Graves’ orbitopathy (GO) is pathologic manifestation of Graves’ disease, an autoimmune disorder of the thyroid gland. Also known as thyroid eye disease (TED), the presentation of GO includes eye pain, eyelid retraction, edema of periorbital tissues, proptosis, and compressive optic neuropathy. Orbital fibroblasts have been identified as the key to disease process. Excessive amounts of hydrophilic glycosaminoglycan, predominantly hyaluronan, accumulate, and fibroblasts differentiate into adipocytes at an abnormally high rate. Such tissue expansion within the bony orbits is thought to be responsible for progression and complications of GO. At the center of the pathogenesis lies inflammation and oxidative stress. Infiltration of inflammatory cells to the orbit triggers and propagates tissue expansion. Oxygen free radicals trigger fibroblast proliferation, and the abundance of thyroid hormones in Graves’ disease make orbital fibroblasts more vulnerable to oxidative damage.

There is currently no reliable and specific therapeutic agent for GO. Glucocorticoids have thus far been the first-line treatment, but side effects and complications necessitate a search for safer therapeutic alternatives. Curcumin is a plant polyphenol present in the rhizome of turmeric, a well-known spice derived from the roots of the Curcuma longa plant. Numerous studies have proved its anti-inflammatory and antioxidant activities in vitro and in vivo, as well as in animals and in humans. Its effects are being tested in various diseases, including autoimmune disease, cardiovascular disease, neurodegenerative disorder, and cancer. However, no previous studies have reported the effect of curcumin in GO.

In light of the above, the present study was designed to study the effect of curcumin on orbital fibroblasts in vitro. We tested whether curcumin could counter inflammation, oxidative stress, and adipocyte differentiation, the three main pathogenic mechanisms in GO, in orbital fibroblasts taken from patients with GO. We further sought insights into the related mechanisms.

Purpose. We examined the therapeutic effect of nontoxic concentrations of curcumin, a plant polyphenol extracted from Curcuma longae, in primary cultures of orbital fibroblasts from Graves’ orbitopathy (GO).

Methods. The effect of curcumin on interleukin (IL)-1β-induced–proinflammatory cytokine production was determined using quantitative real-time PCR, enzyme-linked immunosorbent assay (ELISA), and Western blot analysis. Adipogenic differentiation was identified using Oil-Red O staining, and levels of peroxisome proliferator activator γ (PPARγ) and CCAAT-enhancer-binding proteins (C/EBP) α/β were determined by Western blot analyses. Antioxidant activity was measured using an oxidant-sensitive fluorescent probe to detect intracellular reactive oxygen species (ROS) generated in response to hydrogen peroxide (H₂O₂) and cigarette smoke extract (CSE).

Results. Treatment with curcumin resulted in a dose- and time-dependent decrease in IL-1β-induced synthesis of inflammatory cytokines, including IL-6, IL-8, MCP-1, and ICAM-1 at both mRNA and protein levels. Decrease in lipid droplets and expression of PPARγ and C/EBPα/β were noted in fibroblasts treated with curcumin during adipose differentiation. CSE- or H₂O₂-induced ROS synthesis was significantly lower in curcumin-treated fibroblasts in comparison with the control. Curcumin significantly suppressed IL-1β-induced phosphorylated extracellular signal-regulated kinase, Akt, c-Jun NH(2)-terminal kinase, and nuclear factor κ-light-chain-enhancer of activated B cells, p65 proteins and stimulated β-catenin translocation into nucleus during adipogenesis.

Conclusions. Curcumin inhibits proinflammatory cytokine production, ROS synthesis, and adipogenesis in orbital fibroblasts of GO patients in vitro possibly related to multiple proinflammatory signaling molecules and β-catenin pathway. The results of the study support potential use of the curcumin in the treatment of GO.

