Antifibrotic Effects of Sakuraso-Saponin in Primary Cultured Pterygium Fibroblasts in Comparison With Mitomycin C

Hun Lee,[1] Hana Jeong,[1] Chang Mok Lee,[1] Jin-A Shin,[1] Sung-Wuk Jang,[2] Jae Hyuck Lee,[1] So Young Park,[1] Jae Yong Kim,[1] and Hungwon Tchah[1]

[1]Department of Ophthalmology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, South Korea
[2]Department of Biomedical Sciences, Asan Medical Center, University of Ulsan College of Medicine, Seoul, South Korea

Correspondence: Hungwon Tchah, Department of Ophthalmology, Asan Medical Center, University of Ulsan College of Medicine, 88, Olympic-Ro 43-Gil, Songpa-Gu, Seoul 05505, South Korea; hwtchah@amc.seoul.kr.

HL and HJ contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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Purpose. To investigate the antifibrotic effects of sakuraso-saponin on a primary culture of human pterygium fibroblasts (HPFs) and normal human Tenon fibroblasts (HTFs) as compared to the effects of mitomycin C (MMC).

Methods. Samples of HPFs and HTFs were acquired during primary pterygium surgery. Cell toxicity, cell migration, and expression of α-smooth muscle actin (α-SMA) and transforming growth factor-β (TGF-β) were evaluated in HPFs and HTFs after treatment with sakuraso-saponin and MMC. To determine the possible mechanisms underlying the antifibrotic effects of sakuraso-saponin, the expression of phosphorylated Smad2/3 was evaluated after treatment with sakuraso-saponin and MMC.

Results. MMC (≥200 μg/mL) significantly reduced cell viability in both HPFs and HTFs, whereas sakuraso-saponin (1.0 μg/mL) decreased cell viability in HPFs only. Both sakuraso-saponin (1.0 μg/mL) and MMC (200 μg/mL) treatment significantly reduced the expression of α-SMA and TGF-β in HPFs ($P < 0.05$). It is interesting that the expression of α-SMA and TGF-β after treatment with sakuraso-saponin was significantly lower than that after treatment with MMC ($P < 0.05$). The expression of phosphorylated Smad2/3 protein was decreased by sakuraso-saponin and MMC in HPFs. Both sakuraso-saponin and MMC inhibited TGF-β1–induced cell migration as compared to the control in HPFs.

Conclusions. Sakuraso-saponin could be more effective than MMC for the reduction of fibrosis in HPFs. Our results might present the basis for its use as a promising candidate drug for adjuvant therapy to prevent recurrent pterygium after surgery.

Keywords: sakuraso-saponin, pterygium, antifibrotic agents, mitomycin C

A pterygium is a triangular-shaped overgrowth of the fibrovascular conjunctiva onto the nasal or temporal cornea, which is a commonly observed condition among outdoor workers and people living near the equator.[1] It may cause conjunctival injection, ocular irritation, cosmetic problems, and visual impairments such as astigmatism. Although the exact pathogenesis of pterygium is unknown, destruction of limbal stem cells resulting from chronic exposure to ultraviolet radiation has been regarded to play a key role for development of pterygium.[2–4] Surgical removal is indicated for these symptomatic cases, but wound healing after surgical excision often leads to fibrotic changes that are characterized by recurrent pterygium.

Transforming growth factor-β1 (TGF-β1) is the primary inducer of fibrosis and is known to also induce the expression of additional fibrogenic mediators, facilitate myofibroblast differentiation, and induce epithelial-mesenchymal transition.[5,6] Myofibroblasts exhibit increased proliferative, migratory, and secretory properties, all of which are associated with remodeling processes.[7,8] Several studies have shown the overexpression of TGF-β1 in pterygium tissues as compared to a normal conjunctival tissue.[9–12] In the same way, overexpression of TGF-β1 in recurrent pterygium fibroblast culture was observed as compared to primary pterygium fibroblast culture.[12,13] Since TGF-β1 signaling can stimulate fibroblast migration, cell proliferation, and myofibroblast differentiation, the presence of myofibroblasts in pterygium would suggest that TGF-β1 is involved in the pathogenesis and progression of pterygium.[14,15]

Clinically, mitomycin C (MMC) has been widely used to reduce postsurgical recurrence of pterygium. However, numerous adverse effects, including scleral stromalysis and corneal melting, have been reported.[16,17] Therefore, there is a need for a safe and precise preventive therapeutic option.[15] Sakuraso-saponin is a compound isolated from the plant *Jacquinia flammea* and has been identified to have antifungal effects as well as cytotoxic effects in cancer cells.[18,19] This compound is isolated as a white amorphous powder, and its molecular formula is $C_{60}H_{98}O_{27}$ (Fig. 1).

In the present study, we aim to evaluate the antifibrotic effects of sakuraso-saponin on a primary culture of pterygium fibroblasts in comparison with MMC and to examine the utility of sakuraso-saponin as a potential alternative treatment to MMC for reducing the recurrence of pterygium after primary surgery.

