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Silver nanoparticles protect human keratinocytes against UVB radiation-induced DNA damage and apoptosis: potential for prevention of skin carcinogenesis

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Abstract

Ultraviolet (UV)-B radiation from the sun is an established etiological cause of skin cancer, which afflicts more than a million lives each year in the United States alone. Here, we tested the chemopreventive efficacy of silver-nanoparticles (AgNPs) against UVB-irradiation-induced DNA damage and apoptosis in human immortalized keratinocytes (HaCaT). AgNPs were synthesized by reduction-chemistry and characterized for their physicochemical properties. AgNPs were well tolerated by HaCaT cells and their pretreatment protected them from UVB-irradiation-induced apoptosis along with significant reduction in cyclobutane-pyrimidine-dimer formation. Moreover, AgNPs pre-treatment led to G1-phase cell-cycle arrest in UVB-irradiated HaCaT cells. AgNPs were efficiently internalized in UVB-irradiated cells and localized into cytoplasmic and nuclear compartments. Furthermore, we observed an altered expression of various genes involved in cell-

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cycle, apoptosis and nucleotide-excision repair in HaCaT cells treated with AgNPs prior to UVB-irradiation. Together, these findings provide support for potential utility of AgNPs as novel chemopreventive agents against UVB-irradiation-induced skin carcinogenesis.

Keywords

Silver nanoparticles; ultraviolet radiations; DNA damage; keratinocytes; apoptosis; nucleotide excision repair (NER); cyclobutane pyrimidine dimers (CPDs)

BACKGROUND

According to the “World Cancer Report,” skin cancer constitutes nearly 30 % of all newly diagnosed cancer cases worldwide and incidence continue to rise at an alarming rate in the United States^{1,2}. Solar ultra-violet (UV) radiation, particularly its UVB component, is an established cause of skin carcinogenesis due to its ability to cause DNA damage in skin cells^{3,4}. If unrepaired, the DNA damage may ultimately lead to accumulation of carcinogenic mutations leading to malignant transformation of the skin cells^{5,6}. To protect the DNA damage, several topical sunscreen formulations have been developed and are being used for protection against UV radiation-induced skin injury and carcinogenesis^{7,8}. These formulations include substances (Zinc oxide or titanium dioxide nanoparticles) that reflect, scatter or absorb UV radiations and thus limit its exposure to the skin cells^{8,9}. Even though the sunscreens in the market have high sun protection factor (SPF) potential, they have failed in limiting the UV-induced skin cancer occurrence⁸. Moreover, the data from several studies also demonstrated that zinc oxide or titanium dioxide may cause inflammatory/toxic effects to the normal skin cells^{10,11}. Therefore, it is highly desirable that we develop a novel, safe and effective formulation to reduce the incidence of skin malignancy, and curb the morbidity and mortality associated with it within the US population and worldwide.

Silver has been used for centuries to prevent and treat a variety of diseases as well as in healing of skin wounds due to its excellent free radical scavenging, antimicrobial and anti-inflammatory properties^{12,13}. In 1990s, silver was introduced in a colloidal form (i.e. silver nanoparticles, AgNPs) in ointments that could be applied to open wounds to kill bacteria and promote wound healing through their powerful anti-bacterial and anti-inflammatory properties^{14,15}. AgNPs are currently being used in various medical devices, consumer products and pharmaceuticals including bandages, wound dressings and ointments^{14,15}. In fact, AgNPs now contribute more than a quarter to the list of the commercially available nano-based products suggesting its wide applicability and safety in human applications¹⁶⁻¹⁸. In the present novel work, we have explored the efficacy of AgNPs as chemopreventive agents against UVB radiation-induced skin carcinogenesis. Our studies reveal that AgNPs are non-toxic to the human immortalized keratinocytes (HaCaT) and protects them from UVB-induced DNA damage. Our data also demonstrate that AgNPs pretreatment significantly reduces the extent of apoptosis caused by UVB radiation in HaCaT cells as well as induces G1/S phase cell-cycle arrest. Furthermore, our study reveals the higher internalization of AgNPs in UVB-irradiated cells and indicates the involvement of nucleotide excision repair (NER) genes in the repair of UVB-induced DNA damage. These are promising observations and provide compelling support for potential novel human

applications of AgNPs as chemopreventive agents against UVB-induced skin carcinogenesis.

METHODS

Reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal-bovine serum (FBS) were obtained from Thermo Scientific (Logan, UT) and Atlanta Biologicals (Lawrenceville, GA), respectively. Penicillin, streptomycin and trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA), and silver nitrate, sodium citrate tribasic dehydrate sodium borohydride and 2', 7'-dichlorofluorescein diacetate (DCFH-DA) were from Sigma-Aldrich (St. Louis, MO). Trypan blue and cell Counting Chamber Slides were procured from Thermo Scientific and Life technologies (Grand Island, NY), respectively. Tris buffered saline (TBS) and Tween 20 were from Boston Bioproducts (Ashland, MA). Cell proliferation reagent (WST-1) was obtained from Roche Diagnostics (Mannheim, Germany) and propidium iodide/RNase staining buffer and PE Annexin V apoptosis detection kit were from BD Bioscience (San Diego, CA). DNAzol was procured from Life Technologies. Osmium tetroxide was obtained from Agar Scientific (Stansted Essex, England, UK). RNeasy purification kit was procured from Qiagen (Germantown, MD) and c-DNA reverse transcription kit was from Applied Biosystems (Carlsbad, CA). SYBR Green master mix was procured from Applied Biosystems. Anti-Bcl-2 (rabbit monoclonal), -Bcl-xL, -Bax (rabbit polyclonal) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against cyclin-dependent kinases 1 (CDK1), CDK2, Cyclin B1, Cyclin E1, p21 (mouse monoclonal) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). β -actin (mouse monoclonal) was purchased from Sigma-Aldrich. Antibody against Cyclobutane pyrimidine dimer (mouse monoclonal) was from KamiYa Biomedical Company (Seattle, WA). All respective anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies were procured from Santa Cruz Biotechnology.

