Biomaterials 57 (2015) 33–40

Contents lists available at ScienceDirect

Biomaterials

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

Alteration of metabolomic profiles by titanium dioxide nanoparticles in human gingivitis model

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ARTICLE INFO

Article history:
Received 12 November 2014
Received in revised form
29 March 2015
Accepted 29 March 2015
Available online 22 April 2015

Keywords:
Titanium dioxide nanoparticle
Inerleukin-1β
Inflammation
Human gingival fibroblast
Metabolomics

ABSTRACT

Although nanoparticles (NPs) has afforded considerable benefits in various fields of sciences, several reports have shown their harmful effects, suggesting the necessity of adequate risk assessment. To clarify the mechanism of titanium dioxide nanoparticles (TiO_2 NPs)-enhanced gingival inflammation, we conducted the full-scale metabolomic analyses of human gingival fibroblast cells treated with IL-1β alone or in combination with TiO_2 NPs. Observation with transmission electron microscope demonstrated the incorporation of TiO_2 NPs into vacuoles of the cells. TiO_2 NPs significantly enhanced the IL-1β-induced prostaglandin E_2 production and COX-1 and COX-2 protein expression. IL-1β reduced the intracellular concentrations of overall primary metabolites especially those of amino acid, urea cycle, polyamine, *S*-adenosylmethione and glutathione synthetic pathways. The addition of TiO_2 NPs further augmented these IL-1β-induced metabolic changes, recommending careful use of dental materials containing TiO_2 NPs towards patients with gingivitis or periodontitis. The impact of the present study is to identify the molecular targets of TiO_2 NPs for the future establishment of new metabolic markers and therapeutic strategy of gingival inflammation.

1. Introduction

Engineered nanoparticles (NP: defined as being less than 100 nm in diameter) have provided considerable benefits in the fields of pharmaceuticals [1], diagnosis and therapeutics [2,3], food and cosmetics [4], and dentistry [5]. On the other hand, several reports on their genotoxic [6] and pro-inflammatory potentials [7,8] suggest the necessity of nation-wide regulation for the safe use of NPs.

In dental science, the incorporation of NPs into the glass ionomer cements (GICs) is known to improve their mechanical and antibacterial properties of cements [5]. However, there is a possibility that TiO_2 NPs, when settled in the deep cavities during operation, near to dental pulp chamber, may induce pulpitis and aggravate the pulpar inflammation. Considering that the effects of TiO_2 NPs on the oral environment have not been well investigated, we initiated a series of experiments that cover this topic. We reported that TiO_2 NPs did not induce cytotoxicity, nor affected the sensitivity of a human oral squamous cell line (HSC-2) to five chemotherapeutic drugs (doxorubicin, melphalan, fluorouracil, docetaxel, gefitinib), even though it was incorporated into vacuoles [9], whereas non-cytotoxic concentration of TiO_2 NPs stimulated the production of prostaglandin E_2 (PGE_2) by human gingival fibroblast (HGF) in synergy with IL-1β [10], suggesting the pro-

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http://dx.doi.org/10.1016/j.biomaterials.2015.03.059
0142-9612/

inflammatory action of TiO_2 NPs. However, the mechanism of pro-inflammatory action of TiO_2 NPs is still unclear.

To clarify the mechanism by which TiO_2 NPs stimulate the IL-1β-stimulated PGE_2 production in HGF cells, we investigated the cyclooxygenase (COX)-1 and COX-2 protein expression. We also conducted metabolomics analysis of the intracellular metabolites to identify which parts of cellular metabolic pathways are most profoundly affected by treatment with IL-1β alone or in combination with TiO_2 NPs.

2. Materials and methods

2.1. Materials

The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Grand Island, NY); fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS); TiO_2 nanoparticles (product number: 637254, nanopowder, anatase phase, particle size 18 nm, surface area 50 m^2/g, purity 99.7% based on trace metals analysis), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma–Aldrich Co. LLC., St. Louis, MO), IL-1β (R&D Systemes Mineeapolis, MN, USA); PGE_2 enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical Co., Ann Arbor, MI); Culture plastic dishes and plates (6-well, 96-well) (Becton Dickinson, Franklin Lakes, NJ).

