Cell-based assay system for high-throughput screening of anti-photo-aging agents in fibroblast transfectants

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Received: 5 February 2015 / Accepted: 27 July 2015 / Published online: 18 August 2015
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Abstract The matricellular protein CCN1 is significantly elevated in acutely ultraviolet-irradiated human skin and negatively regulates collagen homeostasis by suppressing collagen synthesis and increasing collagen degradation. In this study, we established a stable cell line, termed CCN1-GFs, by transfection of the pAcGFP1-1-CCN1 promoter plasmid and examined its usefulness as a cell-based assay system for screening anti-aging ingredients. The promoter of the reporter plasmid pAcGFP1-1-CCN1 promoter was transfected into NIH3T3 cells using the Lipofectamine reagent. G418-resistant cells were selected and further cloned. To confirm whether AcGFP1-1-CCN1 promoter plasmid recombined in the NIH3T3 cells, the level of AcGFP1-1-CCN1 was measured by PCR analysis. To determine if NIH3T3 cells expressed the gene encoding green fluorescence protein in a CCN1 promoter-dependent manner, the reporter enzyme activities were assayed using a fluorimeter and flow cytometer. To determine if CCN1 inhibitor, which was selected through this system, exerted a direct effect on

the downstream signal, mRNA expression of collagen1 and MMP1A was checked by using real-time PCR. UVB irradiation of CCN1-GFs resulted in increased CCN1 promoter activity. Treatment with retinoic acid, a CCN1 inhibitor, inhibited UV-induced CCN1 promoter activity. Subsequent use of this assay system to screen anti-aging ingredients revealed that CCN1-GFs treated with sclareol showed decreased levels of UVB-induced CCN1 expression. Sclareol attenuated UVB-induced photo-aging by an increase in collagen synthesis and decrease in MMP-1 activity.

Keywords Cell-based assay · CCN1 promoter · Transfected stable cell · Anti-photoaging · Sclareol

Introduction

Human skin, like all human organs, undergoes progressive alterations as a consequence of natural aging. Additional damage is superimposed on the natural aging process as a result of chronic exposure to ultraviolet (UV) irradiation from sunlight (Gilchrest and Yaar 1992; Fisher et al. 1996, 1997).

CCN1, also known as CYR61 (Cysteine-rich protein 61), is expressed in the human skin dermis and is substantially elevated in the dermis of naturally aged, photo-aged, and acutely UV-irradiated human skin (Quan et al. 2006). CCN1 is a secreted extracellular matrix associated protein that belongs to the CCN gene family (Lau and Lam 1990). CCN1 protein is

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fundamentally important in growth, differentiation, angiogenesis, migration, and extracellular matrix regulation (Chen et al. 2001; Kireeva et al. 1996; Perbal et al. 2003; Perbal 2004).

In fibroblasts, elevated CCN1 up-regulates MMP-1 protein expression and down-regulates type-1 procollagen protein expression (Ramnath and Creaven 2004). Aberrant dermal collagen homeostasis in UV-irradiated fibroblasts is mediated by CCN1 (Quan et al. 2010). Collagen fragmentation and inhibition of collagen production impair the structural integrity of the dermis and are prominent features of the pathophysiology of premature skin aging (Fisher et al. 2008; Varani et al. 2000; Campisi 2008). Retinoic acid has been used in the prevention and repair of photo-aged skin and reportedly reduces CCN1 expression in skin equivalent cultures (Quan et al. 2011; Orfanos et al. 1997).

High-throughput screening (HTS) using stable cell lines harboring a green fluorescence protein (GFP) reporter gene allows long-term, defined, and reproducible expression of genes of interest (Fisher et al. 2000). While transient transfection and CCN1 promoter luciferase assay for the measurement of CCN1 promoter activity have been established (Quan et al. 2010), stably-transfected cell lines for CCN1 promoter activity have not been established.

