A cell-based assay system for high-throughput screening of anti-wrinkle agents in human dermal fibroblast transfectant cells

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A cell-based assay system for monitoring *COL1A2* [α2(I) collagen gene] promoter activity was developed to determine the influence of activated *COL1A2* promoter in human dermal fibroblast cells. A pLuc-*COL1A2* promoter plasmid that expresses the luciferase reporter gene in response to *COL1A2* promoter activity was constructed. The pLuc-*COL1A2* promoter plasmid and pCI-neo plasmid containing the NPT (neomycin phosphotransferase) gene for Geneticin resistance in host cells were co-transfected into human dermal fibroblast cells. *COL1A2* promoter activities were measured by luciferase reporter gene assay using a luminescence detection method. Fibroblast cell transfectants treated with TNFα (tumour necrosis factor α), known to be an inhibitor of *COL1A2* promoter expression, showed a reduction of *COL1A2* promoter activity in a concentration-dependent manner, whereas TGF-β (transforming growth factor-β), known to be a stimulator of *COL1A2* promoter expression, increased *COL1A2* activity in a concentration-dependent manner. This assay system could be used to quantitatively measure *COL1A2* promoter activity in human dermal fibroblast cells and allow the screening of anti-wrinkle agents from various synthetic chemicals and natural products.

Introduction

Collagens are the major structural components of the skin and represent a large family of extracellular proteins that impart specific physical properties to tissues. These proteins also play important roles during morphogenesis and growth [1,2].

Type I collagen, the most abundantly expressed member of the collagen genes, consists of two α1(I) chains and one α2(I) chain that are produced by two fairly large genes residing on different chromosomes in both the human and the mouse genome; it also plays a significant role in maintaining homoeostasis [3–5].

TGF-β (transforming growth factor-β) is well known to be the principal factor that induces type I collagen gene expression and leads to tissue fibrosis [6–9]. The Smads have been identified as intracellular mediators of the signal transduction pathways of TGF-β superfamily members, functioning downstream of the serine/threonine kinase receptors of the TGF-β family to transduce signals to the nucleus [10–12]. Smad3 promotes *COL1A2* [α2(I) collagen gene] activation [13].

Sp1 (stimulating protein-1) binding is required for TGF-β1-induced type I collagen mRNA expression. Sp1 and Smad proteins form complexes, and their synergy plays an important role in mediating TGF-β1-induced type I collagen expression. Sp1- and Smad-binding elements are present in the *COL1A2* promoter region. Removal of Sp1- and Smad-binding elements inhibited the stimulation of *COL1A2* promoter activity by TGF-β. Accordingly, it has been shown that an increase in partial *COL1A2* promoter activity indicates an increase in type I collagen gene expression. Therefore monitoring partial *COL1A2* promoter activity containing both Sp1- and Smad-binding elements in human skin cells could potentially provide an appropriate means for screening of anti-wrinkle agents.

We established stable human dermal fibroblast cells co-transfected with pLuc-*COL1A2* promoter and pCI-neo plasmids for quantitative measurement of *COL1A2* promoter

Key words: cell-based assay, human dermal fibroblast transfectant cell, luciferase, transfection, transforming growth factor, type I collagen promoter.

Abbreviations used: AP-1, activating protein-1; *COL1A2*, α2(I) collagen gene; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; NPT, neomycin phosphotransferase; Sp1, stimulating protein 1; TGF, transforming growth factor; TNF, tumour necrosis factor.

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activity by various synthetic chemicals or natural products. The pLuc-*COL1A2* promoter plasmid expresses the luciferase gene in response to the level of *COL1A2* promoter activity. Additionally, the pCI-neo plasmid is the dominant selective marker of neomycin resistance. We have further tested whether this assay system could be used for quantitative measurement of the level of *COL1A2* promoter activity induced by TGF-β1.

We report that this human-dermal-fibroblast-cell-based reporter system, in response to *COL1A2* promoter activity, could be introduced for the screening of anti-wrinkle agents.

Materials and methods

Materials

Restriction endonucleases and T4 DNA ligase were purchased from Takara (Osaka, Japan). GoTaq® green Master Mix was obtained from Promega. Oligonucleotides were synthesized from Genotech custom primers. Plasmid pLuc-MCS vector and pCI-neo plasmid were from Stratagene. Asiaticoside (98 %, w/v), a triterpene glycoside from *Centella asiatica* commonly used in wound healing, was purchased from LKT Laboratories (St Paul, MN, U.S.A.). Ginseng extract was prepared according to the method previously described [14].

