Titanium nanoparticles potentially affect gingival tissue through IL-13α2 receptor expression

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Abstract

Purpose: To determine the effects of titanium nanoparticles, that may have been scattered after dental implant placement, on gene and promoter expression, and gingival tissue.

Methods: Ca9-22 cell lines were used as gingival epithelial cells to assess the effects of titanium dioxide nanomaterials as titanium nanoparticles. Cells were cocultured with or without titanium dioxide nanomaterials prior to gene and promoter expression analysis. Expression of interleukin-13α2 receptor was investigated using real-time quantitative reverse-transcription polymerase chain reaction and immunofluorescence staining. Additionally, the enhanced messenger ribonucleic acid (mRNA) expression of transforming growth factor β1 was analyzed using the same method.

Results: Titanium dioxide nanomaterials affected gene and promoter expression in Ca9-22 cells: among the 160 upregulated genes, the upregulation of IL13RA2, which encodes interleukin-13α2 receptor, was the highest (8.625 log2 fold change). Immunofluorescence staining confirmed the increased expression of interleukin-13α2 receptor, which enhanced transforming growth factor β1 expression by stimulation with interleukin-13.

Conclusion: Titanium dioxide nanomaterials applied on the gingival epithelium around the dental implant may increase interleukin-13α2 receptor expression. In turn, this can enhance the secretion of transforming growth factor β1, which is known to promote the differentiation of osteoclasts involved in bone resorption, and potentially affect gingival tissue.

Keywords: dental implant, gingival epithelia, IL13RA2, titanium

Introduction

Titanium has been used for various purposes in the fields of dentistry and orthopedics because of its biocompatibility [1,2]. In terms of dental implants, titanium is considered an integral element in current dental treatment methods as it enhances functional recovery in patients [3,4]; for example, edentulous patients can potentially have recovered function on the day of dental implant placement [5]. A favorable prognosis for the immediate loading of titanium implants has also been reported [6,7]. In contrast, some studies report that peri-implantitis, for which there is no immediate loading of titanium implants has also been reported [6,7]. In dental studies, titanium has been reported as potentially toxic when taken in microgram-sized metal particles have been identified around the implant in or near macrophages, a correlation between titanium or metal content and immune response specificity is yet to be demonstrated [17,19]. Some studies have suggested that titanium nanoparticles from dental implants can affect the tissue surrounding the implant. For example, implant particles may induce “tumor necrosis factor-α” (TNF-α) and “regulated on activation normal T cell expressed and secreted/Chemokine (C-C motif) ligand 5” (RANTES/CCL5), and thereby affect the surrounding tissue [20]; however, the mechanism of action and cellular triggers are yet to be elucidated. Also, for clinical purposes, if titanium particles are associated with bone resorption around the implant, it may lead to the prevention of one of the causes of peri-implantitis.

In the present study, the effects of titanium particles on gingival epithelial cells were investigated with a specific focus on titanium dioxide (TiO2) nanomaterial-induced changes in gene and protein expression, which were assessed via gene and promoter expression analysis, real-time quantitative reverse-transcription PCR (RT-qPCR), and immunofluorescence staining. Overall, the findings shed light on the mechanism by which titanium particles affect osteoclast differentiation and bone resorption, and therefore their effect on periodontal tissue.

Materials and Methods

Cell line and cell culture

Ca9-22 cell lines were used as gingival epithelial cells and obtained from Riken BioResource Center (Tsukuba, Japan). Cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Tokyo, Japan) supplemented with 10% fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin solution (Life Technologies) at 37°C in 5% CO2. Cultured cells were used from 80% confluent cultures with trypsin/ethylenediaminetetraacetic acid (0.025%/1 mM).

Coculture with TiO2 nanomaterials and interleukin 13

Cells were plated onto 24-well plates at 1.0 × 104 cells/well in 500 μL of culture medium for 48 h. After the cells were washed three times with phosphate-buffered saline (PBS), the medium was changed to either fresh medium containing various concentrations of TiO2 nanomaterials (Ishihara Sangyo Kaisha, Ltd., Osaka, Japan) or fresh medium only (negative control); the cells were then maintained under these treatments for 1 or 6 h at 37°C in 5% CO2.

