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Human collagen alpha-2 type I stimulates collagen synthesis, wound healing, and elastin production in normal human dermal fibroblasts (HDFs)

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Skin aging appears to be the result of overlapping intrinsic (including genetic and hormonal factors) and extrinsic (external environment including chronic light exposure, chemicals, and toxins) processes. These factors cause decreases in the synthesis of collagen type I and elastin in fibroblasts and increases in the melanin in melanocytes. Collagen Type I is the most abundant type of collagen and is a major structural protein in human body tissues. In previous studies, many products containing collagen derived from land and marine animals as well as other sources have been used for a wide range of purposes in cosmetics and food. However, to our knowledge, the effects of human collagen-derived peptides on improvements in skin condition have not been investigated. Here we isolate and identify the domain of a human COL1A2-derived protein which promotes fibroblast cell proliferation and collagen type I synthesis. This human COL 1A2-derived peptide enhances wound healing and elastin production. Finally, the human collagen alpha-2 type I-derived peptide (SMM) ameliorates collagen type I synthesis, cell proliferation, cell migration, and elastin synthesis, supporting a significant anti-wrinkle effect. Collectively, these results demonstrate that human collagen alpha-2 type I-derived peptides is practically accessible in both cosmetics and food, with the goal of improving skin condition. [BMB Reports 2020; 53(10): 539-544]

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INTRODUCTION

Skin aging appears to be the result of overlapping intrinsic (including genetic and hormonal factors) and extrinsic (external environment including chronic light exposure, chemicals, and toxins) processes (1). These factors simultaneously lead to histopathological and immunohistochemical changes in each skin layer, as well as changes in skin appearance after UV exposure (2-4). Moreover, both intrinsic and extrinsic factors cause decreased synthesis of collagen type I and elastin in fibroblasts and increased melanin in melanocytes (5-7).

Collagen and elastin are required to maintain the structure of skin in the dermis, and also create its elasticity (2, 8). Interestingly, the collagen, the fibrous protein, plays a major role in supporting the mechanical strength of skin, and constitutes the majority of the dermis. The matured collagen is an alpha chain formed by three collagen strands. The fibrogenesis of collagen is associated with the repeated unique amino acid composition of glycine, proline, hydroxyproline, and alanine (9). The most abundant type of human collagen, collagen type I, is a major structural protein which is prevalent in the fibrous connective tissues such as skin, bone, and tendon (9). The collagen type I is comprised by two identical alpha-1 type I chains and one alpha-2 type I chain, which forms a triple helix (9).

Human dermal fibroblasts (HDFs) play vital roles in wound repair and tissue remodeling through cell proliferation, migration into the wound bed, synthesis of the extracellular matrix (ECM), and expression of thick actin bundles in myofibroblasts (10). Previous studies indicated that growth factors/cytokines such as fibroblast growth factors (FGF), transforming growth factor-β 1 (TGF-β1), and epidermal growth factor (EGF) directly or indirectly affect HDF motility (10-12). The effects of this growth hormone cover a broad range of biological phenotypes including regeneration, cell proliferation, and metabolism (13-15).

In previous studies, many products containing collagen or denatured forms of collagen derived from land animals, marine animals or other sources have been used for a wide range of purposes in cosmetics and food. There is controversy over the

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effects of collagen derived from food or supplements because oral intake of native collagen or its partially hydrolyzed form, gelatin, is not efficiently absorbed in the body (16). However, several evidences have shown beneficial effects of small collagen-derived peptides, which show higher absorbability compared to native collagen in a wide variety of tissues including skin, bone, and muscle in humans (16-21). Moreover, collagen has been isolated from many marine organisms including fish and others (22-24). Collagen-derived peptides from aquatic sources, such as rainbow trout skin, have unique biological properties with respect to antioxidant and anti-skin aging activity when compared to collagen peptides derived from land animals. Moreover, collagen-derived peptides from sea and freshwater rainbow trout skin had similar amino acid compositions and molecular weight (25-27). However, the effects of human collagen-derived peptides on improvements in skin condition have not been investigated. In this study, we found that a human-derived collagen peptide improves skin condition, supporting the practical applicability of this peptide in cosmetic and food compositions.

RESULTS AND DISCUSSION

Stimulatory effect of hCOL1A2 domains on fibroblast cell proliferation and collagen type I synthesis.

