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Effect of fluctuating glucose concentration on osteogenic differentiation of periodontal ligament cells

Xin Deng[1], Hirohito Kato[2], *Yoichiro Taguchi[2], Takaya Nakata[2] and Makoto Umeda[2]

[1]Graduate School of Dentistry (Department of Periodontology) and [2]Department of Periodontology, Osaka Dental University, 8-1 Kuzuhahanazono-cho, Hirakata-shi, Osaka, 573-1121, Japan
**E-mail: taguchi@cc.osaka-dent.ac.jp*

Although fluctuations in glucose concentration have been reported to regulate osteogenic differentiation, the effect on periodontal ligament cells (PDLCs) is not clear. We investigated the effect of fluctuating glucose concentrations on the cellular functions of PDLCs. Variations in glucose concentration were examined in four ways. Group A was incubated in 100 mg/dL glucose for 14 days. Group B was incubated in 100 mg/dL glucose for 7 days, the culture medium was replaced with 0 mg/dL glucose, and the group was then incubated for another 7 days. Group C was incubated in 0 mg/dL glucose for 7 days, the culture medium was replaced with 100 mg/dL glucose, and the group was then incubated for another 7 days. Group D was incubated in 0 mg/dL glucose for 14 days. We evaluated the osteogenic differentiation capacity by alkaline phosphatase (ALP) activity, ALP staining, calcium deposition and alizarin red staining after the 14 days of culturing. We found that the osteogenic differentiation of PDLCs was enhanced in the presence of glucose, suggesting that glucose metabolism regulates the osteogenic differentiation of PDLCs, especially in the early stages of differentiation. (J Osaka Dent Univ 2023; 57: 25-29)

Key words: periodontal ligament cells; glucose; osteogenic differentiation

INTRODUCTION

The periodontium is located between the cementum and the alveolar bone. It is a specialized, vascular-rich fibrous connective tissue composed of fibroblasts, osteoblasts, cementum cells, and mesenchymal cells. PDLCs originate from periodontal tissues, maintain some stem cell properties of PDLSCs, and can differentiate into osteoblasts.[1, 2] PDLCs are also closely related to periodontal tissue regeneration, and are crucial for periodontal tissue regeneration.

Many studies have shown that glucose metabolism regulates numerous cellular functions and is also deeply implicated in bone and hard tissue formation.[3] According to recent studies, high glucose environments such as diabetes inhibit osteogenic differentiation of PDLCs.[4-7] It also has been shown that a high glucose environment has different effects in the early and late stages of osteogenic differentiation.[8] According to our previous studies, a sustained low glucose environment inhibits the osteogenic differentiation of PDLCs. However, the effect of a low glucose environment on the early and late osteogenic differentiation of PDLCs is still unclear.

This study clarifies the effect of fluctuating glucose concentrations on early and late osteogenic differentiation of PDLCs.

MATERIALS AND METHODS

Isolation and culture of PDLSCs

PDLSCs were cultured from three females who were 20 to 28 years of age. We used passages 4 to 7 in this study. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nacalai

Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Marlborough, MA, USA) and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin, and 25 μg/mL amphotericin B; Nacalai Tesque) at 37°C under 5% CO_2. All experiments were approved by the Osaka Dental University Medical Ethics Committee (Approval no.111132).

Culture medium

For stimulation at two glucose concentrations, the PDLCs were cultured in DMEM supplemented with 10% FBS, 50 μg/mL ascorbic acid, 10 mM beta-glycerophosphate and 10 nM dexamethasone, all from Nacalai Tesque. Variation in glucose concentration was examined in the four groups shown in Fig. 1. Group A, the positive control, was incubated in 100 mg/dL glucose for 14 days. Group B was incubated in 100 mg/dL glucose for 7 days, the medium was replaced with 0 mg/dL glucose, and the cells were incubated for another 7 days. Group C was incubated in 0 mg/dL glucose for 7 days, the medium was replaced with 100 mg/dL glucose, and the cells were incubated for another 7 days. Group D, the nagetive control, was incubated in 0 mg/dL glucose for 14 days.

Alkaline phosphatase (ALP) staining and activity

The PDLCs were cultured in 24-well plates at 4×10^4 cells/mL in glucose-supplemented osteogenic medium and incubated for 7 and 14 days.

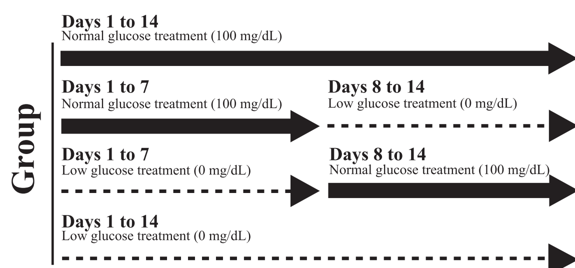

Fig. 1 Protocol for fluctuating glucose treatment during osteogenic differentiation of PDLCs. We examined the variation in glucose concentration in four groups. ALP activity, ALP staining, extracellular calcium deposition, Alizarin red staining and osteocalcin production were examined on day 14.

