Hypoxia Modulates the Differentiation Potential of Stem Cells of the Apical Papilla

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Abstract

Introduction: Stem cells from the apical papilla (SCAP) are a population of mesenchymal stem cells likely involved in regenerative endodontic procedures and have potential use as therapeutic agents in other tissues. In these situations, SCAP are exposed to hypoxic conditions either within a root canal devoid of an adequate blood supply or in a scaffold material immediately after implantation. However, the effect of hypoxia on SCAP proliferation and differentiation is largely unknown. Therefore, the objective of this study was to evaluate the effect of hypoxia on the fate of SCAP. **Methods:** SCAP were cultured under normoxia (21% O_2) or hypoxia (1% O_2) in basal or differentiation media. Cellular proliferation, gene expression, differentiation, and protein secretion were analyzed by live imaging, quantitative reverse-transcriptase polymerase chain reaction, cellular staining, and enzyme-linked immunosorbent assay, respectively. **Results:** Hypoxia had no effect on SCAP proliferation, but it evoked the up-regulation of genes specific for osteogenic differentiation (runt-related transcription factor 2, alkaline phosphatase, and transforming growth factor-β1), neuronal differentiation (2′-3′-cyclic nucleotide 3′ phosphodiesterase, SNAIL, neuronspecific enolase, glial cell-derived neurotrophic factor and neurotrophin 3), and angiogenesis (vascular endothelial growth factor A and B). Hypoxia also increased the sustained production of VEGFa by SCAP. Moreover, hypoxia augmented the neuronal differentiation of SCAP in the presence of differentiation exogenous factors as detected by the up-regulation of NSE, VEGFB, and GDNF and the expression of neuronal markers (PanF and NeuN). **Conclusions:** This study shows that hypoxia induces spontaneous differentiation of SCAP into osteogenic and neurogenic lineages while maintaining the release of the proangiogenic factor VEGFa. This highlights the potential of SCAP to promote pulp-dentin regeneration. Moreover, SCAP may represent potential therapeutic agents for neurodegenerative conditions because of their robust differentiation potential. *(J Endod 2014;40:1410–1418)*

Key Words

Angiogenic factor, apical papilla, dental stem cells, gene expression, hypoxia, neurodifferentiation, regenerative endodontics, stem cells from the apical papilla

In the past decade, regenerative endodontic procedures have emerged as a treatment alternative for immature teeth with pulp necrosis with the goal of pulp tissue regeneration (1, 2). This new treatment modality typically consists of chemical debridement and disinfection of the root canal followed by placement of an intracanal medication (3). On a subsequent appointment, the intracanal medicament is removed, and stem cells are delivered from the apical region by evoked bleeding after instrumentation beyond the apex (4, 5). The delivered stem cells are located in the surroundings of the blood clot, often throughout the whole extent of the root canal to the cementum-enamel junction. Thus, a substantial number of stem cells are placed several millimeters away from nearby apically positioned blood vessels. In the case of immature teeth, these procedures aim at restarting the development of the immature root, possibly increasing its resistance to fracture and survival (6). Many case reports have been published showing that such procedures are able to lead to healing of the periapical lesion and in a certain proportion of cases to an increase of root thickness and/or length (7). Thus, these procedures provided unprecedented clinical benefit in the treatment of immature teeth with pulpal necrosis. Nonetheless, it is unclear whether these procedures are able to recapitulate the once lost pulp-dentin complex.

Multiple animal models of regenerative endodontics have been used to evaluate the nature of the tissue formed. It was found that, in most of these studies, the tissue formed consisted of loose fibrous connective tissue with cementumlike tissues, osteodentin, and bonelike tissue (8, 9). It is important to note that, in certain animal studies using tissue-engineering approaches, complete regeneration of the pulp-dentin complex has been achieved (10, 11). Despite these encouraging reports, histologic examination of human teeth treated with revascularization procedures suggest that the tissues formed lack organization with mineralized tissues that resemble cementum or osteodentin along the dentinal walls and islands of dystrophic calcifications within the canal lumen (12). Therefore, it appears that several factors

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need to be optimized in order to achieve better special and temporal control of tissue regeneration.

For a more orchestrated regenerative process, one of the main factors that needs to be better controlled is mesenchymal stem cell fate. The cells present in the root canal after induced bleeding were shown to present the characteristics of undifferentiated mesenchymal stem cells (expression of CD73+, CD105+, or Stro-1+) (5). Whether these cells are stem cells from the apical papilla (SCAP) or cells of other periradicular sources remains unclear despite the fact that the apical papilla was clearly shown to possess the ability to remain vital for some time even in the presence of periapical radiolucency (4, 13). Shortly after blood clot formation, SCAP are very likely to be in hypoxic conditions until blood vessels grow into the blood clot. Therefore, they are initially subjected to very different conditions compared with those used for their *in vitro* characterization (21% O_2). Culture in hypoxic conditions of dental pulp stem cells (DPSCs) was shown to affect their proliferation (14, 15), migration ability (16), differentiation (14), and angiogenic potential (17). However, DPSCs are not expected to participate in the current regenerative endodontic procedures, and the effect of hypoxic conditions on SCAP has never been previously evaluated. Thus, the objective of the present work was to study the influence of hypoxia (1% O_2) on the proliferation and differentiation potential compared with normoxia (21% O_2).

