Use of Anterior Segment Optical Coherence Tomography to Predict Corneal Graft Rejection in Small Animal Models

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Purpose. To correlate the degree of anterior chamber (AC) inflammation and corneal thickness evaluated by anterior segment optical coherence tomography (ASOCT) with corneal graft rejection status and to explore the value of ASOCT in assisting the diagnosis or prediction of graft rejection using a rat penetrating keratoplasty (PK) model.

Methods. A total of 40 PKs were performed using Fisher rats (allogeneic groups) and Lewis rats (syngeneic group) as donors and Lewis rats as recipients: isograft control group (n = 10), allograft untreated group (n = 10), and allograft with 1% prednisolone acetate treatment group (n = 20). All the grafts were evaluated for 28 days by a scoring rejection index (RI) to assess the graft opacity, edema, and neovascularization using slit lamp biomicroscopy. The AC inflammation and corneal graft thickness were assessed using ASOCT.

Results. All the allogeneic control grafts and four of the 20 allogeneic steroid-treated grafts developed rejection episodes. In the allogeneic treated group, the rejected grafts had a significantly higher mean AC inflammation grade at 1 week (grade 3.25 ± 0.49 vs. 1.83 ± 0.36, P < 0.001), significantly thicker central graft thickness at 2 weeks (355.25 ± 42.42 μm vs. 381.247 ± 12.51 μm, P = 0.047), and a significantly higher RI at 4 weeks (7.75 ± 0.65 vs. 4.60 ± 0.13, P < 0.001) compared to the nonrejected grafts. Eyes with ≥grade 3 AC inflammation at 1 week, or with ≥400 μm central graft thickness at 2 weeks, were significantly associated with graft rejection (odds ratio [OR], 15.15, P = 0.009; and OR, 9.75, P = 0.014, respectively).

Conclusions. The use of ASOCT to evaluate AC inflammation and corneal thickness aids in the early evaluation and diagnosis of graft rejection in animal models. Early increased AC inflammation was an early predictor of graft failure prior to definitive clinical evaluation.

Keywords: anterior segment optical coherence tomography, anterior chamber inflammation, graft rejection, graft thickness

Corneal transplantation is the most prevalent transplant procedure worldwide. Although newer forms of selective tissue transplantation, for example, endothelial keratoplasty (EK) or deep anterior lamellar keratoplasty (DALK), have gained popularity in recent years, penetrating keratoplasty (PK) is still the most common keratoplasty procedure currently. This may be because selective tissue transplantation may not be widely available because of inaccessibility to corneal-trained surgeons worldwide, technical expertise involved in performing the surgery, and problems in donor preparation. PK may also still be the preferred technique in certain indications, such as patients with deep stromal scars, deep-seated corneal infection, or in patients requiring extensive anterior segment reconstruction. In PK patients, despite the use of immunosuppressants, immunological graft rejection remains the major cause of graft failure. Experimental animal models have been used extensively to investigate the mechanisms underlying corneal transplant rejection. Among them, the rat keratoplasty model is a well-established model as the expressions of major histocompatibility complex and leukocyte antigens are similar to those of human corneas and the immunogenetic constitution is well characterized.

The diagnosis of early corneal graft rejection/failure in small animal models can be difficult. In the rat, graft status is often assessed clinically by the rejection index (RI), which includes graft opacity, edema, and neovascularization. Graft rejection is diagnosed when the combined score exceeds 6. However, this is graded subjectively, and it can be difficult, especially in cases of mild edema or when the severity of graft opacity or neovascularization varies in different regions of the transplant- ed cornea. In our previous study using a rat PK model, we also encountered this problem in diagnosing graft rejection. Flynn et al. therefore, reported the use of in vivo graft pachymetry as a useful tool to objectively diagnose graft rejection in a mouse model of corneal transplantation.

