Curcumin enhances the production of major structural components of elastic fibers, elastin, and fibrillin-1, in normal human fibroblast cells

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Curcumin enhances the production of major structural components of elastic fibers, elastin, and fibrillin-1, in normal human fibroblast cells

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Curcumin is the major component of the yellow extract derived from the rhizome of the Curcuma longa, which is also a main bioactive polyphenol and has been generally used as a spice, food additive, and herbal medicine. In this presented study, we found that curcumin can enhance the production of major structural components of elastic fibers, elastin, and fibrillin-1, in normal human fibroblast cells via increasing ELN and FBN1 promoters' activities. With 2 μM curcumin treatment, the enhanced tropoelastin and fibrillin-1 protein amounts in Detroit 551 cells were approximately 134 and 130% of control, respectively. Therefore, our results demonstrated that curcumin may be used as a functional compound and applied to drugs, foods, and cosmetics in the future.

Key words: curcumin; elastin; fibrillin-1; fibroblast cells; promoter activity

The constituents of skin, blood vessels, and lungs are required to withstand various mechanical environments. The mechanical properties of such elastic biological tissues are dominated by an extracellular matrix, in which elastic fibers offer both features of compliance and passive recoil.1) Although elastic fibers are complex assemblies with multiple parts, their structures comprise two main constituents: elastin and fibrillin microfibrils. Elastin is the most important structural element of the elastic fibers that is secreted from cells as a soluble protein (referred to as tropoelastin). The assembly of tropoelastin into a fibrillar matrix is a multi-step process.2) The super-family of fibrillin is a group of large glycoproteins. The most abundant constitution of fibrillin microfibrils backbone is fibrillin-1, which is also released from cells to form fibrillin microfibrils through a complex progression.3)

Denaturations of elastin structure, metabolism or assembly may cause both congenital and acquired diseases, such as the cases of cutis laxa, pseudoaxanthoma elasticum, and elastosis perforans serpiginosa in the parts of connective tissues and skin.4) Similarly, fibrillin-1 gene mutations cause Marfan syndrome, which also leads to various connective tissue disorders in the skeletal, cardiovascular, and ocular systems.5) Therefore, the regulations of elastin and fibrillin-1 expression in cells are the key steps for the formation of elastic fibers. Moreover, discovery of functional compounds that regulate the expressions of elastin and fibrillin-1 in cells is a good method to treat related syndromes.

Curcumin (Fig. 1(A)) is the major component of the yellow extract derived from the rhizome of the Curcuma longa (turmeric, an Indian spice), which is also a main bioactive polyphenol and has been generally used as a spice, food additive, and herbal medicine in Asia.6,7) Many articles have discussed the molecular basis of curcumin for the antioxidant, anti-inflammatory, antibacterial, antiviral, antifungal, and anticancer activities. In addition, many clinical trials have been conducted on this compound, which has effects on various chronic diseases including autoimmune, cardiovascular, neurological, psychological diseases, as well as diabetes and cancer.8–16) Previous studies have demonstrated that curcumin can ameliorate age-associated large elastic artery stiffening, and also protect the developing lung against long-term hyperoxic injury.11,12) However, to the best of our knowledge, no study has reported the effects of curcumin on the expressions of the major structural proteins of elastic fibers: elastin and fibrillin-1.

Therefore, in this study, we use a human cell-based antiaging compound assay system that analyses curcumin through the promoters of elastin (ELN) and fibrillin-1 (FBN1) genes combined with the reporters of secreted alkaline phosphatase (SEAP) in normal human fibroblast cells. Moreover, reverse transcription polymerase chain reaction (RT-PCR) and western blotting analyses were used to confirm the effects of curcumin on the regulations of elastin and fibrillin-1 in fibroblasts.

