Suppression of Transforming Growth Factor Beta/Smad Signaling in Keloid-Derived Fibroblasts by Quercetin: Implications for the Treatment of Excessive Scars

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Background: Keloids are characterized by abnormal proliferation and overproduction of extracellular matrix. Quercetin, a dietary compound, has strong antioxidant and anticancer properties. Previous studies by the authors have shown that quercetin inhibits fibroblast proliferation, collagen production, and contraction of keloid and hypertrophic scar-derived fibroblasts. Quercetin also blocks the signal transduction of insulin-like growth factor-1 in keloid fibroblasts. This study assessed the effects of quercetin on the transforming growth factor (TGF)-β/Smad-signaling pathway in keloid-derived fibroblasts, which may be an important biologic mechanism of this proliferative scarring.

Methods: Keloid fibroblasts were isolated from keloid tissue specimens. Cells were treated with quercetin at different concentrations, then harvested, and subjected to immunoblotting analysis.

Results: Quercetin significantly inhibited the expression of TGF-β receptors 1 and 2 in keloid fibroblasts at three concentrations (low, medium, and high). Quercetin also strongly suppressed the basal expression of Smad2, Smad3, and Smad4 as well as the phosphorylation of Smad2 and Smad3 and the formation of the Smad2-Smad3-Smad4 complex.

Conclusions: Taken together, these data suggest that quercetin effectively blocks the TGF-β/Smad-signaling pathway in keloid fibroblasts. These data indicate that quercetin-based therapies for keloids should be investigated further.

Key Words: TGF-β, Smad, Keloid, Fibroblasts, Quercetin.

Quercetin has a wide range of biologic activity including antiviral, antiinflammatory, and antimicrobial properties. Quercetin causes cell cycle arrest in human leukemic T cells as well as human breast, ovarian, and gastric cancer cells. Quercetin also induces apoptosis through a pathway involving heat shock proteins and down-regulates the mutant p53 gene.

Results from previous studies have demonstrated that quercetin exhibits strong inhibitory effects on keloid fibroblast proliferation and collagen production and suppresses the insulin-like growth factor-1 (IGF-1)–signaling pathway. On the basis of these results, the authors hypothesized that quercetin may have an inhibitory effect on TGF-β/Smad signaling. To pursue this hypothesis, they investigated the effect of quercetin on TGF-β receptors 1 and 2 (TGF-βR1 and TGF-βR2) as well as Smad protein expression and function in keloid-derived fibroblasts. The inhibition of TGF-βR1, TGF-β2, and the function of Smad protein signaling in keloid-derived fibroblasts treated with quercetin suggested a molecular mechanism for the work of this compound to suppress proliferation and collagen production of keloid-derived fibroblasts.

**Inhibition of TGF-β/Smad Signaling by Quercetin**

Cultured fibroblasts under different experimental conditions were lysed in lysis buffer (1 mmol/L CaCl2, 1 mmol/L MgCl2, 1% [v/v] NP-40, 1 μg/mL leupeptin, 1 μg/mL aprotinin, 1 μmol/L PMSF, and 100 μmol/L NaVO₄). Proteins were subjected to Western blot analysis as described. To investigate the effects of quercetin on expression of TGF-βR1, TGF-βR2, Smad2, Smad3, Smad4, phospho-Smad2, CBP, and p300, 50-μg total protein extract was separated by 8% or 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and electroblotted onto nitrocellulose membrane. Blots were incubated with the specific antibodies against TGF-βR1 and TGF-βR2, Smad2, Smad3, Smad4, phospho-Smad2, CBP, and p300 as well as horseradish peroxidase-conjugated donkey antinouse or antirabbit secondary antibody (1:7,500). Blots were visualized with a chemiluminescence detection system (ECL; Amersham Bioscience, New Jersey), as described by the manufacturer.

To investigate the effects of quercetin on the formation of the Smad2-Smad3-Smad4 complex, 2 mg of whole-cell lysate of keloid fibroblasts treated with or without quercetin were immunoprecipitated overnight at 4°C with 6 μg of anti-Smad3, followed by precipitation with 100 μL of protein G-sepharose for 90 minutes at 4°C. After four washes with complete lysis buffer, the immunoprecipitates were eluted by boiling for 5 minutes in 100 μL of ×2 loading dye. The resulting immunoprecipitates were electrophoresed through an 8% SDS-PAGE gel, transferred onto a polyvinylidene difluoride membrane, and immunobotted with anti-Smad3, antiphosphoserine, anti-phospho-Smad2, and anti-Smad4. The blots were developed with chemiluminescence reagents. Qualification of the bands on autoradiograms was performed using densitometric analysis.

