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The effects of gold nanoparticles in wound healing with antioxidant epigallocatechin gallate and α-lipoic acid

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Abstract

Topical applications of antioxidant agents in cutaneous wounds have attracted much attention. Gold nanoparticles (AuNPs), epigallocatechin gallate (EGCG), and α-lipoic acid (ALA) were shown to have antioxidative effects and could be helpful in wound healing. Their effects in Hs68 and HaCaT cell proliferation and in mouse cutaneous wound healing were studied. Both the mixture of EGCG + ALA (EA) and AuNPs + EGCG + ALA (AuEA) significantly increased Hs68 and HaCaT proliferation and migration. Topical AuEA application accelerated wound healing on mouse skin. Immunoblotting of wound tissue showed significant increase of vascular endothelial cell growth factor and angiopoietin-1 protein expression, but no change of angiopoietin-2 or CD31 after 7 days. After AuEA treatment, CD68 protein expression decreased and Cu/Zn superoxide dismutase increased significantly in the wound area. In conclusion, AuEA significantly accelerated mouse cutaneous wound healing through anti-inflammatory and antioxidation effects. This study may support future studies using other antioxidant agents in the treatment of cutaneous wounds.

From the Clinical Editor: In this study, topically applied gold nanoparticles with epigallocatechin gallate and alpha-lipoic acid were studied regarding their effects in wound healing in cell cultures. Significant acceleration was demonstrated in wound healing in a murine model.

Key words: Wound healing; Angiogenesis; Inflammation; Oxidation; Gold nanoparticle

It has been quite some time since the study of wound healing drew public attention, especially in a time when cosmetic dermatology and beauty care are increasingly prized. Poor healing of wounds has resulted in serious complications, even deaths, all across the world.[1] Abnormal biological reactions to the cutaneous injury following disease, trauma, and surgery inevitably can lead to grave complications. In fact, the wound healing process is very complex and involves more than one pathophysiological factor.[2] As we know, the predominant cell populations in mammalian skin are fibroblasts and keratinocytes. Aside from this, the cellular signal transduction mechanism that dominates wound healing is very intricate and not yet fully understood. It has been recently found that antioxidants such as epigallocatechin gallate (EGCG) may have an important role in wound healing.[3] Furthermore, from a clinical point of view, the main reason topical application is popular is that it can reduce a drug's adverse effect on the human body and internal organs. In recent experiments in both animals[4] and humans,[5] the topical application of specific growth factors has proven very effective.

There has recently been great progress in the application of nanotechnology in materials science; nanotechnology has also been widely used in biological technology.[6] Because of their unique properties, gold nanoparticles (AuNPs) can be widely applied in gene transfer and medicine transport.[7] Colloidal AuNPs have been proposed for diverse biomedical applications because of their unique surface, electronic, and optical properties.[8] In recent years, as a result of the nano-bio interaction with skin lipids, AuNPs have been shown to be capable of opening the stratum corneum and penetrating the skin barrier.[9] Accordingly, topical application of antioxidants along with nanogold to enhance absorption is the main subject of this study.

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EGCG, a compound found widely in green tea, is a typical antioxidant. It has recently been discovered that catechin has many biomedical effects such as antioxidative and anticarcinogenic properties.[10] EGCG is a polyphenol that exists abundantly in unfermented teas. Several epidemiological studies have linked the consumption of tea with a lower risk of cardiovascular diseases.[11] However, the mechanism of EGCG's facilitation of cutaneous wound healing complications remains unclear. Another potent antioxidant is α-lipoic acid (ALA), which can be found in animal foods such as meat and liver.[12] ALA was discovered in 1951 as a molecule that assists in acyl-group transfer and as a coenzyme in the Krebs cycle. In addition to eliminating peroxide, ALA is a powerful lipophilic antioxidant both in vitro and in vivo.[13] ALA can interact with other antioxidants and strengthen their antioxidation abilities (e.g., vitamins C and E). As a result, ALA is now used as a dietary supplement in ageing, diabetes mellitus, and vascular and neurodegenerative diseases.[14] Very few attempts have been made at mixing EGCG with ALA in studies of cutaneous wound healing processes. One recent study demonstrated that an AuNP liposomal formulation enhanced the antioxidant activity of vitamin C and topical delivery in the skin.[15]

The AuNPs combined with EGCG and ALA ointment (AuEA) used on cutaneous wound is still not well understood. This study aims to determine the most effective topical formula for wound healing and to unravel its medical mechanism. In the present study we provide evidence that topical application of a mixture of AuNPs, EGCG, and ALA accelerates wound healing in mice by a mechanism that may involve anti-inflammatory and antioxidation actions in the wound area. Furthermore, we tested the hypothesis that AuEA accelerates wound healing by increasing proliferation and migration of epidermal cells.

