Original article

Titanium ion induces necrosis and sensitivity to lipopolysaccharide in gingival epithelial-like cells

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Abbreviations:

Ti: titanium; RANKL: receptor activator of NF-κB ligand; OPG: osteoprotegerin; TLR: toll-like receptor; LPS: lipopolysaccharide; LDH: lactate dehydrogenase; RT-PCR: reverse transcription polymerase chain reaction; *Pg*: *Porphyromonas gingivalis;* ICP: inductively coupled plasma spectroscopy; EGF: epidermal growth factor; PBS: phosphate-buffered saline; ICAM-1: intercellular adhesion molecule 1; CCL2: chemokine (C-C motif) ligand 2;

ABSTRACT

Gingival epithelial-like cells (GE-1) were cultured and used to examine the cellular responses of gingival tissues to varying concentrations of titanium (Ti) ions. Titanium ions at concentrations of more than 13 ppm significantly decreased the viability of GE-1 cells and increased LDH release from the cells into the supernatant, but had no significant effect on their caspase 3 activity. These data suggest that a high concentration of Ti ions induced necrosis of the GE-1 cells. Titanium ions at a concentration of 5 ppm significantly increased the level of CCL2 mRNA expression in GE-1 cells exposed to lipopolysaccharide derived from *Porphyromonas gingivalis* in a synergistic manner. Moreover, the mRNA expression levels of TLR-4 and ICAM-1 in GE-1 cells loaded with Ti ions at 9 ppm were significantly enhanced as compared with those in GE-1 cells without Ti stimulation. We suggest that Ti ions are in part responsible for monocyte infiltration in the oral cavity by elevating the sensitivity of gingival epithelial cells to microorganisms. Taken together, these data indicate that Ti ions may be involved in cytotoxicity and inflammation at the interfaces of dental implants and gingival tissue.

Keywords: titanium ion, cell viability, TLR-2, TLR-4, gingival epithelial cells

1. Introduction

Titanium (Ti) is extensively utilized for numerous medical applications because of its excellent corrosion resistance, mechanical properties and biocompatibility (Wataha, 1996; Long and Rack, 1998). However, Ti ions have been detected in the fibrous membranes encapsulating implants and in synovial fluid (Dorr et al., 1990). The facilitation of osteoclast differentiation by Ti particles has also been reported (Bi et al., 2001). Moreover, clinical studies have shown that Ti particles released during the aseptic loss of orthopedic implants accumulate in the tissues (Jacobs et al., 1998; Gallo et al., 2002). Recently, we revealed that Ti ions had biological and/or adverse effects on the expressions of receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) in osteoblastic cells (Mine et al., 2010). It is well known that the RANKL-RANK pathway and OPG, an antagonist of RANKL function, are essential for the differentiation and development of

osteoclasts, which cause pathologic bone resorption (Koide et al., 2003). The corrosion resistance of Ti is decreased under low dissolved-oxygen conditions such as in the oral cavity, particularly in the presence of small amounts of fluoride (Nakagawa et al., 2002). Taken together, these findings imply that released Ti ions play a pivotal role in bone resorption at the interface of bone and dental implants (Bi et al., 2001; Dorr et al., 1990; Gallo et al., 2002; Jacobs et al., 1998; Mine et al., 2010).

Peri-implantitis accompanies inflamed gingival tissues and is often followed by bone resorption (Lang et al., 2000). We reported that the expressions of RANKL and OPG mRNAs in gingival epithelial-like cells were not changed when the cells were exposed to Ti ions (1-9 ppm) (Mine et al., 2010). However, an *in vitro* experiment showed that Ti ions significantly enhanced the inflammatory cytokine production of macrophages (Taira et al., 2006). In addition, toll-like receptors (TLR), including -4 and -9, increased in the interface membrane around loosening total hip replacement implants, where Ti particles may exist (Takagi et al., 2007). TLRs can be receptors for microbial organisms and endogenous ligands (Kaisho and Akira, 2000; Beutler, 2002). These data indicate that Ti ions, together with other outer stimuli like microorganisms or their products such as lipopolysaccharide (LPS), alter the physiological function of gingival epithelial cells. Therefore, there is a possibility that Ti ions released from dental implants may be involved in, or facilitate the inflammation of gingival tissues with peri-implantitis,

Hence, in the present study, we investigated the effects of Ti ions on the viability of gingival epithelial-like cells using the MTS assay, the lactate dehydrogenase (LDH) release assay, and the caspase 3 activity assay. We also determined the mRNA levels of receptors for microbes and cytokines related to inflammation and bone resorption in the presence or absence of LPS derived from *Porphyromonas gingivalis* using the real-time reverse transcription polymerase chain reaction (RT-PCR).

2. Materials and methods

2.1. Preparation of Ti ions and P. gingivalis LPS

A Ti standard solution for inductively coupled plasma spectroscopy (ICP) was purchased from

Merck (Darmstadt, Germany). For the experiments, the Ti-ICP standard solution was diluted with cell culture medium, under pH monitoring, according to the method described by Taira et al. (Taira et al., 2006). Neither a significant change in the pH of the medium, nor visual precipitations accompanying the supplementation of Ti ions were observed.

P. gingivalis LPS (*Pg*-LPS) was purchased from InvivoGen (San Diego, CA, USA); LPS was diluted with sterile endotoxin-free water and stored at -20°C until use.

