PROLIFERATION AND DIFFERENTIATION OF BONE MARROW CELLS ON TITANIUM PLATES TREATED WITH A WIRE-TYPE ELECTRICAL DISCHARGE MACHINE

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KEY WORDS
Titanium plate
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Proliferation
Differentiation

For successful dental implants, it is necessary to obtain satisfactory osteointegration at the site of both the cortical and trabecular bones in the jaw. Bone marrow stromal cells differentiate into osteoblast-lineage cells and have an important role in bone remodeling. In this experiment, the responsiveness of bone marrow cells to a titanium plate with a rough surface was compared with that of a titanium plate with a smooth surface. The rough surface was created by treating with a wire-type electrical discharge machine, and the smooth plate was produced by polishing with 1,500-grade emery paper. The results indicated that, though bone marrow cells proliferated on both plates, the proliferation pattern and cell growing time on the plates were different. While the cells on the smooth plate proliferated along the grooves produced by polishing, the cells on the rough plate proliferated randomly and more rapidly. As bone marrow cells consisted of heterogeneous cell populations involving hematopoietic cells, we collected bone marrow mesenchymal stromal cells that proliferated on plastic dishes and studied the proliferation and differentiation of these cells. Stromal cells on the rough plate more actively proliferated than those on the smooth plate. In long-term culture, the cells on the rough plate showed higher alkaline phosphatase activity and produced cell nodules. The cells on the smooth plate were stripped off the plate without nodule formation. These results indicated that bone marrow stromal cells on the rough plate could more rapidly proliferate and differentiate into osteoblast-lineage cells compared with those on the smooth plate.

INTRODUCTION
Osteointegration is a major determinant of success for dental implants. Many factors, such as composition or the surface architecture of dental material and bone cell responsiveness of recipients, participate in this process. Pure titanium (Ti) is one of the most frequently used implant materials due to its excellent biocompatibility. Moreover, Ti is very versatile and
can be prepared in many different shapes without affecting its biocompatibility. Martin et al. reported that the surface roughness of titanium plates profoundly affected proliferation and differentiation of osteoblast-like cells (MG63). We developed a titanium plate with a rough surface by treating with a wire-type electrical discharge machine (W-EDM) and compared the affinity of osteoblast-like cells of the rough plate with that of the smooth plate. Cells observed on the plates more rapidly proliferated and differentiated on the rough surface than on the smooth surface. However, Cochran et al. observed the attachment and proliferation of periodontal cells on the smooth and rough surfaces of titanium plates and reported that these cells tended to attach more rapidly to the smooth surface. Another fundamental factor is the responsiveness of the jaw bones. To complete osteointegration, it is important to obtain sufficient compatibility in both the cortical and trabecular bones in the jaws. Although in cases with thin cortical bone and dense trabecular bone, satisfactory osteointegration was obtained, a very high failure rate for osteointegration occurred in cases with thin cortical bone and low-density trabecular bone. At the bone marrow site of the long bone, a high number of osteoblast-lineage mesenchymal stromal cells are present. Some researchers have reported that bone marrow mesenchymal stromal cells differentiated into osteoblast-like cells and produced bone-like cell nodules in long-term cultures. In recent investigations concerning biomaterial in vitro, primary cultured osteoblasts or established osteoblast-like cells have been used. Since the origin of osteoblasts is mesenchymal stromal cells, it is important to investigate the relationship between implant material and bone marrow cells. In this study, we investigated the proliferation and differentiation of bone marrow stromal cells on a titanium plate with a rough surface and compared them with those on a titanium plate with a smooth surface.

**Materials and Methods**

**Preparation of titanium plates**

Two types of titanium plates with different surface architecture were prepared from pure titanium (KS 50, Kobe Steel) according to methods described previously. One was a titanium plate with a rough surface (rough plate), which was treated using a wire-type electric discharge machine (LS 350X, Japax) and trimmed to \(10 \times 10 \times 1.0\) mm³. The machine used to produce the rough plate was adjusted to 6.5 \(\mu\)second \(\tau\) off (pulse off time) and 0.65 \(\mu\)second \(\tau\) on (pulse on time) at 15 amps \(I_p\) (peak current) and 90 V (non-load voltage). The other plate had a smooth surface (smooth plate), which was prepared by polishing with 1500-grade emery paper. The mean height and standard deviation of roughness \(R_{\text{max}}\) of the four individual plates in each group was 20.5 ± 1.56 \(\mu\)m for the rough plates and 0.80 ± 0.14 \(\mu\)m for the smooth plates. The surface produced by electric discharge exhibited a pear-like appearance under scanning electron microscopy and appeared like an accumulation of various sizes of dish-like concavities. However, linear stripes indicating shallow grooves—produced by using the emery paper—were detected on the surface of the smooth plates (Fig 1).
**Preparation of rat bone marrow stromal cells**

