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

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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.

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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-

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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 mm³ 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% CO₂ 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 × 10⁸ 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 14,000g 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 × 10³ cells ml⁻¹ and high: 1 × 10⁵ cells ml⁻¹). Alkaline phosphatase (ALP) activity was expressed as micromoles of reaction product (p-nitrophenol) per 30 min from milligram of cellular pro-

tein as described by Liu et al. [15]. The degree of mineralization was measured by staining with Alizarin Red S (ARS, Sigma, USA) for quantification of matrix calcification that was used as described by Ratisoontorn et al. [16]. Data are expressed as units of ARS released (1 U = 1 U of optical density at 562 nm) in each culture on a parallel well.

Cells suspended on PVA after the culture period 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, ALP activity and the quantification of ARS were determined in the microtube using the same procedure combined with centrifugation for changing the extraction buffer.

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

Total RNA was extracted from TG cell culture using TRI-ZOL (Invitrogen Life Technologies, Carlsbad, CA) after culturing with two kinds of media for 7 days. The RNA (1 µg) was reverse-transcribed into first-stand cDNA using the iScript cDNA Synthesis kit (BIO-RAD, Hercules, CA) for RT-PCR. The oligonucleotide RT-PCR primer for glyceraldehydes-3-phosphohate (GAPDH), osteopontin (OPN) [17] and dentin matrix protein 1 (DMP1) are listed in Table 1b. The PCR amplification was performed as follows: denaturation for 45 s at 94 °C; annealing for 45 s at 56 °C; and extension for 30 s at 72 °C, for 32 cycles. The PCR products were run on 1.5% agarose gel containing 10 µg ml⁻¹ ethidium bromide, electrophoresed at 100 mV, and visualized on a UV transilluminator (Alpha Innotech, San Leandro, CA).

The amount of treated mRNA in TG cells after culturing for 7 days was determined using AlphaEaseFC 4.0 software to quantify the bands. GAPDH was used as an internal control. Then the GAPDH amount in each of the regular media was used to normalize the amounts of OPN and DMP1.

2.6. Addition of Arg-Gly-Asp (RGD)

To examine the effects of RGD on the mineralization of TG cell spheroids, RGD (Sigma, USA, 20 µg ml⁻¹) was added into the regular medium of TG cell spheroids at different densities after culturing on PVA for 7 days. The mineralization of TG cell spheroids on PVA was then assayed by ARS staining.

3. Statistical analysis

Results are presented as means (±standard deviation, SD) of 4–6 independent cultures. Statistical significance was calculated using one-way analysis of variance (ANOVA) followed by post hoc procedure (Bonferoni analysis), with $p < 0.005$ considered significant for all tests.

4. Results

4.1. Formation of TG cell spheroids

For the first experiment, TG cells were cultured on PVA at two different seeding densities (low: 5×10^3 cells ml⁻¹ and high: 1×10^5 cells ml⁻¹) to allow cell suspension and aggregation. For comparison, cells cultured on TCPS were also investigated in this study. After culturing for 7 days, TG cells were grown to confluence on TCPS, regardless of low or high seeding densities (Fig. 1a and b). At the same culturing period, most of TG cells did not attach to PVA-coated surface but formed small clusters and developed into aggregates (Fig. 1c and d). Such a cell aggregation phenomenon is not attributable to differences in the seeding density, since cells with different seeding densities exhibited similar cell behaviors. This suggests that TG cells aggregation was related to the effects of PVA itself and was not only dependent on autocrine and cell-derived factors [18].

