Original

An Investigation of Osteogenesis on Titanium Surfaces using a Type 2 Diabetes Rat Model

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Abstract: The effect of diabetes mellitus on osseointegration capacity has not been addressed using appropriate animal models. The aim of this study was to investigate the proliferation and differentiation of osteoblastic cells using a type 2 diabetes rat model and 3 different titanium surfaces. We used Goto-Kakizaki rats, a Wistar strain which develops type 2 diabetes mellitus, with Wistar/ST rats as controls. Titanium disks of grade 2 commercially pure titanium were prepared with surfaces that were machined, sulfuric acid etched, and hydrofluoric acid etched, and rat bone marrow-derived osteoblastic cells were cultured on the 3 different types of titanium surface. In comparison with the controls, the osteoblastic cells of the diabetes rat were inferior in differentiation. The difference with the controls occurred in a relatively small hydrofluoric acid etched group on one of the 3 different surfaces. These results suggest that type 2 diabetes mellitus impairs the differentiation of osteoblastic cells in diabetic rats.

Key words: Dental implant, Type 2 diabetes mellitus, GK rat, Osteoblast, Titanium surface

Introduction

The use of titanium implant abutments has become a standard and effective treatment modality for restoring missing teeth. However, in patients with type 2 diabetes mellitus (DM), oral implants carry a certain risk, and often there is poorer quality osseointegration than in other patients\(^1\). The effect of DM on fracture healing has been well documented experimentally and clinically. Some reports have shown that DM can affect bone, which causes impaired bone healing\(^2\). DM, which is characterized by high levels of blood glucose, consists of type 1 (insulin dependent) and type 2 (non insulin dependent) varieties. Type 2 diabetes, previously called adult onset diabetes, accounts for about 90% to 95% of all diabetes cases; it is associated with a family history of diabetes and with impaired glucose metabolism\(^3\). The process of osseointegration is associated with proliferation and differentiation of osteoblasts. The aim of this study was to investigate the proliferation and differentiation of the osteoblastic cells on 3 different titanium surfaces. We used Goto-Kakizaki (GK) rats, an established model of type 2 DM, and we used Wistar/ST (ST) rats as controls. The GK rat, a widely used model for type 2 diabetes, was developed by selective breeding over many generations using Wistar rats with high blood glucose. Although there have been reports on the use of GK rats to study the effects of diabetes in experimental bone metabolism\(^4\)-\(^13\), there seem to be no published reports on osteoblastic cells derived from mesenchymal stem cells of GK rats. For the present study, rat bone marrow derived osteoblastic cells were cultured on titanium disks\(^14\),\(^19\). The surface of the titanium was prepared in 3 ways: by sulfuric acid etching, hydrofluoric acid etching, and machining. The hypothesis to be tested was that Type 2 diabetes mellitus impairs cell activity.

Materials and Methods

Titanium samples and surface characterization

Pure grade 2 titanium disks (20mm diameter, 1mm thick) were prepared with 3 different surface treatments: 67% (w/w) sulfuric acid (H\(_2\)SO\(_4\)) at 120 °C for 75 sec; 40 % hydrofluoric acid (HF) at room temperature for 30sec; and machining by means of a lathe. Surface morphology of the disks was examined by scanning electron microscopy (SEM; VE-9800, Keyence, Osaka, Japan).

Cell culture

Bone marrow cells isolated from the femurs of 8-week-old male GK (Japan SLC, Hamamatsu, Japan) and ST rats (Japan SLC)
were placed into alpha-modified Eagle’s medium supplemented with 15 % fetal bovine serum (Biowest, Nuaillé, France), 50 µg/ml ascorbic acid (Gibco, California, USA), 10 mM Na-β-glycerophosphate (Sigma, Missouri, USA), 10^-8 M dexamethasone (Sigma), and antibiotic-antimycotic solution (Gibco). The GK rats were used as type 2 diabetic disease models. The cells were incubated in a humidified atmosphere of 95 % air and 5 % CO₂ at 37 °C, and they were detached using 0.25 % trypsin-EDTA (1 mM EDTA-4Na) and seeded onto the titanium disks at a density of 4x10^4 cells/cm^2 at 80 % confluency. The culture medium was renewed every 3 days. The Aichi Gakuin University Animal Research Committee approved this protocol, and all experimentations were performed in accordance with University Research Guideline (No. 104).

