Anti-inflammatory effect of quercetin in a whole orbital tissue culture of Graves’ orbitopathy

Jin Sook Yoon,1 Min Kyung Chae,1 Sang Yeul Lee,1 Eun Jig Lee2,3

ABSTRACT

**Purpose** The authors previously reported that quercetin significantly inhibits interleukin (IL)-1β-induced increases of proinflammatory cytokines in cultured primary orbital fibroblasts from Graves’ orbitopathy (GO). This study investigated the inhibitory effect of quercetin on inflammation in cultured whole orbital tissue.

**Methods** Orbital fat tissues from GO and normal samples were cultured with or without non-toxic concentrations of quercetin. Lactate dehydrogenase (LDH) release was used to identify non-toxic concentrations of quercetin. Lactate dehydrogenase (LDH) release was used to identify non-toxic concentrations of quercetin. IL-6, IL-8, IL-1α, IL-1β and tumour necrosis factor alpha (TNFα) proteins were measured in tissue culture supernatants by ELISA, and gene transcript levels were determined using quantitative PCR, expressed as relative fold changes of threshold cycle value relative to the control group.

**Results** The maximal non-cytotoxic treatment of quercetin was 100 μM for 72 h, based on the considerably low LDH release with these conditions. IL-1β, IL-6 and TNFα protein levels corrected for tissue weight were significantly higher in supernatants of GO samples than normal controls (p < 0.05). Quercetin reduced IL-6, IL-8 and TNFα protein production in supernatants of all GO samples (n = 4) in a dose-dependent manner; however, only the reduction in IL-6 was statistically significant (p < 0.05). Quercetin had a significant suppression of tissue IL-6, IL-8, IL-1β and TNFα mRNA expression in cultured orbital tissues from three GO samples relative to untreated control tissue (p < 0.05).

**Conclusions** Inhibition of proinflammatory cytokines by the natural product quercetin in both primary orbital fibroblasts and tissue culture provides the basis for its potential use as an anti-inflammatory agent in the treatment of GO.

Graves’ disease is an autoimmune disease of the thyroid gland in which autoantibodies bind to the thyrotropin receptor on thyroid follicular cells, thereby activating gland function and leading to excess production of thyroid hormones. Up to 50% of patients with Graves’ disease develop pathological manifestations in the eye, known as Graves’ orbitopathy (GO).1–3 The most common features of GO include upper eyelid retraction, oedema, erythema of periocular tissues and proptosis. Between 3% and 5% of individuals with GO have intense pain and inflammation, diplopia and vision-threatening compressive optic neuropathy.

An increase in connective and fat tissue within the bony orbits is responsible for most orbital complications in patients with severe active GO.4 Tissue expansion is characterised by marked infiltration of immunocompetent cells, mainly T and B lymphocytes and mast cells. Glucocorticoids have been used for decades and are still the first-line treatment because of their anti-inflammatory and immunosuppressive actions.5,6 However, the drawback of glucocorticoid therapy is long-term side effects, including cushingoid features, diabetes, hypertension and osteoporosis. Because no reliable, specific and safe medical therapeutic agents are available for GO, the development of specific therapies with minimal side effects is essential.

We recently reported that quercetin (3,3′,4′,5,7-pentahydroxy flavanone), a flavonoid phytoestrogen, which is abundant in soybeans, vegetables and fruits, significantly reduces inflammation, hyaluronan production and adipogenesis in primary cultured orbital fibroblasts.6 Quercetin possesses antioxidant, anti-inflammatory and anti-adipogenic properties in other cell systems and animal models.7–11 van Steenel et al.12 recently introduced a whole orbital tissue culture system in GO research to demonstrate the therapeutic effect of imatinib mesylate and adalimumab in an in-vitro system. To the best of our knowledge, reproducible ocular changes resembling those of GO have yet to be reported in animal models, perhaps partly due to differences in orbital anatomy between humans and rodents.3,13 In addition, GO is included among autoimmune-mediated systemic diseases in which the achievement of randomised controlled trials to test new therapeutics is extremely difficult. Therefore, the in-vitro whole orbital tissue culture model seems to be very promising, as it may mimic better the clinical environment than single cell culture. The aim of this study was to confirm the anti-inflammatory effect of quercetin in the whole orbital tissue culture system.

**MATERIALS AND METHODS**

**Reagents**

Quercetin (Q0125) was purchased from Sigma-Aldrich, Inc. (St Louis, Missouri, USA). Dulbecco’s modified Eagle’s medium, fetal bovine serum, penicillin and gentamycin were purchased from Hyclone Laboratories, Inc. (Logan, Utah, USA). ELISA kits for interleukin (IL)-6, IL-8, IL-1α and IL-1β were purchased from Milliplex Map (Millipore Corp., Billerica, Massachusetts, USA).

