Tracheal Reconstruction

Mucosal Survival on Porous Titanium

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Objective: To investigate whether porous titanium can provide a better support for revascularization of a mucosal graft ideal for tracheal reconstruction. In patients with laryngotracheal stenosis or tumor, the mucosa with supporting structures can be damaged, resulting in a defect that has to be reconstructed. Autologous tissues like cartilage and mucosa have been used for reconstruction. The main problem has been incomplete mucosal reepithelialization.

Design: In the first experiment, porous titanium or ear cartilage was combined with mucosa and implanted subcutaneously in athymic mice for different periods of time. In the second experiment, using rabbits, surgically created defects were reconstructed with porous titanium and mucosa on a pedicled fascia flap using a 2-stage procedure. The implants were analyzed with emphasis on angiogenesis and mucosal survival.

Subjects: Male New Zealand white rabbits and nude athymic mice (BALB-c nu/nu).

Results: Normal mucosa having a submucosal layer with vital cells was noted on top of the titanium. Multiple blood vessels were observed extending from the muscle layer through the titanium. Cytokeratin expression was detected in the suprabasal and basal layers of the mucosal epithelium. In contrast, the mucosa on cartilage showed no vital cells and no cytokeratin expression. In the rabbit experiment, all animals survived the reconstruction. The titanium was well integrated to the adjacent tracheal cartilage and surrounding tissues, supporting a fully vital mucosa.

Conclusions: Porous titanium is an inert biomaterial that provides support and allows easy revascularization of a mucosal graft. Titanium, in combination with viable autologous tissues, is a good alternative for tracheal reconstruction.


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cosa have been used in experimental settings. The main problem in all of these studies is the regeneration of the mucosal lining, as mentioned in a review by Tan et al. In cases of minor defects, reepithelialization from the wound edges occurred, but for larger defects this process of reepithelialization proved to be insufficient. Previous attempts to improve revascularization of mucosal grafts using perforated cartilage proved to be futile because perforated cartilage lacked the necessary support to keep the tracheal lumen open. A possible solution to this problem is the use of stable porous biomaterials, which can allow new vessels to grow through the pores and thereby ensure its survival. In addition, the biomaterial of choice should be strong enough to keep the airway lumen open, and most important, the material should not evoke a foreign body reaction or inflammation.

These requirements are met by titanium, a material that has been used for years as an implantation material in both orthopedics and maxillofacial reconstructive surgery. Titanium is chemically resistant to the corrosive and oxidative activities of various agents. It has also been shown to be very well tolerated by animal and human tissues, making it a suitable material for tracheal reconstruction.

We investigated whether human and rabbit buccal mucosa will survive on porous titanium in an athymic mouse model and in a rabbit tracheal reconstruction model. In the athymic mouse model experiment, we determined whether angiogenesis will occur through the porous titanium, allowing graft survival in comparison with cartilage grafts. In the rabbit tracheal reconstruction model experiment, we studied whether a construct of porous titanium and mucosa would be tolerated inside the trachea.

HUMAN MUCOSA EXPERIMENT IN ATHYMIC MICE

Human buccal mucosa (10 × 5 mm) of 2 patients undergoing an intraoral surgical procedure was harvested and used within 1 hour (with approval of the local medical ethics committee (animal protocol No. 232.907/2003/196). The mucosa was split into 2 pieces 3 × 5 mm in size, and each piece was placed on the center of a 10 × 2-mm porous titanium plate and fixed with fibrin glue (Tissucol; Baxter, Utrecht, the Netherlands). To avoid any host-graft rejection, the titanium was sutured on the back muscle of athymic mice (BALB-c nu/nu; Harlan, Horst, the Netherlands) with approval of the local animal ethics committee (animal protocol No. 126-03-01). The mucosa was covered with Neuro-Patch (Braun Aesculap AG & Co KG, Tuttingen, Germany) to prevent adhesion of the mucosa to the inner side of the skin and to limit revascularization of the mucosa from the surrounding tissues aside from the back muscle. The mucosa from 1 patient was implanted for 1 week, and the mucosa from the other patient was implanted for 2 weeks. Two mice were used, each animal carrying 2 pieces of titanium. The mice were killed after 1 and 2 weeks, respectively, and the titanium plates were removed, including a piece of back muscle. These were embedded in plastic for histologic analysis.