It has previously been reported that collagen type I synthesis and fibroblast cell proliferation are required to maintain the elasticity and strength of skin (28-30). To address the biologi-

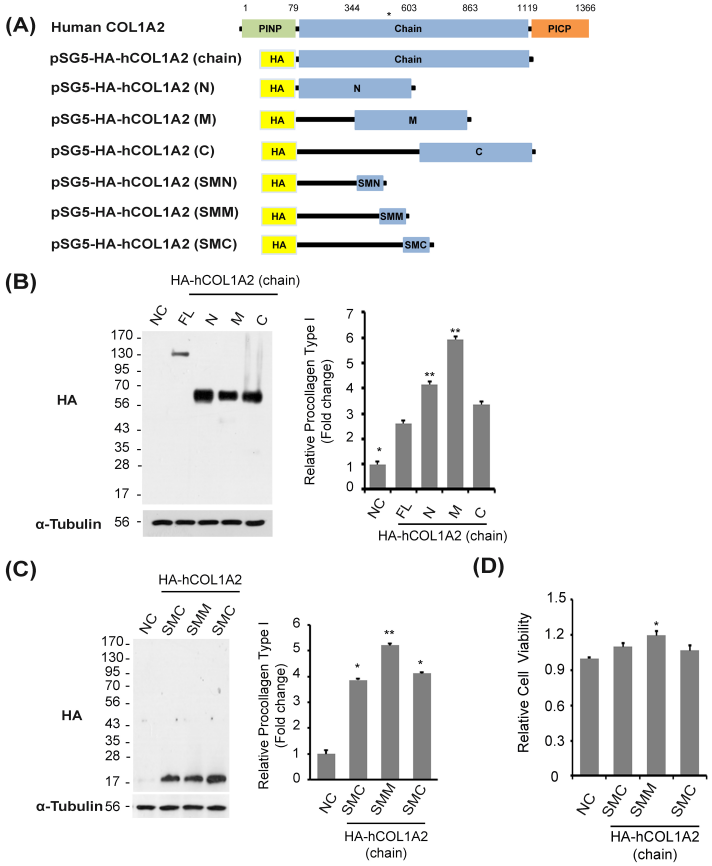

Fig. 1. Defining the domains of Human COL1A2 (hCOL1A2) involved in collagen synthesis. (A) Diagrams of Human full-length COL1A2 (hCOL1A2) and various deletion mutants. (B) HDF cells were transfected with plasmids encoding HA, HA-tagged COL1A2 FL, and each of the deletion mutants (N, M, and C). 48 h after transfection, cells were collected and cell lysates were analyzed by immunoblotting with anti-HA and anti-tubulin antibodies (*left panel*). The amount of collagen Type I was measured with an ELISA kit in transfected HDF cultured media (*right panel*). (C, D) HDF cells were transfected with plasmids encoding HA and each of the indicated mutants (SMN, SMM, and SMC). 48 h after transfection, cells were collected and cell lysates were analyzed by immunoblotting with anti-HA and anti-tubulin antibodies (*left panel*). The amount of collagen Type I was measured with an ELISA kit in transfected HDF cultured media (*right panel*). (D) Cell proliferation, which was induced by transfection with plasmids encoding HA and each of the indicated mutants (SMN, SMM, and SMC), was determined by MTT assay. Results are presented as mean ± SEM of three independent experiments. The student's *t*-test was used for statistical analyses (*P < 0.05, **P < 0.005).

cal function of human alpha-2 type I collagen (hCOL1A2) in skin fibroblasts, we examined the effects of hCOL1A2 domains on collagen type I synthesis and fibroblast cell proliferation. First, we generated a human COL1A2 (hCOL1A2) full-length (chain) and various deletion mutants (Fig. 1A). The effects of collagen type I synthesis were examined in HDF cells transfected with plasmids encoding HA, HA-tagged COL1A2 FL (chain domain), and each of the deletion mutants (N, M, and C). Cells were analyzed 48 h post-transfection by immunoblotting and the amount of collagen Type I was measured with an ELISA kit. The M domain of the COL1A2 chain significantly increased collagen type I synthesis compared to HA, COL1A2 FL (chain domain), and other deletion mutants (Fig. 1B). In addition, HDF cells were transfected with plasmids encoding HA and each of the indicated mutants (SMN, SMM, and SMC). Cells were analyzed 48 h post-transfection by immunoblotting and the amount of collagen Type I was measured with an ELISA kit. Collagen type I synthesis was significantly increased by treatment with the SMM domain of the COL1A2 chain compared to plasmids encoding HA and others mutants (SMN and SMC) (Fig. 1C). Further, the SMM domain of the COL1A2 chain increased fibroblast proliferation, compared to plasmids encoding HA and other mutants (Fig. 1D). These data indicate that the hCOL1A2-derived peptide domain (SMM) significantly stimulates collagen synthesis and fibroblast proliferation.

