Glaucoma is a major cause of irreversible blindness that is associated with increasing age, and is predicted to affect 80 million people by 2020. Elevated intraocular pressure (IOP) is the most important modifiable risk factor for this debilitating disease, and current treatments for glaucoma involve strategies to reduce IOP with medication, laser treatment, or surgery. Glaucoma filtration surgery (GFS) is typically reserved for patients who are refractory to medication, aims to reduce IOP through the creation of a scleral fistula that allows fluid to egress from the anterior chamber into the subconjunctival space, with an overlying pouch of connective tissue known as the bleb. Surgical failure can arise from fibrotic scarring that occurs at the bleb, resulting in poor filtration and uncontrolled rise in IOP.

Prevention of scarring is an important aspect of maintaining satisfactory IOP reduction following GFS. This is currently achieved with topical corticosteroids following surgery, as well as the intraoperative application of antiproliferative agents such as 5-fluorouracil (5-FU) and mitomycin C (MMC). MMC act through the inhibition of DNA synthesis that prevents the proliferation of myofibroblasts, resulting in a dampened scarring response. However, the nonspecific effects of these drugs can result in complications such as corneal toxicity and thin-walled blebs that may lead to hypotonous maculopathy and even loss of sight due to infections such as endophthalmitis. Furthermore, failure of GFS still occurs in a substantial number of patients despite the use of antiproliferative agents.

Macrophages are essential contributors to the healing process by inducing collagen deposition, matrix remodeling, and fibrosis through the regulation of keratinocytes, myoblasts, and fibroblasts. Numerous studies have shown how circulating monocytes that undergo transendothelial migration into tissues provide an important source of macrophages after tissue injury. Monocyte chemoattractant proteins (MCP), particularly MCP-1, also known as CCL2, are potent cytokines produced predominantly by monocytes/macrophages, that mediate leukocyte recruitment to sites of inflammation. They have been found to be expressed at higher levels in...
chronically inflamed eyes and appear to be associated with an early propensity to scar following GFS in glaucoma patients. Histologic studies have also shown that an increased number of inflammatory cells and fibroblasts in the conjunctiva is associated with reduced rates of surgical success in GFS.

MCP inhibition could therefore prove to be an alternative therapeutic target in the prevention of subconjunctival scarring in GFS, through limiting the number of infiltrating monocytes that promote fibrosis following surgery. In fact, two drugs that act as MCP inhibitors are currently being investigated in phase II clinical trials: Bindarit (inhibits MCP-1, -2, and -3; Angelini Pharma; Ancona, Italy) and Emapticap (MCP-1; Noxxon, Berlin, Germany) for kidney diseases such as lupus nephritis and diabetic nephropathy, where they have been shown to be well tolerated with few systemic side effects.

An investigation into whether these pharmacologic inhibitors of MCP could help to promote surgical success in GFS is especially pertinent, given the evidence that monocyte-derived macrophages play key roles in mediating the scarring process and the potential risks associated with the present use of antiproliferative agents.

The specific aims of this study were to evaluate the effect of MCP inhibition in a murine model of GFS in terms of conjunctival fibrosis, bleb survival, and conjunctival health. The results of this study could have important implications for future use in human patients if MCP inhibitors prove to be a safe, effective therapeutic adjunct in GFS.

METHODS

Mouse Model of GFS

C57/Bl6 mice (n = 36) were bred and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were anesthetized by intraperitoneal injections of 0.1 ml ketamine/xylazine mixture comprising 2 mg/ml xylazine and 20 mg/ml ketamine. The conjunctiva in one eye was dissected to expose a subconjunctival filtration site at the superior quadrant, before a 30-gauge needle was used to create a fistula from the anterior chamber of the eye through the sclera. Surgical sponges soaked with 0.4 mg/mL MMC or sterile water (vehicle) were applied for 2 minutes before fixation and cryosectioning to examine the surgical sites for expression of monocyte and monocyte-derived macrophage marker F4/80 (no. 6640; Abcam, Cambridge, UK) using immunohistochemical staining (IHC). TUNEL assay of apoptotic cells (G3250; Promega, Madison, WI, USA) was also performed on these sections. Eyes from D7 were prepared for hematoxylin and eosin (H&E) staining. Numbers of F4/80 and DAPI double-positive cells, as well as the total number of DAPI-labeled cells in the bleb, were quantified.

