Multilineage Cells from Apical Pulp of Human Tooth with Immature Apex

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Abstract: The human tooth with immature apex is a developing organ available for investigation. In this tooth, especially in the apical pulp, the proliferation and differentiation of various cells are activated to make a complete tooth. We investigated the notion that unique cells are included in the apical pulp of human tooth with immature apex. Human impacted third molars with immature apex freshly extracted for orthodontic reasons or treatment were obtained. Histological analyses revealed that BrdU-incorporating cells and cells positive for the mesenchymal stem cell markers SH2 and SH3 were located in the same region. The cells from the apical pulp of a human tooth with immature apex, designated here as apical pulp-derived cells (APDCs), can be cultured easily in vitro under ordinary serum-supplemented culture condition. The expression of surface markers of expanded APDCs is similar to that of bone marrow mesenchymal stem cells, except for CD49d (α4-integrin). APDCs differentiated into mineralized cells, adipocytes, chondroblasts and neural cells in vitro. APDCs have a high capacity for proliferation and multilineage potential in vitro. Our results indicate that human tooth with immature apex is a precious tissue source for the research of human adult stem cells and for the advancement of dental and regenerative medicine.

Key words: apical pulp, dental pulp, developing tooth, multilineage cells

Introduction

Stem cells have the unique capacity to renew themselves and the ability to form specialized cell types. Recently, stem cells have received much attention in the fields of developmental biology, cell biology and clinical research. An adult stem cell is an undifferentiated cell that can differentiate into the specialized cell type of...
the tissue from which it originated. The most commonly studied adult stem cell type is that from bone marrow; hematopoietic stem cells which form blood and immune cells, and mesenchymal stem cells which normally form bone, cartilage and fat. Many researchers believe that adult stem cells are present in far more tissues organs than once thought and that these cells are capable of developing into more kinds of cells than previously thought. Multilineage cells have been isolated from adipose tissue, arterial wall, and umbilical cord blood. Interestingly, pluripotent mesenchymal stem cells demonstrated to be capable of differentiating in vitro to cells of the three germ layers were selected from adult murine bone marrow.

Dental pulp, a soft tissue of mesenchymal origin from neural crest cells, occupies the central chamber of the tooth. Structurally, the dental pulp is a loose connective tissue with fibroblasts, odontoblasts, collagen fibers, extracellular glycosaminoglycans, nerves and blood vessels. Its function is protective and reparative. The tooth responds defensively to severe erosion, caries and restorative procedures by stimulating the secretory activity of odontoblasts to form reparative dentin. When odontoblasts are irreversibly damaged, they are replaced by a second generation of newly differentiated odontoblast-like cells that give rise to a reparative dentin matrix. This suggests the presence of resting progenitor or stem cells in dental pulp, even though the exact origin of the cells responsible for secretion of the reparative dentin matrix has not been clearly identified. Recently, a multilineage population of cells was isolated from the dental pulp of a human mature permanent tooth and a human exfoliated deciduous tooth. The dental pulp is thought to be source of adult stem cells.

In this study, we focus attention on the apical pulp of a human tooth with immature apex. The human tooth with immature apex is a developing organ and a dynamic biological reaction including proliferation and differentiation of stem cells occurs in the apical pulp to complete root formation. The apical pulp of a human tooth with immature apex, therefore, could possibly include unique human adult stem cells. We report here the histological analysis of a human tooth with immature apex, and the proliferation and characterization of cells derived from the apical pulp.

Materials and Methods

1. Tooth and cell culture
The present study was approved by the Ethical Committee of Faculty of Dentistry, Tokyo Medical and Dental University, and all donors gave informed consent. Human impacted third molars with open apex freshly extracted for orthodontic reasons or treatment (n=30) were obtained at the Oral and Maxillofacial Surgery Clinic of Tokyo Medical and Dental University (Fig. 1A ~ C). For primary explant culture, apical pulp tissue not framed in by dentin (Fig. 1C) was separated from the tip of root, and cut into about 1 x 1 x 1 mm pieces. A piece of the apical pulp tissue was plated on a culture dish with Iscove’s Modified Dulbecco’s medium (IMDM) (Sigma Chemical Co., St. Louis, USA) supplemented with 10% fetal bovine serum (FBS) (Moregate FCS, Buckingham, UK) and 2 mM L-glutamine (Wako Pure Chemical Co. Ltd, Osaka, Japan). The medium was changed every 2 days. After 3 weeks of primary outgrowth culture, apical pulp-derived cells (APDCs) were detached by exposure to 0.25% trypsin and 0.05% EDTA. The cells were then seeded at a density of 2.0 * 10^5 cells on a 100-mm culture dish, and cultured with IMDM supplemented with 10% FBS and 2 mM L-glutamine (Wako). When the cells were reaching confluence, they were subcultured as above. The APDCs at third passage was used for each experiment.

