Arch Biol Sci. 2022;74(1):91-96 https://doi.org/10.2298/ABS220215007C

Retinoic acid induces hyaluronic acid production through the klotho-mediated EGFR signaling pathway in human epidermal keratinocytes

Hyangtae Choi[1,2], Yonghee Lee[1], Won-seok Park[1], Beom Joon Kim[2,#] and Chang Seok Lee[3,*]

[1]*Amorepacific R&D Center*, Yongin-si, Republic of Korea
[2]*Department of Dermatology, Chung-Ang University College of Medicine*, Seoul, Republic of Korea
[3]*Department of Beauty and Cosmetic Science, Eulji University*, Seongnam-si, Republic of Korea

Corresponding authors: *cslee2010@eulji.ac.kr; #beomjoon74@gmail.com

Received: February 15, 2022; **Revised**: February 23, 2022; **Accepted**: February 24, 2022; **Published online**: March 16, 2022

Abstract: All-*trans* retinoic acid (RA) is an effective anti-aging chemical substance widely used in skin-care products. RA compromises epidermal differentiation and induces keratinocyte proliferation, causing hyaluronic acid production through mechanisms that are not completely understood. Klotho protein causes the differentiation of human epidermal keratinocytes. Klotho gene expression is mediated by epidermal growth factor (EGF), which inhibits cell apoptosis in aging-related diseases. The klotho gene causes human aging syndrome, including short lifespan, skin atrophy, and osteoporosis. We investigated the relationship between RA and klotho in epidermal keratinocytes for the first time. In human epidermal keratinocytes, RA induced klotho gene expression. Treatment with both RA and recombinant klotho induced hyaluronic acid production in human epidermal keratinocytes. However, in klotho small interfering RNA (siRNA)-transfected keratinocytes, RA produced less hyaluronic acid than in the control group, indicating that RA may partially regulate hyaluronic acid production through a klotho-dependent pathway. Knockdown of klotho gene expression inactivated the EGFR-extracellular signal-regulated kinase (ERK) signaling pathway, which is involved in hyaluronic acid production. We concluded that the effect of RA on hyaluronic acid production is partly regulated through the klotho-mediated EGFR signaling pathway in human epidermal keratinocytes.

Keywords: all-trans-retinoic acid; hyaluronic acid; klotho; epidermal growth factor receptor-extracellular signal-regulated kinase (EGFR-ERK) signaling pathway

INTRODUCTION

Vitamin A and its retinoid derivatives are important regulators of keratinocyte proliferation and differentiation. In normal human skin, RA stimulates keratinocyte proliferation, leading to thickening of the epidermis [1]. RA stimulates hyaluronan production in human skin organ cultures and human keratinocyte cultures [2-3]. RA-induced hyaluronan production is partly mediated by epidermal growth factor receptor (EGFR) signaling in epidermal keratinocytes [4]. EGF upregulates the hyaluronan synthases Has2 and Has3 involved in epidermal proliferation and differentiation in organotypic keratinocyte cultures [5]. However, the molecular mechanism between retinoic acid and the EGFR signaling pathway is not completely known.

The klotho gene was identified in 1997. It encodes for a single-pass transmembrane protein that forms a complex with multiple fibroblast growth factor (FGF) receptors and functions as an obligatory co-receptor for FGF23 [6]. The klotho gene was originally identified as a putative age-suppressing gene in mice that extends the life span when overexpressed; defects in either klotho or *fgf23* gene expression cause phosphate retention and a premature-aging syndrome in mice, unveiling a potential link between phosphate metabolism and aging [7]. The transcriptional activity of klotho is regulated by epidermal growth factor (EGF), and klotho inhibits cell apoptosis via the EGF-dependent pathway [8-9]. Thus, klotho is involved in the suppression of several aging phenotypes because it

functions as part of a signaling pathway that regulates aging *in vivo*, and morbidity in age-related diseases [6,7,10].