Animals also underwent anterior segment slit-lamp biomicroscopy, optical coherence tomography (AS-OCT), and in vivo confocal microscopy at D14 (n = 3 per treatment arm) following surgery.

AS-OCT was used to evaluate cross-sectional bleb survival, while in vivo confocal microscopy was used to detect microcysts and the presence of loose subepithelial connective tissue at the surgical site. These characteristics have been found to be important predictors of the functioning blebs following GFS in glaucoma patients.

In Vitro Mouse Tenon’s Fibroblast Viability

Mouse Tenon’s fibroblasts were isolated from age-matched C57/Bl6 mice (n = 3) based on methods described by Sest et al. Cells were treated with 0.4 mg/mL MMC, 2 μg/mL MCP-Ri, or vehicle for 2 minutes before being plated onto the xCELLigence system to monitor cell viability based on measurements of transmembrane electrical impedance.

MCP-Ri was administered as 2 μg/mL in 0.2% DMSO based on preliminary in vitro experiments that were performed to determine the maximum tolerable drug concentration with minimal effects on cell viability.

Statistical Analysis

Results for qPCR and IHC were analyzed using 1-way ANOVA with Dunnett post hoc correction for multiple comparisons between treatment groups and control. Experiments were performed three times to ensure consistent trends. Conjunctival cell viability was compared using area under the curve (AUC). All statistical analyses were conducted using GraphPad Prism v 6.0 (La Jolla, CA, USA). Graphs represent mean and standard error of the mean, unless stated otherwise where *P < 0.05, **P < 0.01, and ***P < 0.005.

RESULTS

Treatment With MMC and MCP-I Alters the Inflammatory Milieu of Treated Blebs at D2

IHC labeling showed that the percentage of monocytes or monocyte-derived macrophages marked by F4/80 and DAPI and present in post surgery conjunctival blebs was highest in...
control eyes at D2 (Figs. 1A, 1D) while there was a significant reduction of these cells in eyes that had been treated with MMC (Figs. 1B, 1E). MCP-Ri–treated eyes (Figs. 1C, 1F) showed an even greater decrease as compared to control (control: 24.2 ± 3.03; MMC: 11.7 ± 1.2; MCP-Ri: 3.97 ± 0.50) (Fig. 1G). The total number of cells present in postsurgical blebs appeared to be lower in both MMC- and MCP-Ri–treated eyes relative to control, which may reflect changes in the recruitment of myeloid-derived cell populations involved in the acute inflammatory phase, although this did not reach statistical significance (control: 167.3 ± 33.2; MMC: 98.0 ± 5.0; MCP-Ri: 135.0 ± 4.04) (Fig. 1H). TUNEL assay of bleb tissue sections did not show any significant difference between groups (control: 2.3 ± 0.6; MMC: 4.3 ± 1.15; MCP-Ri: 3.7 ± 1.15, results not shown).

Changes in gene expression were also compared using qPCR at D2 following GFS. At this time point, MMC resulted in significantly higher levels of MCP-1 expression than in vehicle-treated control eyes while treatment with MCP-Ri showed a significant reduction in Mcp-1 expression at D2 compared to control (control: 16.7 ± 0.84; MMC: 34 ± 0.84; MCP-Ri: 9.92 ± 0.17) (Fig. 1I).

**Treatment With MCP-I Decreases Expression of Profibrotic Genes at D7**

Quantitative PCR performed on bleb tissues collected at D7 showed that treatment with MCP-Ri resulted in similar decrease in expression of Col1a1 (control: 6.72 ± 0.25; MMC: 2.72 ± 0.67; MCP-Ri: 3.85 ± 0.18) and Sparc (control: 5.60 ± 0.46; MMC: 2.33 ± 0.50; MCP-Ri: 3.42 ± 0.27) compared to vehicle-treated controls. MCP-Ri but not MMC significantly reduced expression of Tgfb1 (control: 1.713 ± 0.075; MMC: 1.3 ± 0.21; MCP-Ri: 1.22 ± 0.055). Conversely, Tgfb2 was decreased in eyes that were treated with MMC but not MCP-Ri (control: 1.64 ± 0.20; MMC: 0.82 ± 0.17; MCP-Ri: 1.45 ± 0.18). Both Fibronectin (control: 11.97 ± 1.94; MMC: 10.15 ± 2.47; MCP-Ri: 7.81 ± 0.35) and MCP-1 (control: 17.03 ± 6.36; MMC: 8.17 ± 1.09; MCP-Ri: 8.52 ± 1.24) did not show any
significant difference in vehicle- and MMC- or MCP-Ri–treated eyes at D7 (Figs. 2A–F). H&E staining showed increased collagen deposition in the blebs of control eyes, but not eyes that had received MMC or MCP-Ri treatments (Fig. 2G).

