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Three-dimensional spheroid culture promotes odonto/osteoblastic differentiation of dental pulp cells

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ABSTRACT

Objective: Three-dimensional (3D) spheroid culture is a method for creating 3D aggregations of cells and their extracellular matrix without a scaffold mimicking the actual tissues. The aim of this study was to evaluate the effects of 3D spheroid culture on the phenotype of immortalized mouse dental papilla cells (MDPs) that have the ability to differentiate into odontoblasts.

Methods: We cultured MDPs for 1, 3, 7, and 14 days in 96-well low-attachment culture plates for 3D spheroid culture or flat-bottomed plates for two-dimensional (2D) monolayer culture. Cell proliferation and apoptosis were detected by immunohistochemical staining of Ki67 and cleaved caspase-3, respectively. Hypoxia was measured by the hypoxia probe LOX-1. Odonto/osteoblastic differentiation marker gene expression was evaluated by quantitative PCR. We also determined mineralized nodule formation, alkaline phosphatase (ALP) activity, and dentine matrix protein-1 (DMP1) expression. Vinculin and integrin signalling-related proteins were detected immunohistochemically.

Results: Odonto/osteoblastic marker gene expression and mineralized nodule formation were significantly up-regulated in 3D spheroid-cultured MDPs compared with those in 2D monolayer-cultured MDPs ($p < 0.05$). Histologically, 3D spheroid colonies consisted of two compartments: a cell-dense peripheral zone and cell-sparse core zone. Proliferating cells with high ALP activity and DMP1 expression were found mainly in the peripheral zone that also showed strong expression of vinculin and integrin signalling-related proteins. In contrast, apoptotic and hypoxic cells were detected in the core zone.

Conclusion: 3D spheroid culture promotes odonto/osteoblastic differentiation of MDPs, which may be mediated by integrin signalling.

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Abbreviations: 2D, two-dimensional; 3D, three-dimensional; ALP, alkaline phosphatase; α-MEM, alpha-modified minimum essential medium; BMP, bone morphogenetic protein; DMP1, dentine matrix protein-1; Dspp, dentine sialophosphoprotein; ECM, extracellular matrix; FAK, focal adhesion kinase; FBS, foetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MSCs, mesenchymal stem cells; Oc, osteocalcin; pFAK, phosphorylated focal adhesion kinase; pPaxillin, phosphorylated paxillin; PLGA, poly (lactic-co-glycolic acid); qPCR, quantitative PCR.

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1. Introduction

Odontoblasts arise from mesenchymal dental papilla cells, and their differentiation is guided by molecular crosstalk between the ectoderm and mesenchyme from the cap stage of tooth development. Various growth factors, such as bone morphogenetic protein (BMP)-2,[1,2] BMP-4,[3,4] BMP-7,[5] epidermal growth factor[6,7] and fibroblast growth factors,[8] are thought to be involved in odontoblastic differentiation of dental papilla/pulp cells. Odontoblastic differentiation is also induced by conditioned medium of cultured tooth germ cells, suggesting that various growth factors secreted from tooth germ cells are essential for odontoblastic differentiation.[9,10]

To induce odontoblastic differentiation of dental pulp cells, most experiments are performed in culture dishes or wells. However, tissues are three-dimensional (3D) and organized by complicated cell–cell and cell–extracellular matrix (ECM) interactions. Therefore, traditional 2D monolayer culture systems have a limitation to reproduce the events occurring in normal cell physiology. To overcome this limitation, various methods have been investigated for cell culture in 3D environments.[11–14] 3D culture systems either use scaffold materials or are ''scaffold-free''. The former culture system is relatively popular, because it is possible to change the cell density and shape of samples easily and differentiation can be directed by the nature of scaffold materials and addition of growth factors. Collagen,[15] gelatin,[16] and poly (lactic-co-glycolic acid) (PLGA)[17] are often used in scaffolds and act as a frame to guide cultured cells. However, it has been reported that scaffold materials can cause infection and/or inflammation during degradation *in vivo*.[18] In addition, cells are surrounded by artificial materials in scaffolds and the environment is different from the *in vivo* cell–cell and cell–ECM attachments in the tissue.