In skin, studies performed to define the function of klotho revealed that epidermal differentiation was disrupted in *kl/kl* mice, and β-klotho was required for the differentiation of human epidermal keratinocytes [11]. (-)-Epigallocatechin-3-O-gallate (EGCG) functions as an inducer of keratinocyte differentiation via transcriptional regulation of klotho protein [12]. Klotho protein protects human keratinocytes from UVB-induced damage, possibly by lowering the expression and nuclear translocation of nuclear factor-κB (NF-κB) [13]. Scleroderma patients exhibit significantly lower serum α-klotho levels than healthy controls [14].

Based on the results of previous studies about RA [1-4] and klotho [11] in keratinocyte differentiation, in the present study, we investigated the biological relation between retinoic acid and klotho as regards the correlation of the production of hyaluronic acid and the EGFR signaling pathway. We report for the first time that RA could serve as an anti-aging reagent, inducing hyaluronic acid production through the klotho-mediated EGFR signaling pathway in keratinocytes.

MATERIALS AND METHODS

Cell culture

Normal human keratinocytes (NHKs) were purchased from Invitrogen (San Diego, CA, USA). Cultured NHKs (early passage cells) were used for all experiments. NHKs were grown in keratinocytes basal media-gold, supplemented with Bullet kit (KBM-GoldBullet kit, Lonza, MD, USA). Normal human dermal fibroblasts (NHF) and HEK293 cells were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and the American Type Culture Collection (Manassas, VA, USA), respectively. Both cells were cultured in Dulbecco's modified Eagle medium (DMEM, #12–604F, Lonza, Walkersville, MD, USA) with 5% FBS and 1% penicillin-streptomycin. Cells were maintained at 37℃ in a humidified atmosphere with 5% CO_2.

Real-time qPCR

Total RNA was extracted using Qiagen RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's manual. The first-strand cDNA was synthesized from 4 μg of total RNA using the Superscript™ III first-strand synthesis system (Invitrogen, CA, USA). Real-time qPCR was performed in ABI 7500HT fast (Applied Biosystems, Foster City, CA, USA) using TaqMan Universal PCR Master Mix. A TaqMan probe for klotho was synthesized by Bioneer (Daejeon, Korea). The sequences of the primers were 5'-TGGAAACCTTAAAAGCCATCAAGC-3' (forward), and 5'-CCACGCCTGATGCTGTAACC-3' (reverse). A TaqMan probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Applied Biosystems. Relative changes in klotho mRNA expression levels were normalized for GAPDH mRNA expression levels in the same samples.

Western blotting

To prepare intracellular protein samples, cells were lysed in lysis buffer containing 150 mM NaCl, 2% sodium dodecyl sulfate (SDS), and 50 mM Tris (pH 8.0). Lysed samples were heated at 95°C for 5 min to denature nuclear DNA. The obtained protein samples were then separated by SDS polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes. The blots were incubated with anti-klotho (Abcam, Cambridge, MA, USA), anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA), anti-phospho-EGFR, anti-EGFR, anti-phospho-ERK, and anti-ERK (Cell Signaling Technology, Beverly, MA, USA). The blots were developed by an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Klotho gene knockdown

For gene knockdown, klotho-specific siRNA was transfected into NHK cells using the 100 μM lipofectamine 2000 reagent (Invitrogen). Scrambled siRNA for the negative control and klotho-specific siRNA were purchased from Dharmacon (Chicago, IL, USA). Cells were harvested after 5 days of incubation.

Hyaluronic acid (HA) enzyme-linked immunosorbent assay (ELISA)

Conditioned media for HA ELISA were harvested 5 days after treatment with recombinant klotho (R&D systems, Minneapolis, MN, USA), klotho siRNA, and RA (Sigma-Aldrich). HA ELISA was performed using the Corgenix (Broomfield, CO, USA) HA test kit according to the manufacturer's manual.

Immunocytochemistry

Immunocytochemistry for klotho detection in NHK cells was performed after formaldehyde fixation. Briefly, cells were fixed for 20 min with 4% formaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.2 % Triton X-100 solution. Fixed cells were then blocked with bovine serum albumin (BSA) in PBS and incubated with Klotho antibody for 1 h, followed by 4',6-diamidino-2-phenylindole (DAPI) counterstaining. Fluorescence images of klotho were obtained by confocal microscopy (Zeiss, Germany).