Cell proliferation assay

At culture days 1 and 3, the cells were rinsed with phosphate buffered saline (PBS) and treated with 0.25 % trypsin-EDTA for 15 min at 37 °C. The number of cells was counted using a hemocytometer.

Cell morphology

The morphology of cells cultured on titanium surfaces was examined by SEM. At culture day 3, samples of 2 types of surface were washed with PBS and fixed with 2.5 % glutaraldehyde for 2 hours. Afterwards, the cultures were washed 2 times with PBS. The samples were postfixed in 1% osmium tetroxide for 2 hours and dehydrated in a graded series of ethanol (50 % to 100 %), and the disks were carbon sputter-coated before SEM examination.

Alkaline phosphatase (ALP) activity

The ALP activity of cultured osteoblastic cells was examined by a culture area based assay. At days 3 and 7, the cultured osteoblastic cells were washed with PBS, and incubated with 120 mM Tris buffer (pH 8.4) containing 0.9 mM naphtol AS-MX phosphate (SIGMA) and 1.8 mM FAST RED TR (SIGMA) for 30 min at 37 °C. The ALP positive area was quantified as a stained density (%) using an analyzer (ImageJ, NIH, Bethesda, ML, USA).

Mineralized nodule formation assay

The von Kossa staining was utilized to visualize the mineralized nodule formation of the cultured osteoblastic cells. At days 14, 21 and 28, the cells were fixed with 10 % buffered formaldehyde solution for 30 min at room temperature. After that, the cells were incubated with 5 % silver nitrate (Kanto Chemical, Tokyo, Japan) under UV light for 40 min. Finally, the cultured cells were washed with ddH₂O twice and incubated with 5% sodium thiosulfate solution (Yoneyama Chemical industry, Osaka, Japan) for 5 min. The mineralized nodule area was quantified as a stained density (%) using ImageJ.
Data analysis

The significance of differences between groups was calculated with the unpaired Student’s t test corrected by Microsoft Excel (for Mac 2004 Version 11.6). The level of statistical significance was defined as p < 0.05. All statistical analyses were conducted using a computer program (Microsoft Excel for Mac 2004 Version 11.6).

Results

Surface character of titanium disks

SEM images showed that the surface of the machined titanium disks had machine grooves. The machined surfaces had relatively smooth morphology with turned traces; the sulfuric acid-etched surfaces exhibited typical micro-roughened surfaces; and the hydrofluoric acid etched surfaces had acicular micro-roughened surfaces. The machined surfaces showed striations roughly 0.5 µm in width. The sulfuric acid-etched surfaces showed a pore size of 0.7 µm in diameter. The hydrofluoric acid surfaces did not possess any nanotubular or nanoporous features, but they did display numerous nanometer rough features (Fig. 1a, b, c).

Increased cell density

The number of cells was significantly affected by the culture group at both culture days 1 and 3 on the machined surfaces. The cells were reduced to a small number in the control group as compared to the diabetes group at both time points. The cell density was significantly reduced to one-half in the diabetes group as compared to the controls at day 3 (Fig. 2a). At both culture days 1 and 3, the number of cells was not significantly different between the control and diabetes groups on the sulfuric acid etched surfaces or the hydrofluoric acid etched surfaces (Fig. 2b, c).

Cell morphology

At culture day 3, SEM images showed that the cells cultured onto the machined surfaces had a spindle shape and flat morphology with extended cytoplasmic elongation on the control group, and the cells cultured for the diabetic group appeared to be small with a round morphology and thin cytoplasmic elongation (Fig. 3a, b). In Figures 4a and 4b, the image at day 3 showed that the cultured cells on the hydrofluoric acid etched surfaces had an elongated dendritic form with extended cell processes for the
control group, while the cells cultured for the diabetic group were small and they had a round morphology with short cytoplasmic elongation.

Alkaline phosphatase activity

The ALP positive areas evaluated at days 3 and 7 were significantly lower for the diabetes group than for the control group on the machined surfaces (Fig. 5a). The positively stained areas were lower for the diabetic group as compared to the controls on the sulfuric acid etched surfaces at days 3 and 7 (Fig. 5b). Likewise, the ALP positive areas were significantly lower for the diabetic group as compared to the controls on hydrofluoric acid etched surfaces at days 3, 7 and 14 (Fig. 5c). In terms of the number per unit of cells, ALP positive areas were significantly lower for the diabetic group as compared to the controls on the hydrofluoric acid etched surfaces at day 3 (Fig. 6a, b, c).