Methods

Preparation and characterization of AuNPs

AuNPs provided from Gold NanoTech (Taipei, Taiwan) were prepared by a proprietary molecular beam epitaxy process as follows. AuNPs were produced by physical manufacturing and contained no surface modifiers or stabilizers. Briefly, gold bulk material was cut or ground into the target material. Then the Au target was vaporized to the atomic level by an electrically gasified method under vacuum. The vapor was condensed in the presence of inert gas and then piled up to form AuNPs. The AuNP sizes can be effectively managed depending on the evaporation time and electric current used. The AuNPs were collected in a cold trap and centrifuged to obtain the final product. The initial concentration of these AuNPs was determined by an inductively coupled plasma mass spectrometer (PE-SCIEX ELAN 6100 DRC; Perkin-Elmer, Waltham, Massachusetts). The sizes of the various AuNPs were observed by a JEOL JEM-1200 transmission electron microscope (Tokyo, Japan) operated at 110 kV. The size distribution of the AuNPs was computed by software based on more than 100 particles in the images. The ultraviolet-visible (UV-vis) absorbance of AuNPs of various sizes was measured by UV-vis spectroscopy (Hitachi U-2000; Hitachi, Tokyo, Japan). The negative surface charge of AuNPs was determined by zeta potential (Zetasizer Nano-ZS; Malvern Instruments, Worcestershire, UK). The stability of the AuNPs in the cell culture medium with serum was examined by transmission electron microscopy. The diameters of the utilized AuNPs were classified into 1–3 nm, 3–5 nm, and 15–30 nm.

Cell culture

Human foreskin fibroblasts (Hs68) and human keratinocyte cells (HaCaT) were cultured in Dulbecco's modified Eagle medium (Cambrex Bio Science, Walkersville, Maryland) containing 20% fetal bovine serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin (pH 7.6). Cultures were passaged on reaching 80% confluence, using 0.05% trypsin-EDTA (GIBCO, Invitrogen, Carlsbad, California), and the medium was changed every 2 days. The experiments were performed at passage 3–5 for cell proliferation and migration assays.

Quantification of cell proliferation

The proliferation of cultured Hs68 and HaCaT cells was measured using a colorimetric assay. Cells were incubated with either vehicle, 2.2 mM EGCG (E), 8 mM ALA (A), 2.2 mM EGCG + 8 mM ALA (EA), 1 ppm AuNPs, or AuNPs + EGCG + ALA (AuEA) for 48 hours. Then 10 mL of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (R&D Systems, Minneapolis, Minnesota) were added to each well, and the cells were incubated for a further 4 hours at 37°C. After the cells were washed three times with phosphate buffered saline (pH 7.4), the insoluble formazan product was dissolved by incubation with 100 mL detergent for 2 hours. The absorbance of each well was measured on an enzyme-linked immunosorbent assay microplate reader at 570 nm.

Cell scratch wound healing assay

Cultured Hs68 and HaCaT cell were seeded into 60-mm plates and grown to confluence. After 24 hours of serum starvation, the cell monolayer was then subjected to a mechanical scratch wound induced using a sterile pipette tip, and cells were treated with either vehicle, E, A, EA, AuNPs, or AuEA for 48 hours. Cells were then fixed in a solution of 4% paraformaldehyde in phosphate buffered saline and stained with crystal violet. Cells in the injury area were visualized under phase-contrast optics (10× objective), and the number of cells that had migrated into the initially cell-free scratch area was counted.

Full-thickness wounds and wound measurement

BALB/c mice were obtained from the Experimental Animal Center of National Taiwan University. All mice were maintained on a standard laboratory diet and water ad libitum. All mice were used experimentally when 8 weeks old at the time of wounding. Mice were anesthetized using 2 to 2.5% vaporized inhaled isoflurane, and the dorsal skin was cleansed with betadine. Under sterile conditions the dorsal area was totally depilated, and a single full-thickness excisional linear wound (1 cm) was created on each side of the upper back of each mouse using a sharp scissors and a scalpel. The left-side wound served as control, and the right-side wound was treated with vehicle, 1 mg/g EGCG (E), 30 mg/g ALA (A), 1 mg/g EGCG + 30 mg/g ALA (EA),