4.2. Cell proliferation

The time course of MTT assay of TG cells cultured on PVA and TCPS were displayed in Fig. 2. At low seeding density, increased formazan absorbance indicates that TG cells cultured on TCPS were able to convert the MTT into a blue formazan product and continued to proliferate during the culture period. However, at high seeding density, TG cells cultured on TCPS almost had the same MTT absorbance, so the obvious growth was not observed during 7 days of culture. This can be ascribed to the fact that cell confluence could be easily obtained to inhibit the cell proliferation when cells were seeded at higher density. Actually, TG cells seeded at high density on TCPS had reached confluence at the first day of culture, but cell seeded at low density did not grow to confluence until the seventh day (not shown here). Compared to TCPS,

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

Target cDNA	Primer sequence (5'–3')	T_{hyp} (°C)	Product size (bp)	NCBI no. or Ref.
GAPDH	F ATGGGAAGCTGGTCATCAAC R CCACAGTCTTCTGAGTGGCA	51.8	375	NM017008
DMP	F CTGGTATCAGGTCGGAAGAATC R CTCTCATTAGACTCGCTGTAC	55	499	NM203493
OPN	F TCCAAGGAGTATAAGCAGCGGGCCA	58	200	[17]

Fig. 1. Phase-contrast photomicrographs of rat tooth germ cells after culture on TCPS and PVA in the regular medium for 7 days. (a) TG cells cultured at low density (5×10^3 cells ml^{-1}) on TCPS, (b) TG cells cultured at high density (1×10^5 cells ml^{-1}) on TCPS, (c) TG cells cultured at low density on PVA, (d) TG cells cultured at high density on PVA. TG cells at high density of 1×10^5 ml^{-1} on TCPS had a flat spreading appearance. In addition, TG cells suspended and aggregated to form spheroids in PVA-coated wells.

PVA almost had no difference in MTT absorbance during the culture period, regardless of low or high seeding density. This finding indicates that the aggregated cells remained viable but the intimate contact within the aggregate was unfavorable for proliferation due to the cell-contact inhibition of growth [19].

4.3. Cell death and apoptosis

The cell death of TG cells on PVA was quantitatively determined by measuring the release of LDH into the culture medium after 7 days of incubation. As shown in Fig. 3a, the significant difference was observed between low and high seeding densities, regardless of PVA or TCPS, which is consistent with the previous report [20]. However, there was no significant difference in LDH assay between TG cells cultured on PVA and on TCPS, regardless of low or high seeding densities. Furthermore, the apoptosis analysis of TG cells on PVA after culturing for 7 days was determined by measuring the presence of caspase-3 activity, an intercellular cystein protease that exists as a proenzyme and is activated during the cascade of events associated with apoptosis [21]. In agreement with results from LDH assay, the caspase-3 activity in TG cells on PVA did not differ significantly from that on TCPS as shown in Fig. 3b. This indicates that PVA inhibited the growth of TG cells, but it did not induce more cell death and apoptosis compared to TCPS. Therefore, the lower

MTT value of cells on PVA should be related to lower proliferation rate of cells on this substrate, which may have implications for better differentiation ability for TG cells [22] as discussed in the following section.

4.4. ALP activity

ALP activity, an early marker for odontoblast differentiation [23], was measured for TG cells cultured on TCPS and PVA following 7 days of induction with differentiation medium. Fig. 4 shows TG cells grown on PVA at high seeding density of 1×10^5 cells ml^{-1} expressed ALP activity increased with culturing time and exhibited significantly higher levels compared to that on TCPS after 7 days of incubation ($p < 0.005$). At low seeding density of 5×10^3 cells ml^{-1} , TG cells exhibited similar ALP activity on PVA and TCPS. We therefore investigated whether other cell densities (1×10^4 and 5×10^4 cells ml^{-1}) also have significantly different influences on the ALP activity of TG cells cultured on different substrates. Clearly, both TG cells on PVA and TCPS produced increasing ALP activity with increasing seeding density. However, it appeared that only higher seeding density (1×10^5 and 5×10^4 cells ml^{-1}) could significantly enhance the ALP activity of TG cell spheroids on PVA than that on TCPS after 7 days of incubation. This suggests the existence of a threshold for cell seeding density to promote ALP activity of TG cell spheroids on PVA.

Fig. 2. MTT assay of tooth germ cells seeded at different densities were cultured on TCPS and PVA in regular medium for 7 days (a) and significant differences between each experiment at day 7 (b). Results are means $\pm$ SD. Increased formazan absorbance indicated that cells seeded at the density of $5 \times 10^5 \text{ ml}^{-1}$ on TCPS were able to continue proliferation throughout the culture period. Conversely, when cells were cultured on PVA, the MTT value was not increased on the fourth and seventh days relative to the first day, indicating that the aggregated cells remained viable and in a rest state.

