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Proliferation and differentiation of human osteoblasts from a type 2 diabetic patient *in vitro*

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ABSTRACT. We investigated the proliferation and differentiation potential of human osteoblasts from a type 2 diabetic patient *in vitro*. Human osteoblasts were obtained from a healthy subject and a type 2 diabetic patient and were cultured *in vitro* using the tissue explant adherent method. Differences in cell morphology were observed under a phase contrast microscope. The osteogenic differentiation capacity was evaluated by alizarin red staining, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, alkaline phosphatase (ALP) staining, and detection of bone Gla-protein (BGP) and Col-1. Expression of Runx-2 and Col-1 was detected using RT-PCR and western blot. Our data indicated that alveolar bone osteoblasts from the type 2 diabetic patient exhibited poorer growth, smaller calcium nodule formation, slower proliferation, and lower ALP, BGP, and Col-1 concentrations in the cell culture supernatant, as compared to control

cells ($P < 0.05$). Combined, our study indicated that alveolar bone osteoblasts from a type 2 diabetic patient exhibited slower proliferation and decreased differentiation, as compared to healthy control, when cultured *in vitro*.

Key words: Type 2 diabetes; Alveolar bone; Osteoblast; Osteoblast proliferation and differentiation

INTRODUCTION

Diabetes is a systemic metabolic disorder (Kinane and Marshall, 2001). Most patients have type 2 diabetes mellitus (T2DM) (Hasegawa et al., 2008; L'Heveder and Nolan, 2013). Recent studies showed that diabetes can damage the balance of bone metabolism (McCracken et al., 2000). Furthermore, clinical studies showed that the failure rate of dental implants in diabetic patients with missing teeth is higher than in healthy controls (Fiorellini et al., 2000; Bugea et al., 2008); however, the mechanism remains unclear. Lack of insulin in diabetic patients can reduce the number of osteoblasts and the synthesis of bone matrix (Tervonen and Oliver, 1993; Taylor, 2001). Additionally, sustained hyperglycemia may also inhibit osteoblast differentiation (Santana et al., 2003). However, the formation and differentiation of osteoblasts play a key role in bone growth and repair. Thus, evaluation of the biological characteristics of osteoblasts from diabetic patients is necessary to understand the mechanism by which diabetes affects bone metabolism.

This study aimed to establish an *in vitro* osteoblast culture system and compare the proliferation and differentiation of osteoblasts derived from diabetic patients and healthy humans to better understand the absorption and regeneration of alveolar bone and its regulatory mechanisms.

MATERIAL AND METHODS

Ethical statement

Ethical approval was granted by the Ethics Committee of Beijing Stomatological Hospital. Patients were provided with a written information letter about the study, and participants provided their written informed consent to participate in the study.

Human alveolar bone osteoblast isolation and culture

Alveolar bone debris from oral implants in healthy humans and a T2DM patient were used in this study. The patient was diagnosed with type 2 diabetes more than 2 years prior, and had no other serious diseases, complications, inflammation, or recent operations. The bone debris was obtained during the preparation of a planting nest during an aseptic operation. The obtained bone debris was washed with 1X PBS with double antibiotics (100 g/mL penicillin and streptomycin) and centrifuged 3 times. Cells were cultured in α-minimal essential medium containing 10% fetal bovine serum at 37°C with 5% CO_2. The cells were passaged when the cell density reached approximately 80%.

Alizarin red staining

Changes in cell growth and morphology were observed under an inverted microscope. The culture medium was discarded after incubation for 21 days, and the cells were washed with PBS 2 times. The cells were fixed with 4% paraformaldehyde for 20 min and stained with alizarin red for 10 min. The formation of calcium nodules was observed and pictures were taken.

Alkaline phosphatase (ALP) chemical staining and quantitative detection

ALP was stained and detected using the ALP staining kit and quantitative detection kit according to the manufacturer protocols. Fourth generation osteoblasts from healthy humans and patients with T2DM were digested and cultured on a 48-well plate, and the optical density (OD) value at a wavelength of 520 nm was determined.