Keywords: Graves’ orbitopathy, curcumin, inflammation, adipogenesis, oxidative stress.
METHODS

Reagents

Curcumin was obtained from Sigma Aldrich, Inc. (Merck KGaA, Darmstadt, Germany). The 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay and Oil Red O were purchased from Sigma-Aldrich, Inc. (Merck KGaA, Darmstadt, Germany). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin, and gentamicin were purchased from Hyclone Laboratories, Inc. (Logan, UT, USA). Recombinant human interleukin (IL)-1β and the enzyme-linked immunosorbent assay (ELISA) kits for IL-6 and IL-8 were purchased from R&D Systems (Minneapolis, MN, USA), and that for MCP-1 from R&D Systems (Abingdon, United Kingdom). Antiperoxisome proliferator activator γ (PPARγ), anti–CCAAT-enhancer-binding protein (C/EBP)α, anti–CCAAT-enhancer-binding protein (C/EBP)β, anti-lamin A/C, anti–β-actin antibodies, and anti–nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against extracellular signal-regulated kinase (ERK), β-catenin, glycogen synthase kinase 3β (GSK3β), Akt, c-Jun NH(2)-terminal kinase (JNK), and cyclin D1, and anti–intercellular adhesion molecule 1 (ICAM-1) antibody were produced by Cell Signaling Technology (Beverly, MA, USA).

Cell Culture and Differentiation Protocol

Orbital adipose/connective tissue explants were obtained from surgical wastes from GO patients (seven females and three males; age, 44.8 ± 6.7 years) undergoing decompression operation. Normal control tissues were harvested during orbital wall fracture reduction or evisceration from patients without history or clinical evidence of any thyroid disease (four females and three males; age, 40.8 ± 7.8 years). The clinical characteristics of participants are illustrated in the Table. The study adhered to the tenets of Declaration of Helsinki, and the study protocol was approved by the Institutional Review Board of Severance Hospital. All patients provided written informed consent. All of the GO patients had achieved a stable euthyroid state for at least 3 months, and their clinical activity scores at the time of surgery were <4. No GO patients had received steroid or radiotherapy treatment for at least 3 months prior to surgery.

Orbital fibroblasts were isolated from the harvested tissue and cultured as described previously.7 After being minced, the tissue was placed directly in DMEM: F12 (in 1:1 ratio) medium containing 20% FBS, penicillin (100 U/mL), and gentamicin (20 μg/mL). The medium was placed in a humidified 5% CO2 incubator and kept at 37°C. The cells were then maintained in two 80-mm flasks containing DMEM, antibiotics, and 10% FBS.

After confirming the growth of fibroblasts, the cells were

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**TABLE. Clinical Information of Patient Samples Used in This In Vitro Study**

<table>
<thead>
<tr>
<th>Age (y)</th>
<th>Sex</th>
<th>CAS</th>
<th>Smoker</th>
<th>Duration of GO (y)</th>
<th>Proptosis R/L (mm)</th>
<th>Surgery Performed</th>
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<tr>
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<td>M</td>
<td>3/7</td>
<td>Y</td>
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<td>21/21</td>
<td>Decompression</td>
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<tr>
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<td>3/7</td>
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<td>Decompression</td>
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</tbody>
</table>

Non-GO control subjects

| 49      | M   | N/A | Y      | N/A              | N/A                | Orificial wall fracture |
| 32      | F   | N/A | N      | N/A              | N/A                | Evisceration          |
| 39      | F   | N/A | N      | N/A              | N/A                | Evisceration          |
| 45      | M   | N/A | N      | N/A              | N/A                | Evisceration          |
| 44      | M   | N/A | Y      | N/A              | N/A                | Evisceration          |
| 32      | F   | N/A | N      | N/A              | N/A                | Evisceration          |
| 45      | F   | N/A | N      | N/A              | N/A                | Orbificial mass excision |

CAS, clinical activity scores; F, female; L, left eye; M, male; N, no; N/A, not applicable; R, right eye; Y, yes.

**FIGURE 1.** Effect of curcumin on viability of orbital fibroblasts. Orbital fibroblasts of GO patients (A) and non-GO patients (B) were seeded in 24-well culture plates, 1 × 10^5 cells/well, and treated with different concentrations (0–100 μM) of curcumin for 24 and 48 hours. Then, the cells were subjected to MTT assay to assess viability.
serially passaged in monolayers by treating them with trypsin/ethylenediaminetetraacetic acid (EDTA). Strains were stored in liquid nitrogen and only those between the third and fifth passages were used for experiments.

