Anti-glycation Effect of Gold Nanoparticles on Collagen

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Gold nanoparticles (GNPs) have been reported to exhibit a variety of biological effects including anti-inflammatory and anti-oxidant activities. The extent of an *in vitro* glycation reaction mixture of collagen and glycolaldehyde was assayed to investigate the inhibition of glycolaldehye-derived advanced glycation end products (glycol-AGEs) formation with GNPs in collagen, which is a major protein component of the human dermis. GNP-treated collagen showed significantly less glycation (56.3±4.2%) than an untreated glycation control. Moreover, GNP-treated glycation in a collagen lattice model significantly decreased the AGEs distribution in the model system. Taken together, these results suggest that GNPs have the potential for use in the prevention of glycation-induced skin aging.

Key words gold nanoparticle; glycation; collagen; dermis; aging

Skin aging is the result of genetic intrinsic chronological aging and extrinsic aging due to external factors. One of the causes of aging is the appearance of advanced glycation end products (AGEs).[1)] AGEs are formed by chemical reaction of carbohydrates with protein in a process known as the Maillard or glycation reaction.[2)] This reaction begins with adduction of a reducing sugar to an amino group in protein, typically the ε-amino group of a lysine residue, to form a Schiff base, which then rearranges to an Amadori compound. The Amadori product is a precursor to AGEs, which are a more permanent, irreversible modification of proteins. AGEs modifications of proteins may lead to alterations in normal function by inducing cross-linking of extracellular matrices.[3)] In the current study, glycolaldehye-derived AGEs (GA-AGEs) was used as a source of AGEs. It appears that short chain sugars such as GA could play an important role as intermediates in the formation of AGE structures in the glycation reaction.[4)] Based on immunoreactivity, GA-pyridine having a GA-AGE structure is reported to be the most significant AGE for cartilage degredation though AGE-its specific receptor (RAGE)-oxidative stress axis.[5)]

A number of compounds have been introduced as AGE inhibitors based on their inhibition of AGE formation during incubation of proteins with glucose *in vitro*. These inhibitors vary widely in structure, the common theme being their nucleophilicity or reactivity with reactive carbonyl intermediates in AGE formation. Aminoguanidine (AG) has been suggested as a representative agent for the inhibition of glycation,[6)] although clinical trials for this compound were stopped due to reported side effects. Gold nanoparticles (GNPs) have been reported to exhibit a variety of biological activities, including anti-imflammatory and anti-oxidant activities.[7—10)] GNPs, which represent an emerging nanomedicine, are renowned for their promising therapeutic possibilities, which include biocompatibility, high surface reactivity, anti-oxidation and plasmon resonance.[11)]

In the present study, we investigated the inhibitory effect on AGE formation with GNPs in glycation between collagen, a major protein component of the human dermis and GA.

MATERIALS AND METHODS

Preparation of GNPs GNPs were prepared using the method described by Storhoff *et al.*[12)] All glassware was cleaned in aqua regia (3 parts HCl and 1 part HNO_3), rinsed with nanopure water, and then oven dried prior to use. A total of 500 mL of 2 mM chlorauric acid ($HAuCl_4$) aqueous solution was brought to a reflux while stirring, after which 50 mL of 38.8 mM trisodium citrate solution was rapidly added. This resulted in a change in solution color from pale yellow to deep red. After the color change, the solution was refluxed for an additional 15 min, allowed to cool to room temperature, and subsequently filtered through a 0.45 μm nylon filter (Micro Filtration System Inc., CA, U.S.A.). The absorption spectra was measured for characterization of GNPs.[13)]

Transmission Electron Microscopic Studies Transmission electron microscopy (TEM) conducted using a JEOL 100CX transmission electron microscope (JEOL, Tokyo, Japan) was used to determine the size and monodispersity of the resulting nanoparticle solutions. Images of GNPs in aqueous dispersions were examined after placing drops of the dispersion onto 400 mesh gold grids and allowing the liquid to dry at 28°C.[14)]