Detection of cell proliferation by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cells were cultured on 96-well plates and measured over 11 continuous days. MTT (20 μL, 5 mg/mL) was added to the cells and was incubated for 4 h at 37°C. The culture medium was discarded, and 100 mL dimethylsulfoxide was added to each well. After shaking at low speed for 10 min, the MTT solution was aspirated and the OD values of the supernatant were read at 490 nm using a microplate reader. The experiments were repeated 3 times and a growth curve was obtained.

Bone Gla-protein (BGP) concentration determination

Cells were treated as described above and the culture supernatants were collected on days 3, 7, and 9. The BGP concentration was determined using the 125I osteocalcin RIA Kit following the manufacturer protocol. The sedimentation cell per milliliter number for each tube was measured with a γ counter, and the BGP concentration was determined according to a standard curve.

Col-1 concentration determination

Cells were treated as described above and the culture supernatants were collected on days 3, 7, and 9. The Col-1 concentration was determined using the Col-1 enzyme-linked immunosorbent assay (ELISA) kit. OD values were read at 450 nm using a microplate reader.

Evaluation of Runx-2 and Col-1 levels by real-time PCR and western blot

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer protocol. Total RNA (1 μg) was subjected to reverse transcription using a reverse transcription system (Promega, Madison, WI, USA). Real-time PCR was performed using an RT-PCR kit (Takara, Shiga, Japan). β-actin gene expression was used as an internal control for normalization. The PCR

products underwent electrophoresis on a 1.0% agarose gel and were visualized under UV illumination using ethidium bromide staining. Primer sequences were as follows: β-actin F: 5'-TGGCACCCAGCACAATGAA-3', R: 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'; Runx-2 F: 5'-CACTGGCGCTGCAACAAGA-3', R: 5'-CATTCCGG AGCTCAGCAGAATAA-3'; Col-1 F: 5'-TCCACATACCTTTATTCCAGGAATC-3', R: 5'-CCCGGGTTTAGAGACAACTTC-3'.

Cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The separated proteins were electrotransferred to polyvinylidene fluoride membranes. The membranes were used for immunoblotting with the required antibodies. The protein bands were scanned and quantified using the ratio to β-actin.

Statistical analyses

The data were analyzed with the SPSS 19.0 software. The results are reported as means ± SD. The *t*-test was used to evaluate differences between groups. A P value less than 0.05 was considered to be statistically significant.

RESULTS

Cell culture and observation of osteoblasts *in vitro*

In the control group, cells grew out of the tissue blocks 5 days after culture. Morphologically, the cells were irregular strips or spindles. The cell density reached approximately 80% after 14 days in culture. In the T2DM group, cells grew out of the tissue blocks after 8 days in culture. The cellular morphology was similar to the control group. The T2DM cells reached 80% confluence on day 21. Calcium nodules in the T2DM group were smaller than that of the control group (Figure 1).

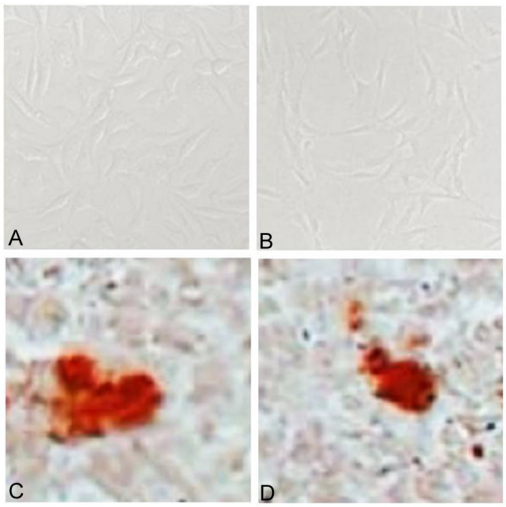

Figure 1. Osteoblast morphology and calcified nodules (100X). **A.** Osteoblasts from human alveolar bone in the control group. **B.** Osteoblasts from human alveolar bone in the T2DM group. **C.** Alizarin red staining of osteoblasts from the control group. **D.** Alizarin red staining of osteoblasts from the T2DM group.