In this study, we established a stable cell line by transfection with the AcGFP1-1-CCN1 promoter applicable for the quantitative measurement of CCN1 promoter activity. CCN1 promoter activity was stimulated by UVB radiation and decreased by retinoic acid. This cell-based reporter system was evaluated to determine its utility as a screen for agents that potentially suppress photo-aging from CCN1. Sclareol was identified as potential agent that suppresses CCN1 in CCN1-GFs irradiated with UVB.

Materials and methods

Materials

Retinoic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Phloretin, Emodin, Morin hydrate, Bilobalide, Kaempferol, Biochanin A, Sclareol, Taxifolin, Isoliquiritigenin were purchased from Selleckchem (Houston, TX, USA).

Cell culture

NIH3T3 (KCLB (Seoul, Korea), 21658) cells were grown in Dulbecco's modified Eagle's medium (DMEM, Hyclone Logan, UT, USA) supplemented with 10 % fetal bovine serum (FBS, Hyclone), 1 % penicillin streptomycin (Pen Strep, Gibco, Grand Island, NY, USA). The cells were grown at 37 °C in a 95 % air/5 % CO₂ environment. Transfected NIH3T3 cells were enriched with 2000 µg/mL G418 (200 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) for selection and maintenance of stable transformants.

Construction of CCN1 promoter plasmid

The CCN1 promoter (−928/−29) construct was a PCR product from human genomic DNA using 5'-GGG AATT CGA TTA A AA GCT TCT CCC CGC GT-3' as the forward primer (EcoRI site is underlined, Bioneer, Daejeon, Korea) and 5'-GGG TCG ACC GGA GGC GCC GCG TGT TGC AG-3' as the reverse primer (SalI site is underlined, Bioneer, Daejeon, Korea). After digestion by EcoRI and SalI (NEB, Ipswich, MA, USA), the CCN1 promoter DNA fragment was extracted using a MiniElute® Gel Extraction Kit (Qiagen, Chatsworth, CA, USA). The purified fragments were subcloned into the pAcGFP1-1 vector (Clontech, Mountain View, CA, USA).

Establishment of a cell line for the CCN1 promoter-based reporter gene assay

NIH3T3 cells were transfected with the recombinant expression vector pAcGFP1-1-CCN1 using Lipofectamine LTX and PLUS Reagent (Thermo Scientific, Pittsburgh, PA, USA). Twelve hours post-transfection, stable transfectants were isolated by selection with 2000 µg/mL G418. The transfectants were maintained in DMEM containing 10 % FBS and 2000 µg/mL G418 for 2 weeks. The G418-resistant cells were selected and further cloned. Consequently, the CCN1-GFs stable cell line expressing CCN1 promoter and GFP was obtained.

DNA isolation and genomic DNA PCR analysis

Cells were harvested and transferred to a 1.5 mL centrifuge tube. Lysis buffer (500 µL containing 100 mM Tris (pH8.0), 5 mM EDTA (pH 8.0), 0.2 %

sodium dodecyl sulfate (SDS), 200 mM NaCl, and 100 mg/mL Proteinase K) was added to the cell pellet and incubated at 55 °C with rocking or rolling overnight. Centrifugation at 12,000 rpm for 10 min was done to pellet and to remove debris. The supernatant was poured into a fresh tube avoiding the transfer of debris. Five hundred microliters of isopropanol was added and mixed by inversion. The DNA appeared as a long thread-like precipitate. A clean and sterile micropipette tip was used to spool out the DNA and transfer it to a new tube. The tube was stored open on the bench until the remaining alcohol evaporated. The genomic DNA was dissolved in 200 µL of TE buffer [10 mM TrisCl, TE (pH8.0)] or distilled deionized water. For genomic DNA-PCR, DNA was amplified using Prime TaqTM (TaKaRa, Shiga, Japan) and CCN1 promoter specific primers (Table 1, CCN1 pro F1, Bioneer, Daejeon, Korea) and GFP gene primer (Table 1, EGFP R1, Bioneer, Daejeon, Korea). PCR amplifications were performed in a total volume of 20 µL containing 1 µL genomic DNA sample, 1 µL of each primer (10 pmol/µL), 10 µL 2 × PCR solution, and 7 µL distilled water. Cycling conditions were one cycle of 10 min at 95 °C; 35 cycles of 20 s at 95 °C, 20 s at 54 °C, and 40 s at 72 °C, followed by one cycle of 5 min at 72 °C.

