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ORIGINAL RESEARCH PAPER

Human dermal fibroblast proliferation activity of usimine-C from Antarctic lichen *Ramalina terebrata*

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Abstract Type I collagen is the major structural protein in dermis and its presence is used to monitor skin cell proliferation and aging. Recently, novel usimine compounds have been found in the Antarctic lichen *Ramalina terebrata*. In the present study, usimine-C induced cell proliferation of human dermal fibroblast, CCD-986SK, up to 1.6-fold after treating with 90 μg/ml for 48 h. Type I procollagen synthesis was significantly increased 1.3-fold, 3-fold, and 5-fold after treating with 0.14, 0.72, and 3.6 μg usimine-C/ml for 24 h, respectively, whereas no significant increase in type I procollagen was observed after treating with usimine-A or -B. Usimines are usnic acid derivatives. Considering that the difference among the derivatives is a side chain, the proliferation activity may be related to this side chain, triggering an internal signal for type I procollagen expression. Further studies still remain to clarify the signaling pathways for the type I procollagen induction, which is activated by usimine-C.

Keywords Type I procollagen · Dermal fibroblasts · CCD-986SK · Usimine · Cell proliferation · Antarctic

Sung Gu Lee and Hye Yeon Koh have contributed equally to this manuscript.

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Introduction

Mammalian dermal skin undergoes deterioration processes during chronological aging. The normal human skin undergoes degenerative changes, including reduced skin thickness, wrinkling, and enhanced dryness (Castelo-Branco et al. 1992, 1994; Sator et al. 2001; Schmidt et al. 1994). Type I collagen is the major structural protein in skin connective tissue and provides strength and flexibility to the skin. It is synthesized as a soluble precursor, type I procollagen, which is secreted from fibroblasts within dermis and proteolytically cleaved to form insoluble collagen fibers (Uitto 1993). In normal human dermal tissues, both type I and III collagens are predominant, composing 80 and 15%, respectively (Oikarinen 2000). However, the ratio of collagen type III to type I changes significantly as skin ages (Affinito et al. 1999; Lovell et al. 1987).

Human dermal fibroblasts are widely used as a model system to investigate or evaluate novel anti-skin

aging compounds in vitro on collagen biosynthesis and deposition. Human skin is a target tissue for various hormones since dermal fibroblasts have hormone receptors on the surface of the cells. Recently, hormone replacement therapy has been adapted to accelerate studies on skin aging (Phillips et al. 2001; Qu et al. 2006; Sator et al. 2001). Clinical studies have reported that hormone replacement therapy increases the amount of dermal collagen (Castelo-Branco et al. 1992; Maheux et al. 1994; Surazynski et al. 2003). Topical application of 17β-estradiol increased both mRNA expression and protein production of type I procollagen in human aged skin in vivo (Son et al. 2005).

Our group has recently discovered novel compounds, usimines, from Antarctic lichen *Ramalina terebrata* and successfully determined the chemical structure (Seo et al. 2008). These three compounds are derivatives of usnic acid and possess a nitrogen-bearing side chain, which is possibly derived from glutamic acid (Fig. 1). In the present study, usimines were subjected to human dermal fibroblast cells to investigate the cellular proliferation activity with the result that usimine-C induced cell proliferation and type I procollagen synthesis.

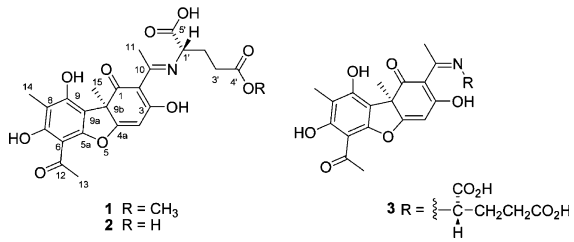

Fig. 1 Chemical structure of usimine-A, -B, and -C. 1, usimine-A; 2, usimine-B; 3, usimine-C (Seo et al. 2008)