**Western Blotting and Immunoprecipitation**

**Materials and Methods**

Media and Chemicals
Dulbecco’s Modified Eagle’s Medium (DMEM), fetal calf serum (FCS), Hank’s balanced salt solution, streptomycin, penicillin, gentamycin, and fungizone were supplied by Invitrogen (Carlsbad, CA). Quercetin was purchased from Sigma Chemical Company (St. Louis, MO).

Rabbit anti-TGF-βR1 and anti-TGF-βR2 antibodies, rabbit polyclonal anti-Smad3, mouse anti-Smad4, mouse monoclonal anti-CBP, and rabbit polyclonal anti-p300 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-Smad2/3 antibodies were supplied by BD Transduction Laboratories (San Jose, CA). Rabbit anti-phospho-Smad2 antibodies were obtained from Upstate Biotechnology (Lake Placid, NY), and mouse monoclonal anti-phosphoserine antibody was supplied by Calbiochem (San Diego, CA).

**Excessive Scar-Derived Fibroblast Selection**

Fibroblasts from a specimen collection of earlobe keloid scar samples were selected. None of the patients had received previous treatment for the keloids before surgical excision. Before the excision, a full history was taken and an examination was performed, complete with color slide photodocumentation and informed consent.

**Fibroblast Culture From Earlobe Keloids**

Fibroblasts were isolated from earlobe keloid scars using the explant technique. Cells were pelleted and grown in tissue culture flasks. Fibroblast cell strains were maintained in DMEM containing 10% (v/v) fetal bovine serum and stored at −150°C until use. Only cells from the second passage were used for the experiments. Keloid-derived fibroblasts were seeded at a density of 2 × 10⁵ cells per well in 100-mm dishes (Iwaki Glass Co.) in DMEM containing 10% (v/v) fetal bovine serum (DMEM/10% [v/v] FCS) for 24 hours after another 48 hours in serum-free DMEM. The cells then were washed with phosphate buffer saline, and the different concentrations of quercetin in DMEM/10% (v/v) FCS were added simultaneously to cells. Control cells were grown in DMEM/10% FCS without the addition of quercetin. At the different time points, cells were harvested and preserved at −86°C until use.

**RESULTS**

The effect of quercetin on TGF-βR1 and TGF-βR2 expression of keloid fibroblasts was first investigated. Significant reduction of both TGF-βR1 and TGF-βR2 expression was observed in keloid fibroblasts treated with quercetin. This reduced expression was obvious at day 3 after quercetin treatment (Fig. 1). The three different concentrations of the
compound all showed similar levels of inhibition of TGF-βR1 and TGF-βR2 expression (Fig. 1).

The effect of quercetin on expression of Smad2, Smad3, and Smad4 by keloid fibroblasts also was investigated. Quercetin strongly suppressed the expression of Smad2, Smad3, and Smad4, and also reduced phosphorylated Smad2 and Smad3 in keloid fibroblasts (Figs. 2 and 3). Significant down-regulation of these proteins was maximal after 72 hours of incubation with quercetin (Fig. 2). Although similar levels of inhibition were seen with all three concentrations of quercetin, with a concentration of 20 μg/mL appearing to have the strongest effect.

To investigate the inhibitory effect of quercetin on the formation of the Smad2-Smad3-Smad4 complex, total cell lysates of keloid fibroblasts with or without quercetin at 5 μg/mL were immunoprecipitated with anti-Smad3 antibody. The resulting complexes were electrophoresed, followed by Western blotting with anti-phospho-Smad2 and anti-Smad4 antibodies. No expression of phospho-Smad2 or phospho-Smad4 was seen in keloid fibroblasts treated with quercetin (Fig. 3), indicating a blocking effect of the compound on the formation of the Smad2-Smad3-Smad4 complex.

