EVOLVING TECHNOLOGY

TRANSPLANTATION OF TRACHEAL EPITHELIAL CELLS ONTO A PREFABRICATED CAPSULE POUCH WITH FIBRIN GLUE AS A DELIVERY VEHICLE

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Objective: The purpose of this study was to investigate whether in vitro cultured tracheal epithelial cells can be transplanted onto a prefabricated capsule surface in vivo for possible use in tracheal reconstruction.

Methods: Tracheal epithelial cells from 12 donor inbred rats were harvested for culture and expansion. In 16 recipient inbred rats, 2 sterile cylinders made of silicone rubber were implanted in each rat bilaterally in the folds of both the left and right anterior rectus sheath by wrapping the sheaths around the cylinders to induce a capsule formation. Ten days later, the cell cultures were divided and suspended in 1 of 2 delivery vehicles (standard culture medium or fibrin glue) and implanted onto the capsule surface. To compare the 2 delivery vehicles, we used fibrin glue on one side and the standard culture medium on the other.

Results: After 2 (group 1, n = 8) and 4 (group 2, n = 8) weeks, histologic findings, immunohistochemical staining, and electron microscopy demonstrated the capsule to be covered with a tracheal neoeepithelium in group 1 and additional ciliated cells and secretory cells in a confluent layer in group 2 but only on the side with fibrin glue as the delivery vehicle. No viable epithelial cells were identified on the side with the standard culture medium in either group.

Conclusion: We conclude that cultured epithelial cells can be successfully transplanted onto a prefabricated capsule surface with fibrin glue, which will differentiate into morphologic, nearly normal epithelium, showing potential for tracheal reconstruction. (J Thorac Cardiovasc Surg 2001;121:1187-93)

A variety of pathologic conditions, both congenital and malignant, require tracheal reconstruction. Because the supply of tracheal tissue is limited, reconstruction with native tissue is possible only for smaller defects and thus limits surgical options. Reconstructive techniques with local flaps wrapped around a free carti-

lage graft or free-flap operations and prosthetic material replacements are associated with several problems, such as stenosis, perforations, insufficiencies of anastomoses, infection, and displacement.¹⁻⁷ These unsatisfying outcomes encourage the search for further developments in modern reconstructive techniques with cell cultures and tissue engineering.^{8,9} It has recently been shown that an intact epithelial layer might be one factor involved in limiting scar formation and subsequent stenosis,^{9,10} and thus an ideal solution would be to use a de novo prefabricated tissue that is well vascularized, transferable on a single pedicle, and lined with tracheal epithelium for tracheal reconstructions.

Tracheal epithelial cells have been successfully cultured from tissue explants to produce enough cells for reimplantation to engineer intact tracheal epithelium; however, reports in the literature address reinoculation of tracheal epithelial cell cultures without reconstructive purposes into tracheas denuded of their epithelium but with viable stroma and cartilage left intact and with stan-

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Presented at the Second BioValley Tissue Engineering Symposium, Freiburg, Germany, November 25, 1999, and the Eleventh Annual Meeting of the European Association of Plastic Surgeons, Berlin, Germany, June 3, 2000.

Project partially funded by the Austrian Society for Surgery.

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0022-5223/2001 \$35.00 + 0 12/1/113936

doi:10.1067/mtc.2001.113936

Fig 1. Implantation of silicone cylinders in folds of anterior rectus sheaths for prefabrication of capsule.

dard medium as a delivery vehicle.¹¹⁻¹³ A few investigators have reported reimplantation of isolated tracheal epithelial cells *in vivo* with limited success.^{14,15} To our knowledge, there are no reports in the literature about revascularization of *in vitro* cultured and expanded tracheal epithelial cells after reimplantation to a heterotopic recipient area. Encouraged by previous reports about successful transplantation of cultured keratinocytes suspended in fibrin glue^{16,17} and our own experiences with urothelial cell transplantation with fibrin glue as a successful vehicle,¹⁸ we compared fibrin glue versus standard medium as a delivery vehicle. We transplanted tracheal epithelial cells onto prefabricated capsule surfaces created by silicone cylinders because previous *in vivo* studies demonstrated successful results of skin grafting on induced capsular tissue.^{19,20}

