Experimental generation of a tissue-engineered functional and vascularized trachea

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Objective: We sought to grow in vitro functional smooth muscle cells, chondrocytes, and respiratory epithelium on a biologic, directly vascularized matrix as a scaffold for tracheal tissue engineering.

Methods: Ten- to 15-cm-long free jejunal segments with their own vascular pedicle were harvested and acellularized from donor pigs (n = 10) and used as a vascular matrix. Autologous costal chondrocytes, smooth muscle cells, and respiratory epithelium and endothelial progenitor cells were first cultured in vitro and then disseminated on the previously acellularized vascular matrix. Histologic, immunohistologic, molecular imaging, and Western blotting studies were then performed to assess cell viability.

Results: The endothelial progenitor cells re-endothelialized the matrix to such an extent that endothelial cell viability was uniformly documented through 2-(18F)-fluoro-2'-deoxyglucose positron emission tomography. This vascularized scaffold was seeded with functional (according to Western blot analysis) smooth muscle cells and successfully reseeded with viable ciliated respiratory epithelium. Chondrocyte growth and production of extracellular cartilaginous matrix was observed as soon as 2 weeks after their culture.

Conclusions: The fundamental elements for a bioartificial trachea were successfully engineered in vitro in a direct vascularized 10- to 15-cm-long bioartificial matrix. Future experimental work will be directed to give them a 3-dimensional aspect and a biomechanical profile of a functioning trachea.

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Tissue engineering applies the principles of engineering and life sciences toward the development of biologic substitutes that restore, maintain, or improve tissue function.¹ Its advantages over other tissue replacement techniques are several, such as use of autologous cells, nonimmunogenicity, no side-effects related to foreign graft materials, and potential to grow when implanted into children.²

On the basis of our previous experimental work with tracheal allotransplantation,³ generation of bioartificial autologous tissue in vitro,^{4,5} and airway engineering patching in human subjects,⁶ we investigated the feasibility of engineering a directly vascularized bioartificial matrix displaying all cellular functioning elements of the trachea, and the results are presented.

Materials and Methods

All reagents were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (München, Germany), and all experiments were done at room temperature unless indicated

Figure 1. Histologic and functional characterization of the reseeded biologic scaffold. **A**, Decellularized matrix showing highly cross-linked collagen fibers. (Original magnification 40 $\times$, HE stain.) *Inset*, Macroscopic view of decellularized jejunal matrix. **B**, Immunofluorescent staining for CD31 in re-endothelialized scaffold matrix. (Original magnification 400 $\times$.) The CD31-positive endothelial cells appear green (*arrow*). Vascular lumen is depicted by the *asterisk*. The collagen fibers of the matrix appear red (*X*). **C**, Nuclear thin-layer scanning of acellular scaffold (negative control) showing nearly uniform distribution of radioactivity. *Inset*, Macroscopic scaffold image. **D**, Activity distribution in reseeded BioVaM scaffold.

otherwise. Cell-culture media and supplements were from Promocell (Heidelberg, Germany). The applied antibodies were obtained from Dako (Hamburg, Germany).

Animal Experiments

German landrace pigs ($n = 10$; age, 3 months; body weight, 18–25 kg) were obtained from a local dealer (Tierzuchtanstalt Mariensee, Germany) and underwent scaffold harvesting under sterile conditions. All animals received human care in compliance with the “Guide for the Care and use of Laboratory Animals” published by the National Institutes of Health (National Institutes of Health publication No. 85-23, revised 1996) after approval from our institutional animal protection board (experiment No. 02-504). General anesthesia was induced by means of continuous thiopental sodium (Trapanal), and fentanyl infusion. A median laparotomy was used to isolate a 10- to 15-cm-long segment of jejunum, including its artery and vein pedicle. After systemic administration of heparin (300 IE/kg), the feeding artery was cannulated with a 6F catheter and flushed with 100 mL of 0.9% NaCl. The draining vein was cannulated with an 8F catheter, and venous backflow was controlled macroscopically. The intestinal lumen was flushed with 500 mL of 0.9% NaCl at 4°C containing antibiotic solution (3250 IE of neomycin and 250 IE of bacitracin) immediately after explantation. The fourth lumbar vertebral bone was punctured, and 50 mL of bone marrow was gathered to isolate porcine bone marrow–derived precursor cells. Costal cartilage served for chondrocyte isolation. At the end of the operation, the animals were

killed by an anesthesia overdose. Respiratory epithelium (RE) was obtained by postmortem tracheal brushing. The specimens were stored at 4°C until further processing.

