Effect of Vocal Fold Injection of Cidofovir and Bevacizumab in a Porcine Model

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IMPORANTCE Recurrent respiratory papillomatosis (RRP) is a common and often chronic disorder. Intralaryngeal bevacizumab has gained recent interest as an adjuvant therapy for RRP. However, no histologic model has been published describing the effects of bevacizumab on the vocal fold.

OBJECTIVE To investigate the histologic effects of bevacizumab injections into the vocal fold and compare these findings with those for cidofovir and saline control injections.

DESIGN AND SETTING In vivo animal study involving eighteen 1-year-old Yorkshire crossbreed pigs, with a blinded review of pathologic findings conducted in a veterinary research laboratory.

INTERVENTIONS The pigs were randomly divided into six study groups receiving 2.5 or 5.0 mg of cidofovir or bevacizumab alone or in combination. Each pig received an injection of 0.5 mL of the test drug in the right vocal fold and 0.5 mL of saline in the left vocal fold. These injections were performed 4 times during the course of 8 weeks. One pig from each group was killed humanely and the larynges harvested 2 weeks after the last injection. The remaining pigs were killed 4 months after the last injection on the remaining pigs. The vocal folds were fixed and stained with hematoxylin-eosin and trichrome and reviewed for histologic changes by 3 blinded pathologists.

MAIN OUTCOMES AND MEASURES Histologic changes to the vocal folds.

RESULTS Minimal inflammation, edema, and atypia were found in all treatment groups. No appreciable histologic differences were found among the 3 treatment groups and their controls. No difference was seen in the vocal folds that were harvested late (4 months) vs early (2 weeks) after last injection. No fibrosis was found in any of the specimens.

CONCLUSIONS AND RELEVANCE No histologic evidence suggests that intralaryngeal cidofovir or bevacizumab alone or in combination resulted in significant changes to the porcine vocal fold. Future studies may build on this model to test higher dosages and/or may combine injections with potassium titanyl phosphate laser therapy.
Recurrent respiratory papillomatosis (RRP) is a common disorder, affecting 4 per 100 000 children and 1.8 per 100 000 adults. Recurrent respiratory papillomatosis represents the most common benign neoplasm of the larynx and is caused by the human papillomavirus, most commonly subtypes 6 and 11. Juvenile-onset RRP may persist for many years, with physical and emotional consequences. Children with RRP receive the diagnosis at a mean age of 3.1 years and require a mean total of 21.6 lifetime surgical procedures, with a mean of 5.1 procedures per year. Children who receive the diagnosis before 4 years of age have a more aggressive disease and require more frequent surgical treatment. Although rare, more severe disease may also correlate with distal spread of the papillomas to the lower airways or with the requirement of a tracheostomy for airway support. Juvenile-onset RRP usually follows a nonlinear course, and most patients experience a decreasing annual rate of surgical procedures over time.

Therapy goals for juvenile-onset RRP include controlling papilloma burden to provide a safe airway and functional voice. Many patients routinely undergo surgical procedures for debulking using a combination of laser, microdebrider, or microlaryngeal techniques. In approximately 20% of cases, some form of adjuvant therapy is required in addition to standard surgical treatment. Investigators in the literature support the use of adjuvant therapy for patients who need more than 4 surgical procedures per year or who experience rapid regrowth of papillomas with airway compromise or distal multisite spread of disease.

The most commonly used adjuvant therapy is injection of cidofovir (Vistide) to the site of papilloma disease, but its use remains controversial. Animal studies have demonstrated a high level of carcinogenic effects of cidofovir, and case reports have documented progressive dysplasia in patients treated with cidofovir. In a canine model, intralaryngeal cidofovir administration led to dose-dependent irreversible scarring and atrophy of the vocal fold muscle at high doses. Given these concerns, the Multi-Disciplinary Task Force on Recurrent Respiratory Papillomas has published guidelines for the treatment of RRP with cidofovir, which include extended patient counseling regarding the risks of treatment.

