

Original

Effects of High Glucose for Hard Tissue Formation on Type II Diabetes Model Rat Bone Marrow Cells *In Vitro*

Makiko Okuda¹⁾, Yoichiro Taguchi¹⁾, Saitatsu Takahashi¹⁾,
Akio Tanaka²⁾ and Makoto Umeda¹⁾

¹⁾ Department of Periodontology, Osaka Dental University, Osaka, Japan

²⁾ Department of Oral Pathology, Osaka Dental University, Osaka, Japan

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Abstract: Diabetes mellitus (DM) is one of the main etiologies and risk factors for periodontal disease, and is an important concern in periodontal medicine. It has been reported that high glucose concentrations mediate proliferation, differentiation and production of inflammatory cytokines on mesenchymal cells, but there are few reports on periodontal regenerative therapy in type II DM patients. The aim of this study was to examine the biological effects of high glucose conditions on GK rat (type II model) bone marrow mesenchymal cells (GK rat BMMSC). Cell culture experiments were performed with GK rat BMMSC. The effects of glucose at four concentrations (5.5, 8, 12 and 24 mM) were determined by examining cell proliferation, differentiation and production of inflammatory cytokines; the latter three concentrations are higher than the normal physiological glucose concentration, represented by 5.5 mM. High concentrations of glucose promoted proliferation and inhibited hard tissue differentiation and calcification of GK rat BMMSC. Differentiation correlated inversely with the expression of inflammatory cytokines, represented by IL-6. Our data suggest that high extracellular glucose concentrations promote proliferation and inhibit hard tissue differentiation and calcification in periodontal regeneration by causing an inflammatory response dependent on cytokines including IL-6.

Key words: Hard tissue differentiation; High glucose; Inflammatory cytokine

Introduction

Diabetes mellitus (DM) is characterized by high blood glucose, which results from defects in insulin secretion, insulin action, or both. DM has become one of the main threats to human health in the 21st century. The two main types of DM are classified on the basis of their underlying pathophysiology; Type 1 (T1) results from an autoimmune-mediated destruction of the insulin producing β -cells, while type 2 (T2) results from insulin resistance, rather than from a total absence of insulin production. Although the pathogenesis of DM involves complex interrelationships between genetic and environmental or acquired factors, the recent proposal that chronic inflammation or infection can provoke insulin resistance, and thereby contribute to the development of diabetes and its complications, has gained interest¹⁾.

Diabetic complications include bone disorders such as periodontal disease and osteoporosis. In particular, T2 DM is said to have a bidirectional relation with periodontal disease and "periodontal medicine"²⁾. Periodontal tissue wound healing is poor

in patients with diabetes. Moreover, much evidence suggest that diabetes is associated with an increased prevalence (3 to 4-fold), extent and severity of periodontal disease^{3,4)}. It has been argued that diabetes-enhanced inflammation may affect the oral environment and increase the severity of periodontitis by provoking a wider destruction of tissue⁴⁾ and by delaying wound-healing⁵⁾. Many *in vitro* studies of diabetic wound healing are undertaken⁶⁾. Periodontal ligament cells, as undifferentiated mesenchymal cells, are often used as models for periodontal disease. Many recent studies have investigated the relationships between high glucose, diabetes and hard tissue differentiation. Li *et al.*⁷⁾ reported on the effects of high glucose on mesenchymal stem cell proliferation and differentiation. García-Hernández⁸⁾ *et al.* investigated the relationship between high glucose concentration and mineralization in human osteoblastic cells. Their data suggested that hyperglycemia may directly affect biomineralization and inflammatory processes in osteoblasts, but they noted that DM is a complex disease. Despite many studies on hard tissue formation from mesenchymal stem cells in high glucose mediated culture, cells used for these experiments are normal undifferentiated cells such as bone marrow and periodontal ligament cells.

