Integrin α5β1 Inhibition by CLT-28643 Reduces Postoperative Wound Healing in a Mouse Model of Glaucoma Filtration Surgery

Tine Van Bergen,[1] Grit Zahn,[2] Patrizia Caldirola,[2] Mario Fsadni,[2,3] Ninus Caram-Lelham,[2] Evelien Vandewalle,[1,4] Lieve Moons,[1,5] and Ingeborg Stalmans[1,4]

[1]Laboratory of Ophthalmology, KU Leuven, Leuven, Belgium
[2]Clanotech, Stockholm, Sweden
[3]International Pharm-Med Ltd., Bramhall, United Kingdom
[4]Department of Ophthalmology, University Hospitals Leuven, Leuven, Belgium
[5]Unit of Animal Physiology and Neurobiology, Biology Department, KU Leuven, Leuven, Belgium

Correspondence: Ingeborg Stalmans, University Hospitals Leuven, Department of Ophthalmology, Kapucijnenvoer 33, B-3000, Leuven, Belgium;
Ingeborg.Stalmans@uzleuven.be.

Submitted: April 17, 2016
Accepted: October 2, 2016

Citation: Van Bergen T, Zahn G, Caldirola P, et al. Integrin α5β1 inhibition by CLT-28643 reduces postoperative wound healing in a mouse model of glaucoma filtration surgery. *Invest Ophthalmol Vis Sci*. 2016;57:6428–6439. DOI:10.1167/iovs.16-19751

Purpose. To evaluate the therapeutic potential of the small molecule integrin α5β1 inhibitor, CLT-28643, to improve the filtering surgery outcome in a mouse model. Different dose regimens and administration routes of the inhibitor were compared with mitomycin C (MMC), the gold standard in clin ical practice.

Methods. The efficacy of CLT-28643 on surgical outcome was studied in a mouse model for filtering surgery ($n = 40$ eyes from 20 mice per group). Single and repeated subconjunctival (SCJ) injections (1 or 2 μg) and topical eye drops (10 μg) of the integrin inhibitor were compared with 2-minute administration of MMC 0.02%. Bleb size, survival, and signs of toxicity were examined until 28 days after surgery. Immunohistochemical analysis of angiogenesis, inflammation, collagen deposition, and integrin α5β1 expression were performed on postoperative days 3, 8, 14, and 28. A masked observer performed all the assessments.

Results. Immunostaining showed that integrin α5β1 was highly expressed in the bleb at early time-points after surgery and that CLT-28643 inhibited this upregulation. Efficacy was shown to be dose-dependent for the integrin inhibitor CLT-28643 for bleb area and survival, and the wound healing process. While 2-μg single injection of CLT-28643 improved bleb characteristics in a similar way as 10-μg administered by eye drops and MMC, repeated injections of 2 μg showed superior efficacy compared to MMC, with no corneal toxicity.

Conclusions. Administration of the integrin α5β1 inhibitor CLT-28643 has therapeutic potential as an adjunct to glaucoma surgery, possibly with a superior efficacy and tolerability compared with MMC when used at the optimal dose.

Keywords: glaucoma surgery, wound healing, integrin

The importance of fibrosis in various pathologic processes in the eye is known. With respect to glaucoma surgery, success depends on the correct balance between adequate wound healing and maintaining a functioning fistula. Surgery immediately triggers a series of cellular and extracellular cascades to promote the wound healing process.[1] Several cell types are involved with interaction with other cell types and various membrane proteins by means of cell surface integrins.[2,3] Integrins constitute an important class of transmembrane receptors responsible not only for cell–cell adhesion, but also for cell–extracellular matrix (ECM) interaction.[4] Among these is integrin α5β1, a well-characterized fibronectin receptor, and known to be an important player in angiogenesis within several organs, including the eye. The α5 subunit is found only in combination with β1 and this determines its receptor activity.[5]

Fibronectin is a major component of ECM and binds to a number of integrins mostly through its arginine-glycine-aspartic acid (RGD). Integrin α5β1 is probably the major fibronectin receptor in most tissues.[5] The knockout of the α5 subunit in mice resulted in nearly the same defects as the fibronectin-null mutation,[5,6] suggesting that most of the effects of fibronectin result from its binding to integrin α5β1. In quiescent cells, integrin α5β1 is absent or inactive. In many pathologic and some physiological processes requiring the proliferation and migration of cells, integrin α5β1 becomes upregulated and activated. The activated integrin-fibronectin complex is involved in many processes, such as angiogenesis,[7] inflammation,[8,9] wound healing,[2] and contributes to many diseases, for example, psoriasis[10] and cancer.[11–13] In angiogenesis, integrin α5β1 binding to ECM regulates the proliferation, motility, and survival of endothelial cells[14] and antibodies and other inhibitors of integrin α5β1 have an antiangiogenic effect.[15–17] Integrin α5β1 is also involved in the interaction between fibroblast and ECM. In vitro data have shown the upregulation of integrin α5β1 expression after stimulation of fibroblast or other myofibroblast precursor-cells with the profibrotic growth factor TGF-β, platelet-derived growth factor (PDGF), connective

Table 1. Overview of Different Treatment Groups

Group, $n = 120$ Mice	Compound	Dose	Administration
Group 1, $n = 20$	Vehicle	NA	SCJ injections D0, 3, 7, 14, and 21
Group 2, $n = 20$	MMC	0.02%	2-min sponge application + 2 mL rinse NaCl
Group 3, $n = 20$	CLT-28643	2 μg	SCJ injection D0
Group 4, $n = 20$	CLT-28643	2 μg	SCJ injections D0, 3, 7, 14, and 21
Group 5, $n = 20$	CLT-28643	1 μg	SCJ injections D0, 3, 7, 14, and 21
Group 6, $n = 20$	CLT-28643	10 μg	3× drops daily

SCJ, subconjunctival; D, day; NA, not applicable.

tissue growth factor (CTGF), as well as the promotion of fibrotic differentiation by fibronectin.[18–21] The important role of integrin α5β1 in fibrosis was also shown in vivo in kidney, lung, liver, and skin fibrosis models.[22–26]

Moreover, Zahn et al.[27] showed that the integrin α5β1-fibronectin interaction is a promising target for the treatment of fibrotic and inflammatory processes in the eye such as proliferative vitreoretinopathy (PVR). In another study, the administration of nonpeptidic analogues of the RGD sequence specifically inhibited the adhesion of human Tenon's capsule fibroblasts to fibronectin in culture.[28] Furthermore, the benefit of nonspecific integrin inhibition in glaucoma surgery was shown by the positive effect of RGD peptides in improvement of the surgical outcome in rabbit.[29,30] Importantly, the specific role of integrin α5β1 in the postoperative process of wound healing is not fully understood. These findings, however, suggest that integrin α5β1 might be a therapeutic target to prevent filtration failure after glaucoma surgery, because excessive postoperative wound healing of the conjunctiva and Tenon's capsule, with subsequent scarring, frequently leads to surgical failure.[31] In the present study, we demonstrated the expression and the time course of integrin α5β1 in the bleb following glaucoma surgery in a mouse model. The same model was subsequently used to investigate different dose regimens of a novel integrin α5β1 inhibitor, CLT-28643, and compared with the current standard of care, mitomycin C (MMC).

CLT-28643 is a novel small molecule with specific integrin α5β1 inhibiting properties. It inhibits RGD-induced conformational changes of integrin α5β1 to prevent integrin α5β1-fibronectin interaction. In in vivo studies in xeno- and synograft tumor models, CLT-28643 inhibited tumor growth and angiogenesis.[32] The inhibition of fibrosis and inflammation of CLT-28643 was shown in a bleomycin-induced lung fibrosis model.[33]

Materials and Methods

Mouse Model of Glaucoma Filtration Surgery

In this study, C57BL/6J mice (8- to 10-weeks old; Charles River Laboratories, Lyon, France) were used in accordance with the standards in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The Institutional Animal Care and Research Advisory Committee of KU Leuven approved all experimental animal procedures (P084/2014).

All mice were anesthetized with an intraperitoneal injection of 10× diluted (60 mg/kg final dose) sodium pentobarbital (Nembutal, 60 mg/mL; CEVA, Sante Animale, Brussels, Belgium). Filtering surgery was performed on both eyes, using a technique that has been described previously and that results in a filtering bleb.[34–36] Briefly, the conjunctiva was first surgically dissected to expose the underlying sclera and a small filtration subconjunctival space was created by running the surgical scissors underneath the dissected conjunctiva. Next, a 30-G needle was used to make an incision through the sclera into the anterior chamber of the eye to allow aqueous humor to escape into the subconjunctival space. Finally, the conjunctiva was closed at the limbus by suturing over the newly-created fistula (using a 10-0 nylon suture; Alcon, Vilvoorde, Belgium). Tobradex ointment (SA Alcon-Couvreur, Vilvoorde, Belgium) was applied at the end of the procedure.