Materials and methods

Animal model. Twenty-eight male, inbred Wistar Furth rats (weight, 250-300 g; age, 11-13 weeks) were obtained from Harlan Sprague Dawley Laboratories (Indianapolis, Ind) and housed in the animal facilities at Southern Illinois University School of Medicine. The animals were handled in accordance with the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and within the guidelines set forth by the American Association for Accreditation of Laboratory Animal Care. All protocols were approved by the Southern Illinois University School of Medicine Animal Care and Use Committee. Intraperitoneal sodium pentobarbital (42 mg/kg) was administered for anesthesia, with periodic supplementation (10 mg/kg) as needed. Assessment of adequate anesthesia included monitoring for increased respiratory rates and rat response to foot stimulation. All surgical procedures were done under sterile conditions by using steam-sterilized instruments with the surgeon masked and wearing a laboratory coat and sterile gloves. A

Fig 2. Confluent transplanted neoepithelium on fibrous capsule surface 2 weeks after transplantation. The epithelium is composed of cells of a more cuboidal appearance, and no ciliated cells can be detected. (H&E stain; original magnification 400 $\times$.)

total of 12 rats served as tracheal epithelial cell donors, and 16 rats were used as the experiment group. The cervical region of the donor rats was shaved with an electric razor and sterilized with povidone-iodine (Betadine). A longitudinal 2.5-cm incision was made in the anterior cervical region to expose the trachea. The trachea was excised from below the larynx to the bifurcation, resulting in a piece 20 mm in height that was used for tracheal tissue culturing. The animals were killed with a 1-mL intracardiac dose of 26% sodium pentobarbital after the trachea had been harvested.

The abdominal region of the 16 recipient rats was shaved with an electric razor and sterilized with povidone-iodine. A 4-cm incision was made in the midline of the abdomen to expose the anterior rectus sheaths. Two sterile cylinders made of silicone rubber (each 3 cm long and 4 mm in diameter, Dow Corning Corporation, Midland, Mich) were implanted in each rat bilaterally in the folds of the anterior rectus sheaths by wrapping the sheaths around the cylinders to induce a capsule formation. The pouches around the cylinders and the skin defect were closed with 4-0 nylon running sutures (Fig 1).

After 10 days, when cultures were ready, the recipient animals were reanesthetized as above, and the abdomen was reopened along the former scar. In each rat both newly formed capsule pouches were incised, and the suspensions of cultured tracheal epithelial cells were injected along the silicone tubes into the capsule cavity. To compare the 2 delivery vehicles, we suspended and transferred the cultured cells in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Life Technologies, Inc, Rockville, Md) on one side and used fibrin glue (provided by Immuno AG, Vienna, Austria) as the delivery vehicle on the other side of each rat. To randomize the recipient sides, in 8 rats DMEM was used on the left side, and fibrin glue was used on the right side; in the remaining rats DMEM was used on the right side, and fibrin glue was used on the left side. The capsules and skin were then closed with 4-0 nylon running sutures. The silicone cylinders were left inside

the pouch for the whole investigation period to promote close contact between the cells suspended in the delivery vehicles and the capsule surface. After 2 weeks, the rats from group 1 were reanesthetized, the silicone cylinders were removed, tissue samples of the capsule were taken for hematoxylin-and-eosin (H&E) immunohistochemical staining and electron microscopy, and the animals were killed. Histologic sections from the capsule pouch were taken for H&E immunohistochemical staining and electron microscopy from group 2 animals 4 weeks after tracheal cell grafting.

Cell preparations. Primary cell cultures were established from fresh tracheal tissue specimens from the donor rats. The excised trachea was washed twice in Hanks balanced saline solution (Gibco) and incubated overnight at 4°C in 0.5% protease/dispase. The epithelial cells were rinsed from the trachea with DMEM plus 10% fetal calf serum (FCS), and the cell suspension was centrifuged for 10 minutes at 1000 rpm. The cell pellet was washed in Hanks balanced saline solution. Final cell suspensions containing tracheal epithelial cells were plated in Ham's F-12/DMEM.