Fig. 3. LDH activity released (a) and Caspase-3 activity (b) in the culture medium from TG cells cultured at low and high cells density ($5 \times 10^3 \text{ ml}^{-1}$ and $1 \times 10^5 \text{ ml}^{-1}$, respectively) on PVA and TCPS after 7 days of culture. Results represent the means $\pm$ SD from four to five independent cultures and determinations, * $p < 0.005$, n.s.: no significant.

4.5. ARS staining

ARS staining, a traditional approach for evaluating the calcium deposition, was performed to identify mineralization of TG cells on TCPS and PVA during 7 days of culture. Fig. 5 shows cell aggregates suspended on PVA were strongly stained with ARS after 7 days of culture regardless of the seeding density (Fig. 5c and d). Although ARS staining was also observed in confluent cells on TCPS at high seeding density, almost no calcification parts represented by red color were seen at low seeding density (Fig. 5a and b). The intensity of ARS staining in TG cells was analyzed according to the method described in Section 2 (Fig. 6). Regardless of the seeding density, the mineralization of TG cell aggregates suspended on PVA showed rapidly increasing intensity and there were significant differences when compared to those on TCPS ($p < 0.005$).

Based on our results, it is reasonable to assume that when cells aggregated into spheroids, the mineralization of TG cells occurred easily. Previous studies had reported the mineralization and calcification of chondrocytes occurred when spheroidal chondrocytes were formed during culturing in centrifuge tubes [24,25]. Therefore, it is interesting to investigate whether the formation of spheroidal aggregates by centrifugation can also enhance the mineralization of TG

cells. For this purpose, TG cells in density of $1 \times 10^5 \text{ cells ml}^{-1}$ were collected by centrifugation at 1500 rpm for 5 min and then cultured with the differentiation medium in the 15-ml tube at 37°C with 5% CO_2 atmosphere in a humidified incubator for 7 days and ARS staining analysis was performed. However, no calcification part represented by red color was found in the centrifugation-induced TG cell spheroids (Fig. 7). This indicates that the mineralization of TG cells was not related to the formation of spheroidal aggregates; in converse, the mineralization might be dependent on the stimulation of PVA.

4.6. mRNA expression of mineralization- and differentiation-related markers

The gene expression of OPN, and DMP 1 of TG cells cultured on PVA and TCPS for 7 days was examined by semi-quantitative RT-PCR (Fig. 8). To clarify the possibility of direct effects of PVA on the differentiation and mineralization of TG cells, differentiation medium and regular

Fig. 4. ALP activity of tooth germ cells cultured under various conditions. Cells seeded at different densities were cultured in differentiation medium on TCPS and PVA for 7 days (a) and significant differences between ALP activities of each group at day 7 (b). Each value is a mean $\pm$ SD. TG cells grown at density of $1 \times 10^5 \text{ ml}^{-1}$ with differentiation media on the PVA expressed greatest increase and significantly highest ALP activity than that on TCPS after culturing for 7 days.

medium without induction supplements such as dexamethasone, β -glycerophosphate and ascorbic acid were prepared simultaneously. For cells cultured on TCPS, in addition to the high seeding density incubated with the differentiation medium, the TG cells expressed very low or no levels of genes encoding OPN and DMP 1. In contrast, the TG cell spheroids cultured on PVA with the differentiation medium showed high gene-expression patterns, especially for the high seeding density. Even in the regular medium without induction supplements, TG cell spheroids cultured on PVA also showed upregulation of mRNA expression of OPN and DMP 1 more than those on TCPS, regardless of low or high seeding densities. These findings strongly support the elevated calcification levels demonstrated with ALP activity and ARS staining assay.

