Healing of a free tracheal autograft is enhanced by topical vascular endothelial growth factor in an experimental rabbit model

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Objective: In 1996, we introduced the free tracheal autograft technique for repair of congenital tracheal stenosis from complete tracheal rings in infants and children. Sources of possible concern with this procedure include the potential for autograft ischemia, patch dehiscence, and recurrent stenosis. Vascular endothelial growth factor is a potent angiogenic inducer (particularly in the setting of ischemia, hypoxia, or both) and is postulated to promote tissue healing. The purpose of this study was to test the hypothesis that pretreatment of tracheal autografts with topical vascular endothelial growth factor would enhance tracheal healing.

Methods: In a rabbit model of tracheal reconstruction (n = 32), an elliptically shaped portion of the anterior tracheal wall was excised. The excised portion of trachea was one third of the tracheal circumference and 2 cm in length (6 tracheal rings). This portion of trachea (the autograft) was soaked in either vascular endothelial growth factor (5 µg/mL, n = 16) or normal saline solution (n = 16) for 15 minutes before being reimplanted in the resultant tracheal opening. Animals were killed and autografts were examined at 2 weeks, 1 month, and 2 months postoperatively for gross and microscopic characteristics.

Results: By 2 weeks, and progressing through 1 and 2 months, autografts treated with vascular endothelial growth factor, as compared with control autografts, had reduced luminal stenosis, submucosal fibrosis, and inflammatory infiltrate (P < .05). The autografts tended to become malaligned in control animals, whereas the tracheal architecture was preserved in rabbits treated with vascular endothelial growth factor. Microvascular vessel density was significantly greater in all vascular endothelial growth factor groups (P < .05) at all time intervals.

Conclusions: Topical treatment of free tracheal autografts with vascular endothelial growth factor in a rabbit tracheal reconstruction model enhanced healing, as evidenced by accelerated autograft revascularization, reduced submucosal fibrosis, and inflammatory infiltrate (P < .05). The autografts tended to become malaligned in control animals, whereas the tracheal architecture was preserved in rabbits treated with vascular endothelial growth factor. Microvascular vessel density was significantly greater in all vascular endothelial growth factor groups (P < .05) at all time intervals.

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tracheoplasty,9-11 and the free tracheal autograft.12,13 More recently, cryopreserved tracheal allografts have been shown to have encouraging results in salvage procedures after failure of one of the described primary techniques.14,15 Our best clinical results have been obtained with the tracheal autograft technique.

Vascular endothelial growth factor (VEGF) is a secreted glycoprotein and a potent endothelial cell–derived angiogenic factor that acts through 2 receptors, flk-1 and flt-1.16 VEGF is particularly important in the setting of tissue ischemia.17 VEGF has been noted in tracheal granulation tissue in children and in normoxic wound fluid.18,19 VEGF has been implicated in enhanced wound healing, increased vascular permeability, angiogenesis, and the stimulation of nitric oxide release from vascular endothelial cells.20-23 We proposed this study in rabbits to evaluate the revascularization and healing process of the autograft and to examine the potential effect on the autograft of topical VEGF. The excised autograft is completely devascularized before reimplantation for tracheal reconstruction. It is therefore subjected to a period of hypoxia-ischemia that might contribute to imperfect healing, excessive scarring, or both. In a rabbit model simulating repair of congenital tracheal stenosis with a free tracheal autograft, we set out to histologically define the healing process of the autograft. Furthermore, an attempt was made to determine whether pretreating tracheal autografts with topical VEGF before reimplantation would enhance local angiogenesis and thus improve tracheal healing.

