Global Journal of Dentistry and Oral Hygiene

ISSN: xxxx-xxxx Vol. 1 (1), pp. 053-058, December, 2013. © Global Science Research Journals

Full Length Research Paper

Titanium particles enhanced osteoclast differentiation and osteoclast bone resorption activity *in vitro*

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Accepted 26 February, 2013

The aim of this study was to evaluate the effect of titanium particles that detached from dental implants on osteoclast differentiation and their bone resorption activity. Osteoclasts were generated by titanium particles medium and conditioned medium with or without anti-receptor activator of nuclear factor kappa-B ligand (RANKL). The number of osteoclast was counted and the resorption areas on dentin slices were measured. Moreover, the expression of RANKL and the phagocytosis of titanium particles by cells were evaluated by western blot and transmission electron microscopy (TEM), respectively. Osteoclast differentiation and osteoclast bone resorption activity were enhanced both in titanium particles medium and conditioned medium. In addition, titanium particles promote the expression of RANKL in osteoblasts and the phagocytosis of titanium particles by osteoblasts was demonstrated by TEM. In conclusion, titanium particles enhanced osteoclast generation and their activity and could induce bone resorption around dental implants.

Key words: Titanium particles, dental implants, osteoclast, bone resorption.

INTRODUCTION

Titanium is a particular suitable material for dental implants due to its better mechanical properties and better biocompatibility. Osseointegration between dental implant surface and alveolar bone play a crucial role in the stability of titanium implants (Castellani et al., 2010; Vidigal et al., 2009). In clinical use of dental implant, many methods were used to modify dental implant surfaces to enhance osseointegration (Triplett et al., 2003). It has been demonstrated that a rough implant surface could enhance osseointegration as compared to a smooth implant surface (Cheng et al., 2010; Schwarz et al., 2009). To enhance osseointegration, many methods were used to create rough surfaces such as titanium plasma sprayed (TPS), sandblasted large-grit acid-etched (SLA), physical vapor deposition, etc (Le Guehennec et al., 2007).

However, many *in vivo* studies have demonstrated that titanium particles were generated in the implant-bone interface. Some researchers pointed that the detachment of titanium particles were due to the frictional force during implant insertion and micro-motion during functional activity of implant (Franchi et al., 2007; Martini et al., 2003). For example, TPS surface was made by spraying titanium fluid to titanium and the cohesive force of titanium plasma with titanium implant was weak (London et al., 2002). Therefore, titanium particles could easily be detached from implant surfaces and be released to the implant-bone interface. Titanium particles that exited around dental implants would have effects on cell's activity, which would finally influence the osseointegration around dental implants.

Osseointegration was a dynamic balance between bone formation by osteoblasts and bone resorption by osteoclast (Mavrogenis et al., 2009). Many factors could influence this balance, such as bacteria, occlusion force, etc (Renvert et al., 2009). Once this balance is destroyed, bone loss would happen and dental implant would be a failure. Therefore, in this study, we evaluated the effect of titanium particles on osteoclast generation and activity *in vitro*.

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MATERIALS AND METHODS

Preparation of titanium particles

Titanium particles (Alfa Aesar, Milwaukee,WI, USA) with 93% of the particles <20 µm in diameter were suspended in deionized water, vortexed and separated according to the variable sedimentation rates of the variously sized particles. Then, titanium particles were examined using oil-immersion-lens microscopy and the particles with diameter less than 10 µm were separated. After sedimentation, titanium particles were passivated with a 25% nitric acid wash at 70°C for 1 h, washed three times with sterile phosphate-buffered saline (PBS) and then autoclaved at 130°C for 20 min to minimize endotoxin contamination. In preparation for the cell culture, titanium particles were mixed with culture medium (a-MEM, Hycolne, Logan, UT, USA) under sterile conditions. The titanium particles culture media were ultrasonicated for 30 min in sealed sterile containers before being added to the cell culture.

Conditioned medium preparation

Conditioned medium were obtained by culturing osteoblasts with titanium particles medium. Briefly, osteoblasts were cultured with titanium particles medium for 48 h. Then, the culture media were collected, centrifugated and passed through a 0.2 µm filter, and stored were at -80°C. Conditioned medium was obtained by mixing the collected medium and the fresh new medium at the ratio of 1:1.