4.7. The effect of PVA on the ALP activity and ARS staining of TG cells in the absence of differentiation medium

Since PVA could upregulate both OPN and DMP 1 genes of TG cells in the absence of differentiation medium, the effect of PVA on the ALP activity and ARS staining of TG cells in the regular medium was further studied. Fig. 9 shows TG cells grown on TCPS and PVA still expressed ALP activity increased with culturing time. Compared to Fig. 4, TG cells also could exhibit high levels of ALP activity on PVA, regardless of the presence or absence of differentiation medium. This indicates that PVA itself can enhance the ALP activity of TG cell spheroids without the differentiation medium.

Fig. 5. Phase-contrast microscopy of ARS staining of TG cells culture in differentiation medium on TCPS and PVA for 7 days: (a) TCPS, 5E3 (b) TCPS, 1E5 (c) PVA, 5E3 (d) PVA, 1E5.

Fig. 6. Quantification of ARS staining: each well was incubated with 100 mM cetylpyridium chloride for 1 h. The ARS stain released into solution was collected, and read as units of ARS released (1 U is equivalent to 1 U optical density at 570 nm). The quantity of ARS stain of TG cells at different density after 7 days' culture on TCPS and PVA (a) and significant differences were found between ARS staining level of each group at day 7 (b). Each value is the means $\pm$ SD for five experiments with separate samples.

Fig. 7. Phase-contrast microscopy of ARS staining of TG cells which were collected by centrifugation in differentiation medium for 7 days.

Similar to ALP activity, the mineralization of TG cell aggregates suspended on PVA in the absence of differentiation medium showed rapidly increasing intensity and

Figure 8. The mRNA expression of the mineralization related markers GAPDH, OPN and DMP 1 in TG cells after 7 days' culture on TCPS and PVA (a). The number of bases expected for each PCR products is indicated on the right. The histogram of the relative mRNA expression of OPN and DMP 1 was standardized by GAPDH. Bar represents mean $\pm$ SD ($n = 4$) (b). Lane 1, DNA ladder; lanes 2–5, TG cells cultured on TCPS; lanes 6–9 TG cells cultured on PVA. Lane 2, TG cells seeded at low seeding density (5×10^3 cells ml^{-1}) with regular medium; lane 3, TG cells seeded at low seeding density with differentiation medium; lane 4, TG cells seeded at at high seeding density (1×10^5 cells ml^{-1}) with regular medium; lane 5, TG cells seeded at at high seeding density with differentiation medium; lane 6, TG cells seeded at low seeding density with regular medium; lane 7, TG cells seeded at low seeding density with differentiation medium; lane 8, TG cells seeded at high seeding density with regular medium; lane 9, cells seeded at high seeding density with differentiation medium.

there were significant differences compared to those on TCPS (Fig. 10). In addition, the intensity of ARS staining was comparable for TG cell spheroids on PVA in the absence or presence (Fig. 5) of differentiation medium. This further suggests the elevated OPN and DMP 1 mRNA expression level detected by RT-PCR strengthened the observation for ARS staining in the absence of differentiation medium, and this phenomenon could be a result of cellular differentiation under PVA stimulation.

Fig. 9. ALP activity of tooth germ cells cultured. Cells cultured at different densities with regular media on TCPS and PVA for 7 days (a) and significant differences between ALP activities of each group at day 7 (b). Each value is mean $\pm$ SD.

Fig. 10. The quantity of ARS stain of TG cells at different density (5×10^3 and 1×10^5 cells ml^{-1}) in regular media after 7 days culture on TCPS and PVA (a) and significant differences were found between ARS staining level of each group at day 7 (b). Each value is the mean $\pm$ SD.