2.2. Cell culture

HGF cells were established from the first premolar extracted tooth in the lower jaw and periodontal tissues of twelve years old girl, according to the guideline of Institutional Board of Meikai University Ethic Committee after obtaining the informed consent from the patients. These cells were cut into small piece by surgical blade, and placed onto 80-mm plastic dish to allow the outgrowth during 2 weeks in DMEM supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate at 37 °C under a humidified 5% CO_2 atmosphere. The outgrown cells were used as primary culture with population doubling level (PDL) zero. Cells were then harvested by treatment with 0.25% trypsin-0.025% EDTA-2Na in phosphate-buffered saline without calcium and magnesium [PBS(−)]. The subculture of HGF cells were done every week by 1:4 split ratio with a medium change between the subcultures. HGF had an *in vitro* life span (cumulative cell population doubling number) of 47 [11]. HGF cells at 10~15 PDL were used in the present study.

2.3. Determination of viable cell number

Cells were trypsinized and inoculated at 1:3 split ratio in 96-microwell plates and incubated for 48 h to allow complete attachment. Near-confluent cells were pre-treated for 30 min with different concentrations of TiO_2 NPs (0, 0.8, and 3.2 mM) in fresh culture medium, added 0 or 3 ng/ml IL-1β and then incubated for a further 24 h. The TiO_2 NPs were dissolved in distilled water, vortexed and suspended by sonication with a bath-type sonicator (Tokyo Cho-Onpa Giken Co. Tokyo, Japan) for 1 min at room temperature before application. After treatment, the viable cell number was determined by MTT method. Briefly, cells were incubated for 4 h with 0.2 mg/ml MTT in fresh culture medium. The formazan formed was dissolved with 0.1 ml of dimethyl sulfoxide (DMSO), and the absorbance at 540 nm of the lysate was determined by using a microplate reader (Multiskan, Biochromatic, Labsystem, Osaka, Japan) [9]. Viable cell number was also determined by direct cell counting with hemocytometer after detaching the cells and staining with trypan blue.

2.4. Determination of intracellular uptake of TiO_2 NPs

HGF cells were treated without or with 0.8 mM of TiO_2 NPs for 3 h. The cells were washed three times with cold PBS(−) and fixed for 1 h with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4 °C, scraped with a rubber policemen, dehydrated and then embedded in Araldite M (Ciba–Geigy Swiss; NISSHIN EN Co., Ltd., Tokyo Japan). Thin sections were stained with uranyl acetate and lead citrate, and were then observed under a JEM-1210 transmission electron microscope (JEOL) at an accelerating voltage of 80 kV [9,10].

2.5. Determination of PGE_2 production

Near-confluent cells were pre-treated for 30 min with different concentrations of TiO_2 NPs (0, 0.8, and 3.2 mM) in fresh culture medium, added 0 or 3 ng/ml IL-1β and then incubated for a further 24 h. The culture supernatants were then collected, and the concentration of PGE_2 released into the culture medium was determined by ELISA, according to the manufacturer's instruction [10].

2.6. Detection of COX-1 and COX-2 protein expression in HGF

The expression of COX-1 and COX-2 protein was investigated by Western blot analysis. Briefly, the HGF cells were seeded in 6-well dishes (Becton–Dickinson Labware, Franklin Lakes, NJ) and incubated for 48 h. The medium was replaced with fresh medium and the cells were incubated for 30 min at 37 °C in a 5% CO_2 incubator to stabilize the pH and temperature of the culture medium. The cells were pre-treated with 0 (control), 0.8 and 3.2 mM of TiO_2 NPs for 30 min, added 0 or 3 ng/ml IL-1β and then incubated for a further 24 h. The cells were collected and suspended in ice-cold 1 × RIPA buffer solution (Cell Signaling Technology, Beverly, MA) plus protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN). Protein concentrations in the lysates were determined, and equal amounts of protein for each sample were subjected to 8% SDS-polyacrylamide gel electrophoresis. After transferring the proteins onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Richmond, CA), the membranes were blocked with 5% skim milk and then reacted for 1 h with 1:1000 dilution of COX-1 (D2G6, rabbit mAb monoclonal, Cell Signaling technologies, Beverly, MA), 1:1000 dilution of COX-2 (Mouse polyclonal, Ann Arbor, MI) antibody or 1:10,000 of β-actin (Sigma–Aldrich, St. Louis, MO). After washing, the membrane was stained with HRP-conjugated goat anti-rabbit IgG or anti-mouse IgG (1:10,000 dilution) (both from Cell Signaling technologies). The blots and images were developed with ChemiDoc™ MP imaging system (Bio-Rad Laboratories, Hercules, CA).