Materials and Methods

SCAP Culture

SCAP previously isolated and characterized from the apical papilla of immature permanent teeth of a 17-year-old girl (RP-89 cells) were used (18). RP-89 cells between the 3rd and 6th passage were grown at 37°C in 21% O_2 and 5% CO_2 in basal culture medium composed of minimum essential medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum (Gemini, Sacramento, CA), 1% L-glutamine (Gemini), and 1% antibiotics (Gemini). RP-89 cell passages were performed at 80% confluence using Accutase (Life Technologies, Gent, Belgium). Cell culture in hypoxic conditions (1% O_2) was performed as followed. Cells were allowed to attach for 24 hours after seeding in basal culture conditions, referred to as normoxia (humidified cell incubator, 21% O_2, and 5% CO_2), before being transferred to hypoxic conditions (humidified $InVivo_2$ 400 Hypoxia Workstation, Ruskinn, Bridgend, UK; 1% O_2). Medium was replaced every other day by fresh cell culture media previously placed in vented tubes in the hypoxia chamber to allow media equilibration at 1% O_2.

RP-89 Cell Proliferation

RP-89 cell proliferation was quantified by life imaging. Images (n = 3–5 zones/well) of 6-well plates seeded with 220,000 RP-89 cells were acquired with an Axio Observer microscope (Zeiss, Zaventem, Belgium) either in normoxia (n = 9) or hypoxia (n = 6) every hour for 72 hours. Six pictures per condition every 24 hours were selected, and the surface occupied by RP-89 cells was quantified using ImageJ (National Institutes of Health, Besthesda, MD). Results were expressed as a percentage of the total analyzed surface. Cell confluence was set as 100% of the analyzed surface.

Vascular Endothelial Growth Factor A Secretion

Supernatants of RP-89 cells cultured in basal medium for 4 weeks in normoxic and hypoxic conditions (n = 3) were collected and stored at −20°C for human VEGFa quantification using an enzyme-linked immunosorbent assay (PeproTech, Rocky Hill, NJ). Samples were not diluted, and the supplier's instructions were followed. Results were expressed in ng/mL as cumulated VEGFa concentration over time.

Osteo-, Adipo-, and Neurodifferentiation of RP-89 cells

Osteogenesis. RP-89 cells were seeded (12,000 cells/cm^2) in 6- or 12-well plates (n = 3), incubated 24 hours in normoxia, and grown either in hypoxia or normoxia until reaching 80% confluence. Cultures were then induced (day 0) to undergo osteogenesis by replacing the basal medium with the differentiation medium (StemPro Osteogenesis Differentiation Kit, Life Technologies). Cells were maintained in osteodifferentiation medium for 4 weeks, and medium was replaced twice a week. Cultures were stained for calcium phosphate by alizarin red (Sigma-Aldrich) or treated for quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) analysis (discussed later). RP-89 cells cultured in basal medium were used as the negative control.

Adipogenesis. RP-89 cells were seeded (12,000 cells/cm^2) in 6- or 12-well plates (n = 3), incubated 24 hours in normoxia, and grown either in hypoxia or in normoxia until reaching 80% confluence. Cultures were then induced (day 0) to undergo adipogenesis by replacing the basal medium with the differentiation medium (StemPro Adipogenesis Differentiation Kit, Life Technologies). Cells were maintained in adipodifferentiation medium for 4 weeks, and medium was replaced twice a week. Cultures were stained for neutral lipids with oil red O (Sigma-Aldrich) or treated for qRT-PCR analysis. RP-89 cells cultured in basal medium were used as the negative control.

Neurogenesis. RP-89 cells were seeded (3,000 cells/cm^2) in 4-well Nunc LabTek II chamber slides (Thermo Fischer Scientific, Waltham, MA) (n = 2) or 6-well plates (n = 3), incubated 24 hours in normoxia, and grown either in hypoxia or normoxia until reaching 50% confluence. Cultures were then induced (day 0) to undergo neurogenesis by replacing the basal medium with the differentiation medium adapted from Abe et al (19) and composed of Neurobasal A (Life Technologies), B27 (1/50, Life Technologies), epidermal growth factor (EGF) (20 ng/mL, Life Technologies), nerve growth factor (NGF) (50 ng/mL, Life Technologies), basic fibroblast growth factor (bFGF) (20 ng/mL, Life Technologies), cyclic adenosine monophosphate (AMPc) (1 mmol/L, Sigma-Aldrich), 3-isobutyl-1-methylxanthine (IBMX) (0.5 mmol/L, Sigma-Aldrich), retinoic acid (2 μmol/L, Sigma-Aldrich), and penicillin/streptomycin (PEST) (Life Technologies). Cells were maintained in neurodifferentiation medium for 1 week, and medium was replaced twice a week. Cultures were rinsed with phosphate-buffered saline, and neurogenic differentiation was assessed by immunofluorescence for Pan neurofilaments (Covance, Brussels, Belgium, SMI-32R, 1/1000) and NeuN (Millipore; Billerica, MA, MAB377, 1/100). Anti–immunoglobulin G Alexa488 (1/500) was used as a secondary antibody. Actin was stained with rhodamine phalloidin (Life Technologies, R415, 1/40) and the nucleus with 4′,6-diamidino-2-phenylindole (DAPI). Images were acquired with a confocal microscope (LSM700, Zeiss). PanNF and NeuN staining quantifications were realized with an RGB quantification script (Zen, Zeiss). The percentage of green pixels (positive staining) for a known analyzed surface (1.44 mm^2) was measured and divided by the cell number for the same surface (n = 10 picture per condition and staining). The number of cells positive for PanNF and NeuN was also quantified and expressed as a percentage of the total number of cells. RP-89 cells cultured in basal medium were used as the negative control.

qRT-PCR Analysis

RP-89 cells were cultured as described previously (n = 3) either in basal or differentiation media in normoxic or hypoxic conditions during 1 or 4 weeks. Cells were treated with Trizol (Life Technologies), and cell pellets were frozen at −80°C. Total messenger RNA was extracted by the chloroform extraction method and stored at −80°C. Approximately 1 μg of messenger RNA was reverse transcribed into