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Short Communication

Induction of Extracellular Matrix Synthesis in Normal Human Fibroblasts by Anthraquinone Isolated from *Morinda citrifolia* (Noni) Fruit

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ABSTRACT In previous studies we found that *Morinda citrifolia* (Noni) fruit extract up-regulated biosynthesis of type I collagen and glycosaminoglycans in primary cultures of normal human fibroblasts. The objective of this study was to identify the active ingredients in Noni fruit extract. An active single compound having a type I collagen-stimulating effect was isolated and identified as 1,4-dihydroxy-2-methoxy-7-methylanthraquinone by nuclear magnetic resonance, infrared, and mass analysis. It was revealed that anthraquinone showed significantly increased elaboration of procollagen type I C-terminal peptide and glycosaminoglycans and reduced expression of the collagenase matrix metalloproteinase-1 dose-dependently in human dermal fibroblasts. Furthermore, in a clinical trial, a nano-emulsion containing anthraquinone predominantly increased the dermal type I procollagen in nude mouse skin. These results suggest that anthraquinone derived from Noni extract is a good candidate for use as a new anti-wrinkle agent due to its strong induction of biosynthetic activity of extracellular matrix components.

KEY WORDS: • ***anthraquinone*** • ***extracellular matrix*** • ***glycosaminoglycans*** • ***matrix metalloproteinase-1*** • **Morinda citrifolia** • ***type I collagen***

INTRODUCTION

The extracellular matrix (ECM) is the structurally stable material that provides support for tissues. ECM is a complex ordered aggregate composed of a number of different macromolecules whose structural integrity and functional composition are important in maintaining normal tissue architecture, in development, and in tissue-specific function.[1,2] Skin connective tissue is composed primarily of fibrillar collagen bundles and elastic fibers, along with a complex array of proteoglycans and other ECM molecules.[3] Among these proteins making up the ECM, interstitial collagen, which accounts for roughly 90% of the protein in human dermis, is essential for providing strength and resiliency to skin.[4,5] Human dermis predominantly contains type I collagen (roughly 85%), with lesser amounts of type III collagen (10%).[6] Proteoglycans, having an important role in determining viscoelastic features, are abundant in dermal connective tissues. Proteoglycans are composed of core protein and glycosaminoglycans (GAGs), which are covalently bound to core protein.

Breakdown of collagen fibrils is dependent on the action of collagenases. It is known that humans have three distinct collagenases: matrix metalloproteinase (MMP)-1, also known as collagenase 1 or interstitial collagenase; MMP-8, also known as collagenase 2 or neutrophil collagenase; and MMP-13, also known as collagenase 3. All of these collagenases can cleave interstitial collagens such as type I and III collagens found in skin.[7,8]

In aging skin, the dermis becomes thin as a result of reductions in the amount and organization of connective tissue.[9,10] Specifically, it has been demonstrated in photo-aged human skin that multiple exposures to ultraviolet radiation lead to sustained elevation of MMPs that degrade skin collagen.[11] Therefore, there is a need to develop novel materials with notable effects in inducing synthesis of dermal ECM and reducing the expression of MMPs in order to prevent or slow skin aging.

During our investigation into developing new anti-wrinkle agents from plant sources, the extract of *Morinda citrifolia* (Noni) was found to increase the biosynthesis of type I procollagen in human dermal fibroblast cells. Noni, also

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known as Indian mulberry, is a small evergreen tree. It is native to India, Polynesia, Australia, China, Malaysia, and South East Asia.

Recently, we purified and identified an active compound from Noni extract, anthraquinone, that strongly stimulates type I collagen biosynthesis. Accordingly, we examined the contribution of noni-derived anthraquinone to ECM protein synthesis and MMP-1 inhibition *in vitro*. Moreover, we prepared a nano-emulsion containing anthraquinone and measured its collagenic effect *in vivo*.

MATERIALS AND METHODS

Preparation of Noni extract and anthraquinone

To prepare Noni extract, Noni powder was immersed in 70% ethanol at room temperature for 1 week under shaded light. After that, the liquid phase was separated from solids, evaporated, and freeze-dried. Anthraquinone was obtained from Noni extract by using Sil-Gel (70–230 mesh, Merck, Darmstadt, Germany) column chromatography. The elution solvent was a blend of chloroform and methanol (7:3 vol/vol). The detailed structure of anthraquinone was confirmed as 1,4-dihydroxy-2-methoxy-7-methylanthraquinone by instrumental analysis, nuclear magnetic resonance, infrared, and mass spectra (data not shown).