Matrix Preparation and Acellularization

The scaffold was decellularized after mechanical removal of the small bowel mucosa by a modification of the method of Meezan and colleagues⁷ and kept in cell type–specific medium at 37°C until reseeded. Modifications were as follows. For decellularization, the small bowel segment was incubated in 1% sodium azide solution (2 hours at 4°C) under shaking conditions. An incubation in 1 mol/L sodium chloride solution containing 2000 U DNase Type I (2 hours at 37°C) followed to remove the cellular proteins. The tissue was incubated twice in sodium deoxycholate/0.1% sodium azide solution (5 hours at 4°C) to dissolve lipid membrane proteins.

Culture of Bone Marrow–Derived Progenitor Cells

Ten milliliters of porcine bone marrow aspirate was mixed with M199 cell-culture medium in a 1:1 volume and centrifuged (1500 rpm, 5 minutes, room temperature). The supernatant was discarded, and the resulting cell pellet was resuspended in 2 mL of culture medium and transferred onto a Percoll gradient (Amersham Biosciences, Freiburg, Germany) for centrifugation (2200 rpm, 15 minutes, room temperature). The superficial 14 mL was preserved, mixed with 35 mL of culture medium, and rotated for another 5

Figure 2. Region-of-interest activity histogram of thin-layer scans of a reseeded BioVaM: 1, background without graft; 2, median activity of the negative controls; 3, BioVaM at arterial pedicle; 4-6, BioVaM at 20-, 40-, and 70-mm radius from the arterial pedicle.

minutes (1600 rpm). The supernatant was then discarded, and the resulting cell pellet was resuspended in M199 culture medium and disseminated on gelatin-coated culture dishes. For endothelial differentiation, cells were maintained in endothelial cell basal medium 2 supplemented with hydrocortisone (100 μ g/500 mL of culture medium solution), fetal calf serum (FCS; 50 mL/500 mL), porcine vascular endothelial growth factor (0.25 μ g/500 mL), human basic fibroblast growth factor (5 μ g/500 mL), human epidermal growth factor (2.5 μ g/500 mL), insulin-like growth factor (10 μ g/500 mL), ascorbic acid, penicillin (100 IE/mL), streptomycin (100 μ g/mL), and essential amino acids (500 mg/500 mL). For smooth muscle cell (SMC) differentiation, 450 mL of smooth muscle basal medium 2 supplemented with FCS (10%), penicillin (100 IE/mL), streptomycin (100 μ g/mL), human epithelial growth factor (0.25 μ g/500 mL), human basal fibroblast growth factor (1 μ g/500 mL), insulin (2.5 mg/500 mL); and transforming growth factor β 1 (TGF- β 1) (5 ng/mL, Sigma) was used.

Re-endothelialization

For vascular re-endothelialization, the arterial pedicle was filled with 2 mL of endothelial cell basal medium 2 culture medium containing 5×10^6 trypsinized cells. A nonpulsatile medium perfusion rate of 0.8 mL/min was implemented with a roller pump (IPC; Ismatec, Glattbrugg, Switzerland) and steadily increased for 72 hours to 1.5 mL/min. This increased rate was maintained unchanged throughout the experiments.

