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Immobilized-OPG-Fc on a titanium surface inhibits RANKL-dependent osteoclast differentiation in vitro

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Abstract The purpose of the present study was to examine the effect of osteoprotegerin (OPG)-Fc fusion protein immobilized on a titanium surface on the initial differentiation of osteoclast precursor RAW264.7 cells. These cells were cultured on titanium specimens over which OPG-Fc was immobilized. The enhancement of tartrate-resistant acid phosphatase (TRAP) and cathepsin K mRNA expression in RAW264.7 cells exposed to receptor activator of NF-κB ligand (RANKL) stimulation on OPG-Fc-coated titanium was significantly lower than that in RAW264.7 cells exposed to RANKL on titanium specimens without immobilized OPG-Fc (ANOVA, $P < 0.01$). Preincubation of OPG-Fc-coated titanium, in a medium supplemented with 10% fetal bovine serum at 37°C for two days before the cells were seeded, had no significant effect on the decrease in mRNA expression (ANOVA, $P < 0.01$). Taken together, these results indicate that OPG-Fc immobilized on a titanium surface blocks the differentiation of RAW264.7 cells induced by RANKL stimulation.

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1 Introduction

Osseointegration is defined as a direct structural and functional connection between living bone and the surface of an implant. At the interface, continuous and dynamic remodeling, replacement of old bone, repair of fatigue-damaged foci and maintenance of interface structural integrity occurs [1]. Successful bone remodeling in dental implants requires coordinated activities of both osteoblasts and osteoclasts. Accelerated adherence of osteoblast cells onto a dental implant surface is an important process for osseointegration [2]. The implant surface is prepared by an increased number of multinucleated gigantic cells, including precursor cells of macrophages and osteoclasts, during the initial period after implant placement [1]. Thus, initial osteoblast-mediated bone formation and osteoclast-mediated bone resorption at the interface between bone and dental implants plays a crucial role in determining the prognosis of dental implants [1].

The effects of the surface properties of titanium or its modification with proteins on osteoblast proliferation or differentiation have been well documented [3, 4]. However, little information has been obtained on the effect of these modifications on osteoclast proliferation or differentiation. Recently, we examined the effect of titanium surface roughness on the differentiation of osteoclast precursor cells, RAW264.7 cells [5]. We found that roughened titanium surfaces accelerated osteoclast differentiation that was induced by the stimulation of NF-κB ligand (RANKL), a receptor activator [1].

Investigations of the molecular mechanisms underlying osteoclast differentiation have demonstrated that the macrophage-colony stimulating factor (M-CSF), the RANKL-RANK pathway and osteoprotegerin (OPG), an antagonist of RANKL function, are all essential for osteoclast

differentiation and development [6]. In response to M-CSF, hematopoietic stem cells differentiate into macrophage colony-forming units, which are the common precursor cells of macrophages and osteoclasts. The osteoclast precursor cells are mainly induced by RANKL and its downstream molecules and consequently become mature osteoclasts acquiring bone-resorbing activity [7]. OPG acts as a competitive inhibitor of RANKL [8]. This pathway might also play a role in the osseointegration of implants.

RANKL, derived from T and B cells, elevates osteoclast activity in cases of periodontal disease and causes local bone loss in rheumatoid arthritis [9]. Given that a significant amount of soluble RANKL (sRANKL) exists in the crevicular fluid of patients with peri-implantitis [10], there is a possibility that osteoclast precursor cells around dental implants are exposed to RANKL derived from activated lymphocytes or osteoblasts, resulting in bone loss through osteoclasts.