Cell Viability Assays

Cell viability was assessed with an MTT assay, following the manufacturer’s (Sigma-Aldrich, Inc.) protocol. Orbital fibroblasts obtained from healthy and GO patients were seeded into 24-well culture plates (1 × 10^5 cells/well), and each well was treated with different concentrations of curcumin (10, 20, 50, 70, and 100 μM) for 24 and 48 hours. Then, cells were washed and incubated with 5 mg/mL MTT solution for 3 hours at 37°C. After solubilization of the converted dye with ice-cold isopropanol, the absorbance was measured with a microplate reader (EL 340 Bio Kinetics Reader; Bio-Tek Instruments, Winooski, VT, USA) at 560 nm, with background subtraction at 650 nm.

Quantitative Real-Time PCR

RNA was extracted from cells using TriZol (Invitrogen, Carlsbad, CA, USA), 1 g of which was reverse-transcribed into cDNA (Qiagen, Valencia, CA, USA). The cDNA was then amplified with TaqMan universal PCR master mix in an ABI 7300 real-time PCR thermocycler (Applied Biosystems, Carlsbad, CA, USA). All PCRs were performed in triplicate using Hs00985639_m1 for IL-6, Hs00174103_m1 for IL-8, Hs00234140_m1 for monocyte chemotactic protein (MCP)-1, Hs00164932_m1 for ICAM-1, and H299999905_m1 for glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) as primers. GAPDH was used to normalize PCR results, and the results are expressed as fold change in the Ct value relative to the control group using the 2^-ΔΔCt method.11

ELISA

Supernatants were collected from cell cultures for ELISA assays, which were performed according to the manufacturer’s instructions to detect levels of IL-6, IL-8, and MCP-1 in the fibroblasts. After measuring the absorbance at 405 nm and determining the percentage of binding for each sample, a standard binding curve was generated to determine the cytokine concentrations. Samples were diluted 1:10 before analysis, and the average value of three assays was used for statistical analyses.

Western Blot Assay

Confluent orbital fibroblasts were pre-exposed to different concentrations of curcumin for 1 hour and then were stimulated with IL-1β for another 16 hours. After they were washed with PBS, cells were treated with cell lysis buffer on ice for 30 minutes.12 The cell lysates were centrifuged to obtain cell homogenate fractions and resolved by 10% SDS-PAGE after boiling. Then, they were transferred to polyvinylidene fluoride membranes (Immobilon; Millipore Corp., Billerica, MA, USA) and treated with primary antibodies overnight in tris-buffered saline and Tween 20 (TBST). Immunoreactive bands were detected with horseradish peroxidase–conjugated secondary antibody with chemilluminescence (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA). The bands were detected on an X-ray film (Amersham Pharmacia Biotech, Inc.), quantified via densitometry, and normalized to the level of β-actin in each sample.

Intracellular Reactive Oxygen Species Measurement

Measurements of reactive oxygen species (ROS) generation have been described previously.7 In short, cigarette smoke extract (CSE) was prepared using two commercially available filtered cigarettes containing 8.0 mg tar and 0.7 mg nicotine (Marlboro 20 class A; Philip Morris Korea, Inc., Seoul, Korea) according to the published methods.7,13 Based on the results from a previous study that 2% to 5% of CSE induced proliferation in GO cells without affecting viability and that 200 μM H_2O_2 did not
significantly lower fibroblast viability. \textsuperscript{14} 4% CSE and 200 \textmu M H\textsubscript{2}O\textsubscript{2} were chosen to generate oxidative stress in fibroblasts.

Fibroblasts were seeded in six-well plates, \(1 \times 10^5\) cells/well, for final volume of 2 mL. To each well, curcumin was added in different concentrations (5, 10, 15, and 20 \textmu M) for 1 hour. The cells were treated with 2% CSE or 200 \textmu M H\textsubscript{2}O\textsubscript{2} for 30 minutes. Then, cells were washed with PBS and incubated with an oxidant-sensitive fluorescent probe, 5-(and 6)-carboxy-2',7'-dichlorodihydrofluorescein acetate (H\textsubscript{2}DCFDA), at 37\textdegree C for 30 minutes. Thereafter, cells were trypsinized, washed, and resuspended in PBS. They were processed by flow-cytometer (ELITE flow cytometer; Coulter Cytometry, Inc., Hialeah, FL, USA) for measurements of fluorescence intensity and examined under an IX71-F22PH inverted fluorescence microscope (Olympus Corp., Tokyo, Japan). Cells were gated and only live cell populations analyzed.

