation into mineralized tissue-forming cells of SHED, as compared with BMMSCs. The pathway analysis of the gene expression profile revealed that the BMP receptor signaling pathway in SHED was up-regulated as compared with that in BMMSCs (Table 1). The pathways that were found to be accelerated in SHED are the Smads-independent pathways, such as the p38, ERK, and c-Jun N-terminal kinase (JNK) cascades, which play crucial roles in osteoblast differentiation (28, 29) (Fig. 1B). In addition, the BMP-2, BMP-4, JNK (MAPK10), and PRKAR2B genes were found to be up-regulated (Table 2, Fig. 1C). This finding is consistent with BMPs acting via activation of the mitogen-activated protein kinase (MAPK) or JNK pathways (30). Moreover, BMP-2, which was reported to have an inductive effect in osteogenesis and dentinogenesis (22, 23), was used in this study to stimulate the BMP signaling pathway. After BMP-2 stimulation, the expression levels of osteogenic/odontogenic-related genes increased in both SHED and BMMSCs (Fig. 2A–E). BMP-4, which promotes osteoblast and odontoblast differentiation and is marker for bone/dentin (17, 24, 25), was specifically expressed in SHED. BMP-2 is known to enhance the expression levels of other BMP genes during bone formation with a paracrine effect (31); thus, the higher expression of BMP-4 in SHED might be induced by this BMP-2 effect. In addition, immunofluorescence staining for BMP-4 after stimulation revealed that the BMP-4 expression was strongly increased in SHED but only mildly in BMMSCs.
Human recombinant BMP-2 (150 ng/mL) was added to the culture medium of SHED and BMMSCs to promote expression of genes related to the BMP signaling pathway. (A–E) Total RNA was extracted at the time points of 0, 3, 7, 14, and 21 days. Real-time RT-PCR analyses were performed to validate the expression of osteogenic/odontogenic related genes (*P < .05; **P < .01, Student’s t test). (F) Immunofluorescence staining was performed to examine BMP-4 protein expression in SHED and BMMSCs at days 0 and 7 after BMP-2 induction. DAPI was used to visualize the nuclei (blue). Scale bar = 40 μm. BMP-2 stimulation induced high expression of the BMP-4 protein in SHED.
Therefore, BMP-4 also might play a specific role in osteoblast and odontoblast differentiation of SHED into mineralized-forming cells.

Since the expression level of Runx2, which is a necessary bone-related transcription factor, was significantly higher in SHED than in BMMSCs. Because the BMP signaling pathway is related to the regulation of Runx2 (32, 33), accelerating the BMP signaling pathway and having a high expression of BMP-2 and BMP-4 might induce a higher Runx2 expression in SHED (Fig. 2). Furthermore, DSPP expression was significantly higher in SHED as compared with BMMSCs. DSPP is highly expressed in odontoblasts and plays a role in the mineralization of dentin (22). Therefore, our results indicate that SHED might have a stronger tendency to induce into odontoblasts than BMMSCs. This is consistent with the previous reports that dental pulp stem cells expressed higher levels of genes associated with odontogenesis and tooth morphogenesis as compared with BMMSCs (11, 27).

Taken together, this study indicates that SHED have specific characteristics that differ from those of BMMSCs. These results of the gene expression profiles suggest that osteogenic/odontogenic differentiation of SHED and BMMSCs is regulated by different mechanisms, and that BMP-4 might play a crucial role in SHED. Therefore, an effective cell-based mineralized tissue regeneration, including that of bone, pulp, and dentin, could be developed by applying the characteristics of SHED in the future.

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The authors deny any conflicts of interest related to this study.

References
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