Titanium Surface Roughness Accelerates RANKL-dependent Differentiation in the Osteoclast Precursor Cell Line, RAW264.7

Seicho MAKIHIRA, Yuichi MINE, Eduardo KOSAKA and Hiroki NIKAWA
Department of Medical Design and Engineering, Division of Oral Health Engineering, Institute for Oral Health Science, Hiroshima University Faculty of Dentistry, 1-2-3 Kasumi Minami-ku, Hiroshima 734-8553, Japan

Received February 27, 2007/Accepted May 25, 2007

The present study was a molecular analysis of the initial differentiation of osteoclast precursor RAW264.7 cells on titanium specimens. RAW264.7 cell line was cultured on titanium specimens of which the surfaces were finished by wet grinding with 2000-, 1200-, 600-, or 180-grit waterproof abrasive paper. Total RNA was extracted from cells cultured in the presence or absence of Receptor Activator of NF-κB Ligand (RANKL), prior to cDNA synthesis for real-time quantitative reverse transcriptase-polymerase chain reaction analysis. Titanium surfaces initially enhanced the expression of osteoclast differentiation markers including tartrate-resistant acid phosphatase and cathepsin K in RAW264.7 cells cultured with RANKL stimulation, in a roughness-dependent manner. The mRNA expressions of both RANKL receptor, RANK, and its adapter protein TNF receptor-associated factor 6 (TRAF6) increased when RAW264.7 cells were cultured on titanium specimens with roughened surfaces, as compared with that of control specimen with a polished surface. These results, taken together, suggested that titanium surface roughness facilitated osteoclast differentiation through the activation of the RANK-TRAF6 signaling network.

Keywords: Titanium, Osteoclast, Implant

INTRODUCTION

The bone-dental implant interface is a critical factor to the maintenance of long-term osseointegration. It is defined as a direct structural and functional connection by Brånemark. At the interface, the following events take place: continuous and dynamic remodeling, bone replacement, fatigue damage repair, and structural integrity maintenance.

The dynamic balance between the synthesis of bone matrix by bone-forming cells/osteoblasts and bone resorption by bone-resorbing cellsosteoclasts controls the normal bone remodeling process that occurs throughout life. In contrast, skeletal abnormalities such as osteopetrosis or osteoporosis can be caused by increased or decreased bone mass resulting from an imbalance of osteoblast and osteoclast activities. In inflammatory diseases, the emergence of lymphocytes, known to be primary sources of Receptor Activator of NF-κB Ligand (RANKL), causes an imbalance of osteoclast and osteoblast activities, resulting in bone loss. Therefore, successful bone remodeling in dental implants requires coordinated activities of both osteoblasts and osteoclasts.

Initial osteoclast-mediated bone resorption, at the interface between bone and titanium, has played a crucial role in determining the prognosis of dental implants. However, increase in osteoclast activity leads to failure in osseointegration and rigid connections. To date, previous studies on osseointegration of dental implants have focused primarily and only on osteoblast differentiation on titanium and its molecular mechanisms. Little information is available on the factors involved in osteoclast differentiation on titanium surface.

In the investigation of the molecular mechanisms of osteoclast differentiation, it was demonstrated that macrophage colony-stimulating factor (M-CSF), the RANKL-RANK pathway, and osteoprotegerin (OPG), an antagonism of RANKL function, were essential for osteoclast differentiation and development. RANKL derived from T and B cells, elevates osteoclast activity in periodontal diseases and causes local bone loss — as in the case of rheumatoid arthritis. A pilot study showed that soluble RANKL was detected in the crevicular fluid of peri-implantitis, and correlated with clinical parameters of the inflammation. From the findings of these studies, it could be suggested that osteoclast precursor cells around dental implant surfaces were exposed to RANKL derived from either activated lymphocytes or osteoblasts, and/or the primary RANKL. These phenomena must be a major cause of the imbalance in bone remodeling. In other words, RANKL-induced osteoclasts may play a key role in the prognosis of dental implants.

