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Induction of differentiation and mineralization in rat tooth germ cells on PVA through inhibition of ERK1/2

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ABSTRACT

Poly(vinyl alcohol) (PVA) has been widely used in the field of biomedical applications because of its hydrophilic properties for desired functions. Nonetheless, the role of PVA in tooth germ (TG) cell differentiation and mineralization has seldom been explored. To test the capacity of PVA in regulating TG cell differentiation and mineralization, TG cells obtained from 4-day-old Wistar rats were cultured on the PVA substrate. It was found that PVA was able to promote TG cell exhibiting high levels of alkaline phosphatase (ALP) activity, mineralization, and mRNA expression of osteocalcin (OCN), osteopontin (OPN), dentin matrix protein 1 (DMP1) and enamelin. Even when the additives routinely administrated in the differentiation medium such as dexamethasone, β-glycerophosphate and ascorbic acid were removed from the culture system, PVA itself still stimulated TG cells with the differentiation and mineralization ability. By showing the direct suppression of extracellular signaling-regulated kinase1/2 (ERK1/2) of TG cells treated with U0126, known to suppress the activation of ERK1/2, and significant synergistic effects between PVA and U0126, we demonstrated the suppression of ERK1/2 pathway is one of the effects of PVA-promoted TG cell differentiation and mineralization. Taken together, this study demonstrated a novel role of PVA in promoting the differentiation and mineralization of TG cells through ERK1/2 acting as a negative regulator.

1. Introduction

The tooth germ (TG), sometimes called the tooth bud, contains a heterogeneous population of cells including ameloblasts, odontoblasts and dental pulp cells. During tooth development, TG cell differentiation and mineralization proceed synchronously eventually forming a tooth [1]. Thus, similar to other osteoblast-like cells [2–4], TG cells also can serve as a model to elucidate the cell differentiation and mineralization in vitro and to determine the effects of growth factors, cytokines, and mechanical stimulus implicated in cell differentiation and mineralization.

We recently reported that the adhesion and proliferation of TG cells were sensitive to changes in surface hydrophilic properties of biomaterial [5]. Especially, the very hydrophilic biomaterial poly(vinyl alcohol) (PVA) could maintain TG cells with a three-dimensional spherical structure, resembling in vivo physiological condition. It is well known that cell behavior on biomaterial is crucial to many biomedical applications, yet the molecular pathways responsible for converting PVA signals into TG cell responses are still being elucidated. Therefore, the purpose of the study was to investigate the effect of PVA on TG cell differentiation and mineralization, and the possible signaling pathway involved in regulating TG cell change in response to PVA biomaterial.

The mitogen-activated protein kinase (MAPK) signaling pathway is tightly related to the regulation of cell proliferation, differentiation, motility and death [6,7]. Three central elements of the MAPK family have been identified in mammalian cells, referred to as extracellular signal-regulated kinase1/2 (ERK1/2), p38 kinase, and c-Jun-N terminal kinase (JNK) [8–10]. This study made use of Western blot analysis to examine the role of individual MAPK pathway in enhancing alkaline phosphatase (ALP) activity and mineralization of TG cells by blocking the pathway using specific inhibitor. To our knowledge, TG cells were not employed to examine how MAPK signaling pathways can be triggered by PVA. Our findings show PVA is an effective substrate that promotes TG cell differentiation and mineralization and the ERK1/2 dependent

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pathway plays an important role in negatively mediating function of TG cells.

2. Materials and methods

2.1. Preparation of PVA substrate

A 5 wt% solution of PVA (Chemika Fluka, MW = 72,000 g/mol, Switzerland) was prepared by dissolving PVA in distilled water at 95 °C. For preparing PVA-coated wells, 140 μl of PVA solution was added into 24-welled tissue culture polystyrene (TCPS) plates (Costar, USA). The solution was then allowed to dry at 60 °C for 24 h to form a thin membrane. Before cell culture, the PVA-coated wells were sterilized in 70% alcohol overnight and rinse extensively with phosphate buffered saline (PBS). As controls, uncoated TCPS wells were treated by the same way as PVA-coated wells.