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The cells were maintained in a humidified incubator at 37 °C with 5% CO₂. The cells were subcultured every 3–4 days to maintain logarithmic growth and were allowed to grow for 24 h before transfection or treatment were applied.13

**Materials and methods**

**Materials.** Curcumin, retinol, dimethyl sulfoxide (DMSO), diethanolamine, magnesium chloride (MgCl₂), L-homoarginine and para-nitrophenyl phosphate (pNPP), ethidium bromide, dithiothreitol (DTT), agarose, and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA). Minimum essential medium alpha (α-MEM), L-glutamine, penicillin-streptomycin, deoxy-nucleotide triphosphate (dNTP), oligo(dT), Pfu and Taq DNA polymerase, and M-MLV reverse transcriptase were purchased from Gibco BRL/Invitrogen (Carlsbad, CA, USA). 3(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Affymetrix/USB (Cleveland, OH, USA). Xfect™ transfection reagent was purchased from Clontech Laboratories, Inc. (Mountain View, CA, USA). Restriction enzymes were purchased from New England BioLabs (Beverly, MA, USA). The antihuman tropoelastin, antihuman fibrillin-1, and antifibrillin antibodies were purchased from GenTex Inc. (Irvine, CA, USA). Deionized distilled water (ddH₂O) used to prepare solutions was purified using a Milli-Q system (Millipore, Bedford, MA, USA).

**Cell line and cell culture.** The Detroit 551 cells (BCRC 60118, normal human fibroblast cells) were obtained from the Bioreource Collection and Research Center (BCRC, Hsinchu, Taiwan, ROC). Detroit 551 cells were cultured in α-MEM medium supplemented with 10% FBS and 1% penicillin-streptomycin (100 U/mL penicillin and 100 μg/mL streptomycin). The cells were maintained in a humidified incubator at 37 °C with 5% CO₂. The cells were subcultured every 3–4 days to maintain logarithmic growth and were allowed to grow for 24 h before transfection or treatment were applied.13

**MTT Assay for cell viability.** The Detroit 551 cells were seeded in 96-well plates (8 × 10³ cells/well) for 24 h incubation. The prepared cells were subsequently treated with different concentrations of curcumin in DMSO for 24 h. The used concentration of DMSO in medium is 0.1% (v/v). Subsequently, 100 μL (0.5 mg/mL) of MTT solution was added to cells, which were then incubated at 37 °C for 4 h and washed twice with phosphate buffered saline. Finally, the cleaned cells were lysed with 100 μL DMSO, and the absorbance was measured spectrophotometrically at 540 nm using an ELISA reader.14

**Plasmid construction and transfection.** The genomic DNA from Detroit 551 human normal fibroblast cells were extracted by FavorPrep™ Blood/Cultured Cell Genomic DNA Extraction Mini Kit (Favorgen, PingTung, Taiwan, ROC). Human ELN and FBN1 promoters DNA fragments were amplified through the extracted genomic DNA by Pfu DNA polymerase with designed primers (Table 1). The prepared promoter DNA fragments were then cloned into pSEAP2-control vectors (Clontech, Mountain View, CA, USA) by XhoI and EcoRI restriction sites to generate pSEAP2-ELN and pSEAP2-pFBN1 plasmids (Fig. 2(A)). The PCR reaction was performed using 1 μg plasmid DNA, 1.5 mM MgCl₂, 0.2 mM dNTP, 2.5 units of Taq DNA polymerase, and 0.1 μM each of the primers (Table 1). For transfection, Detroit 551 cells were cultured in 24-well plates with 8 × 10³ cells/well and the prepared plasmids were transfected by XfectTM transfection reagent into cells at concentration of 1 μg/well according to the protocol. After 4 h transfection at room temperature, the transfection reaction was terminated by replacing of fresh medium.15 The transfection efficiency of tested plasmid was measured by the co-transfected control plasmid (pEGFP-1, Clontech) at the ratio of 10:1.