It is well known that bone resorption is coupled with bone formation [11]; therefore, pathological phenomena may be dependent on a cellular imbalance in the interaction between osteoblasts forming bone and osteoclasts resorbing bone. OPG, produced by osteoblasts, is a crucial regulator of osteoclast development and function. Consequently, OPG knockout mice displayed osteoporosis owing to enhanced bone resorption at local sites [12]. Recombinant OPG-Fc increased cortical and trabecular bone mass in young gonad-intact cynomolgus monkeys, while the addition of OPG resulted in significant suppression of bone turnover markers, such as urine *N*-telopeptide and serum osteocalcin [13]. Likewise, exogenous OPG suppressed murine calvarial osteolysis induced by ultra-high molecular weight polyethylene particles [14]. Furthermore, approaches using the OPG gene therapy revealed that injection of the gene prevents wear debris-induced osteolysis [15, 16]. Similarly, RANK blockade using RANK-Fc, prevented and ameliorated titanium-induced osteolysis in a mouse calvaria model [17]. Recently, Dunn et al. showed that OPG-Fc supplementation at the medial first molar prevented mechanically induced osteoclastogenesis in the same portion, suggesting that the decreased activity in osteoclast cells induced by exogenous OPG may improve bone quantity [18]. RANKL is present in soluble and membrane-bound forms, and OPG is expressed constitutively or specifically in remodeling or inflammatory tissues [19]. It was reported that, during the loosening of a total hip replacement, cells activated by foreign stimuli at the interface tissues overproduced both RANKL and RANK, and also produced OPG. However, RANKL and RANK can interact without interference by OPG at the interface tissue, since the functional site of OPG is clearly different from those of RANKL and RANK [19]. These reports suggest that the site of an exogenous OPG injection is important, although exogenous OPG application may be only one of the approaches for anti-bone loss.

Bone resorption around dental implants leads to loosening. Bisphosphonate is known to be a potent inhibitor of bone resorption [20, 21], and bisphosphonate–immobilized titanium has potential for the treatment of implant wound healing [20, 21]. However, systemic bisphosphonate has proven to have converse effects in bone lytic diseases, including osteonecrosis of the jaw [22], implicating that alternatives to inhibit bone loss are needed.

As such, we hypothesize that, exogenous OPG, delivered as soluble OPG or immobilized on titanium, would improve any imbalance in the RANKL/RANK/OPG system that triggers the disruption of the coupling mechanism between bone formation and bone resorption at the implant site. Moreover, the abutment, immobilized with OPG-Fc, may offer a therapeutic approach to inhibit bone loss around dental implants in peri-implantitis. Thus, the purpose of the present study was to examine whether OPG-Fc immobilized on titanium surfaces inhibits RANKL-dependent osteoclastogenesis, using RAW264.7 cells.

2 Materials and methods

2.1 Preparation of titanium specimens

Pure wrought titanium (cp-titan) discs (JIS, Japan Industrial Specification H 4600, 99.9 mass% titanium, diameter 15-mm, thickness 1-mm; Kobelco, Kobe, Japan) were purchased and used in the experiments. The cp-titan discs were sandblasted (HI ALUMINAS, Shofu, Kyoto) and washed with acetone and ethanol in an ultrasonic bath for 30 min before use. The diameter of the cylindrical-shaped titanium disc fit within the well of a standard 24-well tissue culture plate [23].

2.2 Immobilization of OPG-Fc on titanium surfaces

Immobilization of recombinant OPG-Fc (R&D Systems) on titanium surfaces was performed in accordance with previous studies [24–27]. Briefly, titanium specimens were immersed in 5% γ-aminopropyltriethoxysilane in acetone for 15 min at room temperature and then washed with acetone. Next, specimens were treated in 5% glyoxylic acid monohydrate for 2 h and then washed with ultrapure water. The surfaces of the specimens were subsequently treated with 0.4% sodium borohydride ($NaBH_4$) for 24 h, to reduce the imines to amine groups. After this series of pretreatments, titanium discs were washed with ultrapure water and autoclaved. The carboxyl groups on the disc surfaces were activated with *N*-hydroxylsuccinimide (NHS)/*N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide (EDC) (BiaCore

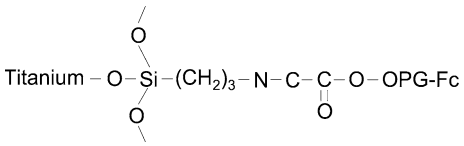

Fig. 1 Scheme of the final product of OPG-Fc immobilized on titanium surfaces

AB, Uppsala, Sweden) and treated with 2.0 μg/ml OPG-Fc (Sigma, St. Louis, MO, USA) in sodium bicarbonate buffer (pH 8.0, 300 μl) for 30 min at 37°C and for 16 h at 4°C to immobilize OPG-Fc on the surfaces (OPG-Fc-titanium). After washing with phosphate buffered saline (PBS) (500 μl) twice to remove any excess OPG-Fc, the activated carboxyl groups were blocked by a 5 min treatment with 1 M ethanolamine-HCl (BiaCore AB). A scheme of the final product is shown in Fig. 1. OPG-Fc-free titanium was prepared by treatment of the titanium specimens with 1 M ethanolamine-HCl immediately after the carboxyl groups were activated by NHS/EDC treatment. Untreated titanium specimens were also used as control specimens (control titanium).