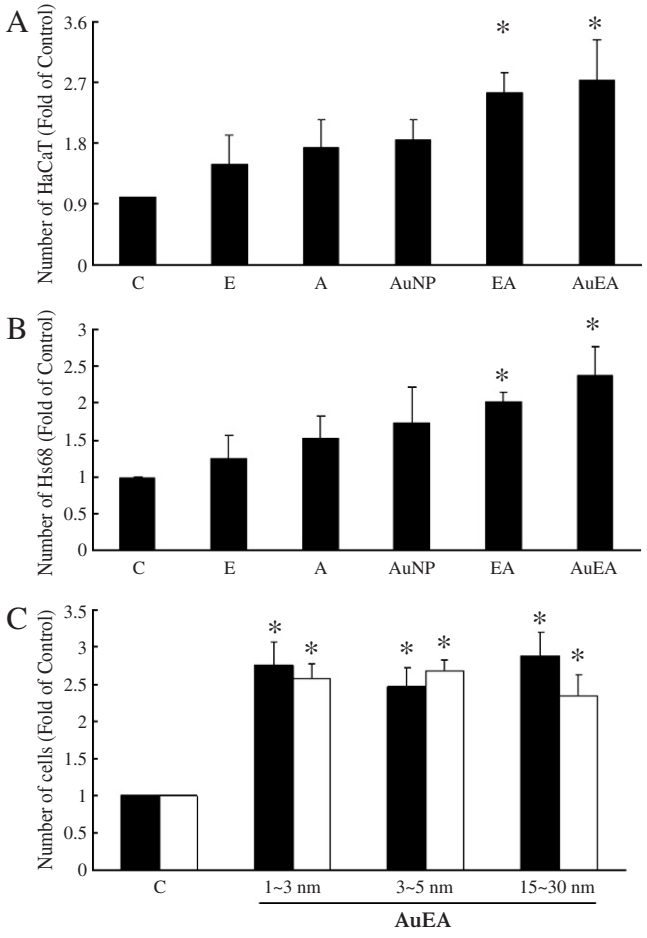

Figure 1. EA and AuEA enhance proliferation of HaCaT and Hs68 cells. Cultured HaCaT cells or Hs68 cells were treated with vehicle (Control), EGCG (E), ALA (A), EGCG + ALA (EA), AuNPs, or AuNPs + EGCG + ALA (AuEA) for 48 hours. After EA and AuEA incubation, the numbers of HaCaT cells **(A)** and Hs68 **(B)** cells significantly increased. **(C)** AuEA with different sizes of AuNPs did not significantly change the cell proliferation in HaCaT and Hs68 cells. Black bars indicate the HaCaT cells study. White bars indicate the Hs68 cells study. Values were obtained from six independent experiments. *$P < 0.01$ when compared to control.

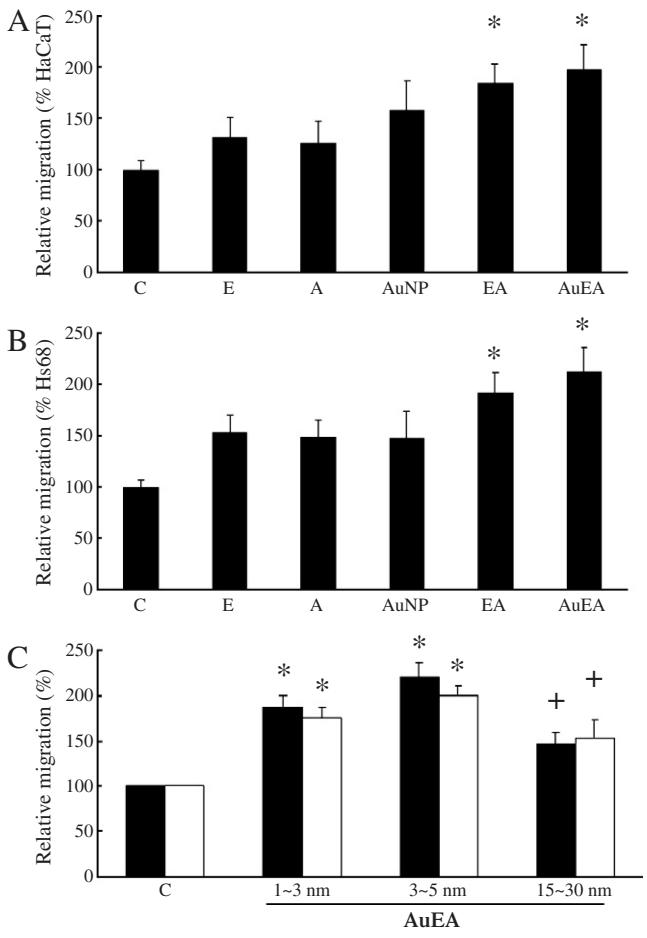

Figure 2. EA and AuEA enhance motility of HaCaT and Hs68 cells. Monolayers of cultured HaCaT cells or Hs68 cells were mechanically wounded with a sterile 200-mL pipette tip following treatment with vehicle (Control), EGCG (E), ALA (A), EGCG + ALA (EA), AuNPs, or AuNPs + EGCG + ALA (AuEA). Images of the wound area were acquired 48 hours after scratch wounding, and the number of cells per field that had migrated into the cell-free wound zone was determined for each culture. Cultured HaCaT cells **(A)** and Hs68 **(B)** cells incubated with EA and AuEA migrated in significant numbers into the wound area ($n = 6$). **(C)** AuEA with 3- to 5-nm AuNPs migrated in higher numbers than other sizes of AuNPs in both types of cells. Black bars indicate the HaCaT cells study. White bars indicate the Hs68 cells study ($n = 6$). *$P < 0.01$ when compared to control. +$P < 0.05$ when compared to 3- to 5-nm group.

0.07 mg/g AuNPs, or AuEA ointment applied directly to the wound site once daily in a blinded manner. All ointments were made with 2.5 mL of glycerol (Sigma-Aldrich, St Louis, Missouri), 1 mL Creagel emulsifier (First Chemical, Taipei, Taiwan), and 45.5 mL double-distilled water. To determine different ointment-mediated healing efficiency, the residual wound size was measured from the unclosed wound area after 3, 5, and 7 days of topical treatment using digital Dino calculation software (AM3013T Dino-Lite Premier; AnMo Electronics, Taipei, Taiwan). Mice in each group were euthanized on days 1, 3, 5, and 7 post wounding, and skin tissue samples from the wound site were excised in full depth and bisected from all of the mice for biochemical analyses or H+E histological staining. The investigations conform to the "Guide for Care and Use of Laboratory Animals" published by the US National Institutes of Health and the Animal Care Committee of the Institute for Frontier Medical Sciences, Fu-Jen University (A9658).