Treatment Scheme

In a first experiment, five mice (10 eyes) per time-point were used to evaluate integrin α5β1 expression in the bleb on postoperative days 3, 8, 14, and 28 by immunohistochemistry (see section Immunohistologic Investigation). In a second experiment, 120 mice were divided in 6 groups of 20 mice each (40 eyes/group) in order to investigate the in vivo efficacy of CLT-28643 on bleb characteristics and compare its effects with MMC. An overview of the different groups is given in Table 1. For study medication, ready-to-use formulations of vehicle and integrin inhibitor, small molecule CLT-28643 (0.2%; provided by Clanotech AB, Stockholm, Sweden), were used. In groups 1, 4, and 5, repeated subconjunctival (SCJ) injections (1 μL) of vehicle or CLT-28643 (2 or 1 μg), respectively, were given on days 0, 3, 7, 14, and 21 after surgery. In group 2, surgical sponges soaked with MMC (0.02%) were placed on the exposed sclera for 2 minutes before creating the channel. After removing the sponge, the ocular tissue was extensively rinsed with 2 mL of NaCl. In group 3, single injection (1 μL; 2 μg) of the integrin inhibitor was administered immediately after surgery. Group 6 was treated with CLT-28643 drops (5 μL, 10 μg) 3× daily until killed. All injections were performed in both eyes by using an analytic science syringe (SGE Analytic Science, Victoria, Australia) and glass capillaries with a diameter of 50 to 70 μm at the end, controlled by the UMP3I Microsyringe Injector and Micro4 Controller (all from World Precision Instruments, Inc., Hertfordshire, UK).

Clinical Investigation

Mice were clinically examined on day 1 after surgery and then every 2 days until they were killed. Bleb area and bleb survival were analyzed under topical anesthesia (Unicain, Théa Pharma, France). Commercial software (KS300; Zeiss, Brussels, Belgium) was used to determine the bleb size from bleb images of mice. More information on the measurement of the bleb area is provided in the Supplementary Material. These pictures were taken using a digital camera (Canon PowerShot S50; Canon, Brussels, Belgium) using a ×3 optical zoom lens at a magnification of ×4. Of note, pictures were taken before repeated administration of the compound, when the two procedures took place on the same day. The boundaries of the bleb on the images were defined based on the appearance of the conjunctiva. Whitish conjunctiva indicates that the conjunctiva was separated from the sclera and was filled with fluid. On the other hand, a bluish/grayish conjunctiva indicates that the conjunctiva was attached to the sclera without any

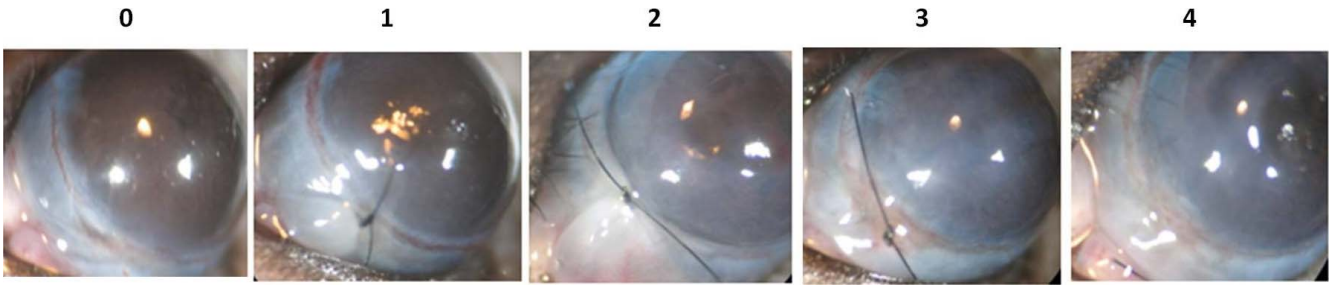

FIGURE 1. Corneal opacity grading, ranging from 0 for a clear/transparent cornea to 4 for complete corneal opacity.

fluid between the two layers (Supplementary Fig. S1A). Bleb survival was taken as the end-point of the study, while bleb failure was defined as the appearance of a scarred and flat bleb on two consecutive measurements.[34] Failure of the bleb was recorded at the second measurement. Also, corneal opacity was graded on these pictures at days 1, 3, 5, 7, 13, 21, and 27 after surgery, using a previously published scoring system,[37,38] ranging from 0 for a clear/transparent cornea to 4 for complete corneal opacity. Figure 1 shows representative pictures of the different scores of corneal opacities. The examiner of the mice was masked to the treatment allocation for all analyses. At the end of the study, all images were randomized and the analysis was performed on the pictures in a masked observatory way, all by one blinded observer. Mice were also checked for pain-distress every other day and body weight was measured on day 0 before surgery and every week until killed.

Immunohistologic Investigation

On postoperative days 3, 8, 14, and 28 after surgery, mice were killed by cervical dislocation (5 mice [10 eyes]/group/time point). Both eyes were enucleated and whole eyes were fixed in 1% paraformaldehyde overnight and rinsed three times for 5 minutes in PBS. The tissues were then dehydrated overnight in the Shandon Excelsior ES (Thermo Fisher Scientific, Waltham, MA, USA) and embedded in paraffin, all eyes in the same orientation. Serial sagittal sections were cut at 7-μm thickness on five series of five glass slides (total number of slides per eye is 25). Hematoxylin and eosin (H&E) staining was performed on the first slide of each series (slide 1, 6, 11, 16, and 21) to localize the bleb on the various sections (see section 'Imaging and Analysis'). The consecutive slides were used for the other immunohistologic staining. To investigate integrin α5β1 expression, an integrin staining was performed using rabbit anti-mouse integrin antibody (1/200, AB1928; Millipore, Overijse, Belgium). Slides without primary antibody were used as a negative control and the cornea was used as a positive control, because it is known that integrin α5 is expressed in the normal cornea.[39,40] Inflammation was analyzed by a CD45 staining as previously described by our research group,[34] and a CD31 staining was performed to visualize the blood vessels. The eye sections were incubated overnight with rat anti-mouse CD45 antibody (1/100, 553076; Pharmingen, Erembodegem, Belgium) or rat anti-mouse CD31 (1/500, 557355; Pharmingen), respectively. The following day, the bound antibodies were visualized using the Perkin Elmer kit (Renaissance TSA Indirect, NEL704A; Waltham, MA, USA) and with cyanin 3 as fluorophore. Deposition of collagen was analyzed in all groups by Sirius Red staining.

Imaging and Analysis

Images were obtained using a microscope (Leica Microsystems, Wetzlar, Germany), equipped with a digital camera (Axiocam MrC5; Carl Zeiss, Meditec, Jena, Germany), at a magnification of ×20 and a resolution of 2584 × 1936 pixels. Morphometric analyses were performed based on literature,[34] using commercial software (KS300; Zeiss). As described above, H&E staining was performed on the first slide of each series to localize the bleb on the various sections. Morphologic borders on the H&E staining were used to delineate and localize the filtrating blebs (Supplementary Fig. S1B). The localization of a failed bleb was defined by the scar tissue present between the conjunctiva/sclera. After localizing the bleb on the H&E sections, adjacent sections present on the slides within the same series, were used to perform the different (immuno) histologic stains. For each bleb, the middle on the H&E section was first defined, based on the sections including a bleb. Analysis of the different processes of wound healing was only performed in the bleb (5 sections in the middle of each filtration bleb, unless the bleb covered fewer sections) and was calculated as a proportion of the total bleb area. The area of the analyzed sections was averaged to provide one value per eye. Integrin expression was determined by calculating the integrin-positive area as a proportion of the total bleb area on 10 slides per time point (5 sections per slide). The density of blood vessels and leukocytes was determined by calculating the CD31-positive and the CD45-positive area as a proportion of the bleb area. Deposition of collagen was determined by measuring the percentage of the collagen positive area in the bleb area. Polarized light was used to distinguish mature from immature collagen fibers. Mature collagen fibers appear bright yellow or orange, whereas immature collagen fibers appear green.

Statistical Analysis

All histologic data were analyzed using the Student's *t*-test for independent samples. Data at individual time-points were analyzed using mixed-model analysis for repeated measures and overall *P* values were calculated (GraphPad Prism 5; GraphPad Software, San Diego, CA, USA). Kaplan-Meier survival analysis was performed for bleb failure using the log rank test (GraphPad Prism 5). *P* values less than or equal to 0.05 were considered to be statistically significant. Data are represented as mean ± SEM.

RESULTS

Ocular Expression of Integrin α5β1 After Glaucoma Surgery

To investigate the localization and expression level of integrin α5β1 in the eye and filtering bleb, immunohistochemical staining was performed at different time-points in a mouse model for trabeculectomy. Integrin α5β1 was not observed in the conjunctiva of naive, nonoperated eyes but its expression increased after filtration surgery. Integrin α5β1 was indeed