The portion of trachea dissected yielded 9×10^4 to 4×10^5 cells counted with a hemocytometer (Bright-line; Cambridge Instruments, Buffalo, NY). The cells obtained from each sample were plated in one T25 flask with 2 to 3×10^5 irradiated 3T3 mouse fibroblasts (ATCC No. CCL92). 3T3 cells were maintained in DMEM with 10% FCS and passaged when the cells were subconfluent by using 0.25% trypsin. For use as a feeder layer, the cells were trypsinized, resuspended in media, counted, and irradiated with 6000 rad from a ¹³⁷Cs source.

The epithelial cultures were incubated at 37°C in 5% carbon dioxide. The medium was changed 3 times a week. The primary cultures became confluent after 8 days, at which time the cells were stripped by using 0.05% trypsin/0.02% ethylenediamine tetraacetic acid solution. Quantitation of cell recovery by counting with a hemocytometer indicated that this method resulted in an average recovery of 6.5×10^6 viable cells (6×10^6 to 9×10^6) per sample of tracheal tissue ($n = 12$).

To inject the tracheal epithelial cells into each capsule pouch, 2.6×10^6 cells were resuspended in 0.7 mL of DMEM or in the 0.35-mL thrombin component of fibrin glue.

Medium and reagents. Dispase (0.5%; Boehringer Mannheim, Indianapolis, Ind) in Hanks balanced saline solution (Gibco) was used to create a single-cell suspension for cell plating. The culture medium for tracheal epithelial cells contained Ham's F-12 medium/DMEM (Gibco) in a 1:1 ratio supplemented with 10% FCS (Gibco), 10 ng/mL epidermal growth factor (Collaborative Biomedical Products, Bedford, Mass), 100 U/mL penicillin, 100 µg/mL streptomycin solution (Sigma Chemical Company, St Louis, Mo), 10 ng/mL cholera toxin (Sigma), 0.4 µg of hydrocortisone (Sigma), 5.0 µg/mL of insulin (Sigma), 5.0 µg/mL transferrin (Sigma), and 0.25 µg/mL Fungizone (Gibco). The (human) freeze-dried sealer protein concentrate (Immuno AG Vienna) contained 100 to 115 mg/mL total protein (70-110 mg/mL fibrinogen, 2-9 mg/mL plasminogen, 10-50 U/mL factor XIII, and 0.04-0.12 mg/mL plasminogen) and was dissolved with a bovine aprotinin solution (3000 KIU/mL). The

Fig 3. Four weeks after transplantation with DMEM, no epithelial cells can be detected along the fibrous capsule surface. Note mild inflammatory response. (H&E stain; original magnification, 150×.)

thrombin component (500 IU/mL) was mixed with a 40 µmol/mL calcium chloride solution. The tracheal epithelial cells were homogeneously suspended in the thrombin/calcium chloride solution, which was then mixed with the fibrinogen solution to form a clot on the capsule-pouch surface.

Histomorphologic studies. Samples were fixed with 10% formalin and routinely processed, embedded in paraffin, and cut for H&E staining. Additionally, immunohistochemical staining specific for epithelial cells was performed on paraffin-embedded tissues with AE1 and AE3 anticytokeratin monoclonal antibodies from mouse-mouse hybrid cells²¹ (Boehringer Mannheim) to detect the reimplanted tracheal epithelium on the capsule surface. The mouse-specific streptavidin biotin (LSAB) 2 Kit (DAKO Corporation, Carpinteria, Calif), in which a biotinylated secondary antibody reacts with several peroxidase-conjugated streptavidin molecules, was used for antibody detection.²²

