Antifibrotic Effects of Quercetin in Primary Orbital Fibroblasts and Orbital Fat Tissue Cultures of Graves’ Orbitopathy

Jin Sook Yoon, Min Kyung Chae, Sun Young Jang, Sang Yeul Lee, and Eun Jig Lee

PURPOSE. We investigated the effects of quercetin on fibrotic markers and matrix metalloproteinases (MMPs) in primary cells and whole orbital tissues from Graves’ orbitopathy (GO).

METHODS. Orbital fat tissues were harvested from GO for primary cell and tissue cultures during orbital fat decompression. To determine noncytotoxic dose and time of quercetin treatment, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay and LDH release assay were performed. The effects of quercetin on fibrosis were evaluated according to a scratch wound closure assay, and Western blotting for expression of fibronectin, collagen Iα, α-smooth muscle actin with or without TGF-β stimulation, and MMP-2, -7, -9, and tissue inhibitor of metalloproteinase-1 with or without IL-1β stimulation. The gelatinolytic activities of MMP-2 and MMP-9 were measured using gelatin zymography. In tissue cultures, MMP secretion and collagen Iα mRNA levels were determined by enzyme-linked immunosorbent assays and reverse transcription-polymerase chain reaction (RT-PCR), respectively.

RESULTS. Quercetin significantly inhibited cell migration at nontoxic concentrations. In primary cells, quercetin dose-dependently downregulated expression of TGF-β-stimulated fibronectin and collagen Iα, and IL-1β-enhanced MMP-2 and MMP-9. However, without IL-1β stimulation, 10-50 μM of quercetin increased MMP-2 expression and activity, but dose-dependently suppressed MMP-9 expression and activity. In tissue cultures, quercetin dose-dependently inhibited MMP-2 and -9 activity and secretion, but 30 and 50 μM of quercetin increased tissue MMP-2 mRNA. MMP-9 and collagen Iα mRNA levels were dose-dependently suppressed.

CONCLUSIONS. Quercetin inhibited fibrotic markers and affected MMP-2 and MMP-9 activities in primary cell and orbital fat tissue cultures from GO at nontoxic concentrations. Our results support the potential use of quercetin for active inflammation and treatment or prevention of chronic fibrosis in GO. (Invest Ophthalmol Vis Sci. 2012;53:5921–5929) DOI: 10.1167/iovs.12-9646

Graves’ disease is an autoimmune disease of the thyroid gland in which autoantibodies bind to the thyrotropin receptor on thyroid follicular cells, activating gland function and leading to excessive production of thyroid hormones. Up to 50% of Graves’ disease patients develop pathological manifestations in the eye, known as Graves’ orbitopathy (GO). Increased connective/fatty tissue within the bony orbits is responsible for most orbital complications in patients with severe active GO. These cells are primarily CD4+ T cells, but there are also minor populations of CD8+ cells, B cells, and macrophages. Current evidence points to orbital fibroblasts as the target cells in GO, which secrete large amounts of hyaluronan in response to various cytokines. A subgroup of orbital fibroblasts can differentiate into mature adipocytes. Fibrosis is a consequence of the repair of damaged tissue, with various causes such as trauma, infection, allergy, and inflammation. This can result from situations persisting for several weeks or months in which inflammation, tissue destruction, and repair occur simultaneously. Eyelid retraction, restrictive myopathy, and longstanding proptosis associated with GO are all believed to be caused first by inflammation of Muller’s muscle, the extraocular muscle, and orbital connective adipose tissues, but these events can be followed by chronic fibrosis in connection with wound healing. This is associated with excessive deposition of extracellular matrix (ECM) components, including collagen and fibronectin. Recently in 2011, Zhao et al. reported that in a mouse model of Graves’ disease, induced by genetic immunization of thyrotropin receptor A-subunit using intramuscular injection combined with electroporation, histological analysis of the orbital tissues demonstrated moderate connective tissue fibrosis and deposition of Masson’s trichrome-staining material. The electroporation technique could result in enhanced transfection efficiency and generate long lasting immunity.