RABBIT MUCOSA EXPERIMENT IN ATHYMIC MICE

Rabbit buccal mucosa (20 × 10 mm) was harvested from dead rabbits (acquired from another experiment) within 1 hour after euthanasia. The mucosa was split into 2 pieces 10 × 10 mm in size and then sutured (with Ethilon 6-0; Johnson & Johnson, Brussels, Belgium) on porous titanium plates or rabbit ear cartilage (from the same dead rabbits) of 10 × 10 mm (Figure 1A). The constructs were sutured subcutaneously on the back of athymic mice with approval of the local animal ethics committee (animal protocol No. 126-03-01). The mucosa was covered with Neuro-Patch to prevent adhesion of the mucosa to the inner side of the skin and to limit revascularization from the surrounding tissues aside from the back muscle. Each mouse carried a titanium construct and a cartilage construct on the back (Figure 2). Both constructs were covered with mucosa from the same rabbit. A total of 3 mice were used. The mice were killed after 1, 2, and 4 weeks, respectively. Half of the mucosa was dissected carefully from the titanium or the cartilage for cytokeratin (CK) staining to evaluate the basal layer and vital keratinocytes of the epithelium. The rest of the mucosa still attached to the graft was embedded either in plastic for the titanium constructs or in paraffin for the cartilage grafts for histologic analysis with emphasis on angiogenesis and mucosal survival.

METHODS

POROUS TITANIUM PLATES

Porous titanium implants were produced by the polymeric sponge replication method. Titanium powders (75% by weight) with a spherical shape and mean diameter of 45 µm (Bongen Titanium Co Ltd, Xi’an, China) were mixed with water (18.5% by weight) to make a titanium slurry. Polyethylene glycol 4000 (Fluka Chemie GmbH, Delsendorf, Germany) and methylcellulose (Fisher Scientific B.V., Landsmeer, the Netherlands) were used as binders (3.5% by weight). One percent by weight of alkali-free carbonic acid–based polyelectrolyte, Dolapix (Aschimmer & Schwarz GmbH, Rhein, Germany), as dispersant; 1% by weight of ammonia solution (25% by weight, Merck, Darmstadt, Germany), and 1% by weight of 1-octanol (Acros Organics, Pittsburgh, Pennsylvania) were mixed in to improve the rheological property of the slurry. Porous titanium green bodies were made by impregnation of polymeric sponges (Coligen Europe B.V., Breda, the Netherlands). The polymeric sponges were thoroughly dipped into the titanium slurry. This dipping process was repeated until all the struts of the polymeric sponge were covered with slurry. Excess slurry was removed by using a roller-pressing device. After drying, the samples were heated to 500°C in argon to burn out the foam. Finally, the porous bodies, with pores ranging from 400 to 700 µm, were sintered in a vacuum furnace (10−3 mbar) at 1250°C for 2 hours. Porous tracheal implants with a porosity of 90% were sliced using a wire electric discharge machine (Figure 1).
TRACHEAL RECONSTRUCTION EXPERIMENT IN RABBITS

Three male New Zealand white rabbits (each weighing 3 kg) were used in a 2-stage procedure with approval of the local animal ethics committee (animal protocol No. 126-03-01). In the first stage, the rabbits were anesthetized intramuscularly with 4 cm³ of xylazine hydrochloride, 2% (Rompun; Brussels, Belgium), and ketamine hydrochloride, 10% (Ketalin; Apharmo, Duiven, the Netherlands). The left thoracic skin was incised, and a concave-shaped titanium plate 20 x 10 x 2 mm in size, together with a section of harvested full-thickness buccal mucosa 20 x 5 mm in size, was sutured with Ethilon 6-0 on the left lateral thoracic fascia flap. The titanium was covered with Neuro-Patch to prevent adhesion of the mucosa to the skin. The skin was closed in 1 layer using Vicryl 2-0 (Ethicon).

