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Glaucoma

Inhibitory Effects of 3′,4′-Dihydroxyflavonol in a Mouse Model of Glaucoma Filtration Surgery and TGFβ1-Induced Responses in Human Tenon's Fibroblasts

Jennifer C. Fan Gaskin[1–3], Roy C. K. Kong[4], Manisha H. Shah[1,3], Amanda J. Edgley[4], Hitesh M. Peshavariya[1,*], and Elsa C. Chan[1,3,4]

[1] Centre for Eye Research Australia, Royal Victorian Eye and Ear Hospital, Melbourne, Australia
[2] Glaucoma Investigation and Research Unit, Royal Victorian Eye and Ear Hospital, Melbourne, Australia
[3] Ophthalmology, Department of Surgery, University of Melbourne, Melbourne, Australia
[4] Department of Medicine, St. Vincent's Hospital, University of Melbourne, Melbourne, Australia

Correspondence: Jennifer Fan Gaskin, Centre for Eye Research Australia, 32 Gisborne Street, East Melbourne, Victoria 3002, Australia. e-mail: fan.j@unimelb.edu.au

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Purpose: Cytotoxic agents such as mitomycin C (MMC) are part of the mainstay treatment for limiting subconjunctival scarring following glaucoma filtration surgery (GFS). However, a safer antifibrotic therapy is clinically needed. The anti-scarring properties of 3′,4′-dihydroxyflavonol (DiOHF) were evaluated in a mouse model of GFS and in cultured human Tenon's fibroblasts (HTFs).

Methods: GFS was performed in C57BL/6 mice receiving daily intraperitoneal injections of DiOHF or vehicle or a single intraoperative injection of MMC. Eyes were harvested on day 14 for assessment of collagen deposition, expression of alpha-smooth muscle actin (α-SMA), cluster of differentiation 31 (CD31), and 4-hydroxy-2-nonenal (4HNE) in the conjunctiva/Tenon's layer. The inhibitory effects of DiOHF on transforming growth factor β (TGFβ)-induced responses were also assessed in HTFs.

Results: Treatment with DiOHF demonstrated a reduction in collagen deposition at the GFS site compared to vehicle-treated mice. The degree of 4HNE-positive fluorescence was significantly reduced in DiOHF-treated eyes compared to the other groups, indicating a decrease in oxidative stress. A reduction in expression of α-SMA and CD31 was seen in DiOHF-treated conjunctiva compared to those treated with vehicle. Concordant results were demonstrated in cultured HTFs in vitro. Furthermore, treatment of cultured HTFs with DiOHF also displayed a reduction in the proliferation, migration, and contractility of HTFs.

Conclusions: Treatment with DiOHF reduces scarring and angiogenesis in the conjunctiva of mice with GFS at a level comparable to MMC. The reduction in oxidative stress suggests that DiOHF may suppress scarring via different mechanisms from MMC.

Translational Relevance: DiOHF may be a safer and superior wound modulating agent than conventional antifibrotic therapy in GFS.

Introduction

Glaucoma is the leading cause of irreversible blindness. The prevalence of glaucoma is projected to increase from 76 million in 2020 to 111.8 million by 2040 worldwide.[1] Glaucoma filtration surgery (GFS) is widely acknowledged to be the most effective and cost-effective treatment for medically uncontrolled glaucoma.[2] It works by creating an opening that bypasses the trabecular meshwork and allows aqueous to drain into the subconjunctival space, thereby lowering the intraocular pressure (IOP) to protect the optic nerve. The surgical process is known to activate a key cell type known as Tenon's fibroblasts in the Tenon's layer and the conjunctiva. Activation of these cells enhances their proliferation and production of fibrotic proteins, leading to excess scar formation and

fibrosis in the conjunctival tissue, which is the principal cause of glaucoma surgery failure and inadequate control of IOP.[3] Despite most patients receiving adjunctive therapy with cytotoxic antiproliferative agents mitomycin C (MMC) and 5-fluorouracil (5-FU) during and following GFS in an attempt to reduce scarring, the failure rate remains at around 50% at 5 years.[4] The long-term use of these agents is limited due to the risk of complications such as endophthalmitis and corneoscleral damage.[5–7] The recent development of less invasive glaucoma stenting procedures is leading to a reconsideration of the role of surgery in glaucoma management, and it is anticipated that these new drainage devices will lead to earlier adoption of surgery.[8,9] Postoperative fibrosis is expected to substantially reduce the long-term benefit of these devices, and improved methods for fibrosis control are being keenly sought. Hence, there is an urgent unmet clinical need for nontoxic and effective therapeutic alternatives that could be used in conjunction with current surgical strategies to prevent excessive scar formation and improve long-term success following glaucoma surgery.

Transforming growth factor β (TGFβ) is a major profibrotic growth factor known to promote scar formation and fibrosis in ocular tissue.[10,11] The TGFβ family (isoforms β1, β2, and β3) plays a key role in fibrosis by converting fibroblasts into pathological myofibroblasts that express alpha-smooth muscle actin (α-SMA) and synthesize excess extracellular matrix components such as collagens and fibronectin.[12] TGFβ stimulation is essential for the activation of human Tenon's fibroblasts (HTFs) into myofibroblasts and induction of collagen production.[13] It has also been shown that, after glaucoma surgery, the filtering bleb (a reservoir in the conjunctiva) contains TGFβ,[13,14] highlighting the central role of TGFβ in surgical scarring. Accumulating evidence pinpoints a role of oxidative stress in the cellular processes that lead to fibrosis, and TGFβ is known to induce oxidative stress.[15] Moreover, an antioxidant flavonol quercetin has been found to reduce the release of collagen in human corneal fibroblasts and keratoconus cells under the stimulation of TGFβ1,[16] suggesting the beneficial effects of antioxidants in suppressing fibrotic responses.

Flavonoids are a group of polyphenolic compounds commonly found in colored vegetables, fruits, and green tea and are the most abundant antioxidants in the diet. The daily intake of flavonoids exceeds that of other known dietary antioxidants.[17] Thus, the amount of flavonoid intake could provide pharmacologically significant concentrations in body fluids and tissues. Flavonoids exhibit beneficial effects on multiple diseases, including cancer, cardiovascular diseases, and neurodegenerative disorders.[18] Many flavonoids act directly as antioxidants to neutralize toxic reactive oxygen species (ROS).[19] Systemic administration of a synthetic flavonol 3′,4′-dihydroxyflavonol (DiOHF)[19,20] has shown beneficial effects in rats with diabetes or ischemic–reperfusion damages, and the protection is attributed to the antioxidant activity.[19,20] We hypothesize that DiOHF limits scarring in the conjunctival tissue after GFS. The study objectives were to compare the antifibrotic effects of DiOHF against conventional MMC treatment in a mouse model of GFS and to evaluate the inhibitory effect of DiOHF on TGFβ1-induced responses in HTFs.