Matrix metalloproteinases (MMPs) are enzymes with proteolytic activities toward several ECM proteins such as collagens, proteoglycans, elastin, laminin, fibronectin, and other glycoproteins, and are involved in tissue remodeling during fibrotic and inflammatory processes. The activities of most matrixins are very low or negligible in the normal state tissues, but expression can be increased by inflammatory cytokines, growth factors, hormones, and cell-cell interaction. Tissue inhibitors of metalloproteinases (TIMPs) are...
controlled during tissue remodeling and physiologic conditions to maintain a balance in ECM metabolism.11,12

Only a few studies have reported the association of MMPs and TIMPs in GO. Han et al. reported that TIMP-1 was induced by interleukin (IL-1) in GO orbital fibroblasts, with gene promoter activity modulated by IL-1 and interferon (IFN)-γ.13 Kim et al. reported that pirfenidone, a novel agent that has shown antifibrotic properties in animal models and clinical trials in tissues such as lung, liver, and kidney, inhibited IL-1β-induced increases in TIMP-1 and decreased hydroxyproline levels in orbital fibroblasts.14 In another report, soluble serum levels of MMP-9 were significantly higher in GO patients compared with both Graves’ disease and control individuals, and decreased significantly after steroid administration; but serum MMP-2 levels were not different between GO and normal individuals and were unaffected by steroid treatment.17

Glucocorticoids have been used for decades and are still the first-line treatment for GO; however, glucocorticoids are primarily effective in active inflammatory states and have a limited role in treating fibrosis of extraocular muscles or orbital fatty connective tissues.2,18 Few effective medical treatments are available, once the fibrotic change develops. Furthermore, their long-term side effects, including cushingoid features, diabetes, hypertension, and osteoporosis, are drawbacks.

We recently reported that quercetin (3,3,4,5,7-pentahydroxy flavanone), a flavonoid phytoestrogen found abundantly in soybeans, vegetables, and fruits, significantly reduces diabetes, hypertension, and osteoporosis, are drawbacks.30,31 Quercetin has been shown to possess antifibrotic properties in hepatic fibrosis in rats25,26 and kidney fibroblasts,27 and it suppressed collagen production, correlated with heme oxygenase-1 induction, in lung fibroblasts.28

Here, we investigated the antifibrotic effects of quercetin in an in vitro culture system of GO and demonstrated suppressive effects of quercetin on the production of fibrotic markers, including fibronectin and collagen. We also assessed the effects of quercetin on MMP and TIMP protein production and activity in cultured primary orbital fibroblasts and whole orbital tissues.

**MATERIALS AND METHODS**

**Cell and Tissue Culture Protocols**

Orbital adipose/connective tissue explants were obtained from 13 GO individuals undergoing surgical decompression for severe proptosis associated with increased orbital fat volume (Table 1). All Mourits’ clinical activity scores were below 4. All patients were euthyroid at the time of surgery and had not been treated with steroids or radiation for at least 3 months. Normal control fat tissues were harvested during upper lid blepharoplasties from three individuals with no history of GO or autoimmune thyroid disease. Mean age was 56 (range, 51–63) and all were female.

The protocol for obtaining orbital adipose/connective tissue was approved by the Institutional Review Board of Severance Hospital. Written informed consent was obtained from all patients.

For primary cell culture, tissue explants were minced and placed directly in plastic culture dishes in Dulbecco’s modified Eagle’s medium (DMEM) containing 20% fetal bovine serum (FBS), penicillin (100 U/mL), and gentamicin (20 μg/mL) from Hyclone Laboratories (Logan, UT), allowing preadipocyte fibroblasts to proliferate. After fibroblasts had grown out from the explants, monolayers were passaged by gentle treatment with trypsin/EDTA, and cultures were maintained in 80-mm flasks containing DMEM with 10% FBS and antibiotics. Cell cultures were grown in a humidified 5% CO₂ incubator at 37°C. Cells were stored in liquid N₂ until needed and used between the third and seventh passages.

