In Vivo Confocal Microscopy of Filtering Blebs After Trabeculectomy

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Objectives: To analyze filtering blebs after trabeculectomy by means of in vivo confocal microscopy and to correlate the images with clinical bleb appearance and function.

Method: In vivo confocal microscopy using the Heidelberg Retina Tomograph/Rostock Cornea Module (Heidelberg Engineering, Heidelberg, Germany) was performed in 53 filtering blebs in 45 patients 6 days to 30 years postoperatively.

Results: In vivo confocal microscopic findings significantly correlated with good bleb function included the number of epithelial microcysts ($P = .03$), a large total stromal cyst area ($P = .009$), the absence of encapsulated stromal cysts ($P = .002$), minimal vascularization ($P = .05$), and the absence of tortuous conjunctival vessels ($P = .01$). In contrast, a hyperreflective condensed bleb stroma was significantly associated with bleb failure ($P < .001$). Bleb stroma mainly consisting of a rarified collagenlike network was significantly linked to trabeculectomy performed with mitomycin C ($P = .001$). Epithelial and stromal inflammation were observed at a median of 1 and 4 months after surgery, respectively.

Conclusions: In vivo confocal microscopy using the Heidelberg Retina Tomograph/Rostock Cornea Module permits diagnostic imaging of filtering blebs and differentiation between good and insufficient bleb function. Moreover, the postoperative inflammatory reaction can be monitored directly for adapted postoperative anti-inflammatory treatment.

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bleb. According to Picht and Grehn,\textsuperscript{16} filtering blebs with favorable outcomes show more microcysts, fewer conjunctival and corkscrew vessels, a lower prevalence of encapsulation, and decreased height compared with filtering blebs with unfavorable postoperative result. Low, localized blebs with high IOP predict subconjunctival fibrosis, whereas high-domed blebs with high IOP are typical of Tenon cysts.\textsuperscript{18} However, functional areas may exist around the base of a Tenon cyst, and an increase in IOP does not always accompany the development of such a cyst.\textsuperscript{19} Whereas Wells et al\textsuperscript{3} were able to describe blebs with horizontally mixed architecture, no grading system thus far is available to describe the architecture of blebs in their vertical composition.

Inasmuch as the clinical evaluation of filtering blebs remains difficult and the appearance of an established bleb alone is an insufficient guide to function,\textsuperscript{7} further diagnostic tools are required to assess functional outcome. Ultrasound biomicroscopy was used to study filtering blebs after trabeculectomy. While eyes with good IOP control had mainly type L (low inside reflectivity) blebs, type E (encapsulated) and type F (flattened) blebs at ultrasound biomicroscopy were observed in eyes with moderate function.\textsuperscript{20} Slitlamp-adapted optic coherence tomography was also used to analyze postoperative filtering blebs that appeared to be either cystic, bullous, or mixed.\textsuperscript{21,22} Functioning blebs showed low reflectivity within the bleb cavity, with multiple hyporeflective microcystic spaces at optical coherence tomography.\textsuperscript{23} However, none of these new techniques enabled imaging of the filtering bleb on a cellular level, as does near-infrared laser light confocal microscopy. Confocal microscopic findings of normal conjunctiva, conjunctival inflammation, and conjunctival tumors have been described.\textsuperscript{24,25} A small series of in vivo confocal images of filtering blebs has been published in the French literature, demonstrating epithelial microcysts in functioning blebs and the absence of these cysts in nonfiltering blebs associated with dense connective tissue.\textsuperscript{26} We report in vivo confocal findings in filtering blebs and correlate the images with function several days to years after trabeculectomy with and without the use of antimetabolites.

