The Combination of PlGF Inhibition and MMC as a Novel Anti-Scarring Strategy for Glaucoma Filtration Surgery

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PURPOSE. The complementary effects of mitomycin-C (MMC) and anti-placental growth factor (PIGF) therapy were explored and compared to the combined administration of MMC and aflibercept. Additionally, the effect of PIGF (inhibition) on IOP was investigated, since aqueous PIGF is known to be upregulated in glaucoma patients.

METHODS. In the trabeculectomy mouse model, intracameral injection(s) of the PlGF inhibitor (5D11D4) were compared to MMC or aflibercept and to the combination of both compounds. Treatment outcome was studied by bleb investigation and by Sirius Red staining. The effect of subconjunctival PIGF administration and topical 5D11D4 on IOP was investigated in normotensive mice and was compared to topical administration of latanoprost, the gold standard for IOP-lowering.

RESULTS. Combination of MMC and 5D11D4 was able to significantly improve surgical outcome compared to monotherapy of MMC or 5D11D4 (n = 20; P < 0.001). Compared to combined treatment of MMC with aflibercept, the simultaneous administration of MMC and 5D11D4 was equally efficacious in improving surgical outcome (n = 15; P = 0.88). In normotensive mice, 5D11D4 was able to significantly reduce the IOP-elevation induced by PIGF (n = 10; P < 0.05), whereas no effect of 5D11D4 was seen in naive mice, which was in contrast to latanoprost.

CONCLUSIONS. The current data suggest that application of MMC together with PIGF inhibition may have complementary effects in the improvement of surgical outcome and is equally efficacious as the combined treatment of MMC and aflibercept. Inhibition of PIGF also might open alternative perspectives as IOP-lowering strategy for glaucoma patients with increased aqueous PIGF levels.

Keywords: glaucoma filtration surgery, wound healing, placental growth factor, mitomycin-C, intraocular pressure

Since the aqueous humor (AH) of glaucoma patients is known to contain increased levels of various proteins1-3 that can increase the proliferation of Tenon fibroblasts (TF), the most important players during postoperative wound healing (Karalekas DHA, et al. IOVS 1994:35:ARVO Abstract 1898), glaucoma patients might be at high risk of bleb failure after glaucoma filtration surgery (GFS). Therefore, these upregulated molecules can be considered as potential targets in the development of new wound modulation agents.

One of the most described upregulated aqueous growth factor related to GFS is VEGF.3-5 It is known that inhibition of VEGF by bevacizumab can improve the surgical outcome in a rabbit model of glaucoma surgery5,6 and in prospective randomized clinical trials.7,8 Moreover, bevacizumab in combination with mitomycin-C (MMC) showed complementary effects in the improvement of surgical outcome in a mouse model of GFS.9 Although bevacizumab, with or without MMC, can improve surgical outcome by inhibiting angiogenesis and deposition of collagen, it is not able to reduce inflammation, another important process in postoperative healing.5 This can probably be explained by the concomitant upregulation of other proinflammatory proteins, like placental growth factor (PIGF), after bevacizumab administration.10 As such, PIGF might be another important player in GFS, besides VEGF. Indeed, we previously described that PIGF is significantly upregulated in the AH of glaucoma patients and in AH of operated rabbits after GFS.10 Moreover, intracameral (IC) injection of an anti-PIGF antibody10 (5D11D4; ThromboGenics NV, Leuven, Belgium) effectively improved surgical outcome in a mouse model of trabeculectomy. This effect on surgical outcome was associated with a decreased angiogenesis, fibrosis, and importantly, also inflammation.10 Therefore, inhibition of PIGF may be more effective than inhibition of VEGF in the improvement of GFS outcome, due to its additional effect on inflammation.

Although anti-PIGF treatment is effective in targeting different phases of wound healing, it remains unclear whether PIGF inhibition can replace the gold standard in clinical practice, MMC, or should rather be used as an adjunctive to the antimetotic agent. Moreover, treatment with a single antiangiogenic agent can lead to drug resistance, because of upregulation of other growth factors, such as PIGF after VEGF-therapy.10 Therefore, combination of VEGF- and PIGF inhibi-
tion, by the use of aflibercept, might be another therapeutic strategy, although this has never been tested.