Correspondence to: Dr. Yoichiro Taguchi, Department of Periodontology, Osaka Dental University, 8-1, Kuzuha-hanazono-cho, Hirakata, Osaka, Japan; Tel: +81-72-864-3084; Fax: +81-72-864-3184; E-mail: taguchi@cc.osaka-dent.ac.jp

In the present study, we investigated undifferentiated mesenchymal cells isolated from a type II diabetes mellitus model when cultured in high concentrations of glucose. We examined the mineralization effects at four different glucose concentrations, as well as analyzing cell proliferation and gene expression related to osteoblastic differentiation and inflammatory cytokines.

Materials and Methods

Cell culture

GK rat bone marrow mesenchymal cells (GK rat BMMSC) were isolated from the femurs of 8-week-old GK rats, a model type II diabetes rat. This study was performed under the Guidelines for Animal Experimentation at Osaka Dental University (Approval No. 14-08001). Briefly, rats were euthanized using 4% isoflurane, and bones were aseptically excised from the hind limbs. The proximal end of the femur and the distal end of the tibia were clipped. A 21-gauge needle (TERMO, Tokyo, Japan) was inserted into the hole in the knee joint of each bone, and the marrow was flushed from the shaft with culture medium (Eagle's minimal essential medium, MEM; Wako Pure Chemical Industrials, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Life Technologies Corp., Carlsbad, CA, USA), penicillin (500 U/ml) (Cambrex Bio Science Walkersville Inc., Walkersville, MD, USA), streptomycin (500 µg/ml) (Cambrex Bio Science Walkersville Inc.) and fungizone (1.25 µg/ml) (Cambrex Bio Science Walkersville Inc.). The resulting marrow pellet was dispersed by trituration, and the cell suspensions from all bones were combined in a centrifuge tube. GK rat BMMSC were cultured in 75 cm² culture flasks (Falcon, Becton Dickinson Labware, NJ, USA) in culture medium as above. The cells were cultured at 37 °C in a humidified 5 % CO₂/95 % air atmosphere. At confluence, cells were removed from flasks by trypsinization and washed twice in phosphate buffered saline (PBS), resuspended in culture medium and seeded. Cells were used in experiments after 3–5 passages.

Normal and high glucose conditions

The glucose concentrations for this study were chosen to reflect normal, postprandial and high glucose values, similar to those seen in DM. Specifically, the normal glucose concentration of 5.5 mM is equivalent to 99 mg/dl, the postprandial concentration of 8 mM corresponds to 144 mg/ml and the high glucose concentrations of 12 and 24 mM are approximately equal to 216 and 432 mg/dl, respectively.

Cell Proliferation Assay

GK rat BMMSC were harvested and seeded at a density of 4×10³ cells/well into 96-well microplates (Falcon). After 24 h, the medium was replaced by each glucose condition medium, consisting of MEM supplemented with 5.5, 8, 12 or 24 mM glucose (Nacalai Tesque Inc., Kyoto, Japan), 10% FBS, penicillin

(500 U/ml), streptomycin (500 µg/ml) and fungizone (1.25 µg/ml). Microplates were washed with PBS and cell proliferation was determined using the CellTiter-Blue™ Cell Viability Assay (Promega, Madison, WI, USA) as MTS assay after 3, 24, 48 and 72 hours of incubation. Assays were conducted according to the manufacturer's protocol. Briefly, following aspiration of supernatant, 100 µl of CellTiter-Blue™ Reagent diluted 6-fold in PBS was added to each well, followed by 1 h of incubation at 37 °C. Fluorescence intensity (excitation 560 nm, emission 590 nm) was measured using a multi-microtiter reader (SpectraMax M5, Molecular Devices Inc., Sunnyvale, CA, USA).

Cell differentiation

At confluence, cells were removed from flasks by trypsinization, washed twice with PBS, resuspended in culture medium and seeded at a density of 4×10⁴ cells/cm² into 24-well tissue culture plates (Falcon). The cells were incubated until they reached confluence. The medium was then removed and replaced with differentiation medium containing 10 % FBS, osteogenic supplements (10 mM β-glycerophosphate (Wako), 80 mg/mL ascorbic acid (Nacalai Tesque Inc., Kyoto, Japan), and 10 nM dexamethasone (Nacalai Tesque Inc.)) and glucose (at one of the four concentrations that were tested). This differentiation medium was replaced every second day.