The content of OPG-Fc remaining on the titanium specimen was predicted by calculating the excess OPG-Fc left in the supernatant and OPG-Fc in the first and second washing buffers. The protein contents of the supernatant and washing buffers were calculated by UV absorbance at 280 nm [28], using bovine serum albumin as a standard.

2.3 RAW264.7 cell culture

The RAW264.7 mouse macrophage/monocyte cell line (TIB-71; American Type Culture Collection) was used in the present study. Cells were seeded into 24-well plates or onto the surfaces of titanium specimens placed on the bottoms of 24-well plates, at a density of 5.0×10^3 cells/well, in alpha-MEM supplemented with an antibiotic mixture (Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum (FBS) (Biological Industries, Haemek, Israel) and 1.5 g/l sodium bicarbonate (Invitrogen). The cells were then cultured at 37°C under 5% CO_2/95% air for 3–8 days and the medium was changed every day. For the differentiation assay, cells were cultured similarly in the presence of 50 ng/ml recombinant soluble murine RANKL (PeproTech, London, UK).

2.4 Real-time quantitative reverse transcriptase-polymerase chain reaction analysis

Total RNA was extracted using TRIzol® reagent (Invitrogen). Real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analyses for tartrate-resistant acid phosphatase (TRAP) and cathepsin K were performed using a line-gene real-time monitoring fluorescent quantitative detection system and software (BioFlux, Tokyo, Japan). First-strand cDNA was synthesized from total RNA (100 ng) using ReverTra Ace reverse transcriptase (Toyobo).

The sequences for the primers used in these analyses were as follows: 5′-GGAGCTTAACTGCCTCTTGC-3′ and 5′-CCGTGGGTCAGGAGTGG-3′ for TRAP cDNA amplification, and 5′-GGAAACAAAGGATATGCTCTCTTGG-3′ and 5′-GCTGGCTGGCTGGAATCAC-3′ for cathepsin K amplification. The sequences of the TaqMan fluorogenic probes used were 5′-6FAM-TTTGTAGGCCCAGCAGC ACCACCC-TAMRA-3′ and 5′-6FAM-AACAACGCCTG CGGCATTACCAACA-TAMRA-3′ for TRAP and cathepsin K, respectively. Primers and probes were purchased from Sigma.

2.5 Scanning electron microscopy (SEM) observation

Cells cultured on titanium specimens were fixed in a buffered solution of 2% glutaraldehyde at 4°C after being rinsed with PBS. Then, the samples were washed with PBS three times to eliminate glutaraldehyde and dehydrated through a graded series of ethyl alcohols (50–99.5%). The surfaces of specimens were sputter-coated with a layer of gold to a thickness of 20–25 mm and observed under a scanning electron microscope using standard procedures.

2.6 Western blot analysis

OPG-Fc-immobilized titanium surfaces were preincubated in culture medium including 10% FBS (500 μl) at 37°C for 0 or 4 days. Each media was collected and lysed in 2× Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) and stored overnight at −80°C. After centrifugation of samples at 15,000×*g* for 20 min at 4°C, the supernatants, from which dissolved proteins were removed, were transferred to new microtubes, boiled for 5 min and stored at −20°C. The samples (2 μl/lane) were separated by SDS-PAGE gel and transferred to PVDF membranes (Immun-Blot PVDF Membrane, Bio-Rad). The membranes were probed with primary antibodies against mouse OPG (polyclonal antibody, Santa Cruz Biotechnology, Inc., CA, USA), followed by incubation with anti-rabbit IgG conjugated with horseradish peroxidase for the detection of mouse OPG (KPL, Gaithersburg, MD, USA). Immune complexes containing horseradish peroxidase deposited to specific target molecules on the PVDF membrane were detected using an enhanced chemiluminescence detection kit (ECL Plus Western Blotting Detection System, GE Healthcare, Buckinghamshire, UK).