UV irradiation and treatment

CCN1-GFs transfected cells were cultured in wells of a 6-well plate (Thermo Fisher Scientific, Carlsbad, CA, USA) for 24 h for UV irradiation experiments. The cells were incubated in 2 % serum containing medium for 2 h and exposed to UVB irradiation using a LZC-UVB lamp (Luzchem, Ottawa, ON, Canada) having an emission spectrum of 280–370 nm and a peak at 312 nm. The UV dose was measured with a UV-340 light meter (Lutron, Coopersburg, PA, USA). After irradiation, the cells were replenished with a 2 % serum containing medium containing retinoic acid or sclareol, and were followed-up for 24 h.

Flow cytometric analysis

CCN1-GFs cells were plated in 6-well plates. The cells were then washed with phosphate buffered saline and were detached with trypsin at 37 °C. After being rinsed in PBS the cells were analyzed by flow

cytometry using AccuriC6 FACS and BD Accuri C6 Software (BD Biosciences, Franklin Lakes, NJ, USA).

Fluorescence intensity measurements

CCN1-GFs were cultured in 96-well black-microplates (Greiner Bio-One, Frickenhausen, Germany) at a density of 8000 cells/well with 100 µL of 10 % FBS-DMEM. Twenty-four hours later the cells were incubated in 2 % serum containing medium for 2 h and exposed to UVB irradiation. After irradiation for 12 h, the cells were washed with PBS. Dulbecco's PBS replaced PBS in the cell culture 96-well black-microplate and fluorescence was measured using an Infinite 200[®] PRO fluorescence reader (TECAN, Männedorf, Switzerland). GFP was detected using an excitation and emission wavelength of 485 and 535 nm, respectively. All fluorescence data were reported as the mean of repeat measurements.

RNA isolation and quantitative real-time PCR

Total RNA was isolated using a RNeasy Midi kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's protocol. One microgram RNA was reverse transcribed using a PrimeScriptII 1st strand cDNA Synthesis kit (TaKaRa, Shiga, Japan). The cDNA samples generated were diluted and used for real-time PCR analysis. Briefly, 2 µL of diluted cDNA was mixed with both forward and reverse primers (Table 1) and SYBR Green Supermix (Thermo Scientific, Pittsburgh, PA, USA) in 20 µL final volumes.

Table 1 Primer sequences

Primer	Sequence
<i>GAPDH</i>	
Forward	5'-TGCACCACCAACTGCTTAGC-3'
Reverse	5'-GGCATGGACTGTGGTCATGAG-3'
<i>CCN1</i>	
Forward	5'-CTCCACACGAGTTACCAATGACAAC-3'
Reverse	5'-CTTGGTCTTGCTGCATTTCTTGCCC-3'
<i>CollA1</i>	
Forward	5'-CGGCAACGATGGTGCTAAGG-3'
Reverse	5'-TCAGACCACGGACGCCATCT-3'
<i>MMP1A</i>	
Forward	5'-GTGCTCTCCTTCCACAGAGGAGAC-3'
Reverse	5'-CTGTTGGTCCACGTCTCATCAAGGTC-3'

Amplification was performed using the real-time PCR system (SDS 7300; Applied Biosystems, Foster City, CA, USA). Amplified PCR products were quantified by measuring each gene, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA calculated cycle thresholds (C_T). The amount of specific mRNA in samples was calculated from the standard curve and was normalized with GAPDH mRNA. Results were expressed as an n -fold difference relative to normal controls (relative expression levels).

Statistical analysis

All data are expressed as mean $\pm$ SD. Differences between the control and treatment groups were evaluated by one way ANOVA using SPSS software, version 22.0 (IBM Corporation, New York, USA). A $P < 0.01$ was considered statistically significant.