**Materials and Methods**

Thirty-two New Zealand White rabbits (16 male and 16 female; weight range, 1.8-3.3 kg; mean weight, 2.5 kg) underwent a tracheal autograft procedure according to the study protocol. All animals received humane care in compliance with the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996. Anesthetic induction was done by using intramuscular acepromazine (1-2 mg/kg), followed by intramuscular ketamine and xylazine (35-50 mg/kg and 5-10 mg/kg, respectively). The rabbits then had their necks shaved, had an intravenous line placed, and underwent pulse-oximetry monitoring. Rabbits at this point were breathing spontaneously. After sterile preparing and draping, a midline collar incision was made, with dissection of the muscular planes down to the trachea. A horizontal tracheal incision was then made, opening one third of the tracheal circumference 3 to 4 rings below the vocal cords. A sterile endotracheal tube (3.5F) was introduced without a cuff and in a retrograde manner through the tracheotomy, through the vocal cords, and out of the mouth. The tube was connected to the ventilator, and anesthesia was continued with inhaled isoflurane (1%-2%). This technique of retrograde intubation was developed after the third case, when repetitive attempts to intubate in a standard antegrade fashion were judged to be unnecessarily traumatic to the vocal cords and larynx, as well as excessively time consuming. The endotracheal tube tip was positioned just above the carina and below the area where the procedure was being performed. The tracheal incision was then carried inferiorly to complete the excision of an elliptical anterior autograft (Figure 1). The autograft consisted of one third of the tracheal circumference and included 6 cartilage rings (2 cm in length). The endotracheal tube tip remained well beyond the most distal part of the defect thus created, so that mechanical ventilation of the animal proceeded without difficulty. The excised autograft was then immersed in either a solution containing human recombinant VEGF (5 µg/mL; n = 16; R&D Systems, Minneapolis, Minn) or normal saline solution (n = 16) for 15 minutes. Randomization was performed by using VEGF on every other animal. During this time,
TABLE 1. Difference in luminal stenosis of the tracheal orifice between control (n = 12) and VEGF-treated (n = 12) rabbits at 2 weeks, 1 month, and 2 months after a tracheal autograft procedure

<table>
<thead>
<tr>
<th>Luminal stenosis</th>
<th>2 wk Control</th>
<th>VEGF*</th>
<th>1 mo Control</th>
<th>VEGF*</th>
<th>2 mo Control</th>
<th>VEGF*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No stenosis</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>&lt;10% stenosis</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>10%-25% stenosis</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>26%-50% stenosis</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>51%-100% stenosis</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

*All P < .05 (exact Wilcoxon P value = .0286).

interrupted 6-0 PDS (polydioxanone) sutures (Ethicon Inc, Somerville, NJ) were placed circumferentially around the defect (Figure 1). The sutures were then passed through the edges of the circumference of the autograft, the autograft was lowered into the defect, and all the sutures were tied and cut. Use of intermittent squirts of either normal saline or VEGF solution, according to the treatment protocol, kept the autograft from desiccating during this phase. During continued ventilation, the surgical site was immersed with saline solution to assess for air leaks, and additional interrupted PDS sutures were added as necessary. The wound was then closed with 2 layers of continuous 4-0 Vicryl sutures (Ethicon, Inc) for the muscular layer and continuous 4-0 Vicryl sutures in the subcuticular layer. The procedures were done under enrofloxacin prophylaxis (5 mg/kg) administered intravenously before the skin incision and administered intramuscularly every 12 hours postoperatively for 72 hours. Postoperative pain management consisted of subcutaneous buprenorphine (0.05 mg/kg) every 12 hours as required.

Rabbits were killed with intravenous pentobarbital (150 mg/kg) in 3 groups of 10 animals each at 2 weeks, 1 month, and 2 months, respectively. In each group, 5 animals were treated with VEGF, and 5 were control animals. The tracheas were excised from larynx to carina and divided along the posterior wall longitudinally to keep the autograft patch intact. At the aforementioned intervals, healing characteristics were compared between the 2 groups to determine the effect of topical VEGF on a devascularized free tracheal autograft. The autografts were placed in 10% buffered formalin overnight. The specimens were embedded in paraffin, and 5-µm sections were placed on glass slides. Each slide was stained with hematoxylin-phloxine-saffarin to highlight fibrous tissue. The pathologist (S.C.) grading the specimens was blinded to whether the specimens were treated or untreated. In 4 rabbits in each group, macroscopic assessment was performed for luminal stenosis and tracheal wall configuration, and microscopic scoring was performed by evaluating the degree of submucosal fibrosis and inflammation. In one rabbit in each group, microvascular density (MVD) was assessed by counting the number of endothelial cell–lined channels in 5 separate high-power fields. Immunohistochemical localization of VEGF was performed on fixed tissue sections by a standard ABC protocol and antibody to VEGF (R&D Systems). The slides were counterstained with hematoxylin, and the intensity of VEGF staining was graded in a blinded fashion.