To examine the effects of interleukin (IL)-13, cells were treated with or without TiO2 nanomaterials (as above) for 24 h. After three washes with PBS, the media in each treatment was changed to fresh medium with 20 ng/mL of IL-13; the cells were then incubated for 6 h at 37°C in 5% CO2.
Ribonucleic acid (RNA) extraction and complementary deoxyribonucleic acid (cDNA) synthesis

Total RNA was extracted from cells cocultured with TiO₂ nanomaterials or those incubated with IL-13 using the RNeasy Mini Kit (Qiagen, Copenhagen, Denmark) according to the manufacturer’s instructions. Subsequently, cDNA was synthesized using the PrimeScript RT Master Mix (Takara, Kusatsu, Japan) and used as template samples for real-time RT-qPCR.

Gene and promoter expression analysis

Gene and promoter expression analysis was conducted using previously described methods [21]. Cap analysis of gene expression (CAGE) library preparation, sequencing, mapping, and gene expression were conducted for each RNA sample (K.K.DNAFORM, Yokohama, Japan). Briefly, RNA quality was assessed using a Bioanalyzer (Agilent, Santa Clara, CA, USA) to ensure that RNA integrity was >7.0 and the A260/280 and 260/230 ratios were >1.7. First-strand cDNAs were transcribed to the 5′ ends of capped RNAs. The CAGE-tag 5′ coordinates were input for CAGEr clustering (v0.5.9) after discarding ribosomal or non-A/C/G/T base-containing RNAs and attached to CAGE “bar code” tags; the sequenced CAGE tags were then mapped to the mouse vM24 genome using BWA software (v0.5.9) and used as template samples for real-time RT-qPCR.

Table: CAGEr clustering

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<tr>
<th>Gene</th>
<th>Base mean</th>
<th>Log fold change</th>
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<td>8.62507066</td>
</tr>
<tr>
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Fig. 1 Gene and promoter expression induced by titanium dioxide (TiO₂) nanomaterials. Top 10 upregulated genes in Ca9-22 cells cocultured with TiO₂ nanomaterials (A). A Bokeh scatter plot of gene expression (B). X-axis showing the log fold change of gene expression in the control and the Y-axis showing the log, fold change of gene expression in cells cocultured with TiO₂ nanomaterials. CPM, count per million; FC, fold change

Statistical analysis

Data are presented as means and standard deviations. Sample numbers were decided by using G*Power application (Ver. 3.1.9.4). Significant differences were determined via parametric methods using Student’s t-tests, and a P value of <0.05 was considered statistically significant. Normality was confirmed by Kolmogorov-Smirnov test for all sample groups before the parametric test. Each sample group was compared with the control group only.

Results

Enhancement of gene and promoter expression by TiO₂ nanomaterials

The gene and promoter expression analysis revealed that 160 genes were upregulated in the cells after coculturing for 1 h with 10 µg/mL of TiO₂ nanomaterials. The top 10 upregulated genes (Fig. 1A) and a Bokeh scatter plot are shown (Fig. 1B). The two genes that were upregulated to the greatest extent were IL13RA2 and pyruvate kinase M1/2 pseudogene 1 (PKMP1) with 8.625 and 8.5677 log₂ fold changes, respectively.

Effects of IL13RA2 mRNA expression by TiO₂ nanomaterials

In cells cocultured with TiO₂ nanomaterials, IL13RA2 mRNA expression increased in a dose-dependent manner. Although there was no effect of coculturing with 1 µg/mL of TiO₂ nanomaterials, the cells cocultured with 10 and 100 µg/mL of TiO₂ nanomaterials showed a significant increase in mRNA expression by 38% and 42%, respectively (Fig. 2). Kolmogorov-Smirnov test results showed normality, the results (P value) were 0.8746 (for 0 µg/mL), 0.9158 (for 1 µg/mL), 0.4839 (for 10 µg/mL) and 0.8287 (for 100 µg/mL), respectively.