Cell culture

Human dermal fibroblast cells were obtained from AmorePacific and were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % FBS (fetal bovine serum), penicillin (100 units/ml) and streptomycin (100 μg/ml). The cells were grown at 37 °C in an air/CO_2 (19:1) environment. Transfected human dermal fibroblast cells were enriched with 800 μg/ml of neomycin (100 mg/ml) for selection and maintenance of stable transformants.

Plasmid construction

The pLuc-378*COL1A2* construct was generated by PCR amplification using DNA from human dermal fibroblast cells as a template and the following pairs of primers: 5′-AAGCTTGATCTGCAAATTCTGCCCATGTCG-3′ with 3′-GTCGACCATGCAGTCGTGGCCAGTACC-5′. The PCR reaction was carried with the GoTaq® Green Master Mix, used according to the instructions of the manufacturer with the following modification: one cycle of 10 min at 95 °C; 30 cycles of 1 min at 95 °C, 1 min at 60 °C, and 1 min at 72 °C, followed by one cycle of 10 min at 72 °C. After digestion by HindIII and SalI, the PCR products were extracted from the agarose gel. The purified fragments were subcloned into the pLuc-MCS vector, which carried the luciferase reporter gene without a promoter.

Cell transfection

Human dermal fibroblast cells were co-transfected with recombinant expression vector pLuc-COL1A2 and pCI-neo plasmid using Superfect transfection reagent (Qiagen) according to the instructions of the manufacturer. After 24 h of transfection, stable transfectants were isolated by selection with 800 μg/ml Geneticin. The transfectants were maintained in DMEM containing 10 % FBS and 300 μg/ml Geneticin.

Reporter gene assay

A 1 ml portion (1×10^5 cells) of each of the stable transfectants was plated in 12-well plates. Chemicals were added to the culture medium after 24 h of incubation. Following stimulation, cells were lysed, and 120 μl of lysate was used to measure reporter-gene expression. Luciferase activity was assayed by the Luciferase Reagent Assay System (Promega). Luminescence was measured at 450 nm using a 96-well plate luminometer.

Type I collagen synthesis assay

The stable transfectants were seeded on to 24-well plates, and the medium was decanted 24 h later. At this time, cells were washed twice with PBS, and incubations were initiated by the addition of serum-free medium. Chemicals were added to the culture medium after 24 h of incubation. The culture medium was used for determination of collagen synthesis.

The collagen content was determined by an ELISA using anti-(type I collagen) antibody (Takara). Culture medium (20 μl) and 80 μl of serum-free medium were added to each well of a 96-well ELISA plate coated with murine monoclonal antibody to PIP (procollagen type I C-peptide), and the plate was incubated for 90 min at 37 °C. After washing three times with PBS, 100 μl of freeze-dried horseradish peroxidase-conjugated murine monoclonal antibody was put into each well, and the plate was then incubated at 37 °C for 1 h. The plate was washed with PBS; then 100 μl of tetramethylbenzidine in buffered solution, used as a substrate, was added to each well. After 20 min, the absorbance in each well was measured by a microplate reader at 450 nm.

Statistical evaluation

Means ± S.E.M. were calculated; statistical analysis of the results was performed by Student's *t* test for independent samples. Values of $^*P < 0.05$ were considered to be significant.

Results and discussion

To measure the quantitative change of partial *COL1A2* promoter-dependent expression of the luciferase gene in human dermal fibroblasts induced by external stimuli, a

