Stem cells isolated from human dental follicles have osteogenic potential

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Objective. Stem cells isolated from human dental follicles as a potential cell source for bone-tissue engineering were examined for correcting a critical bone defect.

Study design. Impacted third molars were collected and single cell–derived cell populations were cultivated in growth medium. Single cell–derived cell lines were examined in terms of cell shape, gene expression patterns, differentiation capacity in vitro, and osteogenic potential in vivo.

Results. Three distinct cell populations were identified with different morphologies, patterns of gene expression, and differentiation capacity. All 3 cell populations promoted bone formation when transplanted into surgically created critical-size defects in immunodeficient rat calvaria, compared with control animals without cell transplantation, although one of these populations showed a weak capacity for osteogenetic differentiation in vitro.

Conclusions. Human dental follicle can derive at least 3 unique cell populations in culture, all of which promote bone formation in vivo. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;111:700-708)

Recently, mesenchymal stem cells (MSCs) were shown to be an attractive cell source for tissue engineering.1,2 They can be easily isolated from bone marrow (BM) and expanded through several passages while retaining their multipotent differentiation capacity.3 However, the harvesting of bone marrow is invasive, and many alternative sources of MSCs also have potential in tissue engineering, including adipose tissue4,5 and dental pulp cells.6,7 Under appropriate stimulation, MSCs undergo osteogenic differentiation via a well-defined pathway, and bone-tissue engineering using sources such as MSCs is a new tool with the potential to replace autologous tissue grafting for bone defects.8,9

Dental follicle is a loose vascular connective tissue composed of a heterogeneous layer of ectomesenchymal cells surrounding the tooth germ in early stages of tooth development.10-14 In recent years, progenitor cells have been identified in the dental follicle,15-17 and dental follicle cells have been demonstrated to differentiate along osteogenic pathways.18-20 We previously identified single cell–derived stem cells in the dental follicle of porcine developing tooth during early crown formation.21 In that study, the single-cell clonal population combined with β-TCP (β-toricalcium phosphate) formed bone tissue subcutaneously in immunodeficient mice,21 consistent with other studies.12,22 More recently, human dental follicle progenitor cells showed hard tissue-forming potential in immunocompromised rats.23 These results indicate that human dental follicle cells may contribute to bone repair in critical-size defects in vivo. However, no studies have used human dental follicle cells to determine the potential for bone healing, nor has the effect of heterogeneous cell populations derived from human dental follicle tissues on bone repair been addressed.

In the first part of this study, we isolated human dental follicle cells from third molars extracted at the
crown formation stage. Three distinct populations of dental follicle stem cells were derived with different morphologies, patterns of gene expression, and differentiation capacities. Second, we examined the potential of cell source in 3 distinct populations for bone-tissue engineering in surgically created critical-size defects in immuno-deficient rat calvaria.

MATERIALS AND METHODS

The procedures used to acquire all cells from the surgically extracted teeth or from iliac crest conformed to the tenets of the Declaration of Helsinki. This project was approved by the local ethical committee of the Institutional Animal Care and Use Committees (IACUC) at the Institute of Medical Science, the University of Tokyo, and the Ethical Review Committee of the Tokushima University Hospital; all donors gave informed consent.

Isolation of putative human dental follicle stem cells

A total of 3 impacted third molars were collected from 3 healthy patients for orthodontic reasons (18-25 years of age) in the Department of Oral and Maxillofacial Surgery, Tokushima University Hospital. Dental follicle tissue was carefully dissected from the upside of the dental crown and cut into several pieces. These tissue fragments were incubated at 37°C for 30 minutes in a solution containing 0.05% collagenase (Wako, Tokyo, Japan) and 0.125% trypsin (Invitrogen, Life Technologies, Grand Island, NY). After cell populations had adhered to the plastic dish surface, nonadherent cells were removed by change of medium. After cell populations were 80% confluent, cells were suspended at a density of 1 cell per 100 μL and seeded into three 96-well culture plates with the growth culture medium (DMEM; Kohjin Bio, Saitama, Japan) and 0.125% trypsin (Invitrogen, Life Technologies, Grand Island, NY). Rate of cell growth was calculated by cell counting (Cell-counting Kit-8; Dojindo Laboratories, Kumamoto, Japan) according to the protocol.24 The relative cell number was determined by measuring absorbance of light at a wavelength of 450 nm (Model 650 Microplate Reader; BioRad Laboratories, Hercules, CA). Results shown are the average of triplicates from one experiment.