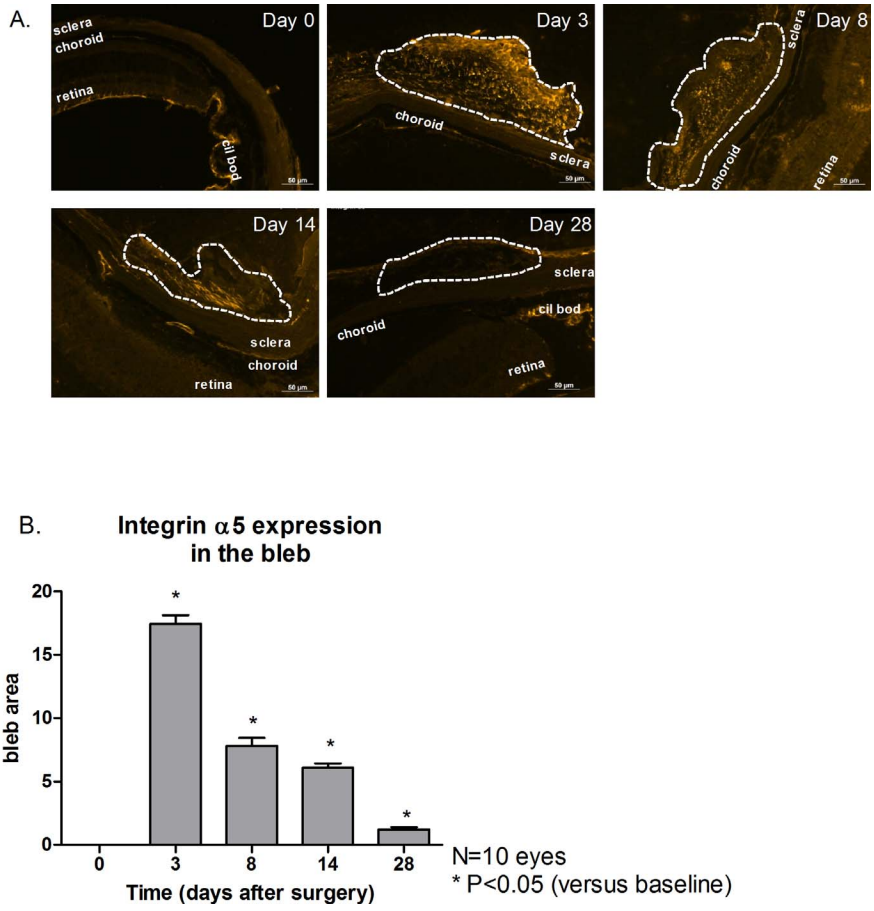

Figure 2. Quantification of integrin α5β1 in the bleb after glaucoma filtration surgery. (**A**) Images of integrin α5β1 immunolabeling in blebs at different time-points after surgery. In naive eyes (no surgery), integrin α5β1 was expressed in the Tenon and conjunctiva (*Panel 1*). At days 3, 8, 14 (and 28), integrin α5β1 was clearly expressed in the bleb (*Panels 2–5*). Edges of the blebs are marked by a *dotted line*, and ocular structures such as sclera, choroid, retina, and ciliare body (cil bod) are labeled. (**B**) Integrin α5β1 protein showed a peak of expression at postoperative day 3 and gradually decreased at later time-points. The expression of integrin α5β1 at all time-points was statistically significant compared with nonoperated levels ($n = 10$ eyes/time point; $P < 0.05$).

detected at every time-point assessed from day 1 to 28 after surgery in the conjunctiva and Tenon's capsule of the bleb (Fig. 2A). Of note, integrin α5β1 was found to be expressed in the epithelium of the murine cornea in nonoperated eyes with an expression pattern that was comparable to the one reported by Zhang et al.[40] (positive control, data not shown). Detailed morphometric analysis of the integrin α5β1 labeling revealed a peak of expression at postoperative day 3 (17.44 ± 0.68%) and a gradual decrease at later time-points (day 8: 7.81 ± 0.63% and day 14: 6.11 ± 0.32 %), with almost no staining left by day 28 after surgery (1.22 ± 0.19%). The expression of α5 integrin at all time-points was significantly higher as compared with nonoperated values (Fig. 2B; $P < 0.05$).

Thus, integrin α5β1 expression was found to be upregulated in Tenon's capsule and conjunctiva of the bleb after trabeculectomy in the mouse eye. Importantly, integrin expression was upregulated as early as day 3 after glaucoma surgery, suggesting it might play a pivotal role during the early stages of postoperative wound healing.

Effect of CLT-28643 on the Surgical Outcome

To determine whether inhibition of integrin α5β1 altered the outcome of glaucoma surgery in vivo, CLT-28643 was assessed for efficacy in preservation of the bleb in the mouse model. Daily clinical examinations revealed that none of the animals showed any pain-distress. No regimen-related differences in pre- and posttreatment body weights during follow-up were detected (data not shown).

Bleb area and survival were analyzed every 2 days after surgery as a measure of filtration surgery outcome. Analysis of the bleb area and survival showed that all vehicle-treated eyes failed by day 17 (Fig. 3). All the other regimens significantly improved bleb area and bleb survival compared with vehicle ($P < 0.05$). The dose regime of repeated injections of 2 μg of CLT-28643 was significantly superior to MMC with respect to bleb area ($P < 0.001$, Fig. 3A) and both these groups showed 100% bleb survival for the duration of the study (Fig. 3B). The effect of a single SCJ injection of 2-μg CLT-28643 immediately after surgery on bleb area and survival was similar to that of MMC ($P = 0.28$, and $P = 0.32$, respectively). Administration of 10-μg drops of CLT-28643, 3× daily, also increased bleb area versus vehicle and was comparable to MMC. By Day 28, 75% of the blebs in the drops treated group failed versus MMC's 100% survival ($P = 0.003$). Repeated injections of a low-dose (1 μg) CLT-28643 was found to be less effective compared with MMC in bleb characteristics and resulted in a significantly reduced bleb area as compared with the antimitotic agent ($P < 0.001$) and increased bleb failure (75% vs. 100%) on day 28 ($P = 0.006$). Figure 4 shows representative photographs of the blebs after treatment on different postoperative days. Repeated injections of CLT-28643 were clearly associated with a large

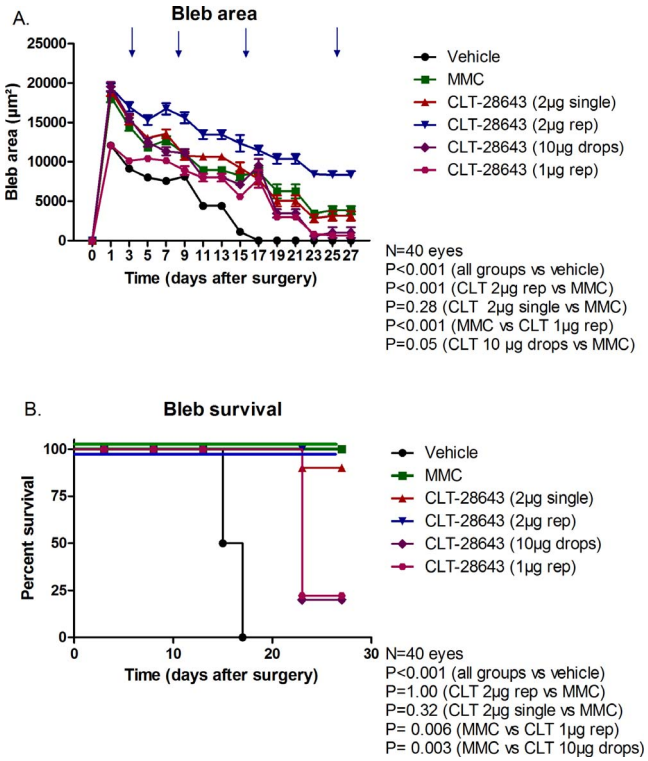

FIGURE 3. Clinical investigation of the bleb after administration of CLT-28643. Bleb area (**A**) and bleb survival (**B**) after treatment with the integrin α5β1 inhibitor (CLT-28643). Repeated injections of CLT-28643 (2 µg) were the most effective in the improvement of surgical outcome, with superior efficacy to MMC ($n = 40$ eyes/compound). *Arrows* indicate time-points of repeated injections.

bleb compared with the flat and scarred blebs observed in the respective vehicle control group. Importantly, grading corneal opacity indicated that 2-minute administration of 0.02% MMC significantly increased corneal toxicity as compared with vehicle ($P < 0.001$), whereas treatment with CLT-28643 was similar to vehicle ($P > 0.05$; Table 2). While all groups showed some transient corneal opacification postoperatively, 8/40 eyes (20%) of MMC-treated eyes had scores of greater than or equal to 1.0 compared with 3/200 eyes in other treatment groups.

Thus, our data revealed a dose-dependent effect in the efficacy of the integrin inhibitor CLT-28643. While a 2-µg single injection of CLT-28643 improved bleb characteristics in a similar way as 10-µg topical administration and MMC, repeated injections of 2 µg induced an even better response in surgical outcome, and were statistically superior to MMC in terms of bleb area. Importantly, MMC increased corneal opacification, to a greater extent compared with the CLT-28643- or vehicle-treated groups.

Effect of CLT-28643 on Postoperative Wound Healing

In order to investigate whether the improved surgical outcome induced by CLT-28643, was associated with altered postoperative healing, different immunohistologic stains for some underlying processes were performed. Specific staining for inflammation, angiogenesis, and collagen deposition in the bleb were analyzed at days 3, 8, 14, and 28. Of note, in the mouse model of glaucoma surgery, it is known that angiogenesis and inflammation peak early after surgery, whereas a gradual increase in collagen deposition occurs at later time-points.[34]

Inflammation

No differences in the inflammatory process were observed between vehicle, MMC 0.02% and 1-µg repeated CLT-28643-treated eyes at the different time-points after surgery ($P > 0.05$). On day 3, administration of a single 2-µg injection or 10-µg eye drops of CLT-28643 reduced the inflammatory response by 30% compared with vehicle treatment ($P < 0.05$). Importantly, 2-µg repeated SCJ injections reduced the leukocyte density by 53%, indicating that an additional reduction of 23% over a single application of CLT-28643 ($P < 0.001$). The differences became less obvious after day 3, because the leukocyte density was decreasing over time in all groups (Fig. 5).