IL-13α2 receptor expression identified by immunofluorescence staining

In cells cocultured with TiO₂ nanomaterials and subjected to immunofluorescence staining, the cell morphology was not prominently altered compared with that of control cells (Fig. 3A, E), and there was no
observable difference according to nuclear counterstaining (Fig. 3C, G). However, IL-13α2 receptor was observed and its expression was increased in cells cocultured with TiO₂ nanoparticles compared with in control cells (Fig. 3B, D, F, H).

Increased TGFβ1 mRNA expression level mediated by IL-13α2 receptor
After coculturing cells with 10 µg/mL of TiO₂ nanoparticles, which affected IL-13α2 receptor expression, and stimulating with IL-13 for 6 h, TGFβ1 mRNA expression levels in these cocultured cells increased significantly by around 50% compared to the cells not treated with TiO₂ nanoparticles (Fig. 4). Kolmogorov-Smirnov test results showed normality, the results (P value) were 0.8287 (for IL-13 only) and 0.8733 (for TiO₂ nanoparticles and IL-13), respectively.

Discussion
In the present study, the effects of TiO₂ nanomaterials on gingival epithelial cells were investigated using gene and promotor expression analysis, immunofluorescence staining, and RT-qPCR. Among the 160 genes upregulated in Ca9-22 cells treated with TiO₂ nanomaterials, the two that were most overexpressed, IL13RA2 and PKMP1, showed similar changes at 8.625 and 8.5677 log₂ fold change, respectively. However, PKMP1 is a pseudogene; it may have encoded a gene product in the past, but it has now lost its function [25]. Therefore, IL13RA2 was focused in this study.

According to RT-qPCR analysis, IL13RA2 mRNA expression in Ca9-22 cells increased following 6 h of coculture with TiO₂ nanomaterials. In contrast, RT-qPCR analysis revealed no change in IL13RA2 expression following 1 h of coculture with TiO₂ nanomaterials (data not shown). This is likely because gene and promoter expression analysis targets RNAs that bind near the promotor region on the gene map; thus, there may have been a time lag of >1 h from the actual mRNA expression. According to immunofluorescence staining analysis, the expression of IL-13α2 receptor, an IL-13 receptor, increased after TiO₂ nanomaterial treatment. IL-13α2 receptor is thought to be a decoy receptor for IL-13 [26,27]. However, it has also been recently suggested that IL-13α2 receptor is involved in signal transduction for the promotion of TGFβ1 expression [28]. In the current study, it was found that coculturing Ca9-22 cells with TiO₂ nanomaterials and stimulation with IL-13 increased the expression of TGFβ1 mRNA in cells, suggesting that the TiO₂ nanomaterials induced IL-13α2 receptor expression and function. The production of IL-13 in several types of immune cells, such as T [29], natural killer [30], mast [31], and dendritic cells has also been previously reported [32]; because these cells are also present around titanium implants, nearby gingival epithelial cells may also be exposed to IL-13. Additionally, an increase in TGFβ1 by IL-13 signaling via IL-13α2 receptor can also trigger fibrosis [28]. Furthermore, TGFβ1 signaling has been associated with osteoclast differentiation [33]. Accelerated osteoclast differentiation can upset the balance of bone remodeling and promote bone resorption, which is associated with inflammatory conditions, such as peri-implantitis [34]. Therefore, the presence of TiO₂ nanomaterials could accelerate the progress of bone resorption; this should be further investigated in future research.

The in vitro nature of this study is a limitation; it is unknown whether the same effects would be observed in vivo. It is also possible that the TGFβ1 produced affects other cells and signaling pathways. Further studies will be required to investigate both possibilities.

TiO₂ nanomaterials affected gene and promoter expression in Ca9-22 cells. In particular, IL13RA2, which encodes IL-13α2 receptor, was strongly upregulated. This increase in IL-13α2 receptor expression seemed to enhance TGFβ1 expression in Ca9-22 cells through stimulation with IL-13. Overall, the findings in this study indicate that periodontal tissue is likely to be negatively affected by titanium particles scattered from dental implants.

![Image](image-url)
Acknowledgments
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Conflict of interest
The authors declare no conflicts of interest.

References