4.8. The effect of PVA on the TG cells in the presence of RGD

Since PVA itself rapidly increases the intensity of ARS in the absence of differentiation medium, it is reasonable

Fig. 11. The quantity of ARS stain of TG cells at different density (5×10^3 and 1×10^5 cells ml^{-1}) with RGD ($20 \mu\text{g ml}^{-1}$) in regular media after 7 days culture on TCPS and PVA. Results represent the means $\pm$ SD from five independent cultures and determinations. Asterisk denotes significant differences of unit of ARS released compared to the absence of RGD.

to assume that PVA could induce the formation of extra-cellular matrix (ECM), which is important for regulating cell differentiation [26], around TG cell spheroids. Therefore, if the formation of ECM around TG cells was inhibited by the specific binding to RGD, the central cell adhesion domain of ECM proteins such as collagen and fibronectin [27,28] the intensity of ARS staining on PVA will be greatly reduced. Clearly, after adding RGD to the regular medium without induction supplements resulted in a decrease in ARS intensity, regardless of low or high seeding density (Fig. 11). This is consistent with the previous study that RGD could inhibit the differentiation and mineralization of osteoblasts [28,29].

5. Discussion

It is well known that the survival, proliferation and function of cells can vary when the density, morphology and intercellular organization of cells are changed. Generally, cells grow as a monolayer on TCPS in vitro. Compared with monolayer culture, the viability, levels of albumin production and detoxificatory function of hepatocytes are enhanced when they form multicellular spheroids [6]. Neuron precursor cells can only maintain their long-term proliferative potential and stem cell characters when they are cultured into neurospheres [7]. In this study, TG cell spheroids from 4-day-old Wistar rats were prepared by using a cell-nonadhesive substrate. Straightforwardly, a wet environment, resembling in vivo physiological condition, is thought to be more favorable for TG cells. However, PVA, a hydrophilic polymer, rejected TG cell attachment. In general, anchorage-dependent cells cultured in vitro would attach onto the substrate surface; otherwise, they underwent apoptosis [12]. Interestingly, suspended TG

cells could aggregate to form a three-dimensional sphere in suspension (Fig. 1). Although the intimate contact within the aggregate was unfavorable for cell proliferation, TG cells remained viable within the spheroid (Figs. 2 and 3). To our knowledge, this is the first time the effect of TG cell spheroids on the cellular mineralization was examined experimentally.

Quarles et al. reported that proliferating osteoblasts decreased the production of ALP during periods of rapid growth [30]. Owen et al. proposed that proliferation was functionally related to the synthesis of bone-specific extracellular matrix and the maturation and organization of the extracellular matrix contributed to the shut down of proliferation [22]. Therefore, it is reasonable to assume that proliferation-unfavorable PVA biomaterial will also enhance mineralization of TG cell spheroids [5,30]. In this study, the in vitro stimulation effect of PVA on differentiation of TG cells was confirmed (Figs. 4–6). PVA was found to increase ALP activity of TG cells in a seeding density-dependent way (Fig. 4). It is hypothesized that the higher ALP activity of cells seeded at higher density on PVA was attributed to the formation of the more complete three-dimensional structure because the cell–cell interaction within spheroids could be greatly strengthened [31]. This suggests that sufficient aggregation of spheroids may play an important role in the ALP activity expression.

TG cell spheroids suspended on PVA also showed rapidly increasing ARS staining intensity (Figs. 5 and 6). This is similar to the results of Hamano et al. that aggregated periodontal ligament fibroblasts were strongly stained by ARS [32]. Therefore, it is reasonable to assume that the level of mineralization is dependent on the cellular morphology of TG cells. In the present study, positive ARS staining in TG cells on TCPS was found only at higher seeding density, but that occurred in TG cell spheroids on PVA at both low and high densities. It is well known that cell shape changes toward a more spheroidal conformation as cells become confluent. This suggests that cell shape within the TG cell aggregation facilitated synthesis of mineralized matrix.

Besides, the elevated OPN and DMP 1 mRNA expression level detected by RT-PCR also strengthened the observation in ALP activity and ARS staining. OPN is a reliable marker for odontoblasts [33–35] and DMP 1, a dentin-specific protein, plays an important role in controlling dentin formation [36,37]. This again demonstrated that TG cell spheroids cultured on PVA can activate mineralization and increase maturation of TG cells. Furthermore, it is interesting to investigate whether PVA per se is a potent stimulator of TG cell differentiation in the absence of a differentiation medium. For promoting differentiation, induction supplements such as dexamethasone, β -glycerophosphate and ascorbic acid were routinely added into the differentiation medium to induce cell differentiation. Surprisingly, it was shown that PVA could upregulate both OPN and DMP 1 genes of TG cells in the absence of differentiation medium (Fig. 8), although it did not have such

profound effects as that in the presence of the differentiation medium. In addition, Figs. 9 and 10 shows PVA was also able to guide ALP activity and calcium accumulation measured by ARS staining. These results indicated that PVA was capable of inducing TG cell differentiation and mineralization directly, not only as a byproduct of functional enhancement of additives.