Adipogenesis

Using a previously published protocol, adipocyte differentiation of fibroblasts was induced to test the effect of curcumin on adipogenesis. \textsuperscript{15} Cells were cultured in serum-free DMEM supplemented with T3, insulin (Boehringer-Mannheim, Mannheim, Germany), carboxyprostaglandin (cPGI2; Calbiochem, La Jolla, CA, USA), and dexamethasone. A PPAR\textgamma agonist, rosiglitazone (10 \textmu M; Cayman, Ann Arbor, MI, USA), was also added from day 1 of differentiation for heightened stimulation of adipogenesis. To evaluate the effect of curcumin on adipogenesis, cells were treated with curcumin for the entire 10-day differentiation period.

Oil Red O Staining of Cells

Cells were stained with Oil Red O as described by Green and Kehinde. \textsuperscript{16} Six milliliters of a stock solution prepared with 0.5% Oil Red O in isopropanol was mixed with 4 mL distilled water and placed at room temperature for 1 hour. The solution was then filtered and added to cells that have been washed with PBS and fixed with 3.7% formalin for 1 hour at 4\textdegree C. The cell-Oil Red O solution mixture was left for 1 hour at room temperature, inspected using a light microscope (Axiovert; Carl Zeiss AG, Oberkochen, Germany), and photographed (Olympus BX60; Olympus Corp., Melville, NY, USA).

Statistical Analysis

All continuous data are expressed as mean \pm SD. All experiments were performed using at least three cell strains from three different individuals. Curcumin-treated samples were compared against the control using repeated-measures ANOVA and independent \(t\)-tests, with Bonferroni test as a post hoc test, using SPSS for Windows, version 20.0 (SPSS, Inc., Chicago, IL, USA). \(P < 0.05\) was considered statistically significant.

RESULTS

Viability of Orbital Fibroblasts

To determine nontoxic concentrations of curcumin in orbital fibroblasts, an MTT assay was performed. More than 95% of GO fibroblasts remained viable when they were exposed to curcumin at a concentration up to 20 \textmu M for 24 hours (Fig. 1A). Cell viability remained greater than 95% when normal fibroblasts were exposed to curcumin at a concentration up to 20 \textmu M even after 48 hours (Fig. 1B). For analysis of the therapeutic effect of curcumin, the orbital fibroblasts were exposed to curcumin at a concentration range of 0 to 20 \textmu M in various conditions throughout the study.
Curcumin Suppresses IL-1β–Induced Expression of Proinflammatory Cytokines

The real-time PCR results showed that when orbital fibroblasts were exposed to curcumin for 1 hour, transcription of the proinflammatory cytokines, including IL-6, IL-8, MCP-1, and ICAM-1, in response to challenge with IL-1β for 16 hours was significantly suppressed in a dose-dependent manner in both GO and normal orbital fibroblasts (Fig. 2). When GO fibroblasts were exposed to 20 μM curcumin for different durations, expression of all four proinflammatory cytokines decreased significantly in a time-dependent manner in both GO and normal fibroblasts (Fig. 3). ELISA tests confirmed the dose-dependent suppressive effect of curcumin on the production of cytokines, including IL-6, IL-8, and MCP-1 in GO fibroblasts (Fig. 4). Expression of ICAM-1 protein stimulated by IL-1β in GO fibroblasts was also inhibited significantly with curcumin (Fig. 5).

