Available online at www.sciencedirect.com
ScienceDirect
Acta Biomaterialia 5 (2009) 1064–1074
Acta BIOMATERIALIA
www.elsevier.com/locate/actabiomat

The behavior of rat tooth germ cells on poly(vinyl alcohol)

Rung-Shu Chen [a], Yi-Jane Chen [b,c], Min-Huey Chen [b,c,*], Tai-Horng Young [a]

[a] Institute of Biomedical Engineering, College of Medicine and College of Engineering, National Taiwan University, Taipei 100, Taiwan
[b] Department of Dentistry, National Taiwan University Hospital, Taipei 100, Taiwan
[c] School of Dentistry, College of Medicine, National Taiwan University, Taipei 100, Taiwan

Received 29 April 2008; received in revised form 3 November 2008; accepted 19 November 2008
Available online 10 December 2008

Abstract

The purpose of this study was to evaluate the behaviors of rat tooth germ (TG) cells cultured on poly(vinyl alcohol) (PVA). It was found that TG cells suspended and aggregated to form three-dimensional spheroids on PVA. Compared with traditional monolayered cells on tissue culture polystyrene, TG cell spheroids on PVA obviously increased the alkaline phosphatase activity, the degree of mineralization, and upregulated both osteopontin and dentin matrix protein 1 genes, regardless of the seeding density. Surprisingly, PVA appears to activate the alkaline phosphatase activity and mineralization effects on TG cell spheroids in the absence of a differentiation medium. Furthermore, the present study indicates that integrins may play an important role in the mineralization on TG cell spheroids by adding Arg-Gly-Asp (RGD) peptides. Therefore, the information presented here should help to clarify the role of PVA in the regulation of the mineralization, differentiation and integrin-mediation of TG cells.

Keywords: Poly(vinyl alcohol); Tooth germ cells; Three-dimensional spheroids; Mineralization; RGD

1. Introduction

Tooth formation is the result of reciprocal instructive interactions between oral epithelium and mesenchymal tissues [1]. In the previous study, we have demonstrated that tooth germ (TG) cells seeded onto the gelatin–chondroitin–hyaluronan scaffold could form tooth-like structures in swine after 36 weeks post-transplantation [2]. Clearly, a supporting material, used as a scaffold, is thought to be needed to act as a template for TG cells attachment, growth and differentiation. For bone tissue engineering, it has been demonstrated that a three-dimensional scaffold can provide a more suitable three-dimensional environment for cell–cell interaction and cell differentiation [3,4]. Similarly, albumin production and detoxificatory function of hepatocytes can be enhanced when they are cultured into multicellular aggregates [5,6]. Compared with monolayer culture, neuron precursor cells can maintain their long-term proliferation and stem cell characters when they are cultured in three-dimensional neurospheres [7].

Poly(vinyl alcohol) (PVA) has been developed for various biomedical applications such as artificial pancreas [8], hemodialysis [9] and implantable medical materials [10]. It is reasonable to assume such a hydrophilic polymer should be favorable for TG cell culture, since it provides a wet environment, resembling in vivo physiological conditions. However, a previous study found that PVA may inhibit TG cell adhesion [11]. Generally, anchorage-dependent cells cultured in vitro would attach onto the substrate surface; otherwise, they underwent apoptosis [12]. Interestingly, TG cells aggregated to form a three-dimensional sphere in suspension on PVA [11]. As mentioned previously, this suggests that TG cell spheroids formed on PVA can also exhibit better function and differentiation potential. Therefore, the purpose of this study was to eval-

* Corresponding author. Address: School of Dentistry, College of Medicine, National Taiwan University, Taipei 100, Taiwan, ROC. Tel.: +886 2 23123456x67701; fax: +886 2 23826145.
E-mail address: minhueychen@ntu.edu.tw (M.-H. Chen).

1742-7061/$ - see front matter © 2008 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.actbio.2008.11.023

uate the behaviors of TG cells cultured on PVA and to study whether floating TG cell spheroids on PVA are similar to other three-dimensional cell aggregates and could adopt different strategies to control their survival or further modify their differentiation.