2.2. Cell culture

The animal study was performed according to a protocol approved by the Review Committee of College of Medicine, National Taiwan University. The method for isolating TG cells from rat mandibular molar TG was described previously [5]. In brief, rat mandibular molar TG was removed from 4-day-old Wistar rats using the explant outgrowth technique without collagenase treatment. Ten first molar TGs in total were isolated from five rats from both sides of lower jaws of each rat. The TGs were placed in PBS and then were cut into small fragments about 1 mm^3 in size, in which the TG cells were released. Subsequently, the excised fragments of TG and released cells were placed into a 15 ml centrifuge tube and centrifuged at 900 rpm for 5 min. After removal of the upper layer solution, cells with tissue fragments were mixed with 10 ml Dulbecco' s modified Eagle medium (DMEM, Chemicon, USA) supplemented with 10% fetal calf serum (Gibco-RBL Life Technologies, UK), antibiotic/antimycotic (penicillin G sodium 100 U/ml, streptomycin 100 g/ml, amphotericin B 0.25 g/ml, Gibco-BRL Life Technologies, UK) placed in a 100-mm cell culture dish (Costar, USA) and then cultured at 37 °C with 5% CO_2 atmosphere in a humidified incubator. TG cells released from the tissue fragments were grown to confluence in approximately 6–8 days. At approximately 90% confluence, tissue fragments were removed and used for another culture to release more TG cells, and subcultured in 100-mm cell culture dishes (Costar, USA) in fresh culture medium for another two weeks. The total number of cells obtained from each primary culture increased to approximately 1×10^8 cells after 30 d in culture. In this work, TG cells used for the subsequent analysis were in the third passage and the medium was changed every 3 or 4 days.

2.3. ALP activity and mineralization assays

TG cells were cultured on PVA and TCPS at a density of 1×10^5 cells/well for 1, 4 and 7 days in the above medium (regarded as the regular medium) and differentiation medium. The differentiation medium was supplemented with 100 nM dexamethasone (Sigma, USA), 10 mM β-glycerophosphate (Sigma, USA) and 50 μg/ml ascorbic acid (Sigma, USA). ALP activity was assayed using *p*-nitrophenylphosphate as a substrate following the method described previously [11]. The amount of *p*-nitrophenol produced was measured spectrophotometrically at 410 nm. The degree of mineralization was measured by staining with Alizarin Red S (ARS, Sigma, USA) as described by Ratisoontorn et al. [12]. Data were expressed as units of ARS released (1 unit = 1 unit of optical density at 562 nm).

2.4. MAPK inhibition studies

The role of individual MAPK pathway in ALP activity and mineralization of TG cells was assessed by blocking the pathways using specific inhibitor. The ERK1/2 pathway was blocked with U0126 (Cell signaling, USA), which inhibits MEK1/2, an upstream molecule of the ERK phosphorylation cascade [13]. The p38 MAPK was specifically inhibited with SB203580 (Sigma, USA) [14] and JNK was inhibited with SP600125 (Sigma, USA) [15]. For assessing ALP activity, mineralization and gene expression of mineralization- and differentiation-related markers, TG cells were treated with or without inhibitor for 7 days. For Western blot analysis, TG cells were cultured in the differentiation medium for 4 h followed by treatment with inhibitor for 30 min.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from TG cells by using Trizol (Invitrogen Life Technologies, CA). The RNA (1 μg) was reverse transcribed into first-strand cDNA using the iScript cDNA Synthesis kit (BIO-RAD, CA) for RT-PCR. The oligonucleotide RT-PCR primers for glyceraldehydes-3-phosphate (GAPDH), osteocalcin (OCN) [16], osteopontin (OPN) [17], dentin matrix protein 1 (DMP1) and enamelin are listed in Table 1. The PCR amplification was performed as follows: 32 cycles of denaturation at 94 °C for 45 s, annealing for 45 s, and extension at 72 °C for 30 s. The amplified products were analyzed by electrophoresis through 1.5% agarose gel containing 10 μg/ml ethidium bromide, electrophoresed at 100 mV, and visualized on a UV transilluminator (Alpha Innotech, CA). All bands were scanned and analyzed using AlphaEase FC 4.0 software.