**METHODS**

The Heidelberg Retina Tomograph II in association with the Rostock Cornea Module (Heidelberg Engineering, Heidelberg, Germany) is a contact confocal microscope originally constructed to examine the cornea in vivo. It operates with a $\times 63$ Zeiss objective (Carl Zeiss, MicroImaging GmbH, Göttingen, Germany) enabling a scanning area of $400 \times 400$ µm with magnification up to $\times 800$ and resolution of approximately 1 µm. After informed patient consent was secured, scans of the conjunctiva were obtained in several areas in the center of a filtering bleb approximately 2 mm distant from the limbus in downgaze (Figure 1). A CCD camera enabled direct
observation of the area scanned. One drop of unpreserved benoxinate hydrochloride (Conjuncain-Edo; Dr Mann Pharma, Berlin, Germany) and carbomer ophthalmic gel (Vidisic; Dr Mann Pharma) was applied to the lower conjunctival sac. Sixty-two in vivo confocal examinations were performed in 53 filtering blebs in 45 patients. Only the final examination of each bleb was considered for statistical analysis. Forty-four to 608 images (mean, 135 images) were obtained during each examination and were analyzed. Images were recorded along the z-axis as single scans or in the movie-motion mode. For the analysis of epithelial and stromal cysts and cystic spaces, 5 representative epithelial images and 5 representative stromal images were selected by an observer (E.M.M.) masked to clinical data. Moreover, 5 representative images of blood vessels were chosen by the same observer to analyze the number, diameter, and tortuosity of the vessels. The number and area of epithelial cysts and stromal cystic spaces were analyzed using freely available ImageJ software (http://rsb.info.nih.gov/ij/download.html) (Figure 2A and B). Blood vessels were graded according to the following scheme: grade 1, 0 to 1 vessel per image; grade 2, 2 to 3 vessels per image; and grade 3, more than 3 vessels per image. The diameter of vessels was measured and stratified into 3 subgroups according to size: smaller than 20 µm, 21 to 50 µm, and larger than 50 µm. Tortuosity of vessels was assessed according to a grading scale with 4 grades: straight (0), mild (1), moderate (2), and severe (3) (Figure 3). The Heidelberg Retina Tomograph/Rostock Cornea Module system enabled imaging of filtering blebs as deep as 200 µm from the epithelial surface in diffuse blebs and as deep as 800 µm in cystic filtering blebs.

Medical records were reviewed to determine demographic characteristics, previous ophthalmic procedures, administration of preoperative glaucoma medication, associated eye disease, and intraoperative and postoperative variables. Demographic data included age, race, sex, and diagnoses, such as primary open-angle, pseudoexfoliation, chronic primary angle closure, low-tension, congenital, juvenile, and secondary glaucomas. Previous surgical procedures consisted of argon laser trabeculoplasty, laser iridotomy, trabeculectomy, viscoanalostomy, cyclophotocoagulation, and cataract surgery. At our institution trabeculectomy was routinely performed with a fornix-based conjunctival flap. Intraoperative use of mitomycin C, surgical and postoperative complications, and the use of fluorouracil injections were documented. For 35 patients, a detailed intraoperative and postoperative history was available. At in vivo confocal microscopy, current medication, time since surgery, logMAR visual acuity, IOP related to target pressure, and corneal thickness were assessed.

Filtering blebs were clinically analyzed according to the grading system published by Picht and Grehn and subdivided into 3 groups according to function: group 1, target pressure reached without antiglaucoma medication; group 2, target pressure
reached with antiglaucoma medication; and group 3, target pressure not reached or only accomplished with oral acetazolamide therapy.

Microcysts were clinically classified into 3 groups according to their presence in 3 sectors of the filtering bleb. Slit-lamp images were obtained of each filtering bleb. Statistical analysis of data was performed using χ² tests and the Spearman correlation.

Table 1. Filtering Bleb Characteristics and Time After Trabeculectomy

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (%) of Blebs</th>
<th>Time Since Surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Mean</td>
</tr>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good bleb function</td>
<td>30 (57)</td>
<td>6 d-30 y</td>
</tr>
<tr>
<td>Group 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate bleb function</td>
<td>11 (21)</td>
<td>31 d-26 y</td>
</tr>
<tr>
<td>Group 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor bleb function</td>
<td>12 (22)</td>
<td>43 d-15 y</td>
</tr>
</tbody>
</table>

Figure 4. Presumed goblet cells (arrows) in epithelium of filtering blebs adjacent to epithelial microcyst (asterisks); scan depth, 2 µm.

Sixty-two in vivo confocal examinations were performed in 53 filtering blebs in 45 patients. All patients were white, 27 (60%) were women, and their mean age was 67 years (age range, 24-90 years). Thirty-two examinations (60%) were performed in the right eye and 30 (49%) were performed in the left eye. The type of glaucoma was predominately primary open-angle (27 patients [51%]), followed by pseudoxfoliation (13 patients [25%]); secondary (5 patients [9%]), including uveitic glaucoma and 1 eye with iridocorneal endothelial syndrome; and normal tension (4 patients [8%]). Trabeculectomy was performed 6 days to 30 years before our examination. Mean and median times since surgery for all patients are given in Table 1. Mitomycin C therapy was used intraoperatively in 11 filtering blebs (21%) and fluorouracil was injected subconjunctivally in 8 (18%) of 44 filtering blebs with known postoperative history.