2. Materials and methods

2.1. Preparation of culture wells coated with PVA

A 5.0% (w/v) solution of PVA (Chemika Fluka, MW = 72,000 g mol^{-1}, Switzerland) was prepared by dissolving in distilled water at 95 °C. For preparing PVA-coated wells, 140 μl of PVA solution was added into 24-well tissue culture polystyrene 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 rinsed extensively with phosphate buffered saline (PBS). For controls, uncoated tissue culture polystyrene (TCPS) wells were treated by the same way as PVA-coated wells.

2.2. Cell culture and assays for cell proliferation

The animal study was performed according to a protocol approved by the Review Committee of the College of Medicine of National Taiwan University. The method for isolating TG cells from rat mandibular molar TG with cultured explant was described in a previous study [11]. In brief, rat mandibular molar TGs were 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 the lower jaw of each rat. 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 TGs 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's medium (DMEM, Chemicon, USA) supplemented with 10% fetal calf serum (Gibco-RBL Life Technologies, Paisley, UK), antibiotic/antimycotic (penicillin G sodium 100 U ml^{-1}, streptomycin 100 g ml^{-1}, amphotericin B 0.25 g ml^{-1}, Gibco-BRL Life Technologies, Paisley, 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 then 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 days in culture. In this work, TG cells used for the subsequent analysis were in the third passage.

The proliferation of TG cells on PVA and TCPS was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma, USA) assay [13] after loading for 1, 4 and 7 days. MTT was prepared as a 5 mg ml^{-1} stock solution in PBS, sterilized by Millipore filtration, and kept in darkness. The 100 μl of MTT solution was added into each well without removal of culture medium. After 3 h incubation at 37 °C, 200 μl of dimethyl sulfoxide (DMSO, Nacalai Tesque, Kyoto, Japan) was added to dissolve the formazan crystals. For TG cells suspended on PVA, the formazan crystals were collected into a 1.5 ml microtube and centrifuged at 1500 rpm for 10 min. After removal of the upper layer of the solution, 200 μl of DMSO was then added to dissolve the formazan crystals. The dissolvable solution was agitated homogeneously for about 15 min by a shaker. The optical density of the formazan solution was read on an ELISA plate reader (ELx 800, BIO-TEK, Winooski, VT, USA) at 570 nm.

2.3. Lactate dehydrogenase (LDH) released and caspase-3 activity

The toxic effect of PVA and TCPS on TG cells was quantitatively determined by measuring lactate dehydrogenase (LDH) released from damaged cells into the extracellular medium after culturing for 7 days. LDH activity was measured by using a LDH kit (Roche, USA) according to the protocol. The optical density of the LDH activity was read on an ELISA plate reader at 490 nm and reference wavelength at 630 nm. Caspase-3 activity was measured by quantifying the cleavage of acetyl-Asp-Glu-Val-Asp p-nitroaniline (Ac-DEVD-pNA) with a colorimetric caspase-3 assay kit (Molecular Probes, USA) according to the protocol. The cells, settled by centrifugation, were rinsed in PBS, incubated at 4 °C with the RIPA buffer (50 mmol l^{-1} Tris–HCl pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mmol l^{-1} NaCl; 1 mmol l^{-1} EGTA), and the lysates were centrifuged at 14,000*g* for 30 min. The supernatant was then assayed for caspase-3 activity. Briefly, 30 μg of supernatant proteins was incubated in the presence of 20 μl of caspase-3 fluorimetric substrate. Relative fluorescence of the sample was determined spectrophotometrically at 342 nm excitation and 441 nm emission after 30 min at room temperature [14].

2.4. Alkaline phosphatase activity and mineralization assay by measurement of Alizarin red S

In this study, TG cells from the third passage were cultured on TCPS and PVA in the regular medium as described previously in Section 2.2 and in the differentiation medium supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate and 50 μg ml^{-1} ascorbic acid at two different seeding densities (low: 5×10^3 cells ml^{-1} and high: 1×10^5 cells ml^{-1}). Alkaline phosphatase (ALP) activity was expressed as micromoles of reaction product (p-nitrophenol) per 30 min from milligram of cellular pro-

ID	Title	Pages
1509	The behavior of rat tooth germ cells on poly(vinyl alcohol)	11