2.2. Culture of GE-1 cells

The GE-1 cell line was obtained from the RIKEN Bioresource Center Cell Bank (Tsukuba, Japan). This cell line was established from temperature-sensitive SV40 large T-antigen gene transgenic C57BL/6 mouse gingival epithelium (Hatakeyama et al., 2001). GE-1 cells were cultured and maintained in SFM-101 culture medium (Nissui, Tokyo, Japan) supplemented with 1% fetal bovine serum and 10 ng/ml epidermal growth factor (EGF) (Sigma-Aldrich, Tokyo, Japan). GE-1 cells were seeded onto 96- or 24- well plates at a density of 4.0×10^3 and 2.5×10^4 cells/well, respectively. In addition, GE-1 cells were inoculated on pure titanium disks at a density of 2.5×10^4 cells/well. The Ti disks were set on the bottom of a 24-well plate. Pure wrought Ti (cp-titan) disks (JIS, Japan Industrial Specification H 4600, 99.9 mass% titanium, 15 mm diameter; Kobelco, Kobe, Japan) were purchased; the diameter of these cylindrical-shaped Ti discs fitted within the wells of a standard 24-well tissue culture plate. GE-1 cells were maintained at 33°C under 5% CO_2/95% air for each experiment.

2.3. MTS assay of GE-1 cells

GE-1 cells were seeded onto 96-well plates, maintained until confluent, and then exposed to Ti ions (1-19 ppm) for 6 h. Suspended cells were removed by gentle rinsing with phosphate-buffered saline (PBS), and the number of adherent cells remaining in each well was quantified using a coupled enzymatic assay which resulted in the conversion of a tetrazolium salt into a red formazan product (MTS assay, CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI, USA). The absorbance at 490 nm was recorded using a microplate reader (Bio-Rad, Hercules, CA) (Makihira et al., 1999).

2.4. LDH release assay of GE-1 cells

GE-1 cells inoculated onto 96-well plates at a density of 4.0×10^3 were maintained until confluent. At confluence, the medium was changed with fresh culture medium and then exposed to Ti ions (1-19 ppm) for 6 h. LDH released into the culture supernatant during the 6 h incubation was isolated from the cultured GE-1 cells and measured using an LDH release assay kit (CytoTox 96 Non-Radioactive Cytotoxicity Assay; Promega). Absorbance of the developed colorimetric substance in a 96-well plate was measured at 490 nm using the microplate reader.

2.5. Measurement of Caspase 3 activity in GE-1 cells

Caspase 3 activity was measured using a kit (CaspACE Assay System; Promega) that detects caspase 3-mediated specific cleavage at the C-terminal side of the aspartate (D) residue in the sequence DEVD (DEVDase activity). GE-1 cells were cultured in 96-well plates in the presence or absence of Ti ions (1-19 ppm) for 6 h. Cells were solubilized with a cell lysis buffer on ice for 15 min, and three repeats of a freeze/thaw cycle. The amount of caspase 3-specific DEVDase activity in the extracted sample was determined by monitoring the release of free chromophore from substrate (OD 405 nm) using the microplate reader.

2.6. Real-time quantitative RT-PCR

Total RNA from each treatment group was extracted using TRIzol reagent (Invitrogen). First-strand cDNA was synthesized from total RNA (100 ng) using ReverTra Ace (Toyobo, Osaka, Japan). The cDNA was then amplified by BIOTAQ DNA polymerase (Bioline, Randolph, MA, USA). Real-time quantitative RT-PCR analyses for TLR-2, TLR-4, intercellular adhesion molecule 1 (ICAM-1), chemokine (C-C motif) ligand 2 (CCL2), RANKL, OPG and β-actin were performed using Rotor-Gene™ 6000 (Qiagen, Tokyo, Japan). β-actin was chosen as an internal control to standardize the variability in amplification owing to slight differences in starting total RNA concentrations. Sequences of primers and probes used in the present study are listed in Table 1. The sequences of primers and probes for β-actin have been described previously (Mine et al., 2010).

2.7 Data analysis

The MTS and LDH release assays were repeated as triplicate independent experiments. Caspase 3 activity assays were repeated as duplicates independent experiments. Data represent the means ± standard deviations (SD) of samples assayed in triplicate. Real-time quantitative RT-PCR analyses for the expression levels of CCL2, RANKL, and OPG mRNAs were repeated as duplicate or triplicate experiments. Data were normalized to the expression levels of β-actin mRNA, which was the internal control. Data represent the means ± SD of samples assayed in triplicate. Differences between average values for groups were subjected to a one-way analysis-of-variance (ANOVA) and Tukey's multiple range tests. Significance was achieved at $p < 0.05$ or $p < 0.01$.

3. Results

3.1. Effects of Ti ions on cell viability

The results of the MTS assays showed that Ti ions at 1–11 ppm had no significant effects on the viability of the GE-1 cells (Fig. 1A), while Ti ions at 13-20 ppm significantly decreased the viability of the GE-1 cells as compared with unloaded controls (ANOVA; $p < 0.05$, 0.01 *vs*. control; Fig. 1A). The LDH release assay (Fig. 1B) was used to determine whether Ti ions caused GE-1 necrotic cell death. The results of the LDH release assay revealed that Ti ions at concentrations higher than 11 ppm significantly increased LDH release from the GE-1 cells into the extracellular medium (ANOVA; $p < 0.05$, 0.01 *vs*. control; Fig. 1B). To examine whether Ti ions caused GE-1 apoptotic cell death, the activity of caspase 3 was measured in cells exposed to Ti ion stimulation. Ti ions at 1-19 ppm had no significant effect on the caspase 3 activity of the GE-1 cells (ANOVA; $p > 0.05$; Fig. 1C). The results from the three cell toxicity assays indicated that Ti ions were non-toxic at concentrations of less than 9 ppm. Therefore, a concentration of Ti ions that did not reduce cell viability (less than 9 ppm) was used for the following experiments.