Rat bone marrow stromal cells were prepared according to the method of Maniotopoulos et al.\(^1\) In brief, bone marrow cells were collected from the tibia of four Sprague-Dawley male rats that were 8 weeks old. The rats were sacrificed by cervical dislocation, and their tibias were dissected free of soft tissues under aseptic conditions and placed in a medium (α-MEM; Gibco BRL) containing 32 U/mL of penicillin (Meiji, Japan) and 50 μg/mL of streptomycin (Meiji). The proximal and distal ends were cut off, and bone marrow tissue from the midshaft was flushed using 18-gauge syringes containing α-MEM until the marrow cavity appeared blanched (about six times). This suspension was then passed through 20–22-gauge syringes several times to produce a largely single-cell suspension. Finally, bone marrow cells were adjusted to a density of 5 × 10^6/mL in α-MEM containing 10% fetal calf serum (Gibco BRL). Aliquots of 10 mL of bone marrow cell suspension were plated on 100 × 20 mm² tissue culture dishes (Corning) and were cultured in an atmosphere of 100% humidity, 5% CO₂, and 37°C. After 4 days in the primary culture, nonadherent cells were removed by changing to a fresh medium, and thereafter the medium was changed every 3–4 days. At a confluent subconfluent stage (about 2 weeks), the adherent stromal cells were collected via treatment with phosphate buffered saline (PBS) containing 0.25% trypsin (Gibco BRL) and 0.2% EDTA (Sigma, St. Louis, MO) and were resuspended at a density of 5 × 10^4/mL in α-MEM containing 10% fetal calf serum.

**Cell culture and plating**

One milliliter of bone marrow cells (5 × 10^6) or dish-adherent mesenchymal stromal cells (5 × 10^6) suspended in α-MEM containing 10% fetal calf serum were seeded on a titanium plate placed in a 24-well plate (Corning) and were cultured in a CO₂ incubator for several weeks. The culture medium was changed every 3–4 days.

**Assessment of cell proliferation and differentiation**

Cells that proliferated on each plate were treated with 0.002% fluorescein diacetate (FDA, Sigma) in PBS, according to the method described by Weinsein et al.,\(^2\) and were observed with fluorescent microscopy. The cell numbers on the plate were estimated by counting the attached cells with 0.2% crystal violet in 2% ethanol.\(^3\) Briefly, after washing the cells with PBS six times to remove excess dye, they were solubilized with 1% sodium dodecyl sulfate (SDS; Wako, Japan) and the density was measured with a spectrophotometer (Dynatech MR5000). On the whole, optical density values of 1 × 10^9 cells/mL corresponded to 1.94 at 540 nm.

The differentiation of mesenchymal stromal cells on the plate was estimated by measuring the alkaline phosphatase (alp-ase) activity of cells and by observing cell nodule formation with FDA. For the differentiation experiment, 5 mM β-glycerophosphate and 200 μg/mL ascorbic acid were added to the medium on day 7 of culture. These agents enhanced alp-ase expression, nodule formation, and mineralization in bone marrow cells,\(^4\) chondroblasts,\(^5\) and osteoblast-like cells \textit{in vitro}.\(^6\) The cells on the plate were solubilized with 1.5 M Tris buffer (pH 9.5) containing 1% triton X-100, and a small aliquot of the solution was utilized for an alp-ase assay. Five millimolar p-nitrophenylphosphate (Sigma) as a substrate was utilized for measurement of this enzyme assay. Alp-ase activity was expressed as nanomoles of p-nitrophenol (p-NP) formed/mg protein/minute. Trichloroacetic acid (TCA) with 10% final concentration was added to the residual solution to precipitate cell protein components. The precipitant was solubilized with 0.1 N NaOH, and the protein content was determined using a Bio Rad Protein Assay Kit (Bio Rad, CA).

**Results**

Bone marrow cells on both plates were observed by FDA staining on days 7, 14, and 28. The cells on both plates were already confluent by day 28. The cells on the rough plate proliferated randomly (Fig 2A–C), whereas cells on the smooth plate proliferated along the grooves produced by polishing (Fig 2D–F). The cell numbers on the smooth plate were less than half of those of the rough plate on days 10 and 16. While the cells on the rough plate had almost reached a subconfluent state on day 16, the cells on the smooth plate were still growing (Fig 3).