Western blot analysis

Total protein samples were mixed with sample buffer, boiled for 10 minutes, separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under denaturing conditions, and electroblotted onto nitrocellulose membranes (Amersham Pharmacia Biotech/GE Health Care, Buckinghamshire, UK). The nitrocellulose membranes were blocked in blocking buffer, incubated with mouse antibodies to CD68, to Ang-1, to Ang-2, to VEGF, to CD31 (Santa Cruz Biotechnology, Santa Cruz, California), and to superoxide dismutase (SOD) (Abcam, Cambridge, Massachusetts), washed, and incubated with horseradish peroxidase–conjugated secondary antibody. Signals were visualized by enhanced chemiluminescent detection.

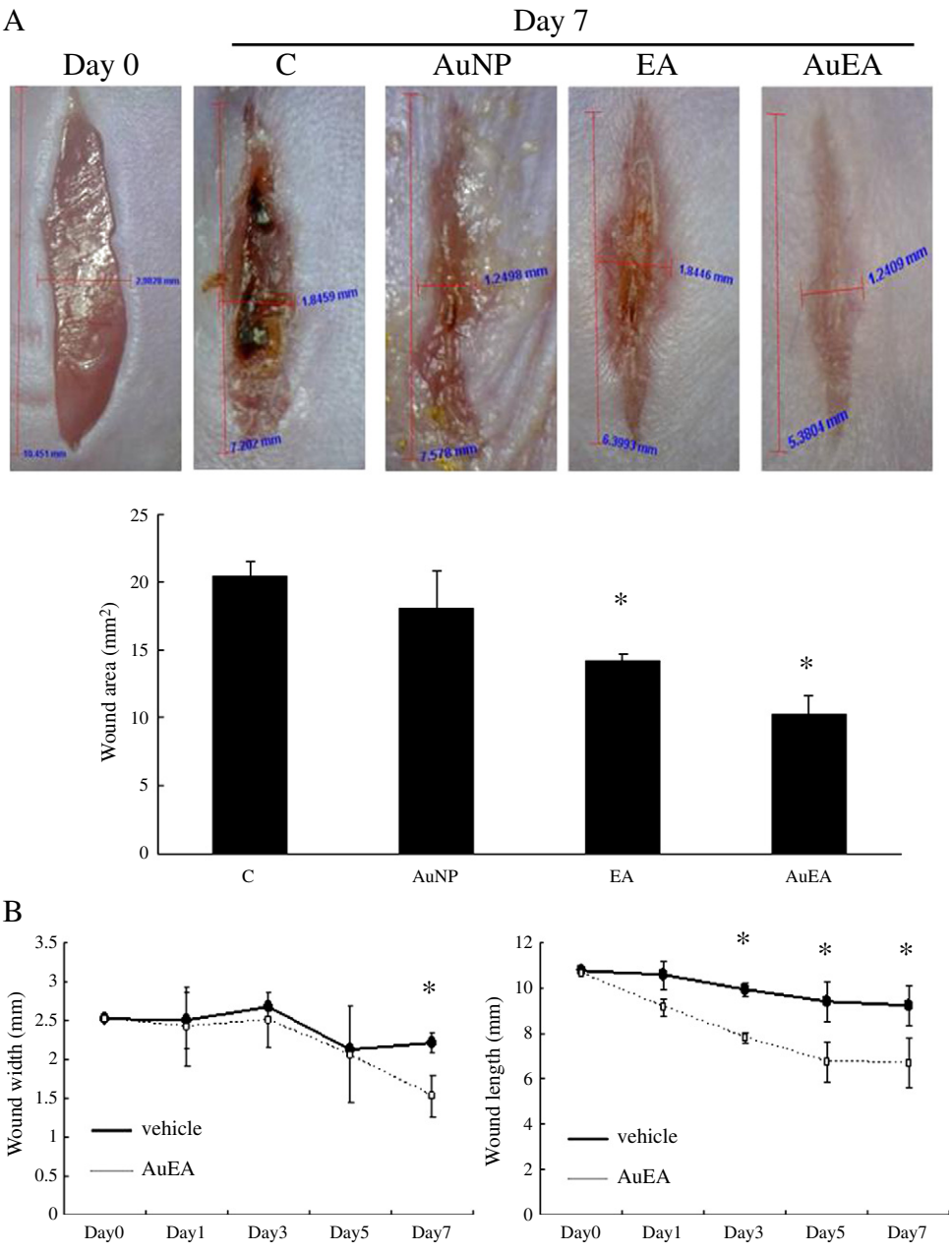

Figure 3. Effects of topical application of AuNPs, EA, and AuEA on the healing of full-thickness wounds in mice. Two full-thickness wounds were induced in vehicle-, AuNPs-, EA-, or AuEA-treated mice. **(A)** Images of representative mouse from each group taken on post-injury day 7 are shown ($n = 6$). Topical application of EA and AuEA significantly accelerated the wound healing in mice. **(B)** Wound width and length at the indicated time points in vehicle and AuEA topically treated mice ($n = 6$). After treatment with AuEA, the cutaneous wound width significantly decreased at day 7 and wound length significantly decreased from day 3 to day 7. *$P < 0.01$ when compared to vehicle.

