Predicting Corneal Graft Rejection by Confocal Microscopy

Viridiana Kocaba, MD,†‡ Caterina Colica, MD,§ Muriel Rabilloud, MD, PhD,†‡¶‖** and Carole Burillon, MD*†‡*

Purpose: This study aims at estimating corneal healing by activated keratocyte (AK) counting with in vivo confocal microscopy after perforating keratoplasty (PK). It assesses the value of AK counting in predicting corneal graft rejection.

Methods: This prospective single-center observational study included 45 patients who benefited from PK in 2013 and were followed up over 2 years. All were monitored by confocal microscopy at day 1, day 7, and monthly thereafter. The AKs were counted in 5 optical sections in each of the anterior, middle, and posterior stroma. The ability of AKs in predicting the occurrence of corneal rejection was assessed by comparison of AK counts between patients with and without clinical signs of rejection.

Results: In patients with graft rejection, the AK counts increased significantly 2 months before the clinical diagnosis of rejection, whereas it remained stable after 4 months in patients without rejection. In patient with graft rejection, the AK count reached a maximum at the rejection diagnosis and antirejection treatment initiation but decreased significantly 1 month after treatment initiation.

Conclusions: This study confirmed the predictive value of AK counting in corneal graft rejection. The increase in the AK count allowed predicting graft rejection 2 months before the clinical diagnosis of rejection; it may then be the first sign of subclinical rejection.

Key Words: perforating keratoplasty, confocal microscopy, cornea graft rejection, activated keratocytes

(*Cornea* 2015;34(Suppl):S61–S64)

Acquired corneal blindness is one of the most common causes for visual loss. Hence, with nearly 40,000 procedures per year in the United States alone, corneal transplant surgery has become the most frequent allograft in human medicine.[1–3] The most important factor in the success of corneal transplantation is cornea avascularity and immunologic privilege.[4,5] Perforating keratoplasty (PK) performed in normal, nonvascularized low-risk beds are highly successful; they showed a 90% graft survival rate at 5 years in first grafts and 53% survival in regrafts.[6] However, these results contrast sharply with the survival rates of corneal grafts in high-risk beds; in such cases, the rejection rates can exceed 70% (up to 90%) despite local and systemic immune suppression.[7–13]

The primary cause of corneal allograft failure seems to be immune-mediated rejection characterized by delayed-type hypersensitivity to donor alloantigens and leukocytic cellular infiltration of the graft site.[5] The normal eye benefits from an anatomical barrier mechanism and from another molecular mechanism of immune suppression in the intraocular microenvironment.[10,14–18] Nonetheless, the exact mechanisms of transplantation failures remain elusive, and there are yet no objective measurements able to predict corneal graft rejection. Actually, rejection has always been clinically diagnosed and the treatment initiated after visible signs on slit-lamp biomicroscopy.

Understanding the rejection mechanisms seems necessary to decrease the number of corneal graft rejections and improve graft survivals.[18–22] Recent microscopy techniques have opened new possibilities for in vivo corneal examination: they are able to reveal activated keratocytes (AKs) that reflect intrastromal inflammation.[23–25] Indeed, in various ocular diseases, keratocytes become activated in response to signals from epithelial cells (cytokines or other mediators).[23,26–29] One hypothesis suggests that the apoptotic death of corneal stromal keratocytes before clinically detectable graft opacification may indicate or predict graft failure.[29]

This study aims at estimating corneal healing by AK counting with in vivo confocal microscopy after perforating keratoplasty. In addition, it assesses the interest of AK counting in predicting corneal graft rejection.

MATERIALS AND METHODS

Study Patients

This prospective single-center observational study included 45 patients who benefited from PK in 2013 and were followed up over 2 years. The study protocol was approved by the Ethical Committee of Hospices Civils de Lyon and adhered to the principles of the Declaration of Helsinki. Signed informed consent was obtained from each patient.

From the *Department of Ophthalmology, Hospices Civils de Lyon, Edouard Herriot Hospital, Lyon, France; †Université de Lyon, Lyon, France; ‡Hospices Civils de Lyon, Eye Bank, Edouard Herriot Hospital, Lyon, France; §Policlinico Umberto I Hospital, "Sapienza" University of Rome, Italy; ¶Université Lyon 1, Villeurbanne, France; ‖Hospices Civils de Lyon, Service de Biostatistique, Lyon, France; and **CNRS, UMR 5558, Laboratoire de Biométrie et Biologie Evolutive, Equipe Biostatistique-Santé, Villeurbanne, France.