2.7. Processing for metabolomic analysis

HGF cells were seeded 10-cm dishes (Becton–Dickinson) and incubated for 48 h. The medium was replaced with fresh culture medium and the cells were incubated for 30 min at 37 °C in a 5% CO_2 incubator to stabilize the pH and temperature of the culture medium. The cells were pre-treated for 30 min with 0 (control), 0.2, 0.8 and 3.2 mM of TiO_2 NPs, added 0 or 3 ng/ml IL-1β and then incubated for a further 24 h. Aliquots of the cells were trypsinized for counting the viable cell number with hemocytometer after staining with trypan blue. The remaining cells were washed twice with 5 ml of ice-cold 5% D-mannitol and then immersed for 10 min in 1 ml of methanol containing internal standards (25 μM each of methionine sulfone, 2-[*N*-morpholino]-ethanesulfonic acid and D-camphor-10-sulfonic acid). The methanol extract (supernatant) was collected. To 400 μl of the dissolved samples, 400 μl of chloroform and 200 μl of Milli-Q water were added and the mixture was centrifuged at 10,000 × *g*, for 3 min at 4 °C. The aqueous layer was filtered to remove large molecules by centrifugation through a 5-kDa cut-off filter (Millipore, Billerica, MA) at 9,100 × *g* for 2.5 h at 4 °C. The 320 μl of the filtrate was concentrated by centrifugation and dissolved in 50 μl of Milli-Q water containing reference compounds (200 μM each of 3-aminopyrrolidine and trimesate) immediately before capillary electrophoresis-time-of-flight-mass spectrometry (CE-TOF-MS) analysis.

2.8. Instrument parameters for metabolomic analysis

The instrumentation and measurement conditions used for CE-TOF-MS were described elsewhere [12,13] with slight modification. Cation analysis was performed using an Agilent CE capillary electrophoresis system, an Agilent G1969A LC/MSD TOF system, an Agilent 1100 series isocratic HPLC pump, a G1603A Agilent CE-MS adapter kit, and a G1607A Agilent CE-ESI-MS sprayer kit (Agilent Technologies, Waldbronn, Germany). Anion analysis was performed using an Agilent CE capillary electrophoresis system, an Agilent G6220A LC/MSD TOF system, an Agilent 1200 series isocratic HPLC pump, a G1603A Agilent CE-MS adapter kit, and a G7100A Agilent CE-electrospray ionization (ESI) source-MS sprayer kit (Agilent Technologies). For the cation and anion analyses, the CE-MS adapter kit included a capillary cassette that facilitates thermostatic control of the capillary. The CE-ESI-MS sprayer kit simplifies coupling of the CE system with the MS system and is equipped with an electrospray source. For system control and data acquisition, 3D-CE ChemStation software (Rev. B.04.01.SP1) for CE and Agilent MassHunter software for TOF-MS (B.02.00, B1128.5) was used. The original Agilent SST316Ti stainless steel ESI needle was replaced with a passivated SST316Ti stainless steel and platinum needle (passivated with 1% formic acid and a 20% aqueous solution of isopropanol at 80 °C for 30 min) for anion analysis.

For cationic metabolite analysis using CE-TOFMS, sample separation was performed in fused silica capillaries (50 μm i.d. × 105 cm total length) filled with 1 M formic acid as the reference electrolyte. The capillary was flushed with formic acid (1 M) for 20 min before the first use and for 4 min before each sample injection. Sample solutions (approximately 3 nl) were injected at 50 mbar for 5 s and a voltage of 30 kV was applied. The capillary temperature was maintained at 20 °C and the temperature of the sample tray was kept below 5 °C. The sheath liquid, composed of methanol/water (50% v/v) and 0.1 μM hexakis(2,2-difluoroethoxy) phosphazene (Hexakis), was delivered at 10 μl/min. ESI-TOF-MS was conducted in the positive ion mode. The capillary voltage was set at 4 kV and the flow rate of nitrogen gas (heater temperature = 300 °C) was set at 7 psig. In TOF-MS, the fragmentor, skimmer and OCT RF voltages were 75, 50 and 125 V, respectively. Automatic recalibration of each acquired spectrum was performed using reference standards ([^{13}C isotopic ion of protonated methanol dimer $(2MeOH + H)]^+$, m/z 66.0631) and ([protonated Hexakis $(M + H)]^+$, m/z 622.0290). Mass spectra were acquired at a rate of 1.5 cycles/s over a *m/z* range of 50–1000.

For anionic metabolite analysis using CE-TOF-MS, a commercially available COSMO(+) capillary (50 μm × 106.5 cm, Nacalai Tesque, Kyoto, Japan), chemically coated with a cationic polymer, was used for separation. Ammonium acetate solution (50 mM; pH 8.5) was used as the electrolyte for separation. Before the first use, the new capillary was flushed successively with the running electrolyte (pH 8.5), 50 mM acetic acid (pH 3.4), and then the electrolyte again for 10 min each. Before each injection, the capillary was equilibrated for 2 min by flushing with the acetic