Osteoclast culture

Osteoclast were generated by six culture media: (A) common culture medium (containing 10^{-6} mol/L prostaglandin E_2 (PGE_2) and 10^{-8} mol/L 1,25$(OH)_2D_3$), (B) common culture medium + antibody of receptor activator of nuclear factor kappa-B ligand (anti-RANKL), (C) conditioned culture medium (CM), (D) conditioned culture medium + anti-RANKL, (E) titanium particles culture medium, and (F) titanium particles culture medium + anti-RANKL.

Osteoclasts were generated by co-culturing osteoblast and bone marrow macrophages as described by Helfrich and Ralston (2003). Bone marrow cells were obtained from the femora and tibiae of 6-week-old male imprinting control regions (ICR) mice and suspended in a-minimum essential medium (a-MEM)/10% fetal calf serum (FCS, Hyclone). After bone marrow cells were cultured on culture dishes for 48 h, the culture medium was removed and most of the adherent cells on culture dishes were bone marrow macrophages. Osteoblasts were prepared from growing calvarial cells from neonatal ICR mice in a-MEM (Hyclone, Logan, UT, USA) containing 10% (v/v) FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Then bone marrow macrophages and osteoblasts were co-cultured in a culture medium with 10% FCS (Hyclone), a-MEM, 100 U/ml penicillin, and 100 µg/ml streptomycin and 10^{-8} M of 1,25-$(OH)_2$ vitamin D_3 (Sigma, St. Louis, MO, USA) and 10^{-6} M PGE_2 in humidified atmosphere of 5% CO_2 at 37°C. After cultured for 2 days, culture medium was removed and six culture media were added separately. The culture medium was changed every other day. After 6 days, the culture medium was removed and cells were stained with tartrate-resistant acid phosphatase (TRAP). The cells with 3 or more cells were considered as osteoclast.

Osteoclast bone resorption ability

Osteoclasts were generated on dentin slices by co-culturing osteoblasts and bone marrow macrophages. After 7 days, the culture medium was removed and conditioned medium and titanium particles medium were added. After cultured for 48 h, attached cells were completely removed from the dentin slices by ultrasonic cleaning. Then dentin slices were stained with toluidine blue and viewed under an inverted phase contrast microscope. Five images were selected seldom and the resorption area was measured by image pro plus 6.0.

Western blot

Osteoblasts were cultured with titanium particles for 48 h. Then, culture medium were removed and osteoblasts were homogenized in a lysis buffer containing 20 mM Tris-HCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA) , 0.5 mM ethylene glycol tetraacetic acid (EGTA), 100 mM NaCl, 1% Triton X-100, 100 µM sodium orthovanadate and 1 mM protease inhibitor PMSF. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% non-fat dry milk in PBS/0.15% Tween for 1 h at room temperature, blots were incubated overnight at 4°C with a monoclonal anti-RANKL antibody pv diluted 1:150 to detect RANKL. The secondary antibody (Jackson Immuno Research Labs, Westgrove, PA, USA) was detected. β-actin expression served as an internal control for protein loading.

Transmission electron microscopy (TEM)

After osteoblasts cultured with titanium particles for 48 h, the culture medium were removed and cells were washed three times with PBS. Then 2% glutaraldehyde was added as the primary fixative. Then cells were collected by a cell scraper, centrifugated and fixed by 5% formalin. Then cells were fixed by 1% OsO_4 in PBS. The samples were then embedded in Epon 812 and Ultrathin sections cut with a diamond knife. Sections were mounted on copper grids, stained with 1% uranyl acetate and lead citrate, and examined with a Philips electron microscope (Philips, Leicester, UK) at 60 kV.

Statistic analysis

All data were expressed as mean ± standard deviation (SD). Statistical analysis was performed by analysis of variances (ANOVA), and $P < 0.05$ was considered as significant.

RESULTS

To evaluate the effect of titanium particles on osteoclast differentiation, we studied osteoclast differentiation which was induced by conditioned medium, titanium particles medium and common culture medium, separately. We found that both conditioned medium and titanium particles medium generated more osteoclast when compared with common culture medium, indicating titanium particles promoted osteoclast differentiation. However, after adding anti-RANKL, the numbers of osteoclast were decreased in all groups (Figure 1).

As shown in Figure 2, titanium particles increased bone resorption area on dentin slices, indicating titanium particles could enhance osteoclast bone resorption activity *in vitro*. Moreover, conditioned medium also increased bone resorption area than in the control.

