Effect of FK 506 on the Cornea: Use of Topical FK 506 in Corneal Transplantation in a Guinea Pig-Rat Model

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To evaluate the effect of different concentrations 0.03%, 0.1% and 0.3% of FK 506 on xenograft corneal rejection, guinea pig corneas were transplanted into rats. FK 506 was then applied topically four times a day for 21 days. The grafts were inspected and scored according to opacity, edema, and graft protrusion. All grafts in the control group were rejected by the 14th postoperative day, and grafts in the FK 506 treated groups began to be rejected by the 17th postoperative day. Inflammatory cell infiltration was less dense in the FK 506 treated grafts than in the control group. Higher concentrations of FK 506 appeared to be more effective in preventing and decreasing the severity of the graft rejection. Topical FK 506 can delay the development of xenograft corneal rejection and decrease its severity in this animal model.

Key words: xenograft, corneal graft, topical FK 506, rejection

Corneal transplantation is a procedure for restoring vision to the eyes when the cornea has become opacified as a result of injury, infection or hereditary diseases. The success rate in obtaining clear transplantation has increased because of a better understanding of endothelial pump function, new corneal storage media, and improved surgical skills and instruments. However, 6%-40% of all corneal transplants still fail. Allograft immune rejection is the most common cause of corneal graft failure. It occurs more frequently, up to 50%, in eyes with a history of graft rejection or eyes with corneal neovascularization. Several immunosuppressive agents have been used to prevent and treat this type of graft rejection. Topical and systemic steroids have been widely used to reduce the frequency and severity of graft rejections. In addition, cyclosporin CSA is starting to be used for the same purposes with good results.

Recently, a new drug FK 506, which is known to be 100 times stronger than CSA, has been used to prevent graft rejection after liver, kidney and heart transplantation. FK 506 is a neutral macrolide, extracted from the fungus, Streptomyces tsukubaensis, which suppresses the production of lymphokines from T cell, interleukin 2 receptor, and cytotoxic T cells. The systemic side effects of this drug found in animal models were diarrhea, body weight loss, liver and kidney toxicity, and neurotoxicity.

If topically applied FK 506 can decrease the frequency and severity of the graft rejection after corneal transplantation, it can be more easily applied and severe systemic side effects can be decreased. This study was conducted in order to evaluate the effects of topically applied FK 506 at
different concentrations on corneal transplantation rejection in an animal xenotransplantation model.

**MATERIALS AND METHODS**

1. **Topical FK 506 preparation**

   FK 506 (Fujisawa Pharmaceutical Co.) was dissolved in 45% hydroxy-propyl-beta cyclodextrin (WKCO Pure Chemical Co.) and 3.5% gamma cyclodextrin to make FK 506 solution in concentrations of 0.3%, 0.1% and 0.03%. These were then preserved at 4°C. Before instillation, the FK 506 solution was shaken well.

2. **Corneal transplantation in animal model**

   Hartley guinea pigs, weighing between 200-300 g, were used as donors and Sprague Dawley rats, weighing between 150-200 g, were used as recipients. All animals were female. The corneal transplantation procedure used has been described elsewhere. In brief, the rats were anesthetized with intramuscular injections of ketamine (25 mg/kg) and xylazine (3 mg/kg). After anesthesia, 10% phenylephrine and 2.5% mydriacyl were instilled in one eye of the recipient animal for maximal dilation of the pupil. The guinea pigs were killed by lethal injection of barbiturates. With the aid of an operating microscope, a 3.5 mm diameter trephine was used to cut the central corneal button from the guinea pig. The button was left sitting on the donor eye with drops of a balanced salt solution while the recipient was being prepared. The recipient rat cornea was prepared by using 3.0 mm trephine. The donor button was then transferred and sutured to the recipient wound with eight interrupted 10-0 nylon sutures. The loose ends were cut as short as possible. The cornea and lens were moistened throughout the procedure with balanced salt solution. Subconjunctival and topical gentamycin were administered at the end of the procedure. No steroids were given. The sutures were not removed postoperatively.