Cell culture

Human dermal fibroblasts derived from neonatal foreskin were purchased from BioWhittaker (Walkersville, MD) and maintained in RPMI 1640 medium (HyClone Laboratories, Logan, UT) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA), 2 m*M* glutamine (Invitrogen), and 1% antibiotic-antimycotic (Invitrogen) in a humidified atmosphere of 5% CO_2 at 37°C. The cells were used in passages 3–8 only.

Cell viability

Cell viability was measured using the reduction of 3-(4,5-dimethylthiazol-2-yl)-2-diphenyl-2*H*-tetrazolium bromide (MTT) to the corresponding blue formazan. Skin fibroblasts treated with anthraquinone and Noni extract were incubated with MTT (1 mg/mL) in medium for 4 hours. The reaction was stopped by addition of 200 μL of 10% (wt/vol) sodium dodecyl sulfate/0.01 *M* HCl per well, and the formazan released from the cells after incubation at 37°C overnight was measured. Absorbance of the supernatant was measured at 570 nm against a background.

Cell treatment

Human dermal fibroblasts were plated at a density of 0.5×10^6 cells/mL into 100-mm-diameter Petri dishes in complete medium. Confluent cells were washed three times with phosphate-buffered saline. They received fresh RPMI 1640 medium without fetal bovine serum prior to sample treatment.

Enzyme-linked immunosorbent assay (ELISA)

The concentrations of procollagen type I C-terminal peptide (PICP) and GAGs contained in the cell-free supernatant of each well from each time point were analyzed by solid-phase ELISA. The ELISA kits used were the PICP ELISA kit (Takara Bio Inc., Otsu, Japan) and the Blyscan™ sulfated GAG assay kit (Biocolor Ltd., Newtownabbey, Ireland).

Immunohistochemistry

Forty-week-old nude mice photo-aged by ultraviolet radiation for 30 weeks were employed in the test. We applied emulsions containing either 0.05% or 0.5% anthraquinone to the back of the mice twice a day for 2 weeks. After application, newly synthesized type I procollagen was detected by immunostaining. Antibody for mouse type I procollagen (Santa Cruz Biotechnology, Santa Cruz, CA) was used on tissue sections.

Preparation of RNA

For RNA analysis, total cellular RNA was extracted from cultured human dermal fibroblasts. RNA extraction and purification were performed according to the method described in the Trizol kit (Invitrogen). The purity and integrity of the RNA were checked by measurement of the A_{260} nm/A_{280} nm absorbance ratio and agarose gel electrophoresis. The RNA yield was measured by absorbance at 260 nm. Samples were stored at −70°C until used.

Reverse transcription (RT)–polymerase chain reaction (PCR)

Extracted total RNA was incubated with RNase-free DNase I (Promega, Madison, WI). Synthesis of cDNA was carried out with Moloney murine leukemia virus reverse transcriptase (Promega) using oligo(dT) primers with a total RNA template. An aliquot of the RT reaction mixture was directly subjected to PCR amplification. The PCR products were electrophoretically separated according to size on a 2% TAE-agarose gel. The intensity of the bands in each lane was quantitated with a Gel-Pro analyzer (Media Cybernetics Inc., Silver Spring, MD).

Statistical analysis

Data were analyzed with one-sided *t* tests. All differences with $P < .05$ were considered statistically significant.

RESULTS AND DISCUSSION

Cytotoxicity of anthraquinone and Noni extract

The viability of human dermal fibroblasts that were exposed to various anthraquinone and Noni extract doses was measured. As shown in Figure 1, cytotoxicity induced by anthraquinone dramatically developed at doses above 100