Anterior segment optical coherence tomography (ASOCT) has been used in a wide spectrum of corneal and anterior segment conditions. Compared to the use of conventional ultrasound pachymetry to measure corneal thickness, ASOCT is noncontact and generates high-resolution cross-sectional images for different corneal locations at the same time, providing...
more in-depth imaging and micrometric measurements for corneal thickness. The use of ASOCT for evaluating anterior chamber (AC) inflammation has been recently suggested. The AC inflammatory reaction is one of the clinical presentations of allogeneic graft rejection. However, when graft rejection occurs, corneal edema and opacification may hinder the proper assessment of the AC with slit lamp biomicroscopy. Agarwal et al. have recently reported the use of ASOCT for detecting AC inflammatory reaction in uveitis patients whose corneal clarity was compromised. Therefore, we hypothesized that ASOCT may aid in detecting AC inflammation in rejected grafts and may consequently add value in addressing the problems of diagnosis of graft rejection in small animals.

The aim of this study was to demonstrate the use of ASOCT for evaluating the cornea and AC inflammation after corneal transplantation in a rat model. We also correlated the degree of AC inflammation and corneal thickness assessed by ASOCT with corneal graft status to explore the value of ASOCT in assisting the diagnosis or prediction of graft rejection response.

METHODS

Animals and Penetrating Keratoplasty

A total of 40 PKs were performed using Fisher rats (allogeneic groups) and Lewis rats (syngeneic group) as donors and Lewis rats as recipients: isograft control group (n = 10), allograft untreated group (n = 10), and allograft treated group (n = 20; 1% topical prednisolone acetate [PA] eye drops treatment four times daily for 4 weeks). All animals were treated in accordance with the tenets of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and the protocol was approved by the Institutional Animal Care and Use Committee of SingHealth. Rats were adequately anesthetized, and then orthotopic corneal transplantation was performed as previously described. In brief, corneal donor grafts with a diameter of 3.5 mm were obtained. After the recipient corneas were removed with a 3-mm trephine, the grafts were transplanted onto the recipients with eight 10-0 nylon interrupted sutures. The AC was then reformed by injection of balanced salt solution. Both syngeneic and allogeneic groups received topical tobramycin ointment (Alcon, Fort Worth, TX, USA) four times daily for the initial 4 days. The graft sutures were removed at 2 weeks. The eyes complicated by cataract, infection, or hyphema were excluded.

Clinical Evaluation

After surgery, all the recipient eyes were observed by slit lamp biomicroscopy under general anesthesia every other day until day 28. The grafts were evaluated clinically using a previously established scoring system (RI) by assessing the graft opacity (0: no opacity; 1: slight opacity; 2: iris clearly visible; 3: some details of graft are no longer visible; 4: pronounced opacity; pupil still recognizable; 4: total opacity); edema (0: no edema; 1: mild edema; 2: pronounced edema with raised transplant; 3: pronounced edema with small bleb; 4: pronounced edema with large bleb); and neovascularization (0: no vessels; 1: vessels appearing in the corneal bed; 2: vessels appearing in the graft periphery; 3: vessels extending deeper; 4: vessels extending to the center). A graft was considered rejected when the combined score (RI 0–12) was equal to or exceeded 6.

ASOCT Examination and Analysis

All the recipient eyes underwent ASOCT examination under general anesthesia every other day until day 28 (RTVue; Optovue, Inc., Fremont, CA, USA). Four high-resolution corneal cross-sectional scans (8-mm scan length, single scan mode 0-, 45-, 90-, and 135-degree axes) were obtained at each time point by an unmasked ophthalmologist (YCL). For the AC inflammation intensity evaluation, the ASOCT images were analyzed using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD, USA; available at http://rsb.info.nih.gov/ij/index.html). Each image of the AC included the central cornea to the anterior capsule of the lens. A background component was first obtained using the filtering function, and then the background component was subtracted from the original image. A central area of 0.625 × 0.875 mm in AC in the image was selected, and then the hyperreflective spots in this area were quantified by measuring the mean gray value of the reflectivity level of the reflective particles. The mean gray value of each allograft was normalized to the mean gray value of the isografts at the same time point. After normalization, these normalized mean gray values represented different AC inflammation intensity and were graded from grade 1 to grade 6: 0 ≤ value < 2: grade 1 (no or trace AC inflammation); 2 ≤ value < 4: grade 2 (mild AC inflammation); 4 ≤ value < 6: grade 3 (moderate AC inflammation); and 6 ≤ value < 8: grade 4 (severe AC inflammation). For the graft thickness evaluation, the central graft thickness and peripheral graft thickness (the average of the thickness 1 mm away from the central cornea at each side) were used. The AC inflammation intensity and graft thickness were measured by two independent observers (one unmasked and one masked, YCL and NCL).