2.7 Data analysis

Differences between mean values of groups were subjected to a one-way analysis-of-variance (ANOVA) and Tukey's multiple range test.

3 Results

We used the RAW264.7 cell line, which differentiates into osteoclasts in the presence of RANKL, to evaluate the effect of soluble (sOPG-Fc) or immobilized OPG-Fc titanium specimens on the initial differentiation of preosteoclasts. At the RNA level, sRANKL enhanced the expression of TRAP and cathepsin K mRNAs in RAW cells cultured on sandblasted titanium specimens (control titanium). Soluble OPG-Fc of 500 ng/ml significantly inhibited the enhanced expression of TRAP and cathepsin K mRNAs induced by sRANKL (ANOVA, $P < 0.01$) (Fig. 2). A preliminary protein assay to prepare OPG-Fc-coated titanium before culturing RAW cells was performed. The results showed that 40% of the OPG-Fc applied to the titanium surface remained after two washes.

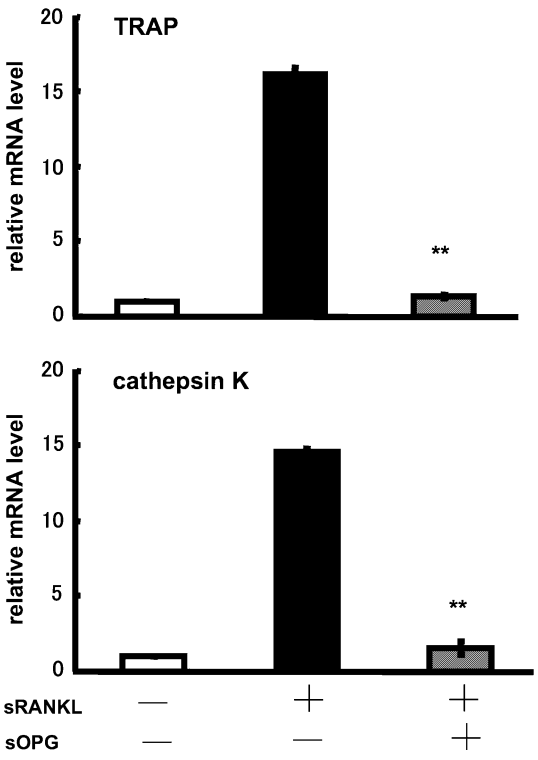

Fig. 2 Effect of 500 ng/ml sOPG-Fc on TRAP and cathepsin K gene expression in RAW cells exposed to sRANKL on tissue culture surfaces, as determined by real-time quantitative RT-PCR analysis. Data represent the means ± SD of triplicate experiments. Three independent experiments were performed

Therefore, we applied 2.0 μg/ml OPG-Fc on the titanium surface to produce OPG-Fc-coated titanium.

Next, to investigate the effects of OPG-Fc-coated titanium on RANKL-induced osteoclast differentiation, RAW cells were grown on OPG-Fc-titanium, OPG-Fc-free titanium or control titanium. Soluble RANKL enhanced the expression of TRAP and cathepsin K mRNAs in RAW cells cultured both on control titanium specimens and on OPG-Fc-free titanium. In contrast, the expression of TRAP and cathepsin K mRNAs in cells exposed to sRANKL on OPG-Fc-titanium was significantly lower than that in cells cultured with sRANKL on OPG-Fc-free titanium (ANOVA, $P < 0.01$) (Fig. 3A). As shown in Fig. 3B, SEM observation indicated a morphological change in RAW cells cultured on titanium specimens for 8 days in the presence of sRANKL. The mononuclear RAW cells on OPG-Fc-free titanium in the absence of sRANKL simply attached to other cells and the surface of titanium (Fig. 3B-a). The mononuclear RAW cells on OPG-Fc-free titanium in the presence of sRANKL fused and spread on the surface of titanium (Fig. 3B-b). Cell spreading on the titanium-surface provoked by sRANKL was not observed in RAW cells cultured on OPG-Fc-titanium with sRANKL (Fig. 3B-c) or on OPG-Fc-free titanium without sRANKL (Fig. 3B-a).

