Emodin Suppression of Ocular Surface Inflammatory Reaction

Ai Kitano,1 Shizuya Saika,1 Osamu Yamanaka,1 Kazuo Ikeda,2 Yuka Okada,1 Kumi Sibrat,1 and Peter S. Reinach3

PURPOSE. To determine whether a Chinese herbal medicine component, emodin, suppresses inflammatory/fibrogenic reaction in cultured subconjunctival fibroblasts and reduces injury-induced increases in ocular surface inflammation in mice.

METHODS. Effects of emodin were measured in human subconjunctival fibroblasts on proliferation and migration with colorimetry and scratch wound assay, respectively. Neovascularization was evaluated using an endothelial cell-fibroblast coculture model. Proinflammatory mediator and extracellular matrix component gene and protein expression was characterized with real-time reverse transcription-polymerase chain reaction, enzyme immunoassay, and immunocytochemistry, respectively. Western blotting and immunohistochemistry evaluated the activation of nuclear factor-κB (NF-κB) and c-Jun N-terminal kinase (JNK). In a mouse corneal alkali-burn model, this herbal component lessened inflammatory and fibrogenic reactions.

RESULTS. Emodin suppressed tumor necrosis factor α (TNF-α)-induced fibroblast migration and fibronectin deposition in vitro. VEGF induced neovascularization but did not affect cell proliferation and collagen type 1 production. Monocyte/macrophage-chemoattractant protein-1 gene and protein expression declined. Emodin inhibited TNF-α-induced NF-κB p65 and JNK activation but did not affect transforming growth factor β1-induced Smad2/3 signaling. In vivo, emodin inhibited proinflammatory and fibrogenic reactions.

CONCLUSIONS. Emodin suppressed in vitro TNF-α-induced stimulation of proinflammatory reaction. In a mouse ocular alkali burn model, this herbal component lessened inflammation and scarring. Additional studies are warranted to evaluate the therapeutic potential of emodin in lessening ocular tissue inflammation and resultant fibrosis after injury.

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Materials and Methods

Primary Cell Culture

Human subconjunctival fibroblasts were cultured as reported.17 In brief, subconjunctival tissue was obtained during strabismus surgery with informed consent from the patients’ parents. The cells were cultured for 2 or 3 passages in Eagle minimum essential medium (MEM; Gibco, Grand Island, NY) supplemented with antibiotics, an antimycotic, and 10% fetal calf serum (MEM-10) before the subsequent experiments. An ethanol-containing emodin stock solution was diluted to obtain final concentrations of 1, 2.5, and 5 μg/mL in serum-free culture medium. The final ethanol concentration was 0.1% in each experiment. Emodin effects were determined in a serum-free condition with...
or without the proinflammatory cytokine, recombinant human TNF-α, at 10 ng/mL (R&D Systems, Minneapolis, MN).

**Evaluation of Cytotoxicity of Emodin**

A commercial kit probed for a cytotoxic effect of emodin by measuring the release of nuclear matrix protein (NMP) 41/719 (Cell Death Detection [Nuclear Matrix Protein] ELISA Kit; Oncogene Research Products, San Diego, CA). The cells were treated with emodin (natural, extracted type, 0–5.0 µg/mL; Sigma, St. Louis, MO) for 0.5, 1, or 2 hours in MEM-10. Culture medium was harvested and processed for the assay according to the manufacturer’s protocol. In brief, 100 µL medium or NMP41/7 standards (0–1021 U/mL) were added to each well of a 96-well plastic plate and left for 90 minutes at room temperature. After washing three times, anti-NMP41/7 antibody reacted for 60 minutes. After another wash, peroxidase-conjugated secondary reagent was added, and then, after 60 minutes, a substrate included in the kit was added to initiate a color reaction. After stopping the reaction, optical densities at 450/570 nm were determined.