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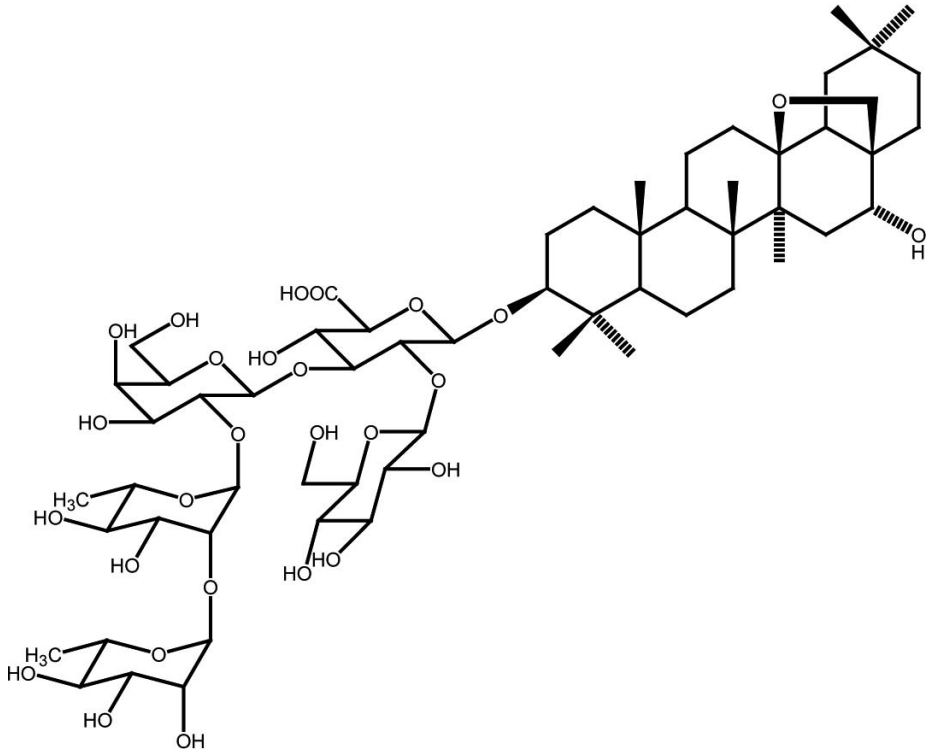

Chemical Formula: $C_{60}H_{98}O_{27}$
Exact Mass: 1250.63
Molecular Weight: 1251.40

Figure 1. Chemical structure of sakuraso-saponin.

Methods

Cell Culture

All human tissues were obtained after approval from the institutional review board of Asan Medical Center in Seoul, Korea. This research followed the tenets of the Declaration of Helsinki. Informed consent was obtained from all the patients after explanation of the nature of the study and its possible consequences. Samples of human Tenon capsule were obtained from patients with primary pterygium during excision surgery for pterygium. Overall, two tissue samples were obtained from each patient; one was excised from the pterygium head (human pterygium fibroblasts [HPFs]), and the other was excised, as far as possible, far from the pterygium (normal human Tenon fibroblasts [HTFs]). In total, five different pterygial and conjunctival tissues were used in this study (two males and three females, aged 50–65 years).

Patients with systemic diseases such as diabetes mellitus, or with other ocular diseases, previous intraocular surgery, or trauma, were excluded from the study. The diagnosis of pterygium was based on clinical findings, and no histologic confirmation was performed.[1] All patients showed a similar grade of severity of pterygium. After surgical excision, tissue samples were propagated in Dulbecco's modified Eagle medium ([DMEM]; Gibco Life Technologies, Karlsruhe, Germany) supplemented with 10% fetal bovine serum ([FBS]; Gibco Life Technologies), 100 U/mL penicillin, and 100 g/mL streptomycin (Gibco Life Technologies). The cells were maintained at 37°C in 5% CO_2 in a humidified condition, and the medium was changed every 3 days thereafter. Cells between the third and sixth passages were used for all experiments.

Cell Morphology

HPFs and HTFs were cultured for 24 or 96 hours in culture medium. After culture, the morphologies of cells were observed and photographed using an upright microscope (Zeiss, Inc., Thornwood, NY, USA) at 100× magnification.

Cytotoxicity Assay

A water-soluble tetrazolium salt (WST)-1 assay was performed to analyze the cytotoxicity at different concentrations of sakuraso-saponin (0.01, 0.1, 0.5, and 1.0 μg/mL) or MMC (1, 50, 200, and 400 μg/mL) on cultured HPFs and HTFs. The cells (10^4 cells/well) were seeded in 96-well plates with FBS-free medium for 24 hours. Then, the cells were incubated with the indicated concentrations of sakuraso-saponin and MMC at 37°C for 24 hours and 5 minutes, respectively. Cells were incubated with the indicated concentrations of MMC. To emulate clinical treatment, MMC was replaced with serum-free medium after 5 minutes, thus the total treatment time with MMC was 5 minutes. Sakuraso-saponin and MMC-treated cells were then incubated with 10 μL/well cell proliferation reagent WST-1 (Roche Applied Science, Penzberg, Germany) in 0.1 mg/well culture medium for 1 hour. After incubation, the absorbance was measured at a wavelength of 540 nm in each well using an ELISA reader (Bio-Rad, Munich, Germany).