Cell culture

The human immortalized keratinocytes, HaCaT (German Cancer Research Center, Heidelberg, Germany) was maintained in a humidified atmosphere of 5 % CO₂ at 37 °C as monolayer culture in DMEM medium supplemented with 10 % FBS, penicillin (100 units/mL) and streptomycin (100 μ g/mL). Cells were continuously monitored for their typical morphology and intermittently tested for mycoplasma using MycoSensor PCR assay kit (Stratagene, La Jolla, CA).

Preparation of silver nanoparticles

AgNPs were synthesized as previously described¹⁹ with some modifications. In brief, aqueous solutions of silver nitrate (100 μ L, 0.1 M) and sodium citrate tribasic dihydrate (100 μ L, 0.3 M) were added to deionized water (9.7 mL) followed by addition of sodium borohydride aqueous solutions (100 μ L, 0.5 M) through slow stirring. The formation of the AgNPs was confirmed when the color of the mixture changed from transparent to golden yellow.

Physicochemical Characterization

AgNPs were characterized by UV-Visible spectroscopy using a Bio-Rad Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA). Hydrodynamic diameter and zeta potential of nanoparticles were measured in distilled water using Malvern Zetasizer (Malvern Instruments, Inc., Westborough, MA). Mean hydrodynamic diameters were calculated for size distribution by volume with the assumption of a lognormal distribution. Surface morphology and size of nanoparticles were characterized with a Zeiss EM10C transmission electron microscope (TEM, Carl Zeiss Microscopy, LLC, Thornwood, NY) equipped with a SIA-L3C CCD camera using MaxIM DL image capture software (Scientific Instruments & Applications Inc., Duluth, GA) operating at 60 kV.

UVB-exposure of HaCaT cells

Cells were irradiated with UVB (40 mJ/cm²) using Daavlin Research Irradiator (Bryan, OH) equipped with four UVB lamps and an electronic controller to regulate UVB dosage. Majority of the wavelengths of UVB radiation were in 290-320 nm range.

Cell viability assay

To examine the toxicity of AgNPs, cells (1×10⁴ cells/well) were seeded in 96 well plates, cultured overnight and treated with various concentration of AgNPs (0.5–80 µg/mL) for different time intervals (12, 24 or 48 h) and percent viability was calculated using WST-1 assay²⁰. The protective role of AgNPs on UVB-induced cell death was examined by trypan blue dye-exclusion method in which cell suspension was diluted 1:1 using a 0.4 % trypan blue solution followed by cell counting using Countess® Automated Cell Counter (Life Technologies) that quantify cell number (live, dead, and total cells) and provides percent viability.

Apoptosis assay

HaCaT cells (1×10⁶/plate) were seeded in glass plates. After overnight incubation, cells were treated with AgNPs (1 µg/mL) for 3 h prior to UVB-irradiation (40 mJ/cm²). Cells were harvested 24 h post UVB-exposure and percentage of apoptotic cell population was calculated as described previously²¹.

Dot-blot analysis

Genomic DNA from HaCaT cells (treated or untreated) was isolated using DNAzol and transferred (500 ng) to a positively-charged nitrocellulose membrane. Immobilized DNA was fixed by baking the membrane for 30 minutes at 80 °C followed by blocking (5 % non-fat dry milk in TBST) to avoid non-specific binding. Subsequently, membrane was incubated with anti-cyclobutane pyrimidine dimers (CPDs) antibody for 2 h at room temperature, washed with TBST and incubated with HRP-conjugated secondary antibody. The membrane was processed with ECL plus detection kit (Thermo Scientific) and signal detected using an LAS-3000 image analyzer (Fuji Photo Film Co., Tokyo, Japan).

Cytostaining of CPD-positive cells

UVB-induced DNA damage in the form of CPD-positive cells was detected as described earlier²².

Cell-cycle analysis

Cells were synchronized using serum free medium for 48 h. Subsequently, treated with AgNPs (1 µg/mL) in regular medium for 3 h prior to UVB-irradiation (40 mJ/cm²). Following 24 h post UVB-irradiation, percentage of cell population in various phases of cell-cycle was determined as described earlier²¹.

Cellular internalization of AgNPs

HaCaT cells (1×10⁶/plate) were seeded in glass plates and allowed to grow overnight. Cells were then treated with AgNPs (1 µg/mL) for 3 h prior to UVB-exposure (40 mJ/cm²) and harvested 24 h post UVB-irradiation. Cell pellets were washed (PBS) and fixed (4 % paraformaldehyde and 2.5 % glutaraldehyde) overnight at 4 °C followed by post-fixation in 1 % osmium tetroxide for 1.5 h. Cells pellets were further dehydrated using graded ethanol (30-100 %; ×2) for 20 minutes at each step. Following ethanol dehydration cell pellets were treated with propylene oxide (PO; ×2; 20 minutes) and stepwise infiltration of Epon resin (3 PO: 1 resin; 1 PO: 1 resin; 1 PO: 3 resin) was performed. After Epon resin infiltration, cell pellets were further infiltrated with fresh resin and polymerized at 60 °C for 18 h. After polymerization, ultra-thin sections (60-70 nm) were cut on a Leica Ultracut T ultramicrotome and picked up on copper grids. Sections were subsequently stained with 2 % uranyl acetate and 0.2 % Reynolds lead citrate and examined under Zeiss EM10C transmission electron microscope equipped with a SIA-LC3 CCD camera at 60 kV.

RNA Isolation and RT-PCR

Total RNA was isolated and cDNA was synthesized using RNeasy purification and high-capacity cDNA reverse transcription kit, respectively. Quantitative real-time PCR was performed in 96-well plates using SYBR Green master mix on an iCycler system (Bio-Rad) as described²³ using following PCR primers: XPA, 5'-AATGTAAAAGCAGCCCCAAA-3'-F and 5'-TTTCCACATTCTTCGCATA-3'-R; XPC, 5'-AGAAACCCCAAGAAGAGC-3'-F and 5'-AGCCCCTCTTCAGATGGT-3'-R; RPA1, 5'-GGCAATCCAGTGCCTATAA-3'-F and 5'-ACTGTGTCCCCAGAAGTG-3'-R; DDB2, 5'-TCAAGGACAAACCCACCTTC-3'-F and 5'-GTGACCACCATTCGGCTACT-3'-R; and GAPDH, 5'-GCTGTGTGGCAAAGTCCAA-3'-F and 5'-GGTCAGGCTCCTGGAAGATA-3'-R.