Statistical analysis

The data were expressed as mean ± SEM. A Student's *t*-test was used for comparing parametric variables between the two groups, and analysis of variance with repeat-measurement design was used for time course changes. Statistical significance was evaluated by Tukey-Kramer multiple comparisons test (GraphPad Software, San Diego, California). A *P* value of less than 0.05 was considered statistically significant.

Results

EA and AuEA mixture increased keratinocyte and fibroblast proliferation

Cultured HaCaT cells or Hs68 cells were treated with either vehicle, EGCG, ALA, EA, AuNPs, or AuEA for 48 hours. Incubation with EA or AuEA significantly increased the number of HaCaT cells (Figure 1, *A*) and Hs68 cells (Figure 1, *B*).

Incubation with EGCG, ALA, or AuNPs alone also increased cell proliferation but not to a statistically significant degree. The proliferation rates of both types of cells in the AuEA group were significantly greater than those in the EA group. In the Hs68 cell line, we found that the cell number of the EA group significantly increased as early as 12 hours and continued to increase until 48 hours after cells were seeded (data not shown). The different sizes of AuNPs did not significantly change the cell proliferation effect of the AuEA group in either cell line (Figure 1, *C*). These results suggested that the mixture of EA and AuEA increased the cell proliferation rate in the in vitro culture system.

EA and AuEA increased the migration of HaCaT and Hs68 cells

Incubation with EA or AuEA significantly increased the migration of HaCaT cells (Figure 2, *A*) and Hs68 cells (Figure 2, *B*). The numbers of migrated cells induced by AuEA were significantly higher than those induced by EA. Neither EGCG nor ALA increased cell migration. Incubation with the AuEA mixture (3- to 5-nm AuNPs) significantly increased migration of both cell types to a higher level than observed for any other group (Figure 2, *C*). We used 5-nm AuNPs for the additional animal study. These data suggest that EA and AuEA treatment not only significantly increased the keratinocyte and fibroblast cell proliferation, but also increased the cell migration in in vitro cell culture systems.

Effect of AuEA on wound closure

For investigating the wound healing process in vivo, two linear full-thickness wounds were created on the dorsum of mice treated with vehicle (control), AuNPs, EA, or AuEA. Topical application of EA and AuEA significantly increased the rate of wound healing over that of vehicle (Figure 3, *A*). Wound healing in the AuEA group was almost complete by 7 days after injury, whereas the wounds of control mice had not yet healed completely. Both wound widths and lengths in AuEA-treated mice were significantly decreased on day 7 after injury when compared with control mice (Figure 3, *B*). No adverse effects of topical treatment were noted on body weight, general health, or behavior of the mice. In histological sections of skin samples from the AuEA group, neutrophil infiltration and inflammation were limited to the site of wounding on day 7 (Figure 4).

The relationship between AuEA and angiogenesis

By producing microvessels that provide nutrients and oxygen to growing dermal cells, angiogenesis may play a critical role in wound healing.[16] VEGF (Figure 5, *A*) and Ang-1 (Figure 5, *B*) in the wound tissues of AuEA-treated mice increased significantly on day 7 after injury. However, on day 3 and 5 Ang-1 protein expression had not increased significantly. Ang-2 expression did not show significant change after 7 days (Figure 5, *C*). Furthermore, CD31 protein expression increased in day 7 wound tissue but did not reach statistical significance after wound injury in AuEA-treated mice (Figure 6, *A*).

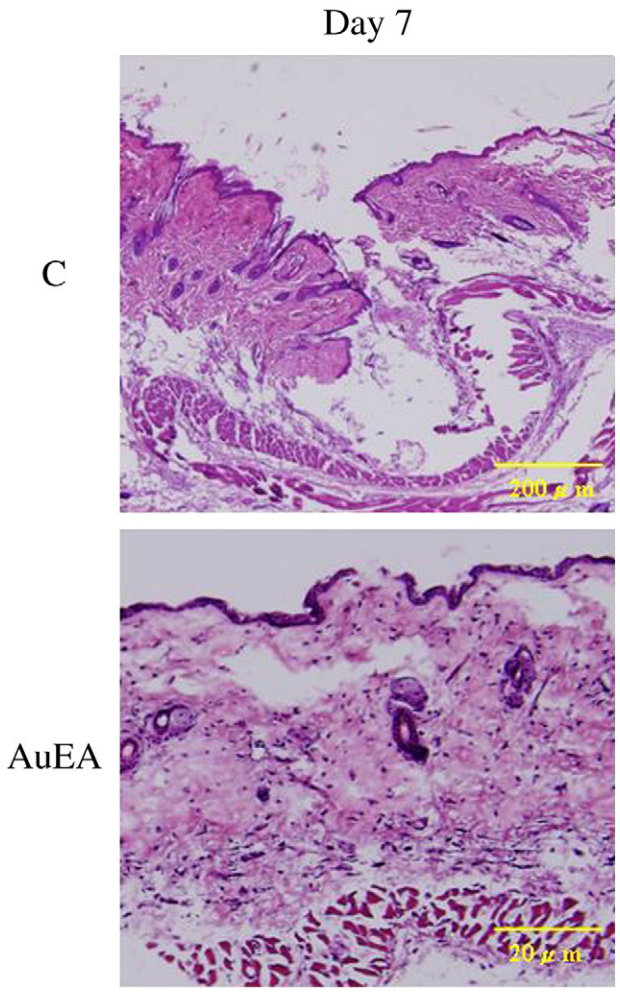

Figure 4. Topical treatment with AuEA did not lead to abnormal host response. H+E-stained skin sections from AuEA-treated mice were examined microscopically 7 days after wounding. No significant increase in neutrophil infiltration was noted in tissues adjacent to the wound site in the AuEA group after wounding. Original magnifications 40× (C, control) and 400× (AuEA).