Matrix mineralization

Mineralized nodule areas of the cells, detected by von Kossa stain, showed a significant difference between the control group and diabetes group at day 14 on machined surfaces, and at days 14, 21 and 28 on hydrofluoric acid etched surfaces (Fig. 7a, c). In contrast, the difference was not seen between the control group and diabetes group on the sulfuric acid etched surfaces (Fig. 7b).

Discussion

At present, the numbers of Japanese “individuals strongly suspected of having diabetes” and “individuals in whom diabetes
can not be ruled out” are estimated at approximately 8.9 and 22.1 million, respectively\(^20\). DM has been increasingly recognized as a risk factor for oral implant treatment in humans. DM, a metabolic disease characterized by abnormal regulation of glucose metabolism with associated disorders such as myopathy, neuropathy, and macrovascular disease, has been associated with impaired osseous wound healing\(^1\). In oral implant treatment, the osseous wound healing has been associated with treatment period and success. Some researchers report an increased risk for implant failure in diabetic patients compared with healthy controls, with a relative risk of 2.75\(^{21,22}\), although others do not support an association between diabetes and implant failure\(^{23,21-23}\). There have been reports of increased failure rates, as well as impaired bone remodeling potential\(^{24,25}\). Although these clinical studies were conducted using type 2 DM patients, it may be concluded that implant placement is not contraindicated in diabetic patients presenting good metabolic control.

In the presence study, we defined the proliferation and differentiation of osteoblastic cells in type 2 diabetic GK rats. The GK rat is non-obese and insulin resistant, with insulin low reaction type 2 diabetes, traits that are very similar to those of many Japanese people with type 2 diabetes. The present study showed that the GK rat was a suitable model for impaired bone healing associated with diabetes\(^17, 26-29\). To the best of our knowledge, there have been no previous reports on the use of GK rats for studying the effects of diabetes in experimental cell cultures. In our study, the cells on the machined surfaces were reduced to a small number for the control group as compared to those for the rats with diabetes at days 1 and 3. However, the number of the cells was not significantly different between the control and diabetes groups on the sulfuric acid etched surfaces and hydrofluoric acid etched surfaces. This finding suggests that a machined surface is advantageous to the cell proliferation of diabetes osteoblastic cells, but that both acid etched surfaces are not as effective. However, the differentiation of the diabetes osteoblastic cells was lower than for the controls on both acid etched surfaces. The ALP positive areas of the all different surfaces in the diabetes group were significantly lower than in the control group. Furthermore, matrix mineralization of the machined and sulfuric acid etched titanium was similar, whereas matrix mineralization of the diabetes group on hydrofluoric acid etched titanium was significantly lower than for the control group. It may be concluded that for mesenchymal stem cells of diabetic rats, there has been a decrease in differentiation ability, although a decrease in proliferation activity has not been seen. One study reported that in osteoblast-like cells derived from rat periosteum, the ALP staining positive rate was less for the sulfuric acid treatment group than for the machined group\(^20\). Other researchers have reported that there is no change in calcification with sulfuric acid treatment and machined surfaces, and that calcification for the diabetic group does not change either with sulfuric acid treatment or with non-treatment. The hydrofluoric acid surface of the pterygoid structure had an effect on cell proliferation and calcification. The number of cells had become greater in the diabetic group than in the control group on 3 different surfaces. However, the ALP positive area/number of cells in the control group had increased, while the ALP positive rate was lower for the diabetic group than for the control group. It is thought that bone differentiation was delayed in the diabetic group.

On surfaces of some titanium implants that have been standardized, the effect for osteoblasts has been established. The surface character affects the biological properties of the titanium substrates\(^{19,29-31}\). In this study, we compared proliferation and differentiation of osteoblastic cells of diabetic rats and control rats on 3 different surface types of titanium disks. For both acid treatment surfaces, when compared to the control group, the diabetic group showed results for cell proliferation, differentiation and mineralization ability that were comparable or inferior. These results suggested that diabetic rats had delayed bone formation. The treatment for dental implants after early fixation is important. The results of this experiment show that for diabetic patients, implant treatment should be done using two-time implantation. At the same time, for implant therapy in diabetic patients, bone formation that occurs early would increase the success of implant treatment. In the future, it is necessary to conduct genetic and in vivo studies, and to further explore implant treatment for diabetic patients.

References