Orthotopic Tracheal Allografts Undergo Reepithelialization With Recipient-Derived Epithelium

Eric M. Genden, MD; Andrew J. Iskander, BS; Jonathan S. Bromberg, MD, PhD; Lloyd Mayer, MD

Background: While the rejection of heterotopic tracheal allografts is characterized by complete airway obliteration, the rejection of orthotopic allografts leads to airway edema and cellular infiltrate of the lamina propria, but is not associated with obliteration. We hypothesized that orthotopic tracheal allografts undergo reepithelialization with recipient-derived mucosa and that this process prevents airway obliteration.

Methods: Thirty mice were randomly assigned to 6 experimental groups. BALB/c donor tracheal segments were transplanted orthotopically or heterotopically into syngeneic BALB/c or major histocompatibility mismatched allogeneic C57BL/6 recipients. Recipients of allogeneic grafts were divided into a nonimmunosuppression group and an immunosuppression group (cyclosporine, 7 mg/kg per day). Twenty-one days after transplantation, histological assessment, immunohistochemistry for CD4 and CD8 lymphocyte infiltration and major histocompatibility-specific immunohistochemistry were performed on the grafts to assess rejection and donor or recipient origin of tissue.

Results: Untreated heterotopic allografts underwent complete airway obliteration by day 21. This response was prevented with cyclosporine immunosuppression. Untreated orthotopic allografts, however, demonstrated edema and lymphocytic infiltrate of the lamina propria resulting in clinical stridor without airway obliteration. Immunosuppressed orthotopic allografts did not develop edema or infiltrate of the lamina propria and consequently stridor did not occur. Immunohistochemical analysis demonstrated migration of recipient-derived mucosa into the donor allograft segment in both the untreated and treated orthotopic groups.

Conclusions: Airway obliteration characteristic of rejecting heterotopic tracheal allografts does not occur in the orthotopic allografts. Migration of recipient mucosa into the donor allograft appears to prevent airway obliteration in the orthotopic allografts. These findings suggest that the orthotopic tracheal transplantation model more accurately represents the biological behavior of clinical tracheal allografts than the traditional heterotopic model.

Arch Otalaryngol Head Neck Surg. 2003;129:118-123

Prior investigation of tracheal transplantation and obliteratorive airway disease in the rodent has been achieved using the heterotopic transplantation model. This model entails placing nonvascularized donor tracheal grafts within the omentum of the recipient rodent and monitoring the graft histologically for rejection. Rejection of heterotopic allografts is characterized by complete airway obliteration, a process that appears to be mediated by fibroblast proliferation. The rejection of orthotopic tracheal allografts, however, is not manifested by airway obliteration, but is characterized by lymphocytic infiltrate and edema of the lamina propria, followed by loss of ciliated columnar epithelium and eventual collapse of the cartilage ultrastructure. We previously described and characterized the murine orthotopic tracheal transplantation model and observed that while rejecting orthotopic allografts sustain moderate airway compromise as a result of edema, obliteration of the airway lumen does not occur. From these studies, it has become clear that the heterotopic model does not accurately reflect the biological behavior of orthotopic tracheal allografts in rodent or large animal models. In the present study, we tested the hypothesis that murine orthotopic tracheal allografts undergo progressive reepithelialization by recipient-derived mucosa and that this process inhibits airway obliteration.
Thirty age-matched mice were randomly assigned to 6 experimental groups (Table 1). BALB/c donor tracheal segments (5 tracheal rings) were orthotopically or heterotopically transplanted into syngeneic BALB/c or class I and II major histocompatibility–mismatched allogeneic C57BL/6 recipients. BALB/c and C57BL/6 mice (weight, 20 g; age, 20 weeks) (Taconic Farms, Germantown, NY). In group 1, BALB/c donor tracheal segments were heterotopically transplanted into syngeneic BALB/c recipients without treatment. In groups 2 and 3, BALB/c donor tracheal segments were heterotopically transplanted into allogeneic C57BL/6 recipients; group 2 was not treated and group 3 was treated with 7 mg/kg per day of intraperitoneal (IP) cyclosporine. In group 4, BALB/c donor tracheal segments were orthotopically transplanted into syngeneic BALB/c recipients without treatment. In groups 5 and 6, BALB/c donor tracheal segments were orthotopically transplanted into allogeneic C57BL/6 recipients; group 5 was not treated while group 6 was treated with 7 mg/kg per day of cyclosporine.