To date, the effects of the surface properties of titanium or scaffolds on cell proliferation or differentiation of osteoblastic cells have been well documented. We hypothesized, therefore, that the physical properties of titanium may also affect the cell differentiation of osteoclast precursor cells. Against this background, the aims of our study were to: (1) Set up, and thereby apply, a simplified osteoclast differentiation assay system using a mouse
macrophage/monocyte cell line as the precursor of osteoclasts; and (2) Clarify whether titanium surfaces with different degrees of surface roughness affected RANKL-dependent osteoclast differentiation.

**MATERIALS AND METHODS**

**Titanium specimens and surface roughness measurement**

Commercially pure wrought titanium (cp-Titan) plates (JIS, Japan Industrial Specification H 4600, 99.9 mass% titanium, 15 mm diameter; Kobelco, Kobe, Japan) were purchased and used in this study. Titanium plates were finished by wet grinding with 2000-, 1200-, 600-, or 180-grit waterproof abrasive paper (WATERPROOF PAPER, Sankyo Rikagaku, Saitama, Japan), and then cleaned with acetone and ethanol in an ultrasonic bath for 30 minutes in each. Titanium plates further polished with a felt wheel (Morita, Tokyo, Japan) prior to washing with acetone and ethanol were used as control. Specimens with different degrees of surface roughness were then named as G2000, G1200, G600, and G180 titanium plates, respectively.

The surface roughness of titanium specimens was measured by a linear variable differential transformer (LVDT)-type contact instrument, Surfcorder (Surfcorder SE3300, Kosaka Laboratories, Tokyo, Japan). Surface roughness was measured at room temperature over a sample length of 15 mm, with at least three measurement points on each specimen, whereby the values were averaged to obtain a representative value for each specimen. A total of six replicated specimens were measured, and the average roughness (Ra) ± S.D. of each sample was calculated.

**RAW264.7 cell culture**

RAW264.7 mouse macrophage/monocyte cell line (TIB-71; American Type Culture Collection), provided by Dr. Atsushi Shimazu (Department of Mucosal Immunology, Hiroshima University Graduate School), was used throughout the study. The cells were inoculated onto the surfaces of titanium specimens placed at the bottom of 24-well plates, at a density of 1.0 ± 10⁴ cells/well, in alpha-MEM supplemented with an antibiotic mixture (Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum (FBS) (Biological Industries, Beit Haemek, Israel), and 1.5 g/L sodium bicarbonate (Invitrogen). The whole assembly was cultured at 37°C under 5% CO₂/95% air for 3–5 days, and the medium was changed every day. For the differentiation assay, the cells were cultured similarly with 50 ng/ml recombinant soluble murine RANKL-dependent osteoclast differentiation.

---

**Table 1 Primers and probes used for real-time PCR**

<table>
<thead>
<tr>
<th>Target name</th>
<th>Primer and probe</th>
<th>Sequence (5’ - 3’)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAP</td>
<td>Primer F</td>
<td>GGAGCTTAACTGCCTCTTG</td>
<td>NM_007388</td>
</tr>
<tr>
<td></td>
<td>Primer R</td>
<td>CCGTGCGTGAGCATG</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>6FAM-TTTGTAGGCCCAAGGCAACCACCC-TAMRA</td>
<td></td>
</tr>
<tr>
<td>cathepsin K</td>
<td>Primer F</td>
<td>GGAAACAAAGTACAGCTTCGTC</td>
<td>NM_007802</td>
</tr>
<tr>
<td></td>
<td>Primer R</td>
<td>GCTGGGCTGGCTGGGAATCAC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>6FAM-AACACGCGCTGCGGACACATACATAC-TAMRA</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Primer F</td>
<td>CCACACTGGGCCGACATCG</td>
<td>NM_007393</td>
</tr>
<tr>
<td></td>
<td>Primer R</td>
<td>GTTGCTTGGAAGGTCTGAGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>6HEX-CCTGCGTGGACCTGGCTGCG-TAMRA</td>
<td></td>
</tr>
</tbody>
</table>

Note. Forward primers (Primer F) and reverse primers (Primer R) are listed.