3. **Study groups**

   Cornea transplanted rats were divided into four groups. Each group consisted of 12 rats 12 eyes. In group A, 10 µl of cyclodextrin (45% hydroxy-propyl-beta cyclodextrin and 3.5% gamma cyclodextrin) was instilled in the surgically treated eye with a micropipette, four times a day after the first postoperative day for three weeks. In groups B, C and D, 0.03% FK 506 solution, 0.1% FK 506 solution and 0.3% FK 506 solution were instilled using the same method for three weeks, respectively.

4. **Evaluation and statistics**

   Each surgically treated animal was inspected twice a week for three weeks under an operating microscope. Quantitative evaluation was based on grades of 0 - 4 for each of following categories; graft opacity, graft edema, and graft protrusion (Table 1). Graft neovascularization was evaluated but not included in grade category. A rejection score, the sum of each grade, was compared between the groups. A rejection score of more than seven was considered to be graft rejection. Statistical analysis was done using the Wilcoxon rank sum test.

   Two rats in each group were killed by inhalation of a lethal dose of ether on the tenth postoperative day, and the eyes were enucleated. The corneas of

| Table 1. Clinical Scoring Scheme for the Severity of Corneal Graft Rejection |
|-----------------------------|-----------------------------|
| **Score** | **Clinical Findings** |
| Opacity | none | slight | moderate iris vessel obscured | marked hardly visible iris | extreme |
| Edema | none | slight | moderate | marked | extreme |
| Protrusion of graft | none | slight | moderate | marked | extreme |
the enucleated eyes were stained using hemotoxylin-eosin, as well as PAS. The remaining ten rats in each group were killed on the 21st postoperative day, and the corneas were stained using the same methods.

**RESULTS**

**Clinical evaluation**

There was a slight corneal edema (grade 1) in all corneas after surgery (Fig. 1A, 1D). Opacification of the corneal graft (grade 1-2) and neovascularization began to appear as early as seven days after surgery in the control group (group A). Opacification of the cornea became more dense (grade 2-3) and neovascularization was present beyond the suture site at ten days postoperatively. We began to notice corneal protrusion between seven and ten days postoperatively in the control group (Fig. 1B). At 14 days postoperatively, all of the rats in the control group showed rejection characterized by moderate corneal edema (grade 2), marked corneal opacity (grade 3), marked neovascularization and moderate corneal protrusion (grade 2) (Fig. 1C).

Opacification of the corneal graft and neovascularization began to appear between ten and 14 days after surgery in the FK 506 treated groups (Fig. 1E), and corneal protrusion was noticed after 14 days. At 17 days postoperatively, corneas began to be rejected in the 0.03% and 0.1% FK 506 treated groups (group B and C). Four grafts out of ten were rejected in group B, and five grafts out of ten were rejected in group C (corneal edema; grade 2, corneal opacity; grade 2-3, and corneal protrusion; grade 2). Corneas began to rejected on the 21st postoperative day in the 0.3% FK 506 treated group (group D, Fig. 1F). The rejection scores of each treated group were lower than that of the control group after ten days. This difference was statistically significant (P<0.05, Table 2).

Grafted corneas appeared more clear when higher concentration of FK 506 solution used. The rejection scores of group D at 14 and 17 days postoperatively were significantly lower than those of group B or group C: 5.5 ± 1.65, 6.1 ± 2.35 and 3.5 ± 1.28 at 14 days postoperatively (p<0.05), 6.0 ± 2.21, 6.8 ± 1.52, and 3.8 ± 0.32 at 17 days postoperatively (P<0.05) in groups B, C and D, respectively.

**Pathology**

Examination by light microscope at ten days after surgery revealed disorganized grafts and heavy infiltration of inflammatory cells in the control group (Fig. 2A). Infiltrated inflammatory cells were mainly polymorphonuclear leukocytes with some monocytes. There was more dense infiltration of inflammatory cells at 21 days after surgery in the control group (Fig. 2B).