**Cell Proliferation**

Subconjunctival fibroblasts (1.5 × 10²/100 µL/well) were seeded into 96-well culture plates and incubated in MEM-10 for 10 to 24 hours before reaching confluence. They were then incubated with different concentrations of emodin for another 24 hours in the presence or absence of 10 ng/mL TNF-α in serum-free medium. Cell proliferation was assayed by using Alamar blue (Trek Diagnostic Systems, West Sussex, UK) according to the manufacturer’s protocol. After a wash with phosphate-buffered saline (PBS), 40 µL Alamar blue was diluted in culture medium (1:2). Three hours later, the optical absorbance was measured at 570 nm.

**Cell Migration**

Cell migration rates were examined by a described scratch wound assay. In brief, the extent of closure of a linear defect produced in a fibroblast monolayer was determined in the presence or absence of 5 µg/mL emodin in a serum-free condition. The remaining distances between migrating cells at the defect boundaries were measured at three different points.

**Immunocytochemistry**

Cells (2.0 × 10³/300 µL/well) were grown to subconfluence in 10% serum-plus medium in the wells of 8-well chamber slides (Laboratory-Tec; Nunc, Rochester, NY). They were incubated in the presence or absence of different concentrations of emodin in serum-free medium supplemented with TGF-β1 (1 ng/mL) for another 24 hours. The cells were then fixed with paraformaldehyde and processed for immunohistochemistry to detect collagen I and fibronectin expression as reported. Antibodies used were goat polyclonal anti-collagen I antibody (1:100 dilution in PBS; Southern Biotechnology, Birmingham, AL) and goat polyclonal anti-fibronectin antibody (1:100 dilution in PBS; Santa Cruz Biotechnology, Santa Cruz, CA). After washing, the cells were first exposed to FITC-conjugated secondary antibodies (1:100 dilution in PBS; ICN Biomedicals, Aurora, OH) and then DAPI stained. Their staining patterns were observed with fluorescence microscopy.

**Real-Time Reverse Transcription-Polymerase Chain Reaction MCP-1 mRNA Expression Detection**

Confluent fibroblast cultures were further incubated for 24 hours in the presence or absence of different emodin concentrations in serum-free medium in the presence or absence of 10 ng/mL recombinant human TNF-α. Total RNA was extracted (Mammalian Total RNA Mini Prep Kit; Sigma, St. Louis, MO). MCP-1 mRNA expression was examined with reported primers and TaqMan probes (Table 1). Three independent experiments were conducted.

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Sequence</th>
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<tr>
<td>hMCP-1</td>
<td>F: 5′-act ctc cgc ccc ttc tgt-3′&lt;br&gt;R: 5′-gca tct tga tga gct-3′&lt;br&gt;P: 5′-ctg atc gca gcc acc ttc att ccc-3′</td>
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<tr>
<td>mTGFβ1</td>
<td>F: 5′-gca aca tgg gga act cta eca gag-3′&lt;br&gt;R: 5′-gac gtc aaa aga cag cca ctc-3′&lt;br&gt;P: 5′-acc tgg tga acc ggc tge tga ccc-3′</td>
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<tr>
<td>mMCP-1</td>
<td>F: 5′-ttg gtc aac cag cat cgg t-3′&lt;br&gt;R: 5′-cca gcc tca tga tgg gaa tca-3′&lt;br&gt;P: 5′-ccc cac tca cct gct gct act cat tca-3′</td>
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F, forward primer; R, reverse primer; P, TaqMan probe.

**Collagen Type I, Fibronectin, and MCP-1 ELISA Determination**

Confluent fibroblast cultures were further incubated for 48 hours in the presence or absence of different emodin concentrations in serum-free medium in the presence or absence of 10 ng/mL recombinant human TNF-α. Culture medium was harvested and a cell layer was sonicated in 500 µL PBS. The samples were stored at −80°C before analysis. Concentration of human procollagen type 1 C-peptide (PnP), human fibronectin, or human MCP-1/CCL2 was determined by using a PNP-EIA Kit (Takara, Tokyo, Japan) according to manufacturer’s protocol. Fibroblast detection used either an EIA kit (Takara) or an ELISA kit purchased from R&D Systems.