**Table 2 Primers used for PCR**

<table>
<thead>
<tr>
<th>Target name</th>
<th>Primer</th>
<th>Sequence (5’ - 3’)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAF6</td>
<td>Primer F</td>
<td>ATGACAGCCACCTCCCCCTGGCGCCCTTC</td>
<td>NM_009424</td>
</tr>
<tr>
<td></td>
<td>Primer R</td>
<td>GGCACTGGCTGGCTGGCAATTGATG</td>
<td></td>
</tr>
<tr>
<td>RANK</td>
<td>Primer F</td>
<td>CCAGGGGACAACGGGAATCA</td>
<td>NM_009399.3</td>
</tr>
<tr>
<td></td>
<td>Primer R</td>
<td>GGGCGTCCGGTCTACATTC</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Primer F</td>
<td>GCTCCGGCTCCGGATGCGATCCATG</td>
<td>XR_002672.1</td>
</tr>
<tr>
<td></td>
<td>Primer R</td>
<td>GGAGGAGCAACCATCCTGGTCTCTTC</td>
<td></td>
</tr>
</tbody>
</table>

Note. Forward primers (Primer F) and reverse primers (Primer R) are listed.
RANKL (PeproTech, London, UK). Cells cultured directly on the plastic bottom of 24-well plates were used as unaffected controls.

**Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis**

Total RNA was extracted using TRIzol reagent (Invitrogen). Real-time quantitative RT-PCR analyses for tartrate-resistant acid phosphatase (TRAP) and cathepsin K were performed using an ABI PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems Inc., Foster City, CA, USA). First-strand cDNA was synthesized using ReverTra Ace (Toyobo, Osaka, Japan) with total RNA (100-500 ng). β-actin was chosen as an internal standard to control for variability in amplification due to differences in the starting total RNA concentrations.

Sequences of all primers and TaqMan fluorogenic probes used in these analyses were listed in Table 1. These primers and probes were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

**RT-PCR analysis**

The cDNA was amplified by BIOTAQ DNA polymerase (Bioline, Randolf, MA, USA). For each gene, a cycle curve experiment was performed and the optimal number of PCR cycles was selected according to the results. Sequences of the primers used in this analysis were shown in Table 2. Amplified products separated on 2.0% agarose gels were visualized by ethidium bromide staining and subsequent ultraviolet light transillumination. Photographs of the gels were taken with a digital camera.

**Assessment of TRAP-positive cells**

RAW264.7 cells were cultured in the presence of soluble RANKL for five days to obtain the differentiated osteoclast cells. To confirm osteoclast differentiation, the cultured cells were stained with TRAP (TRAP Staining kit, Primary Cell, Hokkaido, Japan), and photographs of red-stained (TRAP-positive) and multinucleated cells were taken using a digital camera attached to a phase contrast microscope.

**RESULTS**

The mean surface roughness value (Ra) of each titanium specimen finished with different wet grinding papers, i.e., G2000, G1200, G600, and G180, was confirmed by Surfcorder (Fig. 1). Results showed that the Ra of specimens was influenced by the asperity of polishing papers.

To examine the effect of RANKL stimulation on osteoclast precursor cells, RAW264.7 cells were cultured on titanium specimens, as well as on plastic plates, in the presence of 50 ng/ml soluble recombinant RANKL. To evaluate and standardize osteoclast differentiation, a real-time quantitative RT-PCR strategy and TRAP staining method were employed to measure TRAP and cathepsin K mRNA expressions. TRAP and cathepsin K were used as identification markers for osteoclast differentiation. Soluble recombinant RANKL enhanced TRAP and cathepsin K mRNA expressions, indicating the differentiation of RAW264.7 cells into osteoclasts.