They were then stained using an ALP staining kit (Sigma-Aldrich, St. Louis, MO, USA) in accordance with the manufacturer's protocol. At the same time points, the PDLCs were treated with 0.2% Triton X-100 (Sigma-Aldrich) and ALP activity was measured after addition of 1-step PNPP (Pierce Biotechnology, Rockford, IL, USA). Product formation data were determined by measuring absorbance at 405 nm with a SpectraMax iD 3 spectrophotometer (Molecular Devices, San Jose, CA, USA), and the values were normalized to the DNA content measured by a Pico Green dsDNA Assay Kit (Invitrogen, Thermo Fisher, Paisley, UK) in the respective samples.

Extracellular calcium deposition and Alizarin red S (ARS) staining

The PDLCs were cultured in 24-well plates at 4×10^4 cells/mL for 7 and 14 days. After the osteogenic medium was removed, the cells were washed with PBS and the extracellular calcium was dissolved with 10% formic acid (Nacalai Tesque). The deposition of extracellular calcium was quantified using the Calcium E-test kit (Wako Pure Chemical Industries, Osaka, Japan) according to the manufacturer's protocol. For the ARS assay, PDLCs were cultured for 14 days, then stained for 3 minutes at room temperature with a solution of 1% ARS (Wako Pure Chemical Industries) and washed three times with PBS.

Measurement of osteocalcin (OCN)

PDLCs were cultured in glucose-supplemented osteogenic medium for 14 days. The spent culture medium was then collected and the OCN was quantified using an OCN detection kit (Takara Bio, Shiga, Japan) according to the manufacturer's protocol.

Statistical Analysis

Data are presented as the mean standard deviation. Parametric data from three or more groups were analyzed using a two-tailed one-way Analysis of Variance (ANOVA) with post hoc Tukey's test. Values of $p < 0.05$ were considered significant.

RESULTS

ALP activity and ALP staining

We investigated the effect of glucose variation on ALP by measuring ALP activity and doing an ALP staining assay. As shown in Fig. 2, ALP activity was significantly inhibited in Groups B and C compared with Group A. In the glucose variation groups, the inhibition of ALP activity was more pronounced in Group C than in B. However, the ALP activity was significantly enhanced in Groups B and C compared with D. In other words, ALP activity was greatest in Group A, followed by B, C and D in that order. ALP staining showed the same trend.

Calcium deposition and Alizarin red staining

We assessed the effect of changes in glucose on the late-stage osteogenic differentiation of PDLCs by measuring calcium deposition, osteocalcin production, and Alizarin red staining. As shown in Fig. 3, Calcium deposition was significantly reduced in Groups B and C compared to A. In the glucose variation group, low glucose significantly inhibited calcium deposition more in Group C than in B. Calcium deposition was significantly enhanced in both Groups B and C compared with D. In other words, the calcium deposition was greatest in Groups A, B, C and D, in that order. Alizarin red staining showed the same trend as calcium deposition.

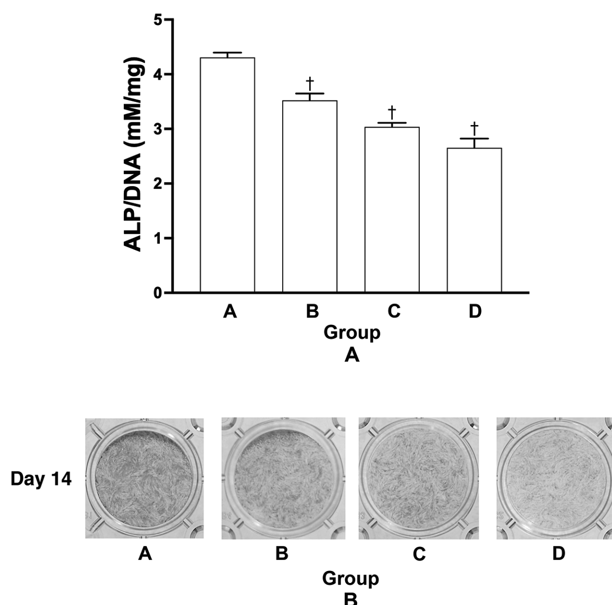

Fig. 2 Effect of fluctuating glucose concentration on the ALP activity in PDLCs. (A) The intensity of alkaline phosphatase (ALP) was decreased in Groups B, C and D after 14 days. (B) ALP staining showed the same tendency as ALP activity in the glucose variation groups (B and C) after 14 days (†$p < 0.05$ versus control, 100 mg/dL glucose at each time point).

