Effects of Extracellular pH on Dental Pulp Cells *In Vitro*

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Abstract

Introduction: The proliferation and migration of dental pulp stem cells (DPSCs), a population comprised of dental pulp cells (DPCs), are important processes for pulp tissue repair. Dental pulp is exposed to changes in extracellular pH under various conditions, such as acidosis and exposure to caries-associated bacteria or a pulp capping agent. The objective of this study was to investigate the effects of extracellular pH on DPC proliferation and migration *in vitro*. **Methods:** To evaluate the proliferation potency of DPCs in various extracellular pH conditions, 2×10^4 cells were seeded into 35-mm dishes. The following day, we changed to $NaHCO_3$-free medium, which was adjusted to different extracellular pH levels. **Results:** After 120 hours, DPCs cultured in media from a pH of 3.5 to 5.5 showed cell death, those cultured in conditions from a pH of 6.5 to 7.5 showed growth arrest or cell death, and those grown at a pH of 9.5 showed mild proliferation. The migratory activity of living DPCs was not affected by extracellular pH. For histologic analysis, human teeth possessing a small abscess in the coronal pulp chamber were sliced for histologic analysis. Proliferating cell nuclear antigen (PCNA) immunolocalization was used as an index of cell proliferation for the sections and cultured cells. Acidic extracellular pH conditions resulted in reduced numbers of PCNA-positive DPCs in the dishes. As for pulp tissue affected by a small abscess, a PCNA-negative pulp cell layer was observed in close proximity to the infectious lesion. **Conclusions:** Together, these results suggest that an acidic extracellular pH condition is associated with DPC growth arrest or cell death. *(J Endod 2016;42:735–741)*

Key Words

Cell death, dental pulp cells, extracellular pH, growth arrest, migration, proliferation

Cell proliferation and migration are necessary for homeostatic tissue maintenance and regeneration of injured tissues (1). In cases of traumatic pulp injury (2) and in response to odontoblast injury (3), pulp progenitor cell proliferation and migration are important repair processes.

Dental pulp is exposed to changes in extracellular pH under various conditions, with a low level of extracellular pH (ie, tissue acidosis) frequently observed in areas of inflamed tissue (4). Furthermore, mineral trioxide aggregate (MTA) has an initial pH of 10.2, which rises to 12.5 at 3 hours after mixing (5). It is thought that dental pulp is exposed to acid from caries-associated bacteria during the process of abscess development. Lactic acid, the predominant microbial by-product in active carious dentin (88%), exhibits a low level of pH (mean = 4.9) (6). As noted previously, it is possible for dental pulp to be exposed to diverse extracellular pH conditions.

The presence of progenitor cells in pulp tissue, known as dental pulp stem cells (DPSCs), has been shown in many studies (7–9). Although it is well-known that bacterial metabolic by-products (10) and cell wall components (11, 12) exhibit toxicity toward pulp cells and elicit pulpal inflammation, little is known about the effect of extracellular pH on pulp cells. DPSCs are a population of mixed pulp cells termed dental pulp cells (DPCs) (13). The objective of this study was to investigate the effect of the extracellular pH level on DPC proliferation and migration *in vitro*.

Materials and Methods

This study was approved by the ethics committees of the National Center for Geriatrics and Gerontology, Obu, Japan; Nagoya University, Nagoya, Japan; and Aichi Gakuin University, Aichi, Japan. All experiments were performed in accordance with the strict guidelines of the gene recombination experiment safety committee.