**Effects of Emodin on the Formation of Neovascularization in an In Vitro Coculture Model**

A commercial kit containing an in vitro coculture system of human vascular endothelial cells (HUVECs) and fibroblasts was used (NV kit; Kurabo, Tokyo, Japan) according to the manufacturer’s protocol. This system cocultures vascular endothelial cells on a fibroblast feeder layer and is used to evaluate new vessel formation based on increases in tubelike tissue formation. The effect of adding emodin (0–2.0 µg/mL) on VEGF-A (10 ng/mL; Kurabo)-stimulated vessel-like tube formation was determined in a serum-free condition according to the protocol provided by the manufacturer. Tubelike tissue was detected by immunostaining for CD31, an endothelial cell marker. Color development was performed by diaminobenzidine (DAB) color reaction, as reported. Five wells were prepared for each culture condition, and the length, number of branch points, and mean value were determined in three different 300-µm² regions.

**Emodin-Induced Changes in NF-κB, JNK, and TGF-β1-Mediated Signaling**

Effects of emodin on TNF-α-induced signaling in subconjunctival fibroblasts were assayed by using immunocytochemistry and Western blotting. The cells (4.5 × 10²/300 µL/well) were grown to subconfluence in 8-well chamber slides (Laboratory-Tec; Nunc) in MEM-10 and were further incubated for 24 hours in a serum-free condition. They were then incubated in the presence or absence of emodin in serum-free medium for 6 hours. Finally, the cells were treated with recombinant human TNF-α (10 ng/mL; R&D Systems) for 0.5, 1, or 2 hours and were fixed with 4% paraformaldehyde for 24 hours, followed by processing for immunocytochemistry. Cell nuclei were labeled with DAPI dye.

To further characterize TNF-α signaling events, the effects of emodin on these responses were characterized by Western blotting. Cells

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were grown to subconfluence in 60-mm culture dishes in MEM-10 and were further incubated for 24 hours in a serum-free condition. They were then incubated in the presence or absence of emodin in serum-free medium for 6 hours, after which they were treated with recombinant human TNF-α (10 ng/mL) for specific intervals and were harvested in buffer (100 μL/dish; Mammalian Cell Lysis; Sigma) and processed for SDS-PAGE and Western blotting. Antibodies used were rabbit polyclonal antibodies against p65 RelA, phosphorylated p65 RelA, c-Jun N-terminal kinase (JNK) and phospho-JNK (Cell Signaling Technology, Beverly, MA).

TGF-β1/Smad2/3 signaling was examined with the same methods as for TNF-α-mediated control. TGF-β1 at 1 ng/mL (R&D Systems) was used, and signal activation was determined by using goat polyclonal anti-Smad2 antibody (1:1000 dilution in PBS; Santa Cruz Biotechnology), rabbit polyclonal anti-phospho-Smad2 (Ser465/467) antibody (1:2000 in PBS; Chemicon, Temecula, CA), goat polyclonal anti-Smad3 antibody (1:1000 dilution in PBS; Santa Cruz Biotechnology), rabbit polyclonal anti-phospho-Smad3 (Ser423/425) antibody (1:1000 in PBS; Biosource, Camarillo, CA).

**Emodin Effects on Ocular Surface Fibrosis in Mice**

Experiments were approved by the Animal Care and Use Committee of Wakayama Medical University and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

A mouse corneal burn model was created by topical application of 0.5% NaOH (3 μL). Alkali was applied to an eye of adult C57BL/6 mice (n = 58) under general and topical anesthesia 24 hours after intraperitoneal injection of emodin (40 mg/kg) or its vehicle. Then these different groups of mice received injections (40 mg/kg) daily for as long as 15 days. The mice were killed at days 5, 10, and 15, and their eyes were processed for fixation in 4% paraformaldehyde or total RNA extraction, as previously reported. The number of eyes used for each time point for all conditions. For RNA extraction, six were used at each time point. Each RNA sample contained two corneas. Immunohistochemistry was conducted by using an antibody against αSMA or F4/80 macrophage antigen, as reported. Real-time RT-PCR was performed for collagen Iα2, TGF-β1, and MCP-1, as described (Table 1).

**Statistical Analysis**

Results are expressed as means ± SD. Student's t-test was used to compare two groups of animals, whereas ANOVA was used in multiple group comparisons. P < 0.05 was considered significant.