2. Tooth organ culture and BrdU labeling
For labeling BrdU, the extracted teeth were immediately cultured in IMDM (Sigma) supplemented with 10% FBS and 2 mM L-glutamine (Wako) and 10 μM BrdU (Sigma) for 24 h. The teeth were then fixed in 4% paraformaldehyde at 4°C for 24 h and dental pulps separated from crown and root were embedded in Tissue Tek O.C.T. compound (Sakura Finetechical Co. Ltd, Tokyo, Japan). The 7 ~ 10 μm sections were used for hematoxylin-eosin and immunohistochemical staining.

3. Growth of APDCs
At primary explant culture, we marked the edge of the cell sheet on the bottom of the culture dish. The growth of APDCs was calculated by measuring the area of cell sheets using Scion Image at days 11, 13, 15, 17, 19 and 21.

MTT assay: The third passage of APDCs was seeded
at a density of 5000 cells/well in 96-well culture plates. MTT assay was performed every 2 days. Briefly, the cells were incubated in a solution containing 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide for 4 h at 37°C. After the solution was removed, 100 μl of 0.04 N HCl-isopropanol was added and the plate was shaken for 30 s. The plate was then subjected to measure absorbance at 570-nm test wavelength and 630-nm reference wavelength using a microplate reader (Bio-Rad Model 550). The calibration curve for the numbers of cells was prepared by the same MTT assay procedure using the same cells.

CFU-F assay: The APDCs was plated at 100, 500, 1000, 2000, 4000 and 6000 cells on 100-mm culture dishes, and cultured with IMDM supplemented with 10% FBS and 2 mM L-glutamine (Wako) for 14 days. The medium was removed, and the cells were rinsed twice with PBS, fixed with methanol and stained with Wright Stain solution (Wako). After staining, the cells were washed with distilled water, and the number of colonies was counted.

4. Cytokines
Recombinant human (rh-) bone morphogenetic protein 2 (BMP2), rh-nerve growth factor (NGF), rh-transforming growth factor β1 (TGF-β1) and rh-basic fibroblast growth factor (bFGF) were purchased from R&D Systems (Minneapolis, MN).

5. Antibodies
Antibodies against human nestin, Tuj-1 and Flk-1/KDR were purchased from R&D Systems (Minneapolis,
MN). Antibodies against human type II collagen were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against human antigens CD13, CD14, CD29, CD31, CD34, CD44, CD45, CD49b, CD49d, CD51, CD56, CD90, CD117, glycopolin-A (Gly-A), von Willebrand factor (vWF), glial fibrillary acidic protein (GFAP), nerve growth factor receptor-p75 (NGFR-p75/NTR) neurotrophic receptor) and BrdU were purchased from Becton Dickinson. Antibodies against human anti antibodies and analyzed with FACS Calibur.

Cells were detached and stained with FITC coupled % skim milk solution for bated in TBST/

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51 90, CD34, CD29, and CD31 expressed in

In vitro.

0 2000 renewal of media was done every 3 days for 3 weeks. We followed the methods previously reported with some modifications. Briefly, APDCs were plated at 2.0 × 10^5 cells on the 100-mm culture dish and cultured at subconfluency. Cells were then cultured in alpha-modified essential medium (α-MEM) (Sigma) supplemented with 10% FBS, 2 mM L-glutamin (Wako), 0.2 mM ascorbic acid (Wako), 1 mM β-glycerophosphate (Sigma), 100 nM dexametasone (Sigma) and 300 ng/ml rh-BMP2 (R&D Systems) for 10 days. Then the cells were cultured in the same medium without rh-BMP2 for up to 3 weeks. The medium was changed every 2 or 3 days for 3 weeks. For evaluation of mineralized matrix, cells were fixed with methanol for 10 min and stained with 1% Alizarin-red S (Wako) solution in 0.25% ammonia water for 2 min at room temperature. In ad-