Finally, cells recognize ECM proteins by their integrins, cell surface receptors, to influence cell proliferation and differentiation. Therefore, it is interesting to investigate whether the addition of small RGD peptide into the medium can regulate TG cell behaviors by changing integrin-mediated function to ECM components. The present data (Fig. 11) indicates the presence of RGD reduced the calcium accumulation of TG cell spheroids. It is likely that the integrin signals from added RGD or ECM proteins produced by TG cells themselves stimulated by PVA could activate different cell behaviors such as cytoskeleton remodeling and cell cycle progression during the mineralization process [38]. Gloria et al. also found that RGD could inhibit the differentiation and mineralization of osteoblasts by binding of specific integrin receptors [28,29]. Since integrins appear to have important roles in mineralization of TG cell spheroids, the relationship between PVA and TG cells is versatile, which needs further investigation to reveal a potential application. Furthermore, it is known that effects of RGD peptides depend on their structure including flanking residues [29]. For example, OPN produced by TG cells has RGD motif and can cause signal transduction via integrin on cell surface such as osteoblasts [39]. In addition to RGD sequence, OPN has a poly Asp sequence and several potential casein kinase phosphorylation sites [34], which is different from the used RGD peptide without other flanking residues. Therefore, the addition of RGD peptide into the medium had a great influence on TG cell differentiation by changing different integrin-mediated signals. As shown in Fig. 12, even the suspended TG cell spheroids gradually attached onto the PVA surface in the presence of RGD after 7 days of culture. TG cell spheroids disintegrated and cells attached onto the PVA surface with a well-spread morphology.

6. Conclusion

The results of this study suggest that PVA has the unique effect on morphology and the mineralization of TG cells. TG cells were aggregated to form three-dimensional spheroids on PVA, regardless of low or high seeding density, although PVA seems to be an unfavorable substrate for TG cell proliferation. However, there are no significant differences of LDH assay and caspase-3 activity between TG cells cultured on TCPS and PVA ($p < 0.005$), which means that death or apoptosis of TG cell spheroids were not significantly increased. PVA can effectively enhance the ALP activity and mineralization of TG cell spheroids with differentiation medium, regardless of low or high seeding density. Interestingly, gene expression of OPN and DMP 1 of TG cells cul-

Fig. 12. Phase-contrast photomicrograph of TG cells culture on PVA at high seeding density (1×10^5 cells/well) in the presence of differentiation medium with RGD after culturing for 7 days.

tured on PVA exhibited a high level, regardless of low or high seeding density in the absence of differentiation medium. Furthermore, the present study provides that integrins may play an important role in mineralization on TG cell spheroids by adding RGD peptides. Therefore, the present study provides evidence that the relationship between PVA and TG cells is versatile. Further studies on integrins in TG cells may elucidate extracellular matrix interaction effects on tooth mineralization, and regulating these effects may have potential applications.