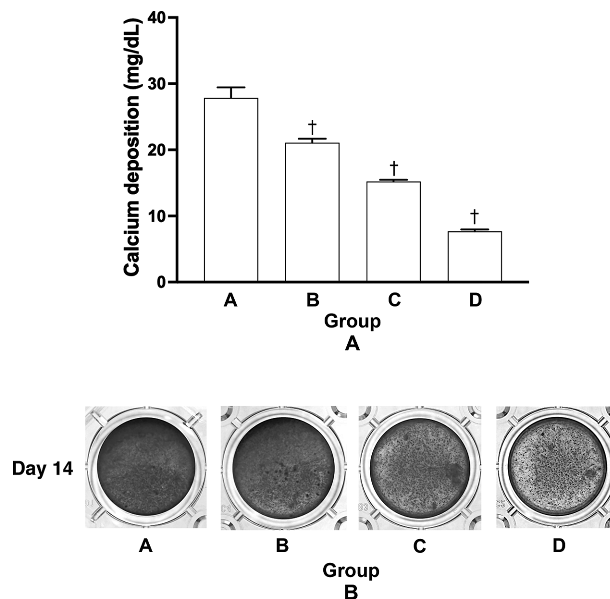

Fig. 3 Effect of fluctuating glucose concentrations on calcium deposition and Alizarin red staining in PDLCs. (A) Calcium deposition was significantly decreased in the glucose variation groups (B and C) after 14 days. (B) Calcified nodule formations stained with Alizarin red showed the same tendency as calcium deposition in the glucose variation groups (B and C) after 14 days (†$p < 0.05$ versus control, 100 mg/dL glucose at each time point).

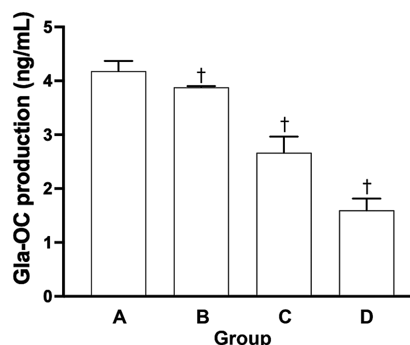

Fig. 4 Effect of fluctuating glucose concentration on osteocalcin production in PDLCs. (A) Osteocalcin (OCN) production was decreased after 14 days in the glucose variation groups (B and C) (†$p < 0.05$ versus control, 100 mg/dL glucose at each time point).

Osteocalcin production

As shown in Fig. 4, Osteocalcin was significantly suppressed in both Groups B and C compared to A. In other words, the osteocalcin production was greatest in Groups A, B, C and D, in that order.

DISCUSSION

The potential for osteogenic differentiation of PDLCs is a key factor for the success of periodontal tissue regeneration. According to previous studies, a prolonged high-glucose environment inhibits the osteogenic differentiation of PDLCs.[4] Bone tissue formation was inhibited and the intracellular glucose concentration in mice decreased in glucose transporter 1 (GLUT1) knockout mice.[9] However, the effect of low glucose concentration is still unclear on the early and late osteogenic differentiation of PDLCs. Therefore, we used ALP activity, ALP staining, calcium deposition, ARS staining, and OCN production to assess the effect of a fluctuating glucose environment on the early and late osteogenic differentiation of PDLCs.

ALP is one of the markers of the early and middle stages of osteogenic differentiation.[10,11] We selected Group A as a positive control and D as a negative control, and confirmed that the ALP activity was significantly inhibited in Group D compared to A. According to our results, Group B, representing the late low glucose environment, inhibited ALP activity in PDLCs compared to Group A, the normal glucose environment. Group C, representing the early low glucose environment, promoted ALP activity in PDLCs compared to Group D, the no glucose environment. This result suggests the importance of glucose for ALP activity, which decreases after glucose starvation in the normal microenvironment. In contrast, glucose in the low-nutrient environment promotes ALP activity. However, Group B showed greater ALP activity than did C. This result indicates that glucose is necessary and essential for the early stage of osteogenic differentiation in PDLCs. ALP staining showed the same trend.

Osteocalcin is one of the markers of late osteogenic differentiation.[12-14] Eextracellular calcium deposition is an essential indicator for assessing osteogenic differentiation.[15,16] As with ALP activity, we first confirmed that calcium deposition was significantly inhibited in Group D compared to A. In the present study, calcium deposition and osteocalcin production were suppressed in Groups B and C compared to A. This result suggests that glucose starvation causes different degrees of inhibition of osteogenic differentiation in PDLCs and a low glucose concentration suppresses the osteogenic differentiation in the middle and late stages of PDLCs. Based on the fact that osteocalcin production and calcium deposition were greater in Group B than in C, we hypothesize that adequate glucose concentration is critical in the early stages of osteogenic differentiation in PDLCs.

In conclusion, this study showed that osteogenic differentiation in PDLCs is promoted in the presence of glucose, which indicates that glucose is an important factor in the early stage of osteogenic differentiation in PDLCs. These results suggest that glucose metabolism is involved in periodontal tissue regeneration and that the presence of glucose is significant in the early stages of osteogenic differentiation. We hope these findings will contribute to a better understanding of the involvement of glucose metabolism in periodontal tissue regeneration.

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