**Patients**

Orbital adipose/connective tissue explants were obtained from six GO individuals undergoing surgical decompression for severe proptosis associated with increased orbital fat volume, and tissue from four control subjects with no history of GO or...
autoimmune thyroid disease was obtained in the course of age of upper or lower eyelid blepharoplasty. Mourits’ clinical activity scores were all less than 4.14 All patients were euthyroid at the time of surgery and had not been treated with steroids or radiation for at least 3 months. The protocol for obtaining orbital adipose/connective tissue was approved by the Institutional Review Board of Severance Hospital, and written informed consent was obtained from all patients.

Orbital tissue culture
Tissue explants were cut into several equal pieces and cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, antibiotics and various quercetin concentrations for 48 h. The medium was then removed and fresh medium containing the same additives was added for another 24 h or 48 h.12 The cultured tissues were subsequently dried on filter paper, snap-frozen and weighed (mean 490.1±123.3 mg, range 204.2–591.5 mg). Supernatants from the cultures were collected for the lactate dehydrogenase (LDH) assay and multicytokine ELISA. Frozen tissues were later used to evaluate IL-6, IL-8, TNFα, IL-1α and IL-1β messenger RNA by real-time reverse transcription (RT)–PCR.

LDH assay
The cytotoxicity of quercetin in the tissue culture was determined using the LDH leakage assay. To prepare for the LDH assay, orbital fat tissues were cultured with media with varying quercetin concentrations (0–200 μM) for 48, 72, or 96 h (media was changed after 48 h). The medium was transferred to a 1.5-ml microcentrifuge tube and centrifuged at 12 000g and 4°C for 15 min to remove cell debris. One hundred microlitres of each sample was added to the substrate solution and the absorbance at 490 nm was measured using a microplate reader (VERSAmax; Molecular Devices, Sunnyvale, California, USA). The LDH activity of the samples was obtained by measuring the decreasing rate of nicotinamide adenine dinucleotide absorbance over time (slopes), and therefore all the slopes obtained were negative.

Multicytokine ELISA
After incubation with various concentrations of quercetin, supernatants from the cultures were collected, and levels of the cytokines IL-6, IL-8, TNFα, IL-1α and IL-1β were determined using a competitive binding ELISA kit according to the manufacturer’s instructions. Values were expressed as pg/ml per gram of tissue weight. Absorbance of reactions 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 cytokines. Samples were diluted 1:10 before analysis, and the average of triplicate assays was determined.

Real-time quantitative RT–PCR
Total RNA (1 μg) was isolated and reverse transcribed into complementary DNA according to the manufacturer’s instructions. The resulting cDNA was amplified on the ABI 7500 real-time PCR thermocycler (Applied Biosystems, Carlsbad, California, USA) using TaqMan universal PCR master mix and recommended PCR conditions to assess gene transcript levels quantitatively in the tissue samples. All PCR were performed in triplicate. The catalogue numbers of primers were Hs00928563_m1 for IL-6, Hs00174103_m1 for IL-8, Hs00174128_m1 for TNFα, Hs00174092_m1 for IL-1α, Hs01555410_m1 for IL-1β and H299999905_m1 for glyceraldehyde-3-phosphate dehydrogenase. All samples were normalised to the values of glyceraldehyde-3-phosphate dehydrogenase and the results expressed as relative fold changes of threshold cycle (Ct) value relative to the control group using the 2−ΔΔCt method.15 Each cytokine was considered for relative quantification and data analysis only if the Ct was less than 35. If the Ct was greater than 35, the cytokine was considered to be present in minimal quantities, but relative quantification in terms of fold change in expression was not performed. In the comparison of GO and normal samples, results from three GO samples were compared with the mean results from the two normal samples.16

Statistical analysis
The effect of quercetin on cytokine levels in culture supernatants and tissue mRNA levels was analysed using the repeated measures analysis of variance and post-hoc Bonferroni method to find differences between control and drug-treated samples using the SPSS program for Windows, V.12.0.1. A p value of less than 0.05 was considered statistically significant.

RESULTS
LDH assay
Quercetin concentrations of 0–200 μM for 48 h in tissue cultures were not cytotoxic based on the LDH assay (figure 1). When tissues were cultured in media with quercetin for 72 h (media was changed at 48 h), LDH release was not significant until 100 μM quercetin was added. LDH release was significantly higher than the control after treatment with 30 μM quercetin for 96 h. A concentration of 100 μM quercetin for 72 h was chosen for subsequent studies because it was the maximal treatment that was not cytotoxic to tissue cultures (figure 1).