For orbital fatty tissue cultures, tissue explants were cut into several equal pieces and cultivated in DMEM containing 10% FBS and antibiotics with quercetin (Sigma-Aldrich, St. Louis, MO) at various concentrations for 48 hours.29 Then, the medium was removed and fresh medium containing the same therapeutics was added for another 24 or 48 hours. The cultured tissues were dried on filtration paper, snap-frozen, and weighed (mean, 384.1 mg; range 128.5–450.2 mg). Supernatants from the cultures were collected and subjected to LDH assays, ELISAs for MMP protein levels, and gelatin zymography for MMP activities. Frozen tissues were later evaluated for mRNA levels of MMPs, TIMP-1, and collagen Iα by real-time reverse transcriptase-polymerase chain reaction (RT-PCR).

**MTT Analysis**

To evaluate the effect of quercetin on cell viability, orbital fibroblasts of GO patients were seeded into 24-well culture plates (1 × 10³ cells/well) and treated with different concentrations of quercetin (10, 30, 50, 100, and 200 μM) for 24, 48, 72, and 96 hours. After treatment, cells were washed; incubated with 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution for 4 hours at 37°C; then solubilized in ice-cold isopropanol and analyzed spectrophotometrically. An MTT assay kit was purchased from Sigma-Aldrich, Inc (St. Louis, MO). Absorbance of the dye was measured at 560 nm, with background subtraction at 630 nm, with a microplate reader (EL 340 Biokinetics Reader; Bio-Tek Instruments, Winooski, VT).

**LDH Assay**

The cytotoxicity of quercetin in both cell and tissue cultures was assessed using the LDH leakage assay. Orbital fibroblasts were incubated in medium containing increasing concentrations of quercetin (10, 30, 50, 100, and 200 μM) for various times (6, 24, 48, and 72 hours), and the medium was transferred to 1.5-ML microcentrifuge tubes and centrifuged (12,000g, 4°C, 15 minutes) to remove any cell debris. Orbital fat tissues were also cultured in medium containing quercetin at increasing concentrations (10, 30, 50, 100, and 200 μM) for various times (48, 72, 96 hours), and cell-free culture supernatants were collected in a same manner. Then, 100 μL of each sample was added to the substrate solution and the absorbance was measured at 490 nm by using a microplate reader (VersaMax; Molecular Devices, Sunnyvale, CA). LDH activity was obtained by measuring the decreasing rate of NADH absorbance over time (slope).

**Wound Healing Assay**

Confluent cell monolayers in a 6-well plate were wounded by mechanical scraping—a straight scratch with a pipette tip, simulating
a wound. Wound width was assessed at the time of scraping to ensure that all wounds were the same width at the start of the experiments. The cell culture medium was then replaced with fresh medium, with or without quercetin (50, 100 μM for 24 hours), and wound closure was observed by phase-contrast microscopy (Nikon, 40x) and photographed. The results are expressed as the percentage of the wound width at the start of the experiment.

**Western Blot**

Cells were washed with ice-cold PBS and lysed on ice for 30 minutes in cell lysis buffer consisting of 20 mM HEPES (pH 7.2), 10% (vol/vol) glycerol, 10 mM Na2VO4, 50 mM NaF; 1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1% (vol/vol Triton X-100). Reagents were obtained from Sigma-Aldrich. Lysates were centrifuged (12,000g, 10 minutes) and cell homogenate fractions were stored at −70°C until used. Protein concentrations in supernatant fractions were determined using the Bradford assay (BioRad, Hercules, CA). Equal amounts of protein (50 μg) were boiled in sample buffer and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis in 10% (wt/vol) gels. Separated proteins were transferred to nitrocellulose membranes (Pall Life Sciences, East Hills, NY); incubated overnight with primary antibodies (antifibronectin antibody [Abcam, Cambridge, UK]; anti-collagen I antibody [Pierce, Rockford, IL]; anti-α-SMA antibody [Dako Corporation, Carpinteria, CA]; anti-MMP-2, anti-MMP-9 antibody [Cell Signaling Technology, Beverly, MA]; anti-MMP-7, and anti-TIMP-1 antibody [Abcam]); and washed three times with Tris-buffered saline-Tween 20. Immunoreactive bands were detected with horseradish peroxidase-conjugated secondary antibody, and the bound peroxidase was visualized using an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ) and exposure to X-ray film (Amersham Pharmacia Biotech, Piscataway, NJ). The relative amount of each immunoreactive band was quantified by densitometry and normalized to the β-actin level in the same sample.