Immunoblot analysis

Cells were processed for protein extraction and Western blotting as described earlier²³ using specific antibodies against various proteins. Primary antibodies were used at 1:200 dilutions except for Bcl-2, Bcl-xL, Bax, and anti-CPD (1:1000), whereas all secondary antibodies were used at 1:2500 dilution. β-actin was used as loading control at a dilution of 1:20000. Blots were processed with ECL plus Western blotting detection kit and the signal detected using an LAS-3000 image analyzer.

Reactive oxygen species (ROS) generation

HaCaT cells (1×10^6 /plate) were seeded in glass plates. After overnight incubation, cells were treated with AgNPs ($1 \mu\text{g/mL}$) for 3 h prior to UVB-irradiation (40 mJ/cm^2). After 24 hours, cells were incubated with DCFH-DA and ROS was measured as described earlier²³.

Statistical analysis

Each experiment was performed at least three times, independently. All the values were expressed as 'mean $\pm$ standard deviation'. Wherever appropriate, the data were also subjected to unpaired two tailed Student's t-test. A value of $p < 0.05$ was considered as significant.

RESULTS

Physicochemical characterization and toxicity evaluation of silver nanoparticles

The synthesized AgNPs showed a characteristic surface plasmon peak at 430 nm (Fig. 1A). TEM studies revealed that AgNPs were spherical in shape ($> 90\%$ particles) and in the size range of 10-50 nm with an average size of 24.24 nm (Fig. 1B). Dynamic light scattering studies showed that average hydrodynamic diameter of nanoparticles was 53.85 ± 1.63 nm (Fig. 1C) with an average zeta potential of -47.7 ± 0.9 mV (Fig. 1D). The stability of synthesized AgNPs was monitored over the course of six months by observing the characteristic plasmon peak. Data demonstrated that formulation of synthesized AgNPs was stable for at least six months (Supplemental Fig. 1). To examine the toxicity of AgNPs, HaCaT cells were treated with different doses of AgNPs (0.5 - $80 \mu\text{g/mL}$). No toxicity was observed after 12 h of treatment with AgNPs even at the highest dose ($80 \mu\text{g/mL}$) and only less than 10 % and 30 % toxicities were observed at this dose after 24 h and 48 h, respectively (Supplemental Fig. 2). Moreover, when we replenished the medium after 12 h incubation of HaCaT cells with AgNPs, no significant toxicities were observed after subsequent 24 h and 48 h (data not shown). Altogether, these data suggest that AgNPs are largely non-toxic to human keratinocytes.

UVB-induced cell death is inhibited by silver nanoparticles

HaCaT cells were incubated with different doses of AgNPs (0.5 - $2.0 \mu\text{g/mL}$) prior to UVB irradiations (40 mJ/cm^2) and the effect on morphology was examined by phase-contrast microscopy. Cells irradiated with UVB showed rounded cell morphology, cell shrinkage and detachment from the substratum. These changes in cell morphology are characteristic features of adherent cells undergoing cell death by apoptosis²⁴. Cells that were pre-treated with AgNPs largely retained their morphology (Fig. 2A). We next examined the protective role of AgNPs on UVB-induced cell death by trypan blue dye-exclusion method. The data show that the number of viable cells was remarkably low ($\sim 63.0 \pm 2\%$) upon UVB-irradiation as compared to control cells ($\sim 95.3 \pm 0.5\%$). Importantly, the viability of UVB-irradiated HaCaT cells was significantly higher ($\sim 82.6 \pm 1.5$, 94.6 ± 1.1 and $94 \pm 0.5\%$, respectively), when pre-treated with AgNPs (0.5 - $2 \mu\text{g/mL}$) (Fig. 2B). This observation was further confirmed by Annexin V staining followed by flow cytometry. We observed that irradiation of HaCaT cells with UVB causes significant higher apoptosis ($\sim 41.6\%$) as

compared to non-irradiated cells (~7.4 %), whereas only ~10.7 % apoptotic cells were observed upon AgNPs pretreatment (Fig. 2C). Together, these findings suggest a chemopreventive role of AgNPs against UVB-induced skin cell injury.

Silver nanoparticles protect human keratinocytes from UVB-induced DNA damage

The direct effect of UVB radiation on cellular DNA is caused by the formation of dimeric photoproducts between adjacent pyrimidine bases on the same strand²⁵. CPDs are considered as a major class of UVB-induced harmful lesions³. To determine if AgNPs are able to protect the HaCaT cells from UVB-induced DNA damage, we examined the formation of CPDs. Cells were irradiated to UVB (40 mJ/cm²) with or without prior treatment of AgNPs (1 µg/mL) and CPD-positive cells were detected by cytostaining after 24 h of UVB-exposure. The numbers of CPD-positive cells were significantly lowered in the AgNPs pre-treated cells as compared to UVB alone (Fig. 3A). The protective effect of AgNPs on UVB-induced DNA damage was further confirmed by performing a dot-blot assay. Our data clearly show that there is no formation of CPDs in UVB-irradiated cells that were pre-treated with AgNPs, thus suggesting a role of AgNPs in UVB-induced DNA damage protection (Fig. 3B). Altogether, these data suggest that AgNPs protect UVB-induced DNA damage in HaCaT cells.

Silver nanoparticles treatment leads to G1/S phase cell-cycle arrest of UVB-irradiated human keratinocytes

Cell-cycle progression is a tightly controlled process. To protect the integrity of the cells, DNA damage resulting from genotoxic insults leads to the activation of DNA repair pathways and cell-cycle arrest to allow time for DNA repair²⁶. Our data on cell-cycle distribution revealed that UVB-irradiation resulted in accumulation of HaCaT cells in the G2-M phase of the cell-cycle (~4.14 folds higher as compared to control cells) (Fig. 4). Interestingly, while treatment with AgNPs alone had a little effect on cell-cycle distribution, AgNP-treatment prior to UVB-irradiation caused significant accumulation of HaCaT cells in the G1/S phase (~6.0 folds higher as compared to UVB-exposed cells) (Fig. 4). These data suggest that AgNPs arrest the cells at first checkpoint (prior to DNA duplication) to facilitate repair of UVB-induced CPDs formation.