The authors have no funding or conflicts of interest to disclose.

Reprints: Viridiana Kocaba, MD, Department of Ophthalmology, Edouard Herriot Hospital, 5, place d'Arsonval, F-69003 Lyon, France (e-mail: viridiana_k@yahoo.fr).

Copyright © 2015 The Cornea Society. All rights reserved.

Copyright © 2015 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

Ophthalmological Procedures

A thorough ophthalmological examination was performed preoperatively, which included slit-lamp biomicroscopy, best-corrected visual acuity (BCVA), applanation tonometry, and fundoscopy or posterior segment B-scan.

All PK procedures were performed by the same surgeon (V.K.) under general anesthesia. Only corneas with an endothelial cell density ≥2500 cells per square millimeter were used for PK. In all PK procedures, the diameter of trephination of the recipient corneal button was 8.0 mm, and that of the donor was 8.2 mm.

Patient Follow-up

All patients were monitored at day 1, day 7, and monthly thereafter over 2 years. At each time point, the patients underwent a BCVA assessment, intraocular pressure evaluation, slit-lamp examination (anterior and posterior segments), and confocal microscopy examination.

The laser scanning confocal microscope used a Heidelberg Retina Tomograph module (HRT III) with a Rostock Cornea Module. AKs were counted in 5 optical sections in each of the anterior, middle, and posterior stroma by spotting the hyperreflective nucleus or the spindle-shaped elongated morphology of each cell. The software computed the cellular density of the AKs according to the depth of the focus. For better reliability, a single physician examined all 15 confocal optical sections per patient and calculated the average of the AK count.

The data collection protocol was the following: (1) AK density in the anterior stroma, image focus from 10 to 130 μm beneath the Bowman membrane, (2) AK density in the middle stroma, image focus from 140 to 260 μm beneath the Bowman membrane, and (3) AK density in the posterior stroma, image focus from 270 μm beneath the Bowman membrane to Descemet membrane.

Patient Treatment

All the patients were treated for 1 year with decreasing doses of topical steroids. More precisely, they were given dexamethasone, neomycin, and polymyxin eye drops 4 times daily for 1 month, 3 times daily for 2 months, twice daily for 2 months, once daily for 4 months, then once every 2 days for 3 months. In case of a high rejection risk, the treatment included 2% topical cyclosporine 3 times daily. Patients with signs of rejection were given subconjunctival betamethasone injection once daily for 3 days and topical steroids hourly.

Statistical Analysis

At each time point, the AK counts were summarized by their mean, minimum, and maximum. The ability of the AK count to predict the occurrence of corneal graft rejection was assessed by comparing the AK counts between patients with and without signs of clinical rejection. In patients without signs of clinical rejection, the AK count made during the stable period (4–12 months and 14–24 months after surgery) was compared with that made at the clinical diagnosis, 1 month before, and 2 months before the diagnosis. To allow for the repeated measurements in each patient, we used a mixed-effects linear model. This model estimated the mean differences in the AK count between patients with and without rejection signs and tested these differences by analysis of variance.

We also compared the AK counts of patients with signs of corneal rejection with those of patients without signs of corneal graft rejection during the peak period (2, 3, and 13 months after surgery). All analyses were performed with R software, version 3.1.3.

RESULTS

Study Population

The population included 25 women and 20 men. Their mean age was 54.8 years (range, 19–70). The indications for PK indications were bullous keratopathy (20 cases, 44.4%), keratoconus (17 cases, 37.7%), and infectious keratitis (8 cases, 17.7%). Among the 45 patients, 5 (11.1%) had a high risk of graft rejection. Six patients (13.3%) rejected the graft within the 2-year follow-up (1 endothelial and 5 stromal rejections), at 6, 8, 9, 11, 15, and 18 months after surgery, respectively. One of them developed irreversible rejection because of herpetic lesions. The other 5 did not present high rejection risks.

AK Counts

In the 39 patients without graft rejection, the mean AK count was 186 cells per square millimeter (range, 154–220) at day 1, 158 cells per square millimeter (154–212) at day 7, 112 cells per square millimeter (77–190) at 1 month, 64 cells per square millimeter (53–99) at 6 months, 57 cells per square millimeter (52–89) at 1 year, and 55 cells per square millimeter (42–62) at 2 years. Overall, in these patients, the AK count decreased over the first 4 months and stabilized thereafter (Fig. 1). During the stable period, the mean AK count was 59 cells per square millimeter [95% confidence interval (CI), 51–67]. A slight but significant increase in AK counts was noticed by the end of steroid treatment at 1 year ($P < 0.001$) (Fig. 1).