To evaluate the duration of immobilized OPG-Fc activity, OPG-Fc-coated titanium specimens were preincubated for 0, 2 or 4 days in fresh culture medium supplemented with 10% FBS at 37°C prior to inoculation of the RAW cells on their surfaces. The expression of TRAP and cathepsin K mRNAs induced by RANKL stimulation was significantly decreased when cells were cultured on OPG-Fc-titanium following 0- and 2-day preincubations (ANOVA, $P < 0.01$), but no inhibitory effects were observed with 4-day preincubated OPG-Fc-titanium compared with OPG-Fc-free titanium (Fig. 4).

Finally, the detection of OPG-Fc in preincubated culture media was performed by using western blot analysis, to examine whether the immobilized OPG-Fc molecules were released from the titanium surface into the culture media supplemented with FBS. The protein of OPG-Fc was detected in preincubated culture media at day 4 (Fig. 5). Also, there was no detection of OPG-Fc in preincubated culture media at day 0 (Fig. 5).

4 Discussion

The roles of immobilized proteins on titanium surfaces, such as collagen and RGD-peptides, in osteoblast responses, have been well documented [29, 30]. Studies on OPG in the contexts of osteoblasts have also suggested that local OPG immobilized on titanium may work more effectively to regulate osteoclast differentiation than systemic OPG