ALP detection

In the control group, many positive ALP cells were observed after staining, with deep staining observed in the cytoplasm. Fewer ALP-stained cells were obtained in the T2DM group, and the cytoplasm was pale. Quantitative detection of ALP showed that ALP was higher in the control group than in the T2DM group (Figure 2).

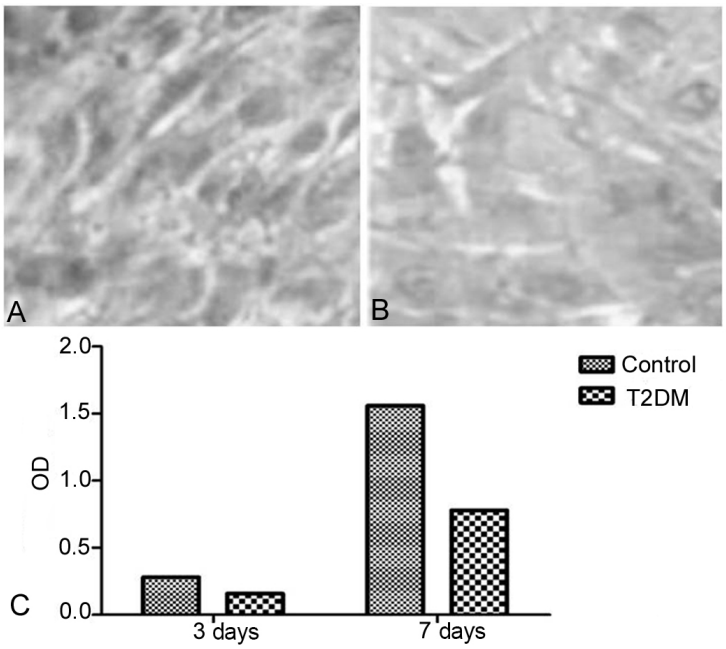

Figure 2. Alkaline phosphatase (ALP) detection. **A.** ALP staining in the control group. **B.** ALP staining in the T2DM group. **C.** Quantitative detection of ALP.

Proliferation of osteoblasts *in vitro*

Cell growth was determined by MTT assay (Figure 3). Proliferation was greatest on the seventh day in the control group and gradually reduced after 10 days. Proliferation was greater in the control group than in the T2DM group at all times.

BGP and Col-1 concentration determination

The concentration of BGP and Col-1 in the control group was greater than in the T2DM group ($P < 0.05$; Tables 1 and 2).

Runx-2 and Col-1 expression

Runx-2 and Col-1 expressed in both groups. However, the expression levels were greater in the control group than in the T2DM group ($P < 0.05$; Table 3 and Figure 4).

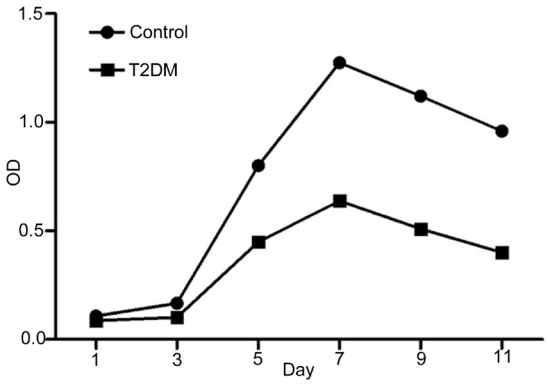

Figure 3. Cell growth curves.

Table 1. Bone Gla-protein (BGP) content in osteoblast culture supernatants (N = 3, means ± SE).

Group	BGP (ng/mL)		
	3 days	7 days	9 days
Control	6.32 ± 0.04	12.95 ± 0.07	10.48 ± 0.11
T2DM	3.20 ± 0.06*	9.87 ± 0.15*	8.04 ± 0.07*

*P < 0.05.

Table 2. Col-1 content in osteoblast culture supernatants (N = 3, means ± SE).

Group	Col-1 (μg/L)		
	3 days	7 days	9 days
Control	4.88 ± 0.42	10.02 ± 0.14	7.28 ± 0.16
T2DM	3.74 ± 0.01*	5.07 ± 0.23*	3.29 ± 0.46*

*P < 0.05.