At examination, target pressure was reached in 30 eyes (57%) without antiglaucoma medication (group 1). In 11 eyes (21%), target pressure was achieved with 1 to 4 glaucoma medications (group 2). Filtering blebs in 12 eyes (22%) were considered failures. Target pressure in these eyes was not reached or was accomplished only with the administration of systemic acetazolamide therapy, and further surgery was planned (group 3) (Table 1).

Architecture at slitlamp examination correlated well with the function of filtering blebs. Microcysts of the conjunctiva (P<.001), moderate elevation of the bleb (P<.001), and diffuse bleb appearance (P=.01) correlated significantly with good filtration. In contrast, scarred (P<.001) or high-domed, encapsulated filtering blebs (P=.02) were associated with insufficient lowering of IOP.

The epithelium varied in thickness from 11 to 80 µm. Epithelial thickness was not associated with filtering bleb function or adjunctive mitomycin C or fluorouracil use. Goblet cells were typically observed throughout the conjunctival epithelium (Figure 4). In vivo confocal microscopy demonstrated encapsulated epithelial microcysts in 60% of filtering blebs (Figure 5A and B). Zero to 34 cysts (mean, 7 cysts) were observed on selected epithelial scans, with a total cyst area of 0 to 63 mm² (mean, 9 mm²). The epithelial cysts occupied 0% to 39% (mean, 6%) per image of the bleb epithelium. The number of epithelial cysts was significantly correlated with bleb function (P=.03) (Table 2). In a few patients, epithelial cysts were filled with amorphous material (Figure 5C). Only the highest clinical grade of microcysts (grade 3) correlated well with the presence of microcysts as seen at in vivo confocal microscopy (P=.02).

Underneath the epithelium a highly reflective basement membrane was observed, as found in normal conjunctiva. Its thickness varied over a wide range, between 2 and 100 µm (mean, 38 µm).

The bleb stroma demonstrated 4 typical patterns, which in some cases occurred concomitantly (Table 2): a loose collagenlike meshwork (Figure 6A), a rarified collagenlike network with large cystic spaces (Figure 6B), condensed and hyperreflective tissue (Figure 6C), and blurred stroma (Figure 6D). In eyes with condensed stroma, additional areas of loose collagenlike stroma could be observed in selected patients in the depth of the filtering bleb (vertically composite blebs; Table 2). Blurred stroma was typically present in the first 2 postoperative months and was mainly associated with a loose collagenlike meshwork. This loose collagenlike stroma was seen in most eyes (39 eyes [74%]), whereas a condensed bleb stroma was evident in 11 eyes (21%). A condensed, hyperreflective bleb stroma was typically associated with bleb failure (P<.001; Table 2). Bleb stroma mainly consisting of a rarified collagenlike network with large cystic spaces was present in 11 filtering blebs (21%). This type of bleb stroma was significantly correlated with trabeculectomies performed with mitomycin C therapy (P=.001; Table 2).

Two kinds of cystic spaces could be observed interspersed in the bleb stroma (Table 2): unencapsulated
Forty-three filtering blebs (81%) contained nonencapsulated stromal cysts and 19 blebs (36%) contained encapsulated stromal cysts. In 7 blebs, stromal cysts were completely absent. In 16 blebs, nonencapsulated and encapsulated cystic spaces were observed concomitantly. The absence of encapsulation was significantly correlated with good bleb function ($P = .002$; Table 2). The total area of stromal cysts ranged from 0.5 to 108 mm$^2$ (mean, 40 mm$^2$) according to a cystic area of 0.3% to 67% (mean, 25%) per stromal image analyzed. A large total cyst area present in the bleb stroma was significantly correlated with good bleb function ($P = .009$; Table 2).

With in vivo confocal microscopy, perfused vessels could be observed directly. Avascular filtering blebs were observed in 8 eyes (15%), whereas grade 1 vascularization was detected in 22 eyes (42%), grade 2 in 21 eyes (40%), and grade 3 in 2 eyes (4%). Vessels lacked tortuosity in 28 eyes (53%), but showed grade 1 tortuosity in 16 filtering blebs (30%) and grade 2 tortuosity in 9 filtering blebs (17%). Both vascularization ($P = .05$) and tortuosity ($P = .01$) as seen at in vivo confocal microscopy were independently significantly correlated with filtering bleb function (Table 1). Hyperreflective structures compatible with lymphatic vessels or compressed veins typically accompanied perfused stromal blood vessels (Figure 8).