**Treatment With MCP-Ri Reduces Bleb Vascularity and Preserves Bleb Morphology In Vivo Following GFS**

Slit-lamp examination of conjunctiva blebs at D14 following GFS showed increased vascularity at the surgical site in eyes that received only vehicle (Fig. 3Ai). Both MMC (Fig. 3Aii) and MCP-Ri (Fig. 3Aiii) treatments resulted in a reduction in the appearance of engorged dilated blood vessels around the bleb. In vivo imaging with AS-OCT also demonstrated improved bleb height (Fig. 3B) while confocal microscopy showed loose subepithelial connective tissue spaces, suggestive of functional blebs, in MMC- and MCP-Ri–treated eyes (Fig. 3C). Statistical analysis of bleb height showed that MMC and MCP-Ri treatments resulted in significantly higher blebs, compared with control (control 20.0 ± 4.6; MMC 82.7 ± 10.4; MCP-Ri 104.0 ± 2.3) (Fig. 4A). All treatment groups were masked at the time of image acquisition and analysis.

**Treatment With MCP-Ri Results in Better Cell viability In Vitro Compared With MMC**

Mouse Tenon’s fibroblasts were cultured in the presence of control medium, MMC, or MCP-Ri and cell viability was monitored over time. Exposure to MMC resulted in a marked reduction in cell viability by 150 hours (AUC: 232.7) in comparison with control (AUC: 1324) and MCP-Ri–treated eyes (AUC: 1248) (Fig. 4B).

**DISCUSSION**

Glaucoma filtration surgery forms an important part of IOP-lowering treatment in order to prevent progression of this sight-threatening disease. Sustained IOP reduction depends on the preservation of a functional, healthy bleb, which facilitates effective aqueous humor egress into the subconjunctival space following surgery. Surgical failure has been reported in between 35% and 43%32–34 of cases, however, due to postoperative scarring and subconjunctival fibrosis. Adjunctive treatment with antiproliferative agents such as MMC or 5-FU is widely used by ophthalmic surgeons to prevent scarring and bleb failure, although these efforts to dampen fibrosis have a broad impact on tissue health and can lead to thin-walled blebs.
FIGURE 3. MMC- and MCP-Ri–treated eyes resulted in less vascular blebs and improved bleb morphology at D14. (A) Slit-lamp images show hypervascularity at the GFS site in control eyes (Ai), which is reduced in MMC- (Aii) and MCP-Ri-treated (Aiii) eyes. (B) AS-OCT imaging demonstrates the presence of prominent superior blebs (bleb area outlined by white dashes; bleb height marked by double-headed arrows) in eyes treated with MMC (Bi) and MCP-Ri (Bii) but not in control (Biii) eyes. (C) Loose subepithelial connective tissue spaces associated with bleb filtration are seen in confocal imaging of MMC- (Ci) and MCP-Ri- (Ciii) but not control-treated eyes (Ci). N = 5 per group.