RNA preparation and Real-time PCR Analysis

After 7, 14 and 21 days of culture, total RNA was isolated using an RNeasy Mini Kit (Qiagen, Venlo, the Netherlands). 10 µL of RNA from each sample were reverse transcribed into cDNA using a PrimeScript RT Reagent Kit (Takara Shuzo Co, Otsu, Shiga, Japan).

mRNA expression was investigated by real-time reverse transcriptase-polymerase chain reaction (RT-PCR), using a StepOne Plus™ Real-Time RT-PCR System (Applied Biosystems, Foster city, CA, USA). Ten microliters of Taqman Fast PCR Master Mix, 1 mL of the each primer (Taqman Gene Expression Assays), 2 ml of sample cDNA, and 7 ml of DEPC water (Nippongene, Tokyo, Japan) were added to each well in a Fast 96-well Reaction Plate (0.1 ml well volume; Applied Biosystems). The plate was subjected to 40 reaction cycles of 95 °C for 1 s, and 60 °C for 20 s. Gene expression levels were calculated employing the $\Delta\Delta C_t$ method⁹, relative to the expression of control genes. Expression of genes including alkaline phosphatase (ALP), osteocalcin (OCN), runt-related transcription factor 2 (RUNX2), collagen type 1 (COL-1), interleukin-1β (IL-1β) and interleukin-6 (IL-6) was quantified. ALP after 14 days of culture and OCN after 21 days of culture were measured as markers of osteogenic differentiation. RUNX2 was measured after 7 days of culture as a marker of transcription factors. COL-1 was measured after 7 days of culture as a marker of the extracellular matrix. IL-1β and IL-6 were

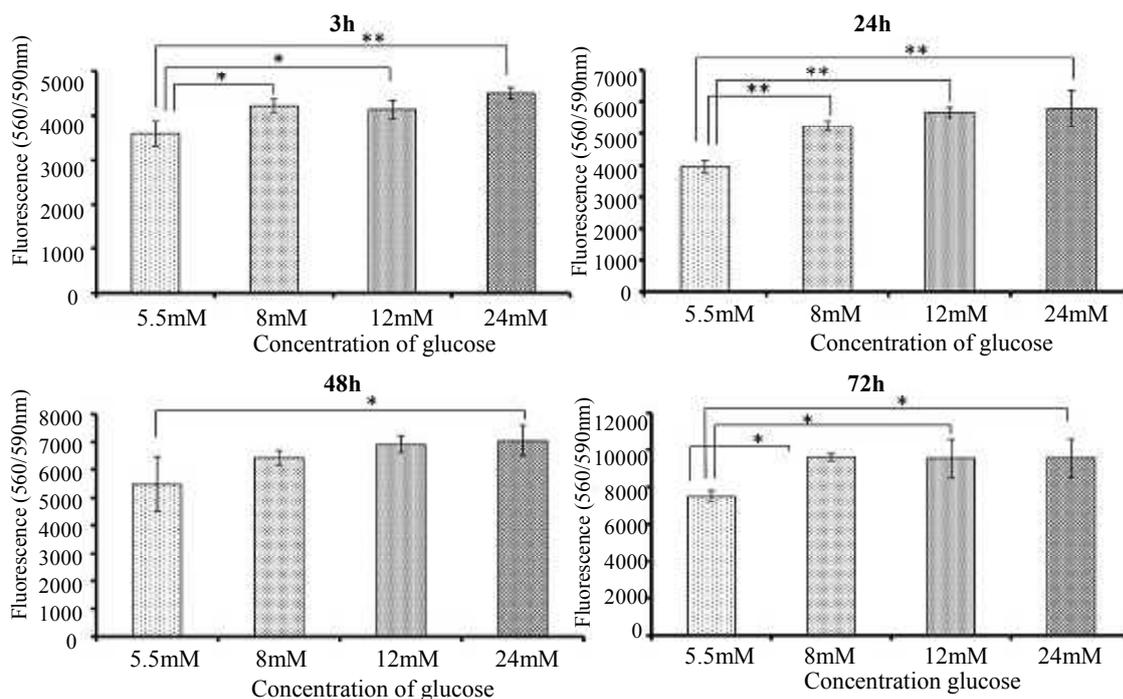


Figure 1. Cell proliferation after 3, 24, 48 and 72 hours of culture (* $p < 0.05$; ** $p < 0.01$).

measured as markers of proinflammatory cytokines in GK rat BMMSC.