There was significantly less infiltration of inflammation cells in the FK 506 treated groups compared to the control group. Minimal cell infiltration was noticed ten days postoperatively (Fig. 2C). The inflammatory cell infiltration was significantly less dense in the FK 506 treated groups than in the control group for all specimens. However, clinically rejected grafts rejection score > 9 at 21 days postoperatively showed heavy inflammatory cell infiltration in spite of FK 506 treatment (Fig. 2D). It appeared that 0.3% of the FK 506 treated groups had at least some inflammatory cell infiltration.

<table>
<thead>
<tr>
<th>Post-op day</th>
<th>cyclodextrin only</th>
<th>0.03% FK506</th>
<th>0.1% FK506</th>
<th>0.3% FK506</th>
</tr>
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<tbody>
<tr>
<td>3 days</td>
<td>3.0 ± 0.81</td>
<td>2.8 ± 0.32</td>
<td>2.8 ± 0.28</td>
<td>2.2 ± 0.25</td>
</tr>
<tr>
<td>7 days</td>
<td>5.5 ± 0.55</td>
<td>4.4 ± 1.56</td>
<td>2.9 ± 1.67</td>
<td>4.1 ± 0.89</td>
</tr>
<tr>
<td>10 days</td>
<td>6.9 ± 1.61</td>
<td>4.33 ± 1.49</td>
<td>4.3 ± 1.69</td>
<td>3.5 ± 1.92</td>
</tr>
<tr>
<td>14 days</td>
<td>8.5 ± 0.45</td>
<td>5.5 ± 1.65</td>
<td>6.1 ± 2.35</td>
<td>3.5 ± 1.28</td>
</tr>
<tr>
<td>17 days</td>
<td>8.8 ± 0.45</td>
<td>6.0 ± 2.21</td>
<td>6.8 ± 1.52</td>
<td>3.8 ± 0.32</td>
</tr>
<tr>
<td>21 days</td>
<td>10.2 ± 1.10</td>
<td>8.0 ± 2.50</td>
<td>7.8 ± 0.71</td>
<td>7.4 ± 0.89</td>
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P: each topical FK506 treated group compared with the cyclodextrin-treated group control group. Wilcoxon rank sum test.
Fig. 1. Clinical pictures of corneal grafts
A. There was slight corneal edema immediately after surgery in the control group.
B. There was moderate corneal edema, more dense corneal opacity, neovascularization beyond the suture site, and slight corneal protrusion ten days after surgery in the control group.
C. At 14 days after surgery, in the control group, all grafts which were characterized by marked corneal opacity, moderate corneal edema, more dense neovascularization, and moderate corneal protrusion were rejected.
D. There was slight corneal edema immediately after surgery in the 0.3% FK 506 treated group.
E. There was moderate corneal opacity at ten days after surgery in the 0.3% FK 506 treated group. However, neovascularization and edema were not severe.
F. All grafts were rejected by the 21st day postoperatively in the 0.3% FK 506 treated group.
Fig. 2. Hemotoxyllion-eosin staining of the corneal grafts. There was marked corneal thickening, inflammatory cell infiltration and neovascularization in the corneal grafts in the control group (A; at ten days postoperatively, B; at 21 days postoperatively) compared to the 0.3% FK 506 treated group (C; at ten days postoperatively, D; at 21 days postoperatively).

**DISCUSSION**

FK 506 is a potent immunosuppressive agent in organ transplantation animal models and in clinical practice.\(^{14}\) It is known to have less systemic toxicity and to reduce the necessary dosages of steroids when used concomitantly.\(^{15,16}\) Kobayashi et al.\(^{17}\) first reported the effect of FK 506 on preventing corneal graft rejection using a rabbit model. They delivered FK 506 subconjunctivally. Nishi et al.\(^{18}\) showed that intraperitoneal injection of FK 506 could prevent corneal graft rejection and Hikita et al.\(^{19}\) showed that topically applied FK 506 also could prevent corneal graft rejection. We have also demonstrated the effect of topically applied FK 506 on corneal graft rejection. We tested topical FK 506 to evaluate its feasibility in the clinical setting. We then used different concentrations of FK 506 in this study in order to evaluate the dose-dependent response of FK 506.