Statistical Analysis

All data were expressed as mean ± standard deviation. Statistical comparisons between the rejected and nonrejected grafts were performed using a 2-tailed Student’s t-test. A Pearson correlation test was used to assess the correlation between the graft thickness measured by ASOCT and clinical graft edema score. The k statistic was used to evaluate the interobserver agreement. Logistic regression analysis was performed to analyze the association of the AC inflammation intensity or graft thickness with graft rejection. Statistical analyses were performed using STATA software (version 13; StataCorp, College Station, TX, USA). P values less than 0.05 were considered statistically significant.

RESULTS

Clinical Evaluation Post Transplantation

All the isografts remained clear during the total follow-up period, with a mean RI of 1.28 ± 0.42 at 4 weeks. All the allogeneic untreated grafts were rejected by 2 weeks at an average of 9.9 ± 0.8 days postoperatively and had the highest RI among different groups during the study period, with a mean RI of 9.86 ± 0.44 at 4 weeks. Four of the 20 allogeneic steroid-treated grafts developed rejection episodes at an average of 21.0 ± 2.2 days. The rejected grafts exhibited pronounced graft edema, opacification, and neovascularization extending from graft–host junction centrally, whereas the nonrejected grafts appeared mildly edematous with a visible pupillary margin (Fig. 1). The changes of the mean scores of the RI, graft opacity, edema, and neovascularization, with time for different groups, are shown in Figures 2A through 2D. In the allograft treated group, the mean RI, opacity, edema, and neovascularization scores at 2 weeks were 3.50 ± 0.57, 2.00 ± 0.00, and 0.25 ± 0.25 for the rejected grafts and 3.46 ± 0.74, 1.75 ± 0.12, 0.00 ± 0.00 for the nonrejected grafts (P = 0.935,
At 4 weeks, the mean RI, opacity, and neovascularization scores were significantly higher in the rejected grafts compared to those in the nonrejected grafts (7.75 ± 0.63, 2.50 ± 0.47, 3.00 ± 0.48 vs. 4.60 ± 0.13, 1.87 ± 0.09, 0.80 ± 0.14; P < 0.001, P ≤ 0.049, P < 0.001, respectively). The edema score was 2.25 ± 0.25 and 1.92 ± 0.07 in the rejected and nonrejected grafts at 4 weeks (P = 0.092).

Anterior Chamber Inflammation Post Transplantation

The representative pictures of grade 1 to grade 4 AC inflammation post transplantation are seen in Figure 3A, and the changes of the mean gray values of the hyperreflective spots in AC after PK in different groups are shown in Figure 3B. The AC inflammation in the nonrejected eyes gradually decreased postoperatively, whereas those in the rejected eyes persisted at the level of grade 3 or grade 4 for at least 1 week. The rejected eyes, either in the allogeneic untreated or allogeneic treated group, had a significantly higher mean AC inflammation grade at 1 week (grade 3.80 ± 0.45 and 3.25 ± 0.49, respectively; Supplementary Table S1) compared to the nonrejected eyes (grade 1.83 ± 0.36; P < 0.001 for both). Logistic regression analysis showed that greater intensity of AC inflammation during the first week was significantly associated with graft rejection (P < 0.001). Eyes with grade 3 or higher AC inflammation at 1 week were 15.15 times significantly more likely to have graft rejection compared to eyes with less than grade 3 AC inflammation at 1 week after adjustment for central corneal thickness (odds ratio [OR], 15.15; 95% confidence interval [CI], 1.98–73.63; P = 0.009). For the allograft untreated group, it was not easy to measure the AC inflammation after 1 week, as the grafts became marked edematous and opaque owing to the graft failure (Fig. 3B). After 2 weeks, all the gray values of the AC inflammation in the three groups were at a range of 0 to 2, corresponding to grade 1 (Fig. 3B and Supplementary Table S1). The logistic regression failed to show a statistically significant correlation between the AC inflammation and graft rejection after 2 weeks (P = 0.682 and P = 0.706).