Western Blot Assay

To detect protein levels of α-smooth muscle actin (α-SMA), TGF-β, and phosphorylated Smad2/3, cultured HPFs and HTFs were treated with or without sakuraso-saponin (1.0 μg/mL) and MMC (200 μg/mL) for 24 hours and then lysed by addition

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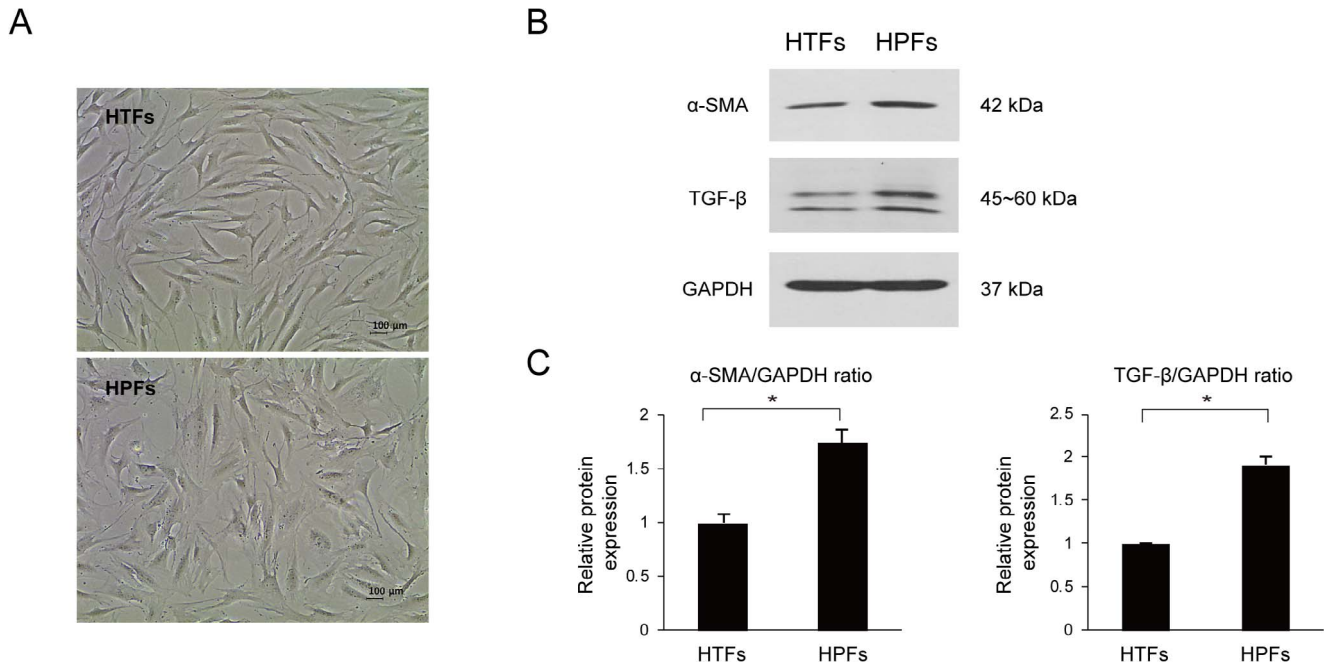

Figure 2. Morphology of normal HTFs and HPFs. (**A**) Phase-contrast micrographs showing normal HTFs and HPFs. (**B**) Representative Western blot images of α-SMA and TGF-β protein levels normalized to GAPDH. (**C**) Quantitative analysis of immunoblots with summary data from three independent experiments. *Error bars* represent SEM (*$P < 0.05$).

of a lysis buffer (10 mM Tris, 10 mM NaCl, 2 mM EDTA, 25 mM NaF, 2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, protease inhibitors, 0.5% Triton X-100, pH 7.0). Cell lysates were subjected to denaturing SDS gel electrophoresis followed by electroblotting and incubation with monoclonal mouse anti-α-SMA antibody (1:1000, no. A2228; Sigma-Aldrich, Inc., St. Louis, MO, USA), anti-TGF-β antibody (1:500, no. 3711; Cell Signaling, Beverly, MA, USA), anti-Smad2/3 antibody (no. 5678; Cell Signaling), anti-phosphorylated Smad2/3 antibody (no. 8828; Cell Signaling), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:1000, no. 5174; Cell Signaling). The membranes were then thoroughly washed, incubated with biotin-conjugated anti-mouse secondary antibodies (1:5000, no. 31430; Gibco Life Technologies), and developed with a detection system (Quantum Dot; Invitrogen, Life Technologies, Grand Island, NY, USA). The band area and intensity of the exposed film were analyzed by densitometric scanning, and mean intensity was quantified using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Normalization was done by calculating the mean intensity ratio of the target protein to that of GAPDH.