Adipogenesis of GO Fibroblasts Is Inhibited by Curcumin

Confluent fibroblasts from GO patients were treated with adipogenic medium for 10 days to induce differentiation into adipocytes. When examined under a light microscope, exposure to adipogenic conditions induced orbital fibroblasts to lose their stellate appearance, assume a spherical adipocyte-like shape, and accumulate intracellular lipid droplets (Fig. 6A). The number of fibroblasts that underwent such differentiation into adipocytes significantly increased in comparison to control when IL-1β was added to the adipogenic medium. Treatment of differentiating cells with curcumin substantially suppressed adipogenesis in a dose-dependent manner as identified with Oil Red O staining (Fig. 6A). When quantified by measuring optical density of Oil Red O–stained cell lysates at 490 nm, the same pattern was observed (Fig. 6B). Curcumin attenuated levels of adipogenic transcription factors, PPARγ, C/EBPα, and C/EBPβ proteins, in a dose-dependent manner in Western blot analysis (Fig. 6C).

Curcumin Shows Antioxidant Properties in CSE and H2O2-Stimulated ROS Production

We investigated whether curcumin acted as an antioxidant in GO fibroblasts. The amount of ROS generated in response to treatment with H2O2 decreased significantly when the cells were pretreated with curcumin 15 μM or greater for 1 hour (Fig. 7A). In situ localization of ROS-sensitive fluorescent probe, DCFDA, showed less ROS in cells pretreated with 15 and 20 μM curcumin (Fig. 7B). The same pattern was observed for GO fibroblasts exposed to CSE for 30 minutes. Fluorescence intensity from ROS (Fig. 7C) and the amount of ROS–specific probes under the microscope (Fig. 7D) in response to

Figure 4. Effect of curcumin on the production of proinflammatory cytokines in GO fibroblasts. Confluent orbital fibroblast cultures from GO patients were pretreated with curcumin in different concentrations (0–20 μM) for 1 hour before stimulation with IL-1β (10 ng/mL for 16 hours). ELISA tests were performed in three GO cell strains from three different individuals. Dose-dependent inhibition by curcumin was noted in synthesis of IL-6 (A), IL-8 (B), and MCP-1 (C) (P < 0.05 versus IL-1β–stimulated cells).

Figure 5. Suppressive effect of curcumin on ICAM-1 protein production in GO fibroblasts. Orbital fibroblasts were pretreated with different concentrations (0–20 μM) of curcumin for 1 hour and then stimulated with IL-1β (10 ng/mL for 16 hours). Cell lysates were subjected to a Western blot analysis. The bands were quantified by means of densitometry and were then normalized to the level of β-actin in the same sample. Measurements for three GO cells from different individuals were averaged, and the results are expressed as mean ± SD (P < 0.05 versus IL-1β–stimulated cells).
FIGURE 6. Effect of curcumin on adipocyte differentiation of GO orbital fibroblasts. IL-1β (10 ng/mL) was added to the adipogenic medium to stimulate differentiation of fibroblasts into adipocytes. Slides from Oil Red O staining show that increasing concentrations of curcumin were able to attenuate adipocyte differentiation in a dose-dependent manner (A). Quantification by measurements of optical density of cell lysates at 490 nm echoed the histochemical results (B). The results are presented as the mean optical density (%) ± SD of three experiments (*P < 0.05 versus IL-1β-stimulated cells). Western blot analyses showed that curcumin attenuated the protein expression of the adipogenic transcriptional regulators, PPARγ, C/EBPα, and C/EBPβ, proteins, in GO cells (C). The results from the quantification of the relative density of Western blot bands are shown as graphs below. Experiments were done in three GO cells from three different individuals.
untreated control values. The fluorescence intensities from ROS in GO quantified using FACS (200 concentrations of curcumin (0–20 μM) for 1 hour. ROS generated in response to exposure with H2O2 was detected using fluorescence and quantified using FACS (A). The results are expressed as percentage of untreated control values. The fluorescence intensities from ROS in GO fibroblasts treated with H2O2 were also localized in situ with a microscope (B). The same procedure was repeated, and ROS generated in response to 4% CSE from GO fibroblasts was quantified (C) and localized (D). Assays were performed in triplicate with cells from three different GO donors (*P < 0.05 versus non–curcumin-treated cells).

4% CSE decreased in cells pretreated with curcumin in a dose-dependent manner.