Materials and Methods

Glaucoma Filtration Surgery and Drug Treatment

Animal care guidelines adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures were approved by the institutional animal care and use committee (St. Vincent's Animal Ethics Committee protocol no. 012/18). GFS was performed only on the right eye of each male C57B/L6 mouse (7–8 weeks old; Animal Resource Centre, Murdoch, WA, Australia) as previously described.[21] The topical antibiotic eye drop Chlorsig (0.5%; Aspen Pharma, St. Leonards, NSW, Australia) was applied to the operated eyes to avoid any infection after surgery. Mice with similar starting weight were then randomly assigned to receive vehicle (n = 7, 22.3 ± 1.0 g or DiOHF (n = 7, 22.0 ± 1.3 g; Sigma-Aldrich, Castle Hill, NSW, Australia;) following surgery or MMC (Sigma-Aldrich) (n = 5, 22.6 ± 0.5 g; Sigma-Aldrich) during the surgery. Vehicle (0.1% dimethylsulfoxide [DMSO]; Sigma-Aldrich) and DiOHF (10 mg/kg bodyweight/d) was administered to mice via intraperitoneal injection (0.1 mL per mouse) on each day for 14 days, commencing on the day of surgery. MMC (0.4 mg/mL, 0.2 mL) was applied directly in the subconjunctival space as a subconjunctival injection through a 30-gauge needle intraoperatively. Dissection into the subconjunctival space was performed after 1 minute of MMC application, and the area was thoroughly irrigated with 5 mL of normal 0.9% saline solution. DiOHF was dissolved in DMSO and then suspended in sterile normal 0.9% saline (Baxter Healthcare, Old Toongabbie, NSW, Australia) containing Captisol (20% w/v; CyDex Pharmaceuticals, Lenexa, KS) for drug administration. Captisol[22] was used to improve the solubility of DiOHF. Vehicle was made up of DMSO (0.1%) in 0.9% saline

containing Captisol (20%). The concentration of DMSO was maintained at 0.1% in both DiOHF and vehicle treatment. To reduce bias, the surgeon did not administer the drug injections and was therefore masked to the treatment groups (except for those in the MMC group). Bleb photographs were taken at day 0 and day 14. At day 14, the blebs were de-identified for analysis in three categories modified from the Moorfields bleb grading system: size, vascularity, and ischemia.[23] A score for each category was given from 0 to 3, where 0 = no bleb/no additional vascularity/no ischemia; 1 = small bleb/mild additional vascularity/mild ischemia; 2 = moderate bleb/moderate additional vascularity/moderate ischemia; and 3 = large bleb/severe vascularity/severe ischemia. The study was terminated at 14 days after GFS. Paraformaldehyde (4%)-fixed eyes were mounted in agar and embedded in paraffin. Serial sections (4 µm thickness) were then used for picrosirius staining, immunohistochemistry, and immunofluorescence. These sections were de-identified, and analyses of these sections were completed by an investigator who was masked to the treatment groups.

Picrosirius Staining

Picrosirius red (ab150681; Abcam, Melbourne, VIC, Australia) was used to detect collagen in the eyes according to manufacturer's instructions. Sections were then imaged with Aperio CS2 (Leica Biosystems, Mount Waverley, VIC, Australia). Picrosirius red–positive staining at the surgical wound sites was identified using a Leica Scanscope based on an Aperio-positive pixel count algorithm. Three different sections from each animal were stained with picrosirius red, and the positive pixel counts to picrosirius red staining were averaged from three sections.

Immunohistochemistry and Immunofluorescence

Sections were subjected to heat-mediated antigen retrieval in citrate buffer for immunohistochemical staining of α-SMA. Following Dako protein block (Agilent, Santa Clara, CA), sections were incubated overnight with α-SMA antibody (1:2000, ab124964; Abcam), which was detected with Dako horseradish peroxidase polymer (K4002). Cluster of differentiation 31 (CD31; 1:150, #553370; BD Biosciences, Bedford, MA)-positive endothelial cells were identified as previously described.[24] Both antibodies were then visualized using Dako 3,3′-diaminobenzidine (DAB) chromogen. Sections were counterstained with hematoxylin and mounted in dibutylphthalate polystyrene xylene (DPX; Labworks, Knox City Centre, VIC, Australia). Rabbit and rat immunoglobulin G (IgG) was used as negative controls. The same method of analysis for picrosirius red was used to determine positive pixels to either α-SMA or CD31. Then, 4-hydroxy-2-nonenal (4HNE) antibody (1:100, ab46546; Abcam) was used to assess the level of oxidative stress in eye sections. Following an overnight incubation with 4HNE, sections were then incubated with a goat anti-rabbit antibody (Alexa Fluor 568; 1:200; Thermo Fisher Scientific, Waltham, MA). Nuclear counterstaining was performed with 4′,6-diamidino-2-phenylindole (DAPI; 10 mg/mL, 28718-90-3; Santa Cruz Biotechnology, Dallas, TX). Dako rabbit IgG was used as a negative control. Sections were mounted in Dako fluorescent mounting media and viewed under a fluorescence microscope (Carl Zeiss Australia, North Ryde, NSW, Australia). All images were taken with uniform exposure settings (400 ms). The fluorescence intensity of 4HNE-positive cytoplasmic staining at the subconjunctival surgical site was measured with Fiji software.[25]

Culturing of Human Tenon's Fibroblasts

The research was conducted in compliance with guidelines approved by the institutional ethics committee (Eye and Ear Hospital Human Research Ethics Committee project no. 16/1294H). Informed consent was obtained from patients after they were given an explanation of the nature of the study. HTFs were propagated from explanted subconjunctival Tenon's capsules collected during GFS performed in patients as previously described.[21] HTFs were maintained in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich) containing Gibco Penicillin–Streptomycin–Glutamine (Thermo Fisher Scientific) and 10% fetal calf serum (FCS; Cytiva, Marlborough, MA) at 37°C with 5% CO_2. The phenotype of the cultured HTFs was verified using anti–fibroblast-specific protein 1 (S100A4) antibody (1:100; ABF32; Merck Millipore, Bayswater, VIC, Australia) (Supplementary Fig. S1). HTFs were seeded in various sizes of Nunc cell culture plates (12-well, 24-well, and 96-well; Thermo Fisher Scientific) and were serum starved in DMEM with 1% FCS overnight for a range of assays as specified below. DiOHF was dissolved and diluted in DMSO, and the final concentration of DMSO in all assays was maintained at 0.1% DMSO. In all cell culture assays, HTFs were assigned to control (medium alone), TGFβ1 (10 ng/mL) + vehicle control (0.1% DMSO), or TGFβ1 (10 ng/mL) + DiOHF groups. Vehicle and

DiOHF were added 1 hour prior to TGFβ1 stimulation, unless otherwise specified.

^{3}H-Proline Incorporation Assay

The ^{3}H-proline incorporation assay was used to assess collagen synthesis by HTFs as previously described.[26] HTFs (30,000 cells/well) were seeded in 24-well plates and serum starved. Cells were treated with either vehicle (0.1% DMSO) or DiOHF (10 µM) for 1 hour. After the addition of TGFβ1 and ^{3}H-proline (1 µCi/mL, L-[2,3,4,5-^{3}H]-proline; Amersham Pharmacia Biotech, Amersham, UK), cells were incubated for a further 44 hours before determination of ^{3}H-proline with a beta counter (PerkinElmer, Rowville, VIC, Australia). Proline incorporation was normalized with protein determined by the Bradford protein assay (Bio-Rad Laboratories, Gladesville, NSW, Australia).