**Gelatin Zymography**

Gelatin zymography was performed to detect MMP activities in quercetin-treated primary cell and tissue cultures using the method of Ambili et al.30 Gelatinase degrades the gelatin substrate, which is impregnated in the gel; thus, clear bands appear against the dark background of Coomassie blue staining. Gelatin was added to visualize the gelatinolytic activities of MMPs. Samples were run on a Tris-glycine SDS-10% polyacrylamide gel. After electrophoresis and a 20-minute incubation with gelatinase, gels were washed in 50 mM Tris-HCl (pH 7.5, 5 mM CaCl2). The gel was then stained using Coomassie blue and destained in water.

**Measurement of Secreted MMP-2, MMP-7, and MMP-9 Proteins by ELISA**

After incubation with various concentrations of quercetin for 48 hours, supernatants from tissue cultures were collected. MMP-2, MMP-7, and MMP-9 were measured using a commercially available ELISA kit (Milliplex MPA kit; Millipore, Billerica, MA) according to the manufacturer’s instructions. Values were expressed as ng/ml per gram of tissue weight for MMP-2 and pg/ml per gram of tissue weight for MMP-7 and MMP-9.31 Absorbance of reaction was measured at 405 nm, and the percentage of binding was calculated for each sample. The concentration in the sample was determined using a standard binding curve generated with known amounts of MMPs. Samples were diluted 1:10 before analysis, and the average of five assays were determined.

**Quantitative Real-Time Polymerase Chain Reaction**

Total RNA (1 μg) was isolated and reverse-transcribed into complementary DNA according to the kit manufacturer’s protocol. The resulting cDNA was amplified using a thermocycler (ABI 7500 Real-Time PCR; Applied Biosystems, Carlsbad, CA) using TaqMan universal PCR master mix and the recommended PCR conditions to quantitatively assess gene transcript levels in the tissue samples. All PCRs were performed in triplicate. The catalog numbers of the primers used were Hs001548727_m1 for MMP-2, Hs01042796_m1 for MMP-7, Hs00234579_m1 for MMP-9, Hs00171558_m1 for TIMP-1, Hs00164004_m1 for collagen Iα, and H299999905_m1 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH expression was used for normalization, and the results were expressed as relative fold changes of threshold cycle (Ct) value relative to the control group using the 2^(-ΔΔCt) method.32

**Statistical Analyses**

All experiments were performed at least three times, and samples were assayed in duplicate each time. For statistical analyses of Western blot and RT-PCR results, mean values and standard deviations (SDs) were calculated for normalized measurements of each protein or mRNA from at least three samples harvested from different individuals. Difference in measured variables between experimental and control groups were assessed using Student’s t test or Wilcoxon rank sum test. Groups containing multiple comparisons were analyzed by the analysis of variance with Tukey’s multiple-comparison test, as a post hoc test. We used statistical software (SPSS version 12.0.1 for Windows; SPSS, Chicago, IL). A P value < 0.05 was considered to indicate statistical significance.

**Results**

**Viability Using MTT Analysis**

Our previous report showed that exposure of cells to quercetin at ≤100 μM for 24 hours did not decrease cell viability below 95% in neither normal or GO orbital fibroblasts in MTT analysis.19 When cells were treated with quercetin for a longer time (48, 72, and 96 hours), a significant decrease in cell viability was noted in a dose- and time-dependent manner (Fig. 1A).