Effect of Curcumin on Multiple Signaling Pathways Including GSK-3β and β-Catenin

To investigate the signaling pathways through which curcumin mediates its effects in GO fibroblasts, the expression of multiple transcription factors was assayed after curcumin treatment. The cells were pretreated with different concentrations of curcumin for 1 hour and were stimulated for 1 hour with IL-1β. Western blot analyses (Fig. 8) showed that curcumin inhibited phosphorylation of signaling molecules including Erk, Akt, Jnk, and NF-xB p65 proteins in a dose-dependent manner. The β-catenin pathway was also evaluated in GO fibroblasts using Western blot analyses. During GO fibroblast differentiation into adipocytes over a 10-day period, the level of inactive GSK-3β (phosphorylated GSK-3β [ser 9]) decreased. When curcumin was added to the adipogenic medium, the average level of phosphorylated GSK-3β was greater than when without curcumin treatment. When the levels of phosphorylated GSK-3β (ser 9) on day 10 of adipogenesis were compared between control and curcumin-treated fibroblasts, the amount of the inactive kinase was significantly greater in the curcumin-treated fibroblasts (Fig. 9A). The overall amount of β-catenin increased in comparison, and the nuclear fraction of β-catenin especially increased after the treatment with curcumin (Fig. 9B). When the density of each band was quantified and calculated as ratios between the nuclear and cytosolic fractions of β-catenin, the ratios increased significantly with curcumin treatment, confirming disproportionate increase in nuclear β-catenin by the plant polyphenol. When the response of these molecules was assessed on the 10th day of adipogenesis across different concentrations of curcumin, the results indicated that increasing concentrations of curcumin led to greater levels of inactive GSK-3β (P-GSK-3β [Ser9]), total β-catenin, and active β-catenin (nonphosphorylated [NP]-β-catenin), leading to increased production of cyclin D1 (Fig. 9C). Given our results, curcumin appears to activate the translocation of β-catenin from the cytosol to the nucleus, resulting in increased expression of antadiopogenic genes downstream.

Discussion

In this study, we assessed cytokine secretion from GO fibroblasts and confirmed that curcumin was able to curtail main pathogenic pathways of GO. Based on the analyses of our results, the ingredient of the C. longa plant appeared to have inhibitory effect on adipogenesis, ROS generation, and inflammatory reaction from GO fibroblasts in response to IL-1β. Curcumin mediated its effects on GO fibroblasts by inhibiting activation of proinflammatory transcription factors.

The pathogenesis of GO is believed to involve interaction of immune cells with orbital fibroblasts.4 Infiltrating T-lymphocytes induce the fibroblasts to secrete cytokines, including IL-6, IL-8, and ICAM-1, which activate and amplify inflammatory reactions.17 IL-6 is thought to stimulate B-cell differentiation and produce functional autoantibodies in GO.18 The chemokine IL-8 is released to recruit more T-lymphocytes to the site.19 In our study, we used IL-1β to simulate the proinflammatory conditions of GO, as the cytokine has been widely used as an inflammation stimulant in many in vitro studies of GO.20 Although GO fibroblasts showed upregulation of IL-6 and IL-8 in response to challenge with IL-1β, the level of these proinflammatory cytokines significantly decreased when cells were exposed to curcumin. The inhibitory effect of curcumin on inflammatory response from GO fibroblasts appeared to be dependent on both the concentration of the natural ingredient and the length of time for which the cells are exposed to curcumin.

Previous reports have partially identified mechanisms by which curcumin alleviates inflammation in various diseases. It was initially suggested that curcumin acts mainly through the nuclear factor erythroid-2 related factor 2 (Nrf2) signaling pathway.10 The transcription factor is thought to play its anti-inflammatory role in part by regulating NF-xB pathway, a crucial mediator of inflammation. Studies have shown that Nrf2 competes against NF-κB for transcriptional coactivator CREB-binding protein.21 Another possible mechanism by which curcumin blocks proinflammatory pathways is binding to TNF directly and covering its site of action.22 One study has suggested that curcumin suppresses the MAP kinases, namely Erk and JNK, which phosphorylate IkBα for subsequent degradation.23 When IkBα is degraded, NF-xB can translocate...
into the nucleus. Some have suggested that curcumin inhibits phosphorylation of Akt and subsequent activation of NF-κB in dose- and time-dependent manners. Other studies have also suggested that curcumin more directly prevents NF-κB activation by inhibiting the action of IκB kinase (IKK). The results of our study confirmed previous reports that curcumin mediated its anti-inflammatory effect by modulating multiple signaling molecules. When GO fibroblasts were treated with curcumin, phosphorylation of NF-κB and Erk, JNK, and Akt was inhibited in a dose-dependent manner.