Silver nanoparticles treatment decreases basal and UVB-induced ROS production

UV radiation-induced ROS generation is known to affect regulation of cell-cycle and apoptosis. Moreover, effects of AgNPs in relation to oxidative stress have been explored in many studies^{27,28}. Therefore, we examined the effect of AgNPs treatment on ROS levels in HaCaT cells. The data suggest that AgNPs treatment leads to the suppression of basal ROS levels in HaCaT cells. Furthermore, as expected UVB exposure significantly enhanced ROS levels in HaCaT cells and this effect was abolished in cells that had been pre-treated with AgNPs (Supplemental Fig. 3).

AgNPs exhibit enhanced internalization upon UVB exposure of human keratinocytes and are biologically active

The cell-cycle arrest at G1/S phase of AgNPs pre-treated HaCaT cells upon UVB-irradiation indicates that AgNPs might have some biological activity. To support this, we next performed TEM analyses of HaCaT cells treated with AgNPs with or without subsequent exposure to UVB radiation. We observed the presence of few dark, electron dense, spherical AgNPs in HaCaT cells treated with AgNPs alone. Interestingly, the number of AgNPs was remarkably higher in HaCaT cells that had been subsequently exposed to UVB radiation (Fig. 5). The majority of AgNPs were found to be distributed throughout the cytoplasm and membrane bound cellular structures including nucleus of UVB-irradiated HaCaT cells (Fig. 5). To further gain evidence that the chemopreventive effect of AgNPs was indeed due to its biological activity and not merely a result of UVB absorption, we treated HaCaT cells with AgNPs post-UVB exposure. We observed that post-treatment of AgNPs was also significantly effective in repairing UVB-induced DNA damage (Supplemental Fig. 4). To provide mechanistic support for the biological activity of AgNPs, we next examined the effect on the expression of key proteins involved in cell proliferation and survival. We observed that expression of cyclin B1, CDK1 and p21 was decreased in UVB-irradiated HaCaT cells in comparison to control cells, however expression of cyclin E1 and CDK2 did not alter significantly (Fig. 6A). On the other hand, when we pre-treated the HaCaT cells with AgNPs prior to UVB-irradiation, expression of cyclin B1, CDK1 and p21 was effectively restored, while we observed a decrease in the expression of cyclin E1 and CDK2 (Fig. 6A). Among the survival proteins, we observed a downregulation of anti-apoptotic proteins, Bcl-2 and Bcl-xL, in UVB-irradiated HaCaT cells, while the expression of pro-apoptotic protein Bax was increased (Fig. 6B). Expression of these proteins in HaCaT cells pre-treated with AgNPs prior to UVB-irradiation was relatively similar to untreated or AgNPs-only treated cells (Fig. 5A), thus maintaining the ratio of Bax/Bcl-2 and Bax/Bcl-xL (Fig. 6B). Moreover, we also analyzed the expression of key genes of NER viz. XPA, XPC, DDB2 and RPA1 involved in CPDs repair. The irradiation of the HaCaT cells to UVB caused slight increase (not significant) in levels of NER genes compared to non-irradiated cells (Supplemental Fig. 5). Whereas, treatment of HaCaT cells with AgNPs prior to UVB-irradiation resulted in significantly ($p < 0.05$) enhanced expression of NER genes (4.1 fold in XPA; 3.6 fold in XPC; 3.8 fold in RPA1; 3.3 fold in DDB2) as compared to untreated UVB irradiated HaCaT cells (Supplemental Fig. 5). Altogether, our data suggests that AgNPs protect the HaCaT cells from UVB-induced DNA damage by modulating the expression of various genes involved in cell-cycle, apoptosis and nucleotide excision repair.

DISCUSSION

Solar UVB radiation is a well-known cause for more than 90 % of skin cancers^{29,30}. Therefore, several approaches have been tested and are currently being used or are under investigation to protect the skin cells from deleterious effects of UVB radiations. Ultimate goal is to minimize the effect of UV either by limiting the exposure of skin cells by use of sunscreen formulations or by interfering with subsequent steps involved in skin carcinogenesis and progression. Here, we tested the efficacy of AgNPs in limiting UVB-

induced DNA damage and apoptosis of skin cells and made several interesting findings that provide strong support for the utility of AgNPs for chemopreventive applications.

It is known that direct exposure of skin to UVB radiation causes DNA damage due to formation of CPDs, pyrimidine dimers (PDs), cytosine photohydrates, and purine photoproduct^{3,25}. Among these, CPDs are the most lethal lesions and their formation is known to trigger skin carcinogenesis by inducing gene mutations^{22,25}. These lesions are usually repaired during the G1 phase of cell-cycle to avoid incorporation of faulty bases during DNA synthesis^{31,32}. The second checkpoint is G2/M phase, which restrict multiplication of genetically corrupted cells³¹. Checkpoint-arrested cells resume cell-cycle progression once the damage has been repaired, whereas cells with unrepairable DNA lesions undergo permanent cell-cycle arrest and subsequent apoptosis or malignant transformation^{5,6}. We observed that pre-treatment of HaCaT cells with AgNPs led to abrogation of CPDs formation, which correlated with induction of G1 phase arrest and suppression of apoptotic cell death upon UVB-irradiation. More importantly, these changes were observed at a low concentration of AgNPs (1 µg/mL), which did not show any toxicity in HaCaT cells even after 48 h of treatment. In some other reports, AgNPs have been shown to exert toxic effects on different types of cells, including keratinocytes^{33,34}. Therefore, it seems that the toxicity of AgNPs may depend on their physicochemical factors including surface-coating. In one recent study, cytotoxicity of uncoated and carbon-coated silver nanoparticles were tested against human epidermal keratinocytes and the latter ones were found to be non-toxic as compared to uncoated AgNPs³⁴. In other published studies, it was demonstrated that citrate-coated colloidal AgNPs were not cytotoxic, genotoxic and phototoxic to HaCaT cells even after treatment with 100 µg/mL for 24 hours³⁵. In the present study, we also used trisodium citrate as a coating agent, which could be one reason for their non-toxic nature. Other concerns related to safety of AgNPs may arise related to its entry into systemic circulation if applied on skin with damaged skin barrier and its possible adverse effect on the skin flora, which is very important for skin homeostasis. However, existing data suggest that AgNPs, if they reach systemic circulation, can be cleared out through urinary and fecal excretion^{36,37}. Moreover, in other studied topical application of AgNPs on damaged skin for wound healing purposes in preclinical mouse models were shown produce no toxic effects^{38,39}. Furthermore, Tyagi et al., (2013) have reported that AgNPs are biocompatible and friendlier to the human body microflora even after their ingestion⁴⁰. Therefore, our findings together with these previous observations generate enthusiasm for potential future human applications of AgNPs as chemopreventive agents.