At day 1, day 7, and during the first 4 months, there were no significant differences in mean AK counts between patients with and without graft rejection. In the 6 patients with graft rejection, the mean AK count was significantly higher than in patients without rejection, 2 months before the rejection, 1 month before, then at the rejection diagnosis [respective differences: 103 (82–125), 149 (127–170), and 237 (216–259) cells/mm^2; $P < 0.0001$].

During the peak AK count period, the mean count was 81 cells per square millimeter (72–89). The mean AK count was significantly higher in the patients with rejection at 2 months and 1 month before rejection and at the rejection diagnosis than in the patients without rejection during the peak period [respective differences: 82 (54–109), 127 (100–155), and 216 (188–243) cells/mm^2; $P < 0.0001$].

Copyright © 2015 The Cornea Society. All rights reserved.

Copyright © 2015 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

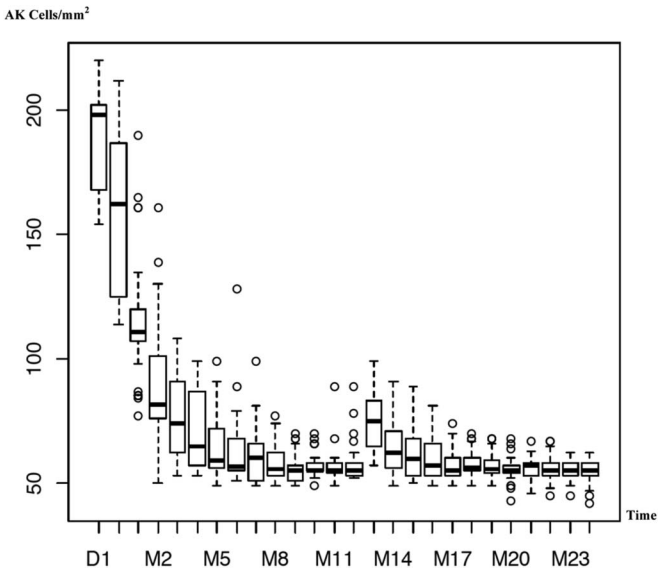

FIGURE 1. Box plots of activated keratinocyte counts (cells per square millimeter) in the 39 patients without rejection up to 2 years after perforating keratoplasty.

Figure 2 shows the kinetics of AK counts in the 6 patients who rejected the graft. In 5 of them, the AK count increased significantly 2 months before the rejection diagnosis but decreased significantly after initiation of the antirejection treatment ($P < 0.001$). After 3 to 6 months, the rejection was regulated and the AK counts became similar to those seen during the stable period. In the patient with irreversible rejection, the AK count increased significantly 2 months before the rejection diagnosis but did not decrease significantly after initiation of the antirejection treatment; this count remained high compared with that of the other 5 patients. In the patients with graft rejection, the AK count reached a peak at the rejection diagnosis but decreased significantly after 1 month.

DISCUSSION

In this study, the percentage of corneal graft rejection was 13.3%, which is in line with the current literature.[1,2] However, to our knowledge, this is the first in vivo study of AK count kinetics up to 2 years after PK. It showed a significant difference in the AK count kinetics between patients with and without rejection; in the former, this count increased significantly 2 months before rejection, whereas in the latter, it remained stable past 4 months. The study highlights the value of the AK count in predicting corneal graft rejection. Indeed, an increase in the AK count may be the first sign of subclinical rejection[23–25,29] and potentially predict graft rejection 2 months before the rejection diagnosis. Our findings support those of Beauregard et al,[29] who studied murine models of high- and low-risk corneal transplantation to determine the role of the keratocyte apoptosis in the failure of orthotopic allogeneic corneal transplants. Their results suggest that the apoptotic death of corneal stromal keratocytes before clinically detectable graft opacification is a predictor of graft failure.