Positron Emission Tomography

Re-endothelialized biologic vascularized matrices (BioVaMs; $n = 3$) were positioned within 20 cm from the center of a high-resolution, dedicated, positron emission tomography (PET), full-ring scanner (HR+; Siemens, Erlangen, Germany, and CPS, Knoxville, Tenn) and perfused with prewarmed culture medium at 30°C. Radiolabeled 2-(18F)-fluoro-2'-deoxyglucose (FDG; 40 ± 7.2 MBq) was injected into the arterial pedicle. Washing in and washing out of the medium was allowed for 90 minutes each, and transmission and emission scans were performed after each step over 10 minutes. Thereafter, the cell-culture medium was replaced with glucose-free phosphate-buffered saline (PBS) containing 1 IU/mL insulin and 40 ± 8.5 MBq

of FDG, and the PET procedure was repeated. Acellular BioVaMs served as controls ($n = 3$). After correction for physical decay, attenuation, and scatter (as measured with a delayed coincidence channel), the data sets were reconstructed by means of an iterative algorithm by a 256 matrix with 2.0-mm Gaussian prereconstruction filtering. After triple washing by PBS rinsing at 25°C (identical for the negative and positive controls), the scaffolds were thin-layer scanned (Instant Imager; Packard, Menden, Conn). The resulting PET images and thin-layer scans were evaluated by the regions of interest technique.

Muscle Tube Formation

The decellularized tubular scaffold matrix was mounted in a specially designed perfusion device (bioreactor), and 1.5×10^6 cells dissolved in 2.5 mL of cell-culture medium was injected into the intestinal lumen. The conduits were rotated for 20 hours at 0.5 rpm and 37°C to facilitate cellular adhesion to the matrix. Then grafts were perfused with SMC culture medium by using a pulsatile flow pump (313U; Watson & Marlow, Rommerskirchen, Germany) at 15 mL/min for 3 weeks.

Chondrocyte Isolation and Culture

Costal chondrocytes were isolated from donor animals and cultured in a 3-dimensional biologic culture system, as previously described.⁸ In brief, costal cartilage was minced into 1- to 3-mm pieces and enzymatically digested with 0.2% collagenase II. The digested cartilage suspension was filtered with a sterile 250-nm nylon filter, and the reaction was stopped with BGJ medium (Sigma) containing 10% FCS. After centrifugation at 6000 rpm, 1×10^6 cells were mixed with 500 μ L of liquid collagen matrix generated from decellularized porcine intestinal segments that were incubated with 0.2% collagenase II solution for 12 hours at 37°C.

RE Cultures

Isolated RE was cultured in 15% FCS Dulbecco's modified Eagle medium solution. For scaffold seeding, 2×10^5 cells/cm² were distributed on the matrix.

Figure 3. Immunohistologic and protein biochemical characterization of bone marrow progenitor cell differentiation into SMCs. **A**, SMCs seeded under nonpulsatile conditions on the scaffold expressing desmin. (Original magnification 400 $\times$.) **B**, Staining for desmin in SMCs seeded under pulsatile conditions (Original magnification 400 $\times$.) The *asterisk* indicates lumen of tubular scaffold. **C**, Western blot analysis for α -actin expression (41-kd band) in seeded SMCs: *lane 1*, molecular weight marker; *lane 2*, porcine aorta (positive control); *lane 3*, primary SMC culture (positive control); *lanes 4 and 5*, SMCs seeded on BioVaM.

Figure 4. Chondrocyte differentiation on the matrix. **A**, Chondrocytes (*black arrow*) are sparsely separated and grow in cartilage-specific lacunas. (Original magnification 200 $\times$, HE stain). **B**, Azan blue staining of bioartificial cartilage synthesized on the matrix: *red*, chondrocytes; *dark blue*, newly synthesized collagen (*white arrow*). (Original magnification 200 $\times$.) **C**, Western blot analysis for collagen II synthesis in cultured 3-dimensional chondrocytes: *lane 1*, molecular weight marker; *lane 2*, porcine collagen II (positive control); *lane 3*, porcine endothelial cells (negative control); *lane 4*, costal chondrocytes seeded on the matrix; *lane 5*, native porcine costal cartilage.