Cell-cycle is regulated by collective activities of cyclins, CDKs and CDK inhibitors^{41,42}. Cyclin E1/CDK2 and cyclin D1/CDK4 complexes play key role in G1/S phase transition, whereas, interaction between cyclin B1 and CDK1 is crucial for cell-cycle progression through G2 to M phase^{42,43}. From the mechanistic standpoint, it has been reported that UVB-irradiation of mammalian cells results in the enhanced levels of p53, a growth suppressor transcription factor⁴⁴. p53, in turn, induces the expression of *p21/WAF1/CIP1*, encoding CDK inhibitor 1 or CDK-interacting protein 1 or p21, which then leads to G1 phase cell-cycle arrest⁴⁴⁻⁴⁶. The present study demonstrated that UVB-irradiation resulted in G2/M and not G1/S phase arrest of cell-cycle in HaCaT cells. This is likely due to the fact

that HaCaT cells have p53 mutations in both the alleles^{46,47}, and therefore are not capable of achieving enhanced p21 levels. However, interesting observation was that the exposure of HaCaT cells to the UVB radiations instead resulted in the downregulation of p21. A similar response has also been reported by other investigators in HaCaT cells upon their exposure to UVB radiations^{48,49}. While we still have to investigate this observation in our future studies, it has been suggested that UVB- exposure activates E3 ubiquitin-protein ligase MDM2 and Glycogen synthase kinase 3 beta (GSK3 β), which then promote p21 degradation⁵⁰.

In another intriguing finding, we observed that while the treatment of AgNPs alone did not significantly alter the expression of cell-cycle-associated proteins, it led to downregulation of cyclin E1 and CDK2 in UVB-irradiated HaCaT cells. Moreover, pretreatment of AgNPs sustained the levels of cyclin B1, CDK1 and p21 in UVB-irradiated HaCaT cells to that observed in untreated or AgNPs- only treated cells. These findings correlated with G1/S phase cell-cycle arrest of HaCaT cells, which had been treated with AgNPs prior to UVB-irradiation, an effect that was likely mediated through the inhibition of G1/S phase- specific cyclin E1 and CDK2, while p21 expression remained unchanged⁵¹. Further studies are; however, required to dissect the mechanisms through which AgNPs led to downregulation of cyclin E1 and CDK2 specifically in HaCaT cells that were later exposed to UVB radiations. It will also be interesting to investigate how AgNPs pre-treated HaCaT cells were able to maintain the expression of cyclin B1, CDK1 and p21 upon UVB-irradiation. It might be possible that AgNPs interferes with UVB-induced MDM2 and GSK3 β activation⁵⁰, or works through other, yet unexplored, pathways responsible for the regulation of these proteins. In various studies, p53-independent induction of p21 expression by several agents including tissue plasminogen activator, dimethyl sulfoxide, trans-retinoic acid, butyrate, okadaic acid, epidermal growth factor and transforming growth factor- β has been reported in cell lines expressing no or mutant p53⁵²⁻⁵⁴. The mechanism of p21 upregulation by these agents was not clear; however, it was suggested that it might be due to activation of pre-existing transcription factors as its level remained unaffected by the treatment with protein synthesis inhibitor, cyclohexamide^{41,42}.

The process of apoptosis is controlled by a fine balance between the expression of pro-apoptotic (e.g., Bax) and anti-apoptotic proteins (e.g., Bcl-2 and Bcl-xL) those subsequently maintain the cell survival^{21,55}. As mentioned earlier, UVB-irradiation of skin cells causes significant apoptotic death of skin cells, which on one hand, serves as a mechanism of photodamaged cell elimination, but also leads to skin injury⁵. In our study, we observed that pre-treatment of HaCaT cells with AgNPs prevented UVB-induced apoptosis to several folds. This is significant considering the fact that AgNPs pretreatment also protected the cells from UVB-induced DNA damage. Our data also suggested that UVB-irradiation resulted in an increased Bax/Bcl-2 and Bax/Bcl-xL ratio, while it remained largely unaffected in AgNPs pre-treated HaCaT cells. Decreased expression of Bcl-2 and Bcl-xL and increased expression of Bax upon UVB-irradiation is supported by similar findings made in earlier studies^{48,49}. However, our data is clearly suggestive of a neutralizing effect of AgNPs on UVB-induced apoptosis-associated protein expression, which could underlie the observed reduction in apoptosis.

Our data also revealed that AgNPs are internalized by the HaCaT cells and this process is promoted in UVB-irradiated cells. Previously, the internalization of AgNPs in other cell types has also been demonstrated^{56,57}. However, it is not yet clear how UVB radiation promote the uptake of AgNPs by HaCaT cells. It is possible that sudden exposure of HaCaT cells with UVB might induce the formation of pores in the cellular membranes making them more receptive to outside elements. This is supported by the fact that the majority of AgNPs inside the cells appeared as individual particles suggesting that they entered the HaCaT cells possibly through direct penetration routes. However, active transport routes might exist as well and further experiments are required to confirm these different possibilities. In relation to observed biological activity of AgNPs, we speculate that AgNPs may influence the function of various proteins directly or indirectly through their interactions with them as has been reported previously⁵⁸. Since, we also observed the presence of AgNPs within the nucleus, we hypothesize their interaction with transcription factor proteins or with DNA⁵⁹ resulting in the altered gene expression. Moreover, AgNPs-pretreatment resulted in suppression of basal as well as UVB radiation-induced production of ROS, which could indirectly result in change in gene expression as demonstrated previously^{3,60}. However, it will be interesting to know in future studies whether AgNPs target specific proteins and if so, what is the underlying molecular mechanism(s).