Besides its role in the postoperative wound healing process, PI GF also is known to be upregulated in the AH of nonoperated glaucoma patients, 10 and a link between high PI GF levels and an increased IOP has been suggested. Indeed, it is known that subconjunctival administration of PI GF can increase the eye pressure in normotensive rabbits (Abdulrazik M, et al. IOVS 2010;51:ARVO E-Abstract 979). Moreover, Carnevale et al. 11 showed that PI GF can regulate the onset of systemic hypertension. Mice deficient in PI GF manifested protection from angiotensin II–induced hypertension, which could be ascribed to modulation of the splenic immune system. Further investigations are needed to clarify the exact role of PI GF in induction of eye pressure.

Therefore, the goal of this study was first to compare the effect of the PI GF antibody, 5D11D4, versus MMC in a mouse model of GFS. Secondly, the complementary effects of combined MMC and anti-PI GF therapy were investigated and compared to the combined administration of MMC and aflibercept (inhibitor of VEGF and PI GF). Thirdly, the effect of PI GF and 5D11D4 on IOP was investigated in normotensive mice and compared to latanoprost, the gold standard in clinical practice for IOP-lowering.

**Materials and Methods**

All experimental animal procedures were performed according the standards in the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and the EC Directive 86/609/EEC for animal experiments. All experiments also were approved by the Institutional Animal Care and Research Advisory Committee of KU Leuven (P139/2011).

**Mouse Model of Glaucoma Filtration Surgery**

In this study, C57Bl/6j mice (8–10 weeks old; Charles River Laboratories, Lyon, France) were used. Intraperitoneal injection of 10 times-diluted (60 mg/kg final dose) sodium pentobarbital (Nembutal, 60 mg/mL; CEVA; Sante Animaile, Brussels, Belgium) was used to induce general anesthesia. Filtering surgery was performed on both eyes, as described in the literature 12 and resulted in a filtration bleb. A topical combination preparation containing steroids and antibiotics (Tobradex; SA Alcon-Couvreur, Vilvoorde, Belgium) was administered at the end of surgery. Pictures of the bleb were administered at the end of surgery. Pictures of the bleb were taken at a 100-mm lens was used at a magnification of 5.4 μg (ThromboGenics) were compared to MMC 0.02% (Kyowa Hakkо Kirin Co., Ltd., Princeton, NJ, USA) and to the combination therapy of both compounds. The effect of 5D11D4 also was compared to equimolar concentrations of aflibercept (3.4 μg; Regeneron Pharmaceuticals, Tarrytown, NY, USA) with or without combined MMC application. Single IC injections were given at day 0 after surgery, whereas repeated administration was performed on days 0, 4, 10, 15, and 21. Mitomycin C 0.02% was administered during surgery by the use of a surgical sponge for 2 minutes. The surgical sponges soaked with MMC were placed on the exposed sclera for 2 minutes and after removing the sponge, the ocular surface and subconjunctival space were extensively rinsed with 2 mL of NaCl. As a negative control, 1C8 (4.8 μg; ThromboGenics), an irrelevant immunoglobulin G (IgG) control antibody, was used. Separate groups of animals were used for each treatment for the different sets of experiments. A detailed overview of the different treatment groups is provided in the Table.

**Processing Eyes and Collagen Staining**

Mice were killed by cervical dislocation on postoperative days 34 and 52 (n = 6–15 per time point). After enucleation, whole eyes were fixed in 1% phosphate buffered paraformaldehyde (Merck, Darmstadt, Germany) overnight and rinsed 3 times for 5 minutes in PBS. Serial paraffin sections were cut (7 μm) in 5 series on 5 glass slides. To localize the bleb (area of analysis), hematoxylin and eosin (H&E) staining was performed on the first slide from each series. Sirius Red staining was performed to analyze deposition of collagen in the bleb.

