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Activity of dental pulp cells in semisolid 3D cultures initiated by transforming growth factor-β1 and bone morphogenetic protein 2, 4

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Abstract

The intention of this study was to investigate the effect of modified 3D culture conditions on dental pulp cells (DPCs). DPCs were isolated from extracted primary molar, premolar, and wisdom teeth. Tooth samples were divided into three groups as control group; plated into methyl cellulose medium without any supplementation, growth factor (GF) group; supplemented with bone morphogenetic proteins (BMP2, BMP4), transforming growth factor-β1 (TGF-β1) and growth factor+conditioned medium (GF+CM) group; supplemented with both growth factors and pulp conditioned medium. The DPCs were tested for colony forming ability, proliferation capacity and morphology. The highest colony forming ability was detected in the GF and GF+CM groups of DPCs isolated from wisdom teeth. The proliferation capacity was higher in GF+CM group of DPCs isolated from primary molars, and in GF and GF+CM groups of DPCs isolated from wisdom teeth. Scanning electron microscope (SEM) observation of the wisdom teeth samples showed cell-cell interactions in the GF and GF+CM groups. Our results indicate that growth factors and pulp conditioned medium in methyl cellulose culture created proper environment to follow the behavior of dental cells three-dimensionally.

Key words: Dental pulp cells, bone morphogenetic proteins, TGF-β1.

Introduction

Dental pulp, which is a soft connective tissue located within the dental crown and root, is an ideal site for the collection of stem cells because of its wealth of such cells and its structure in the form of a niche (1).

Dental pulp stem cells provide a source of cells to replace damaged ones and facilitate repair (2). The dental pulp mesenchymal stem cells are able to regenerate a tissue inside a root canal through activation of growth factors, released after caries, play role in the dental repair (3).

Transforming growth factor-β1 (TGF-β1) and bone morphogenetic proteins (BMPs) belong to the TGF-β superfamily, and are involved in the regulation of cell proliferation, cell differentiation, epithelial-mesenchymal transition and embryonic development. The importance of TGF/BMP signaling is reported in respect of organogenesis during development (4). The role of TGF-β1 signaling in the regulation of differentiation of odontoblasts and homeostasis is crucial (5, 6).

BMPs play an important role on differentiation (7), proliferation, growth inhibition, and the arrest of maturation of many cells under influence of other regulatory factors and cellular microenvironment (8). BMPs also play role in regulating the development of teeth (9).

BMP2 is a potent stimulator of dental cell differentiation. This effect is known both *in vitro* and *in vivo* (10). As a growth factor it is involved in the development of bone and cartilage (11, 12). Tooth formation is based on the expression of BMP4 (13). BMP2 and BMP4 are induced by interactions between dental epithelium and dental mesenchymal stem cells. BMP4 seems to be the dominant regulator of signaling during odontoblast differentiation. This signaling is crucial in the induction of dental mesenchymal cells and their differentiation (14).

With the three-dimensional tissue cultures it is possible to control regeneration of the human tooth and molecular basis of odontogenesis *in vitro.* This type of cell culture allows cell-cell interactions to be investigated (15-17).

Materials and methods

Sample collection

Extracted primary molar (n=11), premolar (n=10) and wisdom (n=11) teeth from healthy patients (mean age 9.3±1.2; 15.1±2.4; 22.5±2.0, respectively) were collected in the department of Pediatric Dentistry and Oral Surgery and were stored in a sterile container containing saline and transported to our lab for sample processing. Informed consent was obtained from the patients or parents. All procedures were performed obeying national guidelines for working with human materials and the study was approved by Istanbul University Istanbul Medicine Faculty Ethics Committee.