Induction of differentiation of dental follicle cells

Three clonal dental follicle cell populations were treated with different media known to induce either osteogenesis, adipogenesis, or chondrogenesis to evaluate their differentiation potential.

Osteogenic differentiation

The osteogenic differentiation capacity of clonal human dental follicle cells was assessed by measurement of ALP activity and staining with alizarin red. The cells were grown on 6-well plates at a density of 5 × 10⁵ cells/well in GCM for 28 days for the assay for ALP activity, and for 14 days for alizarin red staining. For staining of cells with alizarin red, GCM was replaced by a solution containing 0.05% collagenase (Wako, Tokyo, Japan) for 25 to 35 cycles according to the following reaction profile: 95°C for 30 seconds, 45 to 60°C for 30 seconds, and 72°C for 30 seconds. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were used as internal standards. Synthesized cDNA served as a template for subsequent PCR amplification using specific primers as listed in Table I. The designed primers were based on the sequence of the target gene. The experiment was performed in triplicate.
with osteogenic induction medium (OIM; complete medium supplemented with 50 mg/mL L-ascorbic acid, 10 mM β-glycerophosphate, 10−8 M dexamethasone [Wako, Osaka, Japan], and BMP-4 [0.05 μg/mL, R&D System, Minneapolis, MN]) after 14 days in culture. Medium was then changed every 2 days for 28 days. ALP activity was tested using Fast p-nitrophenyl phosphate tablets (Sigma, St Louis, MO) according to the protocol.24 The relative amount of p-nitrophenyl reacted was estimated from the light absorbance at a wavelength of 405 nm (BioRad Laboratories) at days 7, 14, 21, and 28. Each experiment was performed in triplicate.

The presence of mineralized matrix such as calcium deposition was determined by staining with alizarin red. Cells grown to confluence in OIM for 14 days were rinsed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde, stained with 0.3% Oil Red O and counterstained with 1% Fast green dye using standard techniques. Lipid droplets were identified microscopically (BZ-8000, Keyence, Tokyo, Japan).

Table I. Sequence of primer pairs

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequence (5'→3')</th>
<th>Annealing temperature (°C)</th>
<th>Size, bp</th>
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</table>
| runx-2        | F: CAGACCAGCAGACCTCCATA  
R: CAGGCCTCACCACTACATTC | 59  | 178 |
| msx-2         | F: TTACACATCCCCAGTCTCCTC  
R: GAGGGGAGGAAACCCCGGTTTG | 50  | 419 |
| fgfr-2        | F: TGGCAGAAGCTGCAACCATGC  
R: AACGGAAGGATTTAAGCAG | 55  | 500 |
| alp           | F: ATCACTTGGGAGTCTGAGG  
R: GGATGGTCTTCACTCTCCAAGTGA | 49  | 453 |
| Osteonectin   | F: GGAAGAAACCTGGGAGAGGTGAC  
R: TGTGGTCTCCATCCCTCTCATAAG | 50  | 347 |
| Osteopontin   | F: CCAAGTAAAGTCCAAGGAAG  
R: GGTGATGCTGCTCTGTTA | 55  | 186 |
| Biglycan      | F: CAACCAGTACAGATGACAAA  
R: CCCATGGGACAGAAGTCGTTG | 53  | 127 |
| Decorin       | F: GGGAGGTCTGCTGGCACAAACAC  
R: GGGCAAGAGTTCAGTCACTCAACAC | 50  | 417 |
| Periostin     | F: CAAACACCTTACGAGATCTCTC  
R: TGCACTTCAAGGTAGGCTGA | 55  | 220 |
| Collagen-12   | F: GTGCACTATCAGTCCTGGTGTA  
R: AACAGGCTGCTGTTTGGTGC | 50  | 489 |
| Collagen-1    | F: CCAAGGAGTCTTCTGGTGAAA  
R: GGAAACTGCTGCTGCCCTCTT | 55  | 350 |
| Collagen-3    | F: TGGTTGAGGCCGTCGGCA  
R: TTCAGCACTAAATGCTGCC | 50  | 200 |
| Vimentin      | F: GGGACCTTACGAGGAGGAG  
R: CGCATGGTCAACATGCTGCC | 55  | 261 |
| Ameloblastin  | F: GCTAAAACACTATTACCCTT  
R: AATAGTGTCATGTCGTGGTGAAGAG | 50  | 300 |
| GAPDH         | F: CGTCTTCAACACCATGGAGA  
R: AAACGTGCGCGTGATGGCGG | 55  | 261 |

F, forward; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; R, reverse.