Specimens for electron microscopy were fixed in 2% glutaraldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.2) and postfixed in 1% osmium tetroxide for 1 hour in 0.1 mol/L sodium phosphate buffer. Then these were dehydrated in a graded series of ethanol (50%-100%) followed by propylene oxide and were embedded in Epon 812 (Abbott Laboratories, North Chicago, Ill) for transmission electron microscopy. Ultrathin sections of 70 to 80 nm, cut with a diamond knife (Ultratome III 8800; LKB-Produkter AB, Bromma, Sweden), were stained with uranyl acetate and lead citrate and examined with an H-7000 electron microscope (Hitachi, Ltd, Tokyo, Japan). For scanning electron microscopy, specimens critical point-dried after dehydration in ethanol were coated with gold by using vacuum evaporation and were observed with an S-450 scanning electron microscope (Hitachi, Ltd).

Results

Consistently, a highly vascularized capsule in the shape of a cylinder within the anterior rectus sheaths was induced by the silicone tubes.

Fig 4. Re-established tracheal neoeepithelium on fibrous capsule surface 4 weeks after transplantation. (H&E stain; original magnification, 150 $\times$.)

Fig 5. H&E staining of pseudostratified respiratory neoeepithelium 4 weeks after grafting. Compare with Fig 2. (Original magnification, 400 $\times$.)

Group 1 (n = 8): Cells suspended in DMEM. None of the 8 capsule pouches had evidence of viable tracheal epithelium along the surface, as documented by staining with H&E, but revealed a mild inflammatory response consistent with a foreign-body reaction and dense fibrous tissue and vascular proliferation. Accordingly, immunohistochemical staining with AE1 and AE3 anticytokeratin antibodies could not identify any epithelial cells on the capsule surface.

Group 1 (n = 8): Cells suspended in fibrin glue. All 8 capsule pouches suspended in fibrin glue had evidence of viable epithelial cells in a confluent layer, as demonstrated by staining with H&E and AE1 and AE3 anticytokeratin antibodies. The fibrin clot in which the cultures were embedded was already dissolved. The re-established epithelium in all 8 capsule pouches comprised cells of a more cuboidal appearance, and no ciliated cells could be detected (Fig 2).

Group 2 (n = 8): Cells suspended in DMEM. No epithelial cells could be detected after 4 weeks (Fig 3).

Group 2 (n = 8): Cells suspended in fibrin glue. Four weeks after transplantation, staining with H&E and AE1 and AE3 anticytokeratin antibodies showed a confluent layer of morphologic, nearly normal respiratory epithelium in all 8 capsule pouches (Figs 4, 5, and 6). Electron microscopy revealed the capsule to be covered with a pseudostratified mucociliary neoeepithelium, including ciliated and secretory cells in all 8 capsule pouches (Figs 7 and 8). This time, numerous cilia lined the apical surface of ciliated cells. Nonciliated secretory cells appeared next to ciliated cellular areas, and their cytoplasm contained secretory granules of varying electron density. As in the side filled with DMEM, H&E staining showed a mild inflammatory

response, dense fibrous capsule tissue, and vascular proliferation.

Discussion

In our study the rat model was used as a source of tracheal cells because the cells lining the rat trachea have similar morphologic appearance to those found in the major conducting airways of the human trachea.¹³ We demonstrated that tracheal epithelial cell cultures can be expanded ex vivo (with an expansion factor of 10 to 10²) to produce a sufficient quantity of cells for a successful transplantation onto a prefabricated capsule pouch by using fibrin glue as the delivery vehicle. However, harvesting such cell numbers in rats is not possible without killing the animal. Therefore, to avoid serious donor site morbidity, we have used inbred rats in our new animal model. This experiment should clarify the cell culture dynamics, the behavior of transplanted cells in a prefabricated tissue construct, and the morphologic follow-up. The next step would be to test the reconstructive use in respect to epithelial lining incorporation onto the inserted flap. A larger animal that tolerates tracheal epithelial cell harvest without mortality and allows technically feasible tracheal operations similar to those seen in the clinical setting must be used.