6. Immunofluorescence

Cells were fixed overnight with 4% paraformaldehyde at 4°C and washed against TBST. Cells were incubated in TBST/5% skim milk solution for 30 min to prevent non-specific binding of antibodies. The slides and dishes were incubated with mouse primary antibodies against human nestin (1:50), mouse Tuj-1 (1:50), human GFAP (1:50), human type II collagen (1:50), BrdU(1:50), SH2 or SH3 for 1 h, followed by fluorescein isothiocyanate (FITC) or rhodamine-coupled goat antimi- mouse IgG secondary antibodies for 1 h. Between incubations, the slides and dishes were washed with PBS.

7. Flow cytometry

For cell surface antigen phenotyping, the third passage cells were detached and stained with FITC coupled antibodies and analyzed with FACS Calibur (Becton Dickinson).

8. In vitro differentiation

Mineralized cell differentiation: APDCs were plated at 2.0 × 10^5 cells on the 100-mm culture dish and cultured at subconfluency. Cells were then cultured in alpha-modified essential medium (α-MEM) (Sigma) supplemented with 10% FBS, 2 mM L-glutamin (Wako), 0.2 mM ascorbic acid (Wako), 1 mM β-glycerophosphate (Sigma), 100 nM dexametasone (Sigma) and 300 ng/ml rh-BMP2 (R&D Systems) for 10 days. Then the cells were cultured in the same medium without rh-BMP2 for up to 3 weeks. The medium was changed every 2 or 3 days for 3 weeks. For evaluation of mineralized matrix, cells were fixed with methanol for 10 min and stained with 1% Alizarin-red S (Wako) solution in 0.25% ammonia water for 2 min at room temperature. In ad-

dition, mineralized matrix was also evaluated by von Kossa staining using 5% silver nitrate (Wako) in a dark box for 30 min, followed by 3% sodium carbonate (Wako) for 2 min. Alkaline phosphatase (ALP) activity was determined in cell lysates obtained by treating cells with CelLytic™-M mammalian cell lysis/Extraction reagent (Sigma). The ELISA-based method was performed according to the manufacturer’s protocol (Sigma). Results are expressed in 10 μmol of p-nitrophenol produced per min and normalized to protein content. For quantitative Ca^{2+} determination, cell layers were washed three times with PBS, and decalcified with 0.6 N HCl for 24 h at 37°C. The concentration of calcium in HCl supernatants was measured by the o-cresolphthalein complex-one method using a Calcium C-Test Kit (Wako)^13. The protein content was measured using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions.

Adipogenic differentiation: We followed the method previously reported^2,3. The medium was changed every 3 days for 3 weeks. For oil-red O staining, cells were fixed with 4% formaldehyde, stained with oil-red O (Wako) for 15 min, then counterstained with Mayer hematoxylin (Muto Pure Chemicals Co., Tokyo, Japan) for 5 min.

Chondrogenic differentiation: We followed the method previously reported^4. The medium was changed every 3 days for 3 weeks. Chondrogenic differentiation was evaluated after pellets were fixed in 4% paraformaldehyde and embedded in O.C.T. compound. Blocks were cut and sections stained with Toluidin blue (Muto Pure Chemicals Co. Ltd, Tokyo Japan).

Neurogenic differentiation: We followed the methods previously reported^14 with some modifications. Briefly, APDCs were plated at 2.0 × 10^5 cells on the 100-mm culture dish and cultured at subconfluency, then the cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% FBS, 50 ng/ml rh-NGF (R&D Systems), 20 ng/ml rh-bFGF (Genezyme Technne), 1 mM dibutyryl cAMP (Sigma), 0.5 mM 3-isobuthyl-1- methyl xanthine (IBMX) (Sigma), and 10 μM retinoic acid. The medium was changed every 3 days for 2 weeks.