Adipogenesis
To evaluate adipogenic differentiation, 2 × 10^5 cells were plated into each well of a 6-well plate (Becton Dickinson, Franklin Lakes, NJ). At 100% confluence, an adipogenic induction medium was used to stimulate optimal adipogenic differentiation according to the manufacturer’s instruction (CAT: 811D-250, Cell Applications, Inc, Toyobo, Osaka, Japan). The medium was change twice weekly for 2 weeks. Control plates of cells in GCM were also maintained. The cells were fixed with 4% paraformaldehyde, stained with 0.3% Oil Red O and counterstained with 1% Fast green dye using standard techniques. Lipid droplets were identified microscopically (BZ-8000, Keyence, Tokyo, Japan).

Chondrogenesis
The chondrogenic differentiation was induced by the pellet culture of dental follicle cells with a chondrogenic medium (cat: PT4121, LONZA, Walkersville, MD) plus recombinant transforming growth factor (TGF)-β3 (code 243-B3-002, R&D System) for chon-
drocyte differentiation. Briefly, trypsinized dental follicle cells were placed in a 15-mL polypropylene tube and centrifuged at 1000 rpm for 5 minutes to form a pelleted cell mass at the bottom of the tube. The pellet was cultured in the chondrogenic medium for 3 weeks. After cultivation, the pellets were fixed and were stained with 1% alcian blue (Sigma) in 0.1N HCL for 30 minutes.

**Surgical procedure**

We designed a transplantation procedure to generate bone tissue to compare the in vivo osteogenic capacity of human dental follicle cells. A curvilinear sagittal incision was made in the calvarium scalp of anesthetized, immunocompromised rats (total number = 24, F344/NJcl-rnu, Nihon Crea, Tokyo, Japan), allowing reflection in a subperiosteal plane. An 8-mm-diameter full-thickness critical size parietal defect was made unilaterally in the parietal bone using a trephine drill. A critical size defect is defined as the minimal size bone defect that, if left untreated, would not heal spontaneously during the animal’s lifetime. Care was taken to avoid injuring the dura, and the periosteum was eliminated to exclude the possibility of bone formation from periosteum-derived osteogenic cells. The surgical defect was filled with a pellet of dental follicle cells from all 3 clonal populations (n = 3 each) that had been expanded in culture (approximately 2 × 10^6 cells per pellet). The soft tissues were then repositioned and the skin was closed with 4-0 Vicryl suture (Ethicon, Somerville, NJ).

**Tissue processing**

Each group of animals was divided into 2 subgroups at either 1 or 4 weeks after transplantation (n = 3 each). The area of the original surgical defect and the surrounding tissues were removed en bloc. After fixing in Bouin’s solution for 24 hours, the tissue blocks were decalcified with 10% EDTA solution for 1 month, and then processed for embedding in paraffin. Serial sections of 6-μm thickness were cut in a longitudinal direction starting at the center of the original surgical defect. The sections were stained with either hematoxylin and eosin or by immunohistochemistry for analysis by light microscopy.

To increase the data reliability, 3 histological sections were selected for the histomorphometric analysis. Scion image software was used for image analysis (National Institutes of Health, Bethesda, MD). Briefly, the total area corresponded to the entire area of the original surgical defect. The newly regenerated bone area within the confines of the total area was measured and calculated as a proportion of the total area. The measure of regenerated bone in the defect was represented by the mean percentage.

Immunohistochemical analysis was performed using the Vectastain ABC kit (Vector Laboratories, Inc, Burlingame, CA), as previously described with modification. Mouse monoclonal osteocalcin antibody was used to identify osteoblasts (1:200 dilution; ab13420, Abcam plc, Tokyo, Japan).
Statistical analysis

Significance of differences was determined using Student t test and Microsoft Excel software (Microsoft, Redmond, WA). A P value less than .05 was considered significant.

