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Experimental Animal Model for Assessment of Tracheal Epithelium Regeneration

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Objectives/Hypothesis: To develop an experimental model in rabbits for assessment of tracheal epithelium regeneration through application of either natural or artificial polymer scaffolds.

Study Design: First, we identified the size of full-thickness mucosal defect, which does not allow self-healing (a "critical defect"), thus representing an adequate experimental model for regenerative therapy of tracheal epithelium damage. Then, two methods of polymer scaffold fixation at the site of the epithelium defect were compared: suturing and fixation with a stent. This was done through: 1) formation of a full-thickness anterolateral mucosal defect by tracheal mucosa excision; and 2) fixation of the scaffold at the site of the tracheal epithelium defect using sutures (through a tracheal wall “window”) or a vascular stent (through a small tracheal incision).

Results: The dimension of a critical anterolateral mucosal defect of the trachea for rabbits was found to be 1.5 cm in length and more than 50% of the tracheal circumference. Fixation of the scaffold with a stent proved to be more efficient due to a uniform distribution of the pressure over the entire surface of the scaffold, whereas the suturing of the scaffold provided unsatisfactory results. In addition, fixation of the scaffold by suturing required formation of a large “window” in the tracheal wall. Thus, using the stent appeared to be technically less complicated and much less traumatic as compared to suturing.

Conclusion: We present an experimental in vivo animal model of tracheal epithelium injury and recovery. It can be effectively used with certain further modifications as a basis for routine testing of bioengineered constructs.

Key Words: Animal model, regeneration, scaffold, tracheal epithelium.

Level of Evidence: NA

INTRODUCTION

Tracheal stenosis is a significant clinical problem. One of the main causes leading to the development of this pathology is full-thickness damage to the tracheal epithelium due to inflammation, trauma, surgical intervention, or tumors. One potential way to solve the problem is by covering full-thickness mucosal defects with epithelial equivalents comprised of either natural or artificial polymer scaffolds impregnated with autologous cells. Such therapeutically sound equivalents of the respiratory epithelium on polymer scaffolds still do not exist. One of the primary reasons for this circumstance is the lack of adequate scaffolds combining both specific mechanical properties and the ability to support growth and differentiation of the respiratory epithelium. In turn, the search for the most efficient composition and structure of the scaffold requires an adequate experimental in vivo model of tracheal epithelium damage and repair. Such a model would make it possible to test a series of constructs differing in composition and properties. Currently, an animal model meeting the needs of respiratory epithelium bioengineering is not available, which hampers further progress in the field. Thus far, only a few studies have been aimed at the experimental repair of tracheal defects.1,2 In contrast, a significant number of publications have been devoted to experimental animal models for entire trachea replacement (for example, references 3 and 4). Unfortunately, these models are not useful for investigation of the epithelialization processes because they usually lack elements of a vascular system and of a normal cartilaginous framework, key factors in the development and repair of the respiratory epithelium.

The aim of the work was to identify the size of a critical (not self-healing) anterolateral full-thickness mucosal defect in rabbit tracheal epithelium, and then to find a minimally invasive method of fixing the polymer matrix to the damaged area. Two methods of polymer scaffold fixation at the site of the epithelial defect have been compared: scaffold suturing to the edges of the defect and its

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fixation by a vascular stent. The later approach was found to be more efficient and much less traumatic.

The model of rabbit tracheal epithelium damage and defect dressing with polymer scaffold described here is potentially applicable to study the process of tracheal regeneration and to test different approaches to its stimulation, for example, by covering the defect with various dressing materials or with bioengineered epithelial equivalents.

MATERIALS AND METHODS

Animals, Materials, and Experimental Design

All experiments were performed according to the main ethical principles of biomedical experiments on animals, as well as the rules of the Sechenov First Moscow State Medical University Ethics Committee.

For epithelial defect dressing, we used nonwoven scaffold based on the polylactide-chitosan copolymer or collagen sponges cross-linked with glutaraldehyde. The materials were fixed at the site of the tracheal defect, with regular stitches or with vascular stent.

Chinchilla rabbits weighing about 4.5 kg were divided into four experimental groups according to the type of surgical intervention:

1. Prevascularization group (vascularization of the surgical site through fascial flap transfer). This group was formed to evaluate the effectiveness of prevascularization in improving the blood supply to the trachea, and thus the feasibility of a two-stage approach (previously suggested by Hardillo et al.1), which comprises prevascularization of rabbit trachea by fascial flap transfer (step 1) and then creation of mucosal defect itself (step 2). Given that prevascularization did not appear to be critical to the outcome of our experiments, description of the prevascularization technique and discussion of the obtained results can be found in Supporting Figure S1 and S2 (available online).