Mineralization

Calcium deposited in the extracellular matrix after 21 days of culture was measured after dissolution with 10% formic acid. The amount of calcium was quantified using a Calcium E-test Kit (Wako Pure Chemical Industrials Ltd). 1 ml Calcium E-Test reagent and 2 ml kit buffer were added to 50 ml of collected medium, and the absorbance of the reaction products was measured at 610 nm using a 96 well microplate reader (SpectraMax M5). The concentration of calcium ions was calculated from the absorbance value relative to a standard curve.

GK rat BMMSC were plated in 24 well plates and cultured in normal medium until they reached confluence. The culture medium was then switched to osteogenic medium at each glucose concentration and cells were cultured for 21 days. The medium was removed, cells were washed with PBS, and GK rat BMMSC were fixed in 70 % ethanol for 10 min at -20°C . The GK rat BMMSC were next stained with a solution of 1 % alizarin red S for 5 min at room temperature and washed three times with distilled water. The calcified nodules were observed using a fluorescence microscope (BZ9000, Keyence, Osaka, Japan).

Statistical analysis

All experiments were performed in triplicate. Data were analyzed using SPSS 19.0 software (SPSS; IBM, Chicago, IL,

USA). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to determine significance. Values of $P < 0.05$ were considered significant

Results

Cell proliferation

Cell proliferation during the first 3 days of incubation was assessed (Fig. 1). There were significant differences in cell proliferation between 5.5 mM glucose and the other glucose groups after 3, 24 and 72 h. High glucose concentrations tended to promote the proliferation of GK rat BMMSC.

Osteogenic gene expression

Fig. 2 shows the expression of osteogenesis-related genes including RUNX2, ALP, COL-1 and OCN, assessed by quantitative RT-PCR. Similar results were obtained for RUNX-2 and OCN mRNA expression levels; generally, high glucose inhibited mRNA expression for both genes, but 24 mM glucose induced more mRNA expression than any other concentration. Expression of ALP and COL-1 mRNA followed similar profiles; as glucose concentration increased, expression of ALP and COL-1 mRNA significantly decreased.

Extracellular matrix (ECM) mineralization

ECM mineralization was assessed by deposition of calcium and alizarin red staining (Fig. 3). Calcium deposition showed the same glucose concentration dependent pattern as expression of