ROS Detection

The level of ROS was determined by 2′,7′-dichlorofluorescin diacetate (DCFDA) assay (2.5 µM, ab113851; Abcam) in HTFs according to the manufacturer's instruction. Cells were seeded in 96-well plates (30,000 cells/well) and serum starved. Fluorescence intensity was detected with a microplate reader (SpectraMax iD5; Molecular Devices, San Jose, CA) with excitation and emission of 485nm and 535 nm, respectively. Values were normalized using values from vehicle-treated HTFs. A subset of HTFs was imaged with an inverted fluorescence microscope (Nikon Australia, Rhodes, NSW, Australia) with the FITC setting at an exposure of 500 ms.

MTT Assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) reduction assay was used to evaluate the viability of HTFs. HTFs were seeded in 96-well plates (8000 cells/well). Vehicle or DiOHF (5, 10, or 50 µM) was added 1 hour prior to TGFβ1 stimulation and incubated for 25 hours in total. MMC (0.4 mg/mL) was added to the medium for 10 minutes and then washed off prior to TGFβ1 treatment. At 24 hours after the addition of TGFβ1, cells were incubated with MTT (0.5 mg/mL; Sigma-Aldrich) for 4 hours at 37°C. Isopropanol was used to solubilize the formazan. Absorbance was read at 570 nm with a microplate reader. Values were adjusted with values from vehicle-treated HTFs.

Migration Assay

Serum-starved HTFs were seeded in 24-well Transwell plates with 8-µm pore size (Corning Optical Communications, Mulgrave, VIC, Australia) at 37,500 cells/insert. Cells were treated with either vehicle or DiOHF (10 µM). TGFβ1 was added to the receiver well with medium (1% fetal bovine serum [FBS]) to stimulate cell migration. At 24 hours, migrated cells were trypsinized from the bottom side of the insert. Live cells were identified by trypan blue and counted. The extent of migration was expressed as the fold change of migrated cells compared to control.

Collagen Gel Contraction

The contractility of HTFs was assessed by collagen gel assay. A collagen solution was prepared in DMEM with 2% FBS containing NaOH, 10× phosphate-buffered saline, and rat tail collagen I (1 mg/mL, A1048301; Thermo Fisher Scientific) with 400,000 cells/mL. TGFβ1 was added to the collagen solution before seeding in 24-well plates (0.35 mL/well). Collagen was polymerized at 37°C for 30 minutes and then detached from the well edges. Gel (0.5 mL) was floated in serum-free medium with DiOHF (10 µM) or vehicle and imaged. The gel area was quantified using Fiji. Values are expressed as the percentage of the area of the control gel.

Cell Proliferation

The incorporation of bromodeoxyuridine (BrdU) into cells was used to determine the cell proliferation of HTFs, according to the manufacturer's instructions (ab126556; Abcam). Cells were seeded in 96-well plates (2000 cells/well). The absorbance was measured at 48 hours with a microplate reader (450 nm). Values were adjusted with values from the controls.

Determination of Collagen 1, Fibronectin, and α-SMA by Enzyme-Linked Immunoassay

HTFs were seeded in 12-well plates. Cells were harvested in Pierce IP Lysis Buffer with protease inhibitor cocktail (Thermo Fisher Scientific) at 24 hours after TGFβ1 treatment. Lysed cells were used for quantification of collagen 1 (RK01144; ABclonal Technology, Woburn, MA), fibronectin (RK01409; ABclonal Technology), and α-SMA (OKEH04279; Aviva, Tianjin, China) by enzyme-linked immunoassay. Values were normalized with protein determined by the Bradford protein assay (Bio-Rad Laboratories) and were expressed as fold changes of control.

Statistics

Data are expressed as mean ± standard deviation (SD). Mean data were analyzed with one-way or two-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis using Prism 9.0 (GraphPad, San Diego, CA). A value of $P < 0.05$ was regarded as statistically significant.

Results

MMC-Treated Blebs Displayed More Prominent Ischemia Than Mice Treated With DiOHF or Vehicle

Bleb analysis at day 14 did not identify any differences in the sizes and amount of vascularity between the groups; however, blebs treated with MMC were significantly more ischemic than those from the other two groups (Fig. 1).

DiOHF Suppressed Collagen Accumulation in Mice With GFS

Mice from all three treatment groups showed similar extents of weight gain over 14 days (Supplementary Fig. S2). Collagen deposition in bleb was identified with picrosirius staining. Systemic administration of DiOHF (10 mg/kg/d) significantly suppressed collagen accumulation at the wound in the conjunctiva of mice with GFS (Figs. 2A, 2B) at 14 days post-surgery. As depicted in Figure 2A, dense collagen fibers were seen at the surgical site in vehicle-treated mice. In contrast, MMC (0.4 mg/mL) treatment reduced the deposition of collagen, and DiOHF treatment even more so (Fig. 2B).