**RESULTS**

**Cell Viability Insensitive to Emodin**

The possible cytotoxic effects of emodin were evaluated based on measurements of NMP41/7 release into the medium. In the presence or absence of recombinant TNF-α, its levels were not changed at 48 hours (range, 15–17 U/mL) by exposure to emodin at concentrations up to 5.0 μg/mL (Fig. 1a). Therefore, emodin is nontoxic to mouse subconjunctival fibroblasts.

**Selective Retardation of Wound Closure by Emodin**

Another approach to assess for emodin cytotoxicity was to determine whether it inhibited mouse subconjunctival fibroblast proliferation. The results in Figure 1b also show that emodin failed to suppress proliferation over the same concentration range used to evaluate cell viability, irrespective of the presence or absence of TNF-α. However, cell migration was markedly suppressed, as indicated by the results shown in Figure 1c. Under control conditions, the distance separating the encroaching cells was very small 24 hours after wounding, whereas with 5 μg/mL emodin only partial wound closure occurred (Fig. 1c).

**Emodin Suppression of Expression of Fibrogenic Components in Subconjunctival Fibroblast**

The effects of emodin on conjunctival fibroblast phenotype were evaluated based on ELISA measurements of type 1 collagen and fibronectin in the culture medium.
Emodin did not alter the total tissue cellular levels of type 1 collagen (Fig. 2a) or fibronectin (Fig. 2b), but it selectively reduced the fibronectin content in the extracellular space (Fig. 2c). In culture with TGF-β1 (1 ng/mL) and TNF-α (10 ng/mL), collagen type 1 extracellular deposition was not affected by emodin (Figs. 2dA, 2dB). On the other hand, emodin dramatically reduced extracellular deposition of fibronectin (Figs. 2dC, 2dD).

**Emodin Suppression TNF-α–Induced MCP-1 Expression**

MCP-1 is a chemoattractant expressed by various cell types, including fibroblasts. Its upregulation leads to recruitment of macrophages, which in turn induces fibrotic reactions through their expression of TGF-β. As TNF-α is a proinflammatory cytokine expressed by corneal epithelial cells and macrophages, changes in its level, along with those of TGF-β, serve as indicators of pathophysiological changes resulting from injury. To determine whether emodin suppresses the chemoattractive effects of fibroblasts on macrophages, real-time RT-PCR was used to probe for emodin-induced changes in MCP-1 mRNA expression in the presence and absence of TNF-α. Emodin over a concentration range from 1 to 5 μg/mL suppressed the dose-dependent increases by TNF-α on MCP-1 mRNA expression and MCP-1 protein concentration (Figs. 3a, 3b, respectively). These results indicate that emodin suppresses TNF-α–induced pro-inflammatory reactions in fibroblasts.
Effects of Emodin on the Formation of Neovascularization in an In Vitro Coculture Model

Exogenous VEGF-A induced vessel-like tube formation in HUVECs based on increases in CD31 staining. Furthermore, both 1 μg/mL and 2 μg/mL emodin markedly suppressed tube elongation and branching (Fig. 4). At the higher emodin concentration, both responses to VEGF were completely obviated, suggesting that this herbal component is an effective inhibitor of neovascularization.