3.2. Morphological observations of GE-1 cells exposed to Ti ions

When GE-1 cells were cultured with Ti ions at concentrations of 1, 5, and 9 ppm for 6 h, there were no morphological changes compared with the control (data not shown). However, a slight change was observed in GE-1 cells stimulated with 5 and 9 ppm Ti ions (Fig. 2); the borders of cells loaded with 5 and 9 ppm Ti ions became unclear compared with cells treated with 1 ppm Ti or the controls.

3.3. Effects of Ti ions on the mRNA levels of CCL2, RANKL, and OPG in GE-1 cells in the presence of Pg-LPS

The mRNA expression profiles of CCL2, RANKL, and OPG in GE-1 cells were investigated when the cells were exposed to Ti ions and/or *Pg*-LPS.

Pg-LPS (5-25 μg/ml) significantly increased the level of CCL2 mRNA compared with the control (ANOVA; $p < 0.01$). The enhanced level of CCL2 mRNA induced by *Pg*-LPS peaked at 10 μg/ml (Fig. 3A). The level of expression of RANKL mRNA was significantly increased by 10 μg/ml *Pg*-LPS in comparison to the levels at 0, 5, 15, 20, and 25 μg/ml *Pg*-LPS (ANOVA; $p < 0.01$; Fig. 3A). *Pg*-LPS had no significant effect on the abundance of OPG mRNA (ANOVA; $p > 0.05$; Fig. 3A). Similarly, Ti ion (2-9 ppm) stimulation had no significant effect on the mRNA levels of CCL2, RANKL, and OPG (ANOVA; $p > 0.05$; Fig. 3A)

Pg-LPS alone (10 μg/ml) significantly increased the levels of expression of CCL2 and RANKL mRNAs (ANOVA; $p < 0.01$, *vs*. control and Ti alone; Fig. 3B). The addition of Ti ions synergistically elevated the enhanced level of CCL2 mRNA in GE-1 cells stimulated by *Pg*-LPS (Fig. 3B). There were no significant changes in the abundances of RANKL mRNA in GE-1 cells loaded with *Pg*-LPS alone and *Pg*-LPS plus Ti ions, or in the levels of OPG mRNA in GE-1 cells treated with *Pg*-LPS and/or Ti ions (Fig. 3B).

3.4. Comparison of the levels of CCL2 mRNA in GE-1 cells cultured on a plastic dish compared with a Ti disk

CCL2 mRNA in GE-1 cells was investigated to examine the differences in the cellular response to *Pg*-LPS in GE-1 cells culture on a standard plastic culture dish or on a pure Ti disk. *Pg*-LPS increased the abundance of CCL2 mRNA in GE-1 cells cultured on a plastic dish and a Ti disk,

compared with their respective controls; however, these increases in mRNA levels were not significant (ANOVA; $p > 0.05$; Fig. 4).

3.5. Effects of Ti ions on the mRNA levels of TLR-2, TLR-4, and ICAM-1 in GE-1 cells

To examine the cellular responses in the membrane proteins of GE-1 cells when recognizing microorganisms in the presence of Ti ions ranging from 1-9 ppm, the mRNA levels of TLR-2, TLR-4, and ICAM-1 were analyzed by real-time RT-PCR. In the present study, Ti ions at a concentration of 9 ppm significantly enhanced the levels of expression of TLR-4 (Fig. 5, closed square) and ICAM-1 mRNAs (Fig. 5, closed triangle) (ANOVA; $p < 0.05$, *vs*. control), while concentrations of 1 and 5 ppm Ti ions did not increase these levels (Fig. 5). Exposure of cells to Ti ions (1, 5, 9 ppm) did not alter the mRNA level of TLR-2 (Fig. 5, closed circle) in GE-1 cells.

4. Discussion

Titanium ions and/or particles from Ti implants directly and indirectly induce cell death and bone resorption, even though Ti is regarded as an excellent biocompatible metal (Bi et al., 2001; Mine et al., 2010; Sommer et al., 2005; Vamanu et al., 2008). Exposure of primary osteoblast cells derived from rat calvaria to Ti ions at 10 ppm and higher for 24 h was reported to be toxic (Liao et al., 1999). Similarly, two-day exposure to 1 ppm Ti reduced the viability of RAW264.7 cells to approximately 60% as compared with control cells (Taira et al., 2006). Therefore, Ti ions or particles may be involved in the prognosis of implants through bone remodeling. It is generally believed that in the oral cavity, gingival epithelia are constitutively exposed to outer stimuli including oral bacteria. In the present study, to investigate the effects of Ti ions and oral bacteria on gingival epithelial cells, the GE-1 cell line and LPS derived from *P. gingivalis* were used. The GE-1 cell line was established from temperature-sensitive SV40 large T-antigen gene transgenic C57BL/6 mouse gingival epithelium, and possesses the ability to undergo keratinization (Hatakeyama et al., 2001). The present study revealed that 6 h exposure to Ti ions at concentrations over 13 ppm significantly decreased the viability of GE-1 cells. This was thought to be due to the induction of necrosis, since an enhancement of LDH release from GE-1 cells to the supernatant was observed by the LDH release assay, and no change in caspase 3 activity was

detected in the cells (Fig. 1B, C). These results suggest that ionized Ti may be incorporated by GE-1 cells and behave as an inducer of necrosis in the cells at concentrations of greater than 11 ppm; a Ti disk which was not ionized had no effect on the growth of GE-1 (data not shown). However, the mechanism by which the Ti ions induce necrosis in GE-1 cells remains unclear.

