Toxicology 327 (2015) 1–9

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Release of titanium ions from an implant surface and their effect on cytokine production related to alveolar bone resorption

Takanori Wachi, Takahiro Shuto, Yoshinori Shinohara, Yoshinari Matono, Seicho Makihira *

Section of Fixed Prosthodontics, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

ARTICLE INFO

Article history:
Received 29 September 2014
Received in revised form 30 October 2014
Accepted 30 October 2014
Available online 3 November 2014

Keywords:
Titanium ion
RANKL
OPG
Peri-implantitis

ABSTRACT

Although interest in peri-implant mucositis and peri-implantitis has recently been increasing, the mechanisms driving these diseases remain unknown. Here, the effects of titanium ions on the inflammation and bone resorption around an implant were investigated. First, the accumulated amount of Ti ions released into gingival and bone tissues from an implant exposed to sodium fluoride solution was measured using inductively coupled plasma mass spectrometry. Next, the cellular responses in gingival and bone tissues to Ti ions and/or *Porphyromonas gingivalis*-lipopolysaccharide (*P. gingivalis*-LPS) were assessed using a rat model. More Ti ions were detected in the gingival tissues around an implant after treatment with sodium fluoride (pH 4.2) than in its absence, which suggests that the fluoride corroded the implant surface under salivary buffering capacity. The injection of Ti ions (9 ppm) significantly increased the mRNA expression and protein accumulation of chemokine (C–C motif) ligand 2, as well as the ratio of receptor activator of nuclear factor-κB ligand to osteoprotegerin, in rat gingival tissues exposed to *P. gingivalis*-LPS in a synergistic manner. In addition, the enhanced localization of toll-like receptor 4, which is an LPS receptor, was observed in gingival epithelium loaded with Ti ions (9 ppm). These data suggest that Ti ions may be partly responsible for the infiltration of monocytes and osteoclast differentiation by increasing the sensitivity of gingival epithelial cells to microorganisms in the oral cavity. Therefore, Ti ions may be involved in the deteriorating effects of peri-implant mucositis, which can develop into peri-implantitis accompanied by alveolar bone resorption.

1. Introduction

Peri-implant mucositis is characterized by inflamed gingival tissues. Peri-implantitis is often followed by peri-implant mucositis, and alveolar bone resorption is seen in severe cases of peri-implantitis (Mombelli, 1998; Mombelli and Lang, 1998; Lang et al., 2000). The pathological effects of peri-implant mucositis and peri-implantitis may be very different than those of gingivitis and periodontitis because there is no periodontal ligament surrounding a dental implant and the implant is not a tooth, but is mainly composed of titanium (Ti) (Buser et al., 1992; Berglundh et al., 1994). Ti is used extensively in numerous medical applications, including dental implants, 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, as well as in synovial fluid (Dorr et al., 1990). Ti particles have also been reported to facilitate osteoclast differentiation (Bi et al., 2001). Moreover, clinical studies have shown that Ti particles released from orthopedic implants accumulate in tissues, and this release may be caused by aseptic loosening of orthopedic implants (Jacobs et al., 1998; Gallo et al., 2002). The corrosion resistance of Ti is decreased under low dissolved-oxygen conditions, such as in the oral cavity, and particularly in the presence of small amounts of fluoride (Nakagawa et al., 2002; Matono et al., 2006). Together, these findings suggest that Ti ions released from Ti surfaces may play a pivotal role in peri-implant mucositis and peri-implantitis at the interface between the gingiva, bone, and dental implants (Makihira et al., 2010). However, the amount of clinical corrosion around dental implants exposed to fluoride at low pH under salivary buffering capacity and the extent of released Ti accumulation in the tissues around an implant remain unclear.

Abbreviations: RANKL, receptor activator of nuclear factor-κB ligand; OPG, osteoprotegerin; *P. gingivalis*-LPS, *Porphyromonas gingivalis*-lipopolysaccharide.

* Corresponding author at: Section of Fixed Prosthodontics, Division of Oral Rehabilitation, Faculty of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Tel.: +81 92 642 6373; fax: +81 92 642 6374.
E-mail address: makihira@dent.kyushu-u.ac.jp (S. Makihira).

http://dx.doi.org/10.1016/j.tox.2014.10.016
0300-483X/

Recently, we showed that Ti ions exert biological effects on the expression of receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) in osteoblastic cells *in vitro*. The RANKL-RANK pathway and OPG, a decoy receptor for RANKL, are essential for osteoclast differentiation and development because they contribute to pathologic bone resorption (Koide et al., 2003). Soluble RANKL (sRANKL) has been detected in the crevicular fluid of patients with peri-implantitis (Monov et al., 2006). Moreover, Ti demonstrated a synergistic enhancement of chemokine (C–C motif) ligand 2 (CCL2) mRNA expression in a gingival epithelial-like cell line, GE-1, when loaded with *Porphyromonas gingivalis*-lipopolysaccharide (*P. gingivalis*-LPS), a bacterium frequently recovered from peri-implantitis (Quirynen et al., 2002). CCL2 is a chemokine that activates the infiltration of monocytes (Shynlova et al., 2008). These data also suggest that Ti ions may be involved in the inflammation and bone resorption observed in cases of peri-implant mucositis and peri-implantitis. Therefore, Ti ions, as well as other external stimuli such as microorganisms or their LPS production, likely alter the physiological function of cells in the gingiva and bone.