Emodin Inhibits TNF-α–Induced JNK Pathway Activation

Because emodin inhibits inflammatory responses, as described, its effects were determined on cell signaling activated by TNF-α, one of the major proinflammatory cytokines. We examined whether emodin alters recombinant TNF-α–induced activation of the NF-κB and JNK signaling branches known to mediate responses to this proinflammatory cytokine. Figure 5 shows the time-dependent changes in phosphorylation status...
of the NF-κB subunit, p65 RelA, and its nuclear localization induced by TNF-α. Figure 5a shows that exposure to 10 ng/mL TNF-α-induced activation of the NF-κB subunit, p65 RelA, at 1 hour, whereas in the presence of either 2.5 or 5 μg/mL emodin, p65 RelA phosphorylation was reduced at 1 hour. Equivalence of protein loading was documented by the nearly identical intensity of the total RelA bands shown in the bottom portion of Figure 5a. Figure 5b shows the changes in phospho-RelA localization induced by 10 ng/mL TNF-α addition. Irrespective of the presence or absence of emodin, no nuclear localization was detectable of this NF-κB subunit. In the absence of emodin, nuclear translocation was first detectable after 30 minutes, which increased to reach a maximum after 1 hour and was followed by a decline 1 hour later. With 1 μg/mL or 2.5 μg/mL emodin in the medium, phospho-RelA translocation was not inhibited at 30 minutes, whereas 5 μg/mL emodin blocked this response. On the other hand, after either 1 or 2 hours, emodin at all tested concentrations fully blocked phospho-RelA localization. Figure 6 shows the time-dependent changes of JNK phosphorylation status and its nuclear localization induced by 10 ng/mL TNF-α in the presence and absence of emodin. Figure 6a shows that, in the absence of emodin, phospho-JNK formation reached a maximum value after 1 hour, followed by its disappearance after 2 hours. Such activation by TNF-α was fully blocked by all emodin concentrations. Equivalence of protein loading is documented by invariant levels of total JNK. The correspondence between these changes in JNK activation and phospho-JNK nuclear translocation is provided in Figure 6b. In the absence of emodin, TNF-α (10 ng/mL) induced nuclear translocation of phospho-JNK within 30 minutes, which remained evident for the next 30 minutes. At 2 hours, such localization was no longer detectable. On the other hand, with TNF-α and 1 μg/mL emodin together, immunoreactivity at 30 minutes decreased and was more transient because it was no longer detectable at 1 hour.

Injury-induced increases in TGF-β1 mediated fibroblast transformation through Smad2/3 signaling stimulation. Because emodin suppresses injury-induced pathophysiology associated with TGF-β1-mediated myofibroblast transformation, we sought to determine whether emodin suppressed TGF-β1-induced signaling. At emodin concentrations up to 5 μg/mL, neither the TGF-β1-induced increase in Smad2/3 expression levels nor the nuclear immunoreactivity of C-terminal phospho-Smad2/3 was affected (data not shown). Therefore, emodin did not inhibit TGF-β1–induced signaling.

Systemic Emodin Suppresses Ocular Surface Fibrosis in Mice

Given that emodin suppresses TNF-α–induced subconjunctival fibroblast responses, we hypothesized that emodin reduces mouse corneal scarring in an alkali burn model. An alkali burn of the ocular surface resulted in a corneal epithelial defect in the early phase (10 days) and then stromal scarring (opacification) and neovascularization in the later phase (15 days; Fig. 7aA, aC). However, the resultant stromal opacification was much lower in mice that received daily systemic intraperitoneal injections of emodin (Figs. 7aB, 7aD) than in control animals. The injected volume of either emodin or its vehicle was the same as that used for treating malignant tumor in mice.10

To determine whether there is an association between alkali-induced injury and changes in gene expression induced by TNF-α in vitro, real-time RT-PCR was performed with primer pairs for the detection of collagen Iα2, TGF-β1, and MCP-1 RNA in isolated ocular tissue samples. Figure 7b shows that in the nontreated controls, collagen Iα2 expression levels peaked at day 10. TGF-β1 expression was slightly higher at 5 days than at 10 and 15 days. On the other hand, MCP-1 levels decreased markedly from day 5 until day 15 (Fig. 7b). Expression levels of the three genes were lowest in emodin-treated mice.

Histologic examination and immunohistochemistry were performed to determine whether alkali-induced ocular surface changes in emodin-treated and control mice were associated with structural changes, myofibroblast generation, and macrophage infiltration during healing. The markers used to monitor fibroblast transdifferentiation and magnitude of macrophage infiltration were intensity of αSMA and F4/80 staining, respectively. There was a good association in the two groups between immunohistochemical changes and alterations in ocular
surface integrity because less staining correlated well with improved tissue appearance. Hematoxylin and eosin staining showed that there was more cellularity and thickening in the bulbar conjunctiva region in control animals than in emodin-treated mice (Fig. 8a). Overall, systemic emodin treatment suppressed such histologic changes during the healing interval (Fig. 8a). Furthermore, there were fewer myofibroblasts and macrophages in the subconjunctival connective tissue of emodin-treated mice than in control mice at days 10 and 15 (Figs. 8b, 8c). These changes were in marked contrast with the more...
pronounced changes seen in control corneas (Fig. 9). The control group exhibited more cellularity with stromal thickening, probably because of edema, than in the emodin-treated group cornea at days 10 and 15 (Fig. 9a). There were fewer myofibroblasts and macrophages in the healing stroma of the emodin-treated group mice than in control animals at these time points (Figs. 9b, 9c). Therefore, systemic emodin administration could have therapeutic potential in lessening corneal opacification caused by fibrosis and neovascularization during healing.