Figure 1. Clinical evaluation of the corneal grafts by slit lamp biomicroscopy at 4 weeks. The isografts remained clear during the follow-up period (A). The rejected grafts, either in the allogeneic untreated or allogeneic treated group, exhibited severe graft edema, opacity, and neovascularization extending from the graft–host junction centrally (B). The nonrejected grafts in the allogeneic treated group had minimal graft edema with visible pupillary margin (C).

Figure 2. Changes of the mean rejection scores with time for different groups. The mean of the graft rejection index per time point for different groups (A). The mean of the graft opacity scores per time point for different groups (B). The mean of the graft neovascularization scores per time point for different groups (C). The mean of the graft edema scores per time point for different groups (D). Error bars represent standard deviations. Asterisks indicated the time points at which a statistically significant difference was observed between the rejected eyes, either in the allogeneic untreated or allogeneic treated group, and the nonrejected eyes.
The postoperative AC inflammation intensity measured by ASOCT. Representative pictures of grade 1 to grade 4 AC inflammation. Grade 1: no or trace; grade 2: mild; grade 3: moderate; and grade 4: severe AC inflammation (A). The changes of the mean gray values of the hyperreflective spots in AC in rejected and nonrejected allogeneic grafts. The rejected eyes, either in the allogeneic untreated or allogeneic treated group, had a significantly higher mean AC inflammation grade at 1 week compared to the nonrejected eyes ($P < 0.001$ for both, asterisks) (B). Error bars represent standard deviations.

**Graft Thickness Post Transplantation**

All grafted corneas exhibited good anatomic position without graft-host dehiscence or anterior chamber collapse on ASOCT images. The mean changes of the central corneal thickness measured by ASOCT against time in different groups are shown in Figure 4. All the allogeneic grafts showed an increase in corneal thickness during the first 2 weeks, but the rejected grafts underwent more rapid and persistent thickening. For the allograft untreated group, the mean central corneal thickness was $386.63 \pm 21.44$ µm, $518.72 \pm 33.33$ µm, and $501.72 \pm 34.18$ µm at 1, 2, and 4 weeks, respectively. For the allograft treated group, the mean central corneal thickness was $321.75 \pm 35.32$ µm, $455.25 \pm 42.42$ µm, and $421.30 \pm 48.48$ µm at 1, 2, and 4 weeks for the rejected grafts and was $337.40 \pm 17.22$ µm, $381.47 \pm 12.51$ µm, and $256.27 \pm 14.67$ µm at 1, 2, and 4 weeks for the nonrejected grafts ($P = 0.685$, $P = 0.047$, and $P = 0.004$, respectively). Logistic regression analysis showed that thicker central corneal thickness was significantly associated with graft rejection ($P = 0.031$). Grafts with central thickness $\geq 400$ µm at 2 weeks were 9.75 times more likely to have graft rejection as compared to grafts with thickness $< 400$ µm at 2 weeks after adjustment for AC inflammation intensity (OR, 9.75; 95% CI, 1.59–59.69; $P = 0.014$; see the Table). The syngeneic control grafts showed early postsurgery thickening during the first week, but subsided after day 9 and returned to a normal level at 4 weeks. The central thickness measurements of all the grafts were highly correlated with the clinical edema scores ($r = 0.80$). We also measured the peripheral graft thickness, and it showed similar results. For the allograft untreated group, the mean peripheral corneal thickness was $399.25 \pm 30.26$ µm, $556.72 \pm 38.20$ µm, and $589.72 \pm 36.88$ µm at 1, 2, and 4 weeks, respectively.

**Figure 3.** The postoperative AC inflammation intensity measured by ASOCT. Representative pictures of grade 1 to grade 4 AC inflammation. Grade 1: no or trace; grade 2: mild; grade 3: moderate; and grade 4: severe AC inflammation (A). The changes of the mean gray values of the hyperreflective spots in AC in rejected and nonrejected allogeneic grafts. The rejected eyes, either in the allogeneic untreated or allogeneic treated group, had a significantly higher mean AC inflammation grade at 1 week compared to the nonrejected eyes ($P < 0.001$ for both, asterisks) (B). Error bars represent standard deviations.