Isolation of dental pulp cells (DPCs)

Tooth surfaces were washed with iodine and 70% ethanol to prevent contamination from oral bacteria. The teeth were washed five times with sterile phosphate-buffered saline (PBS) to remove iodine and etha-

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nol from the surface. To expose the pulp tissue, the teeth were wrapped in sterile gauze and squeezed in a vice until cracked. The pulp tissue was gently separated from the crown and root and then digested in a solution of 0.66 mg/ml collagenase type I and 0.33 mg/ml dispase in RPMI-1640 medium supplemeted with 10% fetal bovine serum (FBS) and 100 µM L-ascorbic acide for 2 h at 37 °C in 5% CO_2. After centrifugation, the supernatant was removed and the cells were washed 3 times with RPMI-1640 media supplemented with 10% FBS and then the single-cell suspensions of dental pulp were incubated in IMDM supplemented with 10% FBS 2 mM L-glutamine, 100 IU/ml penicilin, 100 µg/ml streptomycin, 100 µM L-ascorbic acid (all purchased from Sigma Chemical Co., St. Louis, MO, USA) for 24 h at 37°C under 5% CO_2 humidified air.

Experimental design

The DPCs from the three types of teeth were divided into three treatment groups. The DPCs in the first group (control) were plated into 3D methyl cellulose medium without any supplementation. The DPCs in the second growth factors group (GF) were supplemented with 250 ng/ml BMP2, 250 ng/ml BMP4, 10 ng/ml TGF-β1 (Sigma Chemical Co., St. Louis, MO, USA). The DPCs in the third group were treated with growth factors and pulp conditioned medium (GF+CM).

Preparation of conditioned mediums (CM) from three kinds of DPCs

Cells from the three kinds of DPCs were separately incubated with 10 ng/ml phytohemagglutinin (PHA, Biochrom KG, Berlin, Germany) in IMDM supplemented with 10% FBS at 25 cm^2 culture flask for 72 h at 37 °C under 5% CO_2 humidified air. Supernatants from culture flasks were filtered from 20 µm pore size syringe type filters, and then stored at -20 °C for subsequent use.

Preparation of methyl cellulose semisolid culture media

The DPCs from the teeth were tested for colony forming ability and proliferative capacity using methyl cellulose media. Methyl cellulose (4.000-centipoise, Sigma Chemical Co., St. Louis, MO, USA) was dissolved in IMDM at a concentration of 3 g per 100 ml. The media was supplemented with 30% FCS, 1% bovine serum albumin (BSA), 200 mmol/l L-glutamine, 10^{-5}mol/l sodium selenite, 10^{-1}mol/l α-monothioglycerol (all purchased from Sigma Chemical Co., St. Louis, MO, USA). In total, 10^4 DPCs were seeded into methyl cellulose semisolid culture media supplemented with or without growth factors and conditioned medium according to the experimental design in triplicate at a final volume of 1.1 ml in 35 mm petri dishes. The petri dishes were incubated at 37 °C in a 5% CO_2 fully humidified atmosphere for 14 days. Colonies were inspected using an inverted microscope.

CFSE analysis of DPCs

The semisolid cultures of DPCs were examined on the initial and the 14th day. DPCs were removed from methyl cellulose culture media by washing three times with PBS containing 1% L-ascorbic acid. After carboxyfluorescein diacetate succinimidyl ester (CFSE) was dissolved in DMSO, cells were incubated with 0.25 µg/ml CFSE dye in PBS for 15 min in the dark and then centrifuged at 4 °C and the supernatant was removed. Cells were resuspended with IMDM containing 0.1% FCS and incubated at 37 °C in a 5% CO_2 fully humidified atmosphere for 5 days. CFSE cell proliferation assays were performed on the 14th day of semisolid cultures to compare the effects of the growth factors and conditioned medium on cell proliferation ability of the DPCs.

Flow cytometric analyses of CFSE-stained cell proliferation were performed using a FACSCalibur (BD Bioscience, USA) flow cytometer equipped with argon laser emitting at a fixed wavelength of 488 nm. The fluorescent filters and detectors used green fluorescence collected in the FL1 channel (530 nm). Samples were gated according to forward scatter/side scatter properties of cells in order to exclude cell debris. CellQuest acquisition and analysis software (BD Bioscience, USA) was used to acquire and analyse data from CFSE stained cells which were gated in FL1 histogram. The histograms displaying CFSE were then used to determine the cell proliferation indexes.