9. Total RNA isolation and RT-PCR

Total RNAs were extracted from cells by ISOGEN (Nippon Gene Co., Toyama, Japan). RT-PCR was per-
formed using SuperScript™ One-Step RT-PCR with a platinum tag (Invitrogen). Complementary DNA synthesis and pre-denaturation was performed for 1 cycle of 50°C for 30 min and 94°C for 2 min. After an initial denaturation, amplifications were performed at 94°C for 15 s, 55-61°C for 30 s, and 72°C for 1 min. Thermal cycling was 30-35 cycles in a Gene Amp PCR System 9700 (PE Biosystems). Primers sequences are shown in Table 1.

<table>
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<th>Table 1 Primers used for RT-PCR</th>
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<td><strong>Target cDNA</strong></td>
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<td><strong>Mineralized cell-specific genes</strong></td>
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<td>parathyroid hormone receptor (PTHr)</td>
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<td><strong>dentin sialophosphoprotein (DSPP)</strong></td>
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<td><strong>Osteocalcin (OCN)</strong></td>
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<td><strong>Neural cell-specific genes</strong></td>
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Results

1. Histological features of apical pulp tissue of a tooth with immature apex

The coronal pulp of young human teeth has four layers; the odontoblastic layer, the cell-free zone (the zone of Weil), the cell-rich zone at the periphery and the center of pulp (Fig. 1 D, E). The cell-free zone separates the cell-rich zone from the odontoblastic layer (Fig. 1 D, E). The apical pulp with immature apex does not, however, form these layer structures (Fig. 1 F). We divided apical pulp into three regions; the dentin-apical pulp junction (DAJ), the central apical pulp (CA) and the tip of the apical pulp (TA) (Fig. 1 G). DAJ is a cell-rich region, bordered by formed dentin, that includes odontoblasts. CA contains large blood vessels and nerve trunks. TA is a cell-poor region, located in the apex of the root and not framed in by formed dentin.

The cells in DAJ and the perivascular region of CA incorporated BrdU (Fig. 2 A, D). Immunofluorescence of the mesenchymal stem cell markers SH2, SH3 showed that cells in DAJ and the perivascular region of CA expressed both SH2 (Fig. 2 B, E) and SH3 (Fig. 2 C, F). Neither BrdU-incorporating cells nor expression of SH2 or SH3 was found in TA (Fig. 2 G).

2. Cell culture

To obtain and characterize cells derived from the apical pulp of immature apex, primary outgrowth cell culture using TA was performed. The fibroblast-like cells were detected around tissue plated on a culture dish at day 4 or 5. The cells formed a cell sheet and grew concentrically, maintaining harmony of division and movement while coming into contact each other (Fig. 3 A). Growth rate was calculated by measuring the area of the cell sheet using Scion Image. The area of the cell sheet at day 21 was nine fold \((8.56 \pm 1.80)\) that at day 11 (Fig. 3 B). On day 21, the total cell number propagated from a tissue explant of apical pulp was about \(3.5 \times 10^6\) \((3.48 \pm 0.70)\) with some differences among individuals (Fig. 3 C). The APDCs exhibited spindle-shaped cells

Fig. 2 Immunohistochemical analysis of apical pulp of human tooth with immature apex. Regions of (A ~ C) dentin-apical pulp junction (DAJ), (D ~ F) centre of apical pulp (CA) and (G ~ I) tip of apical pulp (TA). (A, D, G) BrdU-incorporating cells. (B, E, H) Expression of the mesenchymal stem cell marker SH2. (C, F, I) Expression of the mesenchymal stem cell marker SH3. Some sections (B, C, E, F, H and I) were counterstained with DAPI nuclear staining. Original magnification: B, C, E, F, H, I; \(\times 100\). A, D, G; \(\times 200\).
Fig. 3  Morphology and growth of APDCs.
(A): APDCs around explanted tissue in primary outgrowth culture. (B): Growth of APDCs in primary explant culture, measured by area of cell sheet using Scion Image (N=10). (C): Total cell number from one piece (about 1×1×1 mm) of apical pulp tissue at day 21 in primary outgrowth cell culture. (D F): Morphology of detached and replated APDCs. (G): Colonies of APDCs stained with Wright Stain solution. (H): Cell clusters of APDCs formed a single colony. (I): MTT assay in third passage (N=3). Results are expressed as means ± SD. (J): CFU-F assay in third passage (N=6). Results are expressed as means ± SD. Original magnification: G; ×40, E, F, H; ×100, D; ×200.
(Fig. 3D ~ F), and rapidly grew in vitro (Fig. 3I). The cells had come to confluence about at 8 days and reached high confluence at 10 days, therefore the growth rate from day 8 to day 10 decreased (Fig. 3I). The CFU assay revealed the number of colonies was proportional to the seeded cell number (Fig. 3G, H, J). The colonies were formed even though APDCs were replated at low density, 100 cells per 100 mm dish. These results indicated the high proliferation capacity of APDCs.