**Microscopy Analysis**

A Leica Microsystems microscope (Wetzlar, Germany), equipped with a digital camera (AxioCam MrC5; Carl Zeiss Meditec, Jena, Germany) was used to obtain images, as previously reported. 9,10 Shortly, pictures were taken at a magnification of ×20, a resolution of 2584 × 1936 pixels and morphometric analyses were performed using commercial software (KS500; Zeiss). The percentage of the area of Sirius Red–stained mature collagen fibers to the total wound area was measured to determine collagen deposition in the bleb. Polarized light was used to distinguish mature (appearing bright yellow and orange) from immature (green) collagen fibers. One slide per eye (5 sections per slide) was analyzed and these measurements were averaged. Data are presented as the mean of all eyes within a group.

**Determination of PI GF Levels After MMC Administration in Rabbit GFS Model**

New-Zealand rabbits (n = 10, 12–14 weeks, animal facility; KU Leuven, Leuven, Belgium) were used to investigate PI GF concentrations after MMC application. General anesthesia was induced by intramuscular injection of 50 mg/mL ketamin (Ketalar; Pfizer, Carlisle, PA, USA) and 2% (vol/vol) sedative (Rompun; Bayer Health Care Pharmaceuticals, Whippenny, NJ, USA), as described previously. 3,15 Surgery was performed on both eyes, using a technique similar to trabeculectomy in humans. 7 During surgery, one eye was treated with MMC, whereas the other eye served as a control and was treated with NaCl. A surgical sponge soaked with either MMC 0.02% or NaCl 0.9% was placed on the exposed sclera for 2 minutes, and after removing the sponge, the ocular tissue

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**Treatment Regimen**

In all experiments, IC administration (1 μL) of the compounds was applied 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 UMP31 Microsysyringe Injector and Micro-4 Controller (all from World Precision Instruments, Inc., Hertfordshire, UK). In different groups of mice, IC injection(s) of the PI GF inhibitor (5D11D4; 5.4 μg; ThromboGenics) were compared to MMC 0.02% (Kyowa Hakkо Kirin Co., Ltd., Princeton, NJ, USA) and to the combination therapy of both compounds. The effect of 5D11D4 also was compared to equimolar concentrations of aflibercept (3.4 μg; Regeneron Pharmaceuticals, Tarrytown, NY, USA) with or without combined MMC application. Single IC injections were given at day 0 after surgery, whereas repeated administration was performed on days 0, 4, 10, 15, and 21. Mitomycin C 0.02% was administered during surgery by the use of a surgical sponge for 2 minutes. The surgical sponges soaked with MMC were placed on the exposed sclera for 2 minutes and after removing the sponge, the ocular surface and subconjunctival space were extensively rinsed with 2 mL of NaCl. As a negative control, 1C8 (4.8 μg; ThromboGenics), an irrelevant immunoglobulin G (IgG) control antibody, was used. Separate groups of animals were used for each treatment for the different sets of experiments. A detailed overview of the different treatment groups is provided in the Table.

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IOP-lowering in mice is described by Aihara et al.16 Administered to both eyes at 10 AM. Of note, latanoprost, a positive control, dose based on the study of Aihara et al.16) was washed abundantly with NaCl. Samples (200 µL) of AH were collected using a BD microfine insulin syringe (30 G; Becton Dickinson, Erembodegem, Belgium) from both eyes on the day before and at different time points (days 1, 3, and 7) after GFS. All samples were stored immediately at −20°C until analysis and PlGF protein levels in the AH samples were determined using a quantitative sandwich enzyme immunoassay technique with a detection limit of 1.0 pg/mL (E04P0018; BlueGene, Shanghai, China). Plasma of a pregnant rabbit on day 25 was used as a positive control, since we showed that it contains high PlGF levels.10 Concentrations were expressed as pg/mL.

**IOP Measurements in Naive Mice After PlGF Administration**

Subconjunctival injections of recombinant murine PlGF (1 µg/mL, dose based on the study of Abdurazik et al. [IOVS 2010;51:ARVO E-Abstract 979]); 10 µg, 465-PL/CF; R&D Systems, Inc., Minneapolis, MN, USA) or PBS (1 µL) and topical administration of 5D11D4 (0.5 mg/mL, 500-fold molar excess compared to 1 µg/mL mPlGF) or latanoprost (0.005%; 5 µL, positive control, dose based on the study of Aihara et al.16) was administered to both eyes at 10 AM. of note, latanoprost, a prostaglandin analogue, is the gold standard in clinical practice for lowering the eye pressure. The use of this compound for IOP-lowering in mice is described by Aihara et al.16 Administration was applied by using an analytic science syringe (SGE Analytic Science) and glass capillaries with a diameter of 50 to 70 µm at the end. Mice were anesthetized using isoflurane (WN_8052_09; TechniLab, Inc., Quebec, Canada) and the IOP was measured with a calibrated rebound tonometer (Tono-Lab, iCare).13,14 At baseline (9 AM), and 1 (11 AM), 3 (1 PM), 5 (3 PM), and 7 (5 PM) hours after compound administration, IOP was measured in both eyes.