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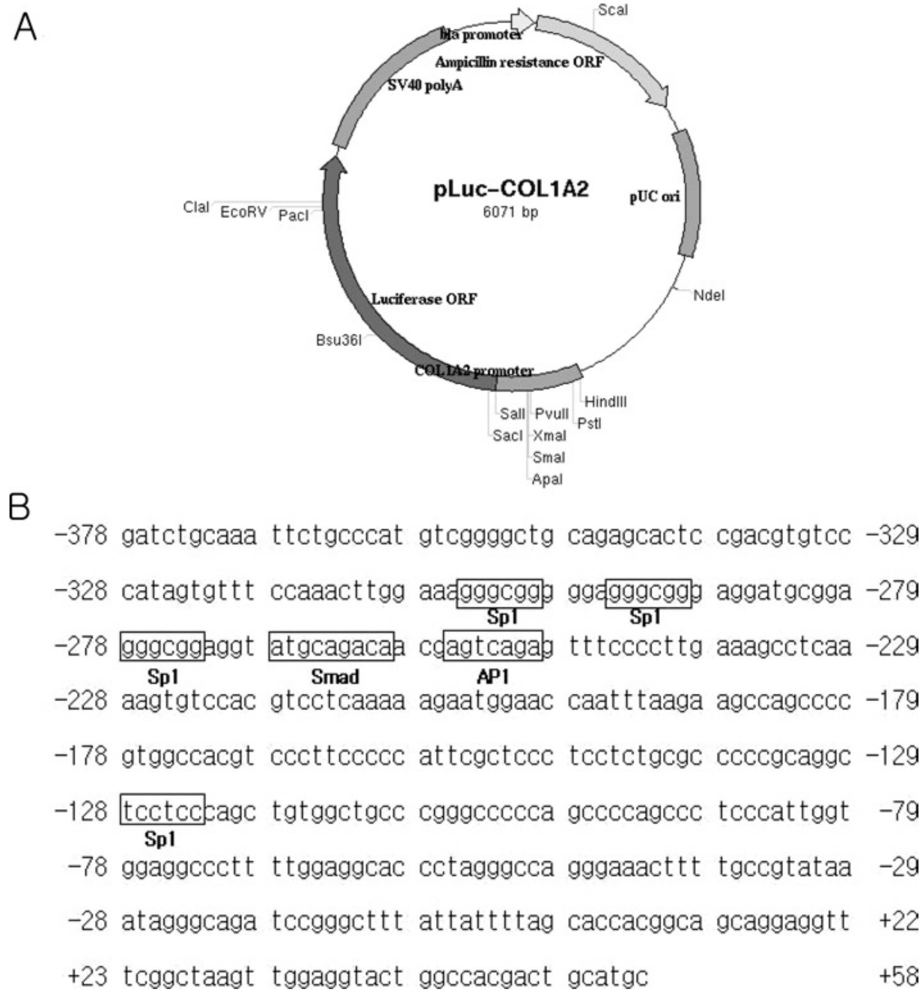

Figure 1 Diagram of pLuc-*COL1A2* promoter plasmid (A) and representation of the region between –376 bp of the α2(I) collagen (*COL1A2*) promoter and +57 bp of the transcribed sequence to the luciferase reporter gene (B)

stable system was developed using human dermal fibroblast cells co-transfected with pLuc-*COL1A2* promoter and pCI-neo plasmids. The pLuc-*COL1A2* promoter plasmid permits expression of the luciferase reporter gene in a partial *COL1A2* promoter-dependent manner (Figure 1), and pCI-neo plasmid expresses the NPT (neomycin phosphotransferase) gene for Geneticin resistance in human dermal fibroblast cells. The human dermal fibroblast cells, derived from neonatal foreskin, are normal fibroblast cells that are capable of producing collagen.

The partial *COL1A2* promoter containing Sp1- and Smad-binding sites was recloned from genomic DNA isolated from the human dermal fibroblast cells using the aforementioned primers (see the Materials and methods section). Sp1 and Smad proteins form a complex, and their synergistic co-operation plays an important role in mediating TGF-β1-induced α2(I) collagen expression. The PCR product and pLuc-MCS plasmid were treated with HindIII and SalI to make them cohesive-ended and linearized respectively. These two DNA fragments were ligated to form a pLuc-*COL1A2* promoter plasmid. The pLuc-*COL1A2* promoter plasmid was transfected into human dermal fibroblast cells by exposing the cell monolayer to DNA complexes mixed with SuperFect reagent for 3 h.