3. Flowcytemetric analysis of APDCs

Flowcytemetric analysis was performed to evaluate the expression of surface markers of APDCs. The expanded APDCs were negative for CD14, CD34, CD45, Gly-A, CD117, indicating that these cells are not of hematopoietic origin. The expanded APDCs were also negative for endothelial cell’s marker (CD31, Flk-1/ KDR, vWF). In contrast, APDCs were found to be positive for integrins CD29 (β1-integrin), CD49b (α2-integrin), CD49d (α4-integrin), CD51/61(αvβ3-integrin), and positive for CD13, CD44, CD56, CD90, NGFR-p75 (NTR, neurotrophin receptor). In addition, h-MSCs markers, SH2 (CD105) and SH3 (CD73) were positive (Fig. 4).

4. Multilineage potential of APDCs

1) Differentiation into mineralized cells in vitro

The cells cultured in mineralization-promoting medium formed nodules, and the nodule stained intensely with Alizarin red stain (Fig. 5A, B) and von Kossa stain (Fig. 5C, D). The treated cells had a 2.5- to 5-fold alkaline phosphatase activity in untreated cells (Fig. 5E). The sensitive colorimetric quantitative calcium assay showed calcium deposition in the treated cells (Fig. 5F).

RT-PCR analysis revealed that various marker proteins characteristic of mineralized cells such as PTHr, Cbfa1, DSPP, and OCN were up-regulated in treated cells (Fig. 5G). These data demonstrated that APDCs possessed the ability to differentiate into mineralized cells such as osteoblasts and odontoblasts. DSPP was regarded as a specific marker for odontoblasts. However, recent studies indicate that DSPP is more widely distributed than originally thought, and the presence of DSPP in osteoblasts was observed. It is not clear whether mineralized cells differentiated from APDCs in vitro were osteoblasts or odontoblasts.

2) Adipogenic differentiation in vitro

To determine whether APDCs undergo adipogenic differentiation, the cells were cultured in adipogenic medium. Multiple, intracellular lipid-filled droplets were formed and visualized by staining with oil-red O, and the oil-red O containing cells exhibited an expanded morphology (Fig. 6B). No lipid droplets were observed in undifferentiated APDCs as negative control (Fig. 6A). These results indicate that APDCs undergo adipogenic differentiation.

3) Chondrogenic differentiation in vitro

A pellet culture technique was employed to trigger APDCs toward the chondrogenic lineage. The cells in the treated group formed a pellet that had a spherical and glistening transplant appearance (Fig. 6C). The development of cartilage matrix, shown by staining proteoglycans with Toluidine blue, revealed the chondrogenic nature of differentiated cells from APDCs (Fig. 6D, E). The cartilage extracellular protein, type II collagen, was detected in the matrix of the cell pellet with immunohistochemistry (Fig. 6F, G).

4) Neurogenic differentiation in vitro

Neurogenic induction resulted in a change in the mor-
Fig. 5  Differentiation of APDCs into mineralized cells. APDCs were cultured in maintenance medium (A, C) or mineralized cell differentiation medium (B, D) for 21 days. (A, B) Arizarrin red staining. (C, D) von Kossa staining. In the differentiation group, a calcified extracellular matrix was present and positive for alizarin red and von Kossa staining (B, D). (E) Alkaline phosphatase (ALP) assay (control: white square, differentiation: black square). Results are expressed as means ± SD. (F) Amount of calcium deposition at 21 days (control: white square, differentiation: black square). (G) Expression of mineralized cell-specific genes. Parathyroid hormone receptor (PTHr), type I Collagen (Co I ), Cbfa-1, osteopontin (OPN), bone sialoprotein (BSP), dentin sialophosphoprotein (DSPP), osterix and osteocalcin (OCN) gene were examined by RT-PCR at 21 days after differentiation induction. Original magnification: A ~ D; ×100