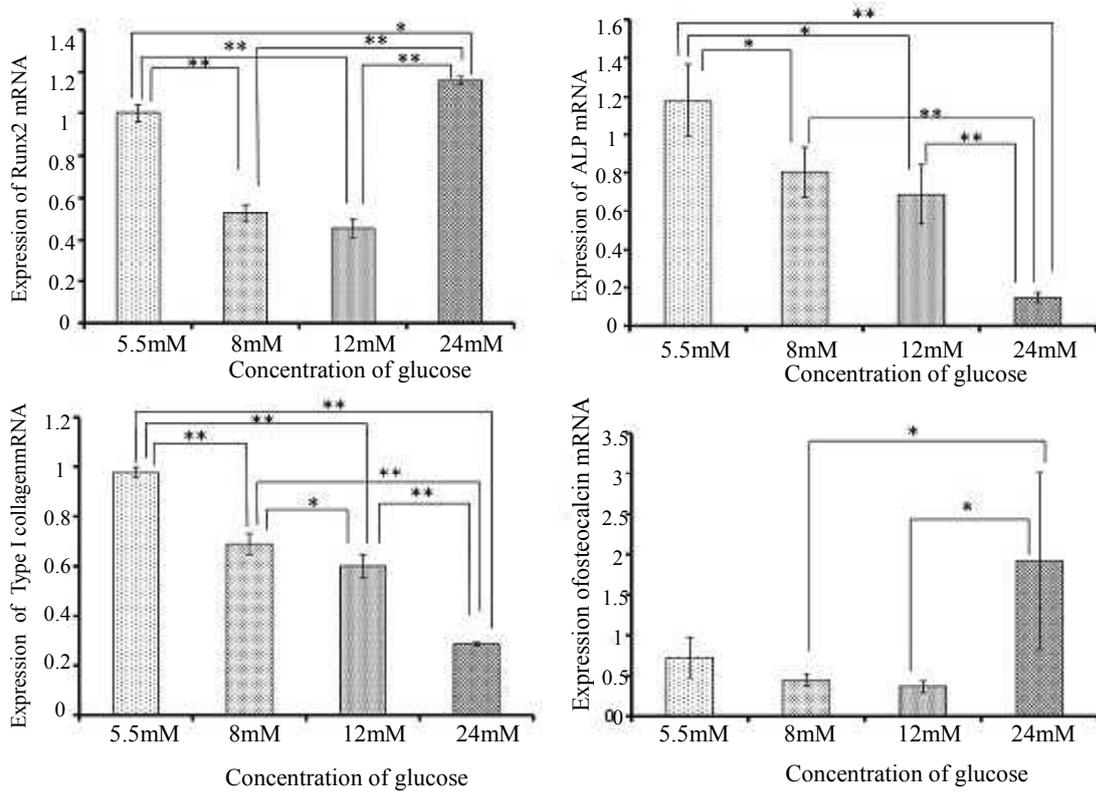


Figure 2. Expression of osteogenesis-related genes. RUNX2, ALP, COL-1 and OCN mRNA levels were assessed by Real-time PCR (* p < 0.05; ** p < 0.01).

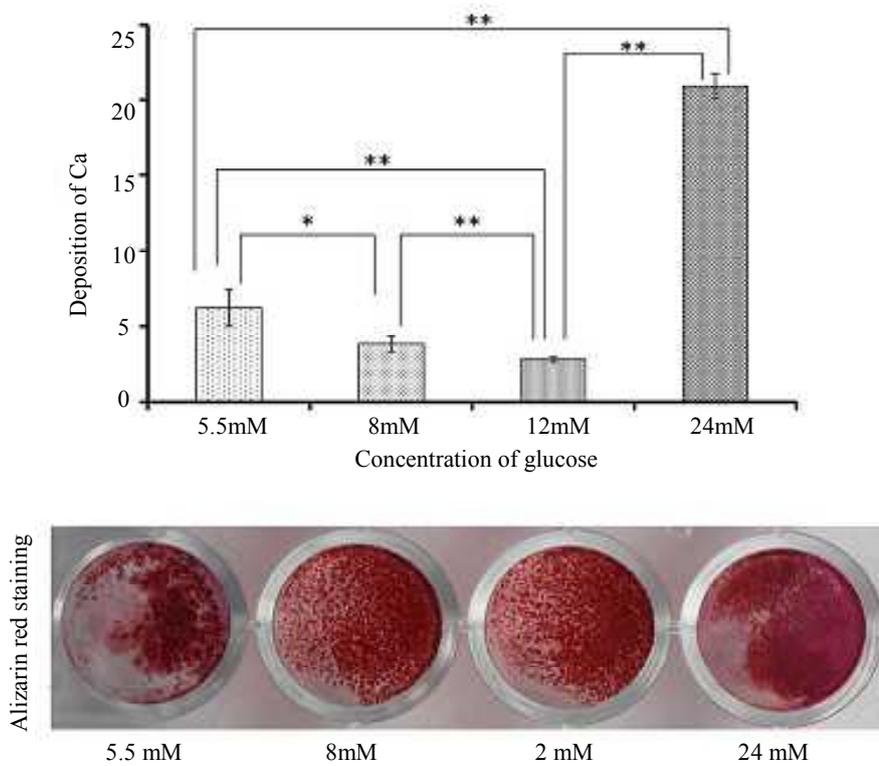


Figure 3. The graph indicates the quantity of extracellular calcium deposition (* p < 0.05; ** p < 0.01). The photograph shows an image of the Alizarin red staining.