Figure 5. Cultured RE. A, Light microscopy showing isolated cells. (Original magnification 400 $\times$.) White arrows indicate fibrillating cilia. B, Immunohistologic staining for villin as a marker for cellular differentiation. The asterisk depicts tubular lumen. (Original magnification 100 $\times$.)

Histologic Staining

For Azan blue staining, 6- μ m paraffin-embedded cross-sections were incubated for 15 minutes at 56°C in an azocamin solution, containing 1 mg/mL azocamin and 1% pure acetic acid. The specimens were first rinsed in sterile water and then differentiated in a 1% aniline-alcohol solution, followed by washing in 1% acetic acid. Sections were stained in 5% phosphotungstic acid and anilin-blue-orange G-acetic acid solution for 3 hours each before final fixation in an increasing ethanol column and incubation with xylol. For hematoxylin and eosin (HE) staining, scaffold samples were embedded in Tissue-Tek (Sakura Finetek, Zoutendoude, Netherlands) and stored at -20°C. Six-micrometer-thick cross-sections were prepared, fixed for 8 minutes in acetone at -20°C, and stained with HE.

Immunohistology

Immunohistochemical staining for characterization of the reseeded scaffold was performed by use of the avidin-biotin-peroxidase technique. Endothelial cells were characterized by the presence of CD31 (MCA 1746; Serotec, Düsseldorf, Germany); SMCs by the presence of desmin (DE-R-11, Dako), actin (clone 1A4, Dako), and myosin (M7786, Sigma); and RE cells by the presence of villin antibody (SM1373P; DPC, Bad Nauheim, Germany). A biotinylated goat anti-mouse antibody (H+L; Vector, Burlingame, Calif) served as a secondary antibody. Streptavidin-peroxidase conjugate was applied, and final staining was performed with diaminobenzidine slides and counterstained with hematoxylin. For fluorescence microscopy, a fluorescein isothiocyanate-conjugated secondary antibody was used.

Western Blotting

For characterization of chondrocytes, matrix production proteins were isolated and separated according to the NuPAGE Bis-Tris Gel instructions (Invitrogen; Life Technologies, Madison, Wis) on days 1, 3, 7, 10, and 14 of the culture period. Quantitative signal detection was performed according to the instructions of the ECL Western blotting detection and analysis system of Amersham Biosciences (Freiburg, Germany). Prestained sodium dodecylsulfate-polyacrylamide gel electrophoresis standard marker with a broad range from 6000 to 196,000 kd was used (Bio-Rad, Munich, Germany). Native porcine cartilage served as a positive control, and porcine endothelial cells and liquid biologic matrix served as negative controls.

Statistics

Results are presented as the number of observations $\pm$ SD.

Results

Biologic Vascularized Matrix

The acellularization procedure resulted in 10- to 15-cm-long (11.9 ± 1.8 cm) vascularized scaffolds composed of a dense layer of highly cross-linked collagen and elastin fibers (Figure 1, A). Three weeks after reseeding the preserved vascular network within the matrix with endothelial progenitor cells (EPCs), the cells expressed the endothelium-specific marker CD31 uniformly (Figure 1, B). PET staining showed an accumulation of the FDG activity in the EPC-reseeded BioVaMs predominantly located within the proximity of the arterial pedicle and nearly uniformly distributed in the acellular negative controls (Figure 1, C and D). FDG uptake was 452 ± 72 Bq/mL in reseeded BioVaMs. Insulin stimulation amplified the FDG uptake in viable cells and resulted in a 2.7 ± 0.3 increased uptake (1225 ± 147 Bq/mL, $P < .05$). Maximum activity of the reseeded BioVaMs was 42 ± 13 Bq/mm² in the thin-layer scanning (Figure 2), 6 ± 3 MBq/mm² in the negative controls, and 0.7 ± 0.4 MBq/mm² in the background (without scaffold; $P < .001$). The accumulated activity in the negative control did not increase after insulin application. The locoregional FDG uptake histologically paralleled the distribution of the endothelial cell marker CD31.