We compared the difference in the degree of luminal stenosis of the tracheal wall between control and VEGF-treated rabbits at 2 weeks, 1 month, and 2 months after a tracheal autograft by using an exact Wilcoxon test.24 Statistical comparisons between control and treated rabbits were done separately at each time period. We conducted similar analyses for differences in the following outcomes: autograft alignment, submucosal fibrosis, and inflammatory infiltrate. To compare the difference in MVD between control and VEGF-treated rabbits, we used a Wilcoxon rank-sum test.25 We used SAS version 7 software for the calculation of exact Wilcoxon P values (SAS Institute, Cary, NC).

Results

The operative procedure was considered successful in 30 (93.8%) of 32 animals, and 30 animals survived until the end of the experimental period. In the early phase of the experiment, there were 2 deaths (1 in the control group and 1 in the treatment group) on the first postoperative day as a result of acute respiratory distress. Autopsy examination revealed macroscopic evidence of tracheal stenosis at the site of repair associated with edema of the vocal cords and larynx. These deaths were attributed to the learning curve involved with the antegrade intubation technique and with the autograft procedure itself.

Tables 1 to 5 summarize the gross and microscopic results comparing control rabbits with VEGF-treated rabbits at 2 weeks, 1 month, and 2 months postoperatively. Gross evaluation of the autograft specimens in the 2 groups revealed essentially no luminal stenosis (Table 1) or autograft malalignment (Table 2) in the VEGF-treated group at all time points. In contrast, the control groups had progressive luminal stenosis (Table 1) and autograft malalignment (Table 2). Microscopic evaluation of the VEGF-treated animals showed a mild degree of submucosal fibrosis at 1 and 2 months (Table 3), although it did not alter the luminal integrity of the autograft (Figure 2). These findings were in striking contrast to those in the control group, in which there was progressive submucosal fibrosis that was severe by 2 months (Table 3). At 2 months, the control animals showed a mild degree of submucosal fibrosis at 1 and 2 months (Table 3), although it did not alter the luminal integrity of the autograft (Figure 2). These findings were in striking contrast to those in the control group, in which there was progressive submucosal fibrosis that was severe by 2 months (Table 3).

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An unexpected histologic finding was a difference in the inflammatory response between the 2 groups. In the control
group, all animals had an early and persistent inflammatory infiltrate located adjacent to the site of repair (Table 4). The inflammatory cell population in both groups was composed of predominantly lymphocytes and occasionally eosinophils. All animals in the VEGF-treated group demonstrated a similar inflammatory response at 2 weeks; however, the inflammatory process appeared to resolve, and by 2 months, no aggregates of lymphocytes were observed in any of the treated autograft specimens (Table 4). These data suggest that VEGF may play a suppressive role in immune modulation, and this may be one mechanism responsible for its ability to enhance wound healing and limit fibrosis.

MVD was assessed by counting the number of endothelial cell–lined channels in 5 high-power fields in one autograft in each group to determine whether VEGF, a potent inducer of angiogenesis, improved the revascularization of the autografts. By 2 weeks, both groups showed evidence of revascularization of the autograft (Table 5). However, the VEGF-treated group had more than a 2-fold increase in MVD when compared with the control group (8.8 vs 21.2; \( P = .012 \), Wilcoxon rank-sum test). The increase in neovascularization of the autografts persisted at 1 and 2 months in the VEGF-treated groups (Table 5 and Figure 3, A vs B). The vessels tended to be arranged in clusters without significant red blood cell extravasation, suggesting that vascular leakage was not a complicating factor.