Fig. 6  Differentiation of APDCs into adipogenic lineage and chondrogenic lineage. Adipogenic differentiation of APDCs; intracellular oil droplets formed and were stained red by oil Red O. Control group (A) was not stained by oil Red O. However, in differentiation group (B), oil Red-O staining of the lipid vesicles performed 3 weeks after stimulation demonstrates ongoing adipogenesis. Chondrogenic differentiation of APDCs; (C) Representative macro picture of chondrosphere. (D, E) Tissue sections stained with Toluidine Blue. (F,G) Immunohistochemistry for type II collagen. In the differentiation group (E), the acidic mucopolysaccharide was stained with Toluidine blue. Type II collagen that is a chondrocyte-specific protein was stained in differentiation group (G). D and F were control groups for each experiment. Original magnification: A, B, D, E; ×200. F, G; ×100
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Phylogeny of cells. Neuro-induced APDCs acquired the morphology exhibiting a refractile cell body with extended cellular processes, and differentiated cells arranged into a neural-network like structure (Fig. 7A ̸ C). Immunofluorescence analysis showed that neuro-induced APDCs were stained for Nestin, an early...
marker of neural progenitor cells (Fig. 7D, G), Tuj-1, a neuron-specific β-tubulin III (Fig. 7E, H) and GFAP, a marker of glial cells (Fig. 7F, I). APDCs in pre-induction culture, however, also were stained for Nestin, Tuj-1 and GFAP (data not shown). RT-PCR analysis confirmed the expression of Nestin, β-tubulin III and GFAP, but the expression of Nestin and GFAP decreased during neurogenic induction (Fig. 7J). Additionally, APDCs in pre-induced culture expressed Musashi1, an RNA-binding protein expressed in neural precursor cells, TrkA, a high affinity nerve growth factor receptor, and MAP2 (microtubule-associated protein 2), a marker for mature neuron. The expression of Nestin, Musashi1 and Trk A decreased during neurogenic induction (Fig. 7J). After 7 days under differentiating conditions, the expression of NeuroD1, a neurogenic basic helix-loop-helix transcription factor, was induced and decreased at 14 days (Fig. 7J). It is known that NeuroD1 induces neuronal differentiation and suppresses glial differentiation28,29. Concurrent with the induction of NeuroD1 expression, we observed increased expression of marker for the mature neuron, MAP2 and decreased expression of the marker of glial cells, GFAP (Fig. 7J). These results indicate that APDCs differentiated into neurons, not glial cells under neurogenic induction.

Discussion

Many adult organs contain populations of stem cells that have the capacity for renewal after trauma, disease, or aging. The frequency of stem cells in adult organs, however, is low. For example, human mesenchymal stem cells are present as a rare population of cells in bone marrow, representing perhaps 0.001% to 0.01% of nucleated cells1. The frequency of stem cells in developing organs may be higher than that in mature organs, but human organs on development is unavailable. The identification, isolation and purification of human stem cells are fraught with difficulty due to their low frequency and ethical issues. The human tooth with immature apex is developing organ, and self-renewal and differentiation of stem cells therein are activated to make a complete tooth. It is conceivable that stem cells or progenitors of various kinds of mature cells at any stage during differentiation are present in a tooth with immature apex. Third molars with immature apex are frequently extracted in orthodontic practice and are, therefore, a valuable and rare developing human organ that is constantly available.

Our histological analysis showed that BrdU-incorporating cells and SH2- and SH3-positive cells were located in the same region of DAJ and the perivascular region of CA. These results suggest that stem cells or progenitor cells exist and proliferate in these regions. This notion is supported by previous studies showing that the perivascular region was a niche of mesenchymal stem cells in bone marrow and dental pulp12,30. The perivascular progenitor or stem cells proliferate in response to dental pulp injury, and these proliferating cells migrate to pulpal injury sites22. On the other hand, TA was a cell-poor region, and there were fewer BrdU-incorporating cells and much weaker expression of SH2 and SH3 compared to DAJ and CA. We thus consider that the signal for cell growth and differentiation was activated in DAJ or CA, but not in TA. TA may be more primitive tissue than DAJ or CA at the thought of the fact that development of root and dental pulp proceeds toward apical site. The cell culture in vitro is considered a factor in the loss of the multilineage potential of stem cells. The primitive tissue is better for cell culture in vitro to obtain multilineage cells. TA is not framed in by hard tissue, therefore it is easy technically to take the pulp tissue from TA. Although it is not known that which kind of cells grow and differentiate in TA during root formation, we attempted cell culture from TA not framed in by dentin to obtain multilineage cells.

