Title: Effects of human collagen α-1 type I-derived proteins on collagen synthesis and elastin production in human dermal fibroblasts

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Running Title: Effects of hCOL1A1-derived peptides on skin cells

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Supplementary Table 1. Identifed peptides of hCOL1A1 by peptide mass fingerprinting analysis

m/z	Range	Amino acid sequences	# PSMs	No. of P residue (s)	No. of Hyp residue (s)
1213.588	746-759	GDAGPKGADGSPGK	2	2	0
1115.550	752-763	GADGSPGKDGVR	2	1	0
2655.351	752-781	GADGSPGKDGVRGLTGPIGPPGPAGAPGDK	1	6	0
2837.412	764-796	GLTGPIGPPGPAGAPGDKGESGPSGPAGPTGAR	6	8	0
1297.615	781-796	GESGPSGPAGPTGAR	2	3	0
2703.188	797-826	GApGDRGEpGppGPAGFAGPPGADGQPGAK	5	8	4
2639.264	797-826	GAPGDRGEPGPPGPAGFAGPPGADGQPGAK	7	8	0
2086.004	803-826	GEPGPPGPAGFAGPPGADGQPGAK	1	7	0
2868.354	803-835	GEPGPPGPAGFAGPPGADGQPGAKGEPGDAGAK	2	8	0
3015.495	827-862	GEPGDAGAKGDAGPPGPAGPAGPPGPIGNVGAPGAK	2	9	0
2233.133	836-862	GDAGPPGPAGPAGPPGPIGNVGAPGAK	31	8	0
2249.168	836-862	GDAGPPGPAGPAGpPGPIGNVGAPGAK	7	8	1
2517.299	836-865	GDAGPPGPAGPAGPPGPIGNVGAPGAKGAR	2	8	0

Hydroxyproline : Hyp, p

Post Translational Modifications : PTMs

Proline : P

Materials and Methods

Antibodies and reagents

The following antibodies were used: anti-α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-HA (Roche, Basel, Switzerland), and anti-His (Cell Signaling Technology, Danvers, MA, USA and ThermoFisher, Waltham, MA, USA). The following reagents were used: EGF (Sigma, St. Louis, MO, USA), L-ascorbic acid/vitamin C (Sigma), TGF-β1 (R&D Systems, Minneapolis, MN, USA), dimethyl sulfoxide (DMSO; AG Scientific, San Diego, CA, USA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma).

Cell culture

Primary HDFs (American Type Culture Collection, Manassas, VA, USA) and immortalized HaCaT cells (Addexbio, San Diego, CA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM; Lonza, Slough, UK) containing 4.5 g/L glucose and supplemented with 2 mM L-glutamine (Sigma), 100 IU/mL penicillin, 100 µg/mL streptomycin (Sigma), and 10% (*v*/*v*) fetal bovine serum (FBS; ThermoFisher). Cells were cultivated in T-flasks at 37 ^{O}C in a humidified atmosphere containing 5% CO_2.

Construction of plasmid expression vectors for hCOL1A1 and its fragments

In this study, we used DNA encoding the full-length (FL) (162 - 1218 a.a) recombinant hCOL1A1, the N-terminal (N; 162-698 aa), the middle (M; 439 - 958 aa), the C-terminal chain (C; 699 - 1218 aa), the small middle A (SMA; 440 - 880 aa), the small middle B (SMB; 439 - 698 aa), the small middle C (SMC; 699 - 958 aa), the small middle D region (SMD; 743 - 958 aa) and the alpha-1 type I collagen effective domain (CED; 743 - 880 aa) of hCOL1A1; these were all amplified by *Pfu* DNA polymerase. hCOL1A1 construct primers were as follows:

FL, forward primer 5′- ACGAAGCTTCAGCTGTCTTATGGCTAT-3′ and reverse primer 5′-TCGAGCGGCCGCAGCCCGGTAGTAGCGGCC-3′;

N, forward primer 5′-ACGAAGCTTCAGCTGTCTATGGCTAT-3′ and reverse primer 5′-TCGAGCGGCCGCCCCTCGGGGACCAGCAGG-3′;

M, forward primer 5′-ACGAAGCTTCCTGGCAGCAAAGGAGAC-3′ and reverse primer 5′-TCGAGCGGCCGCACGCTGTCCAGAATACC-3′;

C, forward primer 5′- ACGAAGCTTGCCAACGGTGCCCCGGC-3′ and reverse primer 5′-TCGAGCGGCCGCAGCCCGGTAGTAGCGGCC-3′;

SMA, forward primer 5′- CTGAAGCTTGCGGCAGCAAAGGAGACA-3′ and reverse primer 5′- CCGGCGGCCGCAGCAGCACCAGGGAAA-3′;
SMB, forward primer 5′-ACGAAGCTTCCTGGCAGCAAAGGAGAC-3′ and reverse primer 5′-TCGAGCGGCCGCCCCTCGGGGACCAGCAGG-3′;
SMC, forward primer 5′-ACGAAGCTTGCCAACGGGCTCCCGGC-3′ and reverse primer 5′-TCGAGCGGCCGCACGCTGTCCAGCAATACC-3′;
SMD, forward primer 5′-CAGAAGCTTGCGGTGACAGAGGTGATG-3′ and reverse primer 5′- CCGGCGGCCGCACGCTGTCCAGCAATA-3′;
and CED, forward primer 5'- ATGGGTCGCGGATCCGGTGACAGAGGT-3' and reverse primer 5′- CTCGAGTGCGGCCGCAGCAGCACCAGG-3′.
Amplified DNA fragments were inserted into the HA-tagged pcDNA3.1 or pET-28a vector.