Table 3. Runx-2 and Col-1 expression levels (N = 3, means ± SE).

Group	Runx-2	Col-1
Control	1.576 ± 0.21	1.469 ± 0.27
T2DM	0.466 ± 0.03*	0.580 ± 0.41*

*P < 0.05.

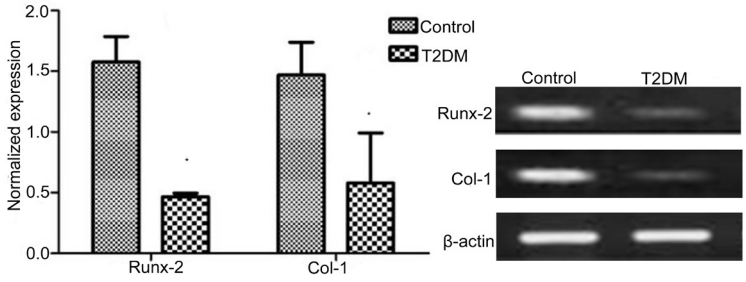

Figure 4. Expression levels of Runx-2 and Col-1. **A.** RT-PCR results. **B.** Western blotting results.

DISCUSSION

Alveolar bone is the most active in human bone and its state has a direct impact on oral health and the function of the stomatognathic system (Heitz-Mayfield, 2005; Holmlund et al., 2006).

Alveolar bone osteoblasts originate from pluripotent bone marrow stromal cells. They are involved in the synthesis and secretion of bone matrix and contribute to matrix mineralization, thereby forming bone tissue (Krentz and Bailey, 2005; Mellado-Valero et al., 2007). It has been reported in animal and clinical experiments that osteoblasts can differentiate and form new bone (Nevins et al., 1998; Zimmet et al., 2003; Seo et al., 2004). Therefore, culturing osteoblasts *in vitro* is of interest for bone tissue engineering. T2DM is a metabolic disorder. Studies have shown that the alveolar bone of rats with T2DM is more severely damaged than normal rats (Wang et al., 2010). Sustained hyperglycemia may reduce ALP and delay mineralization (Chen, 2004; Simao et al., 2007). Damage to the alveolar bone is more serious in patients with poor glycemic control than in healthy controls (Lalla et al., 2000; Gevorgyan et al., 2007). However, the mechanisms of this effect remain unclear.

In this study, we cultured alveolar bone osteoblasts from T2DM patients and healthy humans *in vitro*. We found that there are differences in ALP activity, cellular growth state, proliferation, and differentiation between groups. The alveolar bone osteoblasts from T2DM patients grew slowly and displayed less calcium precipitation by alizarin red staining, indicating that osteoblasts from T2DM patients have weak osteogenic ability *in vitro*. Osteoblast proliferation and ALP activity in T2DM patients were also weaker than in healthy humans.

Runx-2 and Col-1 are early osteogenic genes, and the expression of Runx-2 is the most specific indicator of bone formation (Lind et al., 1995; Takeda et al., 2001). Runx-2 is upstream of Col-1. The Runx-2 and Col-1 mRNA levels in the T2DM group were lower than in normal humans, suggesting that diabetes reduced osteogenic differentiation. BGP and Col-1 are protein frameworks that compose bone tissue, and could be specific markers for bone tissue formation (Sanguineti et al., 2008; Sun et al., 2012). The secretion of BGP and Col-1 in osteoblasts from T2DM patients was lower than in healthy human.

These results showed that proliferation, differentiation, and bone matrix synthesis were decreased in osteoblasts from T2DM patients, as compared to healthy humans, suggesting that diabetes affects alveolar bone metabolic balance. Insulin deficiency or sustained hyperglycemia could inhibit the synthesis and secretion of bone matrix, thus altering peri-implant bone bonding.

Studies on the biological characteristics of osteoblasts of T2DM can provide insight into the mechanism by which diabetes affects bone metabolism, thereby improving the success rate of dental implants in T2DM patients.

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