Table 1
Oligonucleotide primer sequences utilized in the RT-PCR.

Target cDNA		Primer sequence (5′–3′)	T_{hyb} (°C)	Product size (bp)	NCBI no. or Ref.
GAPDH	F	ATGGGAAGCTGGTCATCAAC	51.8	375	NM017008
	R	CCACAGTCTTCTGAGTGGCA			
OCN	F	ATGAGGACCCTCTCTCTFCTC	56.3	293	[16]
	R	GTGGTGCCATAGATGCGCTTG			
OPN	F	TCCAAGGAGTATAAGCAGCGGGCCA	58	200	[17]
	R	CTCTTAGGGTCTAGGACTAGCTTCT			
DMP 1	F	CTGGTATCAGGTCGGAAGAATC	55	499	NM206493
	R	CTCTCATTAGACTCGCTGTCAC			
Enamelin	F	CACCGTACCTTAGAGGCAATAC	54.8	463	NM000106
	R	GAGGTCCATGAAGGAAAGAGAG			

2.6. Western blot analysis

Cells were collected by gentle shaking of the wells and washed twice with PBS. Cell lysates were prepared with ice-cold lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM NaF, 1 mM Na_3VO_4, 1:200 dilution of Protease Inhibitor Cocktail II; Calbiochem, Germany) for 30 min and then were sonicated at 4 °C for 15 s. Lysates were clarified by centrifugation at 14,000 rpm for 30 min at 4 °C and the resulting supernatant was saved for protein analysis and Western blot analysis.

Protein concentration was measured by using the commercial protein assay reagent (Bio-Rad, CA). For Western blotting, the supernatant was added to an equal volume of Laemmli sample buffer (62.5 mM Tris, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% *b*-mercaptoethanol) and heated to 95 °C for 10 min. Proteins (35 μg total protein per lane) were separated by SDS-PAGE on 10% polyacrylamide gels and transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk in TBST buffer (Bio-Rad, CA), probed with primary rabbit antibodies against ERK1/2 and phospho-ERK1/2 (Cell Signaling, USA) at a dilution of 1:1000, and were incubated at 4 °C overnight. After washing, the blots were incubated with anti-rabbit IgG-HRP conjugated secondary antibodies (Cell Signaling, USA) at a dilution of 1:5000 for 2–3 h. Finally, the proteins on the membranes were detected using the ECL Plus chemiluminescence system. Densitometric quantification of Western blots was done using AlphaEase FC 4.0 software.

2.7. Statistical analysis

Results are presented as the mean ± standard deviation (SD) of 3–5 independent cultures. Statistical significance was calculated using one-way analysis of variance (ANOVA) followed by post hoc procedure (Bonferoni analysis) ($p < 0.005$ was considered significant).

3. Results

In the present study, the very hydrophilic PVA substrate with the air–water contact angle of 54.0 ± 2.0° was used to maintain TG cells with a three-dimensional spherical structure [5]. In addition, monolayered TG cells on commercial TCPS with the air–water contact angle of 62.7 ± 2.4° [5] was compared.

3.1. ALP activity and mineralization of TG cells on PVA and TCPS

To determine the effect of PVA on TG cell differentiation, ALP activity, an early marker of odontoblasts differentiation [18,19], and ARS assay, a traditional approach for evaluating the calcium deposition, were measured. In the culture system, cells were confluent on TCPS due to the high seeding density and cells formed aggregates suspending above PVA as reported previously [5] (data not shown). Fig. 1 shows both TCPS and PVA expressed increasing levels of ALP activity and ARS assay through 7 days of culture in the differentiation medium. Nonetheless, TG cells grown on PVA showed greater expression and significantly higher than those on TCPS at every time point, regardless of ALP activity and ARS assay ($p < 0.005$).

Generally, culture medium was routinely changed and added with dexamethasone, β-glycerophosphate and ascorbic acid, which had been reported to be beneficial to differentiation of bone-like cells [20]. Therefore, to further explore whether PVA per se promoted the differentiation and mineralization of TG cells, regular medium without dexamethasone, β-glycerophosphate and ascorbic acid was