RESULTS

Characterization of putative human dental follicle stem cells in vitro

We obtained 12 single cell–derived cell populations that formed colonies that could be expanded in vitro from 3 donors. Each clonal cell population uniformly displayed 1 of 3 distinct morphologies under phase contrast microscope, which we termed HDF1, 2, and 3 (Fig. 1). HDF-1 cells were small and polygonal (Fig. 1, A and B). HDF-2 cells were spindle-shaped with small processes (Fig. 1, C and D), with a similar morphology to PDLC (Fig. 1, G) and BMSC (Fig. 1, H). HDF-3 cells were also polygonal, but were larger than HDF-1 cells (Fig. 1, E and F).

We next examined the expression of specific previously described dental follicle cell markers\(^{28-30}\) by reverse transcription (RT)-PCR in each clone and in controls after 14 days of culture in GCM (Table I). ALP mRNA was expressed at high levels in HDF2 and HDF3 cells, but only weakly in HDF1 cells (Fig. 2). Periostin mRNA was detected only in HDF1 and HDF3 cells. FGFR2 and collagen type XII mRNA were expressed at high levels in HDF1 and HDF3 cells, but only at low levels in HDF2 cells. Decorin mRNA was more highly expressed in HDF1 than HDF2 and HDF3 cells. Furthermore, control PDLC and BMSC cells showed very different patterns of gene expression to HDFs. These control cell populations expressed Msx2, which was not expressed in HDFs, but did not express FGFR2, which was expressed in all HDF cell populations.

Growth potential of dental follicle cells

There were markedly different patterns of cell proliferation among HDF1, HDF2, and HDF3 cells over
the period of observation (Fig. 3). In particular, at day 7, the growth potential of HDF1 cells was significantly higher than that of HDF2 and HDF3 cells.

**Differentiation potential of dental follicle cells**

All experiments were repeated at least 3 times. The differentiation potentials of the 3 HDF cell types were then tested by culturing cells under different conditions. None of the cell types could be driven toward chondrogenesis when cultured with chondrogenesis differentiation medium. HDF1 cells grown in GCM had strong ALP activity, in contrast to HDF2 and HDF3 cells, which had only very low levels of activity (Fig. 4, A). When grown in OIM for 14 days, HDF 1 and HDF 2 cells, but not HDF3 cells, stained strongly for alizarin red, indicating calcium accumulation (Fig. 4, B). There was no precipitation of alizarin red in any cells grown in GCM for 28 days or in those cultured in OIM without BMP-4.

Neutral lipid, a marker of adipogenic differentiation, which stains with Oil Red O, was evident at high levels only in HDF3 cells when cells were cultured with GCM (Fig. 5, upper panel). However, when HDFs were subcultured in adipogenic differentiation medium, multiple intracellular lipid-filled droplets could be seen in all HDFs (Fig. 5, lower panel).

**Osteogenic potential in vivo**

The potential of cells to form bone in vivo was investigated by transplantation of a pellet of dental follicle cells into a surgically created full-thickness critical size parietal defect in rats. In all groups, hematoxylin and eosin staining showed bone formation and evidence of vascular invasion at 4 weeks posttransplantation (Fig. 6). The regions where a defect had been created were reconstituted and tended to be close to the dura surface owing to new bone formation in animals transplanted with HDF1 and HDF2 cells (Fig. 6, A and B). The appearance of new bone formation was similar to that seen in intramembranous ossification. There was some new bone formation in animals transplanted with HDF3 cells, but markedly more than that seen in control animals without cell transplants (Fig. 6, C), which showed fibrous tissues and only very small amounts of bone at the site of the defect (Fig. 6, D). Histomorphometrical analysis confirmed that the bone area generated with cell transplants was more extensive than that with no cell transplants (Fig. 6, E). There was no significant difference among the 3 cell transplants (HDF1, HDF2, HDF3). Osteocalcin immunostaining showed formation of bone matrix surrounded by osteoblasts in all HDF implants (Fig. 7, A-C).

**DISCUSSION**

Dental follicle cells are a relatively recently identified source of multipotent precursor cells that are an attractive source of cells for tissue engineering because of the ease of harvest of cells. Here, we show that human dental follicle obtained between the ages of 18 and 25 years contains at least 3 distinct cell populations that exhibit very different cell morphologies, patterns of gene expression, differentiation potential, and mineralization potential under defined culture conditions.