Electron microscopy

Cells from wisdom teeth were analyzed morphologically using a scanning electron microscope (SEM). The DPCs were seeded on microslides in 24-well culture plates containing 1 ml of DMEM-F12 medium at a concentration of $5x10^4$ cells/well. After cell attachment, 5 ml IMDM was added to the lamella and incubated for 24 h. The DPCs were fixed with 2.5% glutaraldehyde in 0.1 M of sodium cacodylate buffer (pH: 7.4) for 1 h at 4 °C at the 24th h. DPCs were washed twice for 10 minutes with 0.1 M of sodium cacodylate buffer and post-fixed in 1% osmium tetraoxide for 1 h at 4 °C. The cells were dehydrated in a graded acetone series and incubated in amyl acetate. The microslides were then critical-point dried, sputter coated with gold-palladium, and observed using scanning electron microscopy (Jeol-JSM-5200).

Statistical analyses

Data were represented as mean ± standard deviation. Variances between groups were analyzed by ANOVA. Dunnett's t-test was used as post hoc test for comparing control and both GF and GF+CM groups. P values of less than 0.05 were accepted as significant.

Results

Proliferation capacity

The proliferation of DPCs was followed by labeling with the dye CFSE. The average number of cell divisions after 5 days of culture with CFSE is presented as an index of cell proliferation in Figure 1. The proliferation assays with CFSE revealed statistical increases in proliferation capacity of DPCs isolated from primary molar teeth in the GF group (15.4±2.0) ($p>0.05$), and the GF+CM group (16.8± 2.5) ($p<0.05$) compared with the control group (13.5±2.4). The increased capacity of proliferation of DPCs isolated from premolar teeth was not significant. Proliferation capacity of comparison between the control group (11.5±3.3) and induced with the group GF and induced with GF+CM group were

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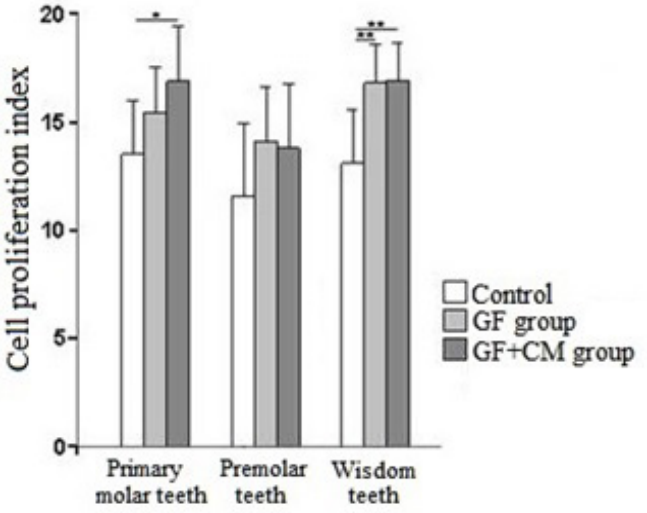

Figure 1. The proliferation index of DPCs. Analysis of proliferation of DPCs isolated from primary molar, premolar and wisdom teeth in response to different modified 3D cultures by CFSE assay. *p<0.05 **p<0.001.

(14.0±2.4), (p>0.05) and (13.7±2.9), (p>0.05), respectively. Statistically significant increases were found in the proliferative capacity of DPCs isolated from wisdom teeth GF group (16.8±1.7) (p<0.001), and the GF+CM group (16.9±1.7) (p<0.001) compared with the control group (13.1±2.4).

Colony formation

The morphology and colony forming abilities of DPCs are represented in Figure 2. DPCs isolated from premolar teeth produced no cell colonies in any group. Some small cell clusters were observed in the GF and GF+CM groups of DPCs isolated from primary molar teeth, and compact colonies in the GF and GF+CM groups of DPCs isolated from wisdom teeth. No colony formation was observed in the control groups.