In a previous study, we revealed that a 24 h exposure of GE-1 cells to Ti ions had no effect on cell viability (as measured by the MTS assay), or on the mRNA expression levels of RANKL and OPG (Mine et al., 2010). In the present study, 9 ppm of Ti ions were not cytotoxic to GE-1 cells after a 6 h-incubation, as indicated by the MTS, LDH and caspase 3 assays, but the borders of cells exposed to 9 ppm Ti ions for 24 h became unclear (Fig. 2). This phenomenon suggests that cellular responses were induced by Ti ions at less than 9 ppm within 24 h, although the effects on cellular viability appeared to be negligible. Thus, we chose three concentrations of Ti ions, 1, 5, and 9 ppm, to examine the cellular response of GE-1 cells to Ti ions within 24 h (Mine et al., 2010). We hypothesized that Ti ions at concentrations which do not affect cell viability may modulate the sensitivity of gingival epithelial cells to oral bacteria, since it is reported that TLRs recognizing bacteria were increased in the interface membrane around loosening total hip replacement implants (Takagi et al., 2007).

The emergence of peri-implantitis has been observed as a problem since the oral implant system, initially proposed by a Swedish group, was established (Mombelli and Lang, 1998). Peri-implantitis, periodontal diseases, and the periodontopathic bacteria *P. gingivalis,* have frequently been detected around and on dental implants (Quirynen et al., 2002). It is noteworthy that *Pg*-LPS is involved in bone resorption and inflammation in gingival tissues in periodontitis (Baker et al., 2000). Similarly, *Pg*-LPS may be involved in the pathological condition of peri-implantitis. The ratio of RANKL to OPG is related to bone resorption (Grimaud et al., 2003). Previously, we reported that GE-1 cells express RANKL and OPG, and that Ti concentrations of 1, 5 and 9 ppm did not induce expression of these proteins (Mine et al., 2010). However, in the present study, *Pg*-LPS increased RANKL mRNA levels in GE-1 cells, while 5 ppm Ti ions alone did not. Synergistically enhanced expression of RANKL mRNA was not observed in GE-1 cells exposed to a combination of 5 ppm Ti ions and 10 μg/ml *Pg*-LPS (Fig. 3). However, Ti ions surprisingly enhanced the expression of CCL2 mRNA in the presence of *Pg*-LPS, in a synergistic

manner (ANOVA; $p < 0.01$); CCL2 is a chemotactic cytokine for monocytes (Kim et al., 2006), which are differentiated multinucleated gigantic cells such as macrophages and osteoclasts (Horowitz et al., 2005). In contrast, the effects of 10 μg/ml *Pg*-LPS on CCL2 mRNA levels in GE-1 cells cultured on a plastic dish and a Ti disk, in the absence of Ti ions, were similar (Fig. 4). Therefore, induction of CCL2 by Ti ions under LPS stimulation may contribute not only to inflammation but also to bone resorption.

Toll-like receptor (TLR)-2 and TLR-4 are competent receptors for LPS (Kaisho and Akira, 2000). We revealed that *Candida albicans* was recognized by ICAM-1 on human primary gingival epithelial cells (Egusa et al., 2005); TLRs and ICAM are important for the recognition of microorganisms in the oral cavity. Therefore, we investigated the expression patterns of TLR2, TLR-4, and ICAM-1 in GE-1 cells exposed to Ti ions. The mRNA expression levels of ICAM-1 and TLR-4 in GE-1 cells stimulated with Ti ions were increased in a dose dependent manner (Fig. 5). Although the mechanism of the synergistic induction of CCL2 by Ti ions and *Pg*-LPS is still unclear, Ti ions may affect the cellular competency of bacterial recognition by altering the sensitivity of epithelial cells to oral bacteria. However, there is inconsistency between the phenomena at the transcriptional and translational levels in cultured cells subjected to external stimuli. Therefore, the influence of Ti ions on the production of cytokines and receptors must be investigated to elucidate the cellular responses to Ti ions within non-cytotoxic concentrations.

5. Conclusion

In summary, we found that the cytotoxic effect of Ti ions on GE-1 cells was partly due to the induction of necrosis. Titanium ions in the presence of *P*g-LPS enhanced the expression of CCL2 mRNA in a synergistic manner. Moreover, Ti ions alone elevated ICAM-1 and TLR-4 mRNAs in GE-1 cells. These results, taken together, suggest that Ti ions might decrease cell viability and affect inflammation by altering the sensitivity of the epithelia surrounding implants to microorganisms.

Conflict of interest statement

The authors declare that they have no competing financial interests.

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Figure legends

Fig. 1 The effects of Ti ions on the cell viabilities of GE-1 cells were assessed by the MTS assay, by measuring LDH release from the cells into the supernatant, and by measuring caspase 3 activity in the cells. When the cells reached confluence they were exposed to Ti ion (1-19 ppm) stimulation for 6 h. Each supernatant containing LDH released from the cells was collected. After gentle rinsing of the cells with PBS, MTS assays were carried out. The content of LDH released from the cells into the extracellular medium was measured using a LDH release assay kit. In an independent experiment measuring caspase 3 activity, confluent cells were prepared and exposed to Ti ion stimulation for 6 h. The activity of caspase 3 in the cells was measured using a caspase 3 assay kit. Independent experiments for the MTS and LDH release assays were repeated three times. Independent experiments for the caspase 3 activity assay were performed twice. Data represent the means ± SD of triplicate samples. * $p < 0.05$, ** $p < 0.01$.