In summary, our findings have suggested a promising role of AgNPs in protection of human skin cells from UVB-induced DNA damage and apoptosis. These effects are correlated with G1 phase cell-cycle arrest as well as altered expression of cell-cycle- and survival-associated proteins. Further, UVB-irradiation results in enhanced cellular uptake of AgNPs. Mechanistically, AgNPs enhances the mRNA expression of NER genes that are involved in DNA repair of UVB-irradiated human keratinocytes. Even though future studies are warranted to test the efficacy of AgNPs in preclinical models and more clearly define the underlying mechanisms, our data is clearly suggestive of their potential as effective chemopreventive agents against UVB-induced skin carcinogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Lazovich D, Choi K, Vogel RI. Time to get serious about skin cancer prevention. *Cancer Epidemiol Biomarkers Prev.* 2012; 21:1893–901. [PubMed: 22962407]
2. Lomas A, Leonardi-Bee J, Bath-Hextall F. A systematic review of worldwide incidence of nonmelanoma skin cancer. *Br J Dermatol.* 2012; 166:1069–80. [PubMed: 22251204]
3. Cadet J, Sage E, Douki T. Ultraviolet radiation-mediated damage to cellular DNA. *Mutat Res.* 2005; 571:3–17. [PubMed: 15748634]

4. Lucas RM, McMichael AJ, Armstrong BK, Smith WT. Estimating the global disease burden due to ultraviolet radiation exposure. *Int J Epidemiol*. 2008; 37:654–67. [PubMed: 18276627]
5. Matsumura Y, Ananthaswamy HN. Toxic effects of ultraviolet radiation on the skin. *Toxicol Appl Pharmacol*. 2004; 195:298–308. [PubMed: 15020192]
6. Murphy G, Young AR, Wulf HC, Kulms D, Schwarz T. The molecular determinants of sunburn cell formation. *Exp Dermatol*. 2001; 10:155–60. [PubMed: 11380610]
7. Burnett ME, Wang SQ. Current sunscreen controversies: a critical review. *Photodermatol Photoimmunol Photomed*. 2011; 27:58–67. [PubMed: 21392107]
8. Krause M, Klit A, Blomberg JM, Soeborg T, Frederiksen H, Schlumpf M, et al. Sunscreens: are they beneficial for health? An overview of endocrine disrupting properties of UV-filters. *Int J Androl*. 2012; 35:424–36. [PubMed: 22612478]
9. Smijs TG, Pavel S. Titanium dioxide and zinc oxide nanoparticles in sunscreens: focus on their safety and effectiveness. *Nanotechnol Sci Appl*. 2011; 4:95–112. [PubMed: 24198489]
10. Ghosh M, Chakraborty A, Mukherjee A. Cytotoxic, genotoxic and the hemolytic effect of titanium dioxide (TiO₂) nanoparticles on human erythrocyte and lymphocyte cells in vitro. *J Appl Toxicol*. 2013; 33:1097–110. [PubMed: 23616399]
11. Yu KN, Yoon TJ, Minai-Tehrani A, Kim JE, Park SJ, Jeong MS, et al. Zinc oxide nanoparticle induced autophagic cell death and mitochondrial damage via reactive oxygen species generation. *Toxicol In Vitro*. 2013; 27:1187–95. [PubMed: 23458966]
12. Bhol KC, Alroy J, Schechter PJ. Anti-inflammatory effect of topical nanocrystalline silver cream on allergic contact dermatitis in a guinea pig model. *Clin Exp Dermatol*. 2004; 29:282–7. [PubMed: 15115512]
13. Jain J, Arora S, Rajwade JM, Omray P, Khandelwal S, Paknikar KM. Silver nanoparticles in therapeutics: development of an antimicrobial gel formulation for topical use. *Mol Pharm*. 2009; 6:1388–401. [PubMed: 19473014]
14. Ip M, Lui SL, Poon VK, Lung I, Burd A. Antimicrobial activities of silver dressings: an in vitro comparison. *J Med Microbiol*. 2006; 55:59–63. [PubMed: 16388031]
15. Jones SA, Bowler PG, Walker M, Parsons D. Controlling wound bioburden with a novel silver-containing Hydrofiber dressing. *Wound Repair Regen*. 2004; 12:288–94. [PubMed: 15225207]
16. Gunasekaran T, Nigusse T, Dhanaraju MD. Silver Nanoparticles as Real Topical Bullets for Wound Healing. *J Am Coll Clin Wound Spec*. 2012; 3:82–96. [PubMed: 24527370]
17. Kokura S, Handa O, Takagi T, Ishikawa T, Naito Y, Yoshikawa T. Silver nanoparticles as a safe preservative for use in cosmetics. *Nanomedicine*. 2010; 6:570–4. [PubMed: 20060498]
18. Munger MA, Radwanski P, Hadlock GC, Stoddard G, Shaaban A, Falconer J, et al. In vivo human time-exposure study of orally dosed commercial silver nanoparticles. *Nanomedicine*. 2014; 10:1–9. [PubMed: 23811290]
19. Chen X, Parker SG, Zou G, Su W, Zhang Q. beta-cyclodextrin-functionalized silver nanoparticles for the naked eye detection of aromatic isomers. *ACS Nano*. 2010; 4:6387–94. [PubMed: 20973513]
20. Bhardwaj A, Srivastava SK, Singh S, Arora S, Tyagi N, Andrews J, et al. CXCL12/CXCR4 signaling counteracts docetaxel-induced microtubule stabilization via p21-activated kinase 4-dependent activation of LIM domain kinase 1. *Oncotarget*. 2014; 5:11490–500. [PubMed: 25359780]
21. Tyagi N, Bhardwaj A, Singh AP, McClellan S, Carter JE, Singh S. p-21 activated kinase 4 promotes proliferation and survival of pancreatic cancer cells through AKT- and ERK-dependent activation of NF-kappaB pathway. *Oncotarget*. 2014; 5:8778–89. [PubMed: 25238288]
22. Katiyar SK, Mantena SK, Meeran SM. Silymarin protects epidermal keratinocytes from ultraviolet radiation-induced apoptosis and DNA damage by nucleotide excision repair mechanism. *PLoS One*. 2011; 6:e21410. [PubMed: 21731736]
23. Arora S, Bhardwaj A, Singh S, Srivastava SK, McClellan S, Nirodi CS, et al. An undesired effect of chemotherapy: gemcitabine promotes pancreatic cancer cell invasiveness through reactive oxygen species-dependent, nuclear factor kappaB- and hypoxia-inducible factor 1alpha-mediated up-regulation of CXCR4. *J Biol Chem*. 2013; 19(288):21197–207. [PubMed: 23740244]