Neovascularization

On day 3, administration of MMC, 2-µg single and repeated injections, or 10-µg drop application of CLT-28643 decreased the neoangiogenic process by 40%, compared with vehicle treatment ($P < 0.05$). This effect was comparable to the effect in the MMC group. Importantly, 2-µg repeated injection induced an additional reduction in blood vessel density of 17% (total reduction of 57%) as compared with 2-µg single integrin inhibitor injection and versus MMC ($P < 0.001$). Blood vessel density in the bleb was not changed in eyes treated with repeated low-dose injections of 1-µg CLT-28643 ($P > 0.05$). On day 8, 14, and 28, MMC, 2-µg single and repeated injections and 10-µg eye drop CLT-28643 administration still resulted in a reduced angiogenic response compared with the vehicle group ($P < 0.05$; Fig. 6).

Collagen Deposition

Analysis of the Sirius Red staining 28 days after surgery showed that collagen deposition was significantly decreased by 31% after MMC 0.02%, 2-µg single injection and 10-µg eye drop treatment with the integrin inhibitor, in comparison to vehicle-treated eyes ($P < 0.05$). Repeated injection of 2-µg CLT-28643 additionally reduced the fibrotic process by 21% (total reduction of 52%), as compared with single injection of 2 µg of the integrin inhibitor ($P < 0.001$). Also at days 3, 8, and 14, CLT-28643 reduced collagen deposition, with an additional reduction after repeated injection of 2-µg CLT-28643 on day 8 and 14, but not on day 3 (Fig. 7). Only small effects were seen after repeated injection of 1-µg integrin inhibitor on day 8 and 14 comparison to vehicle-treated eyes ($P < 0.05$). Repeated 2-µg CLT-28643 use was statistically superior to MMC on day 28 ($P < 0.001$).

Integrin α5β1 Expression

In order to correlate the differences in wound healing processes after CLT-28643 treatment to a possible effect on integrin α5β1 expression in the bleb, an immunostaining for this transmembrane receptor was performed. Analysis on days 3, 8, 14, and 28 after surgery showed no differences in bleb integrin α5β1 expression between vehicle, MMC 0.02%, 10-µg drops and 1-µg repeated CLT-28643-treated eyes ($P > 0.05$). On day 3, the time-point for peak of integrin expression in the bleb, single and repeated injections of 2-µg CLT-28643 both significantly reduced integrin expression, by 27% ($P < 0.001$) and 58% ($P < 0.001$), respectively, as compared with vehicle group and compared with MMC ($P < 0.001$). Moreover, the effect between single and repeated injections was significantly different ($P < 0.001$), indicating that 2-µg repeated injection was able to induce an additional reduction in integrin α5β1 expression. Analysis on day 8, 14, and 28 showed similar

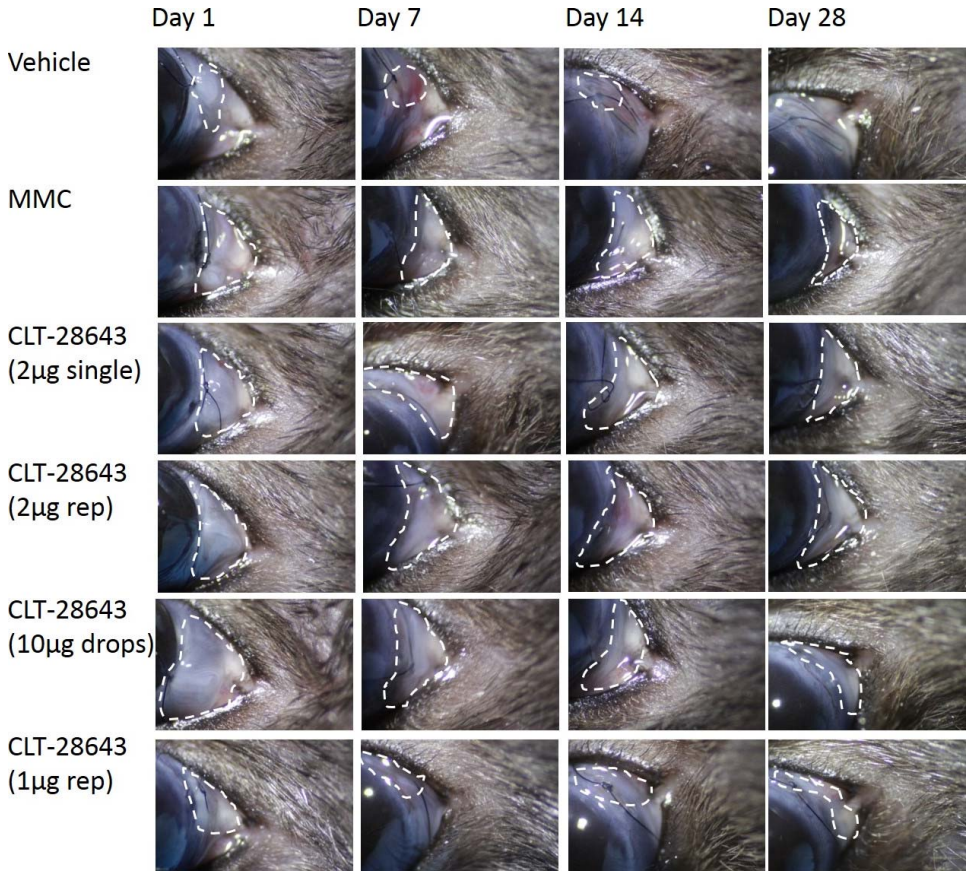

Figure 4. Macroscopic postoperative photographs of eyes after surgery. Representative macroscopic photographs of eyes on days 1, 7, 14, and 28 show blebs. Edges of the surviving bleb are marked with a *dotted line*. Administration of 2 µg CLT-28643 and MMC (*Panels 2–4*) were clearly associated with elevated and surviving blebs, compared with 1-µg administration (*lower panels*) and vehicle controls (*top panels*). Of note, pictures were taken before repeated administration of the compound, when the two procedures took place on the same day.

results as compared with day 3. However, on day 14 and 28 no additional effect was seen after 2-µg repeated injection, since the levels of integrin expression were rather low (Fig. 8).

Overall, the results confirm that single injection of 2 µg or topical administration (10 µg) of the integrin inhibitor result in a comparable effect to MMC 0.02%, by almost equally decreasing the postoperative processes of wound healing. Importantly, repeated injection of 2 µg resulted in a more pronounced reduction of the different wound healing processes, as compared with the antimitotic agent. Repeated injections of 1 µg did not decrease inflammation or angiogenesis and had only a small effect on collagen deposition.

Discussion

Filtration surgery remains the most effective therapy to reduce IOP in glaucoma patients.[41,42] However, this surgery frequently

Table 2. Corneal Opacity After CLT-18643 Treatment

Days After Surgery	1	3	5	7	13	21	27
Group 1: vehicle	0.20 ± 0.04	0.20 ± 0.04	0.17 ± 0.04	0.13 ± 0.04	0.10 ± 0.05	0.20 ± 0.08	0.20 ± 0.08
Group 2: MMC 0.02%*	0.45 ± 0.07	0.56 ± 0.07	0.47 ± 0.07	0.40 ± 0.09	0.35 ± 0.08	0.40 ± 0.16	0.15 ± 0.11
Group 3: 2 µg CLT-28643 single†‡	0.24 ± 0.04	0.21 ± 0.05	0.13 ± 0.04	0.10 ± 0.04	0.05 ± 0.03	0.00 ± 0.00	0.15 ± 0.11
Group 4: 2 µg CLT-28643 repeated§||	0.19 ± 0.04	0.14 ± 0.04	0.13 ± 0.05	0.13 ± 0.05	0.08 ± 0.04	0.00 ± 0.00	0.00 ± 0.00
Group 5: 1 µg CLT-28643 repeated¶#	0.20 ± 0.04	0.16 ± 0.04	0.08 ± 0.03	0.03 ± 0.02	0.03 ± 0.03	0.00 ± 0.00	0.00 ± 0.00
Group 6: 10 µg CLT-28643 drops**††	0.19 ± 0.04	0.15 ± 0.04	0.08 ± 0.03	0.10 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Scoring ± SEM; $n = 10$ eyes/compound/time point.
* Overall $P < 0.001$ (vehicle vs. MMC).
† Overall $P = 0.96$ (vehicle vs. 2 µg CLT-28643 single).
‡ Overall $P = 0.03$ (MMC vs. 2 µg CLT-28643 single).
§ Overall $P = 0.68$ (vehicle vs. 2 µg CLT-28643 repeated [rep]).
|| Overall $P = 0.04$ (MMC vs. 2 µg CLT-28643 rep).
¶ Overall $P = 0.89$ (vehicle vs. 1 µg CLT-28643 rep).
# Overall $P = 0.005$ (MMC vs. 1 µg CLT-28643 rep).
** Overall $P = 0.99$ (vehicle vs. 10 µg CLT-28643 drops).
†† Overall $P < 0.001$ (MMC vs. 10 µg CLT-28643 drops).