In the second stage, 2 weeks after implantation, the skin was incised, and the left thoracic fascia flap was isolated on the lateral thoracic vessels and rotated under the skin into the neck region of the rabbit. Using a midline incision, the anterior cervical trachea was dissected, and a defect 20 x 5 mm in size was created in the anterior cricoid and the first 3 tracheal rings. The defect was one-third of the total diameter. The mucosa/titanium attached to the fascia flap was then sutured with Ethilon 6-0 into the defect with the buccal mucosa facing the tracheal lumen. The skin was closed in 3 layers. After 6 weeks, the rabbits were killed with an overdose of sodium pentobarbital, and the tracheal/cricoid complex was dissected out. The trachea was opened posteriorly in a vertical line and photographed. The trachea was then embedded in plastic for histologic analysis.

HISTOLOGIC ANALYSES

In the human mucosa–athymic mice experiment, all pieces were embedded in plastic, and sections 50 µm in thickness were made using a diamond saw (Leica SP1600; Leica AG, Glattbrugg, Switzerland) and stained with hematoxylin-eosin.

In the rabbit mucosa–athymic mice experiment, half of the mucosa was dissected carefully from the titanium or the cartilage and was snap frozen in liquid nitrogen. Six-micron sections were cut for CK staining (see the next subsection) to evaluate the basal layer and vital keratinocytes of the epithelium. The rest of the mucosa still attached to the graft was embedded in either plastic for the titanium constructs or in paraffin for the cartilage grafts. For the titanium, 50-µm sections were made using a diamond saw (Leica SP1600). For the cartilage, 6-µm sections were made using a microtome (Leica RM 2135). Both the plastic and the paraffin sections were stained with hematoxylin-eosin. In the tracheal reconstruction experiment, 50-µm sections were made using a diamond saw (Leica SP1600) and stained with hematoxylin-eosin.

IMMUNOHISTOCHEMICAL ANALYSES IN THE ATHYMIC MICE

Cytokeratin expression of the mucosa was evaluated using antibodies against CK10 (Euro-diagnostica, Arnhem, the Netherlands), CK13 (Euro-diagnostica), CK14 (Nove Castra, Newcastle, England), and CK16 (Novocastra, Newcastle, England). CK10 and CK13 are markers for the suprabasal layers of stratified squamous epithelium.
fied mucosa, CK14 is a marker for the basal layers of stratified epithelia, and CK16 is a marker for suprabasal cells of hyperproliferative squamous epithelia. Positive controls of these CKs were performed on a piece of rabbit buccal mucosa.

All samples were fixed in formalin, processed in paraffin wax, and sectioned at a thickness of 6 µm. To stain mouse sections with a mouse antibody, the primary and secondary antibody (alkaline phosphatase conjugated goat antimouse IgG; Immunotech) had to be prelinked overnight.14 For CK10, CK14, and CK16, sections were postfixed in formaldehyde, 4%, for 10 minutes after deparaffinization, and antigen retrieval was performed by immersing sections in sodium citrate buffer, 0.01 M (pH, 6.0), and keeping them at boiling temperature for 10 minutes using a microwave oven. Cytokeratin 10 staining required antigen retrieval with trypsin, 0.075%, in phosphate-buffered saline for 30 minutes at 37°C. After retrieval, nonspecific reactions were blocked using 10% normal goat serum (Sigma-Aldrich, St Louis, Missouri) for 30 minutes followed by incubation with the prelinked antibodies for 1 hour and alkaline phosphatase (alkaline phosphatase anti-alkaline phosphatase [ASPAAP], DakoCytomation, Glostrup, Denmark) for 30 minutes. Visualization was performed with an alkaline phosphatase conjugate. Sections were counterstained with Gill hematoxylin (Sigma-Aldrich) for 1 minute. The negative control was performed using a nonspecific mouse IgG1 (DakoCytomation, X0931) also prelinked with the alkaline phosphatase conjugate goat antimouse IgG.15

RESULTS

VIABILITY OF HUMAN MUCOSAL GRAFTS ON TITANIUM

Vital mucosa was seen attached to the titanium (Figure 3A). On histologic evaluation, normal mucosa with a submucosal layer was visible with vital cells. Multiple blood vessels were observed growing from the muscle layer into the titanium in the direction of the mucosa (Figure 3B). This process was seen as early as 1 week after implantation with more pronounced vessel formation after 2 weeks.