Fig. 2 Phase contrast microscopic observations of GE-1 cells cultured in the presence of Ti ions (a: control, b: 1 ppm of Ti ions, c: 5 ppm, d: 9 ppm) for 24 h (magnification: ×100).

Fig. 3 The effects of Ti ions (5ppm) and/or *Pg*-LPS (10 μg/ml) on the mRNA levels of the genes encoding CCL2, RANKL, and OPG in GE-1 cells were examined by real-time quantitative RT-PCR (A and B). Once confluent, the cells were exposed to *Pg*-LPS (5-25 μg/ml), Ti ions (1-9 ppm), or *Pg*-LPS (10 μg/ml) with/without Ti ion (5 ppm) stimulation for 6 h, and total RNA was isolated for analysis. The data from real-time quantitative RT-PCR analyses of CCL2, RANKL,

and OPG mRNA levels were normalized to the expression levels of β-actin mRNA. Independent experiments were repeated three times. Data represent the means ± SD of triplicate samples. * $p < 0.05$, ** $p < 0.01$.

Fig. 4 The effects of *Pg*-LPS on the mRNA expression levels of CCL2 in GE-1 cells cultured on a plastic dish and a Ti disk were examined by real-time quantitative RT-PCR. At confluence, the cells were incubated with or without *Pg*-LPS (10 μg/ml) for 6 h, and total RNA was isolated for analysis. The data from real-time quantitative RT-PCR analyses of the levels of CCL2 mRNA were normalized to the expression levels of β-actin mRNA. Independent experiments were repeated two times. Data represent the means ± SD of triplicate samples.

Fig. 5 The effects of Ti ions on the levels of expression of TLR-2, TLR-4, and ICAM-1 mRNAs in GE-1 cells were analyzed by real-time quantitative RT-PCR. Confluent cells were exposed to Ti ion stimulation at the indicated concentrations for 6 h prior to isolation of total RNA. The data from real-time quantitative RT-PCR analyses of TLR-2, TLR-4, and ICAM-1 mRNA levels were normalized to the expression levels of β-actin mRNA. Independent experiments were repeated three times. Data represent the means ± SD of triplicate samples. * $p < 0.05$ *vs*. the control cells.

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Table. 1

Primers and probes used for real-time RT-PCR

Target name	Primer and probe	Sequence (5'-3')
CCL2	Primer F	ATGCAGGTCCCTGTCATGC
	Primer R	CATCTTGCTGGTGAATGAGTAGC
	Probe	6FAM-TCTGGGCCTGCTGTTCACAGTTGC-TAMRA
TLR-2	Primer F	GGCTTCACTTCTCTGCTTTTCG
	Primer R	AGATCCAGAAGAGCCAAAGAGC
	Probe	6FAM-TCTGGAGCATCCGAATTGCATCACCG-TAMRA
TLR-4	Primer F	CTTCTCCTGCCTGACACCAG
	Primer R	GGACTTTGCTGAGTTTCTGATCC
	Probe	6FAM-AGCTTGAATCCCTGCATAGAGGTAGTTCCT-TAMRA
ICAM-1	Primer F	AGATCCTGGAGACGCAGAGG
	Primer R	ACTGTGGGCTTCACACTTCAC
	Probe	6FAM-ACTTGGCTCCCTTCCGAGACCTCCAG-TAMRA