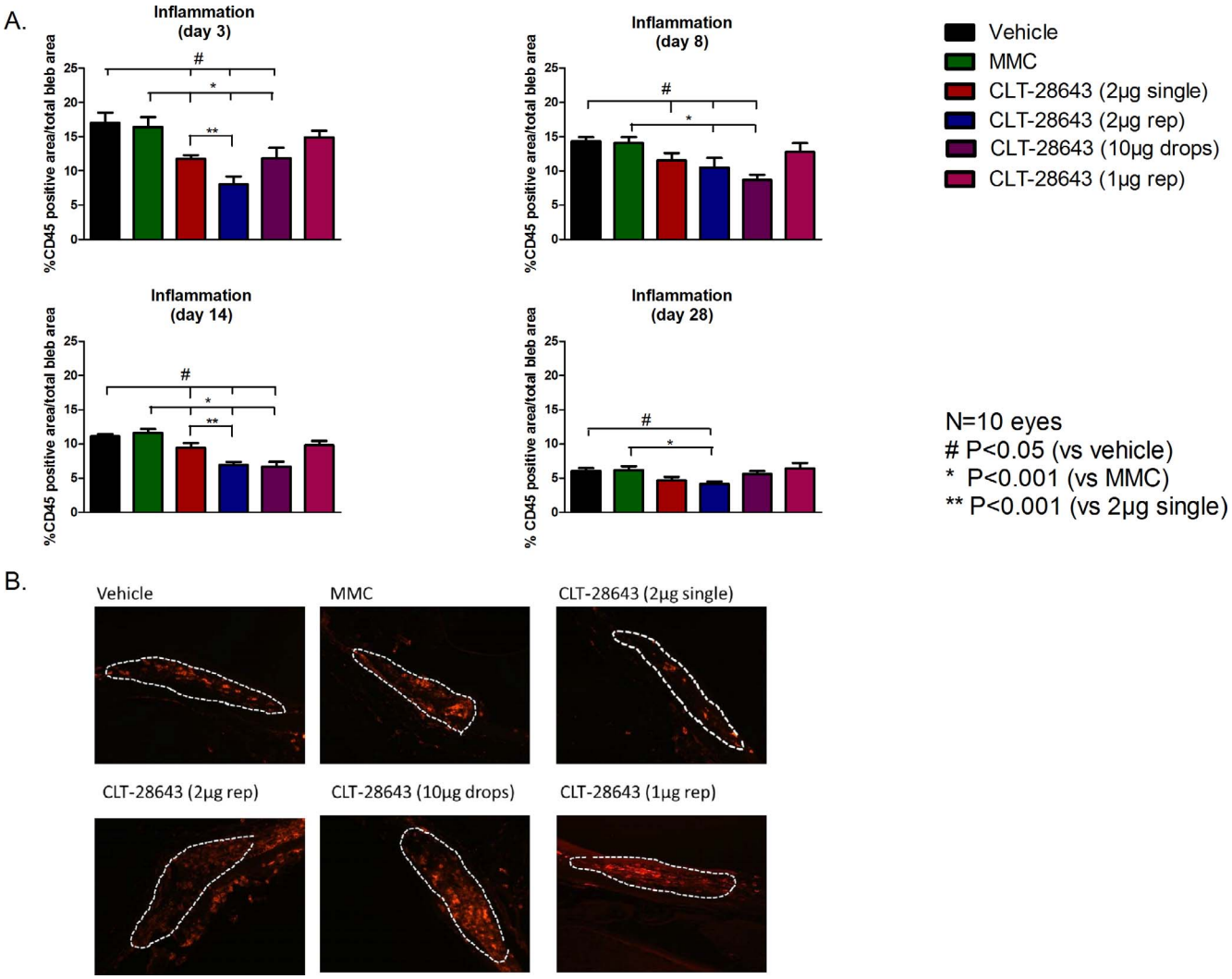

Figure 5. Inflammation in the bleb after treatment with the integrin inhibitor. (**A**) Treatment with injection(s) of CLT-28643 (2 µg) and topical administration significantly decreased the process of inflammation on day 3 after surgery, as compared with vehicle control ($P < 0.05$) and MMC treatment ($P < 0.001$). Repeated injections of 2 µg induced an additional reduction ($P < 0.001$) and showing the highest efficacy in the reduction of inflammation. On days 8, 14, and 28, similar observations were made after repeated administration of 2-µg CLT-28643 ($n = 10$ eyes/compound/time point). (**B**) Representative pictures of the inflammation in the bleb on day 3 after surgery. The edges of the bleb are marked with a *dotted line*.

fails due to subconjunctival wound healing.[31] Pharmacologic enhancement of trabeculectomy using different antiscarring agents, such as MMC, was found to significantly improve surgical success rate.[43] However, this antimitotic agent may result in severe side effects, such as corneal toxicity and thin-walled avascular blebs due to its nonspecific cytotoxic effect.[44] Therefore, it remains necessary to broaden the therapeutic approach and target biologically specific and relevant pathways involved in scar formation.

The integrin-fibronectin complex is known to be involved in different processes underlying wound healing, such as inflammation, angiogenesis, and the activation of residing fibroblasts to myofibroblasts.[8,14,18,19] Cells that are activated during wound healing, such as inflammatory, endothelial, and fibroblasts cells can interact with each other and ECM proteins via cell surface integrin receptors.[2,3] Our data show, for the first time, that integrin α5β1 is upregulated in Tenon's capsule and conjunctiva of the bleb after glaucoma surgery, with expression levels peaking early (day 3) after surgery. These results indicate that integrin α5β1 might be a good molecular target for antiscarring therapy in glaucoma surgery.

Treatment with the small molecule CLT-28643, which inhibits integrin α5β1-fibronectin interaction, indeed significantly increased bleb area and bleb survival compared with vehicle control and the effect correlates with the inhibition of integrin α5β1 expression. Different dose regimen and administration routes of CLT-28643 were investigated and compared with the gold standard in clinical practice, MMC using a mouse model of glaucoma surgery. This mouse model is well described and closely resembles the surgical procedure in clinical practice performed in humans.[35,36] The analysis of bleb area and survival was performed on two-dimensional (2D) bleb images. This read-out technique shows no difference in measuring bleb dimensions (Supplementary Fig. S1C) compared with optical coherence tomography (OCT) analysis of the bleb[35,36]). Analysis showed that a single subconjunctival injection of 2 µg or topical administration three times daily (10 µg) of CLT-28643 were comparable to MMC in surgical outcome for 28 days after surgery. More importantly, repeated injections of 2-µg CLT-28643 showed a statistically significant improvement in clinical outcome compared with MMC and all other groups.

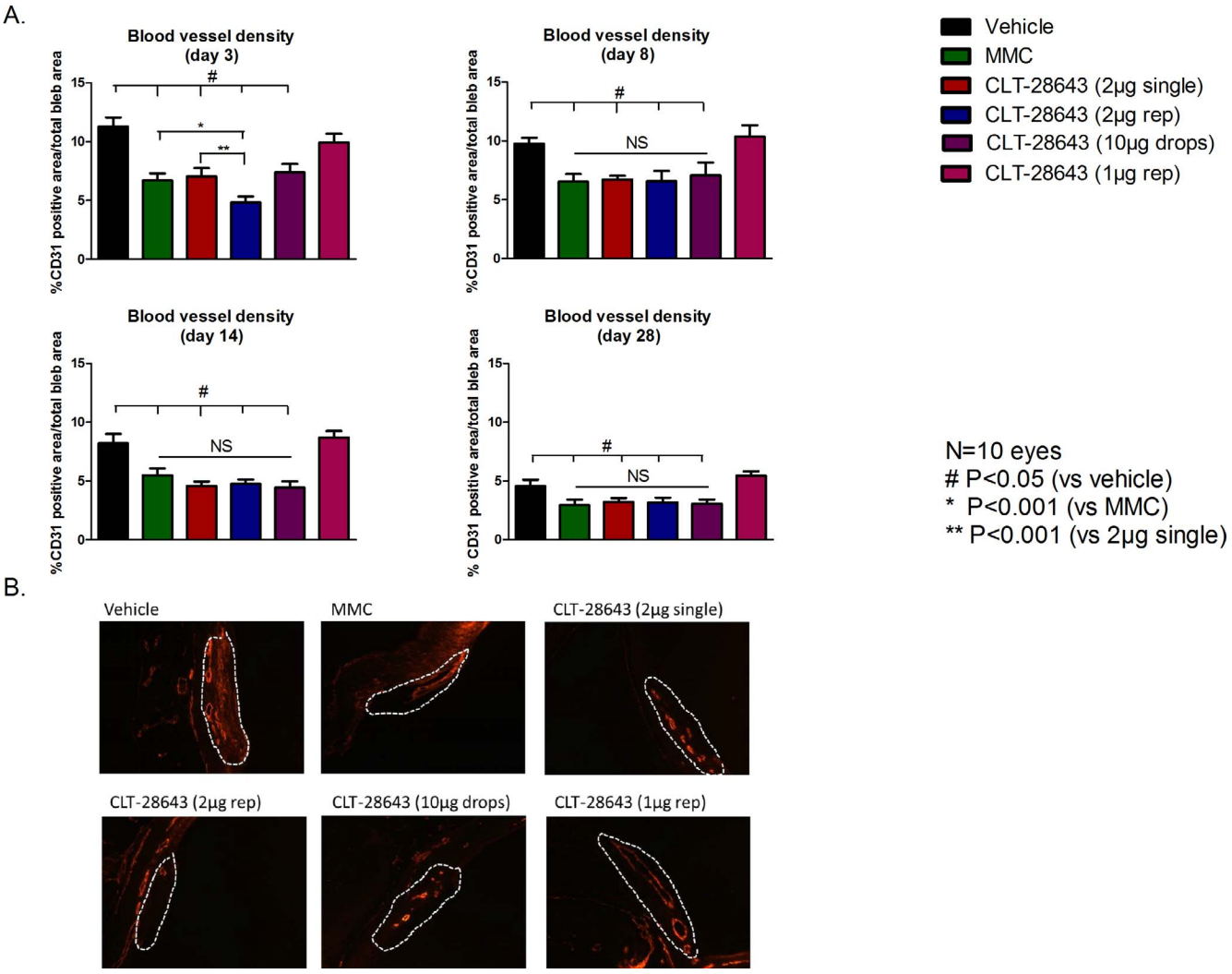

Figure 6. Blood vessel density in the bleb after treatment with the integrin inhibitor. (**A**) Administration of MMC, 2-, or 10-µg application of CLT-28643 were able to decrease the angiogenic process on postoperative day 3, in comparison to vehicle treatment ($P < 0.05$). Importantly, 2-µg repeated administration induced an additional reduction in blood vessel density, compared with single treatment ($P < 0.001$). On days 8, 14, and 28, MMC, 2- and 10-µg CLT-28643 administration resulted in a reduced angiogenic response in comparison to the vehicle group ($n = 10$ eyes/compound/time point). (**B**) Representative pictures of angiogenesis in the bleb on day 3 after surgery. The edges of the bleb are marked with a *dotted line*.