**Table 2. Correlation of Bleb Appearance as Seen at In Vivo Confocal Microscopy With Function and Clinical Appearance**

<table>
<thead>
<tr>
<th>Confocal Microscopy</th>
<th>Filtering Bleb Function</th>
<th>Correlation with Bleb Function</th>
<th>Clinical Feature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Good (n = 30)</td>
<td>Moderate (n = 11)</td>
<td>Poor (n = 12)</td>
</tr>
<tr>
<td>Epithelium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial microcysts per scan</td>
<td>29.7</td>
<td>28.4</td>
<td>17.1</td>
</tr>
<tr>
<td>Stroma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Loose collagenlike meshwork</td>
<td>26 (87)</td>
<td>8 (73)</td>
<td>5 (42)</td>
</tr>
<tr>
<td>Rarified collagen network with cysts</td>
<td>7 (23)</td>
<td>3 (27)</td>
<td>1</td>
</tr>
<tr>
<td>Condensed hyperreflective bleb stroma</td>
<td>0</td>
<td>0</td>
<td>11 (92)</td>
</tr>
<tr>
<td>Blurred stroma</td>
<td>5 (17)</td>
<td>3 (27)</td>
<td>1</td>
</tr>
<tr>
<td>Mean total stromal cyst area, mm$^2$</td>
<td>46.1</td>
<td>37.0</td>
<td>25.6</td>
</tr>
<tr>
<td>Stromal cysts with capsule</td>
<td>11 (37)</td>
<td>3 (27)</td>
<td>5 (42)</td>
</tr>
<tr>
<td>Stromal cysts without capsule</td>
<td>28 (93)</td>
<td>9 (82)</td>
<td>6 (50)</td>
</tr>
<tr>
<td>Vascularization, No. of eyes‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 0</td>
<td>4</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Grade 1</td>
<td>16</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Grade 2</td>
<td>10</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Grade 3</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Tortuosity, No. of eyes‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 0</td>
<td>19</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Grade 1</td>
<td>7</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Grade 2</td>
<td>4</td>
<td>0</td>
<td>5</td>
</tr>
</tbody>
</table>

Abbreviation: NS, not significant.

*Data are given as the number (percentage) of eyes unless otherwise indicated.

†Blood vessels were graded according to the following scheme: grade 1, 0 to 1 vessel per image; grade 2, 2 to 3 vessels per image; and grade 3, more than 3 vessels per image. The diameter of vessels was measured and stratified into 3 subgroups according to size: smaller than 20 µm, 21 to 50 µm, and larger than 50 µm.

‡Tortuosity of vessels was assessed according to a grading scale with 4 grades: straight (0), mild (1), moderate (2), and severe (3).
Postoperative inflammation could directly be monitored using the in vivo confocal microscope. Round, hyperreflective cells, most probably lymphocytes, were typically seen in the epithelium itself and accumulating in epithelial microcysts (Figure 9A) at a median of 1 month postoperatively. Inflammatory cells were seen in the bleb stroma at a median of 3.5 months after surgery and were partly adherent to vessel walls (Figure 9B). Two patients showed epithelial and stromal inflammation 7 and 28 years, respectively, after trabeculectomy. In 1 patient, bleb needling with subconjunctival fluorouracil injection was performed 5 days before in vivo confocal microscopy. The second patient had bacterial conjunctivitis at the time of examination. Two additional patients with secondary glaucoma associated with uveitis experienced prolonged inflammation postoperatively.

In accord with the literature, the clinical architecture of filtering blebs analyzed in our study correlated well with postoperative function. The presence of microcysts, moderate elevation of the bleb, and a diffuse bleb appearance were associated with good bleb function. In contrast, scarred or dome-shaped and encapsulated blebs were typically seen in filtering blebs with insufficient function. We graded bleb function as good when the target pressure was reached. We are aware that the percentage in reduction of IOP after surgery may have been the best criterion for evaluation of bleb function, but this value was unavailable in many of our patients because of prolonged time since surgery or incomplete history after trabeculectomy.
In vivo confocal microscopy proved valuable in the evaluation of filtering blebs after trabeculectomy in this study. The number of epithelial microcysts, the absence of encapsulated cysts in the stroma, and the total stromal cystic spaces as seen at in vivo confocal microscopy correlated significantly with successful filtering blebs. Moreover, less vascularization and tortuosity of the involved vessels as seen at in vivo microscopy were independently significantly associated with good bleb function. A condensed stroma was typical in bleb failure. In scarred or encapsulated blebs, areas of sufficient bleb filtration with loose or rarified collagenlike stroma were observed in selected cases in addition to condensed stroma. Patients with these findings may benefit from cyst puncture or bleb needling with or without fluorouracil.