**Cytotoxicity of LDH**

LDH release from cells treated with quercetin (0–200 μM) for 6, 24, and 48 hours was not significantly different from control levels in primary cell cultures (Fig. 1B). However, when cells were treated with quercetin ≥50 μM for 72 hours, a significant amount of LDH was released compared with control levels (P < 0.05; Fig. 1B). In whole tissue cultures, LDH was not significantly elevated with quercetin treatment at ≤100 μM for 72 hours or ≤200 μM for 48 hours (Fig. 1C). However, when the tissues were treated with quercetin ≥30 μM for 96 hours, significant LDH was released compared with control levels (Fig. 1C).

**Cell Migration**

The effects of quercetin on orbital fibroblast migration were assessed by a scrape wound assay. Photomicrographs taken at 24 hours after wounding showed delayed wound closure in orbital fibroblast cultures treated with quercetin at 50 and 100 μM, compared with untreated control cultures (Fig. 2A). Quantitation of the wound closure area over time revealed a significant inhibitory effect of quercetin on orbital fibroblast motility at both 50 and 100 μM (P < 0.05; Fig. 2B).
Effect of Quercetin on Fibronectin and Collagen Iα in Primary Cell Cultures

First, α-SMA, fibronectin, and collagen Iα protein expression were evaluated in both normal (n = 3) and Graves’ (n = 2) orbital fibroblasts. α-SMA expression was virtually undetectable in all three normal orbital fibroblasts from different donors, but expressed in all two Graves’ orbital fibroblasts (see Supplementary Material and Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-9646/-/DCSupplemental). Fibronectin and collagen Iα protein were detected by Western blot in all normal fibroblasts, but the protein amounts were lesser than in Graves’ fibroblasts.

Quercetin significantly suppressed TGF-β–stimulated fibronectin protein levels dose-dependently in Graves’ orbital fibroblasts (Figs. 3A, 3B). Quercetin downregulated the expression of collagen Iα protein stimulated with TGF-β or not in a dose-dependent manner (Figs. 3A, 3C). Neither quercetin nor TGF-β affected α-SMA protein production (Fig. 3A).

Effects of Quercetin on the Expression of MMP-2, MMP-7, MMP-9, and TIMP-1 Proteins in Primary Cell Cultures

In orbital fibroblast cell cultures, without IL-1β stimulation, MMP-2 protein production was increased by treatment with 10 to 50 μM of quercetin, compared with control cells, but decreased with 100 μM quercetin treatment, whereas quercetin significantly suppressed the expression of MMP-9 protein dose-dependently (Fig. 4A). IL-1β enhanced the expression of MMP-2 and MMP-9 proteins, which quercetin suppressed dose-dependently (Figs. 4B, 4C). TIMP-1 and MMP-7 protein expression was unaffected by quercetin (Fig. 4A).
Antifibrotic Effects of Quercetin in GO

Effects of Quercetin on the Gelatinolytic Activity of MMP-2 and MMP-9 in Primary Cell and Tissue Cultures

In primary cultured orbital fibroblasts, the major band was 84-kDa active MMP-9, and the 67-kDa active MMP-2 band was visible, but weaker than those in whole orbital tissue cultures (Fig. 7A). The 92-kDa proMMP-9 and 72-kDa proMMP-2 bands were barely seen in primary cultured cells (Fig. 7A). In tissue cultures, 84-kDa active MMP-9, 72-kDa proMMP-2, and 67-kDa active MMP-2 bands were clearly detected, although the 92-kDa proMMP-9 band was not (Fig. 7B).

In primary cell cultures, 10 to 50 μM of quercetin increased the activity of MMP-2, but 100 μM quercetin decreased MMP-2 activity. Quercetin significantly suppressed MMP-9 activity, dose-dependently, in gelatin zymography (Figs. 7A, 7C), similar to the data from the Western blot analysis in primary cell cultures (Fig. 4A). In tissue cultures, activities of MMP-2 and MMP-9 in gelatin zymography were inhibited by quercetin at 50 and 100 μM (Figs. 7B, 7D).