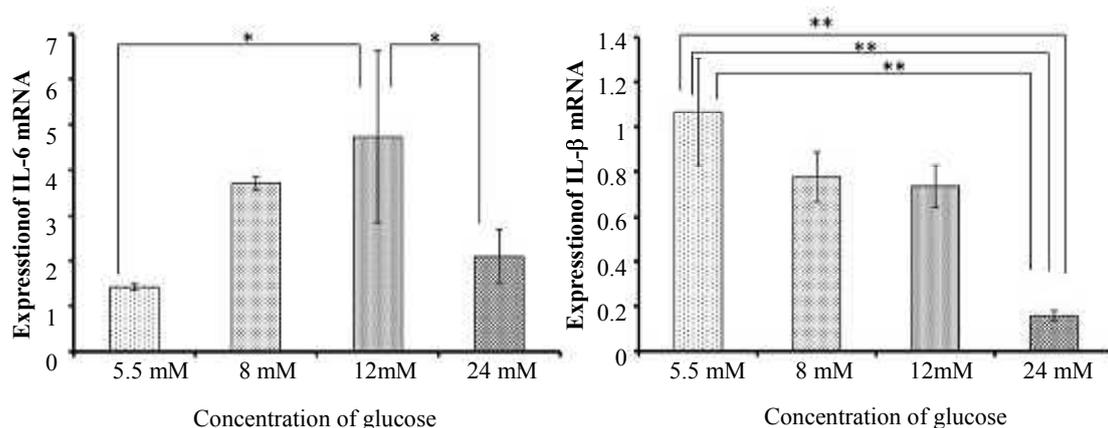


Figure 4. Expression of proinflammatory cytokine genes. IL-6 and IL-1 β mRNA expression levels were assessed by Real-time PCR (* $p < 0.05$; ** $p < 0.01$).

RUNX 2 and OCN mRNA (Fig. 2).

High concentrations of glucose increase IL-1 β and IL-6 mRNA expression

Fig. 4 shows the expression of inflammatory cytokine genes IL-1 β and IL-6, assessed by quantitative RT-PCR. The expression of IL-1 β mRNA was significantly inhibited as glucose concentration increased. The expression of IL-6 mRNA was opposite to that of RUNX2 and OCN mRNA; as glucose concentration increased to 12 mM, expression of the IL-6 mRNA rose, but the mRNA expression level decreased on exposure to 24 mM glucose.

Discussion

In the present study, we assessed hard tissue differentiation of undifferentiated mesenchymal cells from a type II diabetes mellitus model when cultured in high concentrations of glucose. Typical physiological glucose concentration is 5.5 mM; three of the glucose concentrations we tested were higher (8-24 mM). Highly-concentrated glucose promotes cell proliferation and inhibits hard tissue differentiation and calcification of GK rat BMMSC. The observed effect on differentiation depends on production of inflammatory cytokines such as IL-6.

Glucose in the microenvironment markedly affects gene regulation, proliferation, and differentiation of mesenchymal cells. Fig. 1 shows that GK rat BMMSC proliferation is proportional to glucose concentration. Li *et al.*⁷⁾ reported that in human mesenchymal stem cells, no consistent effect on proliferation was seen on short-term (4 day) exposure to 25 mM glucose. In long-term cultures (4 weeks) of cells from individual donors, variable trends were observed, and the overall analysis showed a slight but significant decrease in cell proliferation after 25 mM glucose treatment. Well *et al.*¹⁰⁾ suggested that high glucose concentrations in cell culture medium do not acutely affect human mesenchymal

stem cell growth factor production or proliferation. Zhao *et al.*¹¹⁾ reported that diabetic bone marrow stromal cells demonstrated reduced proliferation ability. Because they described the proliferation of normal rat cells, we cannot compare our results with theirs. The growth speed of the cells extracted from DM seems to tend to decrease, we suggest cell proliferation is proportional to glucose concentration. Li *et al.*⁷⁾ suggested that proliferation and osteogenic differentiation are stimulated by high glucose. However cell proliferation and differentiation are inverse events in general, and all these reports are performed at 25 mM as high glucose concentration.