2. The group with an anterolateral full-thickness mucosal defect without scaffold application. Because tracheal diameter in rabbits is variable, it was essential to find out the optimal size of the defect. To accomplish that, 1.5 cm-long defects varying in width from 1 cm to 1.4 cm were created on the anterolateral wall of the trachea using an ophthalmologic scalpel (Fig. 1). The width limits of the defect were chosen by taking into account the balance between the defect's efficacy (inability to self-heal) and its morbidity.

The surgery was performed under artificial lung ventilation (following intubation) using a Murphy ID2 type neonatal intubation tube (Apex Medical, Malvern, PA), which was manually inserted into the trachea. An infant Ambu bag (Apex Medical, Malvern, PA) was used for oxygen supply. The outline of the anterolateral full-thickness mucosal defect is shown in Figure 1. Macroscopic (at autopsy) and histological assessment of the defect site was performed 2 to 3 weeks after the injury using paraffin-fixed sections.

3. The group with the scaffold fixed at the site of the tracheal epithelium defect by suturing. Because the rabbit trachea is quite narrow, a transverse incision does not provide adequate access to the lumen of the trachea.

Fig. 1. Creation of anterolateral full-thickness mucosal defects in the trachea. (A) A 1.5 cm-long incision is made along the midline of the neck to expose the lower part of the cervical region of the trachea. The trachea is cut transversely (half of its circumference: black arrows). (B) The defects, varying in width from 1 cm to 1.4 cm, are created on the anterolateral wall of the trachea. (C) A hemostasis is performed (without electrocautery). The trachea is sutured with regular U-shaped stitches using 7/0 polypropylene monofilament sutures. Lastly, hemostasis, wound disinfection, and closure are performed, followed by aseptic dressing application. (D) A scheme of defect creation. The full-thickness mucosal defect is indicated by the shaded rectangle. All defects are 1.5 cm long but varying in width to yield noncritical (self-healing) or critical damage. The drawing of the instrument and black arrow show how the epithelial defect was created.
for scaffold fixation by suturing. Therefore, a “window” was formed in the tracheal wall (Fig. 2), and an anterolateral full-thickness mucosal defect was created as described above. The nonwoven polymeric scaffold was fixed to the mucous membrane and to underlying cartilage along its edge by stitching.

4. The group with the scaffold fixed at the site of the tracheal epithelium defect using a stent.

In contrast with the previous group, the lumen of the trachea was accessed through a partial transverse incision (Fig. 3). An anterolateral full-thickness mucosal defect was created, and the collagen sponge scaffold was fixed at the site of the defect using a vascular stent.

Anesthetic Management of the Surgical Interventions and Postoperative Care

All surgeries were performed using artificial lung ventilation. Anesthesia was carried out using a combination of Zoletil (Virbac, Carros, France)/xylazine. The first dose of Zoletil (Virbac)/xylazine was administered intramuscularly, with subsequent doses administered intravenously.

In the postoperative period, special attention was paid to the prevention and treatment of such complications as pulmonary edema, caused by extensive damage to the mucous membrane of the trachea; infectious processes, treated by antibiotics; and atony of the intestine, often observed in rabbits after surgical interventions. Sterile dressings were changed every day for 7 to 10 days and then removed.

RESULTS

1. The group with an anterolateral full-thickness mucosal defect in the trachea and no scaffold (N = 2 + 3). This group was formed to assess the optimal dimensions of a viable defect, which cannot heal by itself, thus requiring regenerative therapy.

First, a 1.5 cm long defect less than 50% of the tracheal circumference in width (between 1.0 and 1.4 cm, depending on trachea size) was created in two rabbits (N = 2). After surgery, fine rales were auscultated in the tracheal region, whereas no other complications were observed. Two weeks after surgery, histological evaluation of the tracheas in both animals showed a slight but noticeable narrowing at the level of the defect. The granulation tissue and a thin newly formed epithelial layer were seen. The islands or newly forming submucosa with apparently normal structure were also visible (Fig. 4A), suggesting self-regeneration of the defect.