Materials and methods

Sample collection and preparation

Antarctic lichen, *Ramalina terebrata* was collected from Barton Peninsula near to King Sejong Station (62°13.3′S, 58°47.0′W) on King George Island, Antarctica in March 2009. Usimines were purified as described previously (Seo et al. 2008). Briefly, an air-dried *R. terebrata* (10 g) was subjected to methanol extraction 2× 200 ml, each for 24 h. The extract was applied to C_{18} silica gel flash column chromatography (3 × 15 cm), followed by eluting with a stepwise gradient of each 20, 40, 60, 70, 80, 90, and 100% (v/v) methanol in H_2O (400 ml). The eluate of 70% (v/v) methanol was subjected to a reverse-phase HPLC using a linear gradient ranging from 50 to 91% (v/v) acetonitrile in H_2O containing 0.1% formic acid over 49 min to obtain usimine-A (t_R = 29.3 min). The eluate of 60% (v/v) methanol was subjected to a reverse-phase HPLC using a linear gradient ranging from 40 to 69% (v/v) acetonitrile in H_2O containing 0.1% formic acid over 29 min to yield pure usimine-B (t_R = 22.2 min) and pure usimine-C (t_R = 27.9 min) (see Supplementary Fig. 1).

Cell culture

Human dermal fibroblasts (CCD-986SK) were from ATCC and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units penicillin/ml, and 100 μg streptomycin/ml at 37°C under 5% CO_2 in humidified atmosphere. Fibroblast cultures were subcultured by trypsinization using Accutase (Sigma) and used between the fifth and eighth passages.

Cell proliferation analysis

The cell proliferation after treatment with usimines was measured by Cell Counting Kit-8 (CCK-8, Dojindo, Tabaru, Japan) assay. CCD-986SK cells were suspended at 5 × 10^3 cells per well and cultured in a 96-well flat-bottomed microplate. After exposure to usimines for 48 h, CCK-8 (10 μl) was added to each well of a 96-well flat-bottomed microplate containing 100 μl culture medium and each usimine (0.14, 0.72, 3.6, 18, and 90 μg/ml) mixture, and the plate was further incubated for 1 h at 37°C. Viable cells were counted by absorbancy measurements at 420 nm using a plate reader. All experiments were performed in triplicate.

Procollagen type I C-peptide (PIP) assay

CCD-986SK (5 × 10^4 cells) were cultured in 24-well culture plates with DMEM plus 10% (v/v) FBS, complemented by various concentrations of usimine-A, -B, and -C (0.14, 0.72, and 3.6 μg/ml, respectively). Fifteen wells were used for each experiment, starting

at day 0. Supernatants of three wells were collected at each time point and stored at −80°C. Procollagen type I amount in each culture supernatant was measured by a procollagen type I c-peptide ELISA kit (Takara Shuzo, Otsu, Japan).

Statistical analysis

Statview 5.0.1 software (SAS Institute, Inc., Cary, NC, USA) was used for statistical analyses. Student's *t* test was used to determine significant differences of relative cell proliferation or procollagen type I c-peptide in response to usimine treatments, accepting $P < 0.05$ as significant. The relative proliferation or the amount of type I collagen were expressed as mean ± 1 SE ($n = 3$).

Results and discussion

Usimine-C significantly induced cell proliferation of human dermal fibroblast, CCD-986SK, around 1.6-fold after treating with 90 μg/ml for 48 h (Fig. 2). Usimine-A and -B activated cell proliferation 1.2-fold at the same concentration and treatment time, but this was not statistically significant (data not shown). The results demonstrate that usimine-C has a cell proliferation activity on human dermal fibroblast cells. The PIP synthesis was significantly increased 1.3-fold, 3-fold, and 5-fold after treating with 0.14, 0.72, and 3.6 μg usimine-C/ml for 24 h, respectively (Fig. 3). However, no significant increase in type I procollagen was observed after treating with usimine-A and -B (data not shown).

TGF-ß induces procollagen synthesis in human dermal fibroblasts through its receptors on the cell surface to phosphorylate and activate transcription factors Smad 2/3 (Cutroneo 2007; Massague 1998; Massague and Wotton 2000; Piek et al. 1999; Schmierer and Hill 2007). However, regulation of type I procollagen expression is not yet fully understood. Previous studies reported successful induction of type I procollagen synthesis, and most of these studies used crude materials and not defined material (Chanvorachote et al. 2009; Kang et al. 2006; Lee et al. 2007; Tanaka et al. 2008). Defining the exact mechanism of type I procollagen synthesis based on the use of a single compound would enable systematic studies to understand regulation of type I procollagen expression. In Fig. 3, the type I procollagen synthesis started at 4 h after usimine-C treatment, and was further