**DISCUSSION**

We previously reported that quercetin can block aspects of the in vitro presentation of inflammation, aberrant accumulation of ECM, and adipose tissue expansion in primary cultured orbital fibroblasts from GO, stimulated with proinflammatory cytokines. Here, we demonstrate antifibrogenic properties of quercetin in both primary cultured orbital fibroblasts and cultured whole orbital tissues. The cytotoxicity of quercetin in our study was minimal in both orbital fibroblasts and tissue cultures, according to the MTT and LDH assay, suggesting that the antifibrotic effects of quercetin occurred in a nontoxic manner.

The fibrotic potential of fibroblasts is characterized by their capability to synthesize ECM compounds, like members of the collagen family and the cell surface-ECM linking protein fibronectin, that act as fibrosis marker proteins in vivo. Recently, different fibroblast subpopulations of the eye, including orbital fibroblasts responsible for fibrosis process associated with trabeculectomy, were characterized. TGF-β1 as well as collagen I, III, and IV and fibronectin proteins were all detected in normal human orbital fibroblasts. Similarly in our study, in all three normal orbital fibroblasts, collagen I and fibronectin were constitutively expressed, albeit in lower amount than in Graves’ fibroblast. Interestingly, in our repeated experiments, α-SMA was constitutively expressed in all Graves’ orbital fibroblasts, even in non-TGF-β-treated conditions, but not in all normal fibroblasts. α-SMA is a characteristic actin isoform expressed by myofibroblasts, a key effector cell in fibrinogenesis. Myofibroblasts localize to fibrotic foci and other sites of active fibrosis, and are the primary cell type responsible for synthesis and deposition of ECM and the resultant structural remodeling. Graves’ adipose tissues are harvested during orbital fat decompression surgery and 3–6 mL of orbital tissues per one orbit are surgically wasted during the surgery. They include not only fat lobules, but also collagenous connective septae surrounding the lobules. We assume that cultured Graves’ orbital fibroblasts could have coexisted with already transdifferentiated myofibroblasts stimulated by increased TGF-β within the orbit. In the report by Kounas et al., α-SMA was not expressed in both Thy-1+ and Thy-1– subsets of orbital fibroblasts from GO. These seemingly contradictory results might be due to heterogeneity of fibroblasts due to different fibrotic potential, reflecting individual patients’ clinical characteristics.
MMPs play a central role in ECM remodeling and are involved in a variety of pathogenic processes, such as inflammation and fibrosis. The clinical relevance of these MMPs, in particular MMP-2 and MMP-9, has been highlighted by the fact that increased hepatic gelatinase levels are associated with fibrosis in chronic liver disease. MMP involvement in pathology involves more than simple excess matrix degradation, or an imbalance between them and their specific TIMPs.

Gelatinase A (MMP-2) and gelatinase B (MMP-9) are also known as 72-kDa gelatinase/type IV collagenase and 92-kDa gelatinase/type IV collagenase, respectively. MMP-2 has the ability to hydrolyze gelatins, types I, IV, V, VII, and XI collagens, fibronectin, laminin, large tenasin-C, aggrecans, and elastin. MMP-9 has the ability to hydrolyze gelatins, types III, IV, V, and XIV collagens, aggrecans, elastin, and entactin. These protease activities can be detected by gelatin zymography; and several bands produced by the proteases can be detected by SDS-PAGE. The proteases recognized by the polyclonal antibodies against MMP-2 and MMP-9 were the 92- and 67-kDa forms of MMP-2 and MMP-9, respectively. Also, 72- and 67-kDa bands, representing pro-MMP-2 and MMP-2, respectively, and 92- and 84-kDa bands, representing pro-MMP-9 and MMP-9, respectively, were evident.

Interestingly, in our study, only active MMP-2 and MMP-9 bands were detected in cultured primary cells, whereas the pro-MMP-2 band was extremely strong, along with active MMP-2 and active MMP-9 bands, in orbital tissue cultures. The bands detected in tissue cultures were far stronger than those detected in cell cultures. Active MMP-2 or MMP-9 to TIMP-1 ratios may be changed by quercetin treatment and may be involved in the antifibrotic mechanism in GO, because high-dose quercetin suppressed MMP-2 and MMP-9 activity, but quercetin did not affect TIMP-1.