To confirm whether pLuc-*COL1A2* promoter and pCI-neo plasmids are recombined in the human dermal fibroblast chromosomal DNA, PCR was carried out with genomic DNA isolate from the transfectant cells and primers (Figure 2). A pair of primers used to verify the partial *COL1A2* promoter and luciferase genes recombinated in the human dermal fibroblast cells was constructed as follows: 5′-AAGCTTGATCTGCAAATTCTGCCCATGTCG-3′ and 3′-CGATAAATAA CGCGCCCAAC-5′. The other primers used to verify the NPT gene recombinated in the human dermal fibroblast were: 5′-AAGATGGATTGCACGCAGGT-3′ and 3′-TCAGAAGAACTCGTCAAGAAGG-5′. As shown

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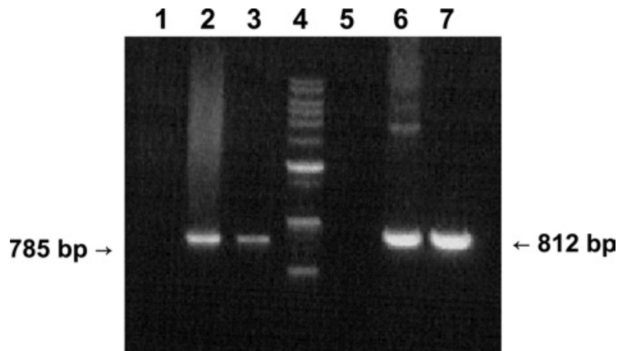

Figure 2 Electrophoretic analysis of transfectant human dermal fibroblast cells

Genomic PCR amplification of the NPT gene and the partial *COL1A2* promoter region. Lane 1, human dermal fibroblast genomic DNA; lane 2, pCI-neo plasmid DNA; lane 3, human dermal fibroblast transfectant genomic DNA; lane 4, 1 kb ladder as a molecular-mass marker; lane 5, human dermal fibroblast genomic DNA; lane 6, pLuc-*COL1A2* promoter plasmid DNA; lane 7, human fibroblast transfectant genomic DNA.

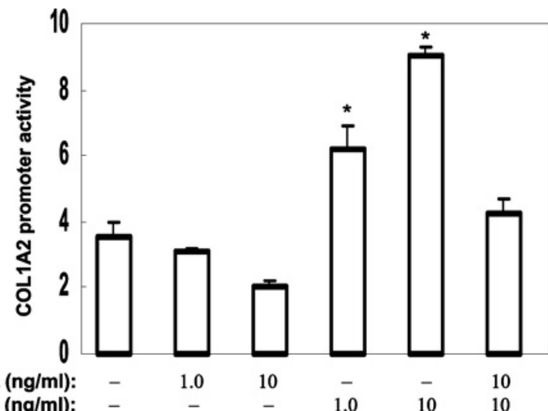

Figure 3 Cellular *COL1A2* activity in response to TNFα and TGF-β in transfectant human dermal fibroblast cells

Transfectant human dermal fibroblast cells were treated with indicated concentrations of TNFα, TGF-β, or TNFα plus TBF-β for 16 h, after which they were harvested and lysed. Supernatants were assayed for luciferase activity, which was determined by the amount of light produced through the conversion of luciferin substrate into oxyluciferin by luciferase. Relative luciferase activity, calculated as the relationship between luciferase activity and the quantity of cells or the efficiency of the transfection, was determined three times in duplicate for each experiment, and the S.D. values are indicated by bars. All values were significant (*$P < 0.05$) compared with values for the untreated control.

in Figure 2, NPT and the partial *COL1A2* promoter region were detected in a genomic PCR experiment, indicating that NPT, partial *COL1A2* promoter, and luciferase genes are stably recombinated in human dermal fibroblast chromosomal DNA.

To further estimate whether the human dermal fibroblast transfectant cells express the luciferase gene in a *COL1A2* promoter-dependent manner, the reporter enzyme activities were assayed using a detection method capable of measuring the enzyme in transfectant cells treated with TNFα (tumour necrosis factor α) and TGF-β (Figure 3). TNFα is known to inhibit *COL1A2* promoter activity [15], and TGF-β is known to promote *COL1A2* promoter activity [7]. As shown in Figure 3, while TNFα reduced luciferase activity in a concentration-dependent manner, TGF-β increased luciferase activity in a concentration-dependent manner. In addition, and as expected, TNFα inhibited TGF-β-induced activation of luciferase activity. Consistent with this, type I collagen production was induced by TGF-β, and this TGF-β-induced collagen production was inhibited by TNFα (results not shown). This result suggests that the transfectant cells may potentially be used to screen agents that affect *COL1A2* promoter activity.