**SEAP activity assay.** Detroit 551 cells were seeded onto the 24-well plates at a density of 8 × 10⁴ cells/well. After incubating at 37 °C for 24 h in 5% CO₂ atmosphere incubator, cells were transfected with 1 μg well plasmids using the Xfect™ transfection reagent. After transfection, the cells were then treated with or without (untreated group) curcumin for 24 h. Cell culture supernatants were collected and then analyzed for SEAP activity. For SEAP activity assay, the cell culture supernatants were placed at 65 °C for 10 min to eliminate endogenous alkaline phosphatase activities, which were then centrifuged at 14,000 x g for 1 min. Add 300 μL supernatants with an equal volume of 2X SEAP buffer (2 M diethanolamine, 1 mM MgCl₂, 20 mM L-homoarginine, pH 9.8) to each well with 20 mM pNPP substrate. The kinetic assay was performed by reading the absorbance at 405 nm at regular
Curcumin enhances elastin and fibrillin-1 productions

Table 1. Information of the primers used in this study.

<table>
<thead>
<tr>
<th>Target</th>
<th>Name</th>
<th>Sequence</th>
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<tr>
<td>ELN promoter</td>
<td>pELN-F</td>
<td>5'-GCTCGAGAAGAGAGGGCTCC-3'</td>
</tr>
<tr>
<td></td>
<td>pELN-R</td>
<td>5'-GGAATTCCTCgggGAAATGCcCA-3'</td>
</tr>
<tr>
<td>FBN1 promoter</td>
<td>pFBN1-F</td>
<td>5'-GCTCGAAGTTGGTCAGGGGAAACGG-3'</td>
</tr>
<tr>
<td></td>
<td>pFBN1-R</td>
<td>5'-GGAATTCAGTCCGTGCCTGGCCCT-3'</td>
</tr>
<tr>
<td>ELN mRNA</td>
<td>ELN-mRNA-F</td>
<td>5'-CTTGGCGAATCCAGCC-3'</td>
</tr>
<tr>
<td></td>
<td>ELN-mRNA-R</td>
<td>5'-CCAAACAGGGAGGAGGACT-3'</td>
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<tr>
<td>FBN1 mRNA</td>
<td>FBN1-mRNA-F</td>
<td>5'-CTGAAGACATGAAAGACTGTTG-3'</td>
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<td></td>
<td>FBN1-mRNA-R</td>
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<tr>
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<tr>
<td></td>
<td>GAPDH-mRNA-R</td>
<td>5'-AGATGATGACCTTTGCGCTT-3'</td>
</tr>
</tbody>
</table>

Fig. 2. Effects of curcumin on the activities of human ELN and FBN1 promoters in the transfected Detroit 551 cells.

Notes: (A) Illustrations of pSEAP2-Control, pSEAP2-pELN, and pSEAP2-pFBN1 plasmids and (B) Relative SEAP activities of curcumin treated Detroit 551 cells. 0–50 min indicates the reaction time of SEAP activity analysis. Each value is expressed as the mean ± S.E. (n = 3). Tukey’s post hoc tests, *Significant difference (p < 0.05) compared with the control group.

RT-PCR analysis. Detroit 551 cells were seeded onto the 6-cm dish at a density of 5 × 10^5 cells. After 24 h incubation, cells were with or without (untreated) curcumin. After 24 h treatment, the lysates were collected and then quantified. For western blotting, each well was loaded with 20 ng of total protein and resolved by 7% or 10% SDS PAGE (7% for fibrillin-1, 10% for tropoelastin), and then electroblotted on to a polyvinylidene difluoride membrane using the Bio-Rad MiniProtein II apparatus (Bio-Rad Laboratories, Carlsbad, CA, USA). The blots were subsequently incubated with an antihuman fibrillin-1, anti-human tropoelastin or anti-brillin-1 antibody as the primary antibody, and the immune complexes were visualized using an ECL reagent. The β-actin was used as an internal control for normalization.17)