In the western blot test, we found that both titanium

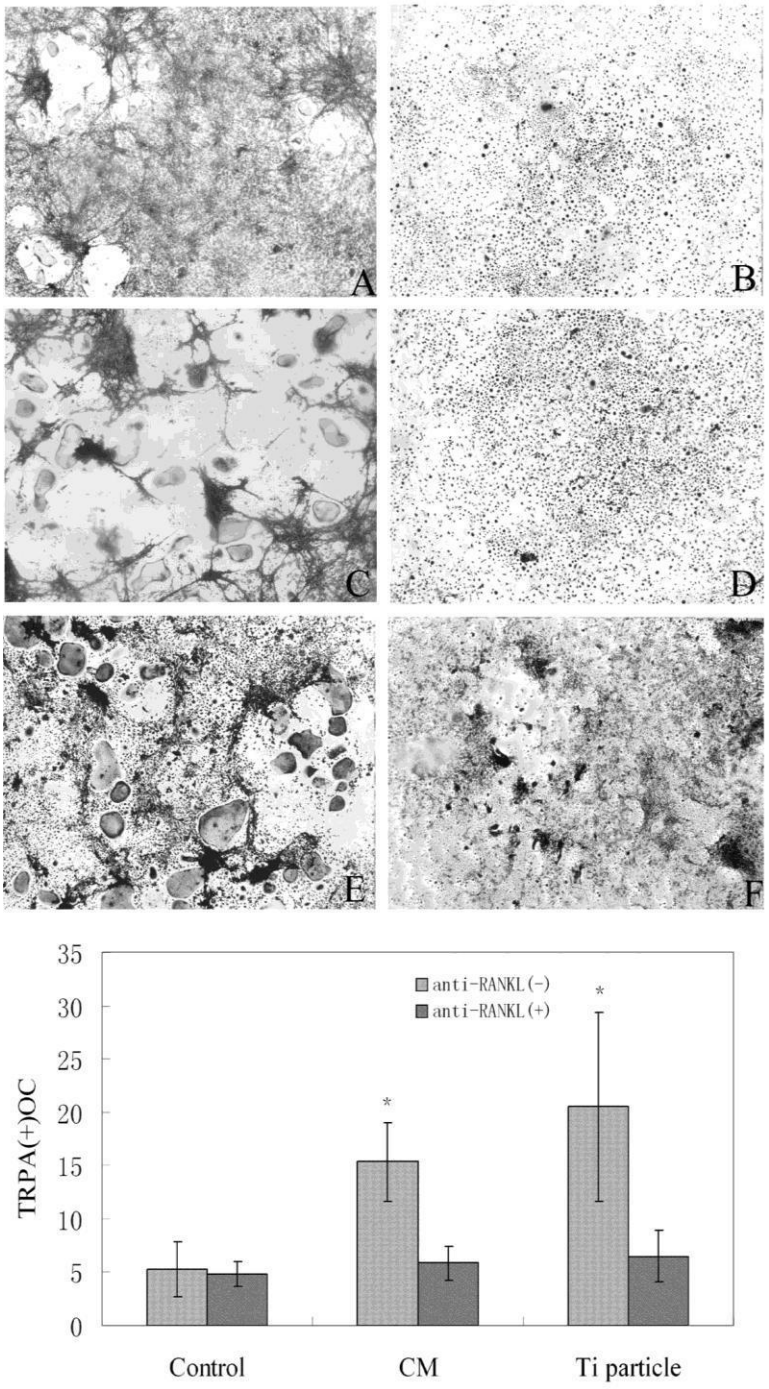

Figure 1. Effect of titanium particles on osteoclast differentiation. (A) control, (B) control+anti-RANKL, (C) CM (conditioned medium), (D) CM+anti-RANKL, (E) titanium particles, (F) titanium particles+anti-RANKL. *P < 0.05.

particles and conditioned medium enhanced the expression of RANKL in osteoblasts (Figure 3). From the TEM examination, we found that many titanium particles were inside cells and some were even inside nucleus, indicating the phagocytosis of titanium particles by cells (Figure 4).