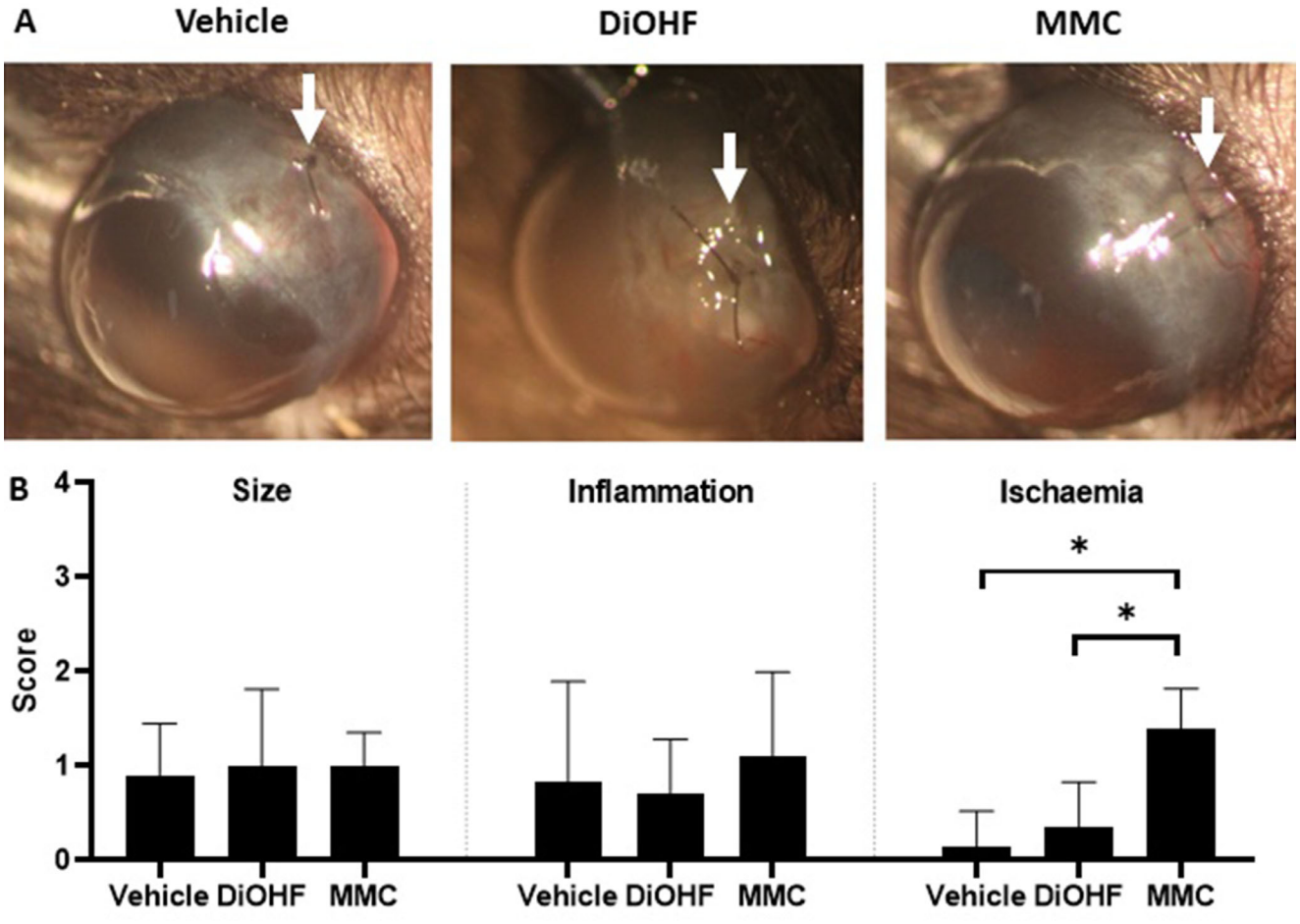

Figure 1. (**A**) Representative bleb images captured at 14 days after GFS from the vehicle, DiOHF, and MMC groups. The *arrow* points at the suture that was used to close the surgical wound after GFS. (**B**) Scoring of blebs based on size, the extent of inflammation, and ischemia at 14 days from mice treated with vehicle ($n = 7$), DiOHF ($n = 7$), or MMC ($n = 5$). *$P < 0.05$ (one-way ANOVA with Tukey's post hoc test).

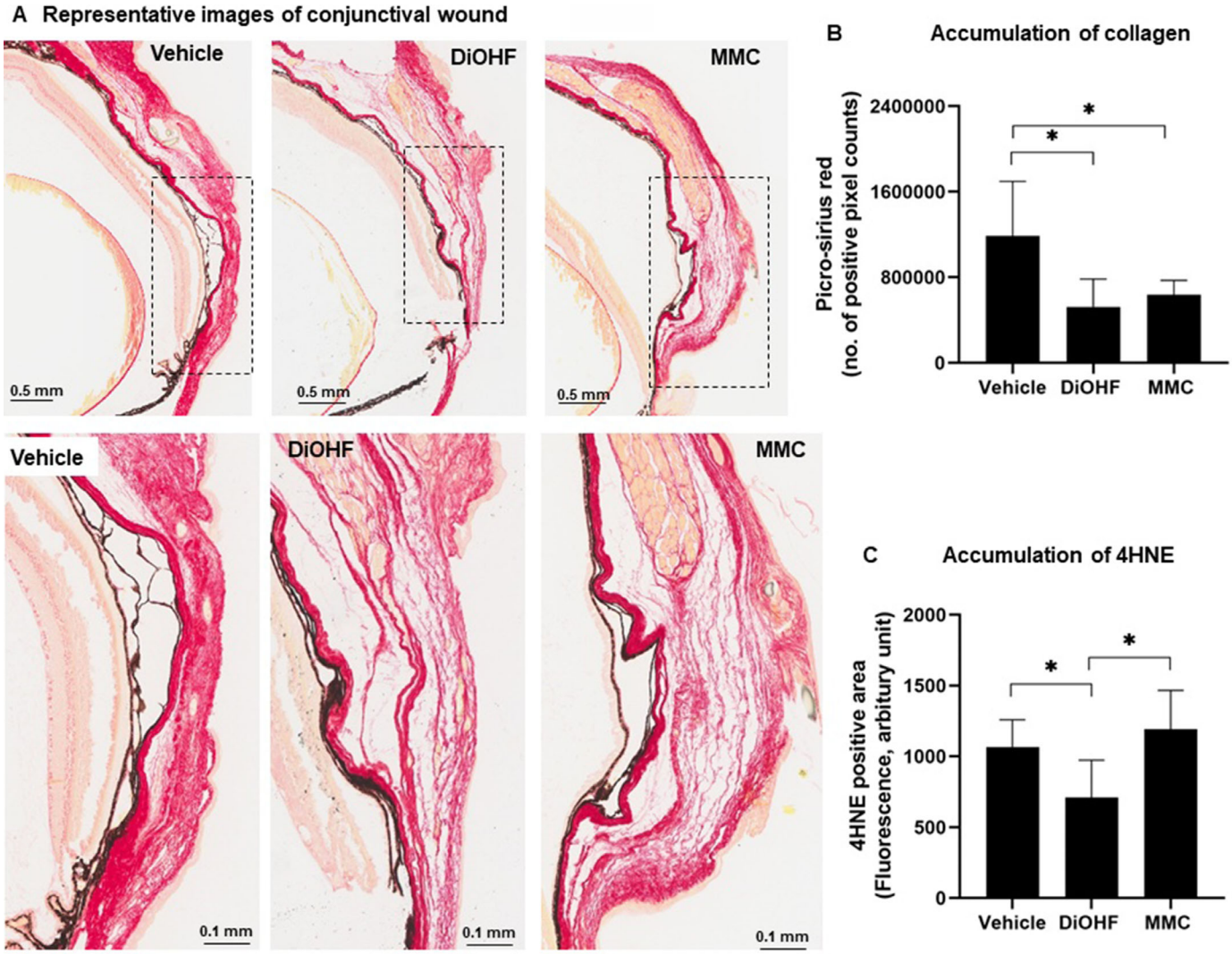

Figure 2. Accumulation of collagen and oxidative stress marker in eyes with GFS. (**A**) Representative picrosirius red–positive collagen at surgical sites in mice receiving intraperitoneal injections of vehicle or DiOHF for 14 days. MMC was given once intraoperatively. Higher magnification images of the areas are delineated by the *boxes*. (**B**) Quantitative data of areas positive to picrosirius red. (**C**) Quantitative measures of areas positive to oxidative stress marker 4HNE in mice with GFS treated with vehicle, DiOHF, or MMC. Vehicle, 0.1% DMSO; DiOHF, 10 mg/kg/d; and MMC, 0.4 mg/mL. *$P < 0.05$ (one-way ANOVA with Tukey's post hoc test; $n = 7$ for vehicle, $n = 7$ for DiOHF, and $n = 5$ for MMC).

Oxidative Stress in Conjunctiva Was Reduced by DiOHF in Mice With GFS

The extent of oxidative stress was evaluated by quantifying the cytoplasmic staining of an oxidative stress marker, 4HNE, at the surgical site. The degree of 4HNE-positive fluorescence was significantly reduced in mice with DiOHF-treated GFS. In contrast, intraoperative treatment with MMC did not suppress the extent of 4HNE-positive fluorescence in mice with GFS when compared to vehicle-treated mice (Fig. 2C).