Isolation and identification of recombinant hCOL1A2 (SMM) protein

We generated a His-tagged hCOL1A2 (SMM) and cloned it into the pET-28a (His-tag) vector. Recombinant proteins were overexpressed in the E. coli Rosetta2 (DE3) strain and purified as previously described (31). To determine the molecular composition ratio, the purified hCOL1A2 (SMM) was analyzed by SDS-PAGE. Purified recombinant hCOL1A2 (SMM) was eluted and visualized as a single band around ~20 kDa (Fig. 2A). Isolated hCOL1A2 (SMM) was identified using an anti-His antibody (Fig. 2B) and was confirmed with an anti-COL1A2 (SMM-specific) antibody using western blot analysis (Fig. 2C).

To confirm whether the protein was actually purified recombinant hCOL1A2 (SMC), we performed in an in-gel digest with trypsin and analyzed the peptides by liquid chromatography–mass spectrometry (LC/MS). Fig. 2D and Supplementary Table 1 show a representative MS/MS spectrum for the identified peptides, which originated from hCOL1A2 (^{554}GEQGPPGPPGFQGLPGPSGPAGEVGKPGER^{573}, 2810.373 m/z) and hCOL1A2 with Hyp residues (^{465}EG*p*VGL*p*GIDGRPG*p*IG*p*AGAR^{486}, 2104.044 m/z).

The collagen biosynthesis and cell proliferation effects of recombinant hCOL1A2 (SMM) protein

A previous study reported that TGF-β1 significantly increases collagen type I synthesis and proliferation in embryonic pulmonary fibroblasts and human dermal fibroblast (HDF) cells in crowded culture (7, 32). To investigate the effect of collagen synthesis, we determined the amount of secreted collagen type I in the culture media of HDF cells stimulated with TGF-β1 or hCOL1A2 (SMM) proteins (1, 10, and 100 μg/ml). Collagen type I synthesis was increased by treatment with hCOL1A2 (SMM) protein in a dose-dependent manner (Fig. 3A). In addition, treat-

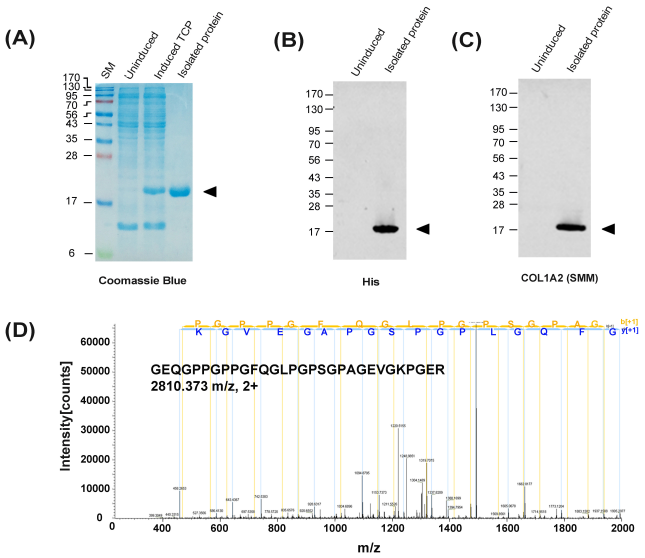

Fig. 2. Identification of purified hCOL1A2 (SMM) by SDS-PAGE and Western blot analysis. (A) SDS-PAGE showing isolated hCOL1A2 (SMM) with a prominent band; arrows indicate that hCOL1A2 (SMM) was identified using (B) anti-His and (C) anti-hCOL1A2 antibodies by western blot analysis. (D) Identification of hCOL1A2 (SMM) protein in the gel band of isolated hCOL1A2 (SMM) by LC-MS/MS analysis. The panels show the representative MS/MS spectrum for the identified peptides of GEQGPPGPPGFQGLPGPSGPAGEVGKPGER (2810.373 m/z, +2).