**Statistical Evaluation**

The Student’s t-test was used to analyze all morphometric data for independent samples. Data at individual time points were studied using mixed model analysis for repeated measures. Kaplan-Meier survival analysis using the log rank test was performed for bleb failure (all using GraphPad Prism 5.3; GraphPad Software, Inc., La Jolla, CA, USA). P < 0.05 was considered statistically significant. Data are represented as mean ± SEM, unless otherwise stated.

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<td>Comparison to MMC</td>
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**RESULTS**

To investigate whether PlGF inhibition can replace MMC or might be more effective when used in combination with the gold standard, the mouse trabeculectomy model was used. In vivo efficacy was determined based on bleb characteristics (area and survival) and collagen deposition in the bleb. Of note, no differences in body weight nor IOP were seen during the experiments in the mouse model of trabeculectomy (data not shown).

**Effect of 5D11D4 on Surgical Outcome Compared to MMC**

Analysis at day 34 after surgery showed that 5D11D4 (5.4 µg, IC injection) and MMC 0.02% (2-minute sponge) administration significantly improved surgical outcome compared to the irrelevant IgG control (1C8; n = 20; P < 0.001). Single application of 5D11D4 significantly improved bleb area with 39% ± 5%, but was significantly less effective compared to the 59% ± 6% improvement after MMC administration (n = 20; P < 0.001). Also bleb survival after MMC 0.02% was prolonged, as shown in the Kaplan-Meier survival curve, with all blebs failed at postoperative day 34 (n = 20; P < 0.001 vs. 1C8), compared to day 19 after 5D11D4 application (n = 20; P = 0.18 vs. 1C8). Importantly, repeated injections of 5D11D4 (5.4 µg) at days 0, 4, 10, 15, and 21 were able to significantly improve bleb area with 78% ± 7% (n = 12; P < 0.001) versus MMC 0.02%. At day 34 after surgery, all blebs survived after repeated administration of the antibody, whereas the MMC-treated blebs started to fail from day 19, with all blebs failed at the endpoint of the study (Figs. 1A, 1B). These effects on surgical outcome were associated with an effect on collagen deposition in the bleb. Application of MMC 0.02% significantly reduced the fibrotic process with 32% ± 3% compared to IgG (n = 10; P < 0.001), while repeated 5D11D4 administration induced an additional reduction of 23% ± 2% (total reduction of 55% ± 2% versus IgG) of collagen deposition (n = 10; P < 0.001). Collagen deposition after single injection of the PlGF antibody was not significantly different from 1C8 administration (n = 10; P = 0.72; Figs. 1C, 1D).

Thus, single administration of the PlGF antibody was able to improve surgical outcome similarly, but was less efficacious compared to administration of MMC 0.02%. Importantly, repeated administration of 5D11D4 seemed to result in improved bleb characteristics and reduced collagen deposition compared to MMC application.

**Upregulation of PlGF in Aqueous Humor After MMC Administration**

The aqueous PlGF levels after MMC administration were investigated, since it is described in the literature that the antimiotic agent can lead to an upregulation of different proangiogenic and profibrotic molecules.9,17,18 The rabbit model for trabeculectomy was used, since the volume of AH that can be collected from a mouse is too small to be used in the PlGF immunoassay. Analysis showed that the first day after surgery, PlGF levels were significantly upregulated after MMC administration (n = 20; P < 0.001). Importantly, repeated administration of 5D11D4 significantly improved bleb area with 59% ± 6% improvement after MMC administration (n = 20; P < 0.001). Importantly, repeated administration of 5D11D4 administration induced an additional reduction of 23% ± 2% (total reduction of 55% ± 2% versus IgG) of collagen deposition (n = 10; P < 0.001). Collagen deposition after single injection of the PlGF antibody was not significantly different from 1C8 administration (n = 10; P = 0.72; Figs. 1C, 1D).