**Fig. 1** Average surface roughness values of titanium specimens. Data are represented as mean ± SD of six specimens.

**Fig. 2** (A) TRAP and cathepsin K gene expressions in RAW264.7 cells exposed to RANKL on plastic surfaces, determined by real-time quantitative RT-PCR. Findings of real-time quantitative RT-PCR of TRAP and cathepsin K expressions were normalized to the expression level of β-actin mRNA. (B) Phase contrast morphological analysis of RAW264.7 cells on plastic plates in the absence and presence of RANKL. Data are representative of at least three experiments.
cathepsin K mRNA expressions in three-day cultured RAW264.7 cells on plastic plates as compared with the control wells without RANKL (Fig. 2A). Further incubation of the sample with RANKL up to five days revealed the emergence of gigantic multinucleated cells, as detected by TRAP staining (Fig. 2B). On the other hand, no TRAP-positive multinucleated cells were observed in RANKL-free control wells (Fig. 2B).

To examine the effect of surface roughness of titanium on the initial differentiation of preosteoclasts, RAW264.7 cells were grown on titanium specimens with different degrees of surface roughness for three days in the presence of soluble recombinant RANKL. Real-time quantitative RT-PCR revealed that TRAP and cathepsin K mRNA expressions occurred in the cultured cells on all titanium specimens, when exposed to RANKL (Fig. 3). Although these expressions were detected, their expression levels on titanium samples were much lower than that of the cells grown on plastic plates (Figs. 2A and 3).

To investigate the effects of surface roughness of titanium on RANKL-related differentiation, the expression of RANK, TRAF6, and ß-actin were analyzed. RANK and TRAF6 are known as key regulators in response to RANKL during osteoclastogenesis. The intensity of RT-PCR products on agarose gels revealed that the levels of RANK and TRAF6 mRNA expressions were enhanced according to the surface roughness of titanium specimens, even in the absence of recombinant soluble RANKL, as compared with the level of ß-actin expression used as an internal control (Fig. 4).
Osteoclasts are multinucleated cells that degrade mineralized bone matrix. They are derived from haemopoietic progenitor cells of a monocyte/macrophage lineage. The control of bone metabolism and remodeling involves the coupling of bone-forming cells (osteoblasts) and bone-resorbing cells (osteoclasts). The coupling is regulated by a complex signaling network mediated through cytokines, cell surface receptors, and various signal transducers. However, several recent studies have identified localization of activated osteoclasts as a major pathological feature, such as focal bone erosion, periodontal disease, arthritis, rheumatism, and peri-implant loosening. These reports suggested that elevated osteoclasts played a crucial role in bone resorption in the lesions of such diseases.

The molecular mechanisms of osteoclastogenesis causing bone remodeling and diseased bone resorption have been elucidated. RANKL (also known as TRANCE) derived from osteoblasts, fibroblasts, and T cells is a key regulator that activates the RANK-TRAF6 pathway and its downstream signaling cascades in osteoclast precursor cells. The responses and differentiation of both osteoblasts and osteoclasts vary during osseointegration, and are affected by implant surface microtopography. In this context, the aspects of cell adhesion, cell morphology, cell surface receptors, and cytokine production have all been well studied. However, the molecular events that take place at the interface between bone and dental implants particularly the role of osteoclasts during osseointegration, long-term bone remodeling, and in implant-related diseases are still unknown.

Against this background of information scarcity, the aim of this study was to address the molecular mechanisms involved in osteoclast differentiation on titanium with different degrees of surface roughness, with respect to the expressions of RANK and TRAF6. The present study offered novel molecular insights into the initial stage of osteoclast differentiation in association with the surface roughness of dental implants during osseointegration and in peri-implantitis.

RAW264.7 cells obtained from murine ascitic fluids were used in this study. This is a very useful cell line because RAW264.7 cells differentiate into functional and mature osteoclasts easily in the presence of certain concentrations of RANKL. Therefore, RAW264.7 cells were cultured on titanium specimens that had been ground with waterproof abrasive papers and which had their surface roughness measured. Indeed, as shown in Fig. 2, differentiation of RAW264.7 cells into osteoclasts was easily achieved.