The early peak of integrin α5β1 expression confirms the prominent role of integrin α5β1 in the early phase of wound healing after filtration surgery. This is also supported by the strong effect of a single injection of CLT-28643, which is comparable to the effect of MMC. Although its expression decreases over the time, α5β1 seems to play a continued role in the later phase of wound healing as shown by the additional efficacy of repeated injections of CLT-28643. On the other hand, MMC has no effect on the integrin α5β1 expression clearly indicating that its antiscarring effect is totally different from the targeted specific mechanism of action of CLT-28643.

Administration of CLT-28643 was nontoxic and well tolerated, whereas corneal toxicity was present after treatment with the antimitotic agent MMC. From clinical practice, we know that the use of MMC can be associated with various complications related to cell toxicity, such as loss of endothelial cells, leading to corneal opacity and swelling.[45–48] Moreover, the use of MMC can also cause changes in ocular surface,[49] corneal epithelial damage,[50] limbal avascularity,[51] and corneal melting.[52] Thus, CLT-28643 was associated with a better safety profile compared with MMC. These results are consistent with previous data showing similar efficacy and safety of CLT-28643 in the rabbit model of glaucoma surgery. In a trabeculectomy-study, 24 rabbits were included that received intraoperative MMC application, SCJ, or topical administration on top of the SCJ injection of CLT-28643, or placebo treatment. Clinical investigation of the bleb showed no toxic effects after integrin inhibition. Surgical outcome was comparable for both administration schemes of CLT-28643 and MMC and superior to vehicle-treated control eyes.[33] Other groups also reported that peptides mimicking the RGD binding domain of fibronectin (GGRGDSPCA) improved surgical outcome in the rabbit model compared with saline treatment, suggesting that the integrin-fibronectin complex plays an important role in the different phases of wound healing.[29,30] However, these peptides were not specific for integrin α5β1.

The beneficial effect on bleb characteristics of CLT-28643 was associated with various immunohistologic changes in the bleb after surgery. Indeed, both injection and topical administration of the inhibitor reduced the processes of inflammation, angiogenesis, and fibrosis by 20%, as compared with vehicle-treated eyes. Single injection also reduced the integrin

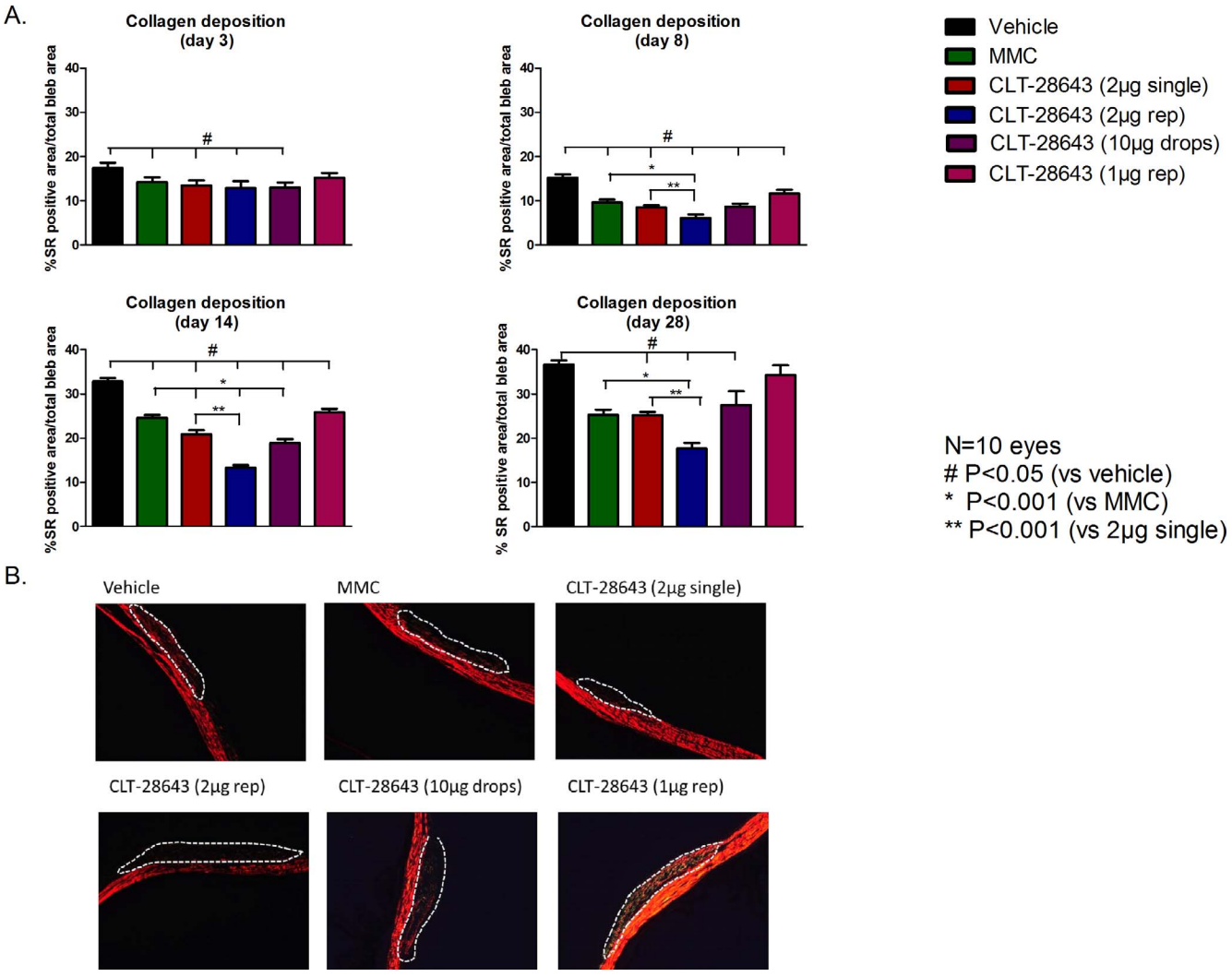

Figure 7. Collagen deposition in the bleb after treatment with the integrin inhibitor. (**A**) Collagen deposition was significantly decreased after MMC 0.02%, 2- and 10-µg treatment of the integrin inhibitor at all time-points, in comparison to vehicle-treated eyes ($P < 0.05$). Repeated application of 2-µg CLT-28643 additionally reduced the fibrotic process ($P < 0.001$) on days 8, 14, and 28, whereas lesser effects were seen after administration of 1-µg integrin inhibitor on day 8 and 14 compared with vehicle ($P < 0.05$). Furthermore, on day 14 treatment with injection(s) of CLT-28643 (2 µg) and topical administration significantly decreased the process of fibrosis compared to MMC ($P < 0.001$; $n = 10$ eyes/compound/time point). (**B**) Representative pictures of collagen deposition in the bleb on day 28 after surgery. The edges of the bleb are marked with a *dotted line*.

expression in the bleb, which can probably be explained by the fact that preventing the integrin α5β1-fibronectin interaction results in less infiltration by inflammatory cells and reduced angiogenic sprouting and proliferation of endothelial cells in the bleb. Surprisingly, integrin expression in the bleb was not reduced after eye drops. These results can be explained by the significantly lower tissue penetration after topical administration (data not shown) and might suggest that drops are not the most suitable way of administration. Of note, and as already described in literature, MMC application did not reduce bleb inflammation.[53,54]

Investigating the effect of repeated administration of the integrin inhibitor, clearly demonstrated a dose-response effect in the efficacy of the SCJ injections. Indeed, repeated application of 1 µg was less effective compared to single 2-µg administration and MMC in the improvement of bleb characteristics until 28 days after surgery. Importantly for surgical outcome, repeated injections of 2 µg CLT-28643 was superior to 2-µg single application, with an even superior effect compared with MMC. These results were also confirmed by immunohistologic analysis of the blebs, showing an additional decrease in integrin expression, inflammation, angiogenesis, and fibrosis, as compared with single CLT-28643 administration. Of note, the reduced integrin expression in the bleb on day 3 after repeated 2-µg administration, as compared with 2-µg single treatment, can be explained by the fact that the second injection on day 3 was given a few hours before mice were killed.

