Hypoxia-amplified Proliferation of Human Dental Pulp Cells

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Abstract

Introduction: Postnatal human dental pulp is a potentially promising source of progenitor cells. Sustaining and amplifying progenitor cell populations would be beneficial for basic science research with application in pulpal regeneration. Hypoxia has been observed to promote the undifferentiated cell state in various stem cell populations. The purpose of this study was to examine human dental pulp cells (DPCs) proliferation in normoxia and hypoxia. **Methods:** Dental pulp cells were obtained from third molars of adult patients and cultured in alpha modification of Eagle's medium culture medium with 10% fetal bovine serum. For cell proliferation, DPCs were divided into two groups: (1) DPCs incubated in normoxic conditions (20% oxygen tension) and (2) DPC incubated in hypoxic conditions (3% oxygen tension). Cell proliferation assays were performed every 2 to 3 days from day 3 to day 14 by trypsinization and quantification of cells with a hemacytometer. Fluorescence-activated cell sorting analysis was completed to investigate stem cell markers, CD133, and STRO-1. **Results:** DPCs proliferated significantly more in hypoxia than in normoxia (ie, two-fold throughout the experiment, $p < 0.0001$). The primitive stem cell marker, CD133, decreased in hypoxia, whereas the osteoprogenitor marker, STRO-1, increased by 8.5-fold. **Conclusions:** This study suggested that hypoxia is an effective treatment to amplify numbers of progenitor cells from human dental pulp. *(J Endod 2009;35:818–823)*

Key Words

Cell proliferation, dental pulp cells, hypoxia, pulp regeneration

Promoting osteogenesis to compensate for physiologic and pathogenic bone loss is a challenging problem. Although bone has the capacity to regenerate and repair, bone loss from endodontic infection, periodontal disease, or trauma may lead to loss of dentition if appropriate treatment is not applied. Numerous approaches have been used to promote bone formation. Bone allograft, xenograft, and alloplast are used extensively to avoid harvesting autogenous bone, yet all these alternatives have their shortcomings. Processed allogenic or xenogenic bone have similar biomechanical stability and elasticity when compared with native bone, yet the lack of osteogenicity is a limitation in these grafts (1). Advances in stem cell research cast hope on the issue. Recently, multipotent cells from adult dental pulp tissue, named dental pulp stem cells (DPSCs), were discovered by Gronthos et al (2). DPSCs have the ability to differentiate into various phenotypes such as osteoblasts, myoblasts, chondrocytes, and adipocytes (2–5). Considering their multipotent ability, researchers have high hope on using DPSC in regenerative endodontics (6). A recent comprehensive review by Hargreaves et al (7) provided a summary of various case reports featuring regenerative endodontics approach to treat necrotic immature permanent teeth. They also suggested prospective applications of tissue engineering concepts to regenerative endodontics. The triad components of regenerative endodontics consist of a cell source, a physical scaffold, and signaling molecules. The appropriate cell source is one that can differentiate into required cell types and is abundant, convenient for harvesting, and autogenous to avoid immunologic reaction (7). Cordeiro et al (8) elaborately showed engineered tissue from stem cells from human exfoliated deciduous teeth that resembled physiologic dental pulp (8). As for a physical scaffold, Gebhardt et al (9) recently showed that polymer and bovine collagen scaffolds provided superior outcomes than calcium phosphate scaffolds in creating pulp and periodontal constructs with DPSCs and periodontal ligament stem cells (9).

Recent evidence also suggests that stem cells are localized in areas with low oxygen tension (10, 11). Work on hematopoietic and neural stem cells showed that culturing progenitors in hypoxic condition (3%-5%) increased the number of multipotent clones when compared with normoxic cultures (21% O_2) (12, 13). In addition to effects on differentiation and cell fate, hypoxia promotes survival and increases the proliferation of multipotent precursors (14). Gustafsson et al (15) have shown that hypoxia blocks neuronal and myogenic differentiation in a notch-dependent manner. Other studies also showed similar results with osteogenic and adipogenic differentiation (16, 17). The effect of low oxygen tension on the proliferation of cells from canine dental pulp has also been studied (18). To date, the hypoxic effect on human dental pulp cells (DPCs) has yet to be investigated.