The effects of AuEA in wound inflammation and Cu/Zn superoxide dismutase (SOD1)

To examine the possible roles of AuEA treatment in wound inflammation, CD68 expression was used as a marker of monocyte infiltration. CD68 protein expression was significantly decreased after AuEA treatment from day 3 to day 7 post injury (Figure 6, *B*). The SOD1 expression, however, increased significantly after AuEA treatment (Figure 6, *C*). These results indicated that AuEA not only reduced inflammation around the wound area but also inhibited the oxidative stress after cutaneous injury.

Discussion

Impaired wound healing continues to be a major health problem in high-risk patients with diabetes, suppressed immune function, or old age.[17] By the development of nanotechnology,

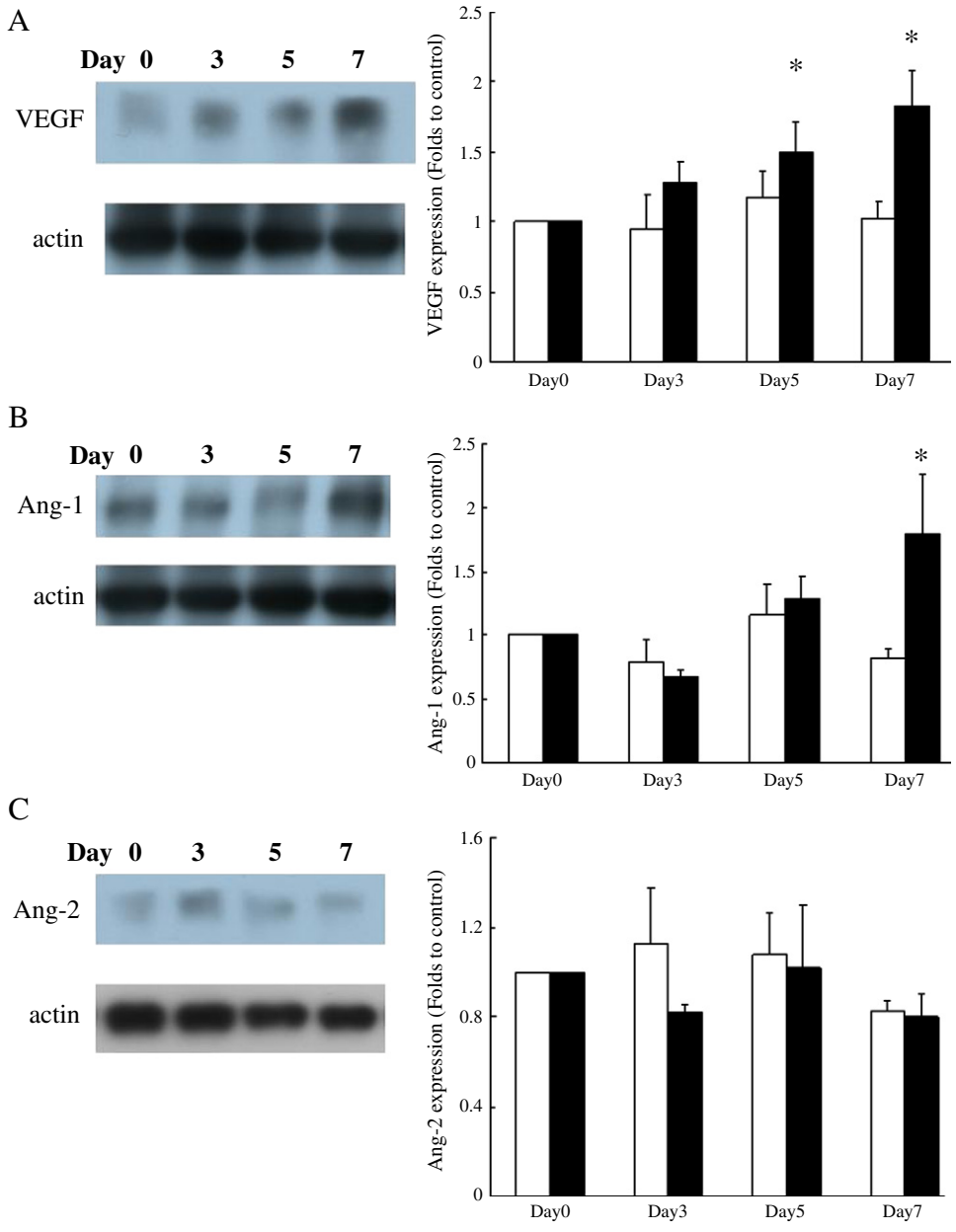

Figure 5. Expression of angiogenesis-related gene and effects of AuEA treatment in wound area. Western blot analysis for cutaneous wound area tissue at different time points after AuEA treatment. VEGF protein expression **(A)** significantly increased from day 5 to day 7 post injury ($n = 6$). **(B)** Angiopoietin-1 (Ang-1) protein expression was significant on day 7 ($n = 6$). **(C)** Angiopoietin-2 (Ang-2) expression did not significantly change after cutaneous wound creation ($n = 6$). White bars indicate the vehicle treatment. Black bars indicate the AuEA treatment. $^*P < 0.01$ when compared to vehicle.