Statistical analysis

The results are expressed as the mean±standard deviation (SD). The data were analyzed using Student's t-test, and the two-tailed value of $P<0.05$ was considered statistically significant. Data were processed by the Statistical Package for the Social Sciences (SPSS) for Windows statistical package (SPSS Inc., Chicago, IL, USA).

RESULTS

RA-induced klotho gene expression in NHK cells

We investigated klotho expressions in NHF and NHK cells. qRT-PCR (Fig. 1A) revealed that both NHF and NHK expressed klotho. HEK293, a representative cell line with klotho proteins, was previously used as a positive cell line to check klotho gene expression [6]. In the present study, we used NHK because the relationship between RA and klotho in epidermal keratinocytes is not yet known. We assessed whether RA induces klotho expression in NHK. In Fig. 1B, it can be seen that RA (1 μM) significantly increased klotho mRNA expression for 2 days in NHK. To further check the expression of klotho protein, we treated NHK with 1 μM RA for 2 or 8 days. After 2 days, klotho protein expression was slightly increased by RA treatment, whereas RA increased klotho protein expression after 8 days (Fig. 1C). Klotho protein

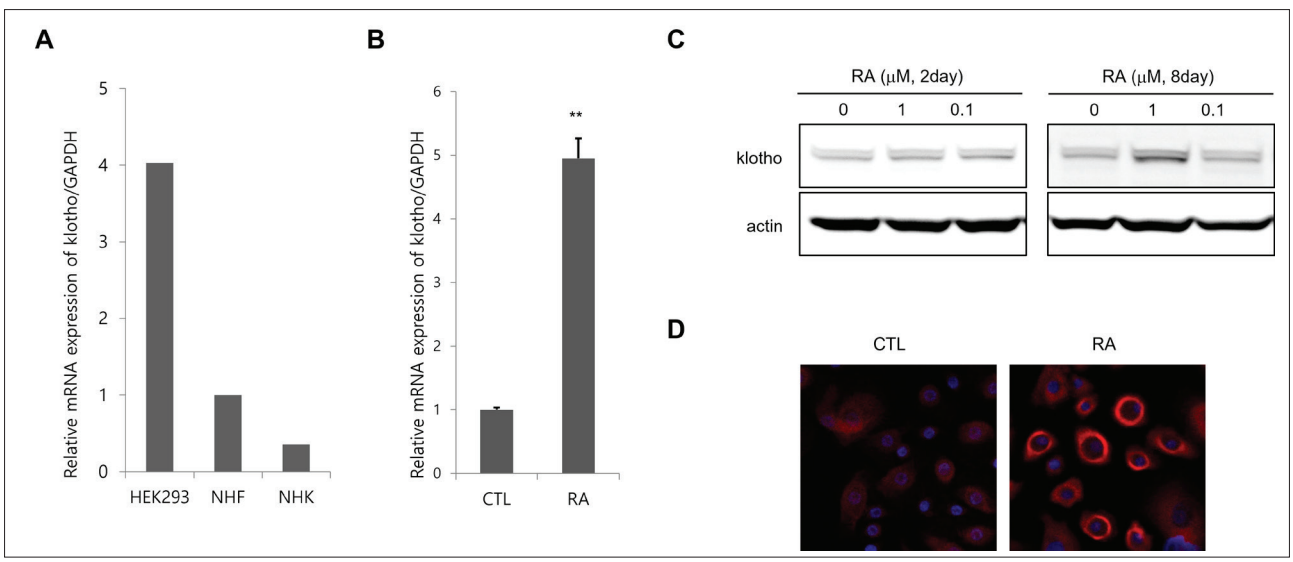

Fig. 1. RA-induced klotho expression in normal human keratinocytes (NHKs). **A** – Klotho mRNA expression in normal human fibroblasts (NHFs) and NHKs. Human embryonic kidney cells (HEK293) were used as the positive control. **B** – Upregulation of klotho mRNA in RA-treated NHKs. **C** – Upregulation of klotho protein measured by Western blotting in RA-treated NHKs. **D** – Upregulation of klotho protein (red color) measured by immunocytochemistry in RA-treated NHKs. The results are expressed as the mean±SD of three independent experiments. **P<0.01 compared to the control (CTL).