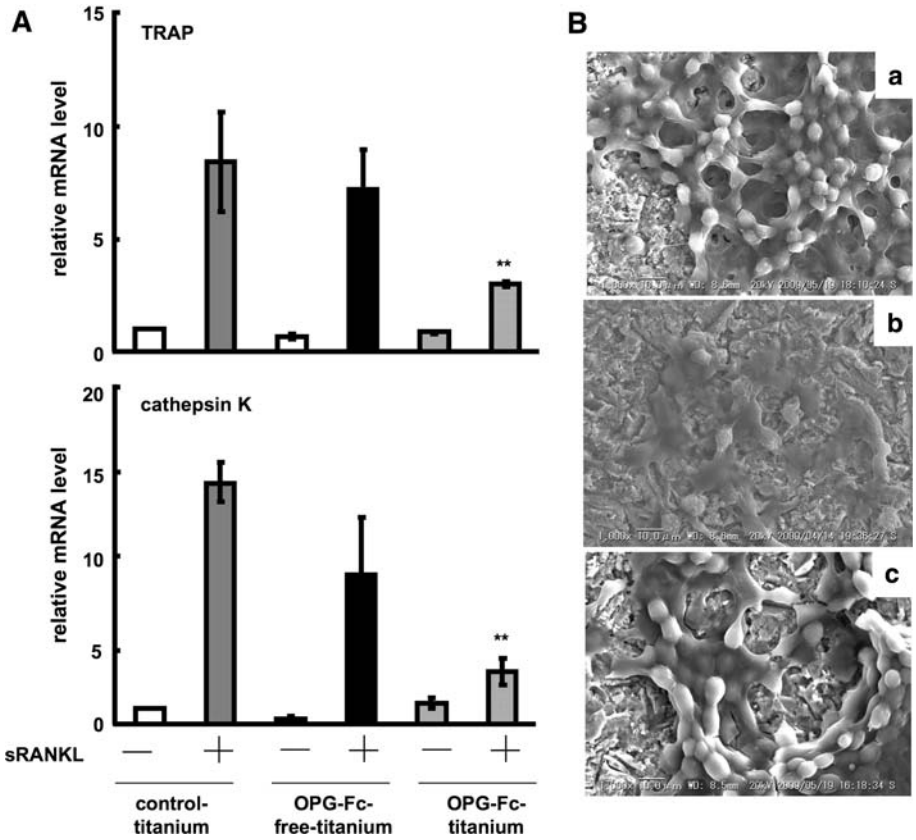

Fig. 3 **A** Effect of immobilized OPG-Fc on TRAP and cathepsin K gene expression in RAW cells exposed to sRANKL. RAW cells were cultured for 3 days on control titanium, OPG-Fc-free titanium or OPG-Fc-titanium in the absence or presence of sRANKL. Data represent the means ± SD of triplicate experiments. Three independent experiments were performed. ** $P < 0.01$ versus control titanium or OPG-Fc-free titanium with sRANKL. **B** A typical SEM view of RAW cells maintained for 8 days on OPG-Fc-free titanium without sRANKL (*a*), on OPG-Fc-free titanium with sRANKL (*b*) or on OPG-Fc-titanium with sRANKL (*c*). Data is representative of duplicate, independent experiments

[19, 31]. Hence, OPG immobilized on titanium surfaces may play a pivotal role in the initial response of precursor osteoclasts to RANKL during osseointegration. Therefore, in the present study, we employed an alkoxy silane compound in order to bind OPG-Fc to the surface of titanium discs. Using RNA extracted from cultured cells, the expression levels of TRAP and cathepsin K mRNAs were investigated in this study by real-time quantitative RT-PCR, because TRAP and cathepsin K are major markers for osteoclast differentiation [5]. As shown in Fig. 3A, OPG-Fc immobilized on titanium significantly decreased the expression levels of TRAP and cathepsin K mRNAs in RAW cells cultured in the presence of sRANKL. The inhibitory effects of OPG-Fc immobilized on titanium surfaces against the actions of sRANKL were active within 2 days in the presence of FBS (Fig. 4). In addition, it is reported that OPG dimer formation is required for the inhibition of the RANKL/RANK receptor interaction [32]. Collectively, these findings suggest that homodimeric immobilized OPG-Fc binds to sRANKL and regulates osteoclastogenesis as a decoy receptor for RANKL. Intact OPG possesses a very brief circulating half-life [8], which is partly due to its heparin-binding domain. Deletion of this domain and fusion of the truncated molecule to the Fc portion of human IgG1 results in a long circulating half-life [33]. Although a comparison of intact OPG and the recombinant OPG-Fc fusion molecule was not carried out here, our results have shown that immobilized OPG-Fc works as a decoy receptor for RANKL, even when immobilized OPG-Fc is preincubated in serum-containing medium for at least 2 days (Fig. 4). Preincubation in a serum-containing medium was expected to simulate the in vivo condition, which caused the release of immobilized OPG-Fc molecules in 4 days (Fig. 5). Therefore, 4-day-preincubated OPG-Fc-titanium had no effect on the expressions of osteoclast differentiation markers enhanced by sRANKL stimulation (Fig. 4). However, it remains possible that RANKL-dependent osteoclast differentiation was inhibited by the released OPG-Fc from the surface of titanium, thus OPG-Fc-titanium is potentially a carrier for a slow delivery of OPG.

The prevention of direct interaction between membrane-bound RANKL and RANK in a co-culture system, in which OPG-deficient mouse-derived osteoblast cells were maintained in the spot portion of culture plates before hematopoietic cells were uniformly inoculated in each well, resulted in no osteoclast differentiation, even in the presence of 1 alpha, $25(OH)_2D_3$ and M-CSF. This report indicates that RANKL, expressed by osteoblasts, functions as a membrane-associated form; however, the function of soluble RANKL cannot be excluded [34]. In this study, sRANKL was used to induce RAW cell differentiation;