Interest has grown recently in bevacizumab (Avastin) for the treatment of RRP. Bevacizumab is a monoclonal antibody that binds to and inhibits vascular endothelial growth factor. Research has found that vascular endothelial growth factor receptors exist in laryngeal papilloma specimens. Clinical reports have also demonstrated early success with using bevacizumab injection as an adjuvant therapy in RRP. In fact, coupling the antiangiogenesis agent bevacizumab with 532-nm pulsed potassium titanyl phosphate (KTP) laser photocoagulation has been proposed to be synergistic. To date, no complications of intralaryngeal bevacizumab have been reported. No study into the possible effects of bevacizumab on the vocal fold has been performed. In light of the increasing use of bevacizumab and the common use of cidofovir for RRP, we sought to investigate the histologic effects of cidofovir and bevacizumab injections into the vocal folds at different predetermined doses and combinations in a porcine model.

### Methods

Our institutional animal research committee approved this study, and institutional guidelines regarding animal experimentation were followed. We used a porcine model because the porcine vocal folds closely resemble those of humans and have proved to be amenable to laryngeal instrumentation and evaluation without significant difficulty.

A total of eighteen 1-year-old pigs (Sus scrofa domesticus, Yorkshire crossbreed) were studied. The pigs were randomly assigned to a treatment group consisting of cidofovir only, bevacizumab only, or combined cidofovir and bevacizumab (Table 1), with 6 pigs per group. To investigate different injection strengths, we chose to use 2.5- and 5.0-mg doses of each drug because they are representative of what is commonly used to treat pediatric RRP at the time of this study. Treatment with higher doses (7.5-88.0 mg) of bevacizumab have been reported but in adult patients only. Half of each treatment group was given 2.5-mg injections of the investigational treatment, and the other half was given 5.0-mg injections of the investigational treatment. The investigational treatments were diluted so that each would be delivered in 0.5-ml aliquots. Each pig served as its own control with an injection of 0.5 ml of saline into the left true vocal fold. All cidofovir and/or bevacizumab injections were performed on the

### Table 1. Outline of Injection and Harvest Protocol

<table>
<thead>
<tr>
<th>Time</th>
<th>Cidofovir, 2.5 mg</th>
<th>Cidofovir, 5 mg</th>
<th>Bevacizumab, 2.5 mg</th>
<th>Bevacizumab, 5 mg</th>
<th>Cidofovir, 2.5 mg, and Bevacizumab, 2.5 mg</th>
<th>Cidofovir, 5 mg, and Bevacizumab, 5 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection</td>
<td>At baseline</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>At 2 wk</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>At 4 wk</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>At 6 wk</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Harvest</td>
<td>At 8 wk</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>At 22 wk</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
right vocal fold. All pigs received a series of 4 injections at 2-week intervals.

Before each injection, the pigs underwent general anesthesia using isoflurane. Direct laryngoscopy was performed with a rigid 0° endoscope attached to a high-definition camera. Exposure was provided with an extended Miller laryngoscope blade. A Bruening syringe with a 27-gauge needle was used to deliver the injections into the subepithelial layer of the midmembranous true vocal fold. The appearance of the larynx was recorded (Figure 1).

Two weeks after the last injection, 1 pig from each treatment subgroup was killed humanely per the established protocol. The remaining pigs were observed for 4 months and then killed humanely. All larynges were harvested (Figure 2), fixed in formaldehyde solution, decalcified using EDTA, coronally sectioned every 0.5 cm from the anterior commissure to the vocal process of the arytenoids, and embedded in paraffin blocks. Microscopic sections 5 μm thick were then cut from the middle areas of each vocal fold and stained with hematoxylin-eosin and trichrome for histologic analysis using routine methods (Figure 3). The sections were reviewed separately by 1 board-certified veterinary pathologist (A.B.) and 2 board-certified head and neck pathologists (M.P., J.K.). The pathologists were all blinded to the study. Each specimen was analyzed for the presence of inflammation in the epithelium and lamina propria and edema, atypia, and fibrosis. The presence of atypia at 2 or 4 months after the last injection would not necessarily presume carcinogenicity.