**Effect of 5D11D4 on Surgical Outcome in Combination With MMC**

Since PlGF levels were significantly upregulated after MMC administration, single and repeated IC injections of 5D11D4
were combined with MMC and follow-up of the bleb was performed until all blebs had failed, that is, day 51. Investigation of the bleb characteristics showed that the combined treatment of the antimitotic agent and the antibody significantly improved bleb area compared to repeated administration of 1C8 (4.8 μg, n = 20; P < 0.001), to single 5D11D4 injection (5.4 μg, n = 20; P < 0.001), to sole MMC 0.02% administration (n = 20; P < 0.001), and, importantly, also a significant increase of 19% compared to repeated injections of 5D11D4 (5.4 μg, n = 20; P < 0.001). No differences were noticed between MMC combined with single or repeated IC injections of the PlGF antibody (n = 20; P = 0.41; Fig. 3A). Comparable effects were observed on bleb survival. All blebs failed by day 20 after 1C8, by day 21 after single 5D11D4, and by day 33 after MMC 0.02% administration, which was similar to the first experiment. Blebs treated with repeated administration of the PlGF antibody started to fail at day 30, and all blebs failed by day 43, whereas 100% bleb failure after both combination therapies was only observed at the end of the study (day 51, n = 20; P = 0.72; Fig. 3B). Figure 4 shows representative photographs of the blebs after treatment on different postoperative days. Analysis of collagen deposition of the groups treated with repeated administration of the PlGF inhibitor and the combined treatments showed that there was an additional reduction of 21% (total reduction of 69% compared to 1C8) after combination treatment (n = 6; P < 0.001; Figs. 3C, 3D). Of note, the levels of collagen observed after combination therapy (n = 6; P = 0.83) were comparable to baseline levels (data not shown).

Thus, the combination of MMC 0.02% with a single or repeated IC injection of 5D11D4 was able to significantly improve surgical outcome compared to monotherapy of MMC and to repeated administration of the PlGF antibody, with no
PIGF Inhibition and MMC in Glaucoma Filtration Surgery

**Effect of PIGF inhibition (5D11D4) in combination with MMC on surgical outcome.** (A, B) Compared to repeated administration of 5D11D4 (5.4 μg), combined treatment of the antimitic agent (MMC 0.02%−2 minutes) together with the PIGF inhibitor was able to induce an additional increase in bleb area and survival (n = 20, P < 0.001), with no differences between MMC together with single or repeated 5D11D4 administration (n = 20, P < 0.05). Arrows indicate treatment scheme for repeated injections. (C) Representative pictures of collagen deposition in the bleb on day 52 after surgery. Scale bar: 20 μm. The edges of the bleb are marked with a dotted line. (D) Analysis of collagen deposition in the bleb of the groups treated with repeated administration of the PIGF inhibitor and the combined treatments with MMC showed that there was a total reduction of 69% ± 3% after combination treatment (n = 6, P < 0.001), compared to 1C8. No significant (NS) differences were seen between the 2 combination groups (n = 6; P = 0.83). Data are mean values ± SEM.

**Effect of 5D11D4 on Surgical Outcome Compared to Aflibercept**

While PIGF levels are known to be upregulated after anti-VEGF therapy, nothing is described in literature about the combined inhibition of PIGF and VEGF. Therefore, the effect of PIGF inhibition was compared to that of aflibercept. Single IC administration of equimolar concentrations of 5D11D4 (5.4 μg) and aflibercept (3.4 μg) showed a significant improvement in bleb area of 56% ± 4%, compared to irrelevant IgG (4.8 μg, n = 15; P < 0.001), without a significant difference between the 2 treatment groups (n = 15; P = 0.99). Bleb failure occurred at approximately day 25 in both treatment groups (n = 15; P = 0.25). The combinations of MMC with single injection of 5D11D4 (5.4 μg) or aflibercept (3.4 μg) were equally effective in improving surgical outcome. Figure 4 shows representative photographs of the blebs after treatment on different postoperative days. A similar increase in bleb area of 52 ± 5% was seen, compared to MMC only (n = 15, P = 0.98) and blebs of both groups failed at day 51 (n = 15; P = 0.88; Figs. 5A, 5B). Also fibrosis was similarly reduced after both combined treatments (aflibercept and 5D11D4) with MMC by, respectively, 73% ± 4% and 71% ± 4% (n = 15; P = 0.49), compared to IgG (n = 15; P = 0.001; Figs. 5C, 5D).