Overall, the current results indicate that by inhibiting integrin α5β1, the small molecule, CLT-28643, affects multiple mechanisms reducing the postoperative wound healing process, thereby offering improved therapeutic opportunities. We already reported improved surgical success in a rabbit model of glaucoma surgery and in a randomized prospective clinical trial after inhibition of VEGF. This improved surgical outcome was associated with a reduction in angiogenesis during the initial phase of healing and with a diminished fibrosis in later stages. However, no effect on bleb infiltration of inflammatory cells was observed.[55,56] Since CLT-28643 has complementary anti-inflammatory effects, it might have therapeutic potential to be used alone or in combination with anti-VEGF therapy. Importantly, it is known that neovascular-

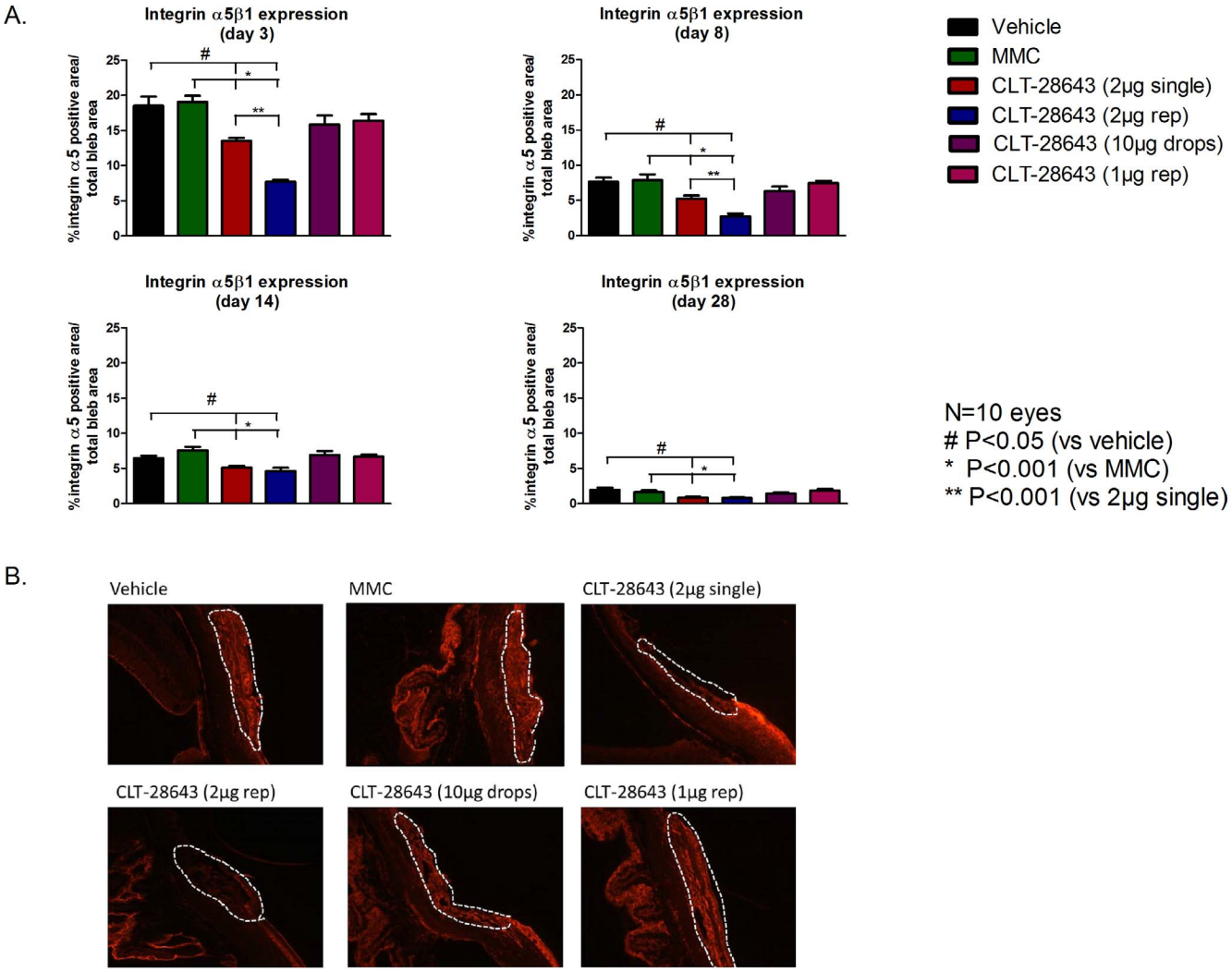

Figure 8. Integrin expression in the bleb after treatment with the integrin inhibitor. (**A**) No differences in integrin α5β1 expression in the bleb was seen between vehicle, MMC 0.02%, 10-µg drops and 1-µg repeated CLT-28643 treated eyes on day 3, 8, 14, and 28 after surgery ($P > 0.05$). Single and repeated administration of 2-µg CLT-28643 were both able to significantly reduce integrin expression on day 3, with a significant difference between single and repeated injections was significantly different ($P < 0.001$). Analysis on day 8, 14, and 28 showed similar results as compared with postoperative day 3 ($n = 10$ eyes/compound/time point). (**B**) Representative pictures of the integrin expression in the bleb on day 3 after surgery. The edges of the bleb are marked with a *dotted line*.

ization and tumor growth are coordinated by a cross-talk between integrin and VEGF,[57,58] and the combined inhibition of both has already been suggested in literature for cancer and choroidal neovascularization (CNV) treatment.[16,59,60]

Conclusions

We showed that integrin α5β1 plays an important role in wound healing after glaucoma filtration surgery, making it a good target candidate in antiscarring therapy. Targeting integrin α5β1 using the small molecule CLT-28643, seems to possess therapeutic potential as an adjunct to glaucoma surgery, possibly with a superior efficacy and tolerability to MMC, when used at the optimal dose.

Acknowledgments

The authors thank Sofie Beckers, Ann Verbeek, and Martine Leijssen for their technical support.

Disclosure: **T. Van Bergen**, None; **G. Zahn**, Clanotech (C); **P. Caldirola**, Clanotech (C); **M. Fsadni**, Clanotech (C); **N. Caram-Lelham**, Clanotech (C); **E. Vandewalle**, None; **L. Moons**, None; **I. Stalmans**, Clanotech (F)

References

1. Seibold LK, Sherwood MB, Kahook MY. Wound modulation after filtration surgery. *Surv Ophthalmol*. 2012;57:530–550.
2. Koivisto L, Heino J, Hakkinen L, Larjava H. Integrins in wound healing. *Adv Wound Care (New Rochelle)*. 2014;3:762–783.
3. Hynes RO. Integrins: bidirectional allosteric signaling machines. *Cell*. 2002;110:673–687.
4. Humphries JD, Byron A, Humphries MJ. Integrin ligands at a glance. *J Cell Sci*. 2006;119:3901–3903.
5. Johansson S, Svineng G, Wennerberg K, Armulik A, Lohikangas L. Fibronectin-integrin interactions. *Front Biosci*. 1997;2:d126–d146.
6. Yang JT, Rayburn H, Hynes RO. Embryonic mesodermal defects in alpha 5 integrin-deficient mice. *Development*. 1993;119:1093–1105.