It has previously been reported that TIMP-1 is expressed highly in IL-1β-activated orbital fibroblasts and that those cells from patients with severe GO exhibited particularly robust induction. Kook et al. also reported that TIMP-1 was enhanced by IL-1β stimulation. However, TIMP-1 expression was not altered by IL-1β (10 ng/mL, 24 or 48 hours) or quercetin treatment in our repeated experiments using GO cells from patients with severe GO.

Table 2. Effects of Quercetin on MMP-2, -7, and -9 Protein Secretions by Whole Orbital Tissue Cultures

<table>
<thead>
<tr>
<th></th>
<th>MMP-2*</th>
<th>MMP-7†</th>
<th>MMP-9†</th>
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<tbody>
<tr>
<td>Control</td>
<td>955.8</td>
<td>1330.1</td>
<td>5183.6</td>
</tr>
<tr>
<td>Q, 100 μM (Q100)</td>
<td>590.9</td>
<td>1128.2</td>
<td>3282.2</td>
</tr>
<tr>
<td>Q, 200 μM (Q200)</td>
<td>338.0</td>
<td>1054.0</td>
<td>1313.6</td>
</tr>
<tr>
<td>*P value</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control, Q100</td>
<td>0.044</td>
<td>0.077</td>
<td>0.013</td>
</tr>
<tr>
<td>Control, Q200</td>
<td>0.034</td>
<td>0.080</td>
<td>0.007</td>
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</tbody>
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Data are presented as mean (minimum–maximum range). The paired Wilcoxon rank sum test was used for statistical comparisons. Q, quercetin.

* ng/mL/g of tissue weight.
† pg/mL/g of tissue weight.
several different human tissue samples in this study. This different result of our study might also be caused by different fibrotic potentials of fibroblasts cultured from clinically heterogeneous GO patients.

The pathological mechanism of the decrease in fibrotic markers and the influence on MMP-2 and MMP-9 protein production and activity in GO orbital fibroblasts or tissues was not clearly defined in this study. Throughout the study, we monitored cell attachment in primary cultures of orbital fibroblasts to assess the credibility of our assays. However, we cannot exclude the possibility of the compound playing an antifibrotic role by inhibiting upstream of TGF-β or IL-1β-induced fibrogenesis, or by directly inhibiting factors inducing fibrogenesis, such as inflammation, because the anti-inflammatory effects of quercetin are well known and have been demonstrated in in vitro-cultured GO cells, as we have reported.19,31 As infiltration and cytokine secretion of inflammatory cells can activate fibroblasts followed by proliferation, inhibition of inflammatory processes indirectly controls fibrotic processes.39 There is a necessity for antifibrotic treatment for fibrotic status of GO, as few effective medical treatments are available once the fibrotic change develops. However, we do not know whether quercetin is capable of reversing clinically evident fibrosis of GO in humans, after fulminant inflammation has already developed and declined. Steensel et al. recently introduced a whole orbital tissue culture system in GO research to test a therapeutic effect of a compound in vitro.29 We recently reported that IL-1β, IL-6, and TNFα protein levels were significantly higher in supernatants of GO tissue cultures than normal controls.31 In gelatin
Figure 7. Effects of quercetin on MMP-2 and MMP-9 activity by gelatin zymography. Confluent orbital fibroblasts from GO (n = 3) and harvested orbital tissue explants (n = 3) were treated or not with quercetin (10, 30, 50, and 100 μM) for 24 and 72 hours, respectively, for gelatin zymography of MMP-2 and MMP-9. (A) Representative figures of MMP-2 and MMP-9 in primary cell cultures. (B) Representative figures of MMP-2 and MMP-9 in whole tissue cultures. In primary cultured orbital fibroblasts, a major band was the 84-kDa active MMP-9 and the 67-kDa active MMP-2 band was visible. In tissue culture, 84-kDa active MMP-9, 72-kDa pro-MMP-2, and 67-kDa active MMP-2 bands were clearly detected. (C) The bar diagram shows the densitometric values corresponding to the zymography in orbital fibroblasts (D) Densitometric values corresponding to the zymography in whole tissue cultures. The data in the column are the mean relative density ratios ± SD of three experiments and differences between control and quercetin-treated cells are indicated. *P < 0.05.

zymography in this study, MMP-2 and MMP-9 activities were much stronger in whole orbital tissue cultures than in cell cultures. The in vitro whole orbital tissue culture model seems to be promising, because it may better reflect the clinical environment than a cell culture system. This model would be very helpful to test therapeutics than single cell systems, especially in rare immune-mediated diseases in which conducting randomized controlled trials is extremely difficult and a suitable animal model is lacking.