**DISCUSSION**

Emodin suppressed proinflammatory and profibrogenic activities in vitro in subconjunctival fibroblasts and in vivo in an alkali-burned mouse ocular surface. The protective effects of emodin are attributable to the inhibition of fibroblast migration, declining MCP-1 chemoattractant gene and protein expression, and the suppression of TNF-α-induced NF-κB and JNK branch signaling. Another attribute of the in vitro studies observed in vivo was that neovascularization was suppressed by emodin in proinflammatory and profibrogenic activities. These effects of emodin account for decreased alkali-induced corneal and conjunctival scarring. Taken together, the findings suggest that this herbal component may have therapeutic value in reducing the losses in corneal structural integrity and transparency that occur during alkali-induced healing.

The mechanism accounting for the suppressive effect of emodin on cell migration was determined by evaluating its effect on TNF-α-induced JNK activation because this pathway can mediate the control of cell migration. Given that emodin suppressed such JNK activation and inhibited cell migration, emodin-induced inhibition of JNK activation could account for its suppression of cell migration. Another contributing factor that could explain the decline in cell migration is that emodin also reduced fibronectin expression and its cell layer deposition. On the other hand, emodin was not toxic, antimitogenic, or inhibitory to in vitro type 1 collagen production. These negative effects suggest that emodin induces its anti-inflammatory fibrinogenetic effects through selective alteration of cell signaling pathways. Even though a previous study showed that emodin suppresses collagen production, such inhibition might be cell type dependent.

Tissue scarring during the healing process is a result of myofibroblast-mediated contraction of connective tissue. TGF-β levels rise during this response to injury, inducing fibroblast-to-myofibroblast transdifferentiation, which is characterized by the appearance of αSMA expression. This response is a consequence of TGF-β stimulation of Smad2 signaling, leading to increases in αSMA gene promoter and extracellular scaffold αSMA activity. In addition, the appearance of αSMA contractile cytoskeletal fibers, or the generation of myofibroblasts, depends on extracellular deposition of fibronectin, especially ED-A type fibronectin. The present in vivo experiment showed that emodin suppressed myofibroblast generation in healing ocular tissue (corneal stroma and subconjunctival tissue). Because emodin did not directly inhibit the Smad signal in fibroblasts, the in vivo phenomenon might have been caused by the secondary reduction of TGF-β1 in tissue with less inflammation with emodin. However, our unpub-
lished data showed that emodin suppressed aSMA-cytoskeletal fiber formation without altering the protein expression level of aSMA, as detected by Western blotting. Although the mechanism of this phenomenon is to be uncovered, a similar phenomenon was reported.\textsuperscript{37,38} The present study leaves unexplained why there were decreases in fibronectin deposition within the fibroblasts, but the fibronectin medium content remained unchanged. Its deposition may be mediated by NF-\kappaB or JNK signaling, but the upstream effectors of their activation are unclear. Even though tissue fibronectin deposition might be favorable for epithelial healing, emodin inhibition of fibronectin deposition instead improved the outcome of the healing process in our mouse corneal alkali burn model, suggesting the therapeutic effects of emodin are essentially of stromal rather than epithelial origin. However, its effects in the stroma are not attributable to a decline in the fibroblast population because in vitro emodin did not inhibit TNF-\alpha-induced stimulation of fibroblast proliferation. The effects of emodin on cell proliferation are cell type specific because it suppresses neoplastic cell growth but has no effect on this process in mesenchymal cell types (hepatic stellate cells). This negative effect is consistent with our observation that emodin did not inhibit fibroblastic cell proliferation because they are also derived from mesenchyme.