DiOHF Attenuated Expression of α-SMA and CD31 in Mice With GFS

GFS has been shown to augment the expression of α-SMA and the density of blood vessels at the surgical site.[3,27] We assessed the expression of α-SMA and endothelial cell marker CD31 in operated eyes with immunohistochemistry. All treatment groups demonstrated accumulation of α-SMA and CD31 (Figs. 3A, 3B). α-SMA appeared to colocalize in CD31-positive blood vessels in all treatment groups (Figs. 3A, 3B). DiOHF (10 mg/kg/d) significantly

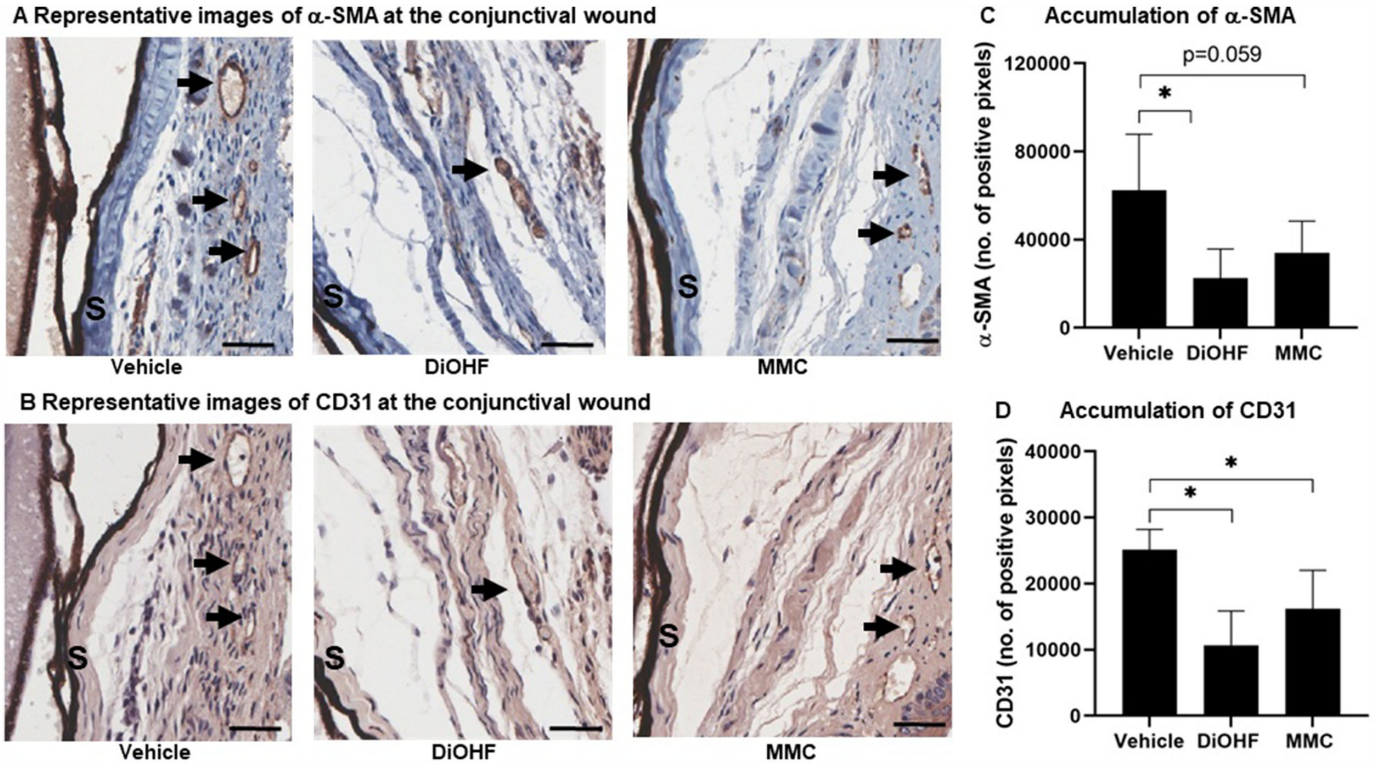

Figure 3. Expression of α-SMA and CD31 in eyes with GFS. (**A**, **B**) Representative images of operated eyes from mice receiving intraperitoneal injection of vehicle (0.1% DMSO) and DiOHF (10 mg/kg/d) for 14 days and a single intraoperative treatment of MMC (0.4 mg/mL). α-SMA and CD31 are identified as *brown*, and sections were counterstained with hematoxylin. α-SMA was mostly localized in CD31-positive blood vessels (identified by *arrows*). (**C**, **D**) Quantitative measures of positive pixels to α-SMA and CD31. S, sclera. *Scale bar*: 50 µm. *$P < 0.05$ (one-way ANOVA with Tukey's post hoc test; $n = 7$ for vehicle, $n = 7$ for DiOHF, and $n = 5$ for MMC).

suppressed the accumulation of both α-SMA and CD31 in the conjunctiva of the operated eye when compared to vehicle (Figs. 3C, 3D). MMC treatment showed a tendency to decrease α-SMA ($n = 5$ mice/group; $P = 0.059$ vs. vehicle, one-way ANOVA) (Fig. 3C) and significantly reduced CD31 expression when compared to vehicle (Fig. 3D).

DiOHF Suppressed Collagen Synthesis and ROS Production in HTFs

The effect of DiOHF on collagen synthesis was evaluated by the incorporation of ^{3}H-proline in HTFs. The induction of ^{3}H incorporation by TGFβ1 (10 ng/mL) was significantly suppressed by DiOHF at both 5 and 10 µM (Fig. 4A). Although the extent of ^{3}H-proline incorporation was normalized to protein, we still wanted to verify whether DiOHF caused any toxicity in HTFs that could contribute to the inhibitory effect on collagen synthesis. As such, we determined the cell viability of HTFs in the presence of DiOHF using the MTT assay. DiOHF at 5, 10, and 50 µM did not affect the cell viability of HTFs (Fig. 4B). We also investigated whether MMC at 0.4 mg/mL affected cell viability. MMC significantly reduced the viability of HTFs ($n = 3$). Given that DiOHF is a known antioxidant, we quantified cellular ROS with the cell-permeable dye DCFDA, which is subsequently oxidized by ROS into 2′,7′-dichlorofluorescin (DCF). As shown in Figure 4C, the level of DCF fluorescence was enhanced by TGFβ1 (10 ng/mL), and this response was reduced by DiOHF (5 and 10 µM). Representative images of HTFs demonstrate that TGFβ1 caused increases in DCF fluorescence in HTFs (Fig. 4D). DiOHF treatment (10 µM) reduced the accumulation of DCF fluorescence in HTFs (Fig. 4D).