FIGURE 4. Treatment with either MMC or MCP-Ri resulted in improved bleb height, but differed in terms of toxicity to mouse Tenon’s fibroblasts. (A) MMC and MCP-Ri resulted in significant differences in bleb height at D14, relative to control. (B) In vitro treatment of mouse Tenon’s fibroblasts with MMC resulted in a marked decrease in cell index over time, while this effect was not seen in MCP-Ri–treated cells.
with risk of bleb leak and infection. Infiltrating monocyte-derived cell populations play a key role in modulating fibrosis and scarring through direct effects on the extracellular matrix as well as influencing other proinflammatory cell types. Ours is the first study to demonstrate that application of a MCP-1 inhibitor results in a targeted reduction in fibroblast migration and stimulates fibrosis through inducing fibroblast migration and proliferation. Both TGFβ1 and B2 isoforms have been implicated in post trabeculectomy scarring, and may reflect their differential effect of MCP-Ri on the inflammatory profile formed below. At D7 post GFS, expression of Coll1a1 and Sparc was reduced with both MMC and MCP-Ri. Collagen 1a1 and Sparc are heavily involved in the extracellular matrix remodeling process associated with fibrogenesis and scarring; therefore our results suggest a lesser degree of scar at the surgical site with MMC and MCP-Ri treatment. TGF-beta is a potent growth factor released by various cell types that make up the inflammatory milieu, including macrophages and fibroblasts, and stimulates fibrosis through inducing fibroblast migration and proliferation. Both TGFβ1 and B2 isoforms have been implicated in post trabeculectomy scarring, although in vitro studies of human Tenon's fibroblasts appear to show the strongest upregulation of TGFβ1. The differential effect of MCP-Ri and MMC to suppress TGFβ1 could reflect their specific actions on different cell populations: TGFβ1 inhibits T cells and B cells and regulates monocyte/macrophage activity, while TGFβ2 may have a predominant action on T cells. Gene expression of fibronectin was reduced with both treatments relative to vehicle; however, this did not reach statistically significant levels. This could be due to relatively high concentrations of plasma fibronectin present in all three groups as compared to cellular fibronectin, or specific effects of MMC or MCP-Ri on leukocytic/fibroblast behavior that remain to be elucidated. However, it is also possible that the lack of statistical significance demonstrated by MMC or MCP-Ri on the different TGFβ isoforms, fibronectin, or MCP-1 arose from the limited numbers of animals used in this study. Further studies with larger groups of animals would be helpful to verify these results. Importantly, in vivo imaging of mice that had undergone experimental GFS with application of MMC or MCP-Ri demonstrated clear evidence of preserved bleb height with characteristic subepithelial cystic spaces that have been associated with functioning trabeculectomies in clinical studies. Vehicle-treated control eyes, on the other hand, showed minimally elevated hypervascular blebs and condensed stromal tissue with the absence of subepithelial spaces by D14 after surgery. These findings imply that both MMC and MCP-Ri treatments maintained bleb filtration after GFS, in contrast to the in vitro experiments, resulting in a marked improvement in cell viability over the first 150 hours. Our results suggest that adjunctive treatment with MCP-Ri in an experimental model of GFS results in a diminished early inflammatory response, leading to an overall reduction in postsurgical fibrosis and the maintenance of subconjunctival filtration in this mouse model. This is consistent with studies showing that the recruitment of large numbers of inflammatory monocytes is one of the earliest wound healing mechanisms to be initiated following injury. Importantly, conjunctival health is also preserved with MCP-Ri treatment compared to MMC. Future studies need to be conducted to determine if our observations are similarly applicable to human eyes following trabeculectomy. Recent phase II clinical studies have successfully demonstrated the safety and therapeutic potential of a specific MCP-Ri inhibitor in diabetic patients; thus it would be extremely pertinent to explore whether this drug is suitable for ocular applications as well. Limitations of our study include the inability to assess if MCP-Ri results in aberrant long-term wound healing responses that may compromise bleb stability, such as damaged acellular conjunctiva/episcleral stroma reported in MMC-treated human eyes that could predispose leaking, hypotony, and infection. TUNEL staining that was performed on tissue sections from eyes that were retrieved on day 2 following surgery also did not show any significant difference among the treatment groups in contrast to the in vitro experiments. This may be due to the extremely low numbers of TUNEL-positive cells counted in each group, or issues with the duration of immersion fixation and the lack of antigen retrieval techniques, which has been reported to interfere with the sensitivity of this assay. We were unable to further our efforts to optimize this, however, due to technical limitations. IOP was excluded as a primary outcome measure, since previous experiments using this model did not demonstrate any significant alterations in IOP before and after surgery. Further studies into the effect of MCP-Ri on larger animal models of experimental GFS that demonstrate more reliable IOP changes are needed. Fibrogenesis or scarring at the surgical site continues to be a major obstacle to achieving sustained, effective IOP control in glaucoma patients who have undergone filtration surgery. Our study suggests that specific inhibition of infiltrating monocytes, which are key regulators of the inflammatory process, by targeting MCP-1 results in prolonged bleb survival following GFS with minimal impact on fibroblast health. These findings could have major clinical and therapeutic implications for improving postoperative outcomes in glaucoma surgery.

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