Under nondifferentiating culture conditions, only HDF2 cells displayed the fibroblastlike morphology that is also seen in PDL and BMSC; the other 2 cell types had very different morphologies. There was marked heterogeneity in terms of cell size, with HDF1 cells being comparatively very small. Cell size has been related to cell cycle, cell proliferation, and cell...
We then assayed the proliferation potential of the 3 HFD clonal populations and found that the HDF1 cells had a higher proliferation potential than HDF2 and HDF3 cells. It has been reported that smaller cells are more likely to have stem cell properties, whereas larger cells are more likely to be differentiated. Our data were consistent with these findings in terms of cell size and cell proliferation.

Mature chondrocytes, osteoblasts, and adipocytes are believed to arise from a common stem/progenitor cell, and resident progenitor cells were recently identified in human dental follicle. Two studies suggested that human dental follicle and periodontal stem cells could specifically differentiate along osteogenic and adipogenic pathways. In contrast, Lindroos et al. reported that human dental follicle cells could differ-
entiate only to the osteogenic lineage, whereas Kemoun et al. showed human dental follicle cells differentiating into osteoblasts, chondrocytes, and adipocytes. Based on these results, the differentiation capacity of these dental follicle stem cells remains poorly characterized. All 3 cell populations identified in the present study could differentiate along the osteogenic lineage, but with very different potentials; HDF1 cells showed the highest osteogenic differentiation potential. All 3 cell populations could also be driven to differentiate into adipocytes; HDF3 cells had the highest propensity to differentiate along this pathway; however, none of the 3 clonal populations showed chondrocyte determination under culture conditions known to produce chondrocyte lineage cells from other stem cell populations. The discrepancies of these data including previous and present results on differentiation capacity in human dental follicle cells may reflect the heterogeneous dental follicle cell populations. Osteoblast, chondroblast, and adipocyte lineages have been shown to express distinct transcription factors during cell specification. These transcription factors include CCAAT (cytidine-cytidine-adenosine-adenosine-thymidine)/Enhancer binding protein (C/EBP) alpha and peroxisome proliferator-activated receptor gamma in adipocytes, core-binding factor alpha 1 (Cbfal/Runx2) and osterix in osteoblasts, and Sox9 in chondrocytes. These lineage-specific transcription factors can also inhibit differentiation of other lineages by suppressing gene expression. We showed that all 3 clonal cell populations expressed the osteoblast marker Runx2, which was not expressed by PDLC or BMSC cells. These 3 HDF cell populations may therefore have a more restricted differentiation potential than PDLCs and BMSCs, which can also be driven to differentiate into chondrocytes, unlike the HDF populations we have identified. HDFs showed other differences to PDLC and BMSC; periodontal ligament marker genes such as peristin and MSX2 were only weakly expressed in HDFs.

One exciting potential application of tissue engineering is bone regeneration. In this study, the clonal HDF populations were remarkably different in terms of mineralization characteristics in vivo (Fig. 6). HDF1 and HDF2 cells were apparently more responsive to in vivo osteogenic differentiation in HDF3 cells using a pellet culture system. The pellet culture system can facilitate a 3-dimensional growth environment in which, for example, the secretion of ECM to form a scaffold may potentiate cell differentiation. On the other hand, we cannot be sure whether the matrix within the transplanted pellet would act as a scaffold for bone formation. Although HDF3 cells produced quantitatively less bone than the HDF1 and HDF2 populations, there was still more bone than seen in controls, suggesting that all 3 HDF populations may be induced to form osteoblasts with osteocalcin expression (Fig. 7). Our results demonstrate that heterogeneity in dental follicles does not affect the ability of this tissue to form bone. Our previous study examined the osteogenic capacity of the porcine dental follicle cells in comparison with that of porcine periodontal ligament-derived cells or bone marrow-derived mesenchymal stem cells. The results suggest that there were no significant differences in areas of new bone formation in the transplant. Based on this and previous studies suggests that dental follicle stem cells may provide a cell source for tissue engineering of bone. However, further studies are needed to determine whether such transplanted cells could directly differentiate into osteoblasts with osteocalcin expression.

REFERENCES