**Proliferation of bone marrow mesenchymal stromal cells and differentiation into osteoblast-lineage cells**

Though mesenchymal stromal cells that differentiate into osteoblast-lineage cells are contained in bone marrow cells, a large population of bone marrow cells consists of hematopoietic cells. Therefore, stromal cells that proliferated on the culture dishes were collected, and the proliferation and differentiation of those cells on each plate were investigated. Stromal cells on both plates were able to proliferate actively, although the extent of proliferation was different. The cell numbers on the rough plate were almost twice those on the smooth plate on day 6 (Fig 4). In order to study the differentiation into osteoblast-lineage cells from stromal cells, alp-ase and nodule formation were investigated. While the alp-ase activity of cells on both plates was low on day 7, after the addition of β-glycerophosphate and ascorbic acid, the en-
FIGURE 2. Fluorescein micrographs of cells on the rough plates (A, B, C) and the smooth plates (D, E, F). A and D, 7-day culture; B and E, 14-day culture; C and F, 28-day culture (A, B, D, and E, original magnification ×100; C and F, original magnification ×40).

FIGURE 3. Proliferation of bone marrow cells on the rough plate and the smooth plate. ■, Rough plate; □, smooth plate.

Enzyme activity increased four- to sixfold 7 days later. The enzyme activity of cells on days 7 and 14 on the rough plate was two- to threefold higher than that on the smooth plate (Table 1). FDA staining on day 21 showed that cell nodules were clearly formed on the rough plate (Fig 5A). However, as cells on the smooth plates were stripped off the plates in the long culture, cell nodules could not be seen on the plates (Fig 5B). In this experiment, four individual plates in each group were observed. Cell nodules were detected on three rough plates, but we could not detect nodules anywhere on the four smooth plates because part of the cells detached along the grooves produced on the plates.

DISCUSSION

To obtain satisfactory osteointegration, the surface roughness of the dental implant is an important factor. The surface roughness of titanium plates treated
Proliferation of Bone Marrow Cells on Titanium Plates

FIGURE 4. Proliferation of bone marrow cells on the rough plate and the smooth plate. *p < 0.01 versus smooth plate.

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tr>
<td>Alkaline phosphatase activity of bone marrow stromal cells on rough and smooth plates*</td>
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<tr>
<td>μM p-Nitrophenol/mg Protein/Minute</td>
</tr>
<tr>
<td>Days</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>14</td>
</tr>
</tbody>
</table>

*5 × 10⁴ stromal cells were placed on both plates and cultured. The attached cells were finally solubilized with 0.2 N NaOH and protein contents were measured. The results are represented as mean ± SD of six plates.
†p < 0.01 versus smooth plate in each culture period.

With W-EDM is relatively homogeneous and reproducible compared with other rough plates. Moreover, it is possible to control the roughness up to an Rₘₐₓ of 20 um by changing the electrical conditions of the machine, as described in Table 2. Previously, we reported that osteoblast-like cells (MC3T3-E1) on plasma-coated plates (Rₘₐₓ = 31.4 μm) or plates treated with W-EDM (Rₘₐₓ = 20.5 μm) showed similar responsiveness in the proliferation and differentiation on both plates. However, the cells on smooth plates (Rₘₐₓ = 0.80 μm) polished with 1,500-grade emery paper were significantly fewer than those on both rough plates. Further, the cells on flat glass plates (Rₘₐₓ = 0.19 μm) were fewer than those on smooth plates. In this experiment, we investigated the efficacy of the rough plates treated with W-EDM using bone marrow cells.

While bone marrow cells consist of heterogeneous cell populations, the progenitors of osteoblast-lineage cells are present. In a 5-year retrospective clinical study, Jaffin and Berman classified jaws into four types based on the cortical bone thickness and the density of the trabecular bone. Their studies showed that, in the group with very thin cortical bone with low-density trabecular bone, 35% of dental implants failed to osteointegrate versus only 3% in the other bone-quality types. These results suggest that the osteoinductive potential of bone marrow stromal cells is an important factor necessary for successful osteointegration. Many investigators showed the formation of bone-like cell nodules using the long-term culture of bone marrow stromal cells. Ozawa and Kasugai reported that bone marrow stromal cells on hydroxylapatite, glass ceramic, or titanium plates were able to proliferate and differentiate into osteoblast-like cells. The present study confirmed that rat bone marrow stromal cells proliferated and formed cell nodules on titanium plates.