Following postoperative recovery, animals were housed in flat-bottomed cages, provided food and water ad libitum, and cared for according to all institutional guidelines. On a daily basis, the orthotopic transplant recipients were monitored clinically for audible respiratory stridor. Twenty-one days after transplantation, 2 animals from each group were euthanized for histological evaluation, 1 animal was assessed for CD4/CD8 immunohistochemistry, and 2 animals underwent immunohistochemical analysis for mucosal phenotype.

TRACHEAL GRAFTING PROCEDURE

Subcutaneous ketamine (50 mg/kg) and xylazine (10 mg/kg) anesthesia was administered preoperatively. Using an operating microscope (Wild M651; Wild Leitz, Willodale, Ontario) while the donor mouse was under ketamine and xylazine anesthesia, we exposed its tracheal segment through an anterior midline neck incision. Division of the strap muscles enabled identification of the entire laryngotracheal complex. The cervical esophagus and vascular structures were separated from the trachea with careful attention to preserve the recurrent laryngeal nerves. A 5-ring circumferential tracheal segment was excised and placed into a glass dish with cooled physiologic phosphate-buffered saline (PBS). The recipient mouse was prepared using a Wild M651 microscope while it was under ketamine and xylazine anesthesia. Heterotopic recipients underwent a midline laparotomy incision followed by the isolation and implantation of the donor trachea segment into the omentum. The tracheal segment was wrapped in omentum, and the abdomen was closed using 7-0 nylon sutures.

The tracheal segment of the orthotopic recipient mouse was exposed through an anterior midline neck incision. Division of the strap muscles enabled identification of the entire laryngotracheal complex. An incision was made in the recipient trachea, which resulted in a tracheal gap that easily accommodated the donor tracheal graft. The donor tracheal graft was orthotopically placed in the recipient tracheal defect, oriented into a position such that the proximal end of the native trachea opposed the proximal end of the graft segment, and secured with 10-0 nylon interrupted transtracheal sutures. The strap muscles were approximated and the skin was closed with 7-0 nylon sutures. No oxygenation was administered to the donor or recipient during the course of the procedure, and postoperatively, the animals were placed under a warming lamp and monitored for 3 hours.

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Donor</th>
<th>Recipient</th>
<th>Graft Type</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (5)</td>
<td>BALB/c</td>
<td>BALB/c</td>
<td>Heterotopic</td>
<td>None</td>
</tr>
<tr>
<td>2 (5)</td>
<td>BALB/c</td>
<td>C57BL/6</td>
<td>Heterotopic</td>
<td>None</td>
</tr>
<tr>
<td>3 (5)</td>
<td>BALB/c</td>
<td>C57BL/6</td>
<td>Heterotopic</td>
<td>Cyclosporine</td>
</tr>
<tr>
<td>4 (5)</td>
<td>BALB/c</td>
<td>BALB/c</td>
<td>Orthotopic</td>
<td>None</td>
</tr>
<tr>
<td>5 (5)</td>
<td>BALB/c</td>
<td>C57BL/6</td>
<td>Orthotopic</td>
<td>None</td>
</tr>
<tr>
<td>6 (5)</td>
<td>BALB/c</td>
<td>C57BL/6</td>
<td>Orthotopic</td>
<td>Cyclosporine</td>
</tr>
</tbody>
</table>

EVALUATION OF TRACHEAL AIRWAY PATENCY

Recipient animals were evaluated once daily for airway compromise as manifested by the development of stridor, labored breathing, and decreased activity. If airway compromise leading to decreased activity was detected, the animal was euthanized.