Thus, the combination of MMC with single 5D11D4 was equally efficacious in the improvement of GFS outcome compared to MMC together with aflibercept.

**Effect of PIGF and 5D11D4 on IOP in Normotensive Mice**

Although no IOP differences were seen in the previous experiments using the model of GFS, there is some evidence from the literature that PIGF can induce an IOP increase (Abdulrazik M, et al. *IOVS* 2010;51:ARVO E-Abstract 979). Moreover, the upregulated PIGF levels in AH of glaucoma patients also might suggest a role of PIGF in IOP regulation. Therefore, the effect of PIGF on eye pressure was investigated further in normotensive mice. Analysis over time showed that SC administration of recombinant murine PIGF (1 μg/mL) significantly increased IOP at 3 and 5 hours after injection with 5 mm Hg, compared to PBS-treated eyes (n = 10; P < 0.001; Figs. 6A, 6B). At 5 hours after administration, a maximum IOP increase of 27% ± 5% was seen after PIGF administration, in comparison with naive noninjected mice (Fig. 6C). Importantly, PBS injection did not alter IOP compared to baseline conditions (n = 10; P > 0.05; Figs. 6A–C). Next, also a possible IOP-lowering effect of topical 5D11D4 was explored. Whereas the positive control, latanoprost, decreased IOP with 29% ± 3% compared to PBS (n = 10; P < 0.001; Figs. 6A, 6B), at 5 hours after administration, a maximum IOP increase of 27% ± 5% was seen after PIGF administration, in comparison with naive noninjected mice (Fig. 6C). Importantly, PBS injection did not alter IOP compared to baseline conditions (n = 10; P > 0.05; Figs. 6A–C). No changes in IOP were seen after 5D11D4 application (data not shown). However, when PIGF was administered in combination with topical therapy of 5D11D4 (0.5 mg/mL), the PIGF induced rise in IOP significantly decreased with 46%, to similar levels found in the PBS treated eyes (n = 10; P < 0.05; Fig. 6C). Latanoprost also was administered after recombinant mPIGF injection, but IOP-change was not different from latanoprost alone (data not shown).
FIGURE 4. Macroscopic postoperative photographs of eyes after surgery. Representative macroscopic photographs of eyes on days 1, 7, 13, 29, and 51 show blebs. Administration of combination therapy of MMC with 5D11D4 or with aflibercept (panels 5, 6) were clearly associated with larger blebs, comparing to the other conditions (panels 1–4). Blebs in all groups were failed at approximately day 51. The edges of the surviving blebs are marked with a white line.

FIGURE 5. Effect of PIGF inhibition (5D11D4) compared to aflibercept on surgical outcome. (A, B) Compared to combined treatment of MMC 0.02% - 2 minutes with single injection of 5D11D4 (5.4 μg), the combined treatment of the antimitotic agent together with aflibercept was equally effective in improving bleb area and survival (n = 15, P < 0.001). (C) Representative pictures of collagen deposition in the bleb on day 52 after surgery. Scale bar: 20 μm. The edges of the bleb are marked with a dotted line. (D) Collagen deposition in the bleb was significantly and equally reduced after the use of the combination therapies (MMC with aflibercept and with 5D11D4) with 73% ± 4% and 71% ± 4%, respectively, at day 52 after surgery (n = 15; P < 0.001). Data are mean values ± SEM.
shown). At 7 hours after administration, the IOP levels of all groups were back to baseline (Figs. 6A–C).

Thus, PlGF inhibition was able to lower PlGF induced IOP increase, which might open new perspectives as IOP-lowering strategy for glaucoma patients with increased PlGF levels in their AH.