Effect of quercetin on proinflammatory cytokines
IL-1β, IL-6 and TNFα protein levels corrected for tissue weight were significantly higher in supernatants of GO samples than normal controls (p=0.048, 0.025 and 0.052, respectively). IL-1α and IL-8 were also higher in GO samples than normal controls.

Figure 1 Effect of quercetin on lactate dehydrogenase (LDH) release. Harvested orbital fat tissues from Graves’ orbitopathy were cut into several equal pieces and cultured in conditioned media including increasing doses (0–200 μM) quercetin (Q) and treatment times (48, 72 and 96 h). Media was changed every 48 h. After treatment, toxicity was determined by measuring LDH release. Assays were performed at least in triplicate. Results are expressed as the percentage of untreated control values and presented as mean ± SD. Differences between treated and untreated tissues (∗p<0.05) are indicated.
and IL-1α and IL-1β protein levels in the media of normal samples were mostly undetectable. IL-6 and IL-8 protein levels were more than 10,000 times higher than the levels of IL-1 in both GO and normal tissues, respectively. TNFα protein levels were higher than IL-1α and IL-1β levels, but much lower than IL-6 and IL-8 levels.

Quercetin reduced IL-6, IL-8 and TNFα protein production in supernatants of all GO samples (n=4) in a dose-dependent manner; however, only the reduction in IL-6 was statistically significant (figure 2). On post-hoc analyses using the Bonferroni method between controls and quercetin-treated samples, there was significant suppression of IL-6 (control vs quercetin 50 µM, p=0.087; control vs quercetin 50 µM, p=0.04; and control vs quercetin 100 µM, p=0.02). Similarly, IL-8 (control vs quercetin 30 µM, p=0.112; control vs quercetin 50 µM, p=0.098; and control vs quercetin 100 µM, p=0.057) and TNFα (control vs quercetin 50 µM, p=0.375; control vs quercetin 50 µM, p=0.250; and control vs quercetin 100 µM, p=0.092) were both decreased by quercetin treatment; however, the reductions were not statistically significant, probably due to a small sample size. IL-1α and IL-1β protein levels were not changed by quercetin treatment.

**Real-time RT–PCR of proinflammatory cytokines**

IL-6, IL-8, TNFα, IL-1α and IL-1β mRNA levels were compared between both uncultured and cultured (48 h) normal (n=2) and GO tissues (n=5). Of these cytokines, the Ct values for IL-1α were over 35, indicative of minimal amounts of target nucleic acid in both GO and normal tissues, thus we could not compare IL-1α mRNA levels between GO and normal tissues. Other cytokines were all expressed significantly higher in GO tissues than normal tissues (table 2).

Quercetin had a significant suppression of tissue IL-6, IL-8, IL-1β and TNFα mRNA expression in cultured orbital tissues from three GO samples relative to untreated control tissue on real-time RT–PCR (repeated measures analysis of variance, p<0.05, figure 3). Ct values for IL-1α were over 35, indicative of minimal amounts of target nucleic acid, thus we could not determine the effect of quercetin on this cytokine expression.

**DISCUSSION**

GO is a potentially vision-threatening ocular disease, which remains difficult to treat. Current evidence points to orbital fibroblasts as the target cells in GO, which recruit T cells resulting in reciprocal activation and subsequent tissue remodelling, which is characteristic of GO.1 4 17 The fibroblasts respond to immune stimulation and actively participate in inflammation through synthesis of chemokines, cytokines and lipid mediators. Deposition of hyaluronan and glycosaminoglycans, and enlargement of the orbital fat volume occur in response to inflammation.

**Table 1** Cytokine levels secreted in tissue cultures from GO and normal tissues

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>GO (n=4)</th>
<th>Normal (n=4)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>188.75 (0.27–259.35)</td>
<td>2.02 (0.17–7.26)</td>
<td>0.086</td>
</tr>
<tr>
<td>IL-1β</td>
<td>4.66 (1.85–11.33)</td>
<td>0.26 (0.17–0.43)</td>
<td>0.048</td>
</tr>
<tr>
<td>IL-6</td>
<td>57.02 (40.54–79.73)</td>
<td>32.08 (26.12–37.33)</td>
<td>0.025</td>
</tr>
<tr>
<td>IL-8</td>
<td>52.60 (30.18–76.03)</td>
<td>32.93 (23.35–43.34)</td>
<td>0.052</td>
</tr>
<tr>
<td>TNFα</td>
<td>202.4 (146.11–416.66)</td>
<td>32.72 (0.43–66.9)</td>
<td>0.032</td>
</tr>
</tbody>
</table>

Data are presented as mean (minimum–maximum range). The t test or Mann–Whitney test was used for statistical comparisons. The IL-6 level in one normal sample was not measurable.