HISTOLOGICAL EVALUATION

Twenty-one days after tracheal transplantation, 2 mice from each group were euthanized and the tracheal graft segments were surgically removed. Tracheal segments were initially fixed in cold 10% neutral buffered formalin solution for hematoxylin–eosin staining. Three-micrometer sections were cut and stained with hematoxylin–eosin. Six random sections from each graft were assessed for cartilage architecture, cilia ultrastructure, and the presence of lymphocytic infiltrate. In addition, measurements of the cartilage thickness and the thickness of the lamina propria were determined from 3 separate predetermined points using computer-assisted morphometry (Axioskop microscope [Carl Zeiss, Thornwood, NY] with a SAMBA 4000 image analyzer [Imaging Products International, Chantilly, Va.]). The ratio of the lamina propria to the tracheal cartilage (LCR) was calculated and averaged for the 3 points. This assessment was performed on 6 random sections from each graft and used as a measure of rejection.

IMMUNOHISTOCHEMICAL ASSESSMENT

A single mouse from each experimental group was evaluated 21 days after tracheal grafting. Routine sections were cut at 5 micrometers and picked up on a glass slide and allowed to dry overnight at room temperature. The sections were then fixed in cold acetone (−20°C) for 2 minutes. The fixed slides were dried for 1 hour at room temperature and then rinsed 2 to 3 times in PBS. Endogenous block of 0.3% hydrogen peroxide solution in PBS was applied for 10 minutes. The slides were rinsed in PBS and blocked with 5% normal rat serum. One of 2 diluted primary antibodies (RM-4, CD4 [L3T4], or 53-6.7, CD8a [Ly-2]; BD Biosciences PharMingen, San Diego, Calif) used at 1:20 was applied and secondary antibody (rabbit, antirat, BD Biosciences PharMingen), was applied at room temperature for 30 minutes. The slides were then rinsed in 3 changes of PBS at 2 minutes each. The enzyme conjugate strepavidin–horse-radish peroxidase (BD Biosciences PharMingen) was applied to each slide and allowed to incubate at room temperature for 30 minutes. The slides were again rinsed in PBS, and 3,3′-diaminobenzidine (DAB) solution was added to the slides and allowed to incubate for 5 minutes. The slides were then rinsed in water and counterstained in hematoxylin–eosin, bluing reagent, and ammonia. The slides were then dehydrated and coverslips were applied.

Six randomly chosen sections from each group were blindly evaluated by a pathologist and scored for CD8 infiltrate. Each
Table 2. Histological and Clinical Results After Tracheal Transplantation

<table>
<thead>
<tr>
<th>Group</th>
<th>Graft</th>
<th>Treatment</th>
<th>Cellular Infiltrate*</th>
<th>LCR</th>
<th>Mucosa Type</th>
<th>Airway</th>
<th>Tracheal Lumen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Heteropic isograft</td>
<td>None</td>
<td>1</td>
<td>0.70±0.10</td>
<td>Donor</td>
<td>NA</td>
<td>Patent</td>
</tr>
<tr>
<td>2</td>
<td>Heterotic allograft</td>
<td>None</td>
<td>5.6</td>
<td>&gt;45</td>
<td>Donor</td>
<td>NA</td>
<td>Obliterated</td>
</tr>
<tr>
<td>3</td>
<td>Heteropic allograft</td>
<td>Cyclosporine</td>
<td>1.3</td>
<td>0.70±0.09</td>
<td>Donor</td>
<td>NA</td>
<td>Patent</td>
</tr>
<tr>
<td>4</td>
<td>Orthotopic isograft</td>
<td>None</td>
<td>1.2</td>
<td>0.66±0.17</td>
<td>Donor</td>
<td>No stridor</td>
<td>Patent</td>
</tr>
<tr>
<td>5</td>
<td>Orthotopic allograft</td>
<td>None</td>
<td>4.6</td>
<td>1.8±0.19</td>
<td>Recipient</td>
<td>Stridor</td>
<td>Patent</td>
</tr>
<tr>
<td>6</td>
<td>Orthotopic allograft</td>
<td>Cyclosporine</td>
<td>2.8</td>
<td>0.75±0.10</td>
<td>Recipient</td>
<td>No stridor</td>
<td>Patent</td>
</tr>
</tbody>
</table>