DISCUSSION

In this study, we studied the effect of titanium particles on osteoclast differentiation and osteoclast activity. Our results showed that both titanium particles medium and conditioned medium enhanced osteoclast differentiation

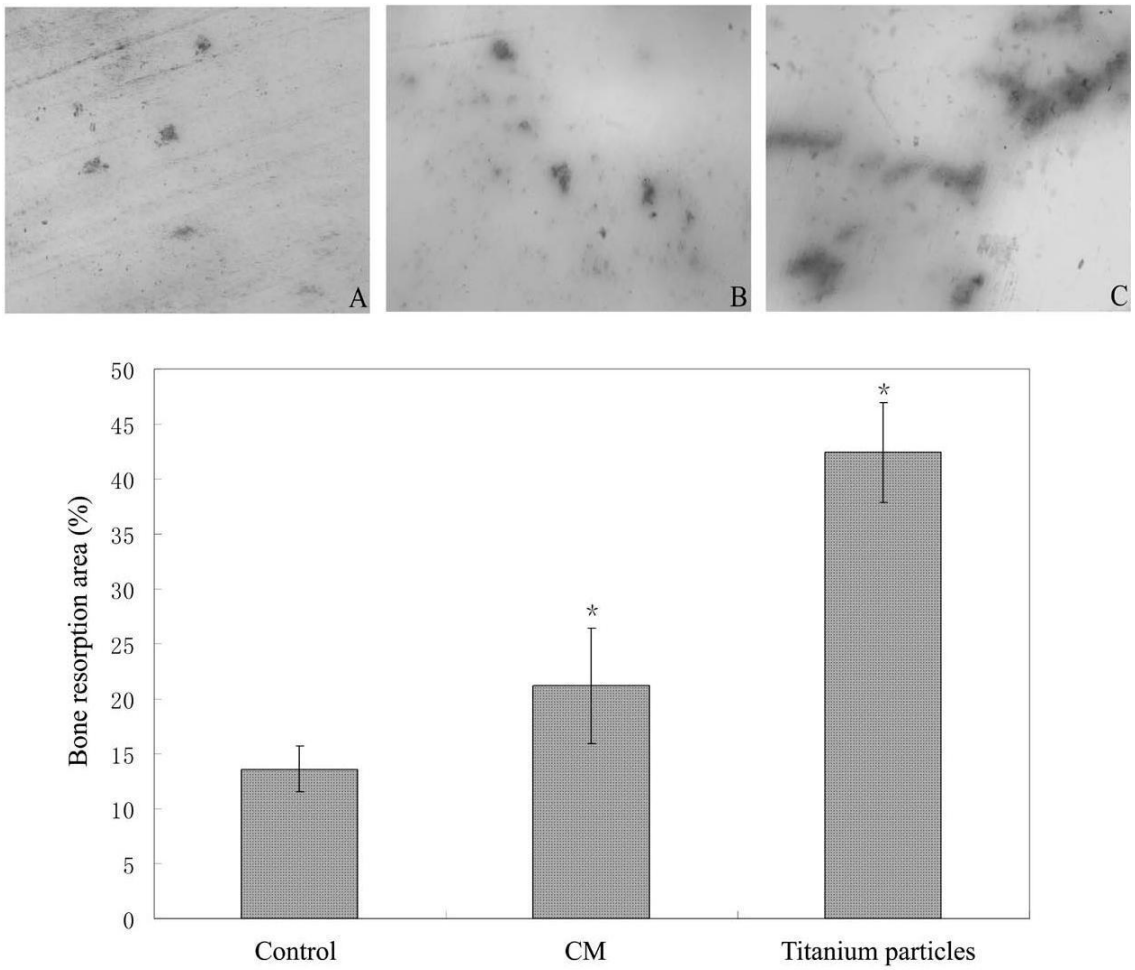

Figure 2. Effect of titanium particles on osteoclast bone resorption activity. Both conditioned medium and titanium particles medium enhanced osteoclast bone resorption area on dentin slices. * P < 0.05.

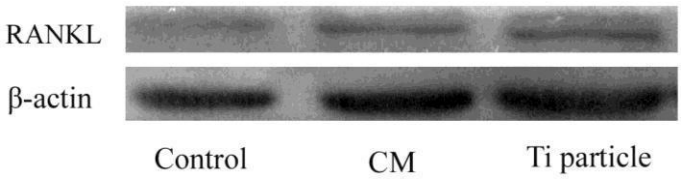

Figure 3. Expression of RANKL in osteoblasts after treated with conditioned medium and titanium particles medium for 48 h.

and resorption activity. Therefore, titanium particles around dental implants have an adverse effect on the osseointegration of dental implants.