24. Desjardins LM, MacManus JP. An adherent cell model to study different stages of apoptosis. *Exp Cell Res*. 1995; 216:380–7. [PubMed: 7843282]
25. Sinha RP, Hader DP. UV-induced DNA damage and repair: a review. *Photochem Photobiol Sci*. 2002; 1:225–36. [PubMed: 12661961]
26. Lindahl T, Wood RD. Quality control by DNA repair. *Science*. 1999; 286:1897–905. [PubMed: 10583946]
27. Patlolla AK, Hackett D, Tchounwou PB. Silver nanoparticle-induced oxidative stress-dependent toxicity in Sprague-Dawley rats. *Mol Cell Biochem*. 2015; 399:257–68. [PubMed: 25355157]
28. Schlinkert P, Casals E, Boyles M, Tischler U, Hornig E, Tran N, et al. The oxidative potential of differently charged silver and gold nanoparticles on three human lung epithelial cell types. *J Nanobiotechnology*. 2015; 13:1. [PubMed: 25592092]
29. Armstrong BK, Krickler A. How much melanoma is caused by sun exposure? *Melanoma Res*. 1993; 3:395–401. [PubMed: 8161879]
30. Saraiya M, Glanz K, Briss PA, Nichols P, White C, Das D, et al. Interventions to prevent skin cancer by reducing exposure to ultraviolet radiation: a systematic review. *Am J Prev Med*. 2004; 27:422–66. [PubMed: 15556744]
31. Branzei D, Foiani M. Regulation of DNA repair throughout the cell cycle. *Nat Rev Mol Cell Biol*. 2008; 9:297–308. [PubMed: 18285803]
32. de LA, van TM, van der Leun JC, van Vloten WA, de Gruijl FR. Cell cycle kinetics following UVA irradiation in comparison to UVB and UVC irradiation. *Photochem Photobiol*. 1996; 63:492–7. [PubMed: 8934764]
33. Mukherjee SG, O'Clonadh N, Casey A, Chambers G. Comparative in vitro cytotoxicity study of silver nanoparticle on two mammalian cell lines. *Toxicol In Vitro*. 2012; 26:238–51. [PubMed: 22198051]
34. Samberg ME, Oldenburg SJ, Monteiro-Riviere NA. Evaluation of silver nanoparticle toxicity in skin in vivo and keratinocytes in vitro. *Environ Health Perspect*. 2010; 118:407–13. [PubMed: 20064793]
35. Lu W, Senapati D, Wang S, Tovmachenko O, Singh AK, Yu H, et al. Effect of Surface Coating on the Toxicity of Silver Nanomaterials on Human Skin Keratinocytes. *Chem Phys Lett*. 2010; 487:10.
36. Prabhu, SaPEK. Silver nanoparticles: mechanism of antimicrobial action, synthesis, medical applications, and toxicity effects. *International Nano Letters*. 2013; 2
37. DiVincenzo GD, Giordano CJ, Schriever LS. Biologic monitoring of workers exposed to silver. *Int Arch Occup Environ Health*. 1985; 56:207–15. [PubMed: 4066049]
38. Jain J, Arora S, Rajwade JM, Omay P, Khandelwal S, Paknikar KM. Silver nanoparticles in therapeutics: development of an antimicrobial gel formulation for topical use. *Mol Pharm*. 2009; 6:1388–401. [PubMed: 19473014]
39. Tian J, Wong KK, Ho CM, Lok CN, Yu WY, Che CM, et al. Topical delivery of silver nanoparticles promotes wound healing. *ChemMedChem*. 2007; 2:129–36. [PubMed: 17075952]
40. Tyagi PK, Tyagi S, Verma C, Rajpal. Estimation of toxic effects of chemically and biologically synthesized silver nanoparticles on human gut microflora containing *Bacillus subtilis*. *Mol Pharm*. 2013; 9:172–7.
41. Kastan MB, Bartek J. Cell-cycle checkpoints and cancer. *Nature*. 2004; 432:316–23. [PubMed: 15549093]
42. Satyanarayana A, Kaldis P. Mammalian cell-cycle regulation: several Cdks, numerous cyclins and diverse compensatory mechanisms. *Oncogene*. 2009; 20(28):2925–39. [PubMed: 19561645]
43. Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif*. 2003; 36:131–49. [PubMed: 12814430]
44. Lu X, Lane DP. Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes? *Cell*. 1993; 19(75):765–78. [PubMed: 8242748]
45. Liu M, Pelling JC. UV-B/A irradiation of mouse keratinocytes results in p53-mediated WAF1/CIP1 expression. *Oncogene*. 1995; 10:1955–60. [PubMed: 7761096]