A recent report by Lisi et al. showed that proliferation of orbital fibroblasts was suppressed by continuous exposure to quercetin (75 μM for 3 days or 30 μM for 5 days) via necrosis and cell cycle blockade. In our study, quercetin ≤100 μM was treated in cell cultures for 24 hours or less, whereas Lisi et al. treated quercetin for at least 3 days, which significantly increased LDH release in a dose-dependent manner. We similarly found that treatment with quercetin ≥50 μM for 72 hours in cell cultures significantly increased LDH release compared with control levels. In whole tissue cultures, as significant LDH was released in tissue cultures treated with quercetin ≥30 μM for 96 hours compared with control levels, tissue cultures were treated with quercetin for 48 hours (≤200 μM) or 72 hours (≤100 μM) to avoid cytocytotoxicity.

We did not compare the effect of quercetin on fibrotic markers between normal and GO cells or tissue cultures. The reason was because in our experience, GO and normal orbital fibroblasts responded similarly to stress condition such as IL-1β treatment or quercetin treatment, as previously reported. Expression of IL-6 and IL-8 mRNA was strongly upregulated by IL-1β in both GO and normal cells and suppressed by quercetin similarly. Adipogenesis induced by adipogenic stimuli and the cell viability affected by quercetin treatment were all similar in GO and normal cultures. Also in the report by Lisi et al., quercetin reduced cell proliferation in both GO and control fibroblasts, with no difference between the two groups. We recently reported that quercetin showed anti-inflammatory action in ex vivo GO orbital tissue cultures. IL-1β, IL-6, and TNFα levels were significantly higher in GO than normal tissue controls, suggesting that ex vivo tissue cultures seem to mimic the clinical environment better than single cell culture. However, one weakness of whole orbital tissue culture is that a large volume of fat tissue is required, and it is not easy to find age-matched normal participants to harvest enough normal fat tissue for ex vivo cultures, whereas Graves’ orbital fat tissues were easily harvested as a surgical waste from orbital fat decompression surgery.

Many questions regarding flavonoids remain to be investigated. Quercetin 3-O-β-D-glucuronide and quercetin-3'-sulfate are known to be the major quercetin conjugates in human plasma, in which the aglycone cannot be detected. Although most in vitro pharmacological studies have been carried out using only the quercetin aglycone form, experiments with the in vivo modified forms of quercetin are important in examining the efficacy of orally administered flavonoids, including quercetin. Additionally, the administration of quercetin has been hampered by its extreme water insolubility, although several reports have demonstrated that a potential solution to this problem is to encapsulate quercetin in liposomes. Above all, it remains unknown whether quercetin may contribute to the clinical benefits seen in the epidemiological setting. However, we believe the results of our studies on quercetin are noteworthy in that these new data lend rationale for future clinical studies to prove the possibility of the usefulness of this compound in GO treatment as an antifibrotic agent. To date, there is no effective and safe strategy for the treatment or prevention of orbital fibrosis caused by GO in clinical practice. Our study results may provide insights into the potential nutritional treatment of the chronic fibrotic stage of GO. Quercetin, one of the most abundant flavonoids in the human diet, showed dose-dependent antifibrotic activity and relatively low cytotoxicity.

Quercetin has previously been reported to be antifibrotic in liver, lung, and kidney in vitro and in vivo. It seems reasonable to propose an increased intake of fruits and/or vegetables rich in quercetin for patients with restrictive myopathy or chronic proptosis caused by GO to prevent the onset and progression of fibrosis.

References