DiOHF Inhibited Proliferation and Migration of HTFs

The inhibitory effect of DiOHF on cellular responses that contribute to fibrosis was further explored using 10 µM of DiOHF. We and others have shown that TGFβ1 induces migration and

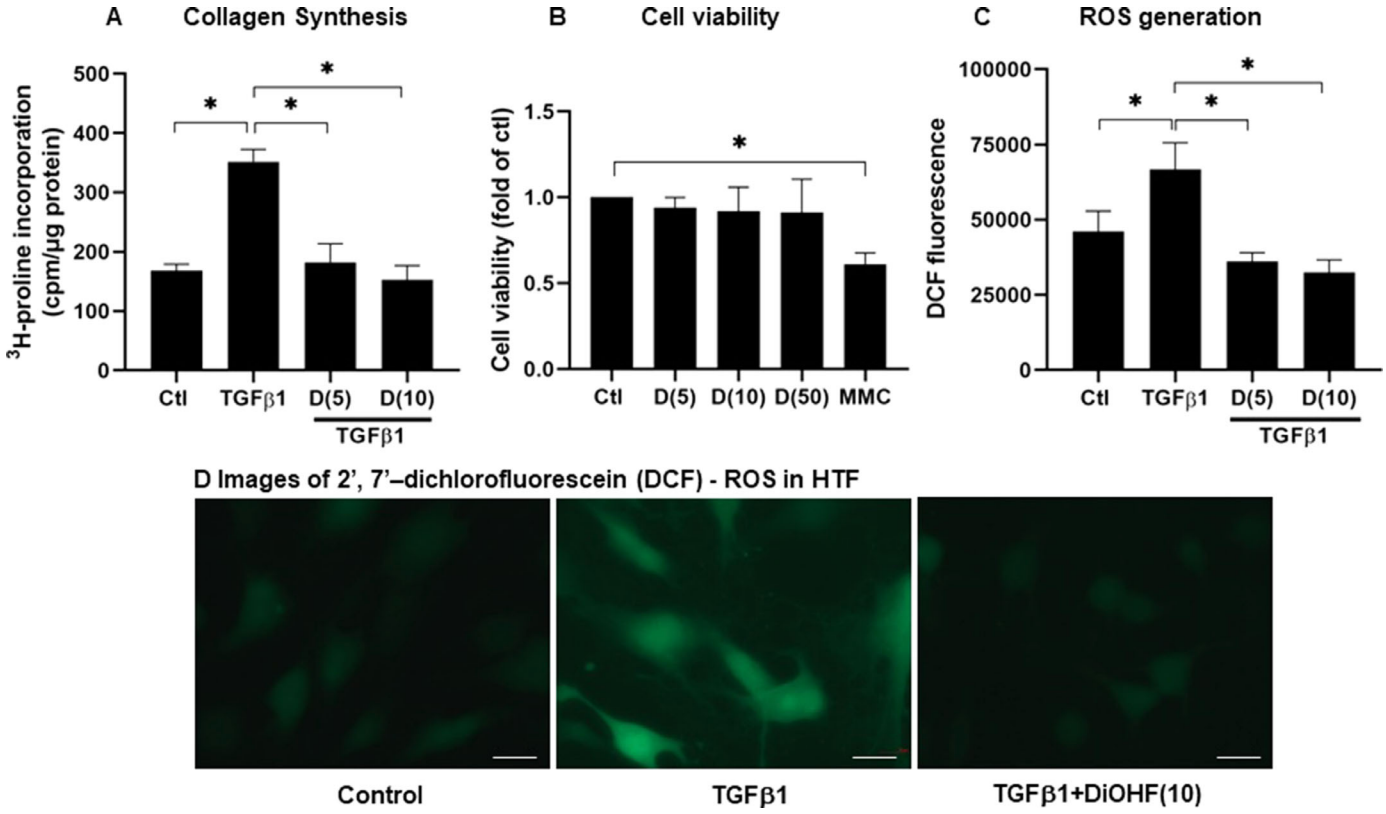

Figure 4. Effect of DiOHF on ^{3}H-proline incorporation and ROS production in HTFs. (**A**) DiOHF (abbreviated as "D" at 5 and 10 µM) significantly inhibited TGFβ1-induced incorporation of ^{3}H-proline. (**B**) DiOHF at up to 50 µM did not affect the cell viability of HTFs. MMC (0.4 mg/mL) reduced cell viability. (**C**) DiOHF (5 and 10 µM) significantly suppressed the TGFβ1 (10 ng/mL) induction of DCF fluorescence as a measure of ROS generation. (**D**) Images demonstrate less fluorescence signal in HTFs treated with DiOHF (10 µM) in the presence of TGFβ1 stimulation (10 ng/mL). Ctl, control. *Scale bar*: 50 µm. *$P < 0.05$ (one-way ANOVA with Tukey's post hoc test; $n = 3$ independent experiments in duplicate). Data are expressed as mean ± SD.

proliferation of cells that are important for fibrosis.[12,28] As such, we determined whether DiOHF affected these responses. TGFβ1 (10 ng/mL) significantly enhanced the incorporation of the cell proliferation marker BrdU in HTFs (Fig. 5A). DiOHF (10 µM) abolished the effect of TGFβ1 on cell prolifera-

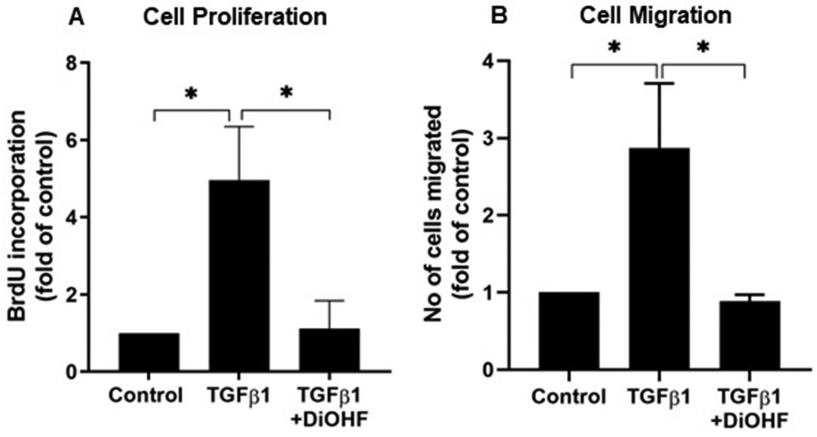

Figure 5. Effect of DiOHF on the proliferation and migration of HTFs. (**A**) DiOHF (10 µM) significantly inhibited TGFβ1 (10 ng/mL)-induced incorporation of the cell proliferation marker BrdU into HTFs. (**B**) DiOHF (10 µM) reduced the migration of HTFs. *$P < 0.01$ (one-way ANOVA with Tukey's post hoc test; $n = 3$ independent experiments in duplicate). Data are expressed as mean ± SD.