Results
All animals tolerated the vocal fold injections without complication. All animals received the full course of 4 injections during a 6-week period. During the treatment schedule, no gross changes to the vocal folds were noted by the operating surgeons. The histologic sections were evaluated and scored on a severity system ranging from 0 to 5, where 0 indicates none; 1, minimal; 2, mild; 3, moderate; 4, marked; and 5, severe. The mean for the 3 different pathologists’ scores was calculated for each variable. A relatively low level of inflammation was present in all slides within the epithelium and lamina propria and for edema, atypia, and fibrosis. The inflammation primarily consisted of lymphocytes with fewer neutrophils, eosinophils, and macrophages present in a multifocal manner (original magnification ×10).

The pig larynx has a larger thyroid cartilage and cricothyroid muscle and a longer vocal fold than that of humans. However, the porcine vocal folds most closely resemble those of humans with regard to the density of collagen and elastic fibers, elastic properties, thickness, and acoustic properties.

This vocal fold was injected with bevacizumab and had a mean pathologic grade of 1.83 for epithelial inflammation, 2.25 for superficial lamina propria inflammation, 0.33 for edema, and 0.00 for atypia and fibrosis. The inflammation primarily consisted of lymphocytes with fewer neutrophils, eosinophils, and macrophages present in a multifocal manner (original magnification ×10).

Effect of Cidofovir and Bevacizumab on Vocal Folds

Figure 1. Endoscopic Image of Injection of the Right Porcine Vocal Cord

Exposure was provided with an extended Miller laryngoscope blade, and injection was delivered via a Bruening syringe.

Figure 2. Total Laryngectomy Porcine Specimen
The pig larynx has a larger thyroid cartilage and cricothyroid muscle and a longer vocal fold than that of humans. However, the porcine vocal folds most closely resemble those of humans with regard to the density of collagen and elastic fibers, elastic properties, thickness, and acoustic properties.

Figure 3. Hematoxylin-Eosin Stain of Right Vocal Fold
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slides. The group mean histologic score with separation by treatment subset is presented in Table 2. The mean histologic score with the animals separated into groups of early (8 weeks) vs late (22 weeks) laryngeal harvest is presented in Table 3. No appreciable histologic differences or trends were found between the different treatment groups and their controls. Likewise, no appreciable differences or trends were found when the pigs were separated by interval to laryngeal harvest.

We also calculated the percentage of agreement among the 3 different pathologists using commercially available statistical software (SPSS Statistics, version 17; IBM). In general, the agreement among the 3 different raters was consistent. Overall agreement for each slide ranged from 43% to 69% in the inflammation categories and 14% to 54% for level of edema. The pathologists had a high level of agreement in the categories of atypia and fibrosis, at 85% and 99%, respectively.

### Discussion

Recurrent respiratory papillomatosis is a challenging disease to treat. The search for adjuvant treatments, such as cidofovir and bevacizumab, is inspiring but often tempered with the concern for safety and interpretation of results based on limited case series. With the recent interest in bevacizumab injections, our study sought to analyze the histologic effects that these injections could have on the vocal fold, similar to analyses already performed for cidofovir. We conducted serial injections of bevacizumab and cidofovir using a dosing scheme common to that reported in the pediatric RRP literature. After histologic analysis, we found no evidence to suggest that these injections cause more inflammation, edema, atypia, or fibrosis compared with saline injections.

In a different study that used a canine animal model, high-dose cidofovir injections (20.0 and 37.5 mg/dose) caused full-thickness damage to the thyroarytenoid muscle. Lower doses were found to be safe and caused only temporary endomysial edema. Our study examined histologic findings in the vocal folds in a similar way but used varying bevacizumab and cidofovir doses. This porcine model and the canine model used by Chhetri et al seem to agree that low-dose cidofovir does not cause scarring or atypia and results only in mild inflammation that we did not find to be any different from inflammation after saline injections.