Western blot analysis. Detroit 551 cells were seeded onto the 10-cm dish at a density of 8 × 10^5 cells. After 24 h incubation, the cells were with or without (untreated group) curcumin. After 24 h treatment, the lysates were collected and then quantified. For western blotting, each well was loaded with 20 ng of total protein and resolved by 7% or 10% SDS PAGE (7% for fibrillin-1, 10% for tropoelastin), and then electroblotted on to a polyvinylidene difluoride membrane using the Bio-Rad MiniProtein II apparatus (Bio-Rad Laboratories, Carlsbad, CA, USA). The blots were subsequently incubated with an antihuman fibrillin-1, anti-human tropoelastin or anti-brillin-1 antibody as the primary antibody, and the immune complexes were visualized using an ECL reagent. The β-actin was used as an internal control for data normalization. The bands were scanned and then quantified by measuring the optical densities using the Vipro Platinum 1.1 software package (Version 12.9; UVitec, UK).18)

Statistical analysis. The quantitative data for the present study were analyzed using Tukey’s post hoc tests or Dunnett’s multiple comparison test. The quantitative data are presented as the means ± S.E. for three independent experiments.

Results and discussion

Cytotoxic effect of curcumin on Detroit 551 cells

First of all, the cytotoxic effect of curcumin on normal human fibroblast cells (Detroit 551 cells) was examined by a standard MTT assay. The result is shown in Fig. 1(B). All tested curcumin concentrations of 0.1 M DTT, and 1 μL of ribonuclease inhibitor (40 U/μL) were added to the mixture. The mixture was incubated at 37 °C for 2 min, and 1 μL of reverse transcriptase was added. The reaction was stopped by heating the solution at 70 °C for 15 min. A 1 μL aliquot of cDNA mixture was used in the subsequent enzymatic amplification. PCR was performed using 1.5 mM MgCl2, 0.2 mM dNTP, 2.5 units of Taq DNA polymerase, and 0.1 μM each of the primers (Table 1). The amplified products were separated in 2% agarose gel in Tris–borate-EDTA (TBE) buffer and stained with ethidium bromide. The GAPDH (glyceraldehyde 3-phosphate dehydrogenase) gene was used as an internal control for normalization.19)
(0.25–2 μM) had no cytotoxic effects on Detroit 551 cells. The presented cell viabilities are all around 100% of control. Thus, we used the highest concentration of curcumin (2 μM) for the following experiments.

Many researchers have confirmed that curcumin possesses antioxidant, anti-inflammatory, antibacterial, anti-amyloid properties, and suppresses proliferation of a wide variety of tumor cells. In our presented study, the concentrations of curcumin lower than 2 μM might be no cytotoxic effect on Detroit 551 cells (Fig. 1(B)). Therefore, this result also suggested that proper concentrations of curcumin have potential to be used as an active ingredient in the applications of drugs, foods, and cosmetics.

Effects of curcumin on the activities of human ELN and FBN1 promoters in Detroit 551 cells

To analyze the effects of curcumin on the expression of elastin and fibrillin-1 in fibroblasts, human ELN and FBN1 promoters were constructed for the assay and shown in Fig. 2(A). Previous studies have verified that the functional human ELN promoter’s region is at the positions between −1 and −2260 bp. Besides, for the promoter of human FBN1, an efficient promoter’s region has been identified from −690 to −1351 bp. Therefore, according to the results from previous researches, in this study, we constructed a human cell-based assay method to analyze the effects of curcumin on the activity of ELN and FBN1 promoters. The results are shown in Fig. 2(B). Our results indicated that curcumin can both increase the promoter activities of ELN and FBN1. From 10 to 50 min analytic conditions, all of the improved SEAP activities for FBN1 in curcumin treated cells are higher than 122% of control. Under the 50 min analytic condition, the enhanced SEAP activities in curcumin treated cells can increase to approximately 120 and 145% of control for ELN and FBN1 activity, respectively (Fig. 2(B)). Although the enhanced activity of ELN promoter is not clearer than that of FBN1 promoter in other analytic conditions, the improvement effects of curcumin on both target genes are still recognized in this experiment. In addition, such enhancement results are not significantly revealed in the group of pSEAP2-control transfected Detroit 551 cells (Fig. 2(B)). To our knowledge, the effects of curcumin on the promoter activities of human elastin and fibrillin-1 are never discussed in any earlier study. Therefore, the improvement effects of curcumin should be further confirmed.