Cell Migration Assay

Cell migration was assessed by mechanically denuding cells from a confluent area (i.e., scratch-wound assay).[7] Initially, HPFs were grown to a confluent monolayer for 24 hours. After the medium was discarded, a scratch was created in a straight line across the cells with a p200 pipette tip. The plates were then rinsed with PBS to remove suspended cells and then incubated with DMEM supplemented with or without 1.0 μg/mL sakuraso-saponin or 200 μg/mL MMC. MMC was replaced with serum-free medium after 5 minutes, thus the total treatment time with MMC was 5 minutes. The cell migration assay was performed once more with or without 1.0 μg/mL sakuraso-saponin or 200 μg/mL MMC by adding TGF-β1 (2.5 ng/mL). Wound closure (i.e., migration of cells) was monitored and photographed after 24 hours under a light microscope, and the number of migrating cells between the edges was counted using image analysis software (Image-Pro Plus 5; Olympus, Inc., Tokyo, Japan).

Statistical Analysis

Data are presented as mean ± standard errors of the mean. Statistical significance across groups was determined by 1-way ANOVA with Tukey's multiple-comparison post hoc test among groups. Paired *t*-test was used to compare two measures within groups. Statistical analyses were performed using statistical software (GraphPad PRISM; GraphPad Software, Inc., La Jolla, CA, USA). A P value < 0.05 was considered to be statistically significant.

Results

Cell Morphology and Expression Level of α-SMA and TGF-β

The primary culture of HTFs showed the typical dendritic shape of fibroblasts, while HPFs showed a more flattened cell body and lost their original shape (Fig. 2A). Compared to HTFs, HPFs showed a significantly higher expression of α-SMA and TGF-β ($P < 0.05$) (Figs. 2B, 2C).

Cytotoxicity

No significant difference in cell viability was observed at any concentration of sakuraso-saponin treatment groups in HTFs compared to the untreated control group (Fig. 3A). Treatment with MMC at concentration of ≥200 μg/mL significantly reduced cell viability in HTFs as compared to the control group ($P < 0.05$; Fig. 3A). In HPFs, treatment with sakuraso-saponin at a concentration of 1.0 μg/mL significantly reduced cell viability compared to the control group ($P < 0.05$; Fig. 3B). Treatment with MMC at concentration of ≥200 μg/mL also significantly

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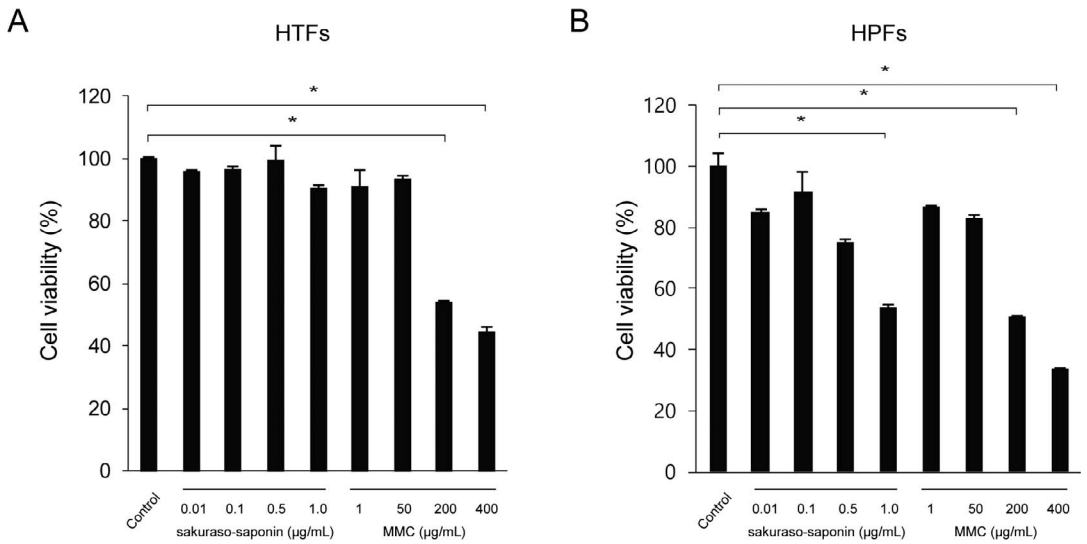

FIGURE 3. The effects of sakuraso-saponin and MMC on cell viability. (**A**) Normal HTFs. (**B**) HPFs. Data are expressed as percentage of control values. *Error bars* represent SEM (*$P < 0.05$).

reduced cell viability compared to the control group ($P < 0.05$; Fig. 3B). Taken together, MMC at concentrations of ≥200 μg/mL significantly reduced cell viability in both HTFs and HPFs, whereas sakuraso-saponin at concentrations at 1.0 μg/mL did not show significant change of cell viability in HTFs and only decreased cell viability in HPFs (Fig. 3).

Effects of Sakuraso-Saponin on Expression Level of α-SMA, TGF-β, and Phosphorylated Smad2/3

In HTFs, the expression of α-SMA decreased following sakuraso-saponin treatment at 1.0 μg/mL when compared to the untreated control group (Figs. 4A, 4B). MMC treatment at 200 μg/mL did not affect the expression level of α-SMA. In HPFs, the expression of α-SMA decreased following both sakuraso-saponin treatment at 1.0 μg/mL and MMC treatment at 200 μg/mL as compared to the control group (Fig. 4B). Sakuraso-saponin treatment also inhibited the expression of α-SMA more significantly than did MMC in HPFs ($P < 0.05$; Fig. 4B).