**Figure 2** Effect of quercetin on secreted interleukin (IL)-6, IL-8 and tumour necrosis factor alpha (TNFα) protein levels. After incubation of orbital fat tissues from Graves’ orbitopathy (GO) with various concentrations of quercetin (Q) (0–100 µM) for 72 h, supernatants from the cultures were collected, and IL-6 (A), IL-8 (B) and TNFα (C) protein levels. After incubation of orbital fat tissues from Graves’ orbitopathy (GO) with various concentrations of quercetin (Q) (0–100 µM) for 72 h, supernatants from the cultures were collected, and IL-6 (A), IL-8 (B) and TNFα (C) concentrations were determined using a competitive binding ELISA kit according to the manufacturer’s instructions. Data represent the protein level corrected for tissue weight (ng/mL per gram of tissue weight for IL-6, IL-8; pg/mL per gram of tissue weight for TNFα) in the individual sample. Experiments were performed in cultured orbital tissues from different GO donors (n=4). p Values regarding differences between treated and untreated control samples are indicated.
We previously reported that quercetin could block the inflammatory pathway, aberrant accumulation of extracellular matrix macromolecules, and adipose tissue expansion in proinflammatory cytokine-stimulated primary cultured orbital fibroblasts from GO. We showed that quercetin inhibited IL-1β-induced intercellular adhesion molecule 1 expression, IL-6 and IL-8 mRNA expression, IL-1β or TNFα-induced hyaluronan production and adipocyte differentiation. However, the cells had to be stimulated with IL-1β to investigate the anti-inflammatory effect of the compound because proinflammatory cytokine gene expression in primary cultured GO orbital fibroblasts without IL-1β stimulation was relatively weak and similar to normal single cell cultures.

Several efforts to mimic the signs and symptoms of GO in a variety of animal species have been attempted; however, no accurate and reliable animal model of GO has been established. An orbital whole tissue culture system was recently introduced to test new therapeutic agents in GO. We used this culture model in this study to confirm the anti-inflammatory effect of quercetin, which was demonstrated in primary cultured orbital fibroblasts. Because randomised placebo controlled clinical trials of GO are very difficult to perform and animal models of GO are lacking, the tissue culture model could provide additional information on the effects of new therapeutics.

Although only the reduction in IL-6 was statistically significant, quercetin suppressed the secretion of IL-6, IL-8 and TNFα protein in a dose-dependent manner. Quercetin also significantly suppressed the gene transcript levels of cytokines including IL-6, IL-8, TNFα and IL-1β in GO tissues. In early stages of GO, infiltrating T cells interact with orbital fibroblasts, potentially resulting in cross-activation, further promoting cytokine production and the secretion of T-cell activating factors such as IL-8 by fibroblasts. In addition, stimulated fibroblasts secrete multiple cytokines, including IL-6, which stimulate B-cell differentiation and thus Graves’ disease IgG. TNFα stimulates the expression of intercellular adhesion molecule 1 in GO orbital fibroblasts. IL-1β increases production of IL-6 and IL-8 in orbital fibroblasts. Considering the possible roles of IL-6, IL-8, TNFα and IL-1β in the immunological pathogenesis of GO, suppression of their production could be an effective treatment for GO.

Quercetin (50 μM for 72 h) significantly reduced IL-6 protein secretion by 50%. IL-6 is known to play an important role in the immunological mechanism of GO. Orbital fibroblasts express high levels of IL-6 when treated with IL-1β and prostaglandin E2. IL-6 promotes synthesis of antibodies by stimulating lymphocyte differentiation and T-cell trafficking to orbital tissues. However, the biology of IL-6 is complex in that IL-6 can exert pro and anti-inflammatory actions through trans-signalling via the soluble IL-6 receptor, or classic signalling via the membrane-bound IL-6 receptor. In several mouse models, it was shown that classic signalling mediates the activation of anti-inflammatory pathways on target cells. The molecular mechanism of IL-6 suppression by quercetin should be further investigated, because long-term global suppression of IL-6 could block both classic and trans-signalling pathways. It was recently reported that specific trans-signalling blockade is under investigation as a target to avert inflammation.