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

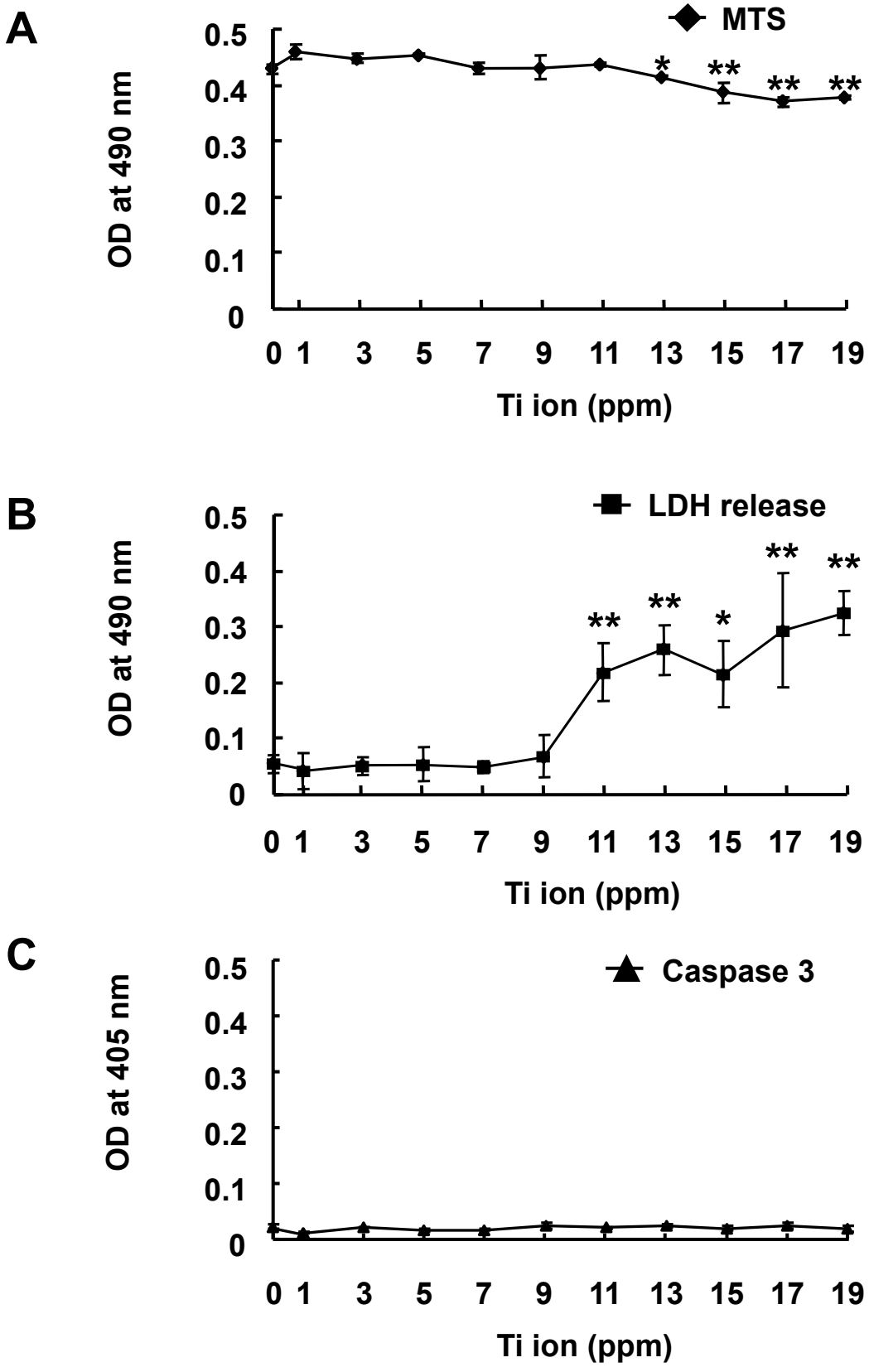

Fig 1. Makihira et al.

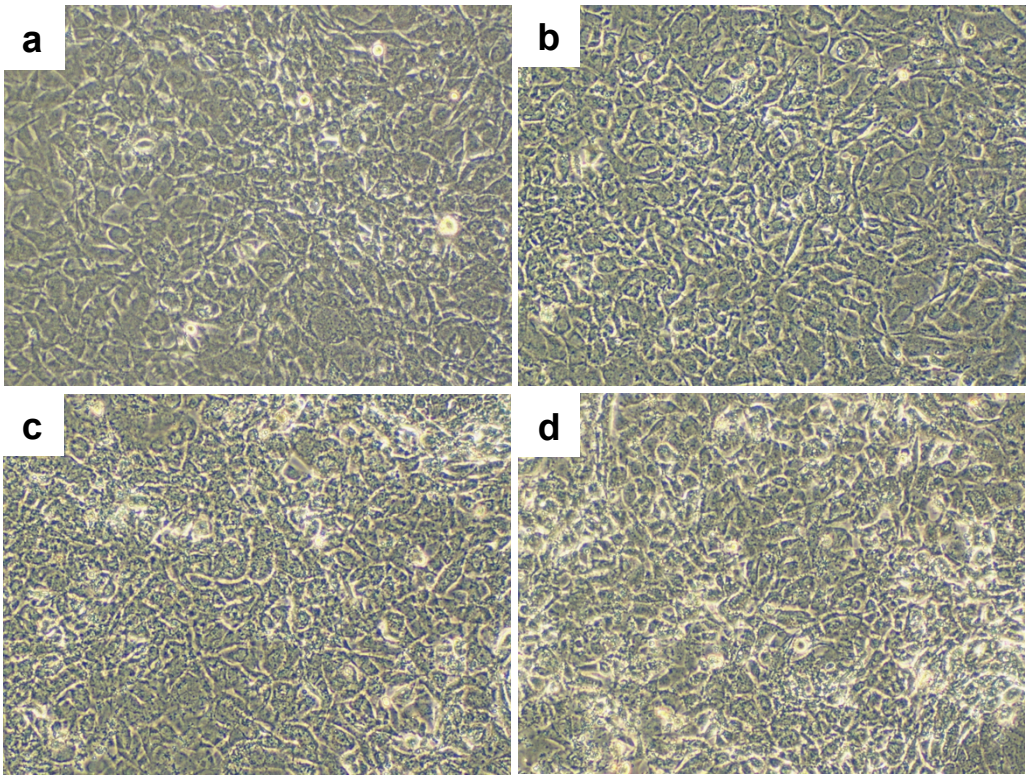

Fig 2. Makihira et al.