The expression of TGF-β decreased following sakuraso-saponin treatment at 1.0 μg/mL and MMC at 200 μg/mL compared to the control group in both HTFs and HPFs. In addition, there was a significant difference between sakuraso-saponin and MMC treatment in HPFs (Fig. 4B). Sakuraso-saponin inhibited the expression of TGF-β more than did MMC.

To determine the possible mechanisms underlying the antifibrotic effects of sakuraso-saponin, the major signal transduction pathways downstream of TGF-β were examined. As shown, the phosphorylated Smad2/3 levels were more than two times higher in HPFs than HTFs ($P < 0.05$). The relative phosphorylated Smad2/3-to-GAPDH ratio was significantly decreased by sakuraso-saponin and MMC treatment in HPFs (Fig. 5B). However, there was no significant difference in the relative phosphorylated Smad2/3-to-GAPDH ratio between sakuraso-saponin and MMC treatment in HPFs (Fig. 5B). Regarding the relative Smad2/3-to-GAPDH ratio, there was no significant difference before and after treatment with sakuraso-saponin or MMC in HTFs and HPFs.

Effects of Sakuraso-Saponin on Cell Migration

In HPFs, cell migration was not significantly different between the untreated control group and cells treated with sakuraso-saponin or MMC. Cell migration was significantly higher in the TGF-β1–treated group than in the untreated control group ($P < 0.05$). In the case of TGF-β1–treated cells, cell migration significantly decreased after sakuraso-saponin or MMC treatment as compared to the control group ($P < 0.05$) (Figs. 6A, 6B).

DISCUSSION

The recurrence rate of recurrent pterygium varies depending on the surgical procedure and duration of follow-up.[20] Previous studies with a minimum follow-up period of 6 months for primary or recurrent pterygium reported recurrence rates of 20.8% and 31.2% after conjunctival autograft, 0.0% and 14.6% after conjunctivolimbal autograft, and 3.0% and 9.5% after amniotic membrane transplantation.[21–27] With regard to MMC, several studies have suggested that increased exposure (dose or duration) to intraoperative and postoperative MMC is associated with greater effectiveness, but concomitantly increased the risk of complications as well.[3,16,23,26] Scleral ulceration and delayed conjunctival epithelialization were associated with intraoperative and postoperative MMC treatment, and there is evidence that increased complications would be directly related to an increased concentration or duration of exposure to MMC.[1,28] Thus, it might be difficult to determine the appropriate agents to prevent tissue scarring effectively and reduce side effects of MMC.

In the current study, we would like to identify the effectiveness and safety of an antifibrotic agent, sakuraso-saponin, for use as adjuvant treatment in pterygium surgery to prevent recurrence with lower toxicity than MMC. When tested, sakuraso-saponin showed cytotoxic activity against mammalian cells at the concentration of 100 μg/mL.[19] It also demonstrated moderate antifungal activity against dermatophytes and very strong antifungal activity against *Colletotrichum gloeosporioides*.[18] However, its antifibrotic effect on ocular fibrotic disorders, such as pterygium, is not well known.

TGF-β1, a primary inducer of fibrosis, is overexpressed in cultured pterygium tissue as compared to normal conjunctiva cultures and in recurrent pterygium fibroblast cultures as compared to primary pterygium fibroblast cultures.[12,13,15] In our study, we measured the expression of TGF-β and α-SMA, a phenotypic hallmark of myofibroblast differentiation.[29] We