Abbreviations: LCR, lamina propria–to–cartilage ratio; NA, not applicable.
*CD4/CD8 immunohistochemistry scored on a 1 to 6 scale: minimal infiltrate (0-2), moderate infiltrate (2-4), and severe infiltrate (4-6).
†Denotes heteropic control.
‡Denotes orthotopic control.
§Denotes significance when compared with control (P=.05, Dunnett test).

RESULTS

EVALUATION OF TRACHEAL AIRWAY

None of the heteropic transplant recipients were assessed for stridor (Table 2). The immunosuppressed mice that received an orthotopic tracheal transplant (group 6) and the isograft recipients (group 4) did not exhibit an audible stridor; however, nonimmunosuppressed recipients of orthotopic allografts (group 5) demonstrated an audible stridor that persisted throughout the 21-day assessment period.

HISTOLOGICAL ASSESSMENT

Twenty-one days after tracheal transplantation, random sections from the proximal portion of the tracheal grafts were evaluated (Table 1). Nonimmunosuppressed heteropic allografts demonstrated complete airway lumen obliteration with a loss of normal airway architecture (Figure 1). No epithelial-lined lumen, basement membrane, or lamina propria could be distinguished, and in several grafts, the cartilage had collapsed. The LCR was greater than 4 in all of the grafts (Figure 2). Immunosuppressed heteropic allografts and heteropic isografts demonstrated a patent lumen with minimal cellular infiltrate of the lamina propria. However, the immunosuppressed heteropic allografts were lined with a nonciliated epithelium (LCR, 0.7±0.09) (Figure 3), while the isograft was lined with a ciliated pseudostratified columnar epithelium (LCR, 0.7±0.10) (Figure 4). In contrast, the nonimmunosuppressed orthopic allograft, the immunosuppressed orthopic allograft, and the orthotopic isograft all demonstrated a patent lumen and ciliated pseudostratified columnar epithelium with normal cartilage architecture. The nonimmunosuppressed graft, however, demonstrated cellular infiltrate and edema within the lamina propria as demonstrated by an elevated LCR (1.8±0.19) (Figure 5), which was significantly increased when compared with the orthopic control (0.66±0.17) and the immunosuppressed orthopic allograft (0.75±0.10) (Figure 6) (P<.05).

Figure 1. Nonimmunosuppressed heteropic allograft demonstrating obliteration of the lumen, collapse of the cartilage, and loss of the normal architecture (hematoxylin-eosin, original magnification ×25).
IMMUNOHISTOCHEMICAL ASSESSMENT

Six randomly chosen sections from each group were blindly evaluated and scored by a pathologist and scored for CD8 infiltrate. Cellular infiltrate scores for the nonimmunosuppressed heterotopic (5.6) and orthotopic allografts (4.6) consistently demonstrated severe cellular infiltrate. Both isograft groups demonstrated minimal cellular infiltrate, while the immunosuppressed orthotopic and heterotopic allografts were scored as mild (1.3) and moderately infiltrated (2.8), respectively. There was no significant difference between CD4 and CD8 scores for each group.

IMMUNOHISTOCHEMICAL ASSESSMENT (TISSUE PHENOTYPE)

Immunohistochemical assessment of tissue phenotype demonstrated that the proximal aspect of the orthotopic allografts was reepithelialized with recipient-derived mucosa in both the immunosuppressed and non-immunosuppressed groups (Figure 7). The staining of the epithelium demonstrates that the adjacent recipient mucosa has migrated into the allograft and prevented the obliteratorative response characteristic of the nonimmunosuppressed heterotopic allografts. While the staining for recipient epithelium was dense in the proximal and distal aspects of the allograft, the central portion of the allograft stained lightly for both the recipient and the donor epithelium.