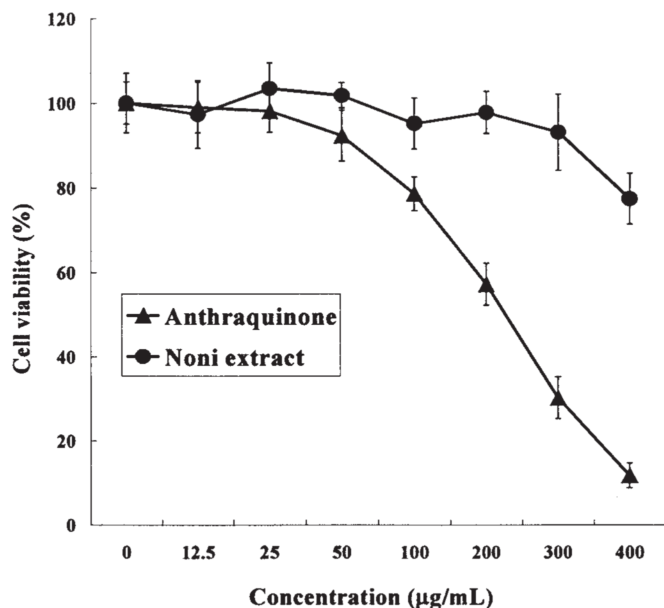

FIG. 1. Effects of various anthraquinone and Noni extract doses on the viability of human dermal fibroblast cells. The cell viability was measured using the MTT method. Data are mean ± standard deviation values ($n = 3$). All differences were statistically significant ($P < .05$).

μg/mL. However, cell death from Noni extract was not detected up to the 300 μg/mL dose.

Effects of anthraquinone and Noni extract on ECM synthesis

We investigated the effect of anthraquinone on ECM synthesis in human dermal fibroblasts. As shown in Figure 2, collagenic activity was increased time-dependently after addition of anthraquinone and Noni extract. When the cells were cultured with 50 μg/mL of anthraquinone and Noni extract, there were 49.6% and 77.2% increases at 24 hours and 110.9% and 127.6% at 36 hours in collagen contents in the culture medium, respectively. These are very significant increases in collagen content when compared with other collagenic activators (data not shown). Figure 3 represents the increase in GAG synthesis by treatment with 50 μg/mL of anthraquinone and Noni extract. There were 32.5% and 47.0% increases at 36 hours in GAG synthesis, respectively.

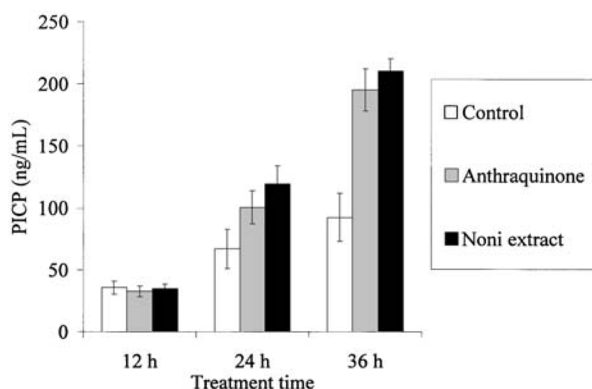

FIG. 2. Anthraquinone and Noni extract increased PICP levels in human dermal fibroblasts. Levels of PICP in the media of normal fibroblasts cultured in a serum-free model with or without anthraquinone and Noni extract were determined at 12, 24, and 36 hours by a specific ELISA. Data are mean ± standard deviation values ($n = 3$). All differences were statistically significant ($P < .05$).

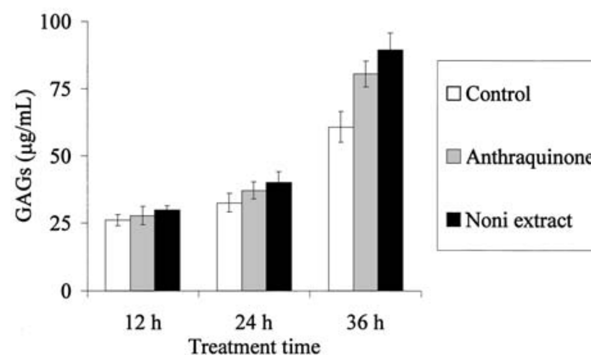

FIG. 3. Anthraquinone and Noni extract increased levels of GAGs for human dermal fibroblasts. Levels of GAGs in the media of normal fibroblasts cultured in a serum-free model with or without anthraquinone and Noni extract were determined at 12, 24, and 36 hours by a specific ELISA. Data are mean ± standard deviation values ($n = 3$). All differences were statistically significant ($P < .05$).