ment with hCOL1A2 (SMM) protein induced fibroblast proliferation in a dose-dependent manner (Fig. 3B). Next, we compared the effects of epidermal growth factor (EGF), which has previously been reported to increase collagen type I synthesis and HDF cell proliferation (33). hCOL1A2 (SMM) protein (at 1 μg/mL) and 0.1 μg/ml EGF showed similar activity for the induction of collagen type I synthesis (Fig. 3C) and fibroblast cell proliferation (Fig. 3D). Together, these results demonstrated that hCOL1A2 (SMM) significantly increased collagen type I synthesis and fibroblast proliferation. Further, hCOL1A2-derived protein (SMN) was detectable in HDF cell lysates after washing out the culture medium containing hCOL1A2-derived

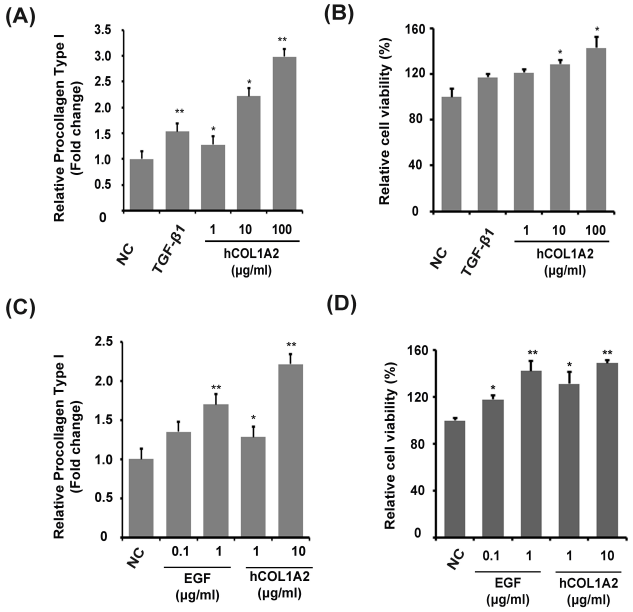

Fig. 3. hCOL1A2 (SMM) stimulates collagen synthesis and cell proliferation in primary human dermal fibroblast (HDF) cells. (A) HDF cells were treated with hCOL1A2 (SMM) (1, 10, 100 μg/ml) and TGF-β1 (5 ng/ml) for 48h. The amount of collagen Type I was measured with an ELISA kit in HDF cultured media. (B) HDF cells were treated with hCOL1A2 (SMM) (1, 10, 100 μg/ml) and TGF-β1 (5 ng/ml) for 48h. Cell proliferation was determined by MTT assay. (C, D) HDF cells were treated with EGF (0.1, 1 μg/ml) and hCOL1A2 (SMM) (1, 10 μg/ml) for 48h. The amount of collagen Type I was measured by ELISA in HDF cultured media (C). Cell proliferation, which was induced by EGF and hCOL1A2 (SMM), was determined by MTT assay (D). Results are mean ± SEM of three independent experiments. A student's *t*-test was used for statistical analyses (*P < 0.05, **P < 0.005).