One possible weakness in this study was the number of pigs used (n = 18), which limited the analysis to descriptive statistics. However, the number is acceptable for animal studies, which commonly are restricted in number secondary to resources available. For comparison, the canine study used only 6 beagles. Also, our study was conducted during a shorter period (22 weeks) compared with the canine study (12 months). As a whole, the levels of inflammation and fibrosis did not change among the groups when compared by time of laryngeal harvest. However, the mean levels of edema and atypia slightly decreased (from 1.25 to 0.26 and 0.17 to 0.01, respectively) when the animals were observed for a longer period. No fibrosis or scarring was found in any of the vocal folds. No available study documents the length of time necessary to see scarring after these kinds of injections in pig larynges. However, given that no changes were seen among the different groups, we believe that scarring was unlikely to occur even if we had a longer observation period.

The most significant weakness in our study remains that it was not a human trial. In gross comparison, the pig vocal fold has been shown to be longer (21 mm) than that of humans (15 mm), and the pig larynx demonstrates a larger thyroid cartilage and cricothyroid muscle (Figures 1 and 2). Also, pigs and dogs have a 2-layered lamina propria structure unlike the 3-layered structure in humans. However, the porcine vocal folds most closely resemble those of humans with regard to the density of collagen and elastic fibers, elastic properties, and thickness. They have also been shown to have a high range of phonation frequencies, making them good candidates for animal studies. Acoustic analysis studies of the natural phonation of animals have found that the fundamental frequency and range of phonation in pigs are closest to those of humans.

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Table 2. Mean Pathologic Grades for Each Treatment Group

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Epithelial Inflammation</th>
<th>SLP Inflammation</th>
<th>Edema</th>
<th>Atypia</th>
<th>Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bevacizumab, 2.5 mg</td>
<td>0.94</td>
<td>1.64</td>
<td>0.72</td>
<td>0.06</td>
<td>0</td>
</tr>
<tr>
<td>Bevacizumab, 5.0 mg</td>
<td>1.56</td>
<td>1.95</td>
<td>0.67</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cidofovir 2.5 mg</td>
<td>1.28</td>
<td>1.56</td>
<td>0.11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cidofovir 5.0 mg</td>
<td>1.06</td>
<td>1.67</td>
<td>0.61</td>
<td>0.22</td>
<td>0</td>
</tr>
<tr>
<td>Bevacizumab, 2.5 mg, and cidofovir, 2.5 mg</td>
<td>1.00</td>
<td>1.80</td>
<td>0.50</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Bevacizumab, 5.0 mg, and cidofovir, 5.0 mg</td>
<td>1.50</td>
<td>1.80</td>
<td>0.50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Saline</td>
<td>1.29</td>
<td>1.74</td>
<td>0.56</td>
<td>0.03</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3. Mean Pathologic Grades for Animals When Grouped by Early vs Late Harvest

<table>
<thead>
<tr>
<th>Time of Harvest</th>
<th>Epithelial Inflammation</th>
<th>SLP Inflammation</th>
<th>Edema</th>
<th>Atypia</th>
<th>Fibrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early (8 wk)</td>
<td>1.08</td>
<td>1.78</td>
<td>1.25</td>
<td>0.17</td>
<td>0</td>
</tr>
<tr>
<td>Late (22 wk)</td>
<td>1.25</td>
<td>1.67</td>
<td>0.26</td>
<td>0.01</td>
<td>0</td>
</tr>
</tbody>
</table>
Conclusions

Based on the prior study by Chhetri et al 9 and the low doses used in our study, we did not expect to see significant changes in the cidofovir group. We had no evidence on which to base such a hypothesis with bevacizumab injections or from which to project how the outcome might change when bevacizumab was combined with cidofovir. Our results clearly document that, at these doses, cidofovir and bevacizumab did not cause significant histologic changes compared with saline injections. Future studies may build on this model to test higher dosages and/or may combine injections with KTP laser therapy because these treatments have demonstrated clinical effectiveness without notable complications. 5, 12 The effects of such treatments on the microstructure of the vocal fold will remain uncertain until a histologic analysis is performed.

REFERENCES