**DISCUSSION**

Placental growth factor, a member of the VEGF family that solely binds to VEGF-R1,19 usually is found only at very low levels under physiologic conditions. However, it contributes to the angiogenic and inflammatory switch in various pathologic conditions, including solid tumor growth, ischemic cardiac and limb revascularization, arthritis, atherosclerosis, and ocular neovascularization.20–22 A monoclonal antibody against mouse PlGF (5D11D4) was shown previously to inhibit tumor growth and choroidal neovascularization in different mouse models, by reducing angiogenesis and inflammation.22–24 It also was shown that intraocular use of the antibody was safe.22 Using a mouse model of glaucoma filtration surgery, we also were able to show that IC injection of 5D11D4 significantly improved surgical outcome by increasing bleb survival and area. This was associated with a significant reduction in postoperative proliferation, inflammation, and angiogenesis during the first postoperative days, and with a decrease of collagen deposition at later stages. Furthermore, inhibition of PlGF was more effective than anti-VEGF-R2 treatment in improving surgical outcome, possibly via its additional effect on inflammation.10

The goal of this study was to determine whether in vivo efficacy of the PlGF inhibitor could be improved further using combination therapy. To investigate this, a well-described mouse model for glaucoma surgery was used.12 This mouse model closely resembles the surgical procedure performed in humans. It achieves the clinical endpoint of aqueous humor flow through the constructed channel and the alterations in bleb structures in the mouse model are similar to what is seen in clinic.12,25 The only major difference in the mouse model is that a needle sclerostomy is performed instead of a partial thickness scleral flap, because this latter step is technically and surgically very challenging due to the very thin sclera of the mouse. The mouse model also is known to be characterized by aggressive scarring resulting in early filtration failure, which generally occurs within 14 to 30 days after surgery, whereas in humans this failure occurs most typically at approximately 2 to 3 months. Notably, an agent that can reduce aggressive scar formation in the mouse is likely to be effective in humans. However, animal disease models never form precise replicas of the human pathology and, as such, one should remain cautious with extrapolation of findings from animal research to the care of human disease.

In this study, the IC administration route of the PlGF antibody was selected, based on previous findings. IC injections allow a diffuse distribution of the antibody in the anterior chamber and throughout the outflow route into the filtration bleb without disturbing conjunctival integrity.7 Moreover, via this route of administration, the PlGF inhibitor may block the elevated aqueous PlGF concentrations described in glaucoma patients.10

To broaden the therapeutic approach, different combination therapies with the PlGF antibody were tested. First, the combination of 5D11D4 with MMC, gold standard in clinical practice, was investigated and secondly, the combined inhibition of VEGF and PlGF, by the use of aflibercept. It already has
been described in the literature that the use of MMC can be correlated with the upregulation of proinflammatory, proangiogenic and profibrotic factors. The upregulation of VEGF after MMC, for example, has been described in cancer models and in the GFS model. It has also been shown that the combination therapy of MMC with bevacizumab resulted in complementary effects on the improvement of surgical outcome after filtration surgery. In this study, we showed for the first time to our knowledge that application of MMC during surgery was associated with higher PlGF levels in the AH, which can probably, or at least partly, explain why MMC is not able to affect bleb infiltration of inflammatory cells, since PlGF is a very strong proinflammatory factor. Therefore, associating MMC with drugs that block the upregulated PlGF may offer promising complementary efficacy.

The first experiment showed that repeated injections of the PlGF antibody were able to significantly improve bleb characteristics and reduce collagen deposition, compared to single application of the antimitic agent or 5D11D4. Combination therapy of MMC with the PlGF inhibitor further enhanced the in vivo efficacy of the antibody in the second experiment, compared to monotherapy of MMC and to single or repeated injections of the antibody. This could be explained by the upregulated PlGF levels after MMC treatment. Importantly, no differences were seen between the combination therapies of MMC with single or repeated 5D11D4 administration. Both treatment groups reduced the fibrosis in the bleb similarly, compared to negative control. The reduction in collagen deposition is comparable to baseline levels (data not shown), indicating a very strong antiﬁbrotic effect.