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References

- [1] Piesco NP, Avery JK. Development of tooth: crown formation. In: Avery JK, Steele PF, Avery NBFA, editors. Oral development and histology. New York, USA: Thieme Medical Publishers; 2002. p. 72.
- [2] Kuo TF, Huang AT, Chang HH, Lin FH, Chen ST, Chen RS, et al. Regeneration of dentin-pulp complex with cementum and periodontal ligament formation using dental bud cells in gelatin-chondroitin-hyaluronan tri-copolymer scaffold in swine. *J Biomed Mater Res A* 2007;85:167.
- [3] Rucci N, Migliaccio S, Zani BM, Taranta A, Teti A. Characterization of the osteoblast-like cell phenotype under microgravity conditions in the NASA-approved rotating wall vessel bioreactor (RWV). *J Cell Biochem* 2002;85:167.
- [4] Botchwey EA, Pollack SR, Levine EM, Laurencin CT. Bone tissue engineering in a rotating bioreactor using a microcarrier matrix system. *J Biomed Mater Res* 2001;55:242.
- [5] Koide N, Sakaguchi K, Koide Y, Asano K, Kawaguchi M, Matsushima H, et al. Formation of multicellular spheroids composed of adult rat hepatocytes in dishes with positively charged surfaces and under other nonadherent environments. *Exp Cell Res* 1990;186:227.

- [6] Kobayashi A, Goto M, Kobayashi K, Akaike T. Receptor-mediated regulation of differentiation and proliferation of hepatocytes by synthetic polymer model of asialoglycoprotein. *J Biomater Sci* 1994;6:325.
- [7] Reynolds BA, Weiss S. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. *Dev Biol* 1996;175:1.
- [8] Young TH, Yao NK, Chang RF, Chen LW. Evaluation of asymmetric poly(vinyl alcohol) membranes for use in artificial islets. *Biomaterials* 1996;17:2139.
- [9] Paul W, Sharma CP. Acetylsalicylic acid loaded poly(vinyl alcohol) hemodialysis membranes: effect of drug release on blood compatibility and permeability. *J Biomater Sci* 1997;8:755.
- [10] Burczak K, Gamian E, Kochman A. Long-term in vivo performance and biocompatibility of poly(vinyl alcohol) hydrogel macrocapsules for hybrid-type artificial pancreas. *Biomaterials* 1996;17:2351.
- [11] Chen RS, Chen YJ, Chen MH, Young TH. Cell-surface interactions of rat tooth germ cells on various biomaterials. *J Biomed Mater Res A* 2007;83:241.
- [12] Bates RC, Buret A, van Helden DF, Horton MA, Burns GF. Apoptosis induced by inhibition of intercellular contact. *J Cell Biol* 1994;125:403.
- [13] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55.
- [14] Necefi A, Tulumoglu B, Giris M, Barbaros U, Gunduz M, Olgac V, et al. The effect of melatonin on TNBS-induced colitis. *Dig Dis Sci* 2006;51:1538.
- [15] Liu HC, Lee IC, Wang JH, Yang SH, Young TH. Preparation of PLLa membranes with different morphologies for culture of MG-63 cells. *Biomaterials* 2004;25:4047.
- [16] Ratisoontorn C, Seto ML, Broughton KM, Cunningham ML. In vitro differentiation profile of osteoblasts derived from patients with Saethre-Chotzen syndrome. *Bone* 2005;36:627.
- [17] Kaneda T, Miyauchi M, Takekoshi T, Kitagawa S, Kitagawa M, Shiba H, et al. Characteristics of periodontal ligament subpopulations obtained by sequential enzymatic digestion of rat molar periodontal ligament. *Bone* 2006;38:420.
- [18] Young TH, Huang JH, Hung SH, Hsu JP. The role of cell density in the survival of cultured cerebellar granule neurons. *J Biomed Mater Res* 2000;52:748.
- [19] Stoker MG, Rubin H. Density dependent inhibition of cell growth in culture. *Nature* 1967;215:171.
- [20] Fujita R, Yoshida A, Mizuno K, Ueda H. Cell density-dependent death mode switch of cultured cortical neurons under serum-free starvation stress. *Cell Mol Neurobiol* 2001;21:317.
- [21] Piro S, Anello M, Di Pietro C, Lizzio MN, Patane G, Rabuazzo AM, et al. Chronic exposure to free fatty acids or high glucose induces apoptosis in rat pancreatic islets: possible role of oxidative stress. *Metab Clin Exp* 2002;51:1340.
- [22] Owen TA, Aronow M, Shalhoub V, Barone LM, Wilming L, Tassinari MS, et al. Progressive development of the rat osteoblast phenotype in vitro: reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extracellular matrix. *J Cell Physiol* 1990;143:420.
- [23] Yokose S, Kadokura H, Tajima Y, Fujieda K, Katayama I, Matsuoka T, et al. Establishment and characterization of a culture system for enzymatically released rat dental pulp cells. *Calcif Tissue Int* 2000;66:139.
- [24] Kato Y, Iwamoto M, Koike T, Suzuki F, Takano Y. Terminal differentiation and calcification in rabbit chondrocyte cultures grown in centrifuge tubes: regulation by transforming growth factor beta and serum factors. *Proc Natl Acad Sci USA* 1988;85:9552.
- [25] Nakagawa Y, Shimizu K, Hamamoto T, Kotani S, Yamamuro T. Electron-microscopy of calcification during high-density suspension-culture of chondrocytes. *Calcif Tissue Int* 1993;53:127.
- [26] Juliano RL, Haskill S. Signal transduction from the extracellular matrix. *J Cell Biol* 1993;120:577.