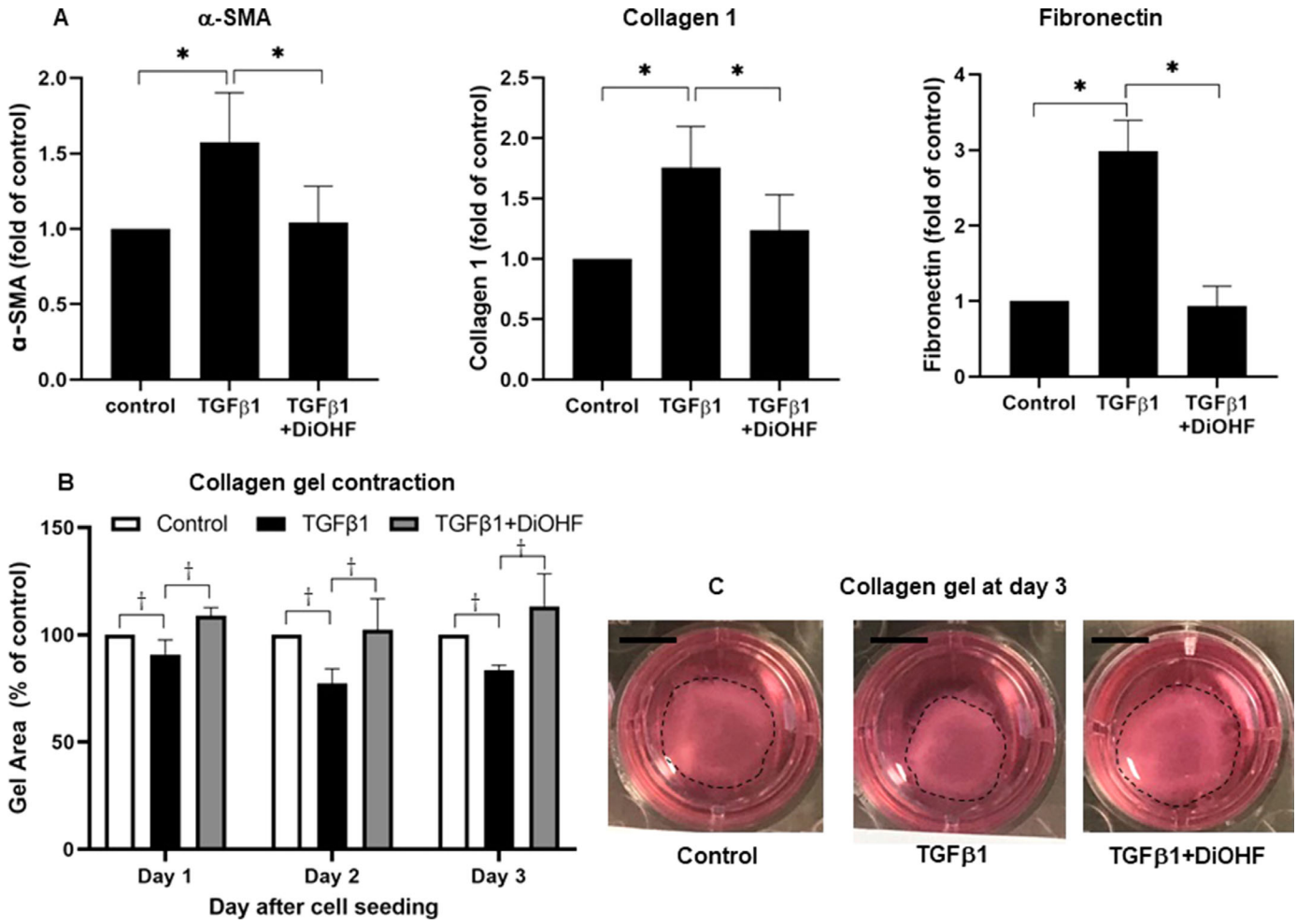

Figure 6. Effect of DiOHF on the contraction of HTFs and TGFβ1-induced expression of fibrotic proteins. (**A**) TGFβ1 (10 ng/mL)-augmented expression of α-SMA, collagen 1, and fibronectin was suppressed by DiOHF (10 µM). (**B**) TGFβ1 (10 ng/mL) significantly enhanced HTF-induced contraction of collagen gel as reflected by decreases in gel area. DiOHF (10 µM) significantly inhibited the stimulatory effect of TGFβ1 on HTF-induced contraction at days 1, 2, and 3 after collagen gel release. (**C**) Representative photographs of collagen gel mixed with HTFs assigned to treatment groups: control, TGFβ1 (10 ng/mL), or TGFβ1 (10 ng/mL) + DiOHF (10 µM) on day 3. *Scale bar*: 0.5 mm. The *outlined area* was measured and used for data comparison in (**B**). *$P < 0.01$ (one-way ANOVA with Tukey's post hoc test); †$P < 0.01$ (two-way ANOVA with Tukey's post hoc test). Data are expressed as mean ± SD ($n = 3$ independent experiments in duplicate).

tion (Fig. 5A). Using the Transwell migration assay, TGFβ1 (10 ng/mL) stimulated migration of HTFs and was blocked by DiOHF (10 µM) (Fig. 5B).

DiOHF Suppressed Contractility of HTFs

During wound healing, fibroblasts have been known to differentiate into the contractile phenotype termed myofibroblasts, increasing the production of extracellular matrix protein.[12] DiOHF (10 µM) significantly suppressed the protein expression of myofibroblast marker α-SMA and extracellular matrix proteins collagen 1 and fibronectin (Fig. 6A) in the presence of TGFβ1 (10 ng/mL) in HTFs. Contraction of HTFs was evaluated by suspending HTFs in a collagen gel. TGFβ1 (10 ng/mL) significantly decreased the area of the collagen gel on days 1, 2, and 3 ($n = 3$; $P < 0.01$) (Fig. 6B). A reduction in gel area indicated gel contraction, as depicted in Figure 6C. There was no statistically significant difference in the gel size exposed to TGFβ1 over the 3-day period (day 1, 91% ± 4%; day 2, 77% ± 4%; day 3, 84% ± 1%; $n = 3$; $P = 0.60$, two-way ANOVA). DiOHF (10 µM) significantly prevented the stimulatory effect of TGFβ1 on HTF-induced collagen contraction at all time points (Fig. 6A).

Discussion

Scarring remains the major cause of surgical failure following GFS. Cytotoxic antiproliferative agents

MMC and 5-FU are routinely administered during and following GFS in an attempt to control scarring, despite considerable potential complications and side effects.[4] We have identified a synthetic flavonol that limits collagen deposition and angiogenesis in the conjunctiva after GFS in mice. The decreases in collagen, α-SMA, and CD31 in the operated eyes were associated with a reduction in the expression of oxidative stress marker 4HNE, pointing to the antioxidant effect of DiOHF in suppressing wound healing responses in the conjunctiva. Using HTFs, we demonstrated that DiOHF inhibited cellular responses that are attributable to scarring, including ROS production, collagen synthesis and proliferation, migration, and contraction of fibroblasts. Unlike MMC, DiOHF did not induce toxicity in HTFs. DiOHF may therefore represent an alternative and superior means of limiting conjunctival scarring associated with GFS.