The mechanism of curcumin’s action reported thus far is also in line with our results that curcumin curtailed oxidative stress in GO fibroblasts exposed to CSE or hydrogen peroxide. Increasing curcumin concentrations appeared to yield greater protective effect against oxidative damage to cells. Curcumin may exert its antioxidant effect largely in two ways. First, by activating the Nrf2, curcumin enables the transcription factor to translocate to the nucleus and activate transcription of genes that contain an antioxidant response element in their promoter regions. The main antioxidant enzyme activated by curcumin is

![Western blot analysis](image)

**Figure 8.** Curcumin inhibits activation of proinflammatory transcription factors. Undifferentiated preadipocytes derived from GO fibroblasts were pretreated with varying concentrations of curcumin for 1 hour. The cells were then treated with 10 ng/mL IL-1β for another hour. The cell lysates were subjected to Western blot analyses. Whereas IL-1β treatment markedly increased levels of phosphorylated Erk, Akt, Jnk, and NF-κB p65 proteins, the levels decreased in cells pretreated with curcumin in a dose-dependent manner. The bands were quantified by means of densitometry and were then normalized to the level of β-actin in the same sample. In the graph, each bar represents a value obtained by dividing the density of the band for a transcription factor in a phosphorylated state by that of the band for the total amount of the same transcription factor (*P < 0.05 versus IL-1β-stimulated cells).
reported to be heme oxygenase-1 (HO-1). A study by Woo et al. showed that when human retinal endothelial cells were treated with hydrogen peroxide and curcumin, HO-1 expression increased and ROS decreased correspondingly. Second, curcumin may be a free radical scavenger on its own, owing to its chemical structure. Priyadarsini et al. have reported that curcumin donates a hydrogen mainly from its phenolic OH to free radicals and strips the unstable ROS of its damaging effect on cells. Mounting evidence indicates that oxidative stress is crucial to pathogenesis of GO. For instance, it is postulated that superoxide radicals stimulate orbital fibroblasts to proliferate and produce glycosaminoglycan and heat shock protein expression.

**Figure 9.** Curcumin activates \( \beta \)-catenin signaling pathway in GO fibroblasts to counter adipogenesis. The lysates of GO fibroblasts undergoing differentiation into adipocytes were taken at different time points and subjected to Western blot analyses. As differentiation progressed, P-GSK-3\( \beta \) (Ser9), the inactive form of the kinase, briefly increased until the third day before decreasing until day 10. When curcumin was applied to the adipogenic medium, the level of P-GSK-3\( \beta \) (Ser9) increased on average, and the level of P-GSK-3\( \beta \) (Ser9) on day 10 of adipogenesis with curcumin was significantly greater than that without curcumin (A). The treatment with curcumin increased \( \beta \)-catenin overall, as well as translocation of \( \beta \)-catenin to the nucleus, resulting in significantly increased ratios between nuclear and cytosolic \( \beta \)-catenin (B). When the signaling molecules were assessed on the 10th day of adipogenesis across different concentrations of curcumin, increasing concentrations of curcumin led to increased levels of inactive GSK-3\( \beta \) (P-GSK-3\( \beta \) [Ser9]), total \( \beta \)-catenin, active \( \beta \)-catenin (nonphosphorylated [NP]-\( \beta \)-catenin), and cyclin D1 (C).
Therapeutic Effect of Curcumin in GO

Protein 72.22,29 Our study demonstrated that curcumin may have a therapeutic effect in patients with GO by also having a protective effect against oxidative damage in GO fibroblasts.

