DPSCs from Inflamed Pulp Modulate Macrophage Function via the TNF-α/IDO Axis

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APPENDIX

Isolation and Culture of DPSCs/I-DPSCs

Extracted healthy teeth were cleaned with iodine and scaled thoroughly to remove all periodontal and periapical tissue. A longitudinal groove was made around the cementum-enamel junction in the extracted teeth by using sterilized dental fissure burs without reaching the pulp tissue. The teeth were fractured with dental surgical extraction forceps and healthy pulp tissue was gently separated with a sterile dentinal excavator from the crown and root. To prevent contamination of the surgical field during the root canal treatment, the rubber dam was applied to only one tooth which needed a root canal treatment. Rubber dam and external surface of tooth were thoroughly cleaned with 5% tincture of iodine before accessing the pulp chamber. Right before accessing the pulp chamber, the external surface and access cavity of tooth were cleaned with 3% sodium hypochlorite. Inflamed pulp tissues were collected from pulp chambers and root canals with a sterile spoon excavator and endodontic file after complete exposure of pulp chamber from teeth diagnosed as teeth with irreversible pulpitis and going through root canal treatment. Extirpated pulp tissues were immediately transferred into sterile culture medium (α-Minimum Essential Medium–α-MEM) with 100 units/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL fungizone (Gibco, Grand Island, NY, USA). Cells were isolated and cultured as described previously (Gronthos et al., 2000). Briefly, collected pulp tissues were washed with phosphate-buffered saline (PBS) three times, minced and digested with 3 mg/mL of collagenase type I and 4 mg/ml dispase (Gibco) for 1 h at 37°C with gentle shaking. After enzymatic digestion, cell suspensions were washed by centrifugation (10 min at 400×g) in culture medium and placed in culture dishes. Cells were then incubated in α-MEM supplemented with 10-20% fetal bovine serum (FBS; Gibco), 2 mM

L-glutamine, 100 µM L-ascorbic acid-2-phosphate, and the above mentioned antibiotics at 37 °C in a 5% CO_2 humidified atmosphere. For all cultures, the medium was renewed every 2-3 days. When sub-confluence (80-90%) was achieved, adherent cells were detached with trypsin (Gibco) and expanded by replating at a lower density. After approximately 7-10 days, primary cells were passed to second passage (named passage 1, P1). The same passage of cells (P3-P5) was used for each experiment. Stem cells from normal pulp tissues were named DPSCs and from inflamed pulps I-DPSCs.

Colony-Forming Unit-Fibroblasts (CFU-F) Assay

Single-cell suspensions of dental pulp tissue were seeded into 6-well culture plates at 3.0×10^3 cells/well in clonogenic growth medium (α-MEM-20% FBS; Gibco). At day 12 cultures were fixed in 1% paraformaldehyde, and stained with 0.1% toluidine blue. Aggregates of greater than 50 cells were scored as colonies using a dissecting light microscope.

Flow Cytometric Analysis

DPSCs and I-DPSCs (1×10^5) were incubated with specific monoclonal antibodies against STRO-1, CD14, CD29, CD34, CD44, CD73 (BD Bioscience, San Diego, CA, USA), CD90, and CD146 (BioLegend, San Diego, CA, USA) for 1 h. After washing with PBS, cells were incubated with fluorescein-isothiocyanate-conjugated (FITC) secondary antibodies for 30 min in the dark. Cells were analyzed on a BD™ LSR II flow cytometer (BD Bioscience).

Multilineage Differentiation Induction Assay

In vitro expanded DPSCs/I-DPSCs (5×10^4) were seeded in triplicate 24-well plates and cultured. When 100% confluency was achieved odonto-osteogenic inductive medium (α-MEM supplemented with 10% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, 100 nM dexamethasone, 100 µM L-ascorbic acid 2-phosphate, and 10 mM β-glycerophosphate) was replaced and changed every 3 days for 3 weeks. Cell cultures were fixed and assayed for mineralized deposits of calcium by Alizarin Red S staining (Fisher scientific, Pittsburgh, PA, USA). For adipogenic differentiation, cells (5×10^4) were seeded in triplicate 24-well plates and cultured. When 80-90% confluency was reached adipogenic inductive medium (α-MEM supplemented with 10% FBS, 50 U/ml penicillin, 50

µg/ml streptomycin, 100 µM L-ascorbate-2-phosphate, 100 nM dexamethasone, 0.45 mM 3-isobutyl-1-methylxanthine, and 60 µM indomethacin) was added and changed every 3 days for 2 weeks. Oil Red O staining (Sigma-Aldrich, St Louis, MO, USA) was used to identify intracellular lipid vesicles in mature adipocytes. For both differentiations, cells in negative control groups were grown in α-MEM with 10% FBS for 3-2 weeks.

THP-1 Cell Culture and Stimulation

The human monocyte cell line, THP-1 cells was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI 1640 containing 10% heat inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), and antibiotics at 37 °C in 5% CO_2. Differentiation of THP-1 cells into macrophages was induced as described previously (Park et al. 2007; Daigneault et al. 2010). Briefly, THP-1 cells were incubated with 50 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St Louis, MO, USA) for 3 h. After the incubation, the PMA-containing medium was removed. Adherent differentiated cells (THP-1 macrophages) were washed with serum-free culture medium twice and then cells were used immediately or subsequent resting in PMA-free medium for 24 h.