Silver Nanoparticles Induce DNA Hypomethylation through Proteasome-Mediated Degradation of DNA Methyltransferase 1

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Received August 7, 2020; accepted September 29, 2020

Nanoparticles are used in many fields and in everyday products. Silver nanoparticles are the most frequently used nanoparticles; for example, in food-related products, owing to their antibacterial activity. However, it has been pointed out that they might have unexpected biological effects, and evaluation of their effects is underway. Although there is a growing body of evidence that nanoparticles can also induce epigenetic changes, there is still little information on the underlying mechanisms. Here, we evaluated changes in DNA methylation induced by silver nanoparticles and attempted to elucidate the induction mechanism. Immunofluorescence staining analysis revealed that silver nanoparticles with a diameter of 10, 50, or 100 nm (nAg10, nAg50, and nAg100, respectively) decreased the content of methylated DNA in A549 alveolar epithelial cells. The level of DNA methyltransferase 1 (Dnmt1) protein, which is involved in maintaining methylation during DNA replication, was significantly decreased, whereas that of Dnmt3b, which is responsible for de novo DNA methylation, was significantly increased by nAg10 treatment. Co-treatment with nAg10 and cycloheximide, which inhibits translation by inhibiting the translocation step of protein synthesis, decreased the level of Dnmt1 in comparison with nAg10-treated A549 cells, indicating a post-translational effect of nAg10. Furthermore, pretreatment with the proteasome inhibitor lactacystin restored the levels of Dnmt1 protein and DNA methylation in nAg10-treated cells. Collectively, these results suggest that nAg10 induced DNA hypomethylation through a proteasome-mediated degradation of Dnmt1.

Key words DNA methylation; DNA methyltransferase 1; nanotoxicology; silver nanoparticle

INTRODUCTION

Nanoparticles are used in products with a diameter of 100 nm or less. They are used in products that are familiar in our daily lives because their properties such as tissue permeability and electronic reactivity are better than those of conventional micrometer-sized materials. For example, silver nanoparticles have been used in products such as food utensils, clothing, masks, and antibacterial sprays owing to the potent antibacterial activity of particles. With the increasing use of silver nanoparticles due to their high potential to improve our QOL, the frequency of human exposure to silver nanoparticles may also increase; in an environment where nanoparticles are released into the atmosphere, such as nanoparticles manufacturing site, risks for unintentional inhalation may arise. On the other hand, it has been pointed out that miniaturization may lead to unexpected biological effects of nanoparticles. Moreover, it was reported that the biological effects of silver nanoparticles via inhaled or nasally instilled silver nanoparticles on respiratory organs such as the trachea and lungs.

Importantly, there is a growing body of evidence that nanoparticles can also induce epigenetic changes. In recent years, attention has become focused on epigenetic changes, a mechanism that affects gene expression without changes in the DNA base sequence and may cause the onset of diseases such as cancer and lifestyle-related diseases. DNA methylation is maintained even in semi-conservative DNA replication during cell division, and there is a mechanism that allows newly methylated sites to be inherited by daughter cells. Therefore, epigenetic alternations have been suggested as possible mediators of health effects of environmental stressors. For instance, it has been reported that atmospheric particles matter with an aerodynamic diameter less than 2.5 µM (PM2.5) induce DNA methylation in the promoter region of Cdkn2a, the gene encoding the tumor suppressor p16 and suppress its expression. Furthermore, inflammatory responses induced by particulates from exhaust gas have been reported to result from the involvement of chromatin modification. These reports indicate that biological effects of environmental fine particles might be related to epigenetic mutations.

Although it has gradually become clear that epigenetic changes are involved in the biological effects of nanoparticle exposure, there is still little information on the mechanisms by which nanoparticles induce epigenetic mutations. To ensure health and environmental safety, it is necessary to examine whether they induce epigenetic changes. Here, as part of our research aimed at characterizing epigenetic variations caused by nanoparticle exposure, we used silver nanoparticles to treat alveolar epithelial cells and assessed their effects on DNA methylation.
MATERIALS AND METHODS

Cell Line and Cell Culture Human alveolar adenocarcinoma cell line A549 was purchased from the RIKEN Cell Bank (Ibaraki, Japan) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (high-glucose) (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (Gibco, Waltham, MA, U.S.A.) and 1% antibiotic–antimycotic (100X) (Thermo Fisher Scientific, Waltham, MA, U.S.A.) at 37 °C in a humidified atmosphere containing 5% CO₂.

Reagents Suspensions of citrate-ligand-capped silver nanoparticles with diameters of 10, 50, and 100 nm (nAg10, nAg50, and nAg100, respectively) were purchased from nanoComposix (San Diego, CA, U.S.A.). We previously reported that all the nanoparticles were smooth-surfaced spheres and that the hydrodynamic diameters of these particles in solvent obtained by dynamic light scattering analysis almost exactly corresponded to the particle sizes was confirmed by the transmission electron microscopy. 5-Aza-2’-deoxycytidine (5-aza-dC), lactacystin, and cycloheximide (CHX) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Immunofluorescence Staining A549 cells (1.0 × 10⁴ cells/200 µL per well) were seeded in an 8-well Chamber slide II (AGC Techno Glass, Tokyo, Japan) and cultured at 37 °C for 24 h. Cells were then treated with silver nanoparticles or 10 µM 5-aza-dC for 24 h. Cells were fixed with 4% paraformaldehyde phosphate buffer solution (Wako) for 30 min, and then treated with 0.1% Triton X-100 (Nacalai Tesque, Kyoto, Japan) for 5 min. Then, 2N HCl was added for 30 min and Tris–borate–ethylenediaminetetraacetic acid (EDTA) (1 × TBE, pH 8.3) for an additional 5 min, followed by blocking in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA-PBS) containing 1% bovine serum albumin (BSA-PBS) for 1 h. Cells were then incubated for 1 h with antibody against 5-methylcytosine (5mC) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Fig. 1. Silver Nanoparticles Induce DNA Hypomethylation in A549 Cells
Cells were treated with nAg10, nAg50, or nAg100 (10 µg/mL) or 5-aza-2’-deoxycytidine (5-aza-dC; 10 µM) for 24 h. Fluorescence intensity of 5-methylcytosine (5mC; red) in the nuclei (4’,6-diamidino-2-phenylindole (DAPI): blue) was evaluated by immunofluorescence staining and quantified from images by using ImageJ. Bars, 50 µm. Data are presented as mean ± standard error of the mean (S.E.M.); n = 20; **p < 0.01, *p < 0.05.
Fluor 488 conjugate (Invitrogen, Carlsbad, CA, U.S.A.) diluted 1:400 in 1% BSA-PBS for 1 h in the dark. Finally, cells were incubated with ProLong Gold Antifade Reagent with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen) and mounted with a coverslip. After 2 d, images were acquired under a confocal microscope (SP5; Leica, Wetzlar, Germany) and fluorescence intensity of 5mC in the nuclei was evaluated with ImageJ (National Institutes of Health, Bethesda, MD, U.S.A.). Three sections per sample were observed, and average fluorescence intensity per nucleus area was calculated.

Western Blotting Analysis
A549 cells (2.5 × 10^5 cells/2 mL per well) were seeded in 6-well plates (Nunc, Rochester, NY, U.S.A.) and cultured at 37°C for 24 h. Cells were then treated with silver nanoparticles, 5-aza-dC, or 1.5 ng/mL CHX. To assess the effect of nAg10 on the proteasome pathway, the cells were pretreated with 10 µM lactacystin for 6 h before treatment with nAg10 for 24 h. Nuclear proteins were isolated with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) with a Halt Protease Inhibitor Cocktail Kit (Thermo Fisher Scientific). Protein extract was mixed with an equal volume of Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, U.S.A.) containing 5% 2-mercaptoethanol, boiled for 5 min and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were electro-transferred onto a poly-vinylidene difluoride membrane (Millipore, Bedford, MA, U.S.A.), which was blocked with 4% Block Ace (KAC Co., Ltd., Kyoto, Japan) in PBST. The membrane was Western blotting with a primary antibody for 1 h and with a secondary antibody for another 1 h. The primary antibodies against DNA methyltransferase 1 (Dnmt1) (1:200, #ab54759) and Dnmt3b (1:200, #ab13604) were purchased from Abcam (Cambridge, U.K.). Peroxidase-conjugated anti-rabbit IgG (1:3000, #7074, Cell Signaling Technology, Danvers, MA, U.S.A.) and anti-mouse IgG (Fab specific) (1:50000, #A9044, Sigma-Aldrich) were used as secondary antibodies. Protein bands were detected by using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and visualized with an ImageQuant LAS 4000 mini biomolecular imager (GE Healthcare Japan, Tokyo, Japan). The images were analyzed with ImageJ.

Statistical Analyses
Statistical analyses were performed by using Graph Pad Prism Macro version 7.0 (GraphPad Software, La Jolla, CA, U.S.A.; www.graphpad.com). Significant differences between control groups and experimental groups were determined by using Tukey’s test with a one-way ANOVA. p-Values lower than 0.05 were considered statistically significant.

RESULTS

Silver Nanoparticles Induce DNA Hypomethylation in A549 Cells
Firstly, we assessed whether silver nanoparticles affect DNA methylation. Since the methylation site of DNA is the 5th carbon of cytosine,20,21) we used the nuclear staining for 5mC as an index of DNA methylation. After 24-h treatment of A549 human alveolar epithelial cells with silver nanoparticles of three different sizes or 5-aza-dC (a DNA methyltransferase inhibitor used as a positive control for decreased DNA methylation22)), the fluorescence intensity of 5mC per nuclear area was decreased in all four treatments. In particular, fluorescence intensity was significantly attenuated in cells treated with nAg10 and nAg50 (Fig. 1). Besides, we used the WST-8 assay to examine viability under conditions with or without the nAg10, nAg50, and nAg100 in A549 cells, and the results showed that no cytotoxic effect at the concentration in this study (Fig. S1). Moreover, treatment of A549 cells with nAg10 at the concentrations of 0.1, 1, and 10 µg/mL resulted in a decrease in methylated DNA in all treated groups in comparison with the control group, with no differences among the three concentrations (Fig. S2). These results revealed that silver nanoparticles reduced the content of methylated DNA in A549 cells, suggesting that exposure to these silver nanoparticles may lead to epigenetic mutations.

nAg10 Represses the Expression of DNA Methyltransferase 1 in A549 Cells
DNA methyltransferases such as Dnmt1 and Dnmt3b are responsible for DNA methylation.21) Dnmt1 is...
mainly involved in maintaining methylation during DNA replication,\textsuperscript{23}) whereas Dnmt3b is responsible for \textit{de novo} DNA methylation.\textsuperscript{24}) To elucidate the mechanism by which silver nanoparticles induce hypomethylation, we assessed the effect of nAg10 on the levels of Dnmt1 and Dnmt3b. Western blot analysis revealed that treatment of A549 cells with nAg10 at 10 µg/mL significantly decreased the level of Dnmt1 in the nuclei in comparison with the control cells (Fig. 2a), but significantly increased that of Dnmt3b (Fig. 2b). The level of Dnmt1 was also decreased by nAg10 at 0.1 and 1 µg/mL, although the effect at 0.1 µg/mL was not statistically significant (Fig. S3). Given that \textit{de novo} DNA methyltransferase Dnmt3b compensates the Dnmt1-deficient intestinal epithelium,\textsuperscript{25}) nAg10-induced increased level of Dnmt3b was complementary. These results suggest that Dnmt1 might be involved in the nAg10-induced reduction in DNA methylation.

Fig. 3. nAg10 Induce DNA Hypomethylation through Proteasome-Mediated Degradation of Dnmt1
A549 cells were treated with nAg10 (10 µg/mL) for 24h (a) in the presence or absence of cycloheximide (CHX; 1.5 ng/mL) or (b, c) after pretreatment with lactacystin (10 µM) for 6h. The level of Dnmt1 was assessed by Western blotting. Lamin was used as an endogenous control. Band intensity was quantified from images by using ImageJ. These experiments were repeated twice or three times with similar results. Data are presented as mean ± S.D.; n = 3–4; **p < 0.01, *p < 0.05. (c) Fluorescence intensity of 5mC (red) in the nuclei (DAPI: blue) was evaluated by immunofluorescence staining and was quantified from images by using ImageJ. Bars, 50 µm. Data are presented as mean ± S.E.M.; n = 19–20; **p < 0.01.
nAg10 Induce Degradation of Dnmt1 Protein via the Proteasome Pathway To further clarify the mechanism of the decrease in Dnmt1 induced by nAg10, we treated A549 cells with nAg10 or 5-aza-dC evaluated the level of Dnmt1 mRNA at 2, 6, 12, and 24 h after the onset of treatment. Trichostatin A (TSA) was used as a positive control of down-regulation of the mRNA level of Dnmt1,25) and decreased the mRNA level of Dnmt1 at 24 h after treatment (Fig. S4). On the other hand, neither nAg10 nor 5-aza-dC significantly affected the expression level of Dnmt1, suggesting that nAg10 act after transcription, likely by inhibiting translation or promoting degradation of Dnmt1 protein.27) To assess the effect of nAg10 on Dnmt1 translation, we treated cells with CHX, which inhibits translation by inhibiting the translocation step of protein synthesis.28) Western blot analysis revealed that, in comparison with nAg10-treated A549 cells, the level of Dnmt1 protein was decreased in cells co-treated with nAg10 and CHX (Fig. 3a), indicating that nAg10 do not act by inhibiting translation. This result suggests that nAg10 affect a mechanism after translation, rather than the translation pathway, which is inhibited by CHX.

Proteolytic degradation of Dnmt1 is induced by exposure to chemicals such as 5-aza-dC and valproic acid, which activates the proteasome.29,30) Hence, we assessed the effect of nAg10 on Dnmt1 degradation. Lactacystin, a proteasome inhibitor that mainly inhibits the chymotrypsin-like activity of the proteasome,31) was added to cells for 6 h before treatment with nAg10. After 24 h treatment with nAg10, the Dnmt1 protein level was restored in nAg10-treated cells that had been pretreated with lactacystin (Fig. 3b), indicating that nAg10 decreased the level of Dnmt1 via the proteasome pathway. In line with these findings, immunofluorescence staining analysis showed that the decrease in 5mC fluorescence intensity per nuclear area observed in nAg10-treated cells was significantly recovered by lactacystin pretreatment (Fig. 3c). Collectively, these results suggest that nAg10 induce DNA hypomethylation through a proteasome-mediated degradation of Dnmt1.

DISCUSSION

Although it has gradually became clear that epigenetic changes are involved in the biological effects of nanoparticle exposure,17,18) there is still little information on the mechanisms by which these environmental factors induce abnormalities in methylation and in DNA methyltransferases. From this viewpoint, the present study showed that silver nanoparticles induce a decrease in the level of Dnmt1 through activation of the proteasome system and thus decrease DNA methylation.

We revealed that nAg10 significantly decreased the content of methylated DNA though there is little relationship with the size of silver nanoparticles. The biological responses induced by nanoparticles has been generally considered that the smaller the particle size, the stronger the responses can be induced. On the other hand, nanoparticles-induced biological responses have begun to be reported, including ours, in which the effect increases and decreases only at a specific nano-sized particle.32,33) Hence, they cannot be explained only by reducing the particle size. From this viewpoint, to elucidate the detailed mechanism of nanoparticles-induced epigenetic changes, further examination is needed not only in the size-dependent effects of nanoparticles but also in the size-specific effect. Moreover, there are several possibilities for reduced methylated DNA regardless of the concentration of nAg10. For example, it is possible that the level of uptake of nAg10 into nuclei, where DNA is methylated and demethylated, hardly changes even at the concentrations in this study. In addition, it is suggested that nAg10 can sufficiently induce DNA demethylation at the concentrations of 0.1 μg/mL. Therefore, it is essential to assess quantitatively the uptake and localization of nAg10 into cells, including nuclear, and to pursue the threshold of nAg10 treatment for DNA demethylation.

Then, we focused on Dnmt1 and Dnmt3b methyltransferases in an attempt to reveal the mechanism of DNA demethylation induced by nAg10. Although further studies are needed to reveal the mechanism of the increase in the level of Dnmt3b and the implications of this increase for DNA methylation, we considered that hypomethylation likely resulted from a decrease in Dnmt1 level because the latter was related to a decrease in methylated DNA induced by nAg10. Elliott et al.25) reported that a mutation in Dnmt1 induced hypomethylation regardless of an increase in Dnmt3b. On the other hand, it was also reported that Dnmt1 maintains methylation during replication,32) whereas Dnmt3b performs de novo DNA methylation,34) and that de novo DNA methyltransferase Dnmt3b compensates the Dnmt1-deficient intestinal epithelium.25) Thus, we considered that nAg10-induced increased level of Dnmt3b was complementary. However, specific DNA regions may be methylated via Dnmt3b upregulation by nAg10 exposure and we have to consider the location of DNA methylated with/without nAg10 exposure to reveal the potential hazards induced by changes in methylation.

We found that nAg10 suppressed Dnmt1 expression due to proteasome-dependent degradation, whereas inhibition of neither transcription nor translation was involved. Proteasome-dependent degradation requires the ubiquitination of target proteins.35) For instance, ubiquitin-like with PHD and RING finger domains 1 serves as a ubiquitin ligase that targets Dnmt1, and ubiquitin carboxyl-terminal hydrolase 7 is reportedly a deubiquitinating enzyme for Dnmt1.36) Also, it is reported that acetylation and deacetylation of Dnmt1 related with its proteasome-dependent degradation.39) Given that silver nanoparticles reportedly promote histone methylation by entering the nucleus and binding to histones,37,38) the mechanism of nAg10-induced DNA demethylation is expected to be elucidate by further examining the effect of nAg10 on factors related to proteasome degradation of Dnmt1 and the interaction with nAg10. Moreover, the involvement of reactive oxygen species (ROS) in epigenetic changes has been reported as a pathway involving the decrease in Dnmt1 expression.39,40) Since the production of ROS is induced by silver nanoparticles,41,42) it is also necessary to investigate the role of ROS produced by nAg10 in reducing Dnmt1 expression.

Furthermore, it is important to assess the mechanism of nAg10-induced DNA demethylation by focusing on not only DNA methyltransferases but DNA demethylating enzymes. For example, another protein that induces hypomethylation such as ten-eleven translocation (TET),43) might be involved in the reduction in methylated cytosine caused by silver nanoparticles. TET have capacity to convert 5mC to 5-hydroxymethylcytosine and actively induces its demethylation via formation of 5-formylated cytosine and 5-carboxylated cytosine.44) Thus, it is necessary to evaluate the effect of silver nanoparticles on
the activity of TET in the future.

Acknowledgments This study was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 16K01437 to K.H. and No. 26242055 to Y.T.); by Health Labor Sciences Research Grants from the Ministry of Health, Labor, and Welfare of Japan (No. H25-kagaku-ippan-005 to Y.T. and No. H28-syokuhinn-wakate-014 to K.H.); and by the Takeda Science Foundation (to K.H.).

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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