Results

UVB irradiation induces CCN1 in NIH3T3 cells

Photo-aging results from cumulative alterations were brought about by chronic exposure to UV irradiation. CCN1 was elevated in dermal fibroblasts in photoaged human skin (Quan et al. 2010). The cell line (NIH3T3) was a suitable transfection host and used for stable expression of transfected genes. The effects of UV irradiation on CCN1 expression in NIH3T3 has not been elucidated to date. Therefore we investigated the effect of UVB irradiation on the expression of CCN1 in cultured NIH3T3 cells to confirm that NIH3T3 cells were suitable for CCN1 screening. Results of a time course study showed that irradiation with 50 mJ/cm² UVB induced an increase in the level of CCN1 mRNA, with a maximum within 30 min (1.72 ± 0.04 -fold) followed by a marked decline by 2 h (0.43 ± 0.02 -fold) (Fig. 1). These data suggested that UVB stimulated CCN1 mRNA expression in NIH3T3 cells.

Construction of the CCN1-GFs stable cell line

A stable system was developed using NIH3T3 fibroblast cells transfected with AcGFP1-1-CCN1 promoter plasmid to measure the quantitative change of CCN1 promoter-dependent expression of GFP in externally stimulated fibroblasts. The pAcGFP1-1-CCN1

Fig. 1 CCN1 is induced by UVB irradiation in NIH3T3. Total RNA and whole-cell lysates were prepared from fibroblasts at the indicated times after UVB irradiation (50 mJ/cm²). CCN1 mRNA levels were quantified by real-time RT-PCR. Data are expressed as mean $\pm$ SEM, $n = 3$, * $P < 0.05$

promoter plasmid permitted expression of the GFP reporter gene in a CCN1 promoter dependent manner. To confirm whether AcGFP1-1-CCN1 promoter plasmid recombined in the NIH3T3 fibroblast chromosomal DNA, PCR was carried out with genomic DNA isolated from the transfected cells and primers. A pair of primers was used to verify the partial CCN1 promoter and GFP gene recombination in the NIH3T3 fibroblast cells (Fig. 2a). As shown in Fig. 2b, the CCN1 promoter region was detected in the genomic PCR experiment, indicating that stable recombination of the CCN1 promoter, and that the GFP gene was resident in NIH3T3 fibroblast chromosomal DNA.

UVB irradiation-induced activation of CCN1 promoter

We further explored whether CCN1-GFs expressed the GFP gene in a CCN1 promoter-dependent manner. GFP reporter gene was assayed using fluorescence-activated cell sorting (FACS) flow cytometry in CCN1-GFs irradiated with UVB. UVB is thought to promote CCN1 promoter activity. As shown Fig. 2c, d, UVB increased GFP in CCN1-GFs. GFP fluorescence intensity measured by a fluorescence

Fig. 2 Confirmation of transfection of CCN1-GFs. **a** Design of the transfection confirmatory PCR primer from AcGFP1-1-CCN1 promoter (−928/−29) plasmid construct. **b** The genomic DNA was isolated and PCR analysis was performed to confirm whether the plasmid construct were stably inserted in the chromosomal DNA of NIH3T3 cell. (**c**, **d**, and **e**). CCN1-GFs cells were irradiated with UVB (50 mJ/cm²) at the indicated

intensity for 12 h. **c** Representation of CCN1 promoter activity in the FACS analysis. **d** Quantitative analysis of the EGFP intensity with or without UVB (50 mJ/cm²). **e** Relative CCN1 promoter activity, calculated as the relationship between GFP fluorescence intensity measuring by a fluorimeter, and cell viability. The data represent the mean $\pm$ SD of three independent experiments. * $P < 0.05$, UVB versus unirradiated control

spectrophotometer was induced by irradiation with UVB at 50 mJ/cm² (Fig. 2d). This result suggested that the transfectants may potentially be used to screen agents that affect CCN1 promoter activity.

Inhibition of CCN1 promoter activity in UVB-irradiated CCN1-GFs

We determined whether UVB induced CCN1 promoter activity in CCN1-GFs. To confirm the utility of CCN1-GFs as a screen for agents that potentially suppress photoaging of CCN1, nine compounds from the Natural Product Library (Selleckchem, Houston, TX, USA) which has been reported to have anti-aging effects were tested by quantitative measurement of the level of the CCN1 promoter activity induced by UVB.