3D spheroid culture is a method for creating 3D aggregations of cells and their ECM without a scaffold,[19,20] which provides physiological cell culture conditions that mimic the actual tissues. Such systems have been applied to studies of tumours,[21] cell–cell interactions,[22] tissue engineering,[23–28] embryonic/induced pluripotent stem cell and mesenchymal stem cell (MSC) differentiation,[14,29,30] and cell death.[31] However, there are only few reports on 3D spheroid culture systems in dental pulp research. Iohara et al. reported that porcine primary pulp cells differentiate into odontoblasts effectively by pellet culture, one of the 3D spheroid culture systems.[23] Nonetheless, little is known about the precise effects of 3D spheroid culture on the properties of dental pulp cells. In this study, immortalized mouse dental papilla cells (MDPs), which possess dental pulp cell properties and the ability to differentiate into odontoblasts,[32] were cultured in a 3D spheroid culture system to evaluate their phenotypic changes.

2. Materials and methods

2.1. Cell culture

We cultured MDPs in alpha-modified minimum essential medium (α-MEM, Wako Pure Chemical Industries, Osaka, Japan) containing 10% foetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA) and an antibiotic and anti-fungus solution (Penicillin–Streptomycin–Amphotericin B suspension; Wako Pure Chemical Industries) at 37 °C/5% CO_2. Cells were cultured for 0 (control), 1, 3, 7, and 14 days in 96-well low-attachment culture plates (PrimeSurface; Sumitomo Bakelite, Tokyo, Japan) at a seeding density of 3×10^4 cells/well for 3D spheroid culture. We used flat-bottomed culture plates for 2D monolayer culture, and the same density of cells. The control cells at day 0 were an aliquot of MDPs prior to seeding. Medium was changed every 3 days. The diameter of each spheroid colony was measured under a microscope (Carl Zeiss, Oberkochen, Germany). For mineralized nodule formation, MDPs were cultured in 96-well low-attachment culture plates at a seeding density of 3×10^4 cells/well for 3D spheroid culture, or in 48-well flat-bottomed culture plates for 2D monolayer culture at a seeding density of 5×10^4 cells/well for 3 days. Then, the medium was changed to mineralization-inducing medium containing 0.2 mM L-ascorbic acid phosphate magnesium salt (Wako Pure Chemical Industries) and 5 mM β-glycerophosphoric acid (Sigma, St. Louis, MO, USA). The cells were then cultured for another 2 or 6 days.

2.2. Analysis of hypoxia

The levels of hypoxia were determined by the hypoxia probe LOX-1 (50 μM; SCIVAX, Kanagawa, Japan). LOX-1 is a phosphorescent light-emitting iridium complex that is quenched by oxygen, and its phosphoresce decreases in response to the level of oxygen.[33] LOX-1 was added to the culture medium at 24 h before detection. 2D monolayer-cultured MDPs in a low O_2 atmosphere (5%) were used as a positive control, and cells cultured in a normal O_2 atmosphere (20%) were used as a negative control.

2.3. Histology and immunohistochemistry

We fixed MDPs cultured on a plastic film (Cell Desk LF; Sumitomo Bakelite) with 4% paraformaldehyde for 15 min at 4 °C. Spheroid colonies of MDPs were fixed for 30 min at 4 °C and then embedded in OCT compound (Sakura Finetek, Torrance, CA, USA) and frozen quickly in dry ice/hexane. The 2D monolayer-cultured MDPs and cryostat sections (7 μm) of spheroid colonies were subjected to haematoxylin and eosin (HE) staining, alkaline phosphatase (ALP) staining, and immunohistochemistry. A mixture of naphthol AS-MX sodium salt (Sigma) and Fast-Blue RR salt (Sigma) was used for ALP staining. For immunohistochemistry, the primary antibodies were anti-Ki67 (1:1000; Thermo Fisher Scientific), anti-cleaved caspase-3 (1:3000; Cell Signaling Technology, Danvers, MA, USA), anti-dentine matrix protein-1 (DMP1, 1:1000; Takara Bio, Otsu, Japan), anti-vinculin (1:20,000; Sigma), anti-phosphorylated focal adhesion kinase (Tyr576/577) (pFAK, 1:2000; Santa Cruz Biotechnology, Dallas, TX, USA), and anti-phosphorylated paxillin (Tyr31) (pPaxillin, 1:500; GeneTex, Irvine, CA, USA). Immunoreactivity was detected by the ABC method (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA) and the colour reaction was developed with 3,3′-diaminobenzidin (Vector Laboratories). For mineralized nodule detection, MDPs were stained