many novel nanomaterials with unique properties such as magnetics, electronics, and photonics are increasingly being exploited. Previous studies showed that AuNPs could be applied therapeutically for intravascular and percutaneous drug or gene delivery.[9] In this study we found that topical application of the mixture of AuNPs, EGCG, and ALA (AuEA) was much more effective than EGCG or ALA alone in promoting the proliferation and migration of dermal fibroblasts or keratinocytes. Topical application of AuEA also effectively accelerated wound healing and enhanced the restoration of normal dermal and epidermal tissue structures in mouse wound areas. These findings support the potential role of AuNPs as an adjuvant compound in the treatment of cutaneous wounds.

The effect of EGCG and ALA in cutaneous wound healing was proved in previous studies. EGCG induces myofibroblast differentiation and connective tissue growth factor gene expression and reduces the expression of the collagen type I gene in mouse cutaneous wounds.[18] Topical application of green tea polyphenols significantly increased healing of photochemical damage wounds in mouse skin induced by psoralen and ultraviolet A.[19] ALA supplementation in combination with hyperbaric oxygen therapy downregulated the inflammatory

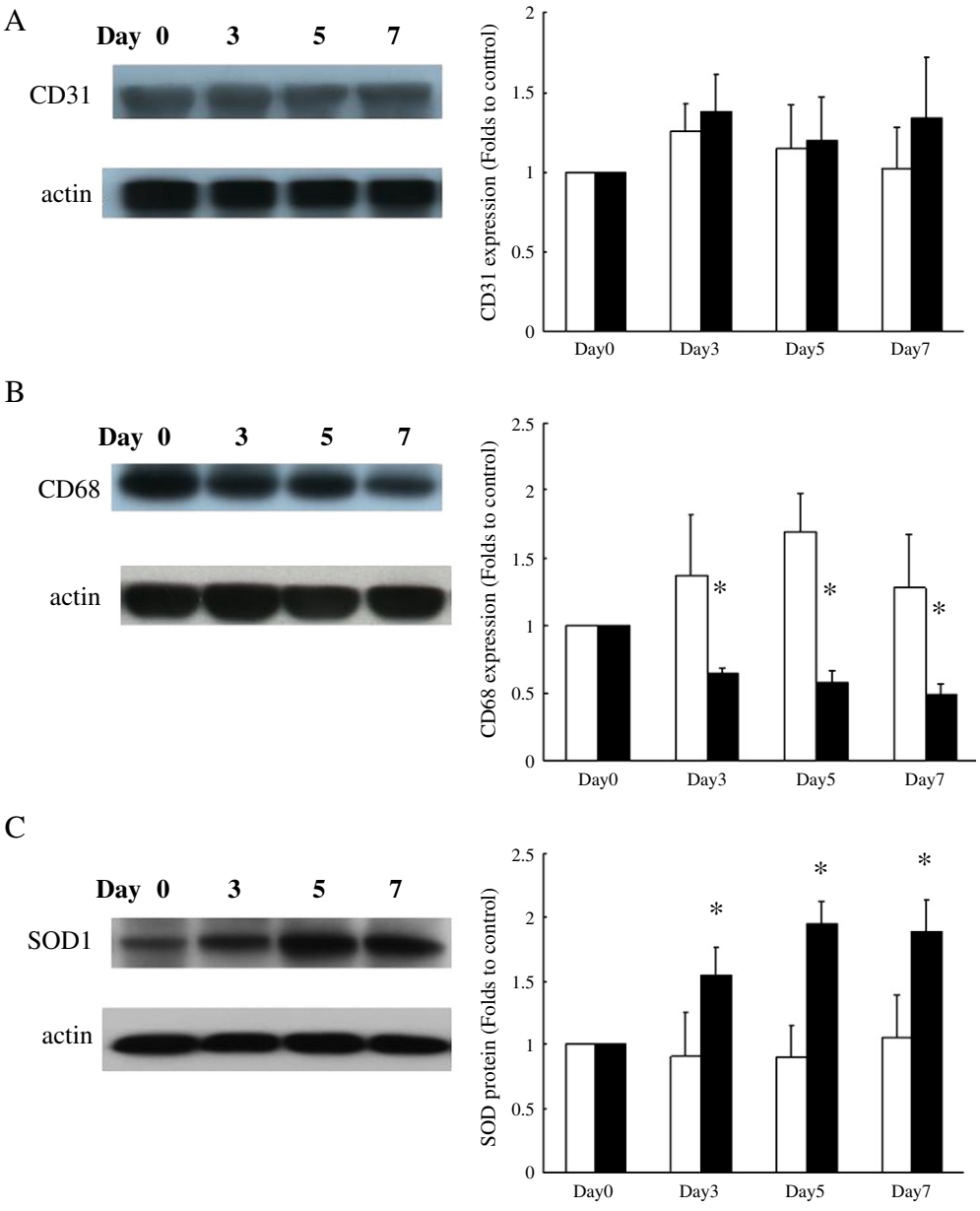

Figure 6. CD31, CD68, and SOD1 expression in wound tissue from mice treated with AuEA. Mice were treated with vehicle or AuEA for the indicated number of days post injury. Wound samples were then removed and were subjected to immunoblot analysis. **(A)** CD31 protein did not differ significantly between vehicle and AuEA treatment after injury ($n = 6$). **(B)** CD68 protein significantly decreased after AuEA treatment from day 3 to day 7 post injury ($n = 6$). **(C)** Immunoblots showed a significantly increased level of SOD1 in the skin tissue from day 3 to day 7 post injury ($n = 6$). White bars indicate the vehicle treatment. Black bars indicate the AuEA treatment. *$P < 0.01$ when compared to vehicle.

cytokines in the nonhealing wounds of human subjects.[20] In streptozotocin-induced diabetic rats, intraperitoneal pretreatment with 100 mg/kg lipoic acid (LA) improved healing of subsequently induced abrasion wounds.[21] Consistent with these findings, a combination of EGCG and ALA also showed beneficial effects on mouse wound healing in our studies. One recent study demonstrated that co-administration with AuNPs enables percutaneous delivery of protein drugs.[9] We suggest that ALA as an antioxidant also enhanced the antioxidant activity of EGCG. Furthermore, AuNPs may increase the specific mixture absorption through the skin by opening the stratum corneum transiently. Consequently, AuEA resulted in beneficial wound healing effects in both in vitro and in vivo studies.

The mechanistic link between angiogenesis and wound healing has been the focus of much research. Recent clinical literature showed that administration of bevacizumab affects the wound healing of the chest wall.[22] This suggested again that angiogenesis has an important role in wound healing processes. However, antiangiogenic effects of EGCG and ALA were shown in previous studies. Previous studies also demonstrated the