Thus, in this study, we first created Ti ion injection and pure titanium screw-type mini-implant models in rats in order to determine their *in vivo* effects. Next, we assessed the accumulation of Ti released from a mini-implant exposed to sodium fluoride solution using inductively coupled plasma mass spectrometry (ICP-MS), and the effects of Ti ions on the expression of cytokines and receptors for microbes related to inflammation and bone resorption with or without *P. gingivalis*-LPS using real-time reverse-transcription polymerase chain reaction (RT-PCR), enzyme-linked immunoabsorbent assay (ELISA), and immunofluorescence analysis.

2. Material and methods

2.1. Preparation of Ti ions and Porphyromonas gingivalis-lipopolysaccharide

A Ti standard solution of $(NH_4)_2TiF_6$ for use during ICP spectroscopy was purchased from Merck (Darmstadt, Germany). The Ti-ICP standard solution was diluted with cell culture medium, under pH monitoring, as previously described (Taira et al., 2006). Neither a significant change in the medium pH nor any visual precipitation accompanying the supplementation with Ti ions was observed. A solution of NH_4PF_6 was purchased (Wako, Osaka, Japan) and used as the Ti-free solution. A solution of sodium fluoride at 1000 ppmF and pH 4.2 was made using previously described methods (Matono et al., 2006). *P. gingivalis*-LPS was purchased from InvivoGen (San Diego, CA, USA), diluted with sterile endotoxin-free water, and stored at −20 °C until use.

2.2. Culture of GE-1 and MC3T3-E1 cells

The MC3T3-E1 cell line was purchased from the European Collection of Cell Cultures (ECACC, Wiltshire, UK). MC3T3-E1 cells were cultured in alpha-MEM supplemented with an antibiotic mixture (Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum (FBS; Biological Industries, Haemek, Israel), and 50 mg/mL L-ascorbic acid (Sigma, St. Louis, MO, USA). The GE-1 cell line was obtained from the Riken Bioresource Center Cell Bank (Tsukuba, Japan) (Hatakeyama et al., 2001). GE-1 cells were cultured and maintained in SFM-101 (Nissui, Tokyo, Japan) containing 1% FBS and 10 ng/mL epidermal growth factor (Sigma). MC3T3-E1 cells were maintained at 37 °C under 5% CO_2/95% air for each experiment. GE-1 cells were maintained at 33 °C under the same gas conditions.

2.3. Injection of Ti with Porphyromona gingivalis-LPS and implantation of the mini-implant into the palatal process

For this study, 8-week-old Wistar rats (Kyudo, Tosu, Japan) were used. Animal selection, management, anesthesia, surgical procedures, and analyses were approved by the animal care and use committee of Kyushu University (No. A25-138, Fukuoka, Japan). The *in vivo* experiments using the rat model were performed in accordance with the procedures allowed by the committee. Rats were anesthetized with isoflurane and pentobarbital, and then 100 μL of *P. gingivalis*-LPS (1 mg/mL) was intermittently injected into the gingival tissues of the palatine around the mini-implant. Nine hours or seven days after the *P. gingivalis*-LPS injection, areas of the gingival tissues and bones in a 2 mm radius around the center of the injection point were carefully isolated for analysis of the cellular responses to *P. gingivalis*-LPS with or without Ti ions. In another experiment, the mini-implants were placed into the palatine process of the maxilla in rats (Atsuta et al., 2005). After seven days, the accessible surface area of each mini-implant in the oral cavity was exposed to NaF solution adjusted to pH 4.2 or PBS controls at physiological pH. After a 30 min-exposure and three washes with pure water to remove any Ti on the surfaces of the gingiva, the mini-implant was gently removed from the palatine. Then, the areas of the gingival tissues and bones in a 3 mm radius from the center of the mini-implant were isolated for ICP-MS.

2.4. Scanning electron microscopy and inductively coupled plasma mass spectrometry

The surfaces of the Ti specimens treated with or without fluoride solution were observed under a scanning electron microscope (SEM) using standard procedures, and images of these surfaces were taken. The ICP-MS data reported here were obtained from the Toray Research Center (Shiga, Japan).

2.5. Preparation of conditioned medium, cell extraction from cultured cells, and gingival and bone tissue homogenization

Cultured cells and tissues were homogenized with RIPA buffer (Thermo Fisher Scientific Inc., Rockford, IL, USA) in order to collect the conditioned medium (CM) and cell extracts (CE) from the cultured cells, and to create tissue-homogenates from the gingival and bone tissues. CE from the cultured cells and the tissue homogenates from gingival and bone tissues were centrifuged at 14 000 rpm for 15 min (Eppendorf, Centrifuge 5424 R, Tokyo, Japan) to remove the cell- or tissue-dissolved fraction with the buffer. The supernatants of the CM, CE, and the tissue-homogenates were carefully collected for further analysis using real-time quantitative RT-PCR or ELISA.

2.6. Real-time quantitative reverse transcriptase polymerase chain reaction analysis and enzyme-linked immunosorbent assay

Total RNA was extracted using TRIzol from the homogenates. First-strand cDNA was synthesized using ReverTra Ace (Toyobo, Osaka, Japan) starting with 100 ng of total RNA. The cDNA was then amplified by BIOTAQ DNA polymerase (Bioline, Randolph, MA, USA). Real-time quantitative RT-PCR analysis for 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. The sequences of primers and probes used are listed below. Forward primer: TGTCCCAAAGAAGCTGTAGTATTTG; reverse primer: GACTG-TAGTTTCTGATCTCACTTGG; and probe: 6CTCAAGAGAGA-GATCTGTGCTGACCCCA-TAMRA for CCl2. Forward primer: