The Expression of Antimicrobial Peptide Lysozyme is Increased by Treatment with Silver Nanoparticle (Atomyball S®) in Mammalian Epithelial Cells

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In recent years, the use of silver nanoparticles (SNPs) is gaining ground especially on its use as disinfectant or antiseptic. The application of SNPs in the manufacture of medical devices and products is attributed to its high antibacterial efficacy. But while most of the studies on SNPs focus on its direct effect on bacteria, investigations have not been done on the effect of SNPs on mammalian innate immune molecules that possess bacterial killing capacity. Thus, in this report, we determined whether SNPs could affect naturally occurring antimicrobial molecules. We used here a silver nanoparticle, termed S (Atomyball S®), which has a size of 5 nanometers (nm), and treated it to lung epithelial cell lines A549 and Calu-3. A low concentration of nanoparticle S (5 μg/ml) did not induce cell toxicity as determined by lactate dehydrogenase assay. Interestingly, we observed that S increased the mRNA and protein expression levels of the antimicrobial peptide lysozyme, which is considered as one of the most important host immune defense molecules in human epithelial tissues. Quantification of the secreted lysozyme in the apical surface fluid obtained from air-liquid interface cultures of Calu-3 cells also revealed a significant increase of lysozyme in cells treated with nanoparticle S. Although the mechanism of its action on lysozyme is yet to be elucidated, these findings firstly suggest that the silver nanoparticle S (Atomyball S®) positively regulated the expression of antimicrobial peptide lysozyme in mammalian cells.

Key words —— silver nanoparticle, lysozyme, apical surface fluid, Calu-3 cells, A549 cells, air-liquid interface culture

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INTRODUCTION

The application of nanoscale materials, usually ranging from 1 to 100 nm, is an emerging area of nanoscience and nanotechnology. Bionanomaterials are already being applied to diagnostic medicine and drug delivery systems and are used or tested in a wide range of consumer products.[1–3] Specifically, silver nanometals are used in many consumer applications, mostly because of its well-documented and safe uses as an antimicrobial agent.[4–6] Silver nanoparticles (SNPs) have found important application in the form of wound dressings and disinfecting agents, which have been reported to effectively reduce bacterial infections in chronic wounds and exhibit better healing capacity.[7–9] Studies have shown that nanomolar concentrations of SNPs significantly inhibited bacterial growth of *Escherichia coli* (*E. coli*), *Vibrio cholera* (*V. cholera*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Syphillis typhus* (*S. typhus*).[10,11] The exact mechanism of action of SNPs on the microbes is still unknown but a possible mechanism has been suggested, that is, SNPs bind to bacterial cell membrane, disturbing permeability and respiration functions of the cell.[11,12] Although numerous reports have provided much information on the direct antibacterial effect of SNPs on microbes,[13] there are no studies on the effect of SNPs on innate immune molecules possessing antimicrobial functions.

Among the antimicrobial peptides, lysozyme, having strong bactericidal capability, is considered as one of the most important host defense molecules in epithelial cells.[14,15] It has muramidase activity that degrades the peptidoglycan in the cell wall of Gram-positive bacteria. In addition, it has been reported that lysozyme can also kill Gram-negative bacteria independently on its muramidase function.[16,17] Antimicrobial peptides such as lysozyme tend to be found in animal tissues that are likely to come in contact with pathogens, namely, the skin, airway epithelia and lungs, among others. They are also highly enriched in airway surface fluid.[18]

In this study, we firstly attempted to investigate whether SNPs impact on the expression level of lysozyme in mammalian cells. We used here a silver nanoparticle having a size of 5 nm and which has the designated name S (for small; or Atomyball S®). We determined the effect of S on lysozyme expression using lung epithelial cells A549 and Calu-3 as well as using the apical surface fluid obtained from air-liquid interface culture of Calu-3. Our data collectively showed that treatment with nanoparticle S at a concentration of 5 μg/ml increased the expression of lysozyme, suggesting that the silver nanoparticle S (Atomyball S®) has the ability to up-regulate lysozyme.

MATERIALS AND METHODS

Cell Culture and Treatment —— Lung carcinoma cell line, A549 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbard, CA, U.S.A.) containing 10% fetal bovine serum (FBS) and antibiotics. Lung adenocarcinoma cell line, Calu-3 cells were maintained in DMEM/Ham's F-12 medium (DMEM/F12) supplemented with 15% FBS and antibiotics. The cell lines were purchased from the American Type Culture Collection (ATCC) and cultured at 37°C in a humidified atmosphere of 5% CO_2.

The nanoparticle S (Atomyball S®) is silver nanoparticle using TiO_2 as inorganic stabilizer or base. The photocatalytic synthesis of S was carried out in a method similar to the one comprehensively described by Cozzoli *et al.*[19] S has a size of 5 nm, is in liquid form and is water-soluble. SAN, the colloidal stabilizer of S, contains TiO_2 but without silver nanoparticle. We used SAN as one of the controls for all experiments. S and SAN were kindly provided by JGC Catalysts and Chemicals Ltd. (Kita-kyushu, Japan). For cell treatment, S and SAN were diluted in filtered distilled water (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). When cells reached 70% confluent, culture media were replaced with fresh media containing distilled water (as control), SAN or S having a final concentration of 5 μg/ml. Treatment time was carried out as indicated for each experiment.

For Calu-3 cells cultured in transwell plates, cells were seeded in 6-well cluster inserts (Costar plates; Corning Inc., Corning, NY, U.S.A.) at a density of 2×10^5 cells with 0.5 ml and 2.5 ml media at the apical and basolateral chambers, respectively. Air-liquid interface (ALI) culture was initiated by removing the medium from the apical chamber 1 week after seeding. ALI culture was maintained by changing the basolateral medium once every 2–3 days for approximately 28 days. The transepithelial resistance (TER) was measured as described previously.[20] Confluent monolayers with a TER value $> 300\,\Omega/cm^2$ were used for nanoparticle treatment. Prior to treatment, the apical and basolateral compartments were washed with pre-warmed, serum-free DMEM/F12 twice. Media were added to both compartments with the apical medium containing 5 μM SAN or S (or water as control) at the indicated time. The apical surface fluid (ASF) and ASF washings were collected following the protocol reported before[21] for analysis of lysozyme. Equal volumes of the collected ASF were used for the assays.

Lactate Dehydrogenase (LDH) Assay —— A549 cells were incubated with water, SAN or S in low-serum medium (1% FBS DMEM) for 24 hr, then the media were isolated and centrifuged to remove the debris. The adherent cells were lysed with low-serum medium containing 1% TritonX-100 at 37°C for 30 min and the lysates were obtained. The media and cell lysates were subjected to LDH assay using a cytotoxicity detection kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Absorbance was measured using a spectrophotometer (BioRad Laboratories, Hercules, CA, U.S.A.). LDH release was computed as LDH in medium/total LDH and expressed as ratio of LDH release relative to control.

Reverse Transcriptase (RT)-PCR Analysis —— Total RNA was isolated from A549 cells treated with SAN or S using TRIzol (Invitrogen) according to the manufacturer's instructions. Semi-quantitative RT-PCR was carried out with RT-PCR Kit (TaKaRa, Ohtsu, Japan) using the recommended protocol. The oligonucleotide primers and conditions used for PCR of lysozyme and GAPDH genes are described previously.[22]

SDS-PAGE and Western Blotting —— To analyze the lysozyme protein expression, cells treated with SAN or S were lysed with radioimmunoprecipitation buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mg/ml sodium deoxycholate, 1% NP-40 and 0.1% SDS) containing 1% protease inhibitor cocktail (Sigma, St. Louis, MO, U.S.A.). Equal amounts of cell lysates or ASF were fractionated on a 15% SDS-PAGE gel and blotted onto polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA, U.S.A.). Blots were probed with goat

anti-lysozyme antibody (W-20; sc-27956, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and rat anti-Hsc70 antibody (SPA-815; Stressgen Bioreagents, Victoria BC, Canada). The HRP-conjugated secondary antibodies used in this study were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, U.S.A.). Blots were visualized using SuperSignal (PIERCE, Rockford, IL, U.S.A.).

Measurement of Secreted Lysozyme —— The amount of lysozyme in the ASF washings was measured using a turbidimetric assay based on the hydrolysis of *Micrococcus lysodeikticus* (*M. lysodeikticus*) cell walls as described in a previous study.[23] *M. lysodeikticus* (M-3770; Sigma) was prepared in 50 mM phosphate buffer (pH 7.4) containing 1 mg/ml sodium azide and 1 mg/ml bovine serum albumin. Purified chicken lysozyme (L-7001; Sigma), dissolved in serum-free DMEM/F12, was used for lysozyme standards (conc: 0-2000 ng/ml). Briefly, 20 µl of ASF or lysozyme standard solution was incubated with 80 µl suspension of *M. lysodeikticus* for 3 hr at 37°C. After incubation, the absorbance was measured using a spectrophotometer (BioRad Laboratories). Lysozyme standard curve was derived and the concentration of lysozyme in each sample was determined.

Statistical Analysis —— Data are presented as mean ± S.E. Significance of the difference between 2 groups was assessed by Student's *t* test. Significance of the difference between 3 groups was assessed by one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparison test (JMP software, SAS Institute, NC, U.S.A.). A p value of < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

We first assessed whether the nanoparticle S (Atomyball S®) is cytotoxic to mammalian cells by determining the LDH release in control and treated A549 cells. We utilized lung epithelial cell line A549 because it had been noted that one of the most important portals of entry and organ target for nanoparticles is the respiratory system.[24] Treatment with 5 µg/ml of nanoparticle S for 24 hr did not induce a change in the level of LDH (Fig. 1), indicating that under this condition, the nanoparticle S is not cytotoxic. This result using A549 cells is consistent with the cytotoxicity test of S

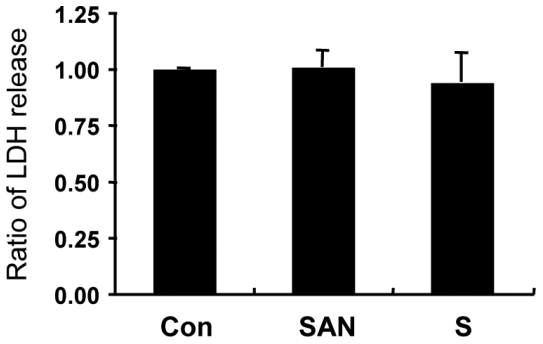

Fig. 1. The Nanoparticle S Did Not Induce LDH Release in A549 Cells

Cells were treated with 5 µg/ml of SAN, S or water (control) for 24 hr. LDH release was determined as described in Methods and expressed as ratio relative to control. Data are mean ± S.E. of 3 independent experiments.

using *in vitro* colony formation assay in which the IC_{50} is 600 µg/ml as assessed in the analytical facility of Japan Food Research Laboratories (Fukuoka, Japan). Recent studies have revealed a dose-dependent toxicity at 10–50 µg/ml of SNPs having a size of 15 nm to germline stem cells and alveolar macrophages when these cells were treated for 24 hr.[25,26] The different nanoparticle size (5 nm), lower concentration (5 µg/ml) and the type of cells used in this study may account for the lack of toxicity observed here. Currently, it is still unclear how SNPs interact and instigate toxicity in mammalian cells. Furthermore, although it is a critical issue to be addressed in nanoparticle applications, the inconsistencies among various studies on nanoparticle toxicity still preclude the making of a generalized conclusion about the toxicological effects of nanoparticles.[27]

SNPs have been shown to be a promising antimicrobial material due to the toxicity of silver to a wide range of microorganisms.[6,28] Because most of these studies examined the direct effect of SNPs on bacteria or viruses, we next asked whether SNPs have any effect on mammalian antimicrobial molecules such as lysozyme, which is one of the most important antimicrobial polypeptides. We treated A549 cells with nanoparticle S and first examined the lysozyme mRNA level. Interestingly, lysozyme transcription was significantly induced by treatment for 30 min with S compared with control or its solvent, SAN (Fig. 2). This was consistently followed by the up-regulation of lysozyme protein after treatment with S for 1 hr (Fig. 3A). We confirmed the increase of lysozyme protein level mediated by S in another lung epithelial cell line, Calu-3