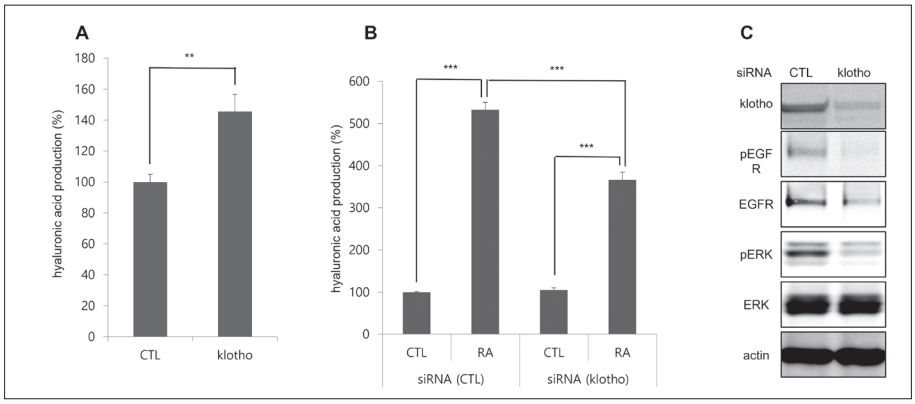

Fig. 2. Alteration of hyaluronic acid (HA) production mediated by klotho expression. **A** – HA secretion measured using HA ELISA in recombinant human klotho protein-treated NHKs. **B** – The effect of RA on HA production depends on the presence or absence of klotho. HA production was measured using HA ELISA. Klotho knockdown was confirmed using Western blotting. **C** – Protein expression of phospho-EGFR, total EGFR, phospho-ERK, and ERK after klotho knockdown in normal human keratinocytes (NHKs), evaluated by Western blotting. Results are expressed as the mean±SD of three independent experiments. **P<0.01, ***P<0.001 compared to the control (CTL) (control group).

expression was upregulated in the cytosol and plasma membrane of RA-treated NHK cells (Fig. 1D). The data suggested that RA induced klotho gene transcription and klotho protein expression in human keratinocytes.

Alteration of hyaluronic acid production mediated by klotho expression

Next, we determined if klotho could induce the production of hyaluronic acid in NHK cells. NHK were treated with recombinant human klotho protein for 2 days, and the ELISA assay was performed to evaluate the amount of secreted hyaluronic acid using NHK-cultured media. In Fig. 2A, recombinant human klotho increased secreted hyaluronic acid production up to 145% compared with the control group. Based on previous reports that RA produces hyaluronic acid in epidermal keratinocytes [2-4], we assumed that RA could stimulate hyaluronic acid production via a klotho-mediated pathway. Klotho siRNA was designed and used to transfect NHK cells. The siRNA-transfected NHK cells were exposed to RA, and after 2 days, the amount of secreted hyaluronic acid was determined. In Fig. 2B it can be seen that RA induced hyaluronic acid production in siRNA-transfected NHK cells. The increase in hyaluronic acid production by RA treatment was significantly suppressed in klotho siRNA-transfected NHK. These findings suggested that RA induced hyaluronic acid production through a klotho-dependent pathway; therefore, we focused on the molecular mechanism underlying the klotho-mediated signaling pathway. To examine whether the effects of klotho are mediated by the EGFR-ERK signaling pathway, Western blotting using both control siRNA and klotho siRNA-transfected NHK was performed. Fig. 2C shows that knockdown of klotho expression inhibited phospho-EGFR, total EGFR, and phospho-ERK protein expression. These results suggest that klotho acts through the EGFR-ERK signaling pathway in epidermal keratinocytes.