A capsule flap was induced by using a silicone cylinder as the recipient side for our cell cultures. The silicone stent creates the shape of a cylindrical capsule pouch, and the induced foreign-body reaction provokes a predictable and directed neovascularization in the capsule tissue around the stent. By placing the silicone cylinder in the vicinity of large vessels, neovascularization can be controlled and directed so that the later capsule pouch will be supplied by a single vascular

Fig 6. AE1 and AE3 staining of tracheal neoeplithelium 4 weeks after grafting. (Original magnification, 400 $\times$.)

Fig 7. Scanning electron photomicrograph of surface of the respiratory epithelium 4 weeks after grafting. Note ciliated cells with numerous cilia that line the surface and nonciliated secretory cells. (Original magnification, 3000 $\times$.)

pedicle. We chose the anterior rectus sheath because of its potential for uptake of cartilage or other materials preventing collapse in reconstruction of a full-thickness, composite, prefabricated trachea and the vertically oriented predictable and anatomically reliable blood supply by both the deep inferior epigastric and the superior epigastric artery. Both of these vessels could be used as a single pedicle for a later pedicled or free-flap transfer in human patients.²³ Although the capsule contains mainly fibrous tissue, it has been shown that in the initial stage of the foreign-body reaction, highly increased blood flow can be shown, which supports successful use of the graft.^{19,20} The capsule surface serves as a basal lamina, which is essential for epithelial development. Immunofluorescence analysis of connective tissue components in such a fibrous capsule demonstrated all essential extracellular matrix molecules of a basement membrane, such as laminin, fibronectin, collagens, procollagens, and associated collagen molecules.²⁴ At epithelial-stromal boundaries, this newly induced capsule serves as a specialized area of extracellular matrix molecules for cell attachment.

We compared the efficiency of 2 different delivery vehicles to optimize the implantation conditions of epithelial cell cultures. Although Terzaghi and associates,¹¹ Steele and colleagues,¹² and Johnson and Hubbs¹³ demonstrated a successful culture implantation into denuded rat trachea by means of standard culture media, in our experiment cells pretreated in identical fashion to the fibrin glue side, but with standard culture medium used as the delivery vehicle conduit, failed to produce an adherent and viable cell layer. Because the recipient side

Fig 8. Transmission electron photomicrograph of pseudostratified respiratory epithelium 4 weeks after grafting showing ciliated and nonciliated secretory cells with secretory granules of varying electron densities in their cytoplasm. (Original magnification, 2500 $\times$.)

in our experiment is not the denuded trachea, all paracrine influences from the neighboring natural environment and possible mesenchymal epithelial induction could be excluded opposite to the described successful reimplantation experiments. Our findings support the fact that transplanted epithelial cell cultures need special

boundaries to survive in an extra-anatomic recipient location. The highly differentiated and specialized cell types of tracheal epithelium and the specific membrane-protein complexes may require a special delivery vehicle after the rapid loss of differentiation during the process of adaptation to the *in vitro* conditions.²⁵ Such a delivery vehicle should contain several important features, including high levels of biocompatibility and biodegradability, less cytotoxicity, and a high affinity to bind to biologic surfaces.

Fibrin glue mainly contains fibronectin, a key protein in the extracellular matrix. It is well known that cellular growth and differentiation, in 2-dimensional cell culture and in the 3-dimensional space of the developing organism, requires the presence of a structured environment with which the cell can interact. This extracellular matrix is composed of fibrous macroproteins, such as collagens, elastin, fibrillin, fibronectin (the main component of fibrin glue), laminin, and hydrophilic heteropolysaccharides. The cells interact with the matrix and communicate with each other through the extracellular matrix molecules. Therefore, the extracellular matrix influences the cell shape, fate, metabolism, and behavior and is considered essential for tissue and organ development. As a delivery vehicle, fibronectin in the form of fibrin glue is obviously the structural constituent and regulator of cell behavior in tracheal epithelial culture reimplantation. Anchorage of the transplanted cells to the extracellular matrix depends primarily on a group of surface receptors that are specialized to recognize and bind linkers, such as fibronectin and laminin. Fibronectin binds to cell surface receptors and regulates cell attachment, cell shape, proliferation, migration, and differentiation. Furthermore, these receptor sites bind growth factors and serve as coreceptors for growth factor–receptor interactions.²⁶