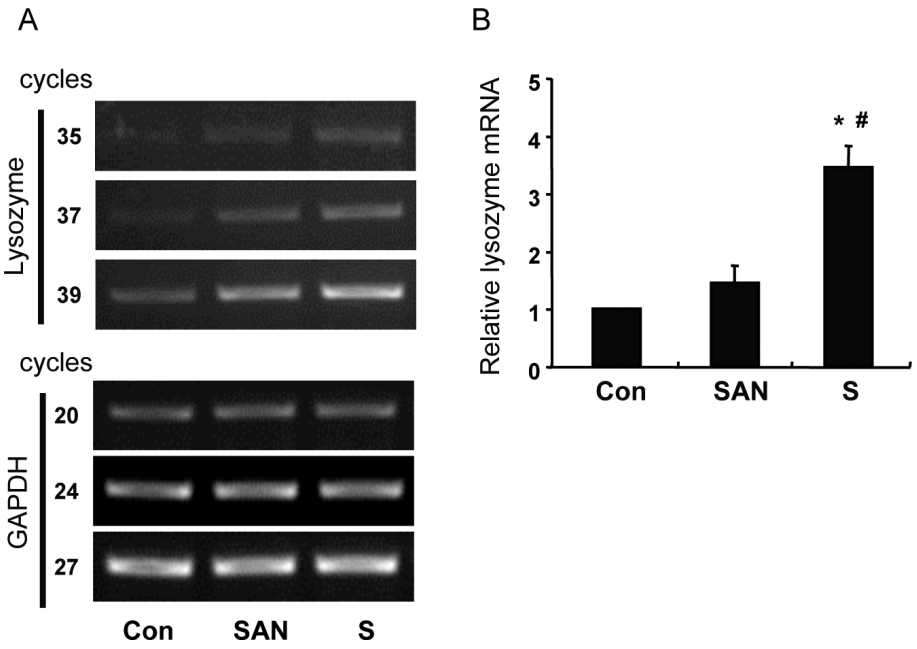

Fig. 2. The Nanoparticle S Increased the Lysozyme mRNA Level in A549 Cells

(A) Cells were treated with 5 μg/ml of SAN, S or water (control) for 30 min. Total RNA was isolated and analyzed for the expression of lysozyme by semi-quantitative RT-PCR. (B) Lysozyme mRNA level (37 cycles) was normalized to GAPDH (24 cycles), which served as internal control, and quantified using Image Gauge software (ver. 4.23, Fujifilm, Japan). Values are mean ± S.E. (n = 3) and presented as relative expression to control. $^{*}p < 0.01$ *v.s.* control, assessed by one-way ANOVA. $^{\#}p < 0.05$ *v.s.* SAN, assessed by Student's t test.

(Fig. 3B). We also attempted to examine the mRNA level of lysozyme in Calu-3 cells. But because the basal level of lysozyme in these cells is relatively high,[29] we did not see a significant difference in the lysozyme mRNA between control and S-treated cells (data not shown). However, it is clear that S enhanced the lysozyme protein expression in Calu-3 cells as mentioned above.

Because lysozyme is a secreted protein, we next examined whether treatment of cells with S will increase the expression of lysozyme in the cell surface fluid. To carry out this investigation, we used ALI culture of Calu-3 cells, a system that has been widely demonstrated to be ideal for determining the various secreted antimicrobial proteins including lysozyme.[20,21,23,29,30] In contrast to ALI culture of Calu-3 cells, which closely resemble that of native lung epithelia, ALI culture of A549 cells was found to be unsuitable for drug permeability experiments and to have less production of surface fluid,[30] thus we utilized here ALI culture of Calu-3 cells to determine the level of secreted lysozyme in airway surface fluid. The ASFs obtained from ALI cultures of control, SAN- and S-treated cells were assessed by Western blotting for the expression of lysozyme. Consistent with the results above, we found that lysozyme was highly expressed in ASF of S-treated cells compared with control and SAN-treated cells (Fig. 3C).

To confirm the Western blotting data, we quantified the amount of lysozyme in the ASF samples by performing a reportedly sensitive turbidimetric *in vitro* enzymatic assay in which the hydrolysis of *M. lysodeikticus*, a lysozyme-specific substrate, leads to a decrease of optical density measured at 450 nm.[31] As shown in Fig. 4, a significant increase in the amount of lysozyme was observed in the ASF from cells treated with S (6.71 ± 0.34 μg/ml) compared with control (4.48 ± 0.15 μg/ml) and SAN-treated (4.49 ± 0.18 μg/ml) cells. Because the solvent SAN, which contains TiO_2 but not the silver nanoparticle, did not significantly up-regulate the expression of lysozyme, we could rule out the effect of TiO_2 (Fig. 4). Overall, these data show that nanoparticle S increased the level of lysozyme in epithelial cells.

In addition, we examined the effect of 1 μg/ml S on lysozyme protein expression in ASF. Although we observed a slight but significant increase in lysozyme by Western blotting analysis, the quantified amount of lysozyme in the ASF samples did not yield a statistically significant difference (data not shown). With regards to the treatment time, we noted that the mRNA level of lysozyme was