High glucose in the body is associated with inflammatory conditions. The inflammatory pathways of Toll-like receptors (TLRs) have been widely studied due to their relationship with proinflammatory cytokines. TLRs recognize pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), lipoproteins and peptidoglycans. The expression levels of TLR-2 and -4 in gingival tissue of patients with chronic periodontitis seem to correlate with the severity of the disease and with an increase in bacterial plaque¹²⁾. Moreover, the expression levels of TLR-2, -4 and -9 are increased in gingival tissue in patients with type 2 diabetes and chronic periodontitis¹³⁾. Recent findings have shown increased TLR-2 and -4 expression, signaling, ligands, and functional activation in monocytes from T1 DM subjects compared with controls; these levels were further accentuated in T1 DM with microvascular complications¹⁴⁾. It has been suggested that inflammation in individuals with diabetes is exacerbated by nonmicrobial mechanisms. In addition to exogenous microbial products, TLRs can recognize endogenous ligands such as necrotic cells¹⁵⁾, heat shock proteins Hsp70 and gp96¹⁶⁾, the oligosaccharides of soluble hyaluronate¹⁷⁾, and advanced glycation end products (AGEs)¹⁸⁾. Causes of diabetes-related periodontitis becoming severe include neutrophil dysfunction in hyperglycemia, microangiopathy, composition suppression of collagen, and

increase in the oxidative stress response¹⁹⁻²¹). One oxidative stress response, production of AGEs, is considered a nonmicrobial inflammatory factor. Takagi *et al.*²²) reported that AGEs promote expression of IL-6 in human bone-derived cells. AGEs have been considered to induce inflammatory reaction in osteoblasts. We reported that hard tissue differentiation from mesenchymal stem cells decreases in inflammatory conditions caused by *Porphyromonas gingivalis* LPS²³). We therefore suggest that the hard tissue differentiation response decreases in conditions of inflammation, particularly increased expression of IL-6. Fig. 2 and 4 show that the expression of Runx2 and osteocalcin mRNA correlated inversely with the expression of IL-6 mRNA. Conversely, Nakajima *et al.*²⁴) reported that AGEs promote hard tissue differentiation in the pulp cell, which is a type of undifferentiated mesenchymal cell. The cells we used in this work are undifferentiated mesenchymal cells obtained from GK rat thighbone.

Our markers of hard tissue differentiation all decreased on exposure to glucose concentrations of up to 12 mM. However, the expressions of Runx2 and osteocalcin mRNA and deposition of calcium increased at a glucose concentration of 24 mM. We suggest that low expression of ALP and COL-1 at 24 mM glucose indicate the weakening of a formed hard tissue. García-Hernández *et al.*⁸) reported high concentrations of extracellular glucose decreased the quality of mineral deposited in culture with a result that was similar to the mineral deposit induced by LPS stimulus. Ding *et al.*²⁵) reported that IL-1 β inhibits expression of Runx2 and collagen in human mesenchymal stem cells. Consistent with this, our results show that IL-1 β expression decreased with increasing glucose concentration, while expression of Runx2 increased in 24 mM glucose (Fig. 4).

The results of this study suggest that proliferation of GK rat BMMSC isolated from T2 DM model rats can be elevated by glucose concentration, however osteogenic differentiation and mineralization from such cells, which play an important role in hard tissue regeneration, can be inhibited. We speculate that this phenomenon is caused by formation of an inflamed state as extracellular glucose concentration increases; osteogenic differentiation is inversely related to expression of the inflammatory cytokine IL-6. These findings will help establish guidelines in periodontal medicine for regenerative therapy in T2 DM patients.

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