Sclareol reduced CCN1 promoter activity dramatically in UVB-irradiated CCN1-GFs but the others showed no significant effects (Table 2). In fact, GFP-positive cells were decreased significantly at 12 h after irradiation with 50 mJ/cm² UVB, on treatment with 10 μ M sclareol (110.8 ± 2.9 %) compared with untreated controls examined after the same treatment for the same time (141 ± 2.6 %). GFP fluorescence intensity was significantly reduced by treatment with sclareol. These data indicated that sclareol reduced UVB irradiation-induced CCN1 promoter activity in CCN1-GFs. Retinoic acid (Sigma-Aldrich, MO, USA) was introduced as a negative control because it is known to inhibit CCN1 expression. Retinoic acid also suppressed CCN1 promoter activity in UVB-irradiated CCN1-GFs.

Table 2 Summary of the CCN1-GFs screening, calculated as the relationship between GFP fluorescence intensity using a fluorescence meter

Compound	Molecular weight (g/mol)	% of Control (10 μ M)	% STDEV
Control	–	100	1.1
UVB 50 mJ/cm ²	–	141	2.6
UVB + retinoic acid	300	118.7	2.9
UVB + phloretin	274	141.1	5.7
UVB + emodin	270	146.6	2.4
UVB + morin hydrate	302	143.1	3.3
UVB + bilobalide	326	137.3	2.7
UVB + kaempferol	286	137	2.7
UVB + biochanin A	284	138.1	3.7
UVB + sclareol	308	110.8	2.9
UVB + taxifolin	304	142.1	6.6
UVB + isoliquiritigenin	256	141.5	4.7

CCN1-GFs were treated with 10 μ M of test chemicals for UVB irradiation at 50 mJ/cm² after 12 h. Retinoic acid was the reference control. Results are expressed as mean $\pm$ standard deviation (SD) (n = 4)

Sclareol inhibits CCN1 expression induced by UVB

To assess the correlation between the CCN1 promoter activity and CCN1 mRNA level, we investigated the effects of sclareol on UVB-induced expression of CCN1 transcript in CCN1-GFs. UVB-induced cell growth inhibition was not verified by treatment with 10 μ M sclareol, as determined by the MTT viability assay (Fig. 3a). As shown in Fig. 3b, UVB-induced transcription of CCN1 was significantly inhibited by sclareol. CCN1 transcript levels were also inhibited by sclareol. These results indicated that sclareol inhibited CCN1 expression induced by UVB.

Regulation of type I procollagen and MMP-1 mRNA in UVB-irradiated CCN1-GFs

Dual regulation of collagen homeostasis occurs by reduction in levels of type I procollagen, the major structural protein in human skin dermis, and promotion of collagen degradation by upregulation of MMP-1, the major collagen-degrading protease in human skin (Ramnath and Creaven 2004). These data raised the possibility that CCN1 promoter activation was reduced by sclareol before exposure to UVB irradiation. Accordingly, the levels of type-I procollagen and MMP-1 were determined. Treatment with sclareol reduced CCN1 promoter activity (Fig. 4). As

Fig. 3 Effects of signal inhibitors on CCN1 expression in UVB irradiated (50 mJ/cm²) CCN1-GFs. **a** Cell viability was measured using the MTT assay 30 min after UVB irradiation. **b** CCN1-GFs were pretreated with 10 μ M sclareol for 4 h, and further irradiated with UVB (50 mJ/cm²) after 30 min. CCN1

expression was analyzed by real-time PCR. Results are expressed as mean $\pm$ standard deviation (SD; n = 3). * P < 0.05 of UVB versus untreated control and ** P < 0.05, UVB versus treated control

expected, UVB irradiation substantially reduced type-I procollagen transcript level and increased MMP-1 transcript level. Importantly, UVB irradiation-induced reduction of type-I collagen and up-regulation of MMP-1 were partially, but significantly, attenuated by treatment with sclareol. This result suggested that the transfected cells may potentially be used to screen anti-photo-aging agents that affect CCN1 promoter activity.