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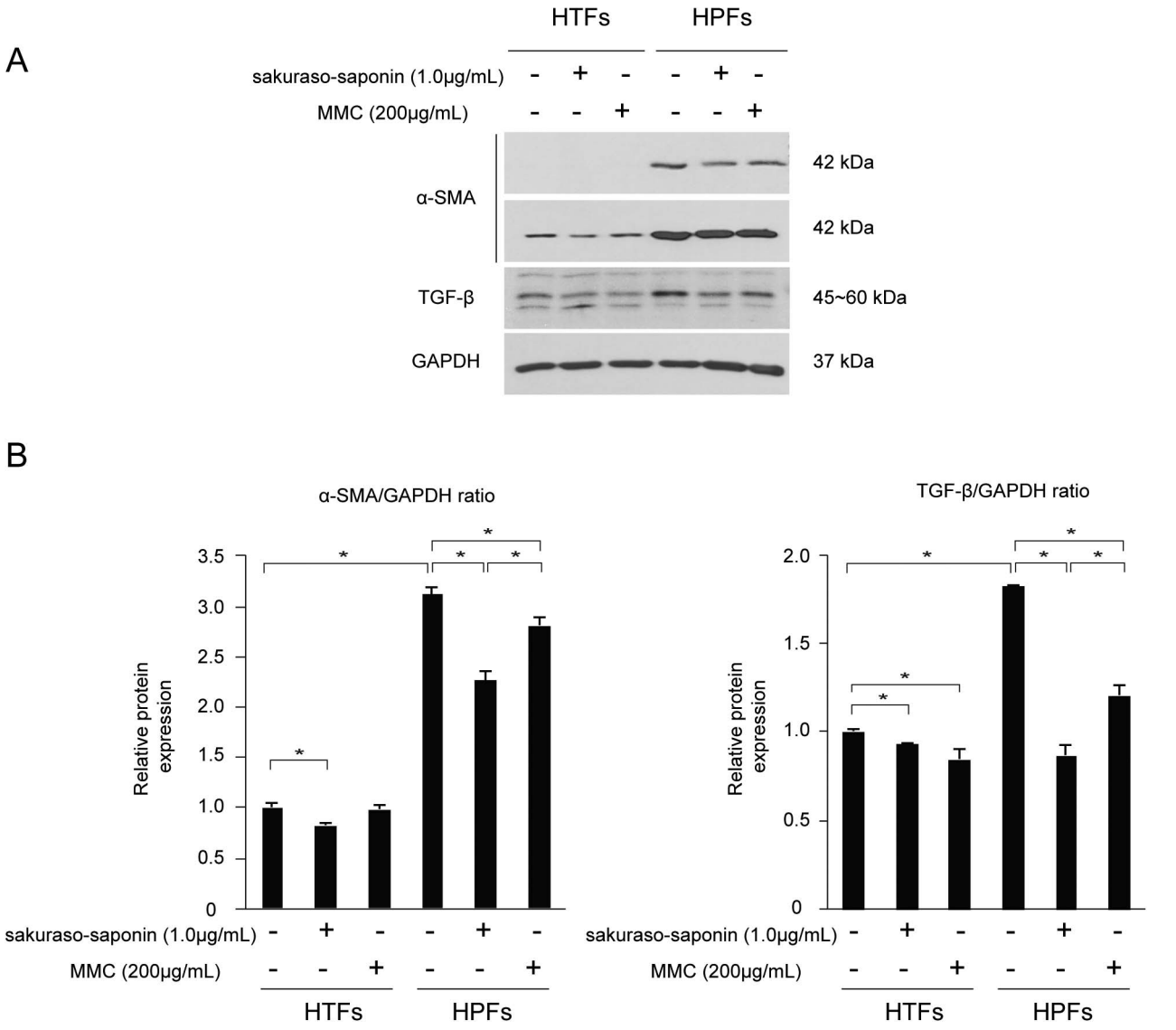

Figure 4. The effects of sakuraso-saponin and MMC on the expression level of α-SMA and TGF-β in normal HTFs and HPFs. (**A**) Representative Western blot images of α-SMA and TFG-β protein levels in normalized GAPDH. (**B**) Quantitative analysis of immunoblots with summary data from three independent experiments. *Error bars* represent SEM (*$P < 0.05$).

found higher expression levels of TGF-β and α-SMA in HPFs as compared to HTFs. These results correlate with our observations of cell morphology in HTFs and HPFs, wherein HTFs showed typical fibroblast morphology and HPFs had a myofibroblast-like appearance.

According to our results, sakuraso-saponin treatment at a concentration of 1.0 μg/mL reduced cell viability in HPFs compared to the untreated control group. In contrast, MMC-treated cells showed significantly lower cell viability at concentrations of ≥200 μg/mL than did the untreated control group in both HTFs and HPFs. Our study confirmed that MMC has dose-dependent toxicity, which is in line with the previous studies.[16] In contrast, sakuraso-saponin has an effect of selective toxicity on HPFs without affecting HTFs.

Our results showed that sakuraso-saponin is an effective antifibrotic agent in pterygium fibrosis in vitro. The ability of sakuraso-saponin to modulate the expression of TGF-β suggests that it could be an alternative option to antimetabolites for the reduction of recurrent pterygium. Notably, our results demonstrated that sakuraso-saponin can reduce the basal level of α-SMA in HTFs. Sakuraso-saponin at 1.0 μg/mL significantly inhibited the expression of α-SMA and TGF-β in both HTFs and HPFs. These effects of sakuraso-saponin at 1.0 μg/mL showed comparable or superior inhibitory effects of α-SMA and TGF-β than did MMC at 200 μg/mL.

In the cell migration assay, sakuraso-saponin and MMC treatment did not suppress the normal cell migration but suppressed cell migration in HPFs cotreated with TGF-β1. The ability of sakuraso-saponin to reduce cell migration in HPFs cotreated with TGF-β1 was comparable to that of MMC.