In another subgroup (N = 3), a defect of the same length (1.5 cm) was made wider, more than 50% of the tracheal circumference (between 1.0 and 1.4 cm, depending on trachea size). All rabbits in this group died within 3 to 4 days after surgery due to aspiration of a large amount of thick exudate formed at the site of the defect. The tracheas of all three animals showed similar anatomical and histological patterns. Autopsy demonstrated that the site of the mucosal defect was edematous and the...
The lumen of the trachea and large bronchi was almost completely obstructed by a large amount of thick, milky mucus. Histologically (Fig. 4B), the zone of the defect appeared quite abnormal as compared to the intact trachea (Fig. 4C). It contained large amounts of detritus consisting of inflammatory infiltrate and erythrocytes. No reepithelization or formation of submucosa was evident, suggesting inability of the defects to heal. Thus, at a given length (1.5 cm), the width of the defect of more than 50% of the tracheal circumference appears to be critical (not self-healing), and thus it can be used as a model for regenerative therapy experiments.

2. The group with the scaffold fixed at the site of the tracheal epithelium defect by suturing (N = 2). This group
was formed to assess the efficiency of fixing the scaffold inside the trachea using sutures. Given the small diameter of the rabbit trachea, a transverse incision does not provide adequate access to the tracheal lumen for scaffold suturing. Therefore, this approach requires to form a “window” in the tracheal wall (Fig. 2).

One of the rabbits in this group died on the first day after surgery, most likely due to excessive damage to the respiratory tract. An autopsy revealed significant edema of the lungs and the entire airway. The second rabbit was euthanized on the 20th day after surgery. The autopsy demonstrated a patent, albeit slightly narrowed, tracheal lumen with a moderate amount of mucus. Histological analysis showed that the scaffold was decomposed and densely infiltrated with inflammatory cells (Fig. 5A). Between the scaffold and the trachea, there was abundant exudate containing inflammatory cells and erythrocytes. Below this exudate, a newly formed submucosal layer was seen sometimes; however, epithelial growth was hindered due to a gap between the infiltrated scaffold and the trachea (Fig. 5B). Nevertheless, at the edges of the defect, the migration of undifferentiated epithelial cells along the surface of the scaffold (facing the lumen) was seen occasionally. We assume that the scaffold failed to promote healing because it was only loosely attached to the tracheal surface. The traumatism of the suturing procedure may have also created unfavorable conditions for optimal healing. Based on these results, the need for a less traumatic and more effective method of scaffold fixation appeared to be evident.

3. The group with the scaffold fixed at the site of the tracheal epithelium defect using a stent (N = 2). The use of the stent requires a much smaller opening of the tracheal wall compared to the approach involving scaffold suturing, making it far less traumatic. The duration and technical difficulty of the surgery are also significantly reduced. Most importantly, however, the use of the stent provides a much tighter and secure attachment of the scaffold to the trachea. During the first 2 days after surgery, mild dry rales were auscultated over the trachea region, whereas no other complications were observed. At autopsy, no pronounced hematoma or edema was observed at the site of the defect (in contrast to the groups with sutured scaffolds). Histological analysis showed that the tracheal lumens were not narrowed. At the same time, multiple foci with ongoing regeneration of the submucosal layer with apparently normal structure were observed with visible regeneration of the epithelium (although not normally differentiated) from the edges of the defect and along the scaffold/trachea interface (Fig. 5C). At the same time, at autopsy some fragments of the scaffold appeared to be damaged by stent pressure and were detached during stent removal. Based on these findings, the sponge scaffolds do not

![Fig. 5. Histological analysis (hematoxylin & eosin): the groups with fixed scaffolds. (A, B) The sutured SC inside the trachea is densely infiltrated with inflammatory cells. (A) In occasional spots (e.g., close to stitches), a scaffold is closely attached to the defect and becomes partially populated by migrating cells or infiltrated by inflammatory cells. (B) Nevertheless, in most places a gap (G) between the scaffold and the trachea is observed. At the edges of the wound (dashed line), a newly formed submucosal layer can be seen, covered with uec migrating from intact r-ep. (C) The stent-fixed SC inside the trachea. Under the scaffold, nearly normal vascularized sm is formed. There is no pronounced hematoma or edema at the site of the defect (compared to the sutured scaffold group). Regenerating ep is evident, although it is not yet differentiated. Scale bars: 200 μm. cr = cartilage; ep = epithelium; r-ep = respiratory epithelium; SC = scaffold; sm = submucosal layer; uec = undifferentiated epithelial cells; v = blood vessel.](image-url)
DISCUSSION

Dimensions of the Critical Defect for Tracheal Epithelium Regeneration Research

To obtain an adequate experimental model for regenerative therapy of tracheal epithelium, a “critical” (not self-healing) defect must be created. Two major factors determine its critical nature: the depth and the area of the defect. According to previous research, tracheal lumen stenosis occurs only if the tracheal damage extends deep into the perichondrium and cartilage.5,6 However, Hardillo et al.1 demonstrated that tracheal stenosis can be induced by damaging the epithelium and the submucosal tissue only, without the involvement of the cartilaginous framework. Our results confirmed these findings.