46. Petrocelli T, Poon R, Drucker DJ, Slingerland JM, Rosen CF. UVB radiation induces p21Cip1/WAF1 and mediates G1 and S phase checkpoints. *Oncogene*. 1996; 12:1387–96. [PubMed: 8622854]
47. Latonen L, Taya Y, Laiho M. UV-radiation induces dose-dependent regulation of p53 response and modulates p53-HDM2 interaction in human fibroblasts. *Oncogene*. 2001; 20:6784–93. [PubMed: 11709713]
48. El-Mahdy MA, Zhu Q, Wang QE, Wani G, Patnaik S, Zhao Q, et al. Naringenin protects HaCaT human keratinocytes against UVB-induced apoptosis and enhances the removal of cyclobutane pyrimidine dimers from the genome. *Photochem Photobiol*. 2008; 84:307–16. [PubMed: 18086244]
49. Reagan-Shaw S, Breur J, Ahmad N. Enhancement of UVB radiation-mediated apoptosis by sanguinarine in HaCaT human immortalized keratinocytes. *Mol Cancer Ther*. 2006; 5:418–29. [PubMed: 16505117]
50. Lei X, Liu B, Han W, Ming M, He YY. UVB-Induced p21 degradation promotes apoptosis of human keratinocytes. *Photochem Photobiol Sci*. 2010; 9:1640–8. [PubMed: 20931139]
51. Abukhdeir AM, Park BH. P21 and p27: roles in carcinogenesis and drug resistance. *Expert Rev Mol Med*. 2008; 10:e19. [PubMed: 18590585]
52. Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y, Wang XF. Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proc Natl Acad Sci U S A*. 1995; 92:5545–9. [PubMed: 7777546]
53. Jiang H, Lin J, Su ZZ, Collart FR, Huberman E, Fisher PB. Induction of differentiation in human promyelocytic HL-60 leukemia cells activates p21, WAF1/CIP1, expression in the absence of p53. *Oncogene*. 1994; 9:3397–406. [PubMed: 7936668]
54. Michieli P, Chedid M, Lin D, Pierce JH, Mercer WE, Givol D. Induction of WAF1/CIP1 by a p53-independent pathway. *Cancer Res*. 1994; 54:3391–5. [PubMed: 8012956]
55. Wood WG, Igbavboa U, Muller WE, Eckert GP. Statins, Bcl-2, and apoptosis: cell death or cell protection? *Mol Neurobiol*. 2013; 48:308–14. [PubMed: 23821030]
56. Arora S, Jain J, Rajwade JM, Paknikar KM. Interactions of silver nanoparticles with primary mouse fibroblasts and liver cells. *Toxicol Appl Pharmacol*. 2009; 236:310–8. [PubMed: 19269301]
57. Gliga AR, Skoglund S, Wallinder IO, Fadeel B, Karlsson HL. Size-dependent cytotoxicity of silver nanoparticles in human lung cells: the role of cellular uptake, agglomeration and Ag release. *Part Fibre Toxicol*. 2014; 11:11. [PubMed: 24529161]
58. Asharani P, Sethu S, Lim HK, Balaji G, Valiyaveetil S, Hande MP. Differential regulation of intracellular factors mediating cell cycle, DNA repair and inflammation following exposure to silver nanoparticles in human cells. *Genome Integr*. 2012; 3:2–3. [PubMed: 22321936]
59. Basu S, Jana S, Pande S, Pal T. Interaction of DNA bases with silver nanoparticles: assembly quantified through SPRs and SERS. *J Colloid Interface Sci*. 2008; 321:288–93. [PubMed: 18346751]
60. Godic A, Poljsak B, Adamic M, Dahmane R. The role of antioxidants in skin cancer prevention and treatment. *Oxid Med Cell Longev*. 2014; 2014:860479. doi: 10.1155/2014/860479. [PubMed: 24790705]

Figure 1. Physicochemical characterization of silver nanoparticles

(A) UV-visible absorption spectra of AgNPs. (B) The transmission electron microscopic image of AgNPs. Scale bar is 100 nm. Dynamic light scattering image of AgNPs colloidal solution showing (C) hydrodynamic diameter distribution and (D) zeta potential. Three curves in panel C and D are reflective of three measurements.

Figure 2. Silver nanoparticles pretreatment results in reduction of UVB-induced cell death and apoptosis

HaCaT cells (1×10^6 /plate) were grown in UV transparent glass plates and treated with AgNPs 3 h prior to UVB-exposure. After 24 h of UVB exposure (A) Cells were examined under phase-contrast microscope. Cells not irradiated with UVB were used as a control. Arrows indicate apoptotic cells. Representative pictures are from one of the random fields of view (magnification X200) of cells. (B) Percent viable cells in various treatment groups were determined using trypan blue dye-exclusion method. Bars represent mean $\pm$ SD, $n=3$; * $P<0.05$; ** $P<0.01$. (C) Percentage of early apoptotic cells in each treatment group was calculated as described in methods. Bars represent mean $\pm$ SD, $n=3$.

Figure 3. Silver nanoparticles reduce the formation and/or repair of CPDs in UVB-exposed Keratinocytes

HaCaT cells (1×10^6 /plate) were grown in UV transparent glass plates and treated with AgNPs 3 h prior to UVB-exposure. Cells were harvested 24 h later, and **(A)** Subjected to cytochemical staining of CPD-positive cells as described in methods. CPD-positive cells are dark brown in color, magnification $\times 100$. **(B)** Genomic DNA from cells was subjected to dot-blot analysis using an antibody specific to CPDs.

Figure 4. Silver nanoparticles treatment leads to cell-cycle arrest in G1 phase
Synchronized cells were treated with AgNPs in serum-containing media for 3 h before UVB irradiation. Cells were harvested 24 h later and distribution of cells in different phases of cell-cycle was analyzed by PI staining followed by flow cytometry.

Figure 5. UVB-exposure enhances the cellular internalization of AgNPs in human keratinocytes Transmission electron micrographs of ultramicrotome slices of HaCaT cells showing cytoplasmic and nuclear localization of AgNPs after treatment with AgNPs (1 $\mu\text{g/mL}$) with or without UVB-irradiation (40 mJ/cm^2). Arrows indicate AgNPs within the cell. Scale bar is 1 μm .

Figure 6. Silver nanoparticles treatment modulates the expression of cell survival and cycle-related proteins in human keratinocytes

Cells were treated with AgNPs for 3 h prior to UVB-exposure. After 24 h, total cellular protein was isolated and subjected to immunoblot analysis for (A) Cell-cycle and (B) survival -associated proteins followed by densitometry of immunoreactive bands. β-actin served loading control. Normalized densitometric values are indicated at the top of the bands. Bar (mean ± SD, n=3) diagram represents the effects of AgNPs treatment prior to UVB-exposure on Bax/Bcl-2 ratio and Bax/Bcl-xL ratio.