A hypoxic condition shows promising results in fostering proliferation and maintaining the stemness of stem cells. The use of low pO_2 levels to enhance the *in vitro* survival and/or self-renewal of stem cells represents an important advance in stem cell research that could be critical for obtaining the great number of cells that would be needed for clinical application. This study was conducted to show that DPCs could be amplified by culturing under hypoxic conditions.

Materials and Methods

DPC Extraction

Impacted third molars were collected immediately after extraction from the oral surgery department and stored in phosphate-buffered saline (PBS). After wiping all teeth with 70% ethyl alcohol on sterile gauze, each root was cracked with a chisel and hammer. All the root pieces were placed in a 50-mL tube with medium (alpha

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modification of Eagle's medium [$\alpha - $MEM; GIBCO, Carlsbad, CA] with 2× penicillin-streptomycin). The pulp was carefully removed from the tooth with sterile pliers, keeping it as intact as possible. The pulp was cut it into small pieces (<1 mm) and resuspended by pipetting several times to shear the tissue. The pulp was put in a 50-mL tube and centrifuged at 1,000 rpm at 20°C for 8 minutes. The medium was removed and resuspended in 10 mL of $\alpha - $ MEM with 2× penicillin-streptomycin at 1,000 rpm at 20°C for 8 minutes. The cell pellet was mixed one more time in 20 mL of $\alpha - $ MEM with 1 × penicillin-streptomycin, seeded in a T-75 tissue culture flask, and incubated in 5% CO_2/95% air at 37°C in an incubator. After 1 week, the medium and the remaining floating pulp fragments were removed and centrifuged. Ten milliliters of fresh warmed medium was added to the pellet of pulp fragments. On day 14, cells that adhered to the flask were trypsinized and counted. These cells would be considered as P1 passage. Cells were stored by freezing in preservation media at −80°C until they were ready to be used.

Cell Cultures

Single-cell suspensions of DPCs were seeded with α-MEM supplemented with 10% fetal calf serum (FCS), 2 mmol/L of L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The medium was changed every 2 to 3 days. For normoxia, the cells were cultured in a controlled atmospheric incubator at 20% O_2 (control 95% air and 5% CO_2 v/v) at 37°C.

For hypoxic groups, DPCs in culture plates were put in the hermetic chamber (Billups-Rothenberg Inc, Del Mar, CA) into which 3% O_2 gas mixture (5% CO_2 v/v and balanced N_2; Airgas, Hingham, MA) was flushed for 5 minutes twice a day. The chamber was placed into a regular incubator as described earlier.

Cell Proliferation Assays

DPCs were plated in triplicate in the presence of the medium at 20,000 cells/cm^2 into 12-well plates. The normoxic group was placed in the regular incubator, and the hypoxic group was placed in the hermetic chamber for 14 days. To assess cell proliferation, cells were rinsed with PBS, detached with trypsin-EDTA solution, and quantified with a hemacytometer every 2 to 3 days from day 3 to 14. The cells were counted 3 times for each sample, and the numbers were averaged. The experiment was repeated 10 times.

Immunofluorescence Staining

DPCs were seeded at 20,000 cells/cm^2 and cultured in normoxic and hypoxic conditions for 3 days. The cells were then washed with PBS and fixed with 4% paraformaldehyde for 10 minutes. A quick rinse with PBS was followed by incubation with STRO-1 antibody (1:200; R&D Systems Inc, Minneapolis, MN) for 3 hours. After being washed twice, the cells were subsequently incubated with 1:100 rabbit antimouse immunoglobulin G Alexa Fluor-488 (Molecular Probes; Invitrogen, Eugene, OR) for 1 hour in the dark. The nuclei were then stained with 4'-6-Diamidino-2-phenylindole (DAPI) 1:1,000 for 30 minutes. Digital images of the cells were then recorded with a Nikon Eclipse TE2000 epifluorescence microscope (Nikon, Inc., Melville, NY) and a SPOT Digital Image System (SPOT Diagnostic Instruments, Sterling Heights, MI). Grayscale image files for different excitation/emission filter sets were stored in TIFF format, and files of the same fields were overlaid and pseudo-colored (RGB) to produce the figures shown in this report.