Since we recently showed that PlGF was upregulated after bevacizumab administration during trabeculectomy, the combined inhibition of VEGF and PlGF was tested, by the use of aflibercept and no signiﬁcant differences on surgical outcome were seen, compared to equimolar concentrations of 5D11D4. These data showed that (the combination of MMC with) inhibition of PlGF and inhibition of VEGF and PlGF are similarly efficacious. This suggested that there is no need for a combined anti-VEGF/PlGF therapy after glaucoma surgery, although it cannot be excluded that the combination of a PlGF with a VEGF-inhibitor (e.g., bevacizumab) might show different results. However, this is an important ﬁnding, since it demonstrates that PlGF seems to be more important than VEGF during glaucoma surgery, indicating the importance of inﬂammation in the postoperative wound healing process. Moreover, it has been shown that higher aequorin levels of inﬂammatory markers, such as TNF-α and IL-6 may be associated with worse outcome of glaucoma surgery. Therefore, inhibiting this inflammatory pathway might be beneﬁcial to improve surgical outcome. Also the expression of the different VEGF-receptors on Tenon ﬁbroblasts is in line with these results. In human and rabbit Tenon ﬁbroblasts, VEGF-R1, the receptor to which PlGF binds, showed a 4-fold higher expression compared to VEGF-R2.6 This might indicate that also the fibroblasts are more PlGF driven during postoperative wound healing, compared to VEGF So, based on all these results, the combination therapy of MMC with a single application of the PlGF antibody can be considered in clinical practice.

In all the experiments performed in the mouse model of GFS, we only demonstrated an effect on bleb characteristics and collagen deposition, but we were not able to show any differences in IOP. Importantly, in this model, mice do not develop glaucoma and validation showed that IOP levels never increased baseline levels, even after bleb failure. It is a model for postoperative wound healing, not for glaucoma. Nevertheless, since PlGF is upregulated in glaucomatous AH and as there is evidence that PlGF is involved in the regulation of IOP (Abdulrazik M, et al. IOVS 2010;51:ARVO E-Abstract 979),11 we further investigated this hypothesis. First, SC administration of PlGF was tested and signiﬁcantly increased eye pressures with 27% at 3 hours after administration. These data are in line with the 24–36% IOP increase seen in rabbits after SC PlGF administration (Abdulrazik M, et al. IOVS 2010;51:ARVO E-Abstract 979). Topical eye drops of 5D11D4 in normal mice were administered, but no changes in IOP were observed (data not shown). If PlGF was administered in combination with the PlGF inhibitor (5D11D4), a 46% decrease of IOP was seen. This conﬁrms that a PlGF induced IOP elevation has to be present for the PlGF inhibitor to be eﬀicacious. The gold standard, latanoprost, induced a decrease in eye pressure compared to baseline, which is comparable to the literature. Latanoprost lowers the pressure in the eye by increasing primarily the uveoscleral outﬂow of the AH. Outﬂow resistance also is affected through the activation of proteinases, which remodel the extracellular matrix. The combination of PlGF and latanoprost also was tested, but was not diﬀerent from latanoprost treatment alone (data not shown). This indicated that the working mechanism of the IOP-increase of PlGF probably is not correlated with the uveoscleral outﬂow, but might be rather linked to the conventional outﬂow regulated by the trabecular meshwork (TM). Nothing has been described about PlGF and the TM, but the TM cells do possesses smooth muscle–like properties, such as their contractility, as evidenced by the expression of α-smooth muscle actin. It is known that PlGF can induce contractile effects in vascular smooth muscle cells by the activation of VEGF-R1. As such, a similar mechanism might be responsible for increasing the resistance to AH outflow, leading to IOP increase. Moreover, as shown in lung cancer cells, PlGF is dependent on the expression of rho kinase (ROCK), a strong regulator of the TM cells contractility. Although the working mechanism of the acute IOP increase after PlGF administration remains unknown, these data might open perspectives for PlGF inhibition as an IOP-lowering therapy for glaucoma patients with increased aqueous PlGF levels.

Conclusions

Our data revealed that MMC and PlGF inhibition may have complementary eﬀects in the improvement of surgical outcome by reducing scarring, and are equally eﬀicacious as the combined treatment of MMC and aflibercept. Moreover, inhibition of PlGF might open perspectives as IOP-lowering strategy in pathologic eye conditions where PlGF levels are upregulated.

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