The results showed that osteoclast differentiation were enhanced by both titanium particles and conditioned medium, which may be partly because titanium particles promote some cell factors released from cells after they phagocytosed titanium particles. When exposed to titanium particles, many cells such as osteoblasts and fibroblasts, could release inflammatory cytokines including interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-a (Bukata et al., 2004; Nakashima et al., 1999; Okafor et al., 2006). In *in vivo* research, cytokines such as IL-6, PGE_2 were also detected around titanium implants (Suska et al., 2005). It is widely accepted that these cytokines could stimulate osteoclast differentiation and promote bone resorption (Colucci et al., 2007; Wei et al., 2005; Yago et al., 2009).

RANKL, as an essential factor for osteoclast differentiation, is usually secreted by osteoblasts and bone marrow stem cells (Anandarajah, 2009; Kobayashi et al., 2009). Therefore, in this study, we studied the effect of titanium particles on the expression of RANKL in osteoblasts and found that titanium particles enhanced the expression of RANKL. The increased expression of RANKL could enhance the fusion of mononuclear cells and finally promote osteoclast differentiation (Lu et al., 2010).

Besides osteoclast generation, we also studied the effect of titanium particles on osteoclast bone resorption

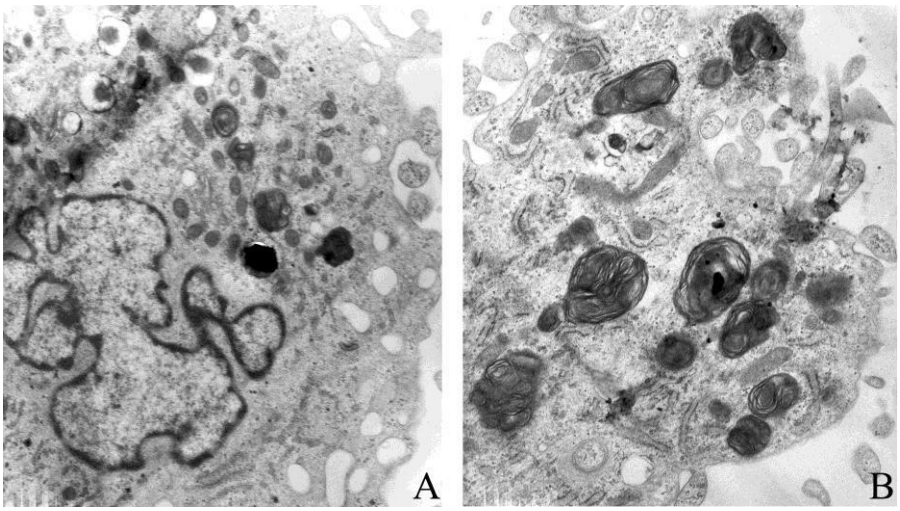

Figure 4. Transmission electron micrographs (×200,000) of osteoblasts after treated with titanium particles for 48 h.

activity. We found that both titanium particles medium and conditioned medium enhanced osteoclast resorption area on dentin slices. The exact mechanism by which titanium particles take their effects on osteoblasts and osteoclast is not clearly known at present. In the TEM image, it was discovered that titanium particles were inside osteoblasts, indicating the phagocytosis of titanium particles by cells. The diameter of titanium particles around dental implants was about 1 to 10 μm and they could be easily phagocytosed by cells. This phagocytosis could enhance the expression of RANKL, which could enhance osteoclast' activity (Leibbrandt and Penninger, 2009). Furthermore, titanium particles could promote osteoclast' activity as an inflammatory factor. This is consistent with the result that macrophages could absorb bone after phagocytosing titanium particles (Fujikawa et al., 2005). Therefore, titanium particles could enhance bone resorption around dental implants due to excessive osteoclast resorption activity.

The enhanced osteoclast generation and their activity would disrupt the balance between bone formation and bone resorption in the bone-implant interface. Moreover, it has been reported that titanium particles could inhibit osteoblasts' and bone marrow stem cells' activity (Choi et al., 2005). As a result, there may be bone loss around dental implants due to the effects of titanium particles, which would finally induce dental implant failure.

Conclusively, titanium particles could promote osteoclast differentiation both in direct and indirect contact and the enhanced osteoclast generation and osteoclast activity could finally induce bone resorption around dental implants.

ACKNOWLEDGEMENT

Thi study was supported by the National Natural Science Foundation of China (No.81100780).

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