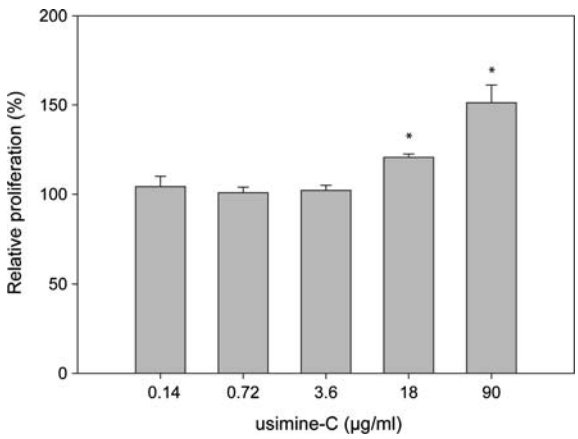

Fig. 2 Cell proliferation activity of usimine-C on human dermal fibroblast CCD-986SK cells. The usimine-C treated CCD-986SK cells were maintained for 48 h in the presence of usimine-C. Viable cell measurement indicated a significant proliferation activity of usimine-C on human dermal fibroblast cells. *Asterisks* denote $P < 0.05$. The proliferation of CCD-986SK cells was expressed as mean ± 1 SE of three samples

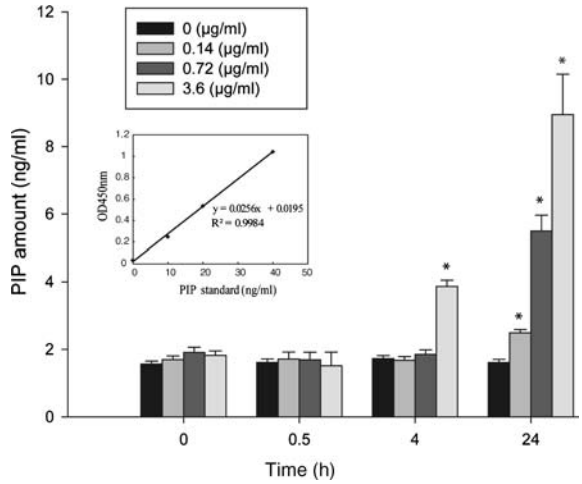

Fig. 3 Procollagen type I C-peptide (PIP) amounts in the supernatants of cultured human fibroblasts for 0.5, 4, and 24 h with usimine-C. The amount of PIP secretion in response to usimine-C treatment increased significantly in dose- and time-dependent manners. The concentrations of usimine-C treatment and PIP standard curve were shown in each box. *Asterisks* denote $P < 0.05$. These data represent the mean ± 1 SE of three samples

enhanced until 24 h post treatment, indicating that the type I procollagen synthesis presumably has been regulated by late-response genes.

Antarctic lichens produce a variety of secondary metabolites and some possess unique structures (Seo et al. 2008, 2009). However, lichens, including Antarctic ones, grow remarkably slowly in either natural or laboratory conditions, so that there is a limit on obtaining sufficient materials for functional studies. Nonetheless, considerable scientific interest in lichen compounds is being shown in the development of potential resources of pharmacological agents (Choi et al. 2009; Seo et al. 2008, 2009). In general, mass harvesting of lichens in Antarctica is restricted according to the Antarctic Treaty to minimize environmental impacts to diverse Antarctic ecosystems, but small size sampling is permitted for scientific research purpose. The lichen samples for the present study were obtained appropriately.

Usimines are usnic acid derivatives (Seo et al. 2008). Considering that the difference among the usimines is a side chain, the proliferation activity of usimine-C may have come from this side chain, triggering internal signal for type I procollagen expression within the cells. Interestingly, usimine-B and -C are geometrical isomers of each another, but showed a distinct biological activity. Further studies still remain to clarify molecular processes and signaling pathways underlying proliferation of the fibroblasts induced by usimine-C. In conclusion, the present study shows that usimine-C from Antarctic lichen exhibits type I procollagen induction activity in human dermal fibroblast cells. Furthermore, understanding the side chain of usimine-C may provide a chemical clue to define mechanisms of biologically active natural compounds.

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