Muscle Tube Formation

The bone marrow-derived progenitor cells seeded on the tubular biologic scaffold differentiated into SMCs under laminar perfusion, showing the tissue-specific expression of intracellular desmin, myosin, and actin (Figure 3, A). SMCs were arranged longitudinally along the matrix fibers. Pulsatile stress supported the development of multiple cell layers in vitro. Tissue maturation was detectable after 10 days by the SMC-induced assemblance of the matrix fibers (Figure 3, B). Histologic findings were confirmed by Western blot analysis staining for α -actin (Figure 3, C).

In Vitro Cartilage Formation

Costal chondrocytes expanded in vitro. The 3-dimensional liquid biologic culture system afforded the spacious separation for the growing chondrocytes, which is essential for their functional differentiation. HE staining documented the formation of cartilage-specific lacunas (Figure 4, A). After 7 days in culture, the chondrocytes started to produce a new extracellular matrix, thus forming cartilaginous tissue (Figure 4, B). The protein-biochemical characterization of this bioartificial cartilage showed the synthesis of collagen II, which represents the major collagen fraction in hyaline cartilage (Figure 4, C). There was no evidence of collagen III and collagen X production. The generated cartilaginous tissue displayed no graft rigidity.

Functional RE

RE cells proliferated in vitro. The applied culture conditions suppressed bacterial overgrowth. Cilial movement as a marker for functionality was detectable for more than 4 weeks in culture (Figure 5, A). Immunohistologic staining for villin remained positive in the RE cytoskeleton throughout cultivation (Figure 5, B).

Discussion

Successful clinical tracheal transplantation is still in its infancy, despite a magnitude of experimental work done over the past century.⁹ One possible explanation relates to the intrinsic technical difficulties of finding an ideal tracheal substitute on the one hand and the very limited clinical indications on the other.¹⁰ Tissue engineering is gaining increasing acceptance in cardiothoracic surgery and represents the most promising technique that might be able to create a functional tracheal substitute in the near future.⁹ Our working hypothesis was to transpose the clinical experience of replacing the cervical esophagus with the free jejunal segment¹¹ in the domain of tracheal tissue engineering.

We first isolated and decellularized a 10- to 15-cm-long porcine jejunal segment and reseeded its vascular network with autologous endothelial cells. This reseeded process is of paramount importance to avoid graft thrombosis and failure.³ Three weeks after the reseeded process, the grafts' vascular network was uniformly re-endothelialized with viable autologous endothelial cells, as suggested by histologic and molecular imaging studies. Future work must clarify whether this vascular network can withstand the systemic arterial pressure in vivo, whether the endothelial cells will shear off on reperfusion, or both. Moreover, it has to be addressed whether the venous draining system has a high- or low-resistance regimen to minimize the risks of venous vascular infarction.³

Next we investigated the feasibility of growing trachea-specific tissue components on the vascularized matrix. Bone

marrow progenitor cells seeded on the matrix differentiated into SMCs and formed a muscular tube, showing the characteristic histologic and biochemical features of muscular tissue. It remains to be explored whether the few cell layers meet the functional and biomechanical demands of the posterior tracheal wall in vivo. It is hoped that muscular tube formation can be optimized by the use of mechanobioreactors during tissue cultivation, inducing mechanical stress on the maturing tissue.¹²

Rigid tracheal hyaline cartilage is a prerequisite for tracheal function. In our experiments we have shown that costal chondrocytes are suitable for tissue engineering tracheal cartilage. They produce cartilage-specific extracellular matrix and show the morphology of hyaline cartilage.^{8,13} Recently, it has been shown that bone marrow precursor cells can be differentiated into chondrocytes.^{14,15} The application of these techniques would make thoracic biopsies to obtain primary costal chondrocytes superfluous, both experimentally and clinically. In this study the generated cartilage shows the morphologic and protein-biochemical features of hyaline cartilage but probably does not have the essential biomechanical properties to stabilize the scaffold matrix to such an extent to prevent airway collapse when implanted in vivo. However, we have recently shown that a tissue-engineered fibromuscular patch implanted into the human airway system undergoes a significant tissue maturation process within the