Because the exposure of the autograft to VEGF was relatively short, it was important to determine whether
TABLE 5. Microvascular density

<table>
<thead>
<tr>
<th>High-power field No.</th>
<th>2 wk Control</th>
<th>VEGF</th>
<th>1 mo Control</th>
<th>VEGF</th>
<th>2 mo Control</th>
<th>VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9</td>
<td>21</td>
<td>14</td>
<td>36</td>
<td>17</td>
<td>41</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>26</td>
<td>18</td>
<td>42</td>
<td>14</td>
<td>46</td>
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<tr>
<td>3</td>
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<td>19</td>
<td>13</td>
<td>31</td>
<td>12</td>
<td>44</td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>22</td>
<td>10</td>
<td>33</td>
<td>19</td>
<td>38</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>18</td>
<td>7</td>
<td>43</td>
<td>25</td>
<td>40</td>
</tr>
<tr>
<td>Mean</td>
<td>8.2</td>
<td>21.2</td>
<td>14.4</td>
<td>37.0</td>
<td>17.4</td>
<td>41.8</td>
</tr>
</tbody>
</table>

MVD was assessed in each autograft by counting the number of endothelial cell–lined channels (vessel density) in 5 separate high-power fields. At 2 weeks, the difference in MVD between control (mean = 8.2) and VEGF-treated (mean = 21.2) rabbits was statistically significant ($P = .012$, Wilcoxon rank-sum test). At 1 month, the difference in MVD between control (mean = 14.4) and VEGF-treated (mean = 37.0) rabbits was statistically significant ($P < .01$, Wilcoxon rank-sum test). At 2 months, the difference in MVD between control (mean = 17.4) and VEGF-treated (mean = 41.8) rabbits was statistically significant ($P < .01$, Wilcoxon rank-sum test).

Figure 2. Photomicrographs of tracheal autografts at 2 months postoperatively at low and high power (hematoxylin-phloxine-saffarin stain): A and C, untreated; B and D, VEGF treated. A versus B: Note the malalignment of the tracheal cartilage in the untreated graft (A) caused by excessive fibrosis causing distortion of the tracheal alignment at the autograft tracheal junction (arrow). C versus D: C, High power showing distortion (arrow) at anastomosis caused by excessive submucosal fibrosis (pink-staining fibroblasts adjacent to arrow). D, Medium power of treated graft shows normal-appearing submucosa. L, Lumen.
this elevated MVD was due to higher tissue localization of VEGF. Immunohistochemical studies confirmed intense immunostaining for VEGF in the submucosal region in the treated group, whereas only minimal VEGF expression was observed in the control group (compare Figure 3, C, with Figure 3, D). The tracheal epithelium in the treated group was uniform, with intact cilia and an occasional goblet-appearing cell. These data suggest that a brief exposure to topical VEGF is sufficient to increase tissue revascularization, and VEGF continues to be secreted by the numerous endothelial cells in the submucosa. We postulate that this is due to a positive feedback loop. It localizes in the interstitial matrix and spares the tracheal epithelial cells. Moreover, 2 potential adverse biologic actions of VEGF, increased vascular permeability and pathologic endothelial cell proliferation, were not observed in this experimental model, suggesting that topical VEGF has minimal unfavorable side effects.

**Discussion**

Tracheal stenosis from congenital complete tracheal rings is a rare anomaly characterized by an absence of the membranous portion of the trachea. The tracheal stenosis may be segmental, funnel-like, or in the form of generalized tracheal hypoplasia, as classified by Cantrell and Guild. Severe respiratory distress is often apparent early in the neonatal period. Various reconstruction techniques and materials for repair have been developed in the treatment of congenital tracheal stenosis. The severity of the tracheal anatomy, especially the length of the stenosed portion, dictates the choice of repair to a large degree. Primary reconstruction with end-to-end anastomosis may relieve short
require more creative techniques. Idriss and colleagues\(^3\) first described this technique because of the above-mentioned complications.\(^{28}\) Various other autologous graft materials have been used in conjunction with or as replacements for pericardium, such as costal cartilage.\(^6\)-\(^8\) Tsang and coworkers\(^9\) introduced the slide tracheoplasty technique. This involves a transverse division of the trachea at the midpoint of the stenosis, spatulating the proximal and distal ends, followed by direct anastomosis of the 2 tracheal segments. Grillo\(^{10}\) and Lang and associates\(^{11}\) have reported good results with this technique. Nakanishi and colleagues\(^{29}\) experimented with tracheal autografts in dogs. They found that autografts longer than 4.0 cm (8-10 rings) showed stenosis, dissolution, or both, in the middle part of the graft. Our group recently published its results with free tracheal autografts with and without pericardium.\(^{12,13}\) Advantages of a free tracheal autograft include the potential for growth, the operation is reproducible and technologically easy, and there is minimal tension on the suture lines. However, in all cases we have been concerned about the potential for autograft ischemia, patch dehiscence, and recurrent stenosis. Despite a survival in our series of over 90\%, a few patients have had prolonged hospitalization as a result of recurrent granulation tissue and stenosis from exuberant scar formation.