**Table.** Binary Logistic Regression Analysis of the Association Between Variables and Graft Rejection

<table>
<thead>
<tr>
<th>Variables</th>
<th>Odds Ratio</th>
<th>Confidence Interval</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eyes with AC inflammation $\geq$ grade 3 at 1 wk*</td>
<td>15.15</td>
<td>1.98–73.63</td>
<td>0.009</td>
</tr>
<tr>
<td>Eyes with AC inflammation $\geq$ grade 2 at 2 wk*</td>
<td>3.49</td>
<td>0.66–18.64</td>
<td>0.364</td>
</tr>
<tr>
<td>Central graft thickness $\geq 400$ µm at 2 wk†</td>
<td>9.75</td>
<td>1.59–59.69</td>
<td>0.014</td>
</tr>
</tbody>
</table>

* After adjustment for central graft thickness.
† After adjustment for AC inflammation intensity.
µm at 1, 2, and 4 weeks, respectively. For the allograft treated group, the mean peripheral corneal thickness was 542.35 ± 28.72 µm, 513.75 ± 38.89 µm, and 556.43 ± 42.58 µm at 1, 2, and 4 weeks for the rejected grafts and was 377.42 ± 24.42 µm, 421.53 ± 23.10 µm, and 345.56 ± 19.82 µm at 1, 2, and 4 weeks for the nonrejected grafts (P = 0.703, P = 0.041, and P = 0.012, respectively; Supplementary Fig. S1). Logistic regression analysis also showed that greater peripheral corneal thickness was significantly associated with graft rejection (P = 0.039).

There was a good agreement on the central thickness measurements (k = 0.86; P = 0.036) and peripheral thickness measurements (k = 0.64; P = 0.004) between two observers.

**DISCUSSION**

The rat keratoplasty model is a well-established technique to study immunopathology associated with corneal transplantation.\(^5\)\(^,\)\(^6\)\(^,\)\(^10\)\(^,\)\(^11\) Compared to larger animal models, rats are genetically identically inbred, allowing control for many experimental variables.\(^5\) In the present study, we demonstrated that moderate to severe AC inflammation during the early stage after PK could predict graft failure earlier than other indicators. In the rat model, eyes with grade 3 or higher AC inflammation at 1 week or with ≥400 µm central graft thickness at 2 weeks (normal rat corneal thickness, 170 µm), were significant predictors of graft failure at 4 weeks. These objective measurements can be used early before distinct clinical signs occur and are especially useful since clinical assessments can be highly subjective. It can also be helpful when there is discrepancy in observers’ clinical assessments or when observers are less experienced. In the present study, a statistically significant difference between the rejected and nonrejected grafts was observed at 1 week for the AC inflammation intensity, 2 weeks for the central graft thickness, and 4 weeks for the clinical RI. Our results showed that ASOCT is a useful tool to predict and diagnose posttransplantation graft rejection in small animal models.

Endothelial rejection is the most common type of graft rejection after PK, and AC inflammation is one of the clinical features of endothelial rejection. Anterior chamber inflammation results from elevated levels of proteins or cells in the aqueous humor that have leaked from the uveal vasculature owing to the increased vascular permeability in an immune-mediated rejection response.\(^12\) Reinhard et al.\(^13\) reported a correlation between the probability of immune cell isolation from the AC of patients who have undergone PK and the severity of the endothelial immune reactions. Similarly, we found that the intensity of early AC inflammation was significantly associated with the probability of graft rejection. In addition, we have also investigated the correlation between the gray values of the AC inflammation in ASOCT images and the cell number and protein concentration in AC, using a previously described rabbit model of inflammation, as the volume of rat aqueous humor was limited. Different concentrations of lipopolysaccharide (0.2 ng/µL, 0.4 ng/µL, 0.6 ng/µL, and 0.8 ng/µL) were injected into the vitreous of rabbit eyes to induce different extents of AC inflammation.\(^14\) The results demonstrated that the gray values of the AC inflammation in ASOCT images positively correlated with the clinical presentations of AC inflammation, smear analysis, and protein amount quantification of the aqueous humor (Supplementary Figs. S2–S6). Furthermore, Igbe et al.\(^8\) showed that ASOCT (Visante OCT; Zeiss Meditec, Dublin, CA, USA) was a promising technique for grading AC cells in noninfectious uveitis patients, and there was a significant correlation between clinical examination and ASOCT grading. Unlike their cross-sectional study design in which the AC inflammation was assessed by counting the hyperreflective spots manually, our longitudinal study assessed the AC inflammation by quantifying the reflectivity of hyperreflective spots objectively. The resolution of the RTVue ASOCT is 5 µm,\(^15\) and therefore particles sized larger than 5 µm can be detected. The sizes of the particles in AC can vary, as inflammatory cells, protein, or pigments in the AC may clump together due to intercellular adhesion.\(^9\) Thus, quantifying the reflectivity that takes into account the sizes of the particles may provide a more accurate way to reflect the degree of AC inflammation than would counting particles alone.