Pre-glycation of Collagen Solution Pre-glycation of collagen solution was modified using the method described by Rahbar *et al.*[15)] Briefly, collagen solution (3 mg/mL, Nitta Gelatin Inc., Osaka, Japan) and 10 mM glycolaldehyde in 0.001 N HCl containing 0.02% sodium azide (NaN_3) and 1 mM diethylene triamine pentaacetic acid (DTPA) to obtain a reaction mixture, and the reaction mixture was incubated at 37°C for 7 d in the presence of GNPs or 5 mM AG. At the end of the incubation period, dialysis was conducted for 24 h against 0.001 N HCl. The glycation of collagen in solution was monitored as follows. An aliquot of the collagen was solubilized with 1% pepsin at 37°C. After pepsin digestion, the samples were centrifuged for 5 min at 10000 ***g*** and the fluorescence intensity of the digested collagen in the supernatant layer was then measured at 370 nm/440 nm.[16)] Table 1 shows the reaction mixture of pre-glycation collagen solution. Collagen solution without glycolaldehyde was used as a negative control. The percent inhibition of AGE formation was calculated using the

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Table 1. Reaction Mixture of Pre-glycation of Collagen Solution

Component (final concentration)	Control (A)	Glycation (B)	Sample (C)	Blank of sample (D)
Collagen (3 mg/mL)	●	●	●	●
Glycolaldehyde (10 mM)		●	●	
HCl (0.001 N)	●	●	●	●
DTPA (1 mM)	●	●	●	●
Sodium azide (0.02%)	●	●	●	●
GNPs			●	●

DTPA: diethylene triamine pentaacetic acid, GNPs: gold nanoparticles.

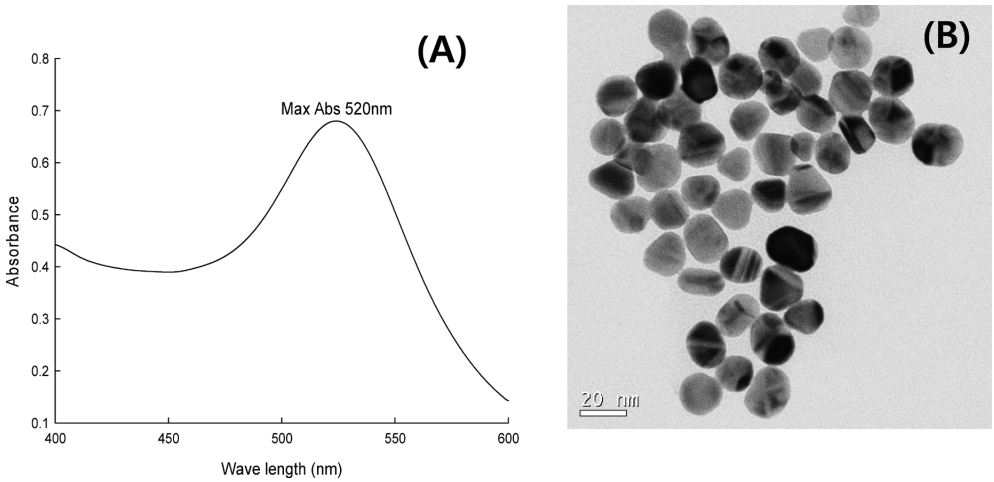

Fig. 1. Absorption Spectra for Gold Nanoparticles (GNPs) and Their Transmission Electron Microscopic (TEM) Image
(A) GNP surface plasmon absorption band was peaked at 520 nm. (B) The diameter of the GNPs was determined to be approximately 20 nm.

following equation:

$$\text{inhibition percentage of AGE formation (\%)} = 100 - \left[\left\{ \frac{(C-D)}{(B-A)} \right\} \times 100 \right]$$

A: Fluorescence of the reaction mixture without glycolaldehyde (negative control). *B*: Fluorescence of the reaction mixture with glycolaldehyde (positive control). *C*: Fluorescence of the reaction mixture with GNPs. *D*: Fluorescence of the reaction mixture with GNPs and without glycolaldehyde.