Discussion

UVB irradiation is deleterious to the extracellular matrix, which confers structural support to the skin. UVB stimulates collagen breakdown and inhibits procollagen production (Fisher et al. 1996, 1997). CCN1 functions as a novel negative regulator of collagen homeostasis by inhibiting type-I collagen production, the major structural protein in the human skin dermis, and promotes its degradation (Quan et al. 2006). The current work focused on constructing a high-throughput screening system of anti-photo-aging agents by evaluating the inhibition of UVB-induced CCN1 promoter activity in a stable CCN1-GFs expressing fibroblast cell line. UVB irradiation markedly up regulated the CCN1 promoter-driven GFP reporter protein expression in CCN1-GFs. Importantly, sclareol reduced UVB-induced CCN1 promoter activity, at least in part, by altering collagen homeostasis.

Sclareol is a fragrant chemical compound found in *Salvia sclarea*, *Prunella vulgaris* and *Salvia officinalis*. It is classified as a bicyclic diterpene alcohol and is an amber-colored solid with a sweet, balsamic scent. Sclareol is used as a fragrance in cosmetics and perfumes and as flavoring in food. Interestingly, sclareol treatment down-regulated UVB-induced CCN1. The mechanisms by which sclareol regulates CCN1 promoter activity are unknown. The CCN1 promoter contains a perfect consensus AP-1 site, TGACTCA. Mutation or deletion of this AP-1 DNA-binding site results in complete loss of induction of CCN1 promoter activity in response to UV irradiation (Quan et al. 2010). Additionally, CCN1 expression is regulated via an Egf-1-dependent mechanism after cigarette-smoke-extract exposure in fibroblasts (Kim et al. 2011). It is likely that sclareol reduces CCN1 by inhibition of the AP-1 transcription factor. CCN1 is transcriptionally activated by a variety of extracellular stimuli. Transcriptional regulation of CCN1 in response to UV irradiation is primarily controlled by the AP-1 transcription factor in primary human dermal fibroblasts (Orfanos et al. 1997). AP-1 transcriptional activity is elevated in both chronologically aged and photo-aged human skin, and is critically important in mediating skin connective tissue damage. Elevated AP-1 is suppressed by sclareol in photo-aged human skin in vivo (Varani et al. 2000; Fisher et al. 2000; Wang et al. 1999; Fisher et al. 1998), suggesting that sclareol down-regulates CCN1 expression by inhibiting AP-1. Interestingly, elevated CCN1 also activates

Fig. 4 Changes in Col1A1 and MMP1A mRNA expression analyzed by real-time PCR 12 h after irradiation (50 mJ/cm²) with UVB. Results for **a** Col1A1 and **b** MMP1A. The data

represent the mean $\pm$ SD of three independent experiments ¶ $P < 0.05$, UVB versus untreated control and ** $P < 0.05$, UVB versus treated control

AP-1 (Quan et al. 2006), suggestive of a positive feedback mechanism in the sustained elevation of CCN1 in aged and photo-aged human skin. Our data suggest that such interactions may be involved in sclareol-mediated regulation of CCN1 expression in fibroblasts, which appears to be a useful model to investigate the nature of these interactions.

In conclusion, we presented a novel and high-throughput screening using NIH3T3 fibroblast cells transfected with AcGFP1-1-CCN1 promoter plasmid. The data acquired in this study demonstrate the ability of sclareol to suppress photo-aging.

Conclusion

The CCN1-GFs cell line is a useful tool for the screening of inhibitors of CCN1 expression induced by UVB irradiation. Sclareol, which was selected as a suppressor of CCN1 expression by a cell-based system that utilizes a pAcGFP1-1-CCN1 promoter, suppressed UVB-induced photo-aging.

Acknowledgments This research was supported by a grant (A004600326) from Jeju Economic Region Diagram Industry R&D project Program funded by Jeju Province.

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