Effects of curcumin on mRNA levels of tropoelastin and fibrillin-1 in Detroit 551 cells

To confirm the effects of curcumin on both mRNA and protein levels of tropoelastin and fibrillin-1 in Detroit 551 cells, RT-PCR, and western blotting analyses were used in the experiments. The well-known enhancer for elastic fibers, retinol, is used as a positive control. Besides, retinol has no cytotoxicity for Detroit 551 cells if the concentrations are lower than 10 μM (data not shown), hence, 10 μM retinol is used for the following experiment. For RT-PCR assay, the image of gel electrophoresis is shown in Fig. 3(A) and the quantified result is presented in Fig. 3(B). The GAPDH mRNA was also analyzed and used as an internal control. For tropoelastin, the mRNA level was evidently increased by the treatment of curcumin in fibroblast cells to about 138% of control. However, the quantity of fibrillin-1 mRNA was slightly increased to approximately 110% of control, but which is not a significant improvement (Fig. 3(B)). In addition, retinol can evidently increase both the levels of tropoelastin and fibrillin-1 mRNA in Detroit 551 cells. Comparing the results of Figs. 3(B)–2(B), it showed that curcumin strongly activate FBN1 promoter compared to ELN, however the expression of mRNA level of tropoelastin were higher than that of fibrillin-1. This situation might be due to the activities of used ELN and FBN1 promoters are different. Therefore, although the enhanced effect of curcumin on FBN1 promoter is obvious, but the improved mRNA level of fibrillin-1 is still slight.

Effects of curcumin on protein levels of tropoelastin and fibrillin-1 in Detroit 551 cells

For western blotting analysis, the results, as shown in Fig. 4(A) and (B) (quantified results), demonstrated that curcumin is similar to retinol, which can clearly increase both the protein levels of tropoelastin and fibrillin-1 in Detroit 551 cells. The β-actin protein was also analyzed and used as an internal control. For tropoelastin, the enhanced protein amount was...
approximately 134% of control. In contrast, the improved fibrillin-1 protein level was also up to about 130% of control (Fig. 4(B)). This result is not similar to the result in Fig. 3(B) for mRNA level. This circumstance might be caused by the analytic time point of the protein amount in cells. Therefore, even the improvement effects of curcumin on mRNA and protein have a little difference and we still believe that curcumin can evidently up-regulate the productions of elastin and fibrillin-1 in fibroblasts.

It is well known that cAMP response element binding protein (CREB) is widely expressed in many cells and can be activated by phosphorylation. When CREB is activated, it will bind to the CREB binding protein and thus activate the target genes. Curcumin has a modulatory effect on the transcription factor CREB expression. Curcumin increases the phosphorylated CREB and which has been mediated by mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI-3 K) pathways in cells. Moreover, a cAMP response element was found between −1811 and −1818 bp within human ELN promoter region. In addition, curcumin also has effect on gene expression through the regulation of CCAAT/enhancer-binding protein (C/EBP) factor function. Similarly, a binding site for C/EBP has been identified in FBN1 promoter region between −1107 and −1114 bp. Thus, these relationships may be a good reason that explains why curcumin can regulate the production of elastin and fibrillin-1 in human fibroblasts. Curcumin is a broad functional compound, which may execute many biological effects. However, this study is the first report about the effects of curcumin on the production of elastin and fibrillin-1 in fibroblast cells.

In summary, our results supposed that curcumin can evidently up-regulate the productions of elastin and fibrillin-1 in fibroblasts. Thus, curcumin may be used as an active compound and may be applied to drugs, foods, and cosmetics to solve problems relating to elastic fibers in the future.

Conflict of interest

The authors declare no conflict of interest.

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