Experimental re-epithelization of the trachea using tracheal substitutes in small laboratory rodents (particularly rats) is usually not associated with any complications.7 However, application of such methods of regenerative therapy in large animal models or clinical practice often results in serious problems, for example.3,8,9 These differences in epithelialization of tracheal substitutes in vivo are likely linked to the dimensions of the trachea and thus to the area of the defect in relation to its diameter. The rates of cell migration over the defect are sufficient to ensure re-epithelization only in animals with small tracheas. Therefore, we assume that experimental animals the size of rabbits or bigger are much more suitable for tracheal defect modeling. The question of the dimensions of a critical (not self-healing) tracheal defect, however, remains open. According to our data, for rabbits the defect of 1.5 cm in length and slightly more than 50% of the tracheal circumference in width is optimal to achieve the balance between animal survival and appropriate severity of the defect. An epithelial defect of this size does not heal spontaneously and is suitable to be used as a model to study tracheal epithelium regeneration in rabbits. For larger animals, the area of a critical epithelial defect should be determined experimentally.

Fixation of the Scaffold to the Site of the Tracheal Defect

In this study, we attempted to attach a scaffold to the site of the defect using two different approaches. The first method, involving suturing the scaffold along its edges to the mucosa at the defect site, yielded unsatisfactory results. It appeared to be extremely difficult to ensure the attachment of the construct to the cartilaginous framework over the entire area of the defect, regardless of the mechanical properties of the scaffold. In all cases, the scaffold was displaced from its initial position due to local edema and exudate at the site of injury. Of note, the incisions in the tracheal wall (for the formation of a defect and scaffold application) did not appear to be detrimental to the tracheal blood supply because the well-developed vascular network of the cartilaginous wall and submucosa was able to sustain the damage and provide sufficient blood supply to the edges of the defect.

The second method (fixation of the scaffold using a stent) proved to be more efficient due to an even distribution of the pressure of the stent over the entire scaffold surface. As a result, the scaffold was tightly fixed to the site of injury. In addition, this method of fixation is technically less complicated and allows for covering larger circumferential defects. Scaffold fixation with a stent can be performed through a minimally invasive endoscopic approach, thus being much less traumatic as compared to suturing with even lower effects upon trachea blood supply. This approach is potentially applicable not only to the trachea but to other hollow tubular organs as well.

Despite the obvious advantages of this approach, currently there are no stents designed specifically for this purpose. Designing a stent suitable for fixation of bioengineered materials to the inner surface of the trachea does not seem to be an easy task. Based on our experience, the major requirements for such stents are the following: 1) They should closely match the diameter of the trachea in order to prevent excessive compression of the construct itself and the tracheal wall, leading to tissue ischemia and necrosis (excessive pressure may also cause cartilage atrophy); 2) the stents should possess sufficient elasticity and strength to fix the scaffold securely from the inside to the tracheal wall; 3) they should be easily and safely removable at the end of the experiment when mechanical fixation is no longer necessary (alternatively, the stents can be biodegradable, with the rates of their biodegradation adjusted to the length of the experiment); and 4) the stents should be fully biocompatible and hypoallergenic.

There are specific requirements for effective scaffolds as well. They should be strong enough not to collapse under the pressure of the stent and have a structure that is sufficiently resilient but at the same time sufficiently flexible to ensure easy manipulation in limited space.

Both the stent and the scaffold should possess antibacterial properties because the lumen of the trachea is a high-risk environment in terms of infection.

CONCLUSION

Over the course of the study, we developed an experimental in vivo animal model of tracheal epithelium injury and recovery. The assessment of this model demonstrated that, with certain further modifications, it could be effectively used as a foundation for routine testing of bioengineered constructs of different composition and properties aimed at tracheal epithelium regeneration. This model would also help to address some basic questions of respiratory epithelium physiology.

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BIBLIOGRAPHY