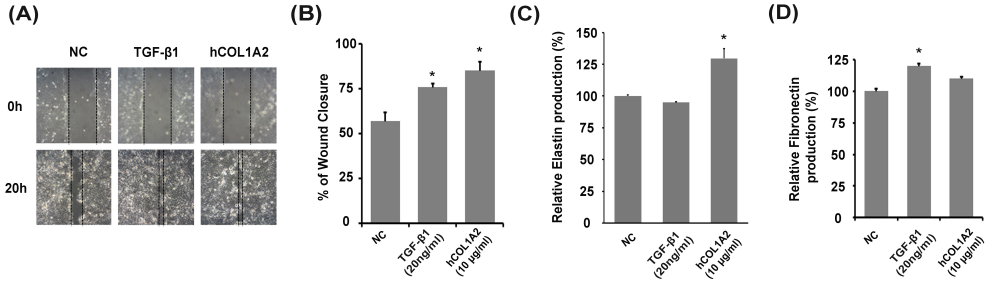

Fig. 4. Human COL1A2-derived protein increased wound healing efficiency and elastin production, but not fibronectin production. (A) HaCaT cells were grown to confluence on six-well tissue culture plates. Cells were serum-starved for 12 hours. Cells treated with TGF-β1 (20 ng/ml) and hCOL1A2 (10 μg/ml) were then used in an *in vitro* wound assay. Migration was monitored for up to 20 hours. The same fields were photographed immediately after wounding and 20 hours later, and pictures were superimposed using Adobe Photoshop. Areas were measured using Image J. A representative result is shown. (B) Cell migration was monitored for up to 20 hours and quantified by image analysis. Results are presented as the mean ± SEM of three independent experiments. The student's *t*-test was used for statistical analyses (*P < 0.05). (C, D) HDF cells were treated with hCOL1A2 (SMM) (10 μg/ml) and TGF-β1 (20 ng/ml) for 48h. The amount of elastin (C) and fibronectin (D) were measured by ELISA in HDF cultured media. Results are presented as the mean ± SEM of three independent experiments. The student's t-test was used for statistical analyses (*P < 0.05) (41).

protein (SMN) (Supplementary Fig. 1), suggesting that SMN is bioactive, similar to hydrolyzed collagen peptides, and has benefits in absorption and utilization.

***In vitro* scratch-wound healing assay and Elastin production**

Previous studies suggested that the abundant amino acid residues in collagen peptides serve as additional nutrients for cellular growth and proliferation (22). To evaluate the effects of hCOL1A2 (SMM) on cell adhesion and growth, we utilized the human keratinocyte cell line HaCaT, which has previously been utilized in *in vitro* skin wound healing models (34). We investigated the effect of TGF-β1 and hCOL1A2 (SMM) on wound closure in HaCaT cell monolayers. As shown in Fig. 4A and 4B, scratch closure occurred more quickly in the presence of hCOL1A2 (SMM) compared to TGF-β1 and the negative control (NC). We examined the wound closure assay and statistically analyzed the data which derived from captured images at least five field in three independent experiments (Fig. 4B). At 20 hours of incubation, the wound closure rate of the cells showed 75-90% by hCOL1A2 (SMM) treatment, while the control group showed less than 60%.

A previous study demonstrated that skin is subject to intrinsic and extrinsic aging, which are both associated with histopathological and immunohistochemical changes (1). Such a skin aging process may include qualitative and quantitative changes, including diminished or defective collagen and elastin synthesis in the dermis (35, 36). To examine the effect on elastin synthesis, we quantified elastin in the culture media of HDF cells stimulated with TGF-β1 or hCOL1A2 (SMM) proteins (10 μg/ml). Elastin synthesis was significantly increased with hCOL1A2 (SMM) protein treatment compared to TGF-β1 and the negative control (NC, media treatment only) whereas fibronectin synthesis was not affected by hCOL1A2 (SMM) (Fig. 4C and 4D). Together, these results demonstrate that a collagen peptide (SMM) from human collagen alpha-2 Type I induces cell migration and elastin synthesis.

According to previous study, collagen peptides inhibited the expression of metalloproteinases (MMP1 and MMP3) release while simultaneously increasing elastin synthesis (7, 37). Matrix metalloproteinases (MMP1 and MMP3), the major collagenases in human, which were induced by ultraviolet irradiation (UV)/sun exposure and natural skin aging, mediate the degradation of fibrillar type I collagen and elastic fiber (38-40). In contrast, treatment of the collagen peptides or derivatives such as a hCOL1A2 (SMM) provides the anti-skin aging effect through collagen expression/activation (collagen chain trimerization) and activation of TGF-β signal pathway (37). In conclusion, the human collagen alpha-2 type I-derived peptide (SMM) enhances collagen type I and elastin synthesis, cell proliferation, and cell migration, supporting a significant anti-wrinkle effect. Additionally, hCOL1A2 (SMM) might be induced by relating hydroxylases such as previously developed collagen derivatives to supporting the anti-skin aging effect. The hCOL1A2 (SMM) induces anti-wrinkle effects, including proliferation of fibroblast and wound healing, but it has not been determined whether SMM can be used as a cosmetic ingredient. Therefore, comparing the functionality of hCOL1A2 (SMM) with the existing functional cosmetic ingredients will be of great interest.

MATERIALS AND METHODS

Detailed information is provided in the Supplementary Information.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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