Recent advances in microscopy have opened new possibilities for in vivo corneal investigations. These noninvasive possibilities allow repeated examinations over time and are ideal for rapid and accurate investigations of corneal wound healing. However, the characteristics of in vivo AKs are not as uniform as those of in vivo experimental AKs phenotypes. The literature still shows disagreements regarding the activated phenotype, and the morphologies described cannot be always recognized on in vivo images.[24]

In this study, the AK count kinetics followed the drop in steroid usage. Indeed, in the 39 patients without rejection, we noticed a significant increase by the end of the antirejection treatment at 12 months. In the patients with rejection, the AK count decreased significantly 1 month after the initiation of the antirejection treatment. These results suggest that the postoperative treatment may be adjusted according to the AK density: an early treatment (at first increases in density; ie, first signs potential graft rejection) may decrease the risk graft

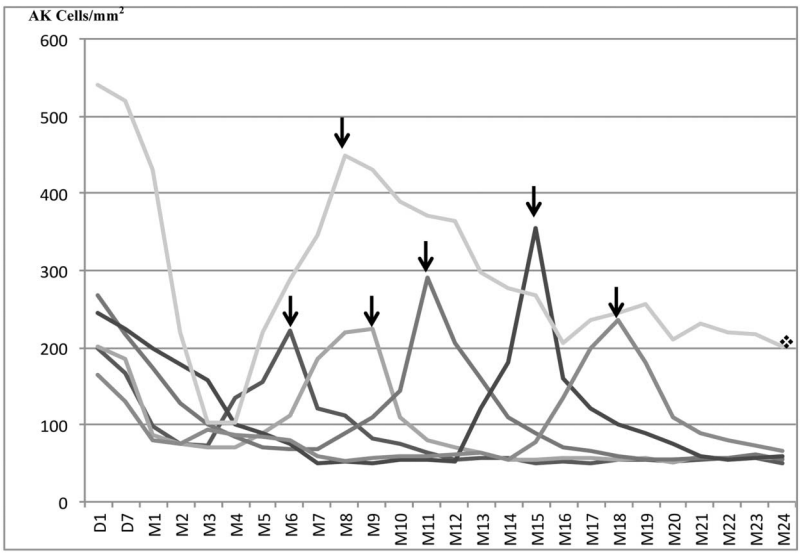

FIGURE 2. AK count kinetics in the 6 patients with rejection over the 2-year follow-up after perforating keratoplasty. ↓Clinical rejection and beginning of antirejection treatment. ❖ Irreversible rejection.

Copyright © 2015 The Cornea Society. All rights reserved.

Copyright © 2015 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.

failure. One of the main difficulties encountered during this study was strenuous AK counting. Because it was demonstrated that this count may change according to the stromal depth,[24] we decided to consider the stroma in 3 parts (anterior, middle, and posterior) and calculate the mean AK count. This required tedious work. Furthermore, the chosen protocol proved to be a complicated and demanding follow-up (monthly confocal microscopy). We deem the counting process was uniform because it was performed by the same physician in all patients. In fact, all patients without rejection had the same AK kinetics profile; however, for easier and more standardized counts, an automated system with image analysis software would be welcome. Finally, although encouraging, the present results stem from a limited number of patients. We hope that further investigations and studies by other teams will confirm these results, allow earlier diagnoses and treatments of corneal graft rejections, and improve the success of corneal grafts.

ACKNOWLEDGMENTS

The authors thank Jean Iwaz (PhD, Hospices Civils de Lyon, France) for the revision of the final versions of the article.