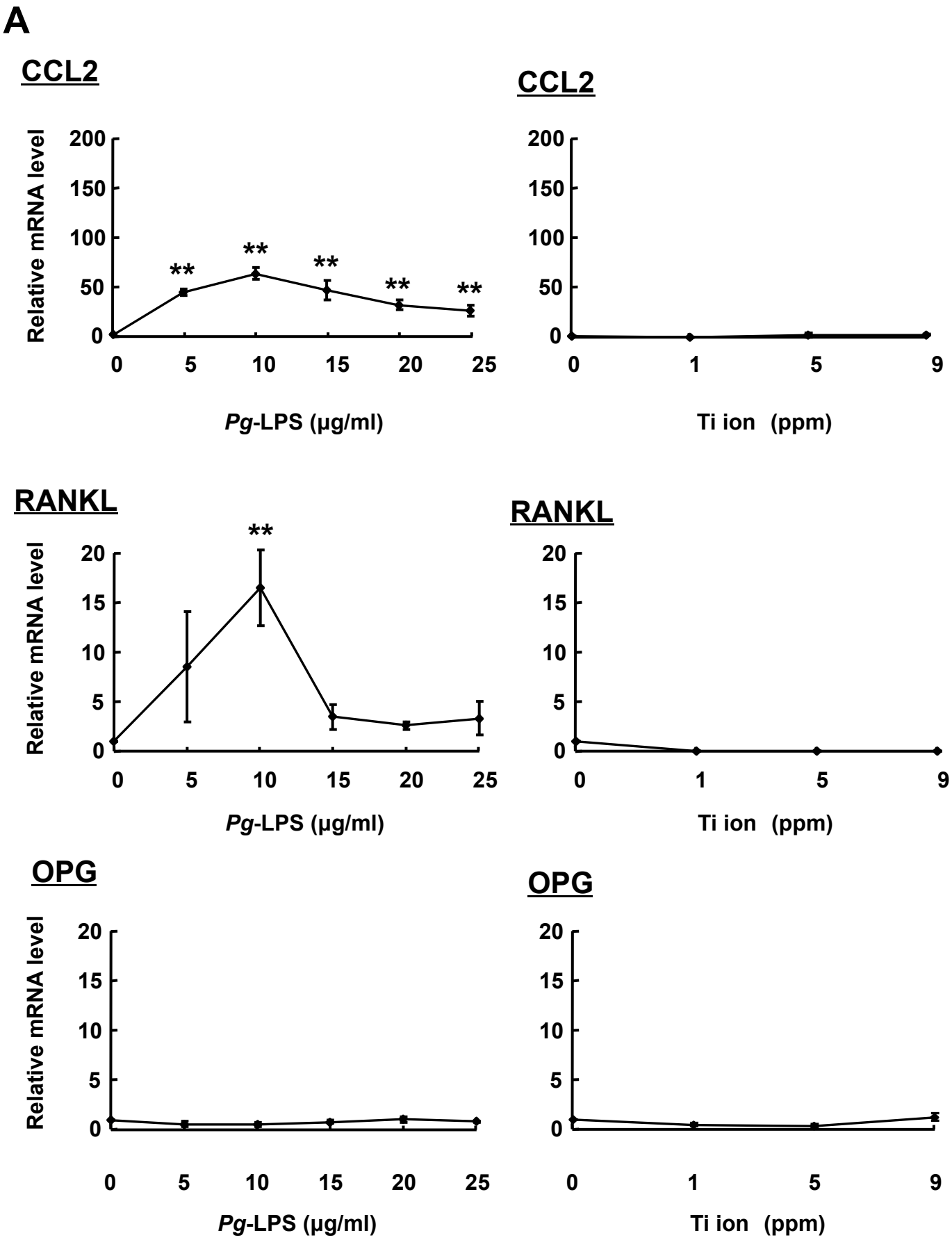

Fig 3. Makihira et al.