Studies of antioxidant flavonoids, including epicatechin[29] and arctigenin,[30] have demonstrated a role in oxidative stress in murine models of pathological scarring such as bleomycin-induced fibrosis in the lung and the skin. In these studies, epicatechin and arctigenin were administered to mice through intraperitoneal and oral routes, respectively,[29,30] and both flavonoids effectively alleviated bleomycin-induced scarring. Presumably, the antifibrotic activity of flavonoids seen in the lung and the skin should also be applicable to ocular tissues. Despite the demonstration of antifibrotic activity of flavonoids such as quercetin and epigallocatechin-3-gallate (EGCG) in fibroblasts cultured from human cornea and Tenon's biopsy,[16,31] no study has yet clarified the protective effects of flavonoids in an animal model of GFS. The present study established that systemic administration of DiOHF reduces the accumulation of collagen in the conjunctiva following GFS. Although the anti-scarring effect of DiOHF is comparable to the therapeutic efficacy of cytotoxic agent MMC in the mice with GFS, MMC did not affect oxidative stress in the conjunctiva, thus suggesting that the anti-scarring effect of DiOHF is attributable to its antioxidant activity. We and others[19,20] have verified that DiOHF reduced the level of ROS in HTFs and mesenteric and aorta from mice and rats and in a cell-free system. Furthermore, MMC works by triggering cell apoptosis and inducing widespread cell death in order to prevent scarring in mice with GFS.[3] As such, our findings show that DiOHF and MMC limit conjunctival scarring in the operated mice via different mechanisms of action.

The activation of Tenon's fibroblasts in the conjunctiva by TGFβ is one of the major causes of scarring following GFS.[13,14,32] In addition, there are increases in TGFβ proteins in an experimental model of GFS.[33] We therefore used HTFs to explore the antifibrotic mechanisms of DiOHF under the stimulation of TGFβ1. DiOHF was found to attenuate the synthesis of collagen by preventing the incorporation of ^{3}H-proline to newly synthesized collagen under the stimulation of TGFβ1 in HTFs, suggesting that DiOHF treatment in the mice inhibits the de novo synthesis of collagen that occurs following GFS. Apart from directly interfering with collagen synthesis, DiOHF also inhibited TGFβ1-induced transformation of fibroblasts to myofibroblasts in HTFs, as reflected by a reduction in the expression of myofibroblast marker α-SMA. Myofibroblasts are known to actively induce the synthesis of matrix proteins that promotes pathological scarring.[34] It has also been known that myofibroblasts promote wound contraction in healing responses.[35,36] Although it was difficult to assess wound contraction in vivo, we used an established three-dimensional collagen gel assay[37] to verify whether DiOHF affected TGFβ1-induced contraction of HTFs. DiOHF markedly prevented contraction of the collagen gel on each day for up to 3 days. Because there was a reduction in TGFβ1-induced α-SMA expression associated with DiOHF, the inhibition of gel contractility may be attributable to a reduced differentiation of fibroblasts into the contractile myofibroblasts. However, it has also been proposed that a large population of individual fibroblasts aggregating at the wound could also induce contraction.[38] It should be noted that DiOHF prevented TGFβ1-induced proliferation of HTFs; therefore, the inhibited gel contraction by DiOHF may not be solely caused by a reduction of myofibroblast differentiation. Having inhibitory effects on both cell contraction and collagen deposition would greatly enhance the potential for DiOHF to improve surgical success because contraction of scarred tissues can block the drainage of aqueous humor, resulting in inadequate control of IOP.[32]

We attempted to evaluate the expression of myofibroblasts in the wound using the myofibroblast marker α-SMA. Interestingly, α-SMA–positive staining appeared to be predominantly seen in regions surrounding lumina that resembled blood vessels. We confirmed with the endothelial cell marker CD31 that α-SMA was indeed primarily localized in CD31-positive vessels. The present findings are consistent with the demonstration of α-SMA expression in cells surrounding blood vessels in operated eyes of animals with GFS.[3] Park et al.[39] previously showed an abundant accumulation of α-SMA–positive myofibroblasts in the conjunctival wound at day 5 after GFS, suggesting that the accumulation of myofibroblasts may occur in the early phase of wound healing following GFS. The inhibitory effect of DiOHF on

GFS-induced angiogenesis is consistent with a previous study that also demonstrated a reduction in the density of CD31-positive vessels in the conjunctiva of rabbits receiving a topical anti-scarring agent following GFS.[27] There have been several investigations on the potential anti-scarring effect of anti-vascular endothelial growth factor (VEGF) agents such as bevacizumab in experimental models of GFS.[33,40,41] The majority of these studies have shown that anti-VEGF agents limited postoperative scarring when given as an adjunct with either MMC[40] or 5-FU,[41] but not as a monotherapy.

The dose selection of DiOHF for evaluating the antifibrotic activity was based on previous studies that showed protection in mice with diabetes, ischemia, and reperfusion injury and in isolated mouse arteries.[19,20] DiOHF at 10 µM displayed maximal responses in suppressing the synthesis of collagen and ROS in HTFs. Such concentrations are comparable to those seen in other flavonoid compounds such as EGCG, quercetin, luteolin, and myricetin in HTFs,[31] as well as human dermal fibroblasts[42] that produce an antifibrotic action. Although the present study demonstrated that daily intraperitoneal administration of DiOHF limited postoperative scarring, future studies investigating topical DiOHF in eye drop form to conform with the post-GFS standard-of-care medication delivery route are planned.

The delivery of flavonoids to tissues has some challenges, such as tissue bioavailability. Captisol has been used as a drug vehicle to improve the solubility of flavonoids in order to facilitate the penetration of flavonoids to target sites.[43] Captisol has also been used to facilitate the incorporation of flavonoids to nanoparticles for slow release.[44,45] As such, we have also used Captisol to improve the bioavailability of DiOHF for systemic administration.[44,45] Although it is conceivable that the vehicle treatments may have some effect on the scarring responses in both the cell culture and animal studies, both experiment models included appropriate vehicle controls; therefore, the potential effect of the vehicles on fibrosis should be accounted for. As such, we are confident in concluding that the antifibrotic effect of DiOHF is attributable to the compound.

GFS is known to cause inflammation, and uncontrolled inflammation can contribute to scarring. As flavonoids have been shown to be antiinflammatory due to their antioxidant properties,[46] the potential antiinflammatory effect of DiOHF in the initial inflammatory phase following GFS may be of significant benefit. Further study would be required to verify if DiOHF affects the infiltration of inflammatory cells in the conjunctival wound following GFS.

We were unable to demonstrate a difference in the size or vascularity of the blebs at day 14 via photographic assessment. This method of assessment provides limited resolution of these clinical features due to the small size of mouse blebs; therefore, the emphasis of our study was on the histological and immunohistochemical differences between groups. Ischemia in blebs is identified as a risk factor for bleb thinning, leakage, and subsequent infection and is an undesirable feature in blebs.[47] It is associated with nonspecific cytotoxic agents such as MMC and 5-FU. The differences seen at day 14 among the DiOHF-treated, vehicle-treated, and MMC-treated blebs support the minimal effect on cell viability seen in DiOHF-treated HTFs in vitro.

In conclusion, our findings demonstrated that DiOHF suppresses pathological angiogenesis and scarring in the conjunctival wound of mice following GFS through an antioxidant effect. Although the anti-scarring effect of DiOHF is similar to the efficacy of standard therapy MMC in mice with GFS, the lack of cytotoxicity and the multiple antifibrotic mechanisms suggest that DiOHF may be a superior alternative in the control of postoperative scarring following GFS.

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