ELSEVIER

Nanomedicine: Nanotechnology, Biology, and Medicine
xx (2014) xxx – xxx

nanomedicine
Nanotechnology, Biology, and Medicine

nanomedjournal.com

Anti-angiogenic effect of bare titanium dioxide nanoparticles on pathologic neovascularization without unbearable toxicity

Dong Hyun Jo, MD[a,b], Jin Hyoung Kim, PhD[a], Jin Gyeong Son, MS[c,d], Nam Woong Song, PhD[c], Yong-Il Kim, PhD[e], Young Suk Yu, MD, PhD[f], Tae Geol Lee, PhD[c,*], Jeong Hun Kim, MD, PhD[a,b,f,**]

[a]*Fight against Angiogenesis-Related Blindness (FARB) Laboratory, Clinical Research Institute, Seoul National University Hospital, Seoul, Republic of Korea*
[b]*Department of Biomedical Sciences, College of Medicine, Seoul National University, Seoul, Republic of Korea*
[c]*Center for Nano-Bio Convergence, World Class Laboratory, Korea Research Institute of Standards and Science, Daejeon, Republic of Korea*
[d]*Department of Chemistry and KI for the NanoCentury, KAIST, Daejeon, Republic of Korea*
[e]*Division of Industrial Metrology, Center for New Functional Materials Metrology, Korea Research Institute of Standards and Science, Daejeon, Republic of Korea*
[f]*Department of Ophthalmology, College of Medicine, Seoul National University, Seoul, Republic of Korea*

Received 19 November 2013; accepted 11 February 2014

Abstract

Local application requires fewer nanoparticles than systemic delivery to achieve effective concentration. In this study, we investigated the potential toxicity and efficacy of bare titanium dioxide (TiO_2) nanoparticles by local administration into the eye. Mono-disperse, 20 nm-size TiO_2 nanoparticles did not affect the viability of retinal constituent cells within certain range of concentrations (~1.30 μg/mL). Furthermore, local delivery of TiO_2 nanoparticles did not induce any significant toxicity at the level of gene expression and histologic integrity in the retina of C57BL/6 mice. Interestingly, at the low concentration (130 ng/mL) without definite toxicity, these nanoparticles suppressed *in vitro* angiogenesis processes and *in vivo* retinal neovascularization in oxygen-induced retinopathy mice when they are administered intravitreally. Taken together, our results demonstrate that even TiO_2 nanoparticles can be safely utilized for the treatment of retinal diseases at the adequate concentration levels, especially through local administration.

Key words: Titanium dioxide; nanoparticles; toxicity; angiogenesis inhibitor; pathologic angiogenesis

Introduction

Inorganic nanoparticles can modulate various biological processes including apoptosis, angiogenesis, and oxidative stress. Although nanoparticles are often utilized as a novel drug delivery system, these characteristics can revolutionize the landscape of therapeutic application of nanoparticles. However, there are still concerns on medical uses of nanoparticles especially for therapeutic purposes because of possible toxicity.[1,2] For instance, titanium dioxide (TiO_2) nanoparticles are known to induce apoptosis and neural toxicity.[3,4] Particularly, systemic treatment requires considerable amount of nanoparticles, resulting in increased risk of toxicity. Nevertheless, inorganic nanoparticles still possess the potential for the therapeutic application. First, it is relatively easy to manipulate size, surface charge, and shape of nanoparticles to improve

This work was supported by the Bio-Signal Analysis Technology Innovation Program (2009-0090895) of MEST/NRF, the Pioneer Research Program (2012-0009544) of MEST/NRF, the Green Nano Technology Development Program (2011-0020504) of MEST/NRF, the Seoul National University Hospital Research Fund (04-2012-0170), the Seoul National University Research Grant (800-20130338), and the Development of Characterization Techniques for the Nano-materials Safety Project of Korea Research Council of Fundamental Science & Technology (KRCF).

Competing interests: There are no competing interests to report.

*Correspondence to: T.G. Lee, Center for Nano-Bio Convergence, World Class Laboratory, Korea Research Institute of Standards and Science, Daejeon 305-340, Republic of Korea.

**Correspondence to: J.H. Kim, Fight against Angiogenesis-Related Blindness (FARB) Laboratory, Clinical Research Institute, Seoul National University Hospital, and Department of Ophthalmology, College of Medicine, Seoul National University, Seoul 110-744, Republic of Korea.

E-mail addresses: tglee@kriss.re.kr (T.G. Lee), steph25@snu.ac.kr (J.H. Kim).

1549-9634/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.nano.2014.02.007

bioavailability and efficacy. Second, conjugation of ligands and receptors to nanoparticles facilitates targeted therapy.[5] Targeting ligands enable effective action of nanoparticles on target cells.[6] Third, inorganic nanoparticles themselves can be good therapeutic candidates. Cerium oxide nanoparticles exert antioxidant effect, decrease reactive oxygen species, and slow down the degeneration of photoreceptor cells.[7,8] Gold, silver, and silica nanoparticles inhibit vascular endothelial growth factor (VEGF)-induced proliferation of endothelial cells, *in vitro* angiogenic processes, and *in vivo* pathologic angiogenesis.[9-12]