Determination of N^{ε}-(Carboxymethyl)lysine by HPLC A reversed-phase-HPLC method[17] with *o*-phtalaldehyde (OPA) pre-column-derivatization for determination of N^{ε}-(carboxymethyl)lysine (CML), a representative AGEs marker was slighltly modified. Briefly, 100 mg of the sample was hydrolyzed with 10 mL of 7.95 M HCl at 105°C for 24 h. After hydrolysis of sample, it was cooled at room temperature under N_2 gas, and subsequently centrifuged at 14000×***g*** for 15 min. Sep-pak C18 cartridge (Waters Associates, MA, U.S.A.) was pre-wetted with 10 mL of methanol and 20 mL of deionized water (DW) before 1 mL of the supernatant was applied to it, and then eluted with 10 mL of 3 M HCl. OPA reagent (20 μL) was added to the 20 μL of sample in vial, and vigorous mixing was applied. After 180 s of reaction, 20 μL of derivatised hydrolysate was analyzed with analytical column (Waters Spherissorb® 5 μm ODS2 column, 4.6×250 mm) at 32°C. Mobile phase were composed of (A) DW and (B) MeOH, and the gradient was linear from 0 to 100% B in 16 min. The OPA-derivatives were detected fluorimetrically at 340 nm excitation and 455 nm emission. The peak retentation time of CML standard (PolyPeptide Lab., CA, U.S.A.) was used to identify the peak of sample constituent.

Construction of Collagen Lattice and Preparation of Frozen Section A collagen lattice was constructed using collagen solution (native collagen for control or a 1 : 1 mixture of pre-glycated and native collagen) and reconstitution buffer (0.05 N NaOH containing 2.2% sodium biscarbonate and 20 mM Hepes buffer solution) at a ratio of 8 : 1.[16,18] Collagen lattices were polymerized by increasing the temperature to 37°C and then incubating the samples for 24 h. The collagen lattices were subsequently detached from the mold and submerged into liquid nitrogen to ensure that they were completely frozen. The lattices were then stored in a deep freezer (−70°C) until ready for sectioning. Frozen collagen lattices were sectioned using cryotome (Leica CM1900, Leica Inc., CA, U.S.A.), and then placed onto glass slides for immunofluorescence staining.

Immunofluorescence Staining for Distribution of AGEs on Glycated Collagen Lattices Frozen sections of collagen lattices were sectioned at 15 μm. Nonspecific binding sites were blocked with a 2% (v/v) dilution of appropriate normal serum in Tris-buffered saline (pH 7.4) in a humidified chamber. Each sample was subsequently treated with specific antibodies against AGEs (TransGenic, Kobe, Japan, 2 μg/mL). Finally, the samples were washed in phosphate buffered saline (PBS), incubated with a goat anti-mouse Alexa 488 fluor

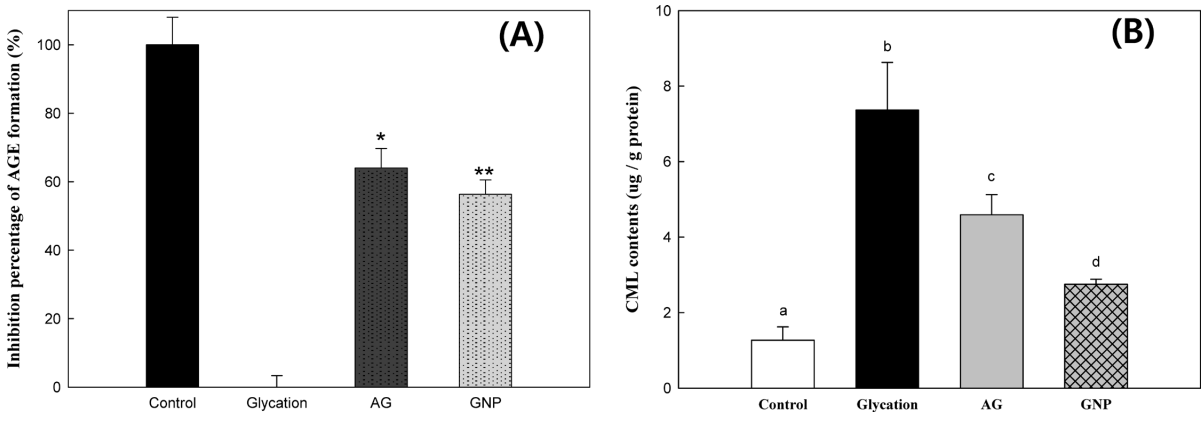

Fig. 2. Inhibitory Effects of GNPs on Glycation and N^{ε}-(Carboxymethyl)lysine (CML) Formation