It is said that the surface architecture of a plate has significant effects on the orientation of cell proliferation. Brunette reported that the long axis of most fibroblasts was found to be parallel with the grooves produced on the titanium surfaces, but fibroblasts cultured on plasma-sprayed rough surfaces were randomly arranged. The surface grooves of the titanium plate also controlled the attachment of epithelial cells. In this experiment, the plate surface polished with 1,500-grade emery paper was relatively smooth, and grooves of Rₘₐₓ = 0.80 μm were produced. The plate treated with W-EDM exhibited a roughness of Rₘₐₓ = 20.5 μm. The bone marrow cells on the smooth plate proliferated along the grooves produced by polishing, similar to fibroblasts and epithelial cells. The cells on the rough plate treated with W-EDM were randomly oriented in comparison with those on the smooth plate. The proliferation of bone marrow cells on both plates was different. Cells on rough plates as well as MC3T3-E1 cells proliferated more rapidly than those on smooth plates. These results suggest that proliferation of bone marrow cells may be self-regulated, depending on the surface architecture of the titanium plate used.

In long-term bone marrow cultures, stromal cells express alp-ase activity and form cell nodules. An increase in the enzyme activity and nodule formation are generally known as markers of differentiation into osteoblast-lineage cells. Ozawa and Kasugai reported that rat bone marrow stromal cells increased alp-ase activity and formed mineralized cell nodules on dental implant materials in long-term cultures. Indeed, the alp-ase activity of cells that had proliferated on a titanium plate increased when cells
were cultured with medium containing β-glycerophosphate and ascorbic acid. Ascorbic acid and β-glycerophosphate are important agents for alp-ase expression, nodule formation, and mineralization of osteoblast-like cells, chondroblasts, and bone marrow cells in vitro. Comparing cells on the smooth and rough plates, the enzyme activity of cells on the rough plates was higher than that on the smooth plates. Cell nodules were clearly observed on the rough plate in long-term culture. However, though the cells on the smooth plate proliferated sufficiently, we could not find cell nodules because of cell detachment from the smooth plate. The exact reason why cells on the smooth plate were stripped from the plate in long-term culture is unclear. The interaction between the topography of the plate surface and extracellular matrix produced by bone marrow stromal cells may be involved. Thomas and Cooks reported that rough-surfaced implants yielded both greater shear strengths and larger direct bone apposition, whereas smooth surface implants exhibited various degrees of fibrous encapsulation. Bowers et al described significantly higher levels of cellular attachment on rough surfaces with irregular morphologies compared to smooth surfaces. Indeed, Martin et al reported that the surface roughness of the plate profoundly affected the matrix synthesis of osteoblast-like cells. On the rough plate, the matrix may be able to tightly bind to its surface, whereas, as the roughness

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**Table 2**

<table>
<thead>
<tr>
<th>W-EDM</th>
<th>$R_{max}$ (μm)</th>
<th>SD (μm)</th>
<th>$\tau_{on}$ (μs)</th>
<th>$\tau_{off}$ (μs)</th>
<th>$I_p$ (Amps)</th>
<th>$V_{no load}$ (Volts)</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>7.7</td>
<td>0.6</td>
<td>0.35</td>
<td>5.0</td>
<td>10</td>
<td>80</td>
</tr>
<tr>
<td>B</td>
<td>11.8</td>
<td>0.9</td>
<td>0.85</td>
<td>7.5</td>
<td>90</td>
<td>80</td>
</tr>
<tr>
<td>C</td>
<td>17.5</td>
<td>1.1</td>
<td>0.85</td>
<td>7.0</td>
<td>110</td>
<td>80</td>
</tr>
<tr>
<td>D</td>
<td>21.9</td>
<td>1.0</td>
<td>0.85</td>
<td>7.0</td>
<td>110</td>
<td>80</td>
</tr>
<tr>
<td>Plasma coated</td>
<td>30.1</td>
<td>0.6</td>
<td>0.85</td>
<td>7.0</td>
<td>110</td>
<td>80</td>
</tr>
</tbody>
</table>

* $\tau_{on}$ pulse on time; $\tau_{off}$ pulse off time; $I_p$ peak current; $V_{no load}$ voltage (Miyazaki et al 15).
of the smooth plate polished by 1.500-grade emery paper was 0.8 μm, the binding force between the plate and the matrix produced by its cells was probably weak. As a result, even if cell nodules were formed on the smooth plate, cells may be easily stripped off the smooth plate. This may be a reason why cell nodules could not be found on the plate.

The roughness produced by W-EDM is relatively homogeneous and reproducible. Bone marrow stromal cells proliferated and differentiated into osteoblast-lineage cells on the rough plate. Furthermore, the machine used can control the surface roughness up to 20 μm. Therefore, it is an effective device in producing the optimal surface roughness of dental implants needed to obtain satisfactory osteointegration.

REFERENCES