Preparation of Acidic and Alkaline Media

Powdered $NaHCO_3$-free Dulbecco modified Eagle medium (DMEM) with a high level of glucose (Life Technologies, Carlsbad, CA) was dissolved in water and sterilized by autoclaving and then supplemented with 10% fetal bovine serum (FBS; AusGenex, Loganholme, QLD, Australia) and used as the cell medium. Media samples were adjusted to pH levels of 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, and 12.5 by titration with hydrochloric acid (Wako, Osaka, Japan), and sodium hydroxide solution (Kanto Chemical, Tokyo, Japan) was added (Fig. 1*A*, *upper*). For migration assays, we adjusted $NaHCO_3$-free and serum-free DMEM to pH levels of 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5, and 12.5 by titration. DMEM containing $NaHCO_3$ (Nakarai, Kyoto, Japan) was prepared following the manufacturer's guidelines. pH was measured using an F-53 pH electrode

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(Horiba, Kyoto, Japan). All media were sterilized by filtration through a 0.22-μm membrane filter (Millex-GV; Millipore, Carrigtwohill, Ireland).

Cell Isolation and Culture Conditions

Normal human third molars were collected from 4 healthy adults (men 20–32 years old) during treatment at Aichi Gakuin University Dental Hospital under guidelines approved by the school of dentistry of that university. Using a form approved by the ethical committee, informed written consent was obtained from all enrolled subjects to participate before sample collection. DPCs were isolated from human dental pulp tissue using an enzymatic method, as previously described (14). Briefly, pulp tissue samples were minced into pieces and enzymatically digested in 0.2% collagenase (Wako) for 30 minutes at 37°C and then passed through a 70-μm nylon mesh (Cell Strainer; BD Biosciences, San Jose, CA). Isolated cells were plated into 100-mm dishes (BD Biosciences) in DMEM supplemented with 10% FBS. DPCs were detached at 60%–70% confluence by incubation with 0.05% Trypsin-EDTA (Life Technologies) and subcultured at a 1:4 dilution under the same conditions. For the present study, all cells were cultured in a humidified atmosphere at 37°C with 5% CO_2 using an incubator. To evaluate the proliferation potency of DPCs under various extracellular pH conditions, 2×10^4 cells at the 4th to 5th passage of culture were seeded into 35-mm dishes in DMEM supplemented with 10% FBS. The next day, they were transferred to $NaHCO_3$-free DMEM supplemented with 10% FBS, which had been adjusted to various pH values by titration. The medium was changed, and the pH was measured every 2 days ($n = 4$) (Fig. 1*A*, *lower*). Viable cells were counted by trypan blue exclusion after 12, 24, 48, and 120 hours.

Flow Cytometric Analysis

DPCs were characterized after the 4th to 5th passage of growth using flow cytometric analysis. They were immunolabeled for 60 minutes at 4°C with mouse immunoglobulin (Ig) G1 (diluted 1:10) (fluorescein isothiocyanate [FITC]) (MOPC-21; BioLegend, San Diego, CA), mouse IgG2 (diluted 1:10) (FITC) (sc-2856; Santa Cruz Biotechnology, Santa Cruz, CA), rat IgG2b (diluted 1:10) (phycoerythrin heptamethine cyanines [PE-Cy7]) (RTK4530, BioLegend), and mouse IgG1 (diluted 1:10) (phycoerythrin [PE]) (MCA928PE; AbD Serotec Ltd, Oxford, UK) antibodies as a negative control as well as with antibodies against CD105 (diluted 1:10) (FITC) (MEM-229; Abcam, Cambridge, UK), CD29 (diluted 1:20) (FITC) (TS2/16; eBioscience, San Diego, CA), CD44 (diluted 1:20) (PE-Cy7) (IM7, eBioscience), CD31 (diluted 1:10) (PE) (WM59, BD Biosciences), and CD45 (diluted 1:10) (allophycocyanin [APC]) (HI30, BioLegend). All antibodies were diluted in Hank's balanced salt solution (Life Technologies) supplemented with 2% FBS and N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) (1:100) (Life Technologies). Flow cytometric analysis was performed using an FACSAria flow cytometer (BD Biosciences).