7. Kim S, Bell K, Mousa SA, Varner JA. Regulation of angiogenesis in vivo by ligation of integrin alpha5beta1 with the central cell-binding domain of fibronectin. *Am J Pathol*. 2000;156:1345–1362.
8. Sampaio AL, Zahn G, Leoni G, et al. Inflammation-dependent alpha 5 beta 1 (very late antigen-5) expression on leukocytes reveals a functional role for this integrin in acute peritonitis. *J Leukoc Biol*. 2010;87:877–884.
9. Dietrich T, Onderka J, Bock F, et al. Inhibition of inflammatory lymphangiogenesis by integrin alpha5 blockade. *Am J Pathol*. 2007;171:361–372.
10. Bata-Csorgo Z, Cooper KD, Ting KM, Voorhees JJ, Hammerberg C. Fibronectin and alpha5 integrin regulate keratinocyte cell cycling. A mechanism for increased fibronectin potentiation of T cell lymphokine-driven keratinocyte hyperproliferation in psoriasis. *J Clin Invest*. 1998;101:1509–1518.
11. Bhaskar V, Fox M, Breinberg D, et al. Volociximab, a chimeric integrin alpha5beta1 antibody inhibits the growth of VX2 tumors in rabbits. *Invest New Drugs*. 2008;26:7–12.
12. Bell-McGuinn KM, Matthews CM, Ho SN, et al. A phase II, single-arm study of the anti-alpha5beta1 integrin antibody volociximab as monotherapy in patients with platinum-resistant advanced epithelial ovarian or primary peritoneal cancer. *Gynecol Oncol*. 2011;121:273–279.
13. Sheldrake HM, Patterson LH. Strategies to inhibit tumor associated integrin receptors: rationale for dual and multi-antagonists. *J Med Chem*. 2014;57:6301–6315.
14. Kim S, Harris M, Varner JA. Regulation of integrin alpha vbeta 3-mediated endothelial cell migration and angiogenesis by integrin alpha5beta1 and protein kinase A. *J Biol Chem*. 2000; 275:33920–33928.
15. Zahn G, Vossmeyer D, Stragies R, et al. Preclinical evaluation of the novel small-molecule integrin alpha5beta1 inhibitor JSM6427 in monkey and rabbit models of choroidal neovascularization. *Arch Ophthalmol*. 2009;127:1329–1335.
16. Umeda N, Kachi S, Akiyama H, et al. Suppression and regression of choroidal neovascularization by systemic administration of an alpha5beta1 integrin antagonist. *Mol Pharmacol*. 2006;69:1820–1828.
17. Ramakrishnan V, Bhaskar V, Law DA, et al. Preclinical evaluation of an anti-alpha5beta1 integrin antibody as a novel anti-angiogenic agent. *J Exp Ther Oncol*. 2006;5:273–286.
18. Thannickal VJ, Lee DY, White ES, et al. Myofibroblast differentiation by transforming growth factor-beta1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase. *J Biol Chem*. 2003;278:12384–12389.
19. Nesbit M, Schaider H, Berking C, et al. Alpha5 and alpha2 integrin gene transfers mimic the PDGF-B-induced transformed phenotype of fibroblasts in human skin. *Lab Invest*. 2001;81:1263–1274.
20. Roberts CJ, Birkenmeier TM, McQuillan JJ, et al. Transforming growth factor beta stimulates the expression of fibronectin and of both subunits of the human fibronectin receptor by cultured human lung fibroblasts. *J Biol Chem*. 1988;263:4586–4592.
21. Weston BS, Wahab NA, Mason RM. CTGF mediates TGF-beta-induced fibronectin matrix deposition by upregulating active alpha5beta1 integrin in human mesangial cells. *J Am Soc Nephrol*. 2003;14:601–610.
22. Norman JT, Fine LG. Progressive renal disease: fibroblasts, extracellular matrix and integrins. *Exp Nephrol*. 1999;7:167–177.
23. Pilewski JM, Latoche JD, Arcasoy SM, Albelda SM. Expression of integrin cell adhesion receptors during human airway epithelial repair in vivo. *Am J Physiol*. 1997;273:L256–L263.
24. Zhou X, Zhang Y, Zhang J, et al. Expression of fibronectin receptor integrin alpha 5 beta 1 of hepatic stellate cells in rat liver fibrosis. *Chin Med J (Engl)*. 2000;113:272–276.
25. Iwamoto H, Sakai H, Nawata H. Inhibition of integrin signaling with Arg-Gly-Asp motifs in rat hepatic stellate cells. *J Hepatol*. 1998;29:752–759.
26. Juhasz I, Murphy GF, Yan HC, Herlyn M, Albelda SM. Regulation of extracellular matrix proteins and integrin cell substratum adhesion receptors on epithelium during cutaneous human wound healing in vivo. *Am J Pathol*. 1993;143: 1458–1469.
27. Zahn G, Volk K, Lewis GP, et al. Assessment of the integrin alpha5beta1 antagonist JSM6427 in proliferative vitreoretinopathy using in vitro assays and a rabbit model of retinal detachment. *Invest Ophthalmol Vis Sci*. 2010;51:1028–1035.
28. Hershkoviz R, Melamed S, Greenspoon N, Lider O. Non-peptidic analogues of the Arg-Gly-Asp (RGD) sequence specifically inhibit the adhesion of human tenon's capsule fibroblasts to fibronectin. *Invest Ophthalmol Vis Sci*. 1994;35: 2585–2591.
29. Avila M, Ortiz G, Lozano JM, Franco P, de PG Patarroyo ME. The effects of RGD (Arg-Gly-Asp) peptides on glaucoma filtration surgery in rabbits. *Ophthalmic Surg Lasers*. 1998;29:309–317.
30. Avila M, Ortiz G, Lozano JM, Franco P, Patarroyo ME. GGRGDSPCA peptide: a new antiscarring agent on glaucoma filtration surgery. *Ophthalmic Surg Lasers*. 2001;32:134–139.
31. Addicks EM, Quigley HA, Green WR, Robin AL. Histologic characteristics of filtering blebs in glaucomatous eyes. *Arch Ophthalmol*. 1983;101:795–798.
32. Caldirola P, Bäckman U, Cao Y. Development of Novel Anti-Angiogenic Drug by Targeting (integrin α5β1) - Potential for Treatment of VEGF-resistant Cancer. EORTC-NCI-AACR Symposium; 2010: poster 476 ed.
33. Schultheiss M, Schnichels S, Knorad EM, et al. α5β1-Integrin inhibitor (CLT-28643) effective in rabbit trabeculectomy model [published online ahead of print August 31, 2016]. *Acta Ophthalmol*. doi:10.1111/aos.13215.
34. Van Bergen T, Jonckx B, Hollanders K, et al. Inhibition of placental growth factor improves surgical outcome of glaucoma surgery. *J Cell Mol Med*. 2013;17:1632–1643.
35. Seet LF, Lee WS, Su R, Finger SN, Crowston JG, Wong TT. Validation of the glaucoma filtration surgical mouse model for antifibrotic drug evaluation. *Mol Med*. 2011;17:557–567.
36. Seet LF, Su R, Barathi VA, et al. SPARC deficiency results in improved surgical survival in a novel mouse model of glaucoma filtration surgery. *PLoS One*. 2010;5:e9415.
37. Lee SH, Leem HS, Jeong SM, Lee K. Bevacizumab accelerates corneal wound healing by inhibiting TGF-beta2 expression in alkali-burned mouse cornea. *BMB Rep*. 2009;42:800–805.
38. Yoeruek E, Ziemssen F, Henke-Fahle S, et al. Safety penetration and efficacy of topically applied bevacizumab: evaluation of eyedrops in corneal neovascularization after chemical burn. *Acta Ophthalmol*. 2008;86:322–328.
39. Elner SG, Elner VM. The integrin superfamily and the eye. *Invest Ophthalmol Vis Sci*. 1996;37:696–701.
40. Zhang H, Li C, Baciu PC. Expression of integrins and MMPs during alkaline-burn-induced corneal angiogenesis. *Invest Ophthalmol Vis Sci*. 2002;43:955–962.
41. Burr J, Azuara-Blanco A, Avenell A. Medical versus surgical interventions for open angle glaucoma. *Cochrane Database Syst Rev*. 2005:CD004399.
42. Hitchings R. Initial treatment for open-angle glaucoma- medical, laser or surgical? Surgery is the treatment of choice for open-angle glaucoma. *Arch Ophthalmol*. 1998;116:241–242.
43. Katz GJ, Higginbotham EJ, Lichter PR, et al. Mitomycin C versus 5-fluorouracil in high-risk glaucoma filtering surgery. Extended follow-up. *Ophthalmology*. 1995;102:1263–1269.
44. Soltau JB, Rothman RF, Budenz DL, et al. Risk factors for glaucoma filtering bleb infections. *Arch Ophthalmol*. 2000; 118:338–342.

Investigative Ophthalmology & Visual Science

45. Sihota R, Sharma T, Agarwal HC. Intraoperative mitomycin C and the corneal endothelium. *Acta Ophthalmol Scand*. 1998; 76:80–82.
46. Derick RJ, Pasquale L, Quigley HA, Jampel H. Potential toxicity of mitomycin C. *Arch Ophthalmol*. 1991;109:1635.
47. McDermott ML, Wang J, Shin DH. Mitomycin and the human corneal endothelium. *Arch Ophthalmol*. 1994;112:533–537.
48. Storr-Paulsen T, Norregaard JC, Ahmed S, Storr-Paulsen A. Corneal endothelial cell loss after mitomycin C-augmented trabeculectomy. *J Glaucoma*. 2008;17:654–657.
49. Dogru M, Erturk H, Shimazaki J, Tsubota K, Gul M. Tear function and ocular surface changes with topical mitomycin (MMC) treatment for primary corneal intraepithelial neoplasia. *Cornea*. 2003;22:627–639.
50. Manche EE, Afshari MA, Singh K. Delayed corneal epitheliopathy after antimetabolite-augmented trabeculectomy. *J Glaucoma*. 1998;7:237–239.
51. Sauder G, Jonas JB. Limbal stem cell deficiency after subconjunctival mitomycin C injection for trabeculectomy. *Am J Ophthalmol*. 2006;141:1129–1130.
52. Coppens G, Maudgal P. Corneal complications of intraoperative Mitomycin C in glaucoma surgery. *Bull Soc Belge Ophthalmol*. 2010:19–23.
53. Minguini N, Monteiro de Carvalho KM, Akaishi PM, De Luca IM. Histologic effect of mitomycin C on strabismus surgery in the rabbit. *Invest Ophthalmol Vis Sci*. 2000;41:3399–3401.
54. Chou SF, Chang SW, Chuang JL. Mitomycin C upregulates IL-8 and MCP-1 chemokine expression via mitogen-activated protein kinases in corneal fibroblasts. *Invest Ophthalmol Vis Sci*. 2007;48:2009–2016.
55. Li Z, Van Bergen T, Van de Veire S, et al. Inhibition of vascular endothelial growth factor reduces scar formation after glaucoma filtration surgery. *Invest Ophthalmol Vis Sci*. 2009; 50:5217–5225.
56. Vandewalle E, Abegao PL. Van BT, et al. Intracameral bevacizumab as an adjunct to trabeculectomy: a 1-year prospective randomised study. *Br J Ophthalmol*. 2014;98: 73–78.
57. Varner JA, Cheresh DA. Tumor angiogenesis and the role of vascular cell integrin alphavbeta3. *Important Adv Oncol*. 1996:69–87.
58. De S, Razorenova O, McCabe NP, O'Toole T, Qin J, Byzova TV. VEGF-integrin interplay controls tumor growth and vascularization. *Proc Natl Acad Sci U S A*. 2005;102:7589–7594.
59. Papo N, Silverman AP, Lahti JL, Cochran JR. Antagonistic VEGF variants engineered to simultaneously bind to and inhibit VEGFR2 and $\alpha_v\beta_3$ integrin. *Proc Natl Acad Sci U S A*. 2011; 108:14067–14072.
60. Uronis HE, Jia J, Bendell JC, et al. A Phase I/biomarker study of bevacizumab in combination with CNTO 95 in patients with advanced solid tumors. *Cancer Chemother Pharmacol*. 2015; 75:343–352.

Investigative Ophthalmology & Visual Science