Histologic and ultrastructural observations in functioning and failed excised filtering blebs correlate well with the images obtained at in vivo confocal microscopy. However, some conflicting findings warrant discussion. Functioning filtration blebs are reported to histologically demonstrate loosely arranged subepithelial connective tissue with clear spaces. This corresponds well with the loose or rarified collagenlike material with cystic spaces observed in vivo at microscopy. In the early postoperative period, the conjunctiva and subconjunctival tissues are clinically edematous and hyperemic. This clinical observation matches our in vivo confocal findings of a blurred stroma up to 2 months postoperatively. Failed blebs demonstrated dense collagenous connective tissue in their walls at histologic analysis and at in vivo confocal microscopy. However, at in vivo confocal microscopy, this condensed stroma was not confined to the bleb wall but made up the entire visible bleb stroma. Most authors describe a morphologically normal bleb epithelium at histologic analysis. The microcysts observed at slitlamp examination are mainly ascribed to the clear stromal spaces seen at histologic analysis. Few authors observed a separation of basilar epithelial cells and intraepithelial spongiotic vesicles. With in vivo confocal microscopy, we were able to demonstrate that the intraepithelial spongiosis observed histologically corresponds to encapsulated cysts of different sizes and shapes throughout the entire epithelium in vivo. Moreover, we could show that inflammatory cells preferably lodge in these epithelial microcysts in the early postoperative phase. Single epithelial cysts are filled with amorphous material compatible with proteinaceous debris observed at transmission electron microscopy in this location. In contrast to published reports, we are confident that epithelial cysts observed at in vivo confocal microscopy, and not stromal cystic

![Figure 7. A, Unencapsulated stromal cystic space; scan depth, 65 µm. B, Encapsulated cystic space (arrows); scan depth, 79 µm.](image)

![Figure 8. Hyporeflective structures compatible with lymphatic or compressed vein (#) accompanying stromal perfused blood vessels (asterisks); scan depth, 90 µm.](image)
spaces, constitute the microcysts observed clinically. Moreover, the presence of microcysts throughout the epithelium adds further anatomical evidence that aqueous humor can move transconjunctivally as a mechanism of filtration, as proposed previously.29,30,32-34 Further outflow mechanisms discussed include absorption by conjunctival and episcleral or lymphatic vessels.35-37 Conjunctival and presumed lymphatic vessels are visible in the bleb stroma at in vivo confocal microscopy; however, their anatomical presence alone does not confirm their role in bleb filtration.

We observed an unusually thick epithelial basement membrane at in vivo confocal microscopy in normal conjunctiva and in selected filtering blebs. Technical features such as gain adjustment time lag or a minimally oblique scanning technique may be responsible for this phenomenon.

Postoperative inflammation can be observed directly in the conjunctiva at in vivo confocal microscopy. Round cell infiltrates persisted longer in patients with uveitis and are associated with bleb needling procedures, the application of fluorouracil, and conjunctival infection.

Mitomycin C is known to reduce postoperative subconjunctival fibrosis and to improve postoperative function, especially in patients at high risk for bleb failure.1,10-12 Several clinico-pathologic reports on large excised mitomycin C–treated filtering blebs causing discomfort have been published.31,38 The epithelium appeared attenuated with intraepithelial microcysts, dyskeratosis, and focal keratinization.31,38 A prominent acellular band of collagen was present immediately subjacent to a thickened epithelial basement membrane. The substantia propria was largely acellular and was composed of loosely arranged hypocellular connective tissue with a paucity of vessels and scattered lymphocytes.31 Episceral fibrotic tissue obtained after trabeculectomy with mitomycin C therapy exhibited few fibroblasts without contractile elements, randomly oriented collagen, and no acid mucopolysaccharide ground substance.39 In vivo confocal microscopy confirmed the presence of a rarified stroma with large cystic, nonencapsulated spaces in filtering blebs after treatment with mitomycin C. In contrast to the histologic observations of Francis et al,40 the bleb epithelium was not thinner in blebs treated with mitomycin C compared with blebs treated without mitomycin C at in vivo confocal microscopy.

CONCLUSIONS

In vivo confocal microscopy of filtering blebs after trabeculectomy enables differentiation of blebs with good and insufficient function. Moreover, this technology enables differentiation between blebs after trabeculectomy with and without mitomycin. It permits monitoring of postoperative inflammation in the filtering bleb directly and may allow adjustment of postoperative anti-inflammatory treatment. In clinically failing blebs with areas of loose collagen-like stroma, cyst puncture or bleb needling with or without fluorouracil therapy may be promising, whereas in filtering blebs with merely condensed stroma, repeat trabeculectomy may be warranted. Our ongoing prospective study is designed to answer the question of whether in vivo confocal microscopy can demonstrate signs of bleb failure earlier than clinical observation of bleb appearance or elevation of postoperative IOP.

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