(A) Anti-glycation effect of GNPs was determined by the collagen–glycolaldehyde assay. (B) CML contents were determined by HPLC. Values represent mean±S.E.M. (n=3 in each group). *p<0.05, **p<0.01, compared with the values of control group. Different letters above the bar in the groups indicate statistically significant differences by Duncan's multiple range test (p<0.05). Control group: collagen only; Glycation group: collagen with glycolaldehyde; AG group: collagen and glycolaldehyde with 5 mM aminoguanidine (AG); GNP group: collagen and glycolaldehyde with GNPs.

antibody (Invitrogen, Carlsbad, U.S.A., 1 : 200), mounted and cover slipped. All slides were then examined and evaluated using a confocal laser scanning microscope (LSM 5 Exciter, Carl-zeiss, Hamburg, Germany).

Statistical Analysis All data ±S.E.M. Statistical analysis was performed by analysis of variance (ANOVA), followed by Duncan's multiple range test for the individual comparisons of the means.

RESULTS AND DISCUSSION

The absorption spectrum of GNPs exhibited a surface plasmon absorption band. The position and shape of the plasmon absorption of metal nano materials are strongly dependent on the particle size, dielectric medium and surface adsorbed species.[19—21] A typical solution of 13—22 nm diameter GNPs exhibited a characteristic surface plasmon absorption band centered at 518—521 nm.[22] Our preparation of GNPs produced a surface plasmon absorption band centered at 520 nm (Fig. 1A). Figure 1B shows the GNPs based on TEM observation having a 20 nm of diameter.

It has been reported that AG has two key reaction centers: the nucleophilic hydrazine group $-NHNH_2$ and the dicarbonyl-directing guanido group $-NH-C(=NH)NH_2$ and these two groups together are involved in preventing the formation of AGEs from α,β-dicarbonyl precursors.[23] On the other hand, the activity of GNPs against glycation may come from competitively binding to the free amino groups of Lys and Arg which are potent sites for glycation.[24] Figure 2A shows the inhibitory effects of AG and GNPs on glycated collagen in the presence of GNPs. AG treated glycation of collagen solution and GNP-treated glycation of collagen showed significant glycation inhibition (64.0±5.7, 56.3±4.2%, respectively) when compared with non-treated glycation control. In addition, the levels of CML on glycated collagen were measured (Fig. 2B). CML has been reported as a representative AGEs marker *in vivo* and foods.[25] The treatments of AG and GNPs significantly inhibited the formation of CML (63.1±8.2, 38.2±7.7%, respectively) compared with non-treated glycation control.

Figure 3 shows immunofluorescence staining for the distribution of AGEs on glycated collagen lattices in each group. The AGEs immunoreactivity in the collagen lattices were found to be elevated in the glycation group (Fig. 3B) when compared with the control group (Fig. 3A). After the addition of 5 mM AG (Fig. 3C) or GNPs (Fig. 3D) to the glycation collagen solution, the intensity of the AGE staining was significantly lower than that of the non-treated glycation group.

AGEs can mediate their effects *via* specific receptors, including generation of reactive oxygen species (ROS). ROS radicals can affect various cellular pathways and gene expression.[26] Through this pathway, AGEs alter the structural properties of tissue proteins and reduce their susceptibility to catabolism.[27] In the preliminary experiment, 2′,7′-dichlorofluorescin diacetate (DCFDA)-loaded keratinocytes (HaCaT) incubated with 200 μg/mL of GA-AGEs increased the increased intracellular reactive oxygen species 1.7-fold compared with bovine serum albumin control. Antioxidant reagents are known to lead to significant decreases in oxidation stress due to ROS. Yakimovich *et al.* demonstrated that GNPs have antioxidant activity.[28]

The dermal matrix of skin consists of collagen, elastin and fibroblast. AGEs alter the mechanical properties of the skin by modifying macromolecules and the biological properties of the resident cells.[1] Alikhani *et al.* reported that AGEs stimulate fibroblast apoptosis *in vivo*,[29] and AGEs accumulation is known to cause chronological skin aging.[30] Collagens are important proteins for the skin because they are essential for the structure and function of the extracellular matrix in the dermis. AGEs accumulation of the dermis collagen contributes to protein aggregation, and increases the expressions of metalloproteinases, which degrade the collagen, and subsequently leads to skin aging.