Before tracheal transplantation can be applied to the clinical reconstruction of extensive circumferential defects of
the airway, it is essential to elucidate the biological behavior of tracheal allografts. The heterotopic tracheal transplantation model has been used extensively for the study of obliterative airway disease associated with lung transplantation and tracheal transplantation. While heterotopic transplantation has been a popular model for rodent experimentation because of its technically simple procedure, undoubtedly the behavior of heterotopic tracheal allografts differ when transplanted orthotopically. Prior work has demonstrated that while rejection of the heterotopic allograft leads to complete airway obliteration, obliteraton does not occur in orthotopic allografts. We have previously demonstrated that nonimmunosuppressed orthotopic tracheal allografts undergo a rejection response characterized by edema and T-cell infiltrate of the lamina propria, loss of the ciliated lummen epithelium, and clinical stridor; however, the grafts remain patent. Genden et al7 and others6,8 have suggested that the presence of recipient-derived epithelium within the graft segment is essential in preventing an obliterative response; however, the specifics of this process have not been elucidated.

In the present study we have confirmed that without persistent immunosuppression, heterotopic tracheal allografts will obliterate. In contrast, the airway of nonimmunosuppressed orthotopic allografts becomes constricted as a result of edema, but remains patent. Nonimmunosuppressed orthotopic grafts sustain a rejection response characterized by airway edema and clinical stridor. However, if the recipient is immunosuppressed, the airway infiltrate and edema can be prevented and the recipient will therefore not become stridorous. Immunohistochemical analysis demonstrates that recipient-derived tracheal epithelium migrates into the proximal donor segment within 21 days after transplantation regardless of the state of immunosuppression, and this process appears to protect the graft lumen from obliteration. The failure of this process to occur in the heterotopic grafts leads to an irreversible obliterative response.

It has been proposed that ischemia, infection, and immune-mediated direct cellular damage associated with an up-regulation of fibroblasts may be responsible for obliterative airway disease in heterotopic grafts; however, orthotopic grafts are exposed to the same conditions yet fail to obliterate. Neuringer et al9 characterized the immune cells associated with the obliterative response and demonstrated that the acute immunologic airway injury that occurs in heterotopic tracheal allograft rejection is followed by a period of intense fibrosis mediated by macrophages and fibroblasts. The recipient epithelial cells that lie adjacent to the orthotopic donor graft may regulate the proliferation of macrophages and fibroblasts. The adjacent syngeneic mucosa appears to confer a protective influence on the adjacent allograft through a mechanism that may be mediated by such anti-inflammatory cytokines as interleukin 10. Boehler et al10 used adenoviral-mediated interleukin 10 gene transfection to mitigate the obliterative response, and subsequently, Dosanjh et al11 demonstrated that epithelial cell-derived interleukin 10 can regulate the proliferation of pulmonary fibroblasts in vitro and suggested that a dysregulation of interleukin 10 may play a role in the fibroproliferative response associated with obliterative airway disease.

We have demonstrated, for the first time, that reepithelialization of murine orthotopic tracheal allografts with recipient-derived mucosa plays an essential role in preventing airway obliteration. Irrespective of immunosuppression, the reepithelialization process occurs in orthotopic grafts; however, when allografts are not subject to the influence of adjacent syngeneic epithelium, a brisk fibroproliferative response results in complete obliteration of the airway. Our findings suggest that the orthotopic tracheal transplantation model more accurately reflects the clinical behavior of tracheal allografts. Future work will be focused on defining the kinetics and mechanism of allograft reepithelialization.

Accepted for publication August 16, 2002.

This study was supported by training grant 1K08DC00199-01 from the National Institutes of Health, Bethesda, Md.

This study was presented at the annual meeting of the American Head and Neck Society, Boca Raton, Fla, May 11, 2002.

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REFERENCES