Induced Differentiation

The differentiation of DPCs after the 4th to 5th passage and human adult dermal fibroblasts (HADFs) (catalogue no. CC-2511; Lonza, Basel, Switzerland) into angiogenic, neurogenic, and odontogenic/osteogenic lineages was determined as previously described (8). Briefly, for endothelial cell differentiation, DPCs and HADFs (1.0×10^4 cells/50 μL) were separately seeded into 96-well plates in Matrigel inducer medium (BD Biosciences). Network formation was observed after 6 hours of cultivation. For neuronal differentiation, DPCs and HADFs (1.0×10^5 cells/dish) were separately cultured in noncoated 35-mm dishes in Neurosphere Progenitor Basal Medium (Lonza) designed for neurosphere formation for 14 days. The resulting neurospheres were triturated using polished glass pipettes, and single-cell suspensions were obtained and cultured in gelatin-coated 35-mm dishes (Iwaki, Shizuoka, Japan) in inducer medium with the medium changed every 3 days. For immunocytochemical analysis, cells were fixed for 15 minutes in 4% paraformaldehyde (Wako) in PBS at room temperature and then reacted with a mouse monoclonal neurofilament protein antibody (2F11; Dako, Carpentaria, CA) (1:100) for 1 hour at room temperature followed by a biotinylated antimouse IgG secondary antibody (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. Cells were developed using VECTASTAIN Elite ABC reagent (Vector Laboratories) and ImmPACT DAB Peroxidase Substrate (Vector Laboratories). For differentiation into an odontoblast lineage, DPCs and HADFs (1.0×10^5 cells/dish) were separately cultured in 35-mm dishes in DMEM supplemented with 10% FBS, 50 mg/mL L-ascorbic acid 2-phosphate (Wako), 1 mmol/L inorganic phosphate (Sigma-Aldrich, St Louis, MO), and bone morphogenetic protein (kindly provided by Astellas Pharma Co, Ltd, Tokyo, Japan) at a final concentration of 100 ng/mL. The medium was changed twice a week. After 28 days, the cell layer was fixed with 4% paraformaldehyde (Wako), and alizarin red (Wako) staining was performed.

Conditioned Medium

We switched the culture medium of DPCs after the 4th to 5th passage and HADFs upon reaching 60%–70% confluence to DMEM without serum. After 24 hours, the conditioned medium (CM) was collected and concentrated approximately 20-fold using an Amicon Ultra-15 centrifugal filter unit equipped with an Ultracel-3 membrane (Millipore). The concentrated CM was adjusted to 5 μg/mL and placed into 96-well plates to analyze the expressions of cytokines and chemokines (Milliplex MAP, human cytokine/chemokine panel, Millipore) in accordance with the manufacturer's guidelines. The quantification of secreted factors was performed using a Bio-Plex 200 reader and Bio-Plex Pro wash station (Bio-Rad, Richmond, CA) and then analyzed with Bio-Plex Manager version 3.0 software (Bio-Rad). All data were normalized to /pg/mL/10^6 cells.

Migration Assays

The migratory activity of DPCs in various extracellular pH conditions was determined by a horizontal chemotaxis assay using a TAXIScan-FL (ECI, Kanagawa, Japan) as previously described (13). Briefly, DPCs were placed into a single chamber of the device, and concentrated DPC CM as a chemotactic factor was placed into the opposite chamber. Serum- and $NaHCO_3$-free DMEM was used as culture media after adjusting to various pH levels. The number of migrating cells and farthermost cell migration distance were determined after 12 hours.

Histologic Analysis of Asymptomatic Irreversible Pulpitis in Teeth with a Small Abscess in the Coronal Pulp Chamber

Human third molars with a small abscess in the coronal pulp chamber were collected for histologic analysis from 3 patients whose clinical presentation was asymptomatic. After extraction, the teeth were fixed in 4% paraformaldehyde (Nakarai) at 4°C for 2 days and embedded in paraffin wax (Sigma-Aldrich) after demineralization with Kalkitox (Wako) at 4°C for 1 week. The