SEM analyses

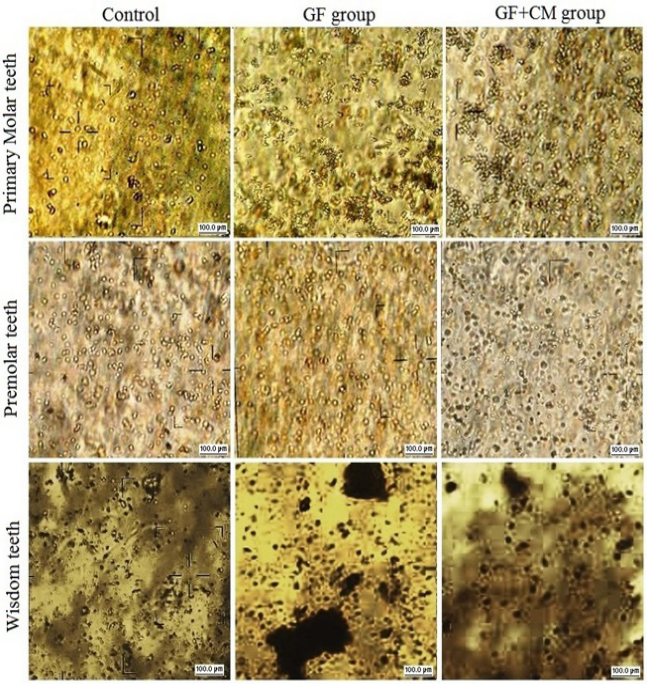

Figure 2. The morphology and colony forming abilities of DPCs isolated from primary molar, premolar and wisdom teeth after 14 days cultures.

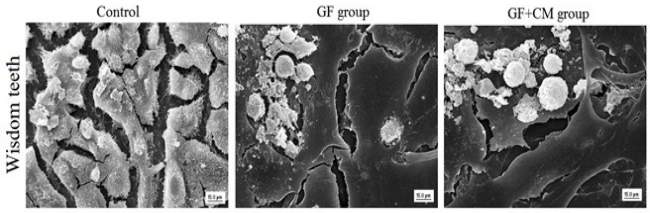

Figure 3. Scanning electron micrographs of DPCs isolated from wisdom teeth after 14 days of methyl cellulose cultures.

SEM observation was carried out for DPCs isolated from wisdom teeth that showed the highest colony forming ability in methyl cellulose cultures. Cell colonies were observed in the GF and GF+CM groups, while the DPCs of the control group were mostly solitary. The micrographs of these groups showed cell-cell interactions in the GF and GF+CM groups (Figure 3).

Discussion

DPCs contain multipotential stem cells whose differentiation is modulated by the local environment they inhabit (18). To produce optimal conditions for cell proliferation of DPCs from primary molar, premolar, and wisdom teeth, we cultured these cells with some growth factors known to be effective on stem cells and conditioned medium. Some studies have shown that conditioned medium prepared from different tissue has positive and negative effects on cell proliferation and differentiation (19, 20). In this study, we observed that the growth factors and conditioned medium cocktail accelerate cell proliferation in all teeth samples, but they did not have the same effect on colony formation.

The proliferation capacity of DPCs isolated from primary molar and wisdom teeth was higher than that of DPCs isolated from the premolar teeth. DPCs from human exfoliated deciduous teeth were different to those from DPCs of premolar teeth, affirming that they were more immature (21).

Cells that are maintained and differentiated in three-dimensional culture can enhance cell-cell interactions (22, 23). In this study, 3D culture allowed DPCs to grow and respond to their environment in a more realistic manner. DPCs induced with growth factors and pulp conditioned medium in methyl cellulose cultures showed better organized colonies containing similar cell size and morphology. The colony formation capacity was not parallel to proliferative capacity in primary molar teeth compared with wisdom teeth. Despite having similar levels of proliferative capacity, the DPCs isolated from primary molar teeth demonstrated lower levels of ability to create colonies than those obtained from wisdom teeth. This may happen because of the more immature premolar teeth that have limited capacity to respond to external stimulators.

Scanning electronmicroscopy studies that were performed for further investigation of DPCs derived from wisdom teeth showed greater colony formation capacity. The observations showed that growth factors alone, and in combination with conditioned medium support cell-cell interactions in DPCs derived from wisdom teeth.

To conclude, the data reported here indicate that growth factors and pulp conditioned medium in methyl cellulose culture create the physiological environment

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to promote DPCs, thereby enhancing their proliferation, and maintenance and differentiation of dental pulp cells in 3D culture support interactions between cells.

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