VEGF, also known as vascular permeability factor, is a soluble dimeric protein existing in 4 isoforms that is secreted by numerous cell types. It is an endothelial cell–specific mitogen and an angiogenic inducer by in vitro and in vivo assays, promoting its effect through the specific receptors flk-1 and flt-1.\(^{16,17,19,20}\) It also induces endothelium-dependent vasodilatory responses through stimulation of the release of nitric oxide from the endothelial cell\(^{19}\) and is implicated in wound healing through its angiogenic activities.\(^{20}\) VEGF secretion is upregulated by hypoxic stress, ischemia, and anemia.\(^{17,20}\)

Pokharel and colleagues\(^{18}\) recently demonstrated augmented expression of VEGF protein and mRNA in tracheal granulation tissue specimens after prolonged intubation in children. Increased VEGF levels were found in those epithelial cells and macrophages that migrated to infiltrate the granulation tissue. They speculated that enhanced VEGF expression may play a pivotal role in granulation tissue development. Albes and colleagues\(^{30}\) investigated whether another angiogenic inducer, basic fibroblast growth factor (bFGF), can enhance the blood supply of an ischemic autograft that was placed in a subcutaneous pouch. They concluded that application of bFGF increased revascularization and resulted in improved epithelial preservation. Corral and associates\(^{31}\) demonstrated, in a rabbit skin ulcer model, that topical VEGF was more effective than bFGF during ischemic wound healing.

We hypothesized that through enhanced local angiogenesis and microvascular vasodilation, devascularized free tracheal autografts treated with topical VEGF would revascularize and heal better than control grafts in a rabbit model of tracheal reconstruction. At 2 weeks, 1 month, and 2 months after reimplantation, gross and histologic examination of the treated grafts demonstrated an enhanced healing process. Fibrous tissue formation leading to potential stenosis and localized airway obstruction was significantly less in the VEGF-treated rabbit autografts. Histologically, MVD was increased and inflammation was decreased in the VEGF group. We postulate that a more abundant blood supply was one mechanism underlying the improved healing. This enhanced healing resulted in less protrusion of the graft into the airway lumen. Of note, at 2 months postoperatively, there was stabilization of the MVD back to normal ranges. This represents an important finding in that a relative quiescence in the vasculature was noted over the relatively short time period of the study.

There are some interesting findings in our experiment for which we do not necessarily have an explanation. We noted an increase in VEGF localization (Figure 3), even at 2 months postoperatively. We speculate that this is due to some form of positive feedback loop but have no data to support this. We have not looked at mRNA expression in the autografts or at immune mechanisms for VEGF action.

Topical VEGF treatment of free tracheal autografts in a rabbit tracheal reconstruction model accelerated revascularization, increased MVD, reduced inflammation, suppressed submucosal fibrosis, and preserved architectural integrity. It is extrapolated from these observations that enhanced graft healing promoted by topical VEGF administration results in an improved airway that could be an important adjunct to the surgical repair of congenital tracheal stenosis in infants and children. Postoperative intubation times could be reduced, as could the number of treatments with a rigid bronchoscope required for granulation tissue debridement. Potentially, topical treatment with VEGF represents a single pulse of a healing boost in the setting of tissue injury at the precise time when hypoxia-ischemia and resultant necrosis can be most menacing. The optimal concentration and exposure time to topical VEGF still need to be determined in an experimental setting. We speculate that topical VEGF may be an...
important adjunctive therapy to improve clinical results of surgical repair of congenital tracheal stenosis.

We thank William Checkley, ScM, Biostatistics, for statistical analysis of the study data.

References