Our Western blot data showed that the mechanism of action of sakuraso-saponin in HPFs involves the suppression of TGF-β expression via the Smad2/3 signaling pathway. TGF-β signaling is activated by the binding of TGF-β to type I and type II TGF-β receptors. The activated receptor complex phosphorylates the downstream transcription factors Smad2 and Smad3, which then form a heterotrimeric complex with Smad4. The Smad complex translocates into the nucleus and regulates the expression of the target genes.[30,31] Canonical Smad2/3 signaling and several other noncanonical pathways are

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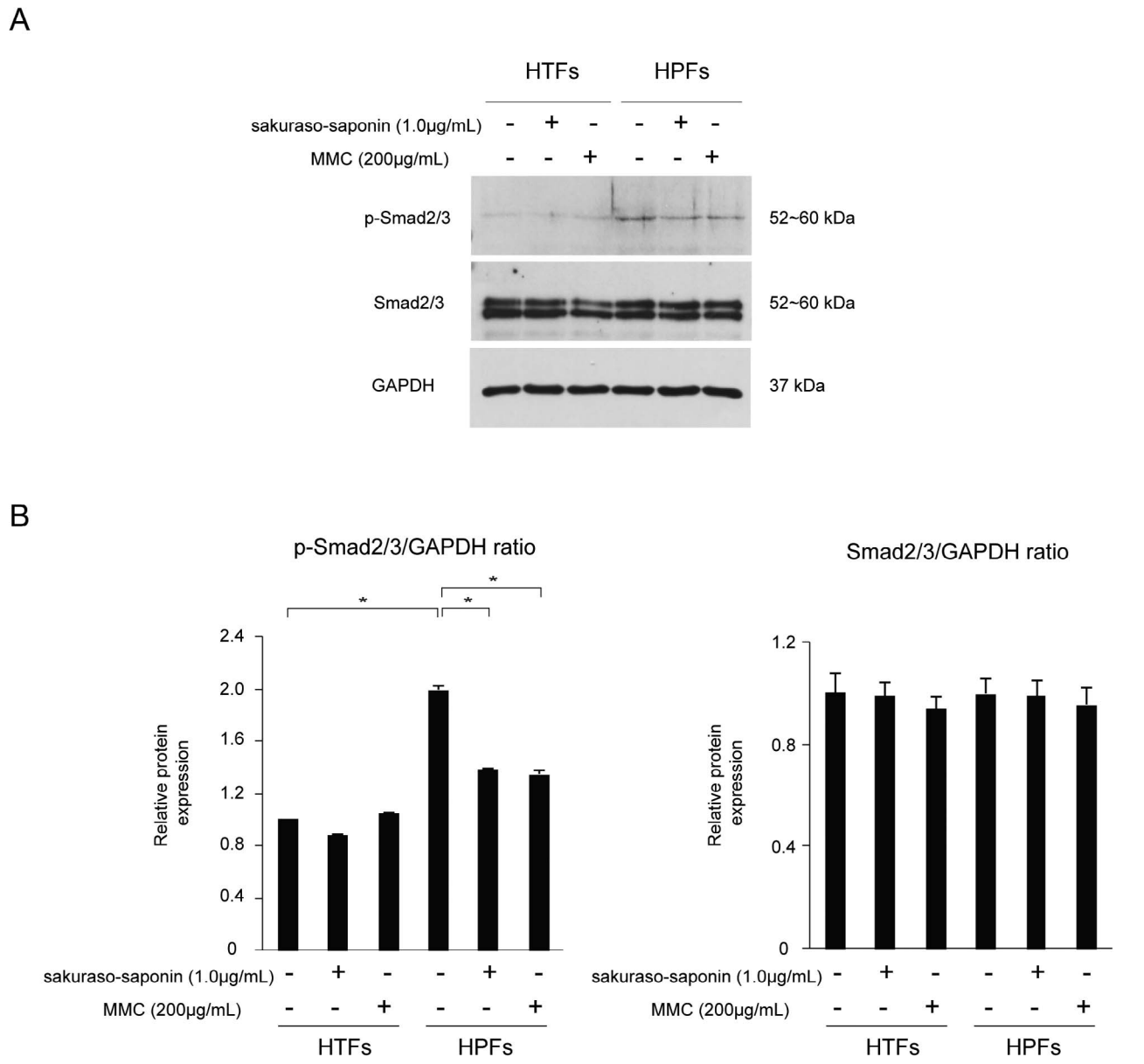

FIGURE 5. The effects of sakuraso-saponin and MMC on the expression level of phosphorylated Smad2/3 in normal HTFs and HPFs. (**A**) Representative Western blot images of phosphorylated Smad2/3 protein levels after treatment with sakuraso-saponin and MMC. (**B**) Quantitative analysis of immunoblots with summary data from three independent experiments. *Error bars* represent SEM (*$P < 0.05$). p-Smad2/3, phosphorylated Smad2/3.

involved in TGF-β1–induced fibrosis.[32,33] Therefore, the decrease in TGF-β expression is associated with the reduction in phosphorylated Smad2 and phosphorylated Smad3. In our study, the expression of phosphorylated Smad2/3 was decreased by sakuraso-saponin and MMC treatment in HPFs. Although we did not identify the specific mechanisms involved in the sakuraso-saponin–induced decrease in the phosphorylation of Smad2 and Smad3, the possible molecular mechanisms underlying the observed antifibrotic effects of sakuraso-saponin indicated that sakuraso-saponin could inhibit TGF-β1–induced fibrosis in pterygium fibroblasts, partially by modulating the Smad2/3 cellular signaling pathway.