IL-1β, IL-6 and TNFα protein levels were significantly higher in GO than normal tissue cultures and IL-8 production in GO tissue was higher than in normal tissue controls without statistical significance. IL-6, IL-8, TNFα and IL-1β mRNA in GO tissue were also higher than in normal tissue controls in both

Table 2: Relative fold expression of various cytokines in orbital tissue from GO patients (n=3) relative to the mean of two normal tissue samples in both cultured (48 h in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, antibiotics) and uncultered conditions

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Condition</th>
<th>GO 1</th>
<th>GO 2</th>
<th>GO 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>cultured condition</td>
<td>11.90</td>
<td>9.74</td>
<td>4.26</td>
</tr>
<tr>
<td></td>
<td>GO 1</td>
<td>5.6</td>
<td>5.3</td>
<td>1.8</td>
</tr>
<tr>
<td>IL-8</td>
<td>GO 2</td>
<td>10.84</td>
<td>9.92</td>
<td>3.81</td>
</tr>
<tr>
<td></td>
<td>GO 3</td>
<td>2.4</td>
<td>3.6</td>
<td>2.9</td>
</tr>
<tr>
<td>TNFα</td>
<td>GO 1</td>
<td>6.50</td>
<td>3.55</td>
<td>1.55</td>
</tr>
<tr>
<td></td>
<td>GO 2</td>
<td>2.8</td>
<td>2.3</td>
<td>1.8</td>
</tr>
<tr>
<td>IL-1β</td>
<td>GO 3</td>
<td>11.13</td>
<td>4.26</td>
<td>3.41</td>
</tr>
<tr>
<td></td>
<td>GO 1</td>
<td>5.4</td>
<td>2.7</td>
<td>1.7</td>
</tr>
</tbody>
</table>

GO, Graves’ orbitopathy; IL, interleukin; TNFα, tumour necrosis factor alpha.

Figure 3: Effect of quercetin on tissue mRNA transcript levels of interleukin (IL)-6, IL-8, tumour necrosis factor alpha (TNFα) and IL-1β. After treatment of tissue cultures with various concentrations of quercetin (Q) for 72 h, total RNA (1 μg) was isolated and reverse transcribed into cDNA, which was amplified for IL-6, IL-8, TNFα and IL-1β mRNA by real-time PCR. Gene transcript levels of IL-6 (A), IL-8 (B), TNFα (C) and IL-1β (D) are shown as mean ± SD fold depression in cytokine mRNA levels relative to the control samples without quercetin treatment. Experiments were performed in triplicate in cultured tissues from different Graves’ orbitopathy donors (n=3). Differences between treated and untreated tissues (*p<0.05) are indicated. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
cultured and uncultured conditions. These results were similar to a previous report by Kumar and Bahn. It was also reported that active GO tissues have higher levels of IL-6 and IL-8 mRNA than inactive GO tissues.

Quercetin, a member of the flavonoids family, is one of the most widely distributed dietary polyphenolic compounds in food. Quercetin is now available in a high-grade purified form, and clinical phase I–III studies can be readily performed in the near future. The beneficial effects of quercetin are supported by the detailed findings at the molecular and cellular levels of the specific pathways and molecules affected. However, many questions regarding flavonoids remain to be investigated. Quercetin 3-O-β-D-glucoridone and quercetin-3′- sulphate are the known major quercetin conjugates in human plasma and the aglycone could not be detected. Although most of the in-vitro pharmacological studies have been carried out using only the quercetin aglycone form. Experiments with the in-vivo forms of quercetin would be important to elucidate the efficacy of orally administered flavonoids. It is unknown whether they may contribute to the clinical benefits seen in epidemiological studies. In addition, the specific dosage of quercetin to achieve the therapeutic window for GO is not known. Quercetin has been marketed as a dietary supplement with recommended daily doses up to 1 g or more by suppliers, which is higher than what can normally be achieved from a flavonoid-rich diet. However, supraphysiological flavonoid levels may have adverse effects, raising concerns about the safe use of dietary flavonoids. Further human clinical study is required to establish the beneficial effect of quercetin on GO and identify the therapeutic range of quercetin in GO patients, bearing in mind that phytochemicals seem to have tissue and concentration-specific effects.

Contributors All authors were qualified for authorship, based on contributions to conception and design (JSY, EJL), acquisition of data (JSY, SYL) and analysis and interpretation of data (JSY, MKC); all authors contributed to drafting the article and conception and design (JSY, EJL), acquisition of data (JSY, SYL) and analysis and interpretation of data (JSY, MKC); all authors contributed to drafting the article and

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Patient consent Obtained.

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Data sharing statement Data are available on request from the corresponding author: Jin Sook Yoon: yoonsj@yuhs.ac.

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