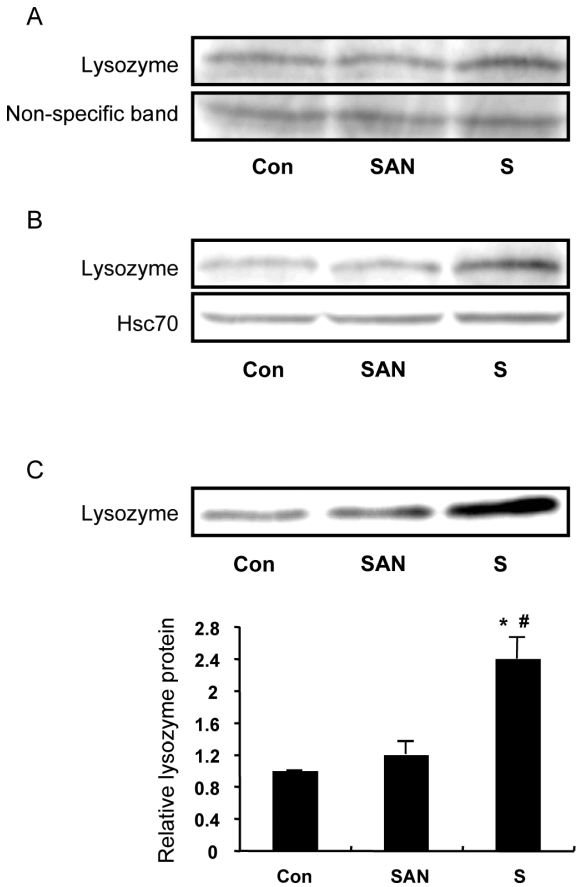

Fig. 3. The Nanoparticle S Increased the Lysozyme Protein Level in Cell Lysates and ASF

(A) A549 cells or (B) Calu-3 cells plated in 6-well plates were treated with 5 μg/ml of SAN, S or water (control) for 1 hr. Cell lysates were extracted using RIPA buffer and analyzed by Western blotting with anti-lysozyme antibody. Non-specific bands or Hsc70 served as loading control. Data shown are representative of 2 independent experiments. (C) Twenty microliter of ASF samples obtained from ALI cultures of Calu-3 cells treated with 5 μg/ml of SAN, S or water for 2 hr were subjected to Western blotting with lysozyme antibody. *Lower panel*, Protein blots were quantified using Image Gauge software. Data are presented as means ± S.E. from 3 independent experiments. $^{*}p < 0.01$ *v.s.* control, assessed by one-way ANOVA. $^{\#}p < 0.05$ *v.s.* SAN, assessed by Student's *t* test.

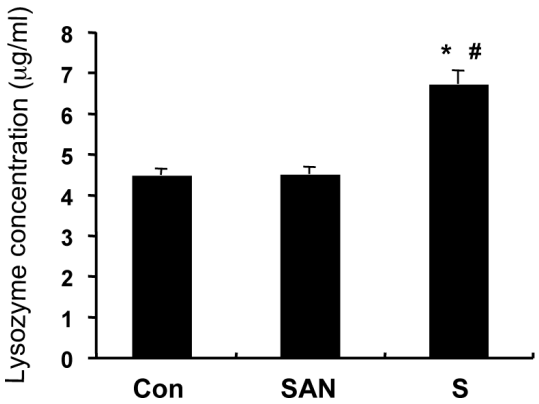

Fig. 4. The Nanoparticle S Increased the Concentration of Secreted Lysozyme in ASF

ASF washings from ALI-cultured cells were collected after treating cells with 5 μg/ml of SAN, S or water for 2 hr. Lysozyme levels were measured using 20 μl of the ASF samples by enzymatic assay described in Methods. Data are presented as mean ± S.E. ($n = 3$). $^{*}p < 0.01$ *v.s.* control, assessed by one-way ANOVA. $^{\#}p < 0.05$ *v.s.* SAN, assessed by Student's *t* test.

first up-regulated within 30 min of treatment with nanoparticle S (Fig. 2). On the other hand, the protein expression of lysozyme in the cell lysate was observed to be up-regulated by S treatment within 1 hr but not within 30 min (Fig. 3A, B and data not shown). Treatment with nanoparticle S for 1 hr slightly increased the secreted lysozyme in the ASF but this up-regulation was observed more significantly within 2 hr of treatment (Fig. 3C). Allowing for the time lag between transcription, translation and secretion of lysozyme, the observed effects at different time treatment for these different assays was reasonable.

We also investigated the effect of S on other innate immune molecules such β-defensin, mucin, IL-1β and IL-8. However, we could not detect any change in their expression level (data not shown). Exactly how this nanoparticle works on lysozyme still remains unclear. It might also be interesting to examine the effects of nanoparticles other than silver on the induction of lysozyme to determine if the up-regulation of lysozyme could be mediated by other nanoparticles as well. The elucidation of the mechanism for lysozyme up-regulation by nanoparticle S needs to be further investigated in future studies, which would be highly relevant considering that the use of nanoparticles is becoming more widespread. In conclusion, our data here firstly suggest that the silver nanoparticle S (Atomyball S®) may be effective as an antibacterial agent in part due to its effect on enhancing the expression of the antimicrobial molecule lysozyme.

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