The results presented here demonstrate that GNPs exhibited an AGE inhibitory effect. In future studies, we propose development of a reconstituted skin model that consists of a fibroblast cell, keratinocytes and collagen lattice. We will then apply GNPs to this reconstituted skin model to investigate the mechanism of the skin anti-aging action of GNPs in detail.

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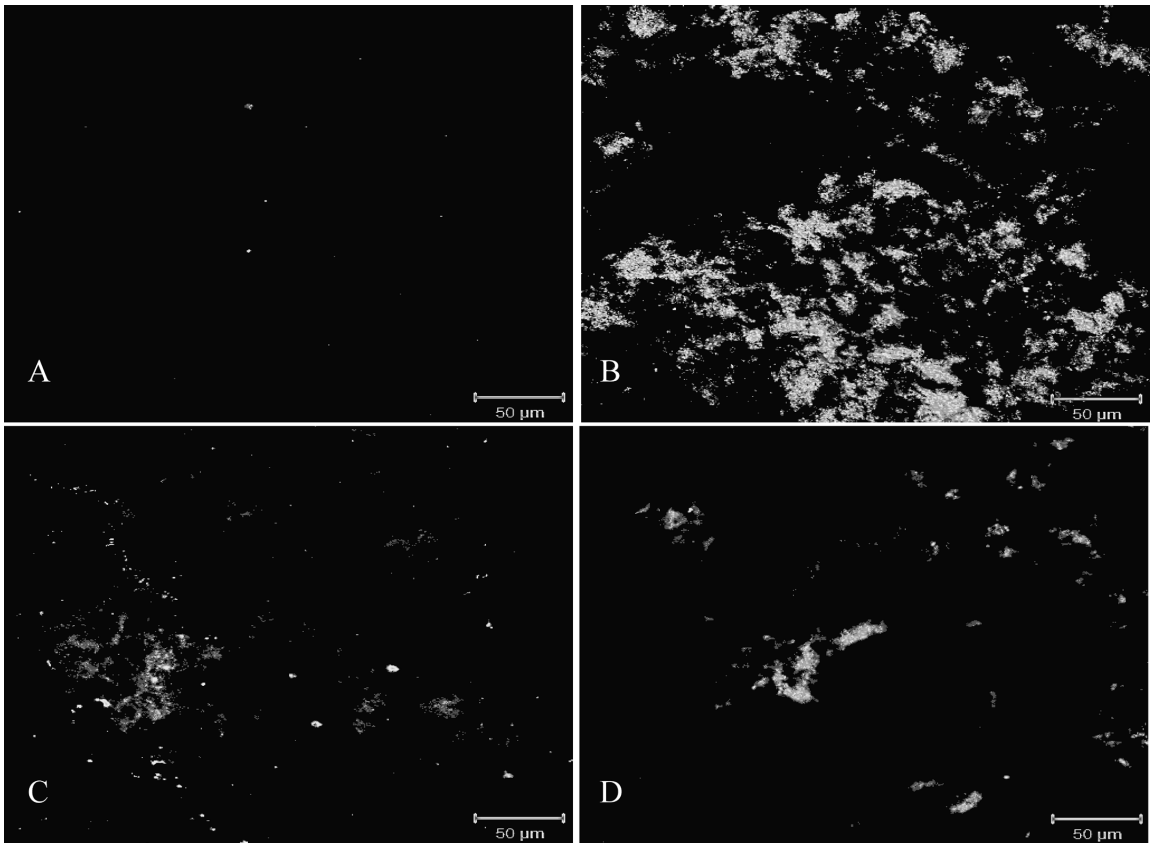

Fig. 3. Distribution of AGEs on Glycated Collagen Lattice

A (control group): collagen only; B (glycation group): collagen with glycolaldehyde; C (AG group): collagen and glycolaldehyde with 5 mM AG; D (GNP group): collagen and glycolaldehyde with GNPs. Gray tone shows the distribution of AGEs. Immunohistochemical stain; original magnification, ×200.

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