DISCUSSION

In the human keratinocyte, RA, a representative anti-aging compound, induces cell proliferation and differentiation and stimulates hyaluronan production through the EGFR signaling pathway [1-4]. In a human clinical study, it was confirmed that RA increases epidermal thickness [15]. Klotho also affects the differentiation of human epidermal keratinocytes [6-11]. A defect in klotho expression in the mouse results in skin atrophy [6]. However, there are only a few reports describing the relationship between RA and klotho,

although klotho was reported to bind to retinoic acid-inducible gene I (RIG-I) and suppress NF-κB signaling [16]. Thus, we hypothesized that the mechanism of RA action would be involved in a klotho-mediated signaling pathway in keratinocytes. We found that RA increased klotho gene expression in keratinocytes and increased hyaluronic acid production. Furthermore, because EGFR signaling in epidermal keratinocytes mediates RA-induced hyaluronan production [4], we checked if klotho mediates the EGFR-ERK signaling pathway. We observed that klotho is involved in the EGFR-ERK signaling pathway, and that knockdown of the klotho gene inhibited not only EGFR phosphorylation but total EGFR expression. Based on the data, we concluded that klotho produces an effect on EGFR gene expression, and subsequently ERK phosphorylation. These results imply that RA exhibited anti-aging activity and they show that RA-induced klotho gene expression increased hyaluronic acid through the EGFR-ERK cascade in keratinocytes. However, we have not presented direct evidence that klotho-mediated EGFR/ERK signaling is involved in hyaluronic acid production, although klotho regulates hyaluronic acid production in RA-treated keratinocytes. Nonetheless, we expect that the klotho-mediated EGFR signaling pathway could also produce an effect on hyaluronic acid production in NHK, as was shown previously [4], although a detailed study is needed. In addition, the knockdown of Egr-1 attenuated EGF-induced klotho promoter activity. The Ras/MEK/ERK signaling cascade is also involved in the EGF-induced activation of the klotho promoter [8]. These results indicated that klotho is activated by the EGF signaling pathway. Therefore, the expression of klotho protein is induced by EGF-ERK signaling, and simultaneously, the klotho protein activates the EGF-ERK signaling pathway, as described herein and in previous studies [8-9].

In conclusion, our results provide preliminary evidence that RA increases hyaluronic acid production through klotho gene expression in keratinocytes. We defined for the first time the relationship between RA and klotho expression, which causes hyaluronic acid production in keratinocytes. We present evidence for a novel anti-aging mechanism of RA mediated by klotho expression. We believe that this study describing the relationship between RA and klotho will be helpful to further work in anti-aging.

Funding: The study was supported by grants from the Amorepacific R&D Center.

Author contributions: Conceptualization, BJ Kim and CS Lee; methodology, H Choi and Y Lee; validation, H Choi and CS Lee; investigation, H Choi and Y Lee; data curation, WS Park and CS Lee; writing – original draft preparation, H Choi; writing – review and editing, BJ Kim and CS Lee; project administration, WS Park and CS Lee; funding acquisition, H Choi and WS Park. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest disclosure: The authors have no conflicts of interest to declare

Data availability: Data underlying the reported findings have been provided as part of the submitted article and are available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Choi%20et%20al_7502_Data%20Report.Fig.2C.pdf