APDCs can be cultured easily in vitro under ordinary serum-supplemented culture condition, and have a high capacity for proliferation. APDCs are capable of providing enough cells for potential clinical application. This may be attributed to the early developmental state of apical pulp. The expression of some markers alters during cell culture in vitro. To take our examples, the expression of SH2 and SH3 was very weak in TA, but these expression of cultured APDCs were detected obviously. And the expression of BSP, which is marker of mineralized cells, was detected in undifferentiated APDCs. It was reported that different isolation or culture methods give rise to different populations or lineage cells during cell culture in vitro31. Cell culture condition is to be noted when the character of cultured cell is evaluated.

From the results of flow cytometric analysis the phenotype of expanded APDCs is similar to that of bone
marrow mesenchymal stem cells, except for CD49d (α4-integrin). APDCs express NGFR-p75 (NTR), CD49d (α4-integrin), Nestin and Musashi1. Neural crest stem cells also express NGFR-p75 (NTR), CD49d (α4-integrin), Nestin and Musashi1. Dental pulp is derived from neural crest cells and other types of cells, and it is known that neural crest cells contribute to odontoblasts, cementoblasts, osteoblasts and chondrocytes in the cranio-facial region. It is not clear that APDCs include mesenchymal stem cells or neural crest stem cells from only the results of marker expression. The origin of APDCs is very interesting and important point for future study.

APDCs exhibited multilineage potential in vitro, differentiating into odontogenic/osteogenic, adipogenic, chondrogenic and neurogenic lineage when cultured in the presence of established lineage-specific factors. This multilineage potential of APDCs may be derived from the presence of (1) multipotent stem cells, (2) several lineage-committed precursor cells, such as preodontoblasts/preosteoblasts, preadipocytes, prechondroblasts or neural precursor cells, or (3) a combination of the above. We cannot conclude the presence of multipotent stem cells in APDCs because APDCs are a heterogeneous cell population. Stem cells may be responsible for the phenomenon of developing multiple cell types. As dental pulp includes odontoblasts, nerves and schwann cells, there is a possibility that preodontoblasts and neural precursor cells are included in APDCs. In contrast, bone, fat or cartilage does not occur naturally in dental pulp. It is unlikely that preosteoblasts, preadipocytes or prechondroblasts are included in APDCs.

Recently, populations of multilineage cells, DPSCs (Dental Pulp Stem Cells) and SHED (Stem cells from Human Exfoliated Deciduous teeth), were found to be derived from whole pulp of human permanent teeth and remnant coronal pulp of human exfoliated deciduous teeth, respectively. Both DPSCs and SHED could differentiate into mineralized cells, adipocytes and neural cells in vitro. The cells derived from human dental papilla were also able to differentiate into mineralized cells. APDCs were able to differentiate into mineralized cells, and cells of adipogenic, chondrogenic and neurogenic lineage in vitro. Previous reports on cells derived from dental pulp have no description about the expression of NGFR-p75 (NTR) or CD49d (α4-integrin), which is related to neural crest stem cells. These differences between APDCs and other dental pulp-derived cells are important points for further study. It is also reported that human periodontal ligament contains a population of multipotent postnatal stem cells that have potential to generate a cementum/periodontal ligament in vivo. These cells from teeth will contribute to the tissue engineering of teeth.

Most of the basic research discoveries concerning embryonic and adult human stem cells come from animal, and particularly mouse models. Although more is known about mouse cells, not all of the information can be translated to the understanding of human cells. For regenerative medicine, research using primary human cells is indispensable. For basic or clinical research using human primary cells, we must obtain cells from an easily accessible tissue resource without ethical problems. As shown in the present report, a human tooth with immature apex is a valuable and rare developing organ that has constant availability, and cells derived from the apical pulp of the tooth exhibited multilineage potential. Human tooth with immature apex will be an important contribution to the research of human adult stem cells and to the advancement of dental and regenerative medicine.

Reference
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