Fluorescence-activated Cell Sorting

To further analyze DPCs characteristics and behavior after normoxic and hypoxic treatment, cells were cultured under hypoxic and normoxic treatment for 7 days. After trypsinization, cells were divided in groups and stained with Hoechst 33342 dye (Immunochemistry Technologies, Bloomington, MN), CD133 (Miltenyi Biotec, Auburn, CA), and Stro-1 (R&D Systems, Minneapolis, MN) antibodies before sorting through a FACSAria (BD Bioscience, San Jose, CA) flow cytometry instrument. For control groups, cells were incubated with fumitremorgin C (5 μL/mL) for 30 minutes at 37°C to block cell membrane pumps that rejected Hoechst 33342. Cells were stained with Hoechst 33342 (10 μL/mL of media) for 1 hour at 37°C. After the Hoechst 33342 treatment, cells were put on ice and rinsed twice with media. Cells were resuspended at 4°C. Supernatant from the suspension was discarded, and fresh media was added to the cell pellets. Prelabeled CD133 antibody was added to the cell pellets on ice for 1 hour. After staining, cell pellets were resuspended in PBS supplemented with 2% fetal bovine serum (FBS) twice.

Stro-1 staining groups started with the same procedure of trypsinizing and suspending cell pellets. Monoclonal antihuman STRO-1 antibody 0.5 μL/mL was added to cell pellets on ice for 45 minutes. Cells were then rinsed with media and resuspended at 4°C. This step was repeated twice. A secondary antibody, Alexa Fluor 647 goat antimouse immunoglobulin M (Molecular Probe, Invitrogen, Eugene, OR), was added to the pellets on ice for another 45 minutes. The samples were rinsed and resuspended at 4°C again. The final solution of PBS supplemented with 2% FBS was added to the samples.

Statistical Analysis

Comparisons between the two groups were analyzed by an unpaired Student *t* test. The data are presented as the mean ± standard deviation (SD).

Results

Hypoxia Promotes Cellular Proliferation

To determine whether a hypoxic condition had an affect on cell proliferation, DPCs were plated at the same density and cultured under normoxic and hypoxic conditions over a span of 14 days. DPCs were collected from passage 6 and 7. From a preliminary study (results not shown), the difference in proliferation between passages was found to be insignificant. For quantification, cells were trypsinized and counted with a hemacytometer every 2 to 3 days from day 3 to 14. A significant increase in cell number was observed in the hypoxic group when compared with its normoxic counterpart on all assay days. From the starting point of 38,000 cells/well at day 3, the mean cell number increased slightly for normoxic groups to 58,750 cells/well (SD ± 15,526) and more than double amount of the original number to 86,520 cells/well (SD ± 10,606) in the hypoxic groups. The difference between the two groups was considered statistically significant ($p = 0.001$). On day 6, a significant difference in cell number between the two groups continued. Cells from the hypoxic group proliferated 2.42-fold more than their normoxic counterpart (normoxic = 82,500 cells/well (SD ± 13,887) vs hypoxic = 200,000 cells/well [SD ± 15,526] [$p < 0.0001$]). From 7 days onward, cells cultured in hypoxic condition (H) proliferated more rapidly than cells cultured in a normoxic condition (N), with the total cell number being 2.08-fold higher at 9 days (N = 147,500 cells/well [SD ± 13,887) vs H = 307,500 cells/well [SD ± 21,213] [$p < 0.0001$])], 2.06-fold higher at 12 days (N = 191,250 cells/well [SD ± 26,958) vs H = 393,750 cells/well [SD ± 61,164] [$p < 0.0001$])], and 2.04-fold higher at 14 days (N = 281,250 cells/well [SD ± 35,632] vs H = 575,000 cells/well [SD ± 121,655] [$p < 0.0001$]). Cells cultured in hypoxia proliferated approximately 2-fold higher than cells cultured in normoxia (Fig. 1).

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