We used the xenotransplantation model, Guinea pig donor-rat recipient orthotopic corneal transplantation, to maximize antigen disparity, like Umberto.\(^{20}\) The guinea pig and rat do not have any identical major or minor histocompatibility antigens. One of the advantages of the xenotransplanta-
tion model is that it provides strong antigenic sensitization, which always results in transplant rejection.

Therefore, the xenotransplantation model is a good model with which to test new immunosuppressive agents for preventing transplant rejection. All corneal transplants in the control group were rejected at 14 days postoperatively.

Topical FK 506 delayed the development of rejection. Although all corneal grafts were rejected within three weeks in spite of FK 506 treatment, there was a four to seven day delay in the development of graft rejection, and less severe infiltration of inflammatory cells in rejected grafts compared to the control group. Antigenic disparity in this model might be too strong to be completely suppressed by the tested FK 506, concentrations of 0.03%, 0.1% and 0.3%. Stronger concentrations of FK 506 may be able to prevent corneal transplant rejection in this model.

Hikita et al.\textsuperscript{19} tested only one concentration of topical FK 506, 0.3%. Three different concentrations were tested in this study and the higher concentration appeared to be better in delaying the development of corneal graft rejection than the lower concentrations, in a tested range. In a previous study,\textsuperscript{21} we found that there was no significant difference in the rejection score-summation of the corneal opacity grade, edema grade, neovascularization grade or corneal protrusion grade between the three different FK 506 concentration groups. This appeared to be because of the grading system. The grading system used in the previous study was modified from Tchah’s system.\textsuperscript{13} That system was not adequate in detecting small differences between the FK 506 treated groups. The graft neovascularization grade was excluded from the grading system in this study. The rejection score of the 0.3% FK 506 treated group was significantly lower than those of 0.03% and 0.1% FK 506 treated groups \textit{p}<0.05. In pathologic specimens, the rejected grafts of the 0.3% FK 506 treated group had less severe inflammatory cell infiltration than those of the 0.03% FK 506 treated group. It appeared that a stronger concentration of topical FK 506 was more effective in preventing corneal graft rejection. But, when the concentration of topical FK 506 is increased further, the possibility of local and systemic toxicity could also be increased. Further study will be needed in order to determine the optimal concentration of topical FK 506.

FK 506 has been known to prevent new corneal vessel formation by inhibiting the secretion of lymphokines and interleukin-2 from T-cells.\textsuperscript{17,22} However, FK 506 exerted no significant effect on new vessel formation in this study. We did not remove the sutures in order to maximize immune reaction. However, Umberto\textsuperscript{20} removed corneal sutures at five days postoperatively, and showed FK 506 induced neovascularization inhibition.

The ocular toxicity of FK 506 is not well known. Kobayashi et al.\textsuperscript{18} found that there was no serious ocular toxicity after subconjunctival injection of FK 506, 0.1 mg/kg for 100 days. Meanwhile, Kawashima et al.\textsuperscript{23} showed that intraperitoneal injection of FK 506 caused systemic side-effects, such as diarrhea, weight loss, and liver toxicity. Topically applied FK 506 may decrease the incidence and severity of these systemic side effects. That is the one of the reasons why we chose to study the topical effect of FK 506. We did not find any local toxicity of topical FK 506 in rats such as punctate corneal erosion and conjunctival injection, during the follow-up period before the development of graft rejection.

Penetration of the FK 506 solution into the cornea is a very important factor in providing therapeutic levels of FK 506 to the cornea. We used cyclodextrin to increase the corneal penetration of FK 506. The other problem, which must be solved before application of topical FK 506 in the clinical setting, is the stability of the potency of the FK 506 solution. We produced FK 506 solution once a week for this study. The potency of FK 506 solution seemed to be maintained for at least one week considering our results. Before clinical trial, however, this will require further study.

REFERENCES

3. Ing, J.J., Ing, H.H., Nelson, L.R., Hodge, D.O., and