REFERENCES

1. Thompson RW Jr, Price MO, Bowers PJ, et al. Long-term graft survival after penetrating keratoplasty. *Ophthalmology*. 2003;110:1396–1402.
2. Dana MR, Moyes AL, Gomes JA, et al. The indications for and outcome in pediatric keratoplasty: a multicenter study. *Ophthalmology*. 1995;102: 1129–1138.
3. Sit M, Weisbrod DJ, Naor J, et al. Corneal graft outcome study. *Cornea*. 2001;20:129–133.
4. Streilein JW. Anterior chamber-associated immune deviation: the privilege of immunity in the eye. *Surv Ophthalmol.* 1990;35:67–73.
5. Hori J. Mechanisms of immune privilege in the anterior segment of the eye: what we learn from corneal transplantation. *J Ocul Biol Dis Infor.* 2008;1:94–100.
6. Dunn SP, Gal RL, Kollman C, et al. Corneal graft rejection 10 years after penetrating keratoplasty in the cornea donor study. *Cornea*. 2014;33: 1003–1009.
7. Yu AL, Kaiser M, Schaumberger M, et al. Donor-related risk factors and preoperative recipient-related risk factors for graft failure. *Cornea*. 2014; 33:1149–1156.
8. Patel SV, Diehl NN, Hodge DO, et al. Donor risk factors for graft failure in a 20-year study of penetrating keratoplasty. *Arch Ophthalmol.* 2010; 128:418–425.
9. Choi JA, Lee MA, Kim MS. Long-term outcomes of penetrating keratoplasty in keratoconus: analysis of the factors associated with final visual acuities. *Int J Ophthalmol.* 2014;7:517–521.
10. Bartels MC, Doxiadis II, Colen TP, et al. Long-term outcome in high-risk corneal transplantation and the influence of HLA-A and HLA-B matching. *Cornea*. 2003;22:552–556.
11. Maguire MG, Stark WJ, Gottsch JD, et al. Risk factors for corneal graft failure and rejection in the collaborative corneal transplantation studies. Collaborative corneal transplantation studies research group. *Ophthalmology*. 1994;101:1536–1547.
12. Sugar A, Tanner JP, Dontchev M, et al; Cornea Donor Study Investigator Group. Recipient risk factors for graft failure in the cornea donor study. *Ophthalmology*. 2009;116:1023–1028.
13. Cornea Donor Study Investigator Group, Gal RL, Dontchev M, Beck RW, et al. The effect of donor age on corneal transplantation outcome results of the cornea donor study. *Ophthalmology*. 2008;115: 620–626.
14. Qazi Y, Hamrah P. Corneal allograft rejection: immunopathogenesis to therapeutics. *J Clin Cell Immunol.* 2013;2013(suppl 9):pii 006.
15. Collaborative Corneal Transplantation Studies (CCTS). Effectiveness of histocompatibility matching in high-risk corneal transplantation. The Collaborative Corneal Transplantation Studies Research Group. *Arch Ophthalmol.* 1992;110:1392–1403.
16. Beekhuis WH, Bartels M, Doxiadis II, et al. Degree of compatibility for HLA-A and -B affects outcome in high-risk corneal transplantation. *Dev Ophthalmol.* 2003;36:12–21.
17. Streilein JW, Arancibia-Caracamo C, Osawa H. The role of minor histocompatibility alloantigens in penetrating keratoplasty. *Dev Ophthalmol.* 2003;36:74–88.
18. Dana MR, Qian Y, Hamrah P. Twenty-five-year panorama of corneal immunology: emerging concepts in the immunopathogenesis of microbial keratitis, peripheral ulcerative keratitis, and corneal transplant rejection. *Cornea*. 2000;19:625–643.
19. Hori J, Streilein JW. Survival in high-risk eyes of epithelium-deprived orthotopic corneal allografts reconstituted in vitro with syngeneic epithelium. *Invest Ophthalmol Vis Sci.* 2003;44:658–664.
20. Chong EM, Dana MR. Graft failure IV. Immunologic mechanisms of corneal transplant rejection. *Int Ophthalmol.* 2008;28:209–222.
21. Niederkorn JY. Mechanisms of corneal graft rejection: the sixth annual Thygeson Lecture, presented at the Ocular Microbiology and Immunology Group meeting, October 21, 2000. *Cornea*. 2001;20:675–679.
22. Bachmann B, Taylor RS, Cursiefen C. Corneal neovascularization as a risk factor for graft failure and rejection after keratoplasty: an evidence-based meta-analysis. *Ophthalmology*. 2010;117:1300–1305.
23. Nishida T. Commanding roles of keratocytes in health and disease. *Cornea*. 2010;29(suppl 1):S3–S6.
24. Hovakimyan M, Falke K, Stahnke T, et al. Morphological analysis of quiescent and activated keratocytes: a review of ex vivo and in vivo findings. *Curr Eye Res.* 2014;39:1129–1144.
25. Abdelkader A, Elewah el SM, Kaufman HE. Confocal microscopy of corneal wound healing after deep lamellar keratoplasty in rabbits. *Arch Ophthalmol.* 2010;128:75–80.
26. Yamagami S, Kawashima H, Tsuru T, et al. Role of Fas-Fas ligand interactions in the immunorejection of allogeneic mouse corneal transplants. *Transplantation*. 1997;64:1107–1111.
27. Stuart PM, Griffith TS, Usui N, et al. CD95 ligand (FasL)-induced apoptosis is necessary for corneal allograft survival. *J Clin Invest.* 1997; 99:396–402.
28. Wilson SE, Li Q, Weng J, et al. The Fas-Fas ligand system and other modulators of apoptosis in the cornea. *Invest Ophthalmol Vis Sci.* 1996; 37:1582–1592.
29. Beauregard C, Huq SO, Barabino S, et al. Keratocyte apoptosis and failure of corneal allografts. *Transplantation*. 2006;81:1577–1582.

Copyright © 2015 The Cornea Society. All rights reserved.

Copyright © 2015 Wolters Kluwer Health, Inc. Unauthorized reproduction of this article is prohibited.