antiangiogenic role of EGCG in many cancer cell lines. EGCG inhibited expression of VEGF, Ang-1, and Ang-2 proteins in human prostate carcinoma cells.[23] Topical use of EGCG decreased hypoxia-inducible factor-1α induction and VEGF expression in human skin.[24] Intraperitoneal injection of 60 mg/kg R-(+)-ALA reduced Ang-2 and VEGF expression in the diabetic retina of rat.[25] In certain studies, nontoxic doses of LA showed no growth inhibition on endothelial or tumor cells.[26] These controversial results may indicate that dosages of EGCG and ALA determine antiangiogenic effects. The antiangiogenic effects of AuNPs were also shown in several studies. AuNPs blocked VEGF-induced autophosphorylation of VEGF receptor-2. Intravitreal injection of AuNPs significantly inhibited retinal neovascularization in a mouse model of premature retinopathy.[27] AuNPs also inhibited proliferation of VEGF-treated retinal endothelial cells by blocking Src signaling pathways.[28] Those studies indicated that dosages of these compounds determine the effects of angiogenesis or antiangiogenesis. In our study the combination of EGCG, ALA, and AuNPs in specific concentrations did not increase angiogenesis significantly on the molecular level in the mouse cutaneous wound area.

Oxidative stress has been shown to be detrimental to multiple cellular processes in wound healing.[29] The anti-inflammatory and antioxidation effects of EGCG and ALA were proved in previous studies. Previous data have shown protective effects of EGCG and a classic natural antioxidant ALA against oxidative stress and ageing.[30] EGCG has been shown to have potent anti-inflammatory effects.[31] Oral administration of 1200 mg of LA to multiple sclerosis patients resulted in significant anti-inflammatory effects by activating the cyclic adenosine monophosphate/protein kinase A signaling cascade.[32] Gold compounds have also received much attention as an anti-inflammatory agent because of their ability to inhibit NF-κB expression and subsequent inflammatory reactions.[33] Gold compounds are effective in quenching reactive oxygen species in a dose-dependent manner.[34] AuNPs catalyzed oxidation of NADH to NAD, enhanced the antioxidant activity of vitamin E,[35] decreased reactive oxygen species induced in a hepatoma cell line,[36] and inhibited osteoclast formation induced by the receptor activator of NF-κB ligand in bone marrow–derived macrophages.[37] In our study, decreased CD68 expression and increased SOD1 expression around the wound area suggest that anti-inflammatory and antioxidative effects of AuEA may have a role in mouse cutaneous wound healing.

In conclusion, we have demonstrated clearly that AuEA increased Hs68 and HaCaT cells proliferation and migration. Moreover, our study provides insight into the molecular action of AuEA in a mouse cutaneous wound healing model. This study showed that AuNPs combined with EGCG and ALA significantly accelerated cutaneous wound healing in mice. The negatively charged AuNPs accumulated in organs more than positively charged AuNPs after oral administration in a rat model.[38] Further studies on positively charged AuNPs are needed to confirm the effects of percutaneous delivery. However, our results provided a rationale for future development of AuNPs mixture with other antioxidant agents in topical treatment of cutaneous wounds.

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