TNF-\alpha and TGF-\beta are major proinflammatory/profibrogenic cytokines expressed by ocular surface tissues. We used them to mimic the inflammatory responses by subconjunctival cells after injury that resulted from their cognate receptor activation. Such responses in turn resulted from invasive infiltration of inflammatory cells mediated by such chemoattracants as MCP-1. The reduction in TNF-\alpha-induced increases in MCP-1 gene and protein expression could lead to declines in invasive monocytes/macrophages from surrounding tissue, in turn decreasing fibroblast activation and transformation by proinflammatory and profibrogenic cytokines (e.g., TNF-\alpha and TGF-\beta). Consistent with emodin inhibition of proinflammatory responses, this herbal component also suppressed TNF-\alpha-induced NF-\kappaB p65 and JNK activation. On the other hand, emodin did not affect the TGF-\beta1/Smad pathway signaling, as determined by its failure to suppress cytokine-mediated stimulation of C-terminal phosphorylation of Smad2/3 (data not shown). However, another effect by emodin on TNF-\beta signaling might still have been possible because we failed to examine whether emodin affected Smad2/3 signal by modulating the phosphorylation of middle linker regions of Smad2/3 molecules.\textsuperscript{39–41}

Another possible factor contributing to the improved outcome of the healing response to ocular surface injury is that emodin therapy suppressed neovascularization. Neovascularization is also an important component in the development of unfavorable scar tissue formation on the ocular surface (i.e., the cornea). This effect is consistent with our in vitro observation in which emodin suppressed cells TNF-\alpha-induced vessel-like tube formation in cultured endothelial. Therefore, emodin appears to be an effective antiangiogenic agent of potential use in a clinical setting.

Tissue fibrosis is thought to be caused by cytokine acceleration of extracellular matrix deposition. Although emodin did not affect type 1 collagen expression by fibroblasts, it suppressed the expression of other components associated with extracellular matrix formation; namely, it inhibited fibronectin deposition, myofibroblast formation, and MCP-1 expression. These latter effects suggest that emodin might have a therapeutic effect on the ocular surface fibrogenic disorders vernal or atopic conjunctivitis or Stevens-Johnson syndrome. To explore this hypothesis, we tested whether systemic administration of emodin might have a therapeutic effect on a mouse alkali burn model of mice fibrogenic diseases. This is a relevant model because during the healing response to injury, corneal or conjunctival opacification or scarring develops. Such pathophysiology is also observed in human ocular surface scarring diseases, in the later phase of healing. Systemic intraperitoneal administration of emodin effectively suppressed fibrogenic reaction in cornea and conjunctiva, as revealed by reduced macrophage infiltration, inhibited myofibroblast generation, and decreased mRNA expression levels of collagen Iα2, TGF-\beta1, and MCP-1. Reductions of MCP-1 expression might account for declines in macrophage infiltration, resulting in declines in TGF-\beta1 levels within the injured site. Although emodin did not affect type 1 collagen production by cultured fibroblasts, systemic emodin did suppress the expression of collagen Iα2 mRNA in a healing cornea. This phenomenon could be explained by the reduced expression of TGF-\beta1 mRNA in the healing tissue.

In conclusion, our current understanding of the mechanism of emodin suppression of inflammation and fibrogenic cytokine levels in fibroblasts presumably by inhibiting JNK and NF-\kappaB signaling. Even though our study provides evidence suggesting that emodin has potential beneficial value in reducing in vivo ocular surface scarring during healing, such symptom lessening occurred 24 hours after the systemic administration of emodin. It is not yet possible to extrapolate with certainty whether emodin is of use in the clinical setting because expression of the ocular surface abnormalities associated with such diseases as vernal conjunctivitis and Stevens-Johnson syndrome occurs over a much longer time. Before emodin can be considered for use in such disturbances, it is first necessary to evaluate whether this herbal component produces adverse effects after repeated systemic administration over long periods of time. The herbal medicine Inchin-ko-to may be used for the treatment of ocular fibrotic diseases.

References

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