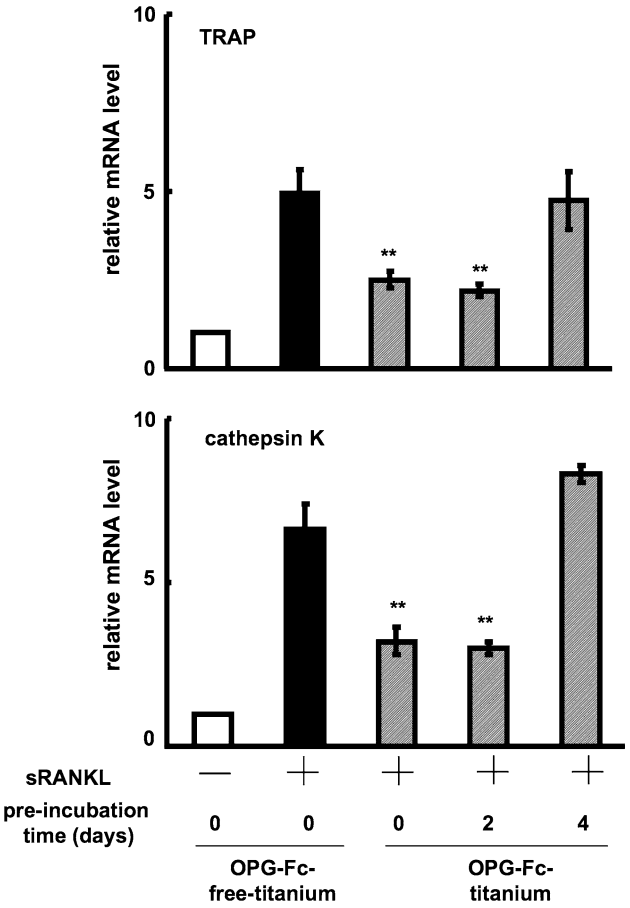

Fig. 4 Effect of preincubated immobilized OPG-Fc-titanium on TRAP and cathepsin K mRNA expression in RAW cells exposed to sRANKL. RAW cells were cultured on OPG-Fc-free titanium or on OPG-Fc-titanium in the absence or presence of sRANKL after each prepared titanium specimen was preincubated in culture medium including 10% FBS at 37°C for 2 or 4 days. Data represent the means ± SD of triplicate experiments. Three independent experiments were carried out. ** $P < 0.01$ versus OPG-Fc-free titanium without preincubation in the presence of sRANKL

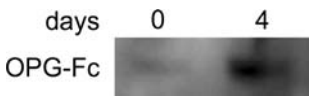

Fig. 5 Detection of OPG-Fc in media. Proteins dissolved by Laemmli sample buffer were separated in SDS-PAGE and transferred onto PVDF membranes. After reaction of a polyclonal antibody to OPG on the membrane, the target-specific band of OPG-Fc was detected using a chemiluminescence-based detection system

although, whether the immobilized OPG-Fc blocked the interaction between membrane-bound RANKL and RANK remains unclear. Further study is necessary to clarify the mechanisms in detail.

In vitro experiments have shown that collagen and RGD peptides immobilized on titanium enhanced the adhesion of osteoblast cells in 1-day [29, 30]. Collagen I coating of dental screw implants enhanced peri-implant bone formation in 1-month [35]. Bone morphogenic proteins (BMPs) are generally known to increase bone formation, and BMPs released from atelopeptide type I collagen (carrier) stimulated a bone response in peri-implants [36]. However, BMP immobilized on titanium did not increase peri-implant bone formation after 1- and 3-months [35]. These above reports, as well as the results in the present study, suggest that the conformation and/or activity of the molecule after the immobilization process are very important for the function of the immobilized protein.

Here, we have shown the following effects of OPG-Fc immobilized-titanium on RAW cells: (i) OPG-Fc immobilized-titanium decreased the expression levels of TRAP and cathepsin K mRNAs in RAW cells exposed to sRANKL stimulation for 3 days. (ii) No morphological changes in RAW cells exposed to sRANKL were observed on the titanium immobilized with OPG-Fc for 8 days. (iii) OPG-Fc immobilized on titanium in serum-containing medium at 37°C remained active for 2 days. Collectively, these results clearly indicate that OPG-Fc immobilized-titanium, as well as sOPG-Fc, inhibit RANKL-dependent osteoclast differentiation. This in vitro study suggests that OPG-Fc immobilized-titanium has potential to regulate osteoclastogenesis during osseointegration. The alternative properties of titanium surfaces affecting osteoclast activity may contribute to the development of implants that improve osseointegration; as such, the effect of titanium surfaces on osteoblast or osteoblastic cell activities should be taken into consideration [3, 30, 37].

5 Conclusions

OPG-Fc immobilized-titanium inhibited RANKL-dependent differentiation of RAW264.7 cells as well as soluble OPG-Fc. Immobilized OPG-Fc remained active on the surface of titanium for 2 days.

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