Our experiment could demonstrate that these properties are the essential difference in the 2 different delivery vehicles tested. Fibrin glue helps attach the transplanted cells to the recipient bed, enhances the migration of growth factors, and is a nutrient medium itself. These anchoring properties and the ability of enhancing communication might bridge the gap during which the implanted cell is nourished by means of diffusion of extracellular fluid until revascularization and definitive incorporation occur. Although transplanted as single cells, the cells spontaneously reoriented into an evenly distributed tracheal epithelial lining on the capsule surface. DMEM conduit, being only a nutrient medium, failed to produce an adherent and viable cell layer because it lacks any anchoring properties and the ability to enhance communication between cells and their surroundings.

After 14 days, the re-established epithelium is composed of cells of a more cuboidal appearance, and no ciliated cells can be detected. It can be assumed that these cells are immature secretory cells and preciliated cells because Johnson and Hubbs¹³ showed that the secretory cells are the major progenitive cell type and are capable of re-establishing a new epithelium composed of secretory and ciliated cells in rats.

In contrast to our findings, Kaschke and coworkers,¹⁴ who seeded isolated respiratory cells into implanted tubular prostheses of porous polyurethane and expanded polytetrafluoroethylene, observed multiple cell layers of squamous epithelium but no mucus or ciliated cells. They stated that chemical properties and surface structure of the material, as well as immunologic and inflammatory reactions, may have influenced the differentiation process of the transplanted cells. For that reason, we think use of a nonbiologic framework or delivery vehicle should be avoided if there is a biologic alternative.

Cellular functions depend on the coexistence of different cell types in a quantitative equilibrium, which will be disturbed by tissue-culturing techniques; the coexistence of fibroblasts is of special importance. Fibroblasts are capable not only of producing components of the extracellular matrix (eg, collagen, fibronectin, and laminin) but also of secreting a number of growth factors (eg, basic fibroblast growth factor, epidermal growth factor, and keratinocyte growth factor) that modulate proliferation and differentiation of the epithelial cells.²⁷⁻³⁰

It can be assumed that the coexistence of fibrin glue and a fibrous capsule make the differentiation of the transplanted cells to specialized mucociliary epithelial cells possible by facilitating communication and interaction between fibroblasts and the transplanted cells.

However, there might be a considerable difference in the potency of differentiation after implantation *in vivo*, depending on tissue species. Further investigations must clarify whether cultured human tracheal epithelium has the same potential as rat tracheal epithelium in complete epithelial regeneration.

We demonstrated, for the first time, the establishment of a continuous layer of tracheal epithelium successfully transplanted onto a *de novo* prefabricated tissue by means of fibrin glue that is well vascularized and transferable on a single pedicle at an extra-anatomic location, which is a necessary prerequisite for the surgeon dealing with tracheal reconstruction. However, for use in the airway, a framework has to be added because the fibrous capsule would not provide enough stability to resist collapse on negative pressure during inspiration. Such a framework could be established by cultured car-

tilage cells, as shown by Vacanti and associates,⁸ which could be implanted onto the capsule surface before or together with the tracheal epithelial cells. Creation of a cartilaginous scaffold in combination with transplantation of tracheal epithelial cells will be the next step in our experiments. Provided that a stable support of the epithelial layer can be established, segments or even the entire trachea could be replaced by using techniques of tissue culturing.

We thank Sharon Lyons, MA, Hans Suchy, MA, and Auruna Mathur, MA for providing technical assistance; Lynn Rhoades, MA, for editing the manuscript; Sonja Wurzer for technical support preparing the manuscript; and Immuno AG, Vienna, Austria, for providing the fibrin glue.

Received for publication Aug 30, 2000; revisions requested Dec 6, 2000; revisions received Dec 21, 2000; accepted for publication Dec 27, 2000.

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