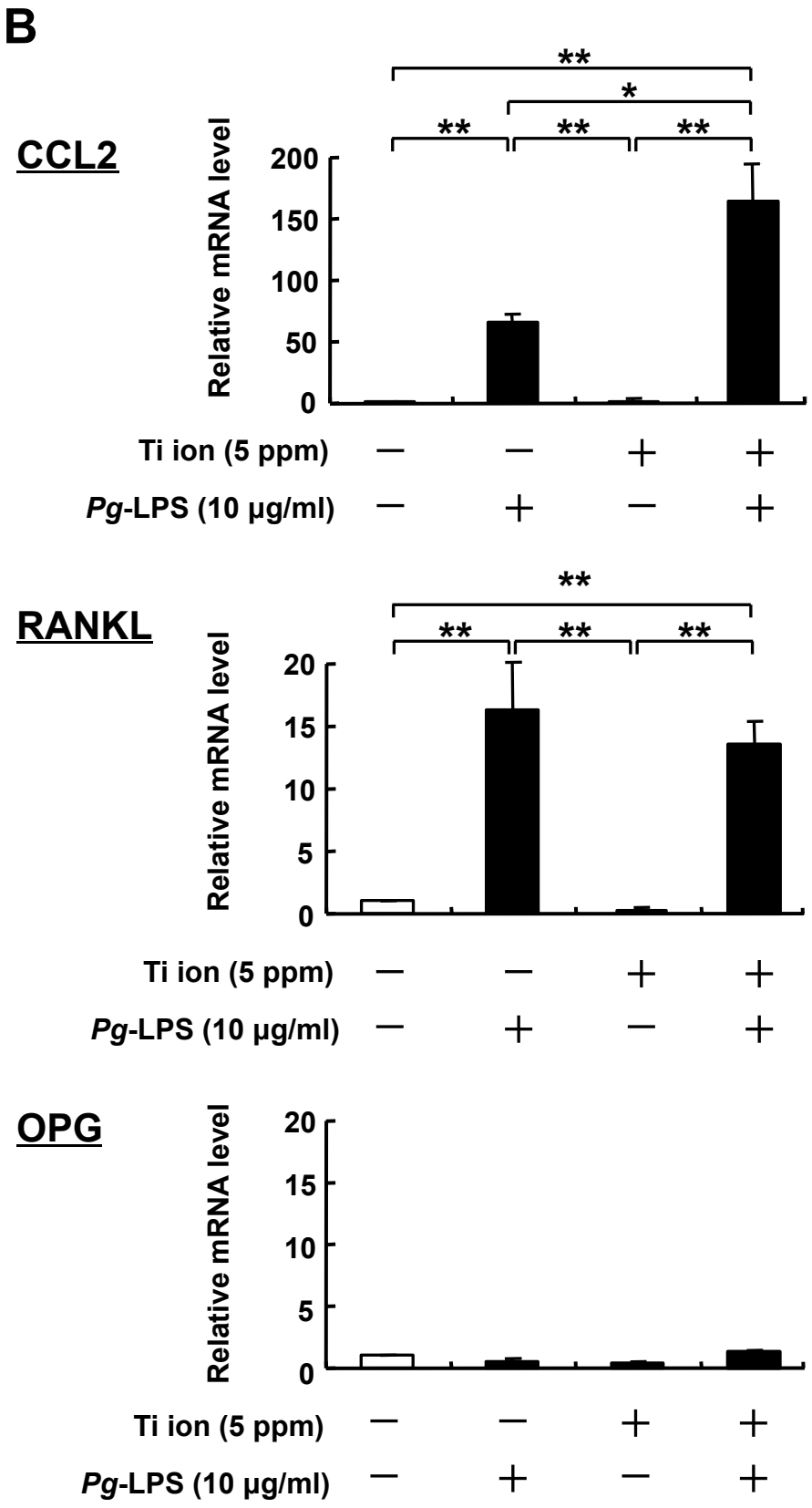

Fig 3.
Makihira et al.

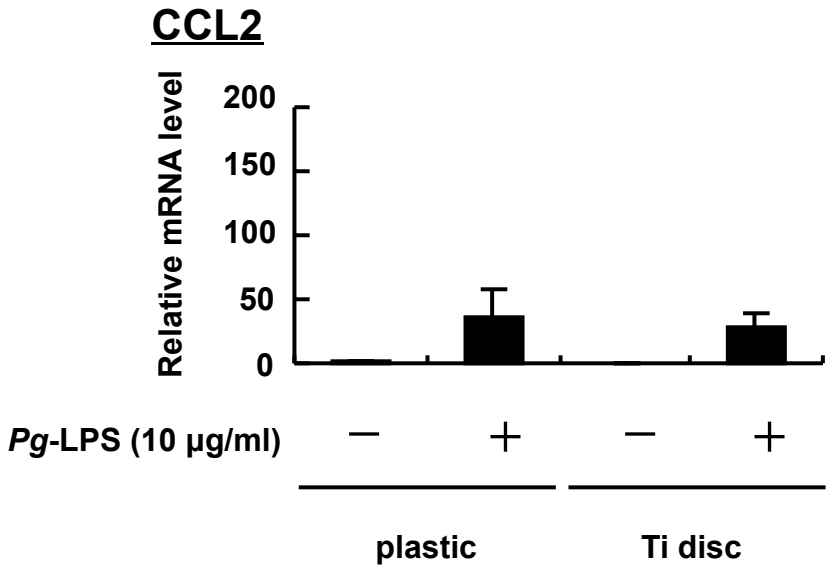

Fig 4. Makihira et al.

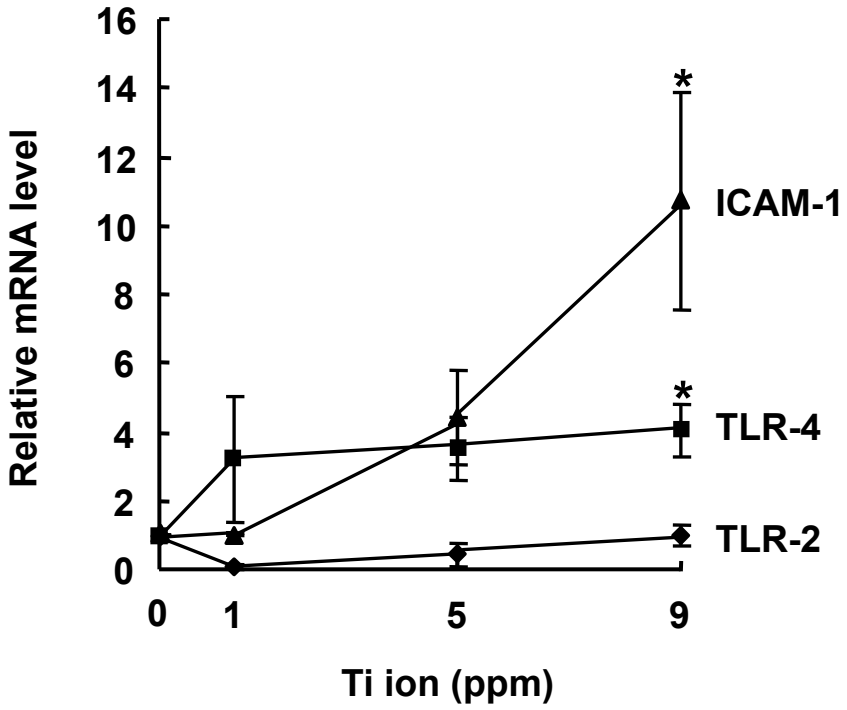

Fig 5. Makihira et al.