The toxicity of high dose nanoparticles is repeatedly reported and might be inevitable.[13,14] We hypothesized that local treatment would be a bypass strategy to maintain biological effect of nanoparticles even at a very low concentration. In this regard, as a proof-of-concept study, we utilized TiO_2 nanoparticles of which toxicity is widely studied. We speculated that we could examine the feasibility of our strategy to minimize the toxicity and maintain the biological effect simultaneously at the presumptive therapeutic concentration (PTC, 10^5 nanoparticles per cell, 130.47 ng/mL). This concentration is lower than the limits of concentration at which there is no definite cellular toxicity in previous studies with TiO_2 nanoparticles (800 ng/mL to 40 μg/mL).[15-18]

Furthermore, it is essential to properly prepare and characterize nanoparticles before utilization in *in vitro* and *in vivo* experiments.[19] Agglomeration and aggregation often lead to overestimation or underestimation of biological effects of nanoparticles.[20] To investigate the potential of TiO_2 nanoparticles in aqueous solution for further applications, researchers previously exchange organic ligands or change pH with hydrothermal treatment.[21,22] However, we speculated that TiO_2 nanoparticles might be prepared in neutral solution for medical application and without functionalization to figure out properties of nanoparticles themselves. In this regard, we prepared TiO_2 nanoparticles in pH 8.2 distilled water (DW),[23] resulting in mono-disperse anatase TiO_2 nanoparticles stabilized in aqueous solution with the size of 20 nm.

In this study, we demonstrate that well-prepared and characterized TiO_2 nanoparticles in aqueous solution do not induce any definite genetic, cellular, and histologic toxicity at 10 times PTC. Surprisingly, they exert anti-angiogenic effect on retinal neovascularization even at PTC. The anti-angiogenic effect of TiO_2 nanoparticles comes from the inhibition of angiogenic processes, not from the toxicity of them. Furthermore, we demonstrate the suppression of VEGF receptor-2 (VEGFR2)/mitogen-activated protein kinase (MAPK) pathway by TiO_2 nanoparticles at PTC. These results suggest that proper preparation and establishment of the adequate concentration range of TiO_2 nanoparticles lead to maintenance of biological effect and minimization of the toxicity at the same time, especially through local administration.

Methods

Preparation and characterization of nanoparticles

To obtain primary nanoparticle suspension of TiO_2 with ~25 nm in diameter, 640 mg of titanium dioxide powder (Degussa/Evonik) was suspended in 10 mL DW (pH 8.2, 18.2 MΩ) by stirring with magnetic bar for 24 hours. Then, the suspension was transferred into a 50 mL conical tube and sonicated for 5 minutes by using a tip sonicator (Sonics & Materials). The suspension was transferred to a 15 mL conical tube and centrifuged for 40 minutes at 9,000 rpm. To get primary TiO_2 nanoparticle suspension, the supernatant was sampled and utilized for further characterization procedures. Dynamic light scattering (DLS) size of TiO_2 nanoparticles was measured from 15 different batches (ELS-Z plus, Otsuka Electronics). Zeta potential was measured by using an electrophoretic mobility analyzer (Zetasizer Nano Z, Malvern Instruments). To obtain a concentrated suspension, the suspension was divided as 1 mL aliquots in 1.5 mL microcentrifuge tubes. Then, the tubes were centrifuged for 30 minutes at 13,000 rpm. The supernatant of 900 μL in each tube was removed and the pellet was resuspended by vortexing. The suspension concentration was measured by weighing dehydrated powders from 3 mL suspensions. Morphology of nanoparticles was observed via transmission electron microscopy (TEM; Technai G2, FEI). TEM image was analyzed for size and their distribution. The X-ray diffraction (XRD) data were obtained from the powder diffractometer (Dmax2200, Rigaku) between 20° and 142° at a 2Θ step of 0.02° using Cu Kα radiation with a graphite monochromator in the reflection geometry at room temperature (RT). To obtain the particle concentration of the stock solution, we theoretically postulated the sphere of which diameter was 18.37 nm as we measured with TEM. Then, we calculated the volume of a sphere nanoparticle and then the mass of a nanoparticle (density, 4.23 g/mL). Based on the mass of a nanoparticle and measured mass concentration of stock solution, we could estimated the number of nanoparticles in the stock solution.

Cell culture

Human retinal microvascular endothelial cells (HRMECs) were purchased from ACBRI and maintained in Medium 199 supplemented with 20% fetal bovine serum (FBS), basic fibroblast growth factor of 3 ng/mL and heparin of 10 IU/mL. SNUOT-Rb1 cells, previously established by our group from the human retinoblastoma tissue,[24] were maintained in RPMI 1640 medium, supplemented with 10% FBS. Human brain astrocytes were purchased from ACBRI and maintained in Dulbecco's Modified Eagle Medium, supplemented with 10% FBS and N-2 Supplement. Cells were incubated at 37 °C in an atmosphere of 95% air and 5% CO_2.

Cell viability assay

Cell viability was evaluated with WST-1 assay using EZ-Cytox Cell Viability Assay kit (Itsbio) according to the manufacturer's instruction. Briefly, HRMECs, SNUOT-Rb1 cells, and human brain astrocytes were plated in 48-well plates and cultured overnight (2×10^4 cells per well). The cells were treated with TiO_2 nanoparticles of different concentrations (10, 10^3, 10^5, 10^7 nanoparticles/cell) for 48 hours. Then, the reagent from EZ-Cytox Cell Viability Assay kit was applied to each well. After 2 hours of additional incubation, absorbance was measured