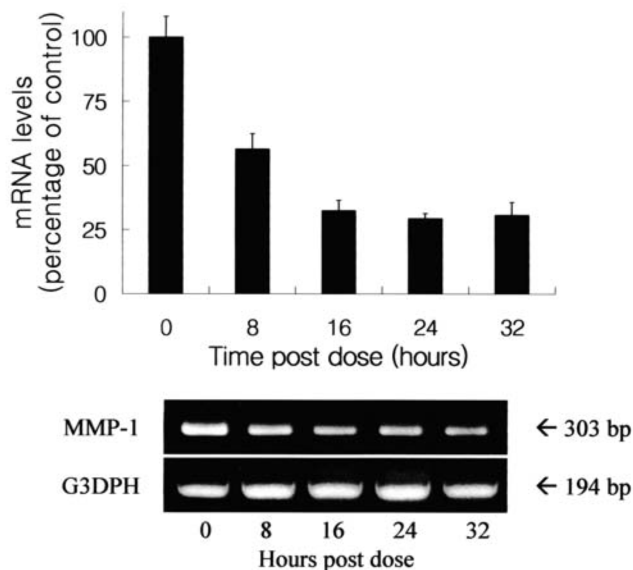

FIG. 4. Anthraquinone suppressed MMP-1 expression in human dermal fibroblasts. Levels of MMP-1 mRNA in fibroblasts applied with anthraquinone were determined at 0, 8, 16, 24, and 32 hours by RT-PCR analysis. A representative blot is shown. Data shown (mean ± standard deviation) are from two independent experiments expressed as a percentage of the zero-time level (defined as 100%) after correcting for loading using the glyceraldehyde 3-phosphate dehydrogenase (G3DPH) mRNA levels. All differences were statistically significant ($P < .05$).

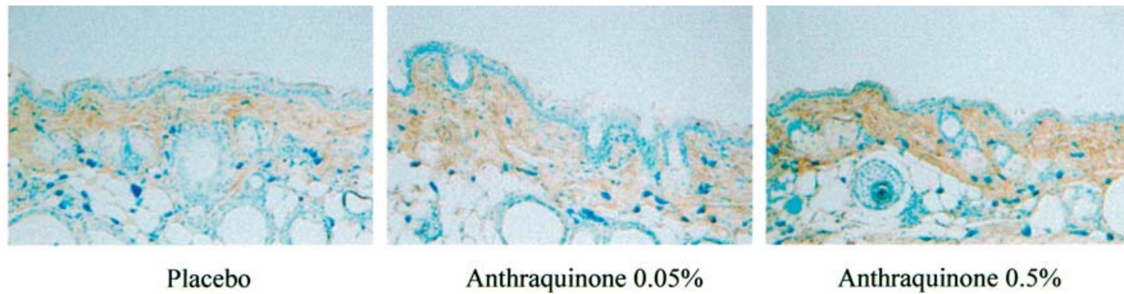

FIG. 5. Dermal type I procollagen expression after 2 weeks of treatment with anthraquinone contained in a nano-emulsion. Type I procollagen was immunohistochemically detected with a monoclonal antibody against type I procollagen in nude mice skin. Original magnification ×100.

Effects of anthraquinone on MMP-1 expression

We applied anthraquinone to human dermal fibroblasts to examine MMP-1 gene expression within 32 hours. RT-PCR analysis revealed that repression of MMP-1 expression could be observed within 8 hours after the addition of anthraquinone into the culture medium (Fig. 4). By 16 hours after the addition, the MMP-1 mRNA level was dramatically decreased and remained so up to 32 hours after exposure.

Effects of anthraquinone on type I collagen synthesis in nude mice

Newly produced type I collagen was detected in mice skin *in vivo* by immunohistochemistry. Anthraquinone was administered in the form of a nano-emulsion particle. As shown in Figure 5, the type I procollagen distributed in the dermal layer of mouse skin was densely stained brown when the emulsions with anthraquinone were applied, signifying an increased expression of type I collagen because of the effect of anthraquinone.

In conclusion, the effect of anthraquinone isolated from Noni extract on the synthesis of ECM components was investigated for efficacy as an anti-aging/anti-wrinke cosmetic ingredient. In normal human fibroblasts, synthesis of type I collagen and GAGs was increased and MMP-1 mRNA expression was decreased by anthraquinone. Furthermore, newly synthesized type I procollagen in nude mouse skin was increased in the dermal layer by the application of an emulsion containing anthraquinone.

CONCLUSIONS

Based on the collagen quantity assay in human dermal fibroblasts, anthraquinone, having strong collagenic activity, was isolated and identified from Noni extract. It was revealed that anthraquinone showed significantly increased elaboration of type I collagen and GAGs in human dermal fibroblasts and reduced expression of MMP-1. These results suggest that anthraquinone would be a good candidate for use as a new anti-wrinkle agent due to its strong synthetic activity of ECM components.

ACKNOWLEDGMENTS

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