In the present study, a simplified assay system was set up and applied to evaluate the effects of surface roughness of titanium specimens on osteoclast differentiation. As shown in Fig. 3, the differentiation of preosteoclasts was facilitated as compared with the control titanium with a polished surface. However, with TRAP and cathepsin K gene expressions used as markers, it was evident that the differentiation of preosteoclasts depended upon surface roughness. This finding agreed with the report of Marchisio et al., who reported that RAW264.7 cells cultured on the machined titanium surface displayed a higher adhesion ability, while cells cultured on a rougher surface disclosed a more evident capability to differentiate. From these results, it could be speculated that RANK, TRAF6, and the signaling network via them might be involved in the enhanced osteoclast differentiation abetted by surface roughness. Nonetheless, further analysis is necessary to clarify these mechanisms in detail.

Surprisingly, the expression levels of RANK and TRAF6 were increased in RAW264.7 cells which were cultured without RANKL and with increase in the surface roughness of titanium (Fig. 4). This implied a possibility of mechanical or contact stimulation by roughened surface which directly affected the molecular signaling of RAW264.7 cells. This phenomenon might be explained by an alteration in cell surface contact for extracellular matrix receptors, such as integrins. This is because integrins control the osteoblastic differentiation in response to titanium surface roughness.

Integrin signaling is known to be related to motility and cell survival. Furthermore, integrin attachment induces calcium signaling in osteoclasts. With due consideration to the aforementioned findings obtained in previous studies and the results obtained in this study, it could be logically suggested that RANK and TRAF6 were induced by roughened titanium surfaces. This was because some integrins were reported to recognize surface structures and subsequently activated their downstream signaling pathways. This then resulted in RANK and TRAP6 induction. It is well known that osteoclasts attach and adhere to surfaces predominantly through IPv3 integrin, forming a ruffled border. In the same vein, a rougher surface may trigger the activation of focal adhesion kinase through IPv3 integrin.

Activation of RANK leads to the enhanced expression of osteoclast-specific genes during differentiation. RANK signaling is mediated by cytoplasmic factors that activate downstream signaling pathways. During osteoclastogenesis and activation, at least five distinct signaling cascades mediated by protein kinases are induced: inhibitor of NF-κB kinase, c-Jun N-terminal kinase, p38 mitogen-activated
protein kinase (p38 MAP kinase), extracellular signal-regulated kinase, and Src tyrosine kinase. Stimulation of p38 MAP kinase results in the downstream activation of a transcriptional regulator, microphthalmia transcription factor (MITF), which controls the expressions of the genes encoding TRAP and cathepsin K. The enhancement of RANK and TRAF6 mRNA expressions by roughened titanium surface (Fig. 4) might be involved in the activation of p38 MAP kinase, thereby triggering the enhanced TRAP and cathepsin K expressions.

In view of the abovementioned results, it could be logically suggested that a roughened surface of titanium indeed contributed to the enhancement of osteoclast differentiation due to the activation of the RANKL-RANK-TRAF6 signaling pathway.

To date, a few studies have favorably reported on enhanced differentiation of osteoblasts on roughened surfaces. As such, conventional wisdom in the dental field is that titanium with a roughened surface is a preferred choice for dental implants. However, our results clearly suggested the risk of roughened surface of titanium. Further investigations will be required to elucidate the molecular mechanisms underlying these responses and to evaluate the effects of surface roughness on the balance of osteoblast-osteoclast ratio during differentiation. An understanding of these mechanisms will lead to the development of more advanced prosthetic interventions with respect to dental implant therapy.

CONCLUSION

The present study showed that titanium surface roughness enhanced the expressions of TRAP and cathepsin K, genes known to be involved in RANKL-dependent differentiation of osteoclast precursors into osteoclasts, through the enhanced expressions of RANK and TRAF6.

ACKNOWLEDGEMENTS

The authors greatly appreciate Dr. Atsushi Shimazu, who was most generous in giving us invaluable advice and technical support. This study was supported in part by a Grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science (No. 18689046).

REFERENCES