A single exposure of MMC to HTFs for 5 minutes at the concentration of 400 μg/mL resulted in 56% cell death after 24 hours via the WST-1 assay. Sakuraso-saponin, however, at the concentration of 1.0 μg/mL in HTFs is likely to be safe, as the WST-1 assay showed no significant change in cell viability between the control and treated groups. However, in vitro experiments are not sufficient for assessing the toxicity of sakuraso-saponin. Thus, in vivo bioassays should be explored to further evaluate the toxicity of sakuraso-saponin.

The antifibrotic effects of sakuraso-saponin on HPFs suggest that sakuraso-saponin may limit the proliferation of pterygium fibroblasts and their transformation into myofibroblasts. Moreover, these effects combined with the noncytotoxic effects in HTFs suggest that sakuraso-saponin can be developed into an alternative treatment modality to prevent recurrent pterygium after surgery.

Our study has some limitations. First, in vitro experiments are not enough to assess the toxicity or antifibrotic effect of agents, and thus the data cannot be extrapolated directly in clinical situations. Second, sakuraso-saponin treatments were administered for 24 hours, which differs from current clinical practice where intraoperative MMC is usually administered for

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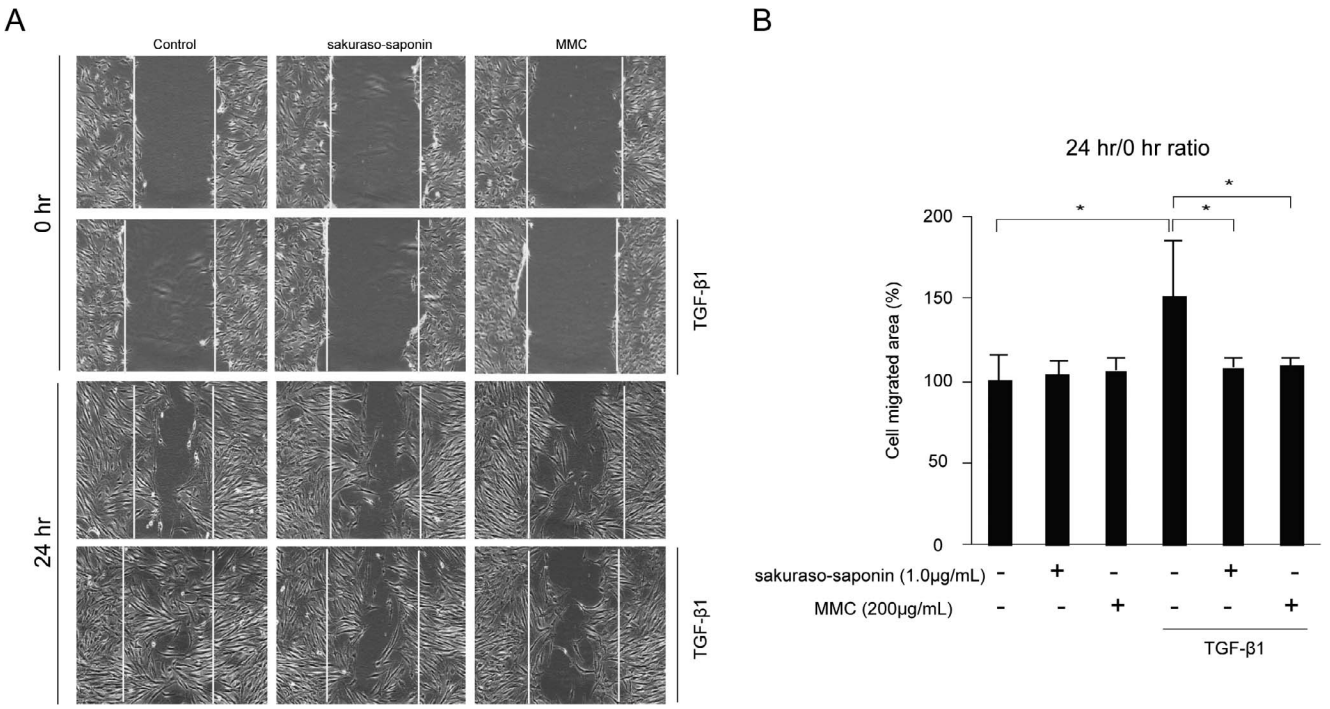

Figure 6. The effects of sakuraso-saponin and MMC in the presence or absence of TGF-β1 on cell migration in HPFs. (**A**) Cell migration assay phase-contrast microscope images at 24 hours. (**B**) Quantitative analysis of the wound area with representative data from three different sites using ImageJ software program. *Error bars* represent SEM (*$P < 0.05$).

a few minutes after pterygium excision. Additionally, the mechanism underlying the antifibrotic effects of sakuraso-saponin needs to be further investigated.

In summary, our study demonstrated that sakuraso-saponin is effective in reducing fibrosis in HPFs. Therefore, our study may support its use as a promising candidate drug for adjuvant therapy to prevent recurrence of pterygium after primary pterygium surgery.

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