Transcriptional coactivators p300 and CBP play a critical role in the stimulation of endogenous type 1 collagen gene expression, and enhance Smad-dependent stimulation of COLA1A2 promoter activity in human dermal fibroblasts, leading to overproduction of collagen and fibrosis. The effect of quercetin on protein expression of CBP and p300 was investigated. The data in Figure 4 show that both CBP and p300 levels were suppressed by quercetin at 5 μg/mL, indicating another potential mechanism of action whereby quercetin may inhibit collagen production in keloid fibroblasts.
It is known that TGF-β/H9252 signaling plays a fundamental role in pathologic fibroproliferation and abnormal scar formation. It has been hypothesized that TGF-β/H9252 stimulates the transcription of the α2(I) procollagen gene (COL1A2), leading to the production of collagen, the major structural component of the extracellular matrix. In contrast to the detailed structural knowledge of TGF-β/H9252 responsive elements in the COL1A2 promoter, little was known about the intracellular signaling mechanisms that enable TGF-β/H9252 to stimulate collagen transcription in vivo and in vitro until recently. Upon phosphorylation by TGF-β receptors, Smad2 and Smad3, known as receptor Smads (R-Smads), form heteromeric complexes with co-Smad or Smad4. Co-Smad acts as a convergent node in the Smad pathways downstream of TGF-β/H9252 receptors, forming a complex with Smad2 and Smad3. The Smad2-Smad3-Smad4 complex then is translocated into the nucleus, where it functions as a transcription factor, binding DNA either directly or in association with other DNA proteins.22–23,33

The transcriptional role of Smad proteins in collagen production by fibroblasts in relation to fibrosis pathogenesis was demonstrated recently by Chen et al.,26,27 who directly correlated Smad3 with the transactivation of a chloramphenicol acetyltransferase reporter gene driven by a 772-bp segment of the human COL1A2 promoter containing the putative TGF-β/H9252 response elements. In these experiments,26,27 Smad3 mutant harboring substitutions that replaced critical serine residues with alanine abrogated the stimulation of COL1A2 promoter activity by TGF-β/H9252. These data further implicated Smad3 as an important component of TGF-β signaling.

The mechanism by which collagen transcription is stimulated by Smad3 recently has been elucidated further by a number of researchers. These researchers showed that Smad3 functionally or synergistically cooperates with the transcription factors of p300/CBP, Sp1 in human fibroblasts, glomer-
quercetin action in preventing scar formation. In the pathogenesis of keloids, the insulin-like growth factor-1 (IGF-1) and transforming growth factor beta (TGF-β) signaling systems both are overactive, stimulating fibroblast overproliferation and production of collagen and ECM. On the basis of the current data, quercetin could potentially have an antiscarring effect by inhibiting the signaling pathway of IGF-1 and TGF-β systems.

Taken together, recent studies strongly suggest a correlation among TGF-β receptor, Smad activity, and fibroblast overproliferation and collagen production that leads to abnormal scar formation and pathologic fibrogenesis. The TGF-β receptors and Smads therefore represent a potential drug target for scar reduction therapy. Blocking of the TGF-β/Smad signaling pathway could theoretically suppress overproliferation of scar fibroblasts and the production of collagen, thus preventing the formation of keloid and hypertrophic scars.

The authors’ previous studies have shown that quercetin can inhibit scar-derived fibroblast proliferation, contraction, and production of collagen. An important potential profibrotic mechanism successfully inhibited by quercetin was the IGF-I-signaling pathway. The current study has shown that quercetin also significantly suppresses TGF-β/Smad signaling in keloid fibroblasts at the concentrations found to be effectively inhibitory to fibroblast proliferation, collagen production, and lattice contraction. Western blot analysis of the TGF-β type 1 and type 2 receptors showed that quercetin reduced expression of TGF-β receptors in keloid fibroblast cell culture. Analysis of the protein levels of Smad2, Smad3, and Smad4 reinforced this finding by showing that quercetin strongly suppresses the expression of these proteins in keloid fibroblasts and also blocks phosphorylation of Smad2 and Smad3.

Together, these data suggest that quercetin negatively regulates TGF-β/Smad signaling in keloid fibroblasts at the receptor level and also inhibits its downstream signaling molecules. In the immunoprecipitation experiments, quercetin totally blocked binding of Smad2, Smad3, and Smad4, with no evidence of Smad2 and Smad4 expressions in complexes immunoprecipitated with Smad3 in keloid fibroblasts treated with the compound. On the other hand, quercetin strongly prevented formation of the Smad2-Smad3-Smad4 complex and nuclear translocation. Finally, quercetin also reduced protein levels of p300 and CBP, which are important for the stimulation of endogenous type 1 collagen gene expression and for the enhancement of Smad activity.

In conclusion, this study demonstrated that quercetin effectively blocks the TGF-β–signaling pathway. Figure 5 summarizes the hypothesized potential of quercetin’s mode of action against abnormal scar formation.

**REFERENCES**


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