Expression and purification of the recombinant hCOL1A1 protein

hCOL1A1 was cloned into the pET-28a (His-tag) vector, and the recombinant DNA fragments were overexpressed in the *E. coli* Rosetta2 (DE3) strain. Transfected bacterial cells were lysed using three rounds of sonication (10-s duration with a 50-s interval). His-tagged proteins were purified from the supernatant using 1 mL of Ni-NTA resin (GE Healthcare), and the filtered supernatant was incubated with the resin for 1 h at 4 °C under continuous rotation (4 rpm). The resin was washed off with a washing buffer (phosphate-buffered saline [PBS], pH 7.4; 50 and 100 mM imidazole), and proteins were eluted with an elution buffer (PBS, 500 mM imidazole, pH 7.4). Eluted proteins were dialyzed at 4 °C overnight against PBS (pH 7.4) in a dialysis tube with cellulose membranes (Sigma). Purified proteins were stored at −80 °C until use.

Selection of anti-hCOL1A1 single-chain variable fragments (scFvs) from a phage library

A phage library representing over 7.6×10^9 independent human scFvs was provided by Prof. Hyun-bo Shim (Ewha Womans University, Korea) (1), and was screened for recombinant hCOL1A1 using recombinant His-tagged hCOL1A1-CED as a soluble competitor (1). After four rounds of panning, phagemid vectors were extracted from high-affinity binding phages and then subjected to DNA sequencing. The expression and purification of isolated scFv clones was performed as previously described (1).

In vitro cell viability assay

HDFs were incubated at 37°C in a 5% CO_2 incubator for 48 h with TGF-β1 (20 ng/ml), EGF (0.1 and 1 μg/ml), vitamin C (100 μg/ml) or hCOL1A1 (1 and 10 μg/ml), and cell viability was estimated. Mitochondrial dehydrogenease activity, an index of cell viability, was evaluated

using MTT, which was reduced to an insoluble formazan dye. Briefly, culture media were replaced with fresh 0.1% FBS media containing 1 mg/ml of MTT and incubated for 3 - 4 h. Next, DMSO (200 μl) as added to each well. After incubation at room temperature for an additional 30 min, the optical density was measured spectrophotometrically at 490 nm. Each experiment was repeated at least three times.

Measurement of collagen biosynthesis and elastin production

HDFs were seeded into 6-well plates (60,000 cells per well) and grown in DMEM containing 5% FBS for 24 h. Next, the medium was replaced with fresh assay medium containing 0.1% FBS and the corresponding treatment to test for collagen production. After 48 h of incubation, the cell culture medium was collected and centrifuged at 3000 rpm for 15 min. The levels of collagen I secreted from treated cells were quantified using an ELISA kit (Takara Bio Inc., Otsu, Japan), according to the manufacturer's recommended protocols. Measurements were repeated at least thrice with an independent cohort of cells. The levels of elastin secreted from treated cells were quantified using an ELISA kit (Abcam, Cambridge, UK), according to the manufacturer's recommended protocols. Measurements were repeated at least thrice with an independent cohort of cells.

Measurement of melanin contents

B16F10 melanoma cell lines were seeded on 24-well plates (20,000 cells per well) and grown in DMEM containing 10% FBS. Next, the medium was replaced with fresh assay medium containing 200 nM α-MSH and/or 125 nM vitamin C or hCOL1A1-CED as indicated concentrations for 24 h. Cells were detached using 0.05% trypsin-EDTA in PBS, harvested, and transferred into 1.5 ml tube. Equal numbers of cells were solubilized in 1 N NaOH for 1 h at 80°C. The dissolved melanin was assessed by measuring the absorbance at 405 nm, and melanin content was measured by ELISA. The results were normalized by protein concentrations and was analyzed as percentage.

Transfection and western blotting

HDFs were transfected with HA, HA-tagged FL hCOL1A1, or various mutant plasmids (N, M, C, SMA, SMB, SMC, SMD, or SME) using the TurboFect transfection reagent (ThermoFisher). Cells were harvested 48 h post-transfection and lysed in nuclear extraction buffer (20 mM HEPES [pH 7.6], 20% glycerol, 250 mM NaCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 1 mM PMSF, 1 mM DTT, and protease inhibitor cocktail [Roche]). Equal amounts of protein were separated using SDS-PAGE and analyzed by immunoblotting with anti-HA and anti-α-tubulin antibodies.

RNA isolation and quantitative RT-PCR

RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA), and cDNA synthesized with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), followed by quantitating reverse-transcriptase PCR with Power SYBR Green PCR master mix (Applied Biosystems) in a CFX96 Real-Time PCR detection (Bio-Rad, Hercules, CA). Primer sequences are available on request.

LC/MS and MS/MS data analysis

The gel band (recombinant hCOL1A1-CED protein) was digested with trypsin enzyme, as described previously (1). All LC/MS analysis samples were analyzed with an LTQ-Orbitrap Velos (ThermoFisher) connected to an Easy-nano LC II system (ThermoFisher) incorporated with an autosampler. All MS/MS samples were analyzed with Sequest (XCorr Only; version v.27, rev. 11) and X! Tandem [version CYCLONE (2010.12.01.1)] using the UniProt human database (version 2014). LC/MS and MS/MS data analyses were performed as previously described (2).

Transwell migration assay

Transwell migration assays were performed using uncoated cell culture inserts with 8-μm pores (Corning Inc., Tewksbury, MA, USA), according to the manufacturer's protocol. The experiments were conducted in triplicate and two fields were analyzed for each replicate (n = 6).

Statistical analysis

The results are presented as the mean ± standard deviation (SD) of at least three independent experiments that were performed in triplicate, unless otherwise specified. Data from the test and control groups were compared and analyzed for statistical significance using the two-tailed, unpaired Student's t-test using GraphPad Prism 5 software.

References

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