VIABILITY AND MORPHOLOGICAL CHARACTERISTICS OF RABBIT MUCOSAL GRAFTS ON TITANIUM VS CARTILAGE

Vital buccal mucosa, adherent on the titanium, was observed in all sections (Figure 4A). The mucosa had a normal structure consisting of epithelial cells and a submucosal layer. As early as 1 week after implantation, new blood vessels were already visible, originating from the back muscle of the mouse and growing into the titanium up to the submucosal layer.

A normal basal layer was present in all 3 mice (1, 2, and 4 weeks after implantation) as shown in Figure 5. The suprabasal layers were more pronounced after 1 and 2 weeks. Cytokeratin expression was detected in the suprabasal (CK10 and CK13) and basal (CK14) layers of the mucosa on the titanium. No expression of CK16 was seen.

The mucosa on the cartilage showed varying degrees of necrosis and no revascularization. The mucosal layer was not integrated with the ear cartilage (Figure 4B). No CK expression was detected in this material.

PERFORMANCE OF TITANIUM-MUCOSAL GRAFT CONSTRUCT IN TRACHEAL RECONSTRUCTIONS

All 3 rabbits survived the 2 operations until the end of the experiment, 6 weeks after tracheal reconstruction. In 2 rabbits, the titanium was covered with mucosa and the airway lumen was open (Figure 6A and B). Granulation tissue was seen in only 1 of the suture points in 1 rabbit. The third rabbit became dyspnoeic at the end of the experiment. On dissection of the cricotracheal complex, a segment of the titanium was noted to have shifted inside the trachea.
Histologic evaluation of the mucosa and the titanium showed revascularization of the mucosa through the titanium and a vital mucosa (Figure 6C). In 1 rabbit, some granulation tissue was found around a suture, as noted on macroscopic inspection. Otherwise, no signs of inflammation or foreign body reaction were visible. The constructs were well integrated with the adjacent cartilage and soft tissues (Figure 6D).

**COMMENT**

Surgical repair of the trachea remains a therapeutic challenge. Although long-segment stenosis is a rare entity, acquired stenosis is a growing problem owing to advances in medical care that have resulted in increased numbers of critically ill patients requiring chronic mechanical ventilation.16

At present, subglottic and short-segment stenosis are mainly repaired by cartilage interposition grafts in cricotracheal split procedures and with cricotracheal resection followed by end-to-end anastomosis.17 Long-segment stenosis, involving more than half of the entire tracheal length and restenosis after an initial resection and end-to-end anastomosis, continues to be a therapeutic problem because further resection is usually not possible.

There are a couple of clinical case reports18,19 in which a neotrachea was formed using autologous costal cartilage or seeded chondrocytes in a Marlex (C. R. Bard Inc) mesh tube with palatal mucosal graft. All patients survived the procedures, but the disadvantage was the need for 2 or 3 steps, which were necessary to create the neotrachea. Another study20 described a series of 112 patients in whom preserved allografts from cadavers were used for tracheal reconstruction in long-segment stenosis. However, they reconstructed only a part of the trachea, and, moreover, the use of homografts in this time of Creutzfeld-Jacob disease and other possible diseases would not be the first choice of therapy.

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**Figure 4.** Rabbit buccal mucosa. A, Rabbit buccal mucosa on top of titanium (hematoxylin-eosin, original magnification ×25). Note the intact basal layer indicated by the black arrows. B, Rabbit buccal mucosa on top of ear cartilage (hematoxylin-eosin, original magnification ×25). In the middle of the picture the cartilage is visible with its chondrocytes (black arrows). There is no connection between the mucosa and the cartilage. The basal layer of the mucosa is not visible (white arrows).