The intensity of AC inflammation in the rejected eyes (both allogeneic untreated and allogeneic treated groups) reached a peak at day 7 to 9, regardless of the continued increase in the RI. The AC inflammation intensity measurement decreased drastically after week 1 in the allogeneic untreated group and was at a low level (grade 1) after week 2 in the allogeneic treated group, with rejection and allogeneic untreated groups (Fig. 3B). We hypothesized that this occurred because the ASOCT beam detector captures data following reflections from the ocular tissue. As the rejected grafts became markedly

![Figure 4](https://iovs.arvojournals.org/ on 12/11/2018)
edematous and opaque, this may interfere with the reflectivity of the beam from the anterior chamber, and hence the correlation between the intensity of AC inflammation and graft rejection was not easily and accurately depicted after 1 week. Furthermore, we found that the AC inflammation intensity only poorly to moderately correlated with the RI (r = 0.30), edema score (r = 0.42), opacity score (r = 0.38), and neovascularization score (r = 0.26). This is similar to the clinical presentation of graft failure in patients, where AC inflammation often presents first before other clinical signs of rejection/failure. In the present study, the AC inflammation intensity of the allogeneic grafts was normalized to that of the syngeneic grafts before the statistical analysis to eliminate the component of the AC inflammation caused by the surgery. To our knowledge, this is the first study to report the usefulness of ASOCT to evaluate AC inflammation in the prediction and diagnosis of posttransplantation graft failure. It may also be used to monitor response to treatment in both animals and patients. For example, if a certain degree of AC inflammation persists postoperatively (in the present study, grade 3 or higher of AC inflammation for more than 5 days), more intensive steroid treatment can be considered.

Graft edema is another clinical feature of corneal allograft rejection. Ultrasonic pachymetry has been evaluated as a possible method for determining allograft rejection in a recent clinical trial, and corneal thickness has been reported to serve as a predictor of graft survival. Flynn et al. reported that in vivo graft ultrasound pachymetry provided a reliable way to objectively diagnose rejection in a mouse model of corneal transplantation. Compared to ultrasonic pachymetry, ASOCT is sterile and noncontact, providing more in-depth imaging and micrometric measurements for corneal thickness. It also generates high-resolution images of the anterior segment, including the AC, allowing for AC evaluation. Additionally, in small animal models, touching the same point of the cornea with the probe when performing the ultrasonic pachymetry measurements is not easy, as rodent’s corneas are even smaller than the probe, whereas the ASOCT measurements may be more reproducible as it scans both central and peripheral cornea at the same time in every measurement. Our results demonstrated that the graft thickness, either central or peripheral thickness, measured by ASOCT could serve as an additional indicator of graft rejection, and it was highly correlated with the conventional, subjective graft edema scores (r = 0.80).

In conclusion, we demonstrated that besides a conventional clinical grading system with slit lamp biomicroscopy, ASOCT provides an objective measurement to predict and diagnose graft failure. Early increased AC inflammation or early increased graft thickness was an early predictor of graft rejection prior to definitive clinical evaluation. The eyes with AC inflammation greater than grade 3 at 1 week, or a graft with central thickness more than 400 μm at 2 weeks, were at significant risk of developing graft rejection at 4 weeks. This information will be useful not only for clinical evaluation, but also for therapeutic response in the experimental models of corneal transplantation research.

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