REFERENCES

1. Fisher GJ, Voorhees JJ. Molecular mechanisms of retinoid actions in the skin. FASEB J. 1996;10(9):1002-13. https://doi.org/10.1096/fasebj.10.9.8801161
2. Tammi R, Tammi M. Influence of retinoic acid on the ultrastructure and hyaluronic acid synthesis of adult human epidermis in whole skin organ culture. J Cell Physiol. 1986;126(3):389-98. https://doi.org/10.1002/jcp.1041260309
3. Akiyama H, Saito M, Qiu G, Toida T, Imanari T. Analytical studies on hyaluronic acid synthesis by normal human epidermal keratinocytes cultured in a serum-free medium. Biol Pharm Bull. 1994;17(3):361-4. https://doi.org/10.1248/bpb.17.361
4. Pasonen-Seppänen SM, Maytin EV, Törrönen KJ, Hyttinen JM, Hascall VC, MacCallum DK, Kultti AH, Jokela TA, Tammi MI, Tammi RH. All-trans retinoic acid-induced hyaluronan production and hyperplasia are partly mediated by EGFR signaling in epidermal keratinocytes. J Invest Dermatol. 2008;128(4):797-807. https://doi.org/10.1038/sj.jid.5701098
5. Pasonen-Seppänen S, Karvinen S, Törrönen K, Hyttinen JM, Jokela T, Lammi MJ, Tammi MI, Tammi R. EGF upregulates, whereas TGF-beta downregulates, the hyaluronan synthases Has2 and Has3 in organotypic keratinocyte cultures: correlations with epidermal proliferation and differentiation. J Invest Dermatol. 2003;120(6):1038-44. https://doi.org/10.1046/j.1523-1747.2003.12249.x
6. Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, Ohyama Y, Kurabayashi M, Kaname T, Kume E, Iwasaki H, Iida A, Shiraki-Iida T, Nishikawa S, Nagai R, Nabeshima YI. Mutation of the mouse klotho gene leads to a syndrome resembling aging. Nature. 1997;390(6655):45-51. https://doi.org/10.1038/36285
7. Kuro-o M. Klotho and aging. Biochim Biophys Acta. 2009;1790(10):1049-58. https://doi.org/10.1016/j.bbagen.2009.02.005
8. Choi BH, Kim CG, Lim Y, Lee YH, Shin SY. Transcriptional activation of the human Klotho gene by epidermal growth

factor in HEK293 cells; the role of Egr-1. Gene. 2010;450(1-2):121-7. https://doi.org/10.1016/j.gene.2009.11.004
9. Ishii M, Yamaguchi Y, Yamamoto H, Hanaoka Y, Ouchi Y. Airspace enlargement with airway cell apoptosis in klotho mice: a model of the aging lung. J Gerontol A Biol Sci Med Sci. 2008;63(12):1289-98. https://doi.org/10.1093/gerona/63.12.1289
10. Kuro-o M. Klotho and the aging process. Korean J Intern Med. 2011;26(2):113-22. https://doi.org/10.3904/kjim.2011.26.2.113
11. Nakai K, Yoneda K, Haba R, Kushida Y, Katsuki N, Moriue T, Kosaka H, Kubota Y, Inoue S. Deranged epidermal differentiation in kl/kl mouse and the effects of βKlotho siRNA on the differentiation of HaCaT cells. Exp Dermatol. 2013;22(11):772-4. https://doi.org/10.1111/exd.12258
12. Kim HJ, Chang H, Han SH, Lee MS, Jung JY, An S, Baek SY, Lee JH, Lee JH, Lee TR, Shin DW, Kim H. Epigallocatechin-3-O-(3-O-methyl)-gallate-induced differentiation of human keratinocytes involves klotho-mediated regulation of protein kinase-cAMP responsive element-binding protein signaling. Int J Mol Sci. 2014;15(4):5749-61. https://doi.org/10.3390/ijms15045749
13. Zhang B, Xu J, Quan Z, Qian M, Liu W, Zheng W, Yin F, Du J, Zhi Y, Song N. Klotho protein protects human keratinocytes from UVB-induced damage possibly by reducing the expression and nuclear translocation of NF-κB. Med Sci Monit. 2018;24:8583-91. https://doi.org/10.12659/MSM.910687
14. Niazy MH, Gaber W, Sayed S, Shaker OG, Gheita TA. The anti-aging protein alpha-Klotho in systemic sclerosis patients: does a relationship to telangiectasia exist? Z Rheumatol. 2020;79(4):404-9. https://doi.org/10.1007/s00393-019-00718-w
15. Kang S, Duell EA, Kim KJ, Voorhees JJ. Liarozole inhibits human epidermal retinoic acid 4-hydroxylase activity and differentially augments human skin responses to retinoic acid and retinol in vivo. J Invest Dermatol. 1996;107(2):183-7. https://doi.org/10.1111/1523-1747.ep12329579
16. Liu F, Wu S, Ren H, Gu J. Klotho suppresses RIG-I-mediated senescence-associated inflammation. Nat Cell Biol. 2011;13(3):254-62. https://doi.org/10.1038/ncb2167