**Figure 5.** Cytokeratin staining (CK13) on rabbit buccal mucosa after 2 weeks of implantation with a clear basal membrane (black arrows) and suprabasal keratinocytes of vital mucosa (original magnification ×100). Visualization with New Fuchsin [Chroma, König, Germany], counterstain with Gill hematoxylin [Sigma-Aldrich, St Louis, Missouri].
To improve reconstruction of the trachea, several experimental approaches have been followed in animal models. As mentioned in our introduction, autologous cartilage with laryngotracheal frameworks from solid titanium, Medpor, or hydroxyapatite\(^5,7\); tissue engineered cartilage with chondrocytes seeded in polypropylene or polyglycolic acid with or without mucosal lining\(^8-10\); and prefabricated tubes with cartilage strips and a vascularized muscle or fascia\(^21\) have all been used. They all had the same problem, that is, the restoration of the mucosal lining through reepithelialization. This occurred from the wound edges in small defects, but for larger defects this process of reepithelialization was not sufficient. The key to the problem is speeding up the process of revascularization. This can be achieved with the use of porous materials, in which new vessels can grow through the pores to reach the epithelializing surface. Porous hydroxyapatite was used in rabbits by Triglia et al.\(^22\) Their main problem was the fixation of the hydroxyapatite to the surrounding tissue. Schultz et al.\(^23\) implanted porous titanium tracheal prostheses into 17 rats. Six rats died, 5 of them because of displacement of the titanium or sealing of the prosthesis from overgrowth of granulation tissue. The titanium, however, was well tolerated by the surrounding tissue, as seen on histologic analysis.

An optimal tissue for tracheal reconstruction should have 3 different components: a support tissue to keep the lumen open, a good epithelial lining, preferably consisting of mucosa, and a vascular supply.\(^3\) The results of our study show that these criteria can be met by using porous titanium in combination with autologous tissues. Titanium is sturdy enough to keep the lumen open, and the porous matrix offers the opportunity for blood vessels to grow into the scaffold. This allows reepithelialization and survival of a mucosal graft. An angiogenic effect of titanium clips in rats has been described by Fos-
chi et al.24 They observed new blood vessels in the mesentery of rats after creating a wound that was closed with titanium clips. Titanium has also been described as an ideal biomaterial in a septic environment like the trachea, and our rabbit study confirmed this. No inflammatory response or foreign body reactions were observed after 6 weeks.

To our knowledge, this is the first study that has used porous titanium in combination with mucosal grafts, and it clearly showed histologic evidence of revascularization of the mucosal graft. After 1 week, new blood vessels were observed growing from the underlying tissue through the titanium scaffold. The presence of the Neuro-patch eliminated possible revascularization of the mucosa coming from the surrounding tissues other than the back muscle. The importance of this revascularization was also highlighted by the observation that the mucosal grafts in combination with cartilage grafts appeared nonvital.

Mucosal grafts can be fixed on the scaffold using stitches or glue. In our first study using human mucosa, human fibrin glue (Tissucol; Baxter GmbH, Utrecht, the Netherlands) was used. Glue is easy to apply, and the mucosa remained well fixed on the titanium, even 4 weeks after implantation. Stitching the mucosa might last longer, but some factors have to be taken into consideration, namely, that the tissue might be harmed by the stitches, it might partly detach and, depending on the material used, granulation tissue may form around the stitches. For the rabbit experiments, we did choose to use stitches to avoid possible reactions against the human fibrin glue. In theory, however, fibrin glue can offer an advantage by stimulating angiogenesis.25

In conclusion, our experiments showed that reconstruction of a rabbit laryngeal defect using composites of porous titanium and mucosal grafts is a promising technique. Porous titanium is an inert biomaterial that allows easy penetration of blood vessels and survival of the mucosa. The morphologic characteristics of the mucosa were preserved, indicating a functional tissue. Future experiments have to fine-tune the use of such composite grafts for clinical use, especially when larger and circular defects have to be reconstructed.

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