Until now, we have found that the human dermal fibroblast cells co-transfected with *COL1A2* promoter and pCI-neo plasmids are able to respond to agents that can activate or inhibit collagen production; these agents include TNFα and TGF-β. In order to further confirm this property of stably transfected NPT and luciferase gene-expressing human dermal fibroblast cells, asiaticoside and ginseng extract [14] were introduced. As shown in Figure 4, when the transfectant cells were treated with asiaticoside and ginseng extract, *COL1A2* promoter activity increased compared with

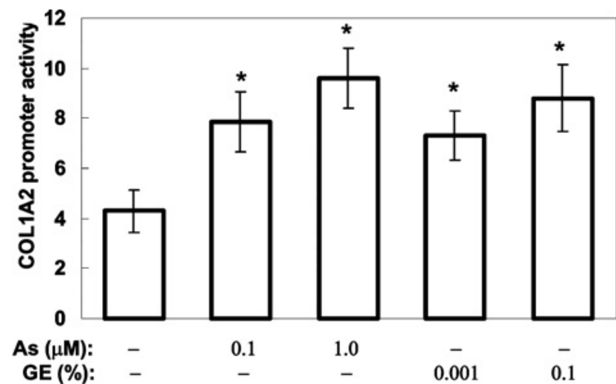

Figure 4 Up-regulation of *COL1A2* promoter activity by treatment with asiaticoside and ginseng extract in transfectant human dermal fibroblast cells

Transfectant human dermal fibroblast cells were treated with indicated concentrations of asiaticoside and ginseng extract for 16 h, after which they were harvested and lysed. Supernatants were assayed for luciferase activity, which was determined by the amount of light produced through the conversion of luciferin substrate into oxyluciferin by luciferase. Relative luciferase activity, calculated as the relationship between luciferase activity and the quantity of cells or the efficiency of the transfection, was determined three times in duplicate for each experiment, and the S.D. values are indicated by bars. All values were significant (*$P < 0.05$) compared with values for untreated control. As, asiaticoside; GE, ginseng extract.

that of the control. Consistent with this, type I collagen production was also induced by asiaticoside and ginseng extract (Figure 5). These results indicate the possibility that the pLuc-*COL1A2* promoter-transfected human dermal fibroblast cells are stable and can be introduced to screen

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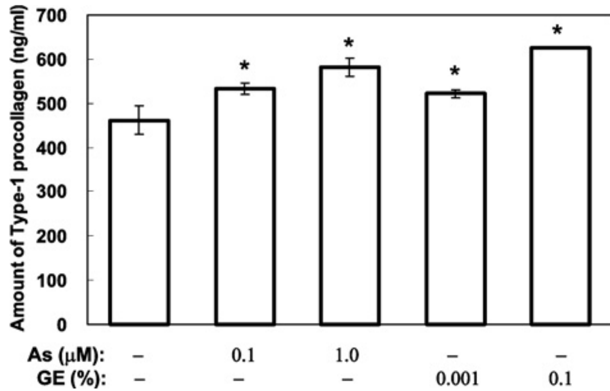

Figure 5 Up-regulation of type I collagen synthesis by treatment with asiaticoside and ginseng extract

Effects of asiaticoside and ginseng extract on type I procollagen synthesis, as determined using a sandwich immunoassay kit (Takara). Results are expressed as means ± S.D. The asterisks indicate $P < 0.05$ compared with controls. The results were verified by the repetition of three experiments, each in triplicate. As, asiaticoside; GE, ginseng extract.

wrinkle-related agents *in vitro*. This possibility is more strengthened by several reports [16–19]. A report showed that a construct containing the sequence from –376 to +58 of human α2(I) collagen gene (*COL1A2*) promoter is responsive to TGF-β1 in human cells, and overexpression of Smad3 stimulates *COL1A2* promoter activity [16]. The TGF-β response element of the α2(I) collagen gene has been mapped to the sequences located between –340 and –183 from the transcription start site [17]. This region contains three Sp1-binding sites and one AP-1 (activating protein-1) consensus sequence. Different groups have implicated either Sp1 or AP-1 as the mediator of TGF-β stimulation of the α2(I) collagen gene [17–19]. When taken together, these results suggest that the pLuc-*COL1A2* promoter-transfected human dermal fibroblast cells can be introduced to screen wrinkle-related agents and to monitor signal-transduction pathways that are involved in human collagen production.

Acknowledgments

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