Multiple studies have proven that curcumin inhibits adipogenesis at both cellular and animal levels.30,31 Sakuma et al. reported that curcumin significantly inhibited level of adipogenesis in murine preadipocytes treated with benzyl butyl phthalate, an endocrine disruptor known to stimulate adipogenesis.32 Other curcuminoids have also shown antiadipogenic effect in mice.33 In our study, curcumin treatment significantly reduced adipogenesis of these cells in a dose-dependent manner. In an attempt to elucidate the mechanism by which curcumin inhibits GO fibroblasts from differentiation into adipocytes under inflammatory conditions, we investigated the expression of PPARγ, C/EBPα, and C/EBPβ. They are known to be key regulators of adipocyte differentiation, ultimately causing expression of adipocyte-specific genes downstream to promote terminal differentiation into adipocytes.34

There is reportedly a positive feedback loop between these transcription factors during the terminal stages of adipocyte differentiation.35 Our results showed that the levels of these transcription factors that initially increased in response to IL-1β, decreased when they were cotreated with increasing amounts of curcumin. The study of Sakuma et al. on curcumin’s effect in benzyl butyl phthalate-treated murine cells showed suppression of PPARγ-C/EBPβ pathway similar to our study.32

Recent studies have shown that additional pathways through which curcumin exerts its antiadipogenic effect may exist. Ahn et al. have shown that suppression of adipogenic differentiation might also involve the Wnt signaling pathway in mice.35 Tian et al.36 came to a similar conclusion. Similar to previous studies, we found that curcumin treatment of GO fibroblasts undergoing adipogenesis reduced the level of active GSK-3β, which is strongly related to Wnt pathway and increased not only the overall amount of β-catenin in cells, but also nuclear fraction of the molecule for expression of effector genes of the pathway. It has also been suggested that curcumin inhibits the induction of PPARγ-C/EBPβ through its effect on β-catenin/Wnt signaling.37 Kumara et al. suggested that PI3K, a known inhibitor of GSK-3β, is essential in terminal adipocyte differentiation in GO.38 Further studies are needed to elucidate the exact mechanism by which curcumin suppresses adipogenesis in human GO fibroblasts.

Even though curcumin holds promise as a future treatment option in GO, its clinical uses have been limited by poor bioavailability. Curcumin showed poor absorption, rapid metabolism, and equally rapid elimination in animal models.39 In rats, intravenous administration of 10 mg/kg of curcumin led to a maximum serum concentration of 0.36 ± 0.05 μg/mL and oral administration of curcumin 50 times greater led to a maximum plasma concentration of 0.06 ± 0.01 μg/mL.40 Of the multiple pharmacokinetic studies on humans, the highest plasma concentration reported to date was 0.051 μg/mL after administering 12 g curcumin.41 Although initial clinical trials showed that curcumin has low toxicity (humans are able to tolerate up to 8 g curcumin/day)42 poor bioavailability remains a problem in curcumin administration. Numerous attempts have been made to address the problem. Recently, microbubble-mediated delivery was found to be promising in cervical cancer cell line of HeLa cells.43 Use of microspheres, nanocapsules, as well as nanodisks and micelles, were reported to increase serum concentration of curcumin.44,45 Structural analogues and curcumin metabolites have shown similar antiinflammatory properties in cells.46 Improvements in bioavailability have allowed clinical trials of curcumin on human subjects. Panahi et al.47 reported that in their randomized controlled trial on patients with type 2 diabetes mellitus, curcuminoids showed significantly elevated serum total antioxidant capacity. To assess whether curcumin may have similar effect in patients with GO in vivo, a protocol for a randomized controlled trial should be developed in the near future.

In conclusion, curcumin had anti-inflammatory, antioxidant, and antiangiogenic properties in GO fibroblasts in vitro. Curcumin suppressed the release of proinflammatory cytokines and inhibited activation of proinflammatory transcription factors. It also suppressed adipocyte differentiation through the PPARγ-C/EBPβ pathway, as well as the β-catenin pathway. The ingredient prevented ROS formation in GO fibroblasts challenged with H₂O₂ and CSE. Further studies are necessary to prove the effect of curcumin in GO in a clinical setting. The optimal dose and more effective means of delivering curcumin to the orbital fibroblasts in vivo also need to be investigated.

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