- [27] Hwang DS, Sim SB, Cha HJ. Cell adhesion biomaterial based on mussel adhesive protein fused with RGD peptide. *Biomaterials* 2007;28:4039.
- [28] Keselowsky BG, Collard DM, Garcia AJ. Integrin binding specificity regulates biomaterial surface chemistry effects on cell differentiation. *Proc Natl Acad Sci USA* 2005;102:5953.
- [29] Gronowicz GA, Derome ME. Synthetic peptide-containing Arg-Gly-Asp inhibits bone-formation and resorption in a mineralizing organ-culture system of fetal-rat parietal bones. *J Bone Miner Res* 1994;9:193.
- [30] Quarles LD, Yohay DA, Lever LW, Caton R, Wenstrup RJ. Distinct proliferative and differentiated stages of murine MC3T3–E1 cells in culture: an in vitro model of osteoblast development. *J Bone Miner Res* 1992;7:683.
- [31] Lin SJ, Jee SH, Hsiao WC, Lee SJ, Young TH. Formation of melanocyte spheroids on the chitosan-coated surface. *Biomaterials* 2005;26:1413.
- [32] Hamano T, Teramoto A, Iizuka E, Abe K. Effects of polyelectrolyte complex (PEC) on human periodontal ligament fibroblast (HPLF) function. I. Three-dimensional structure of HPLF cultured on PEC. *J Biomed Mater Res* 1998;41:257.
- [33] Chou YF, Dunn JC, Wu BM. In vitro response of MC3T3–E1 pre-osteoblasts within three-dimensional apatite-coated PLGA scaffolds. *J Biomed Mater Res B Appl Biomater* 2005;75:81.
- [34] Butler WT, Ritchie H. The nature and functional significance of dentin extracellular matrix proteins. *Int J Dev Biol* 1995;39:169.
- [35] Leali D, Dell’Era P, Stabile H, Sennino B, Chambers AF, Naldini A, et al. Osteopontin (Eta-1) and fibroblast growth factor-2 cross-talk in angiogenesis. *J Immunol* 2003;171:1085.
- [36] Foster BL, Nociti Jr FH, Swanson EC, Matsa-Dunn D, Berry JE, Cupp CJ, et al. Regulation of cementoblast gene expression by inorganic phosphate in vitro. *Calcif Tissue Int* 2006;78:103.
- [37] Baba O, Qin C, Brunn JC, Wygant JN, McIntyre BW, Butler WT. Colocalization of dentin matrix protein 1 and dentin sialoprotein at late stages of rat molar development. *Matrix Biol* 2004;23:371.
- [38] Huveneers S, Truong H, Faessler R, Sonnenberg A, Danen EHJ. Binding of soluble fibronectin to integrin alpha 5 beta 1—link to focal adhesion redistribution and contractile shape. *J. Cell Sci* 2008;121:2452.
- [39] Puleo DA, Bizios R. RGDS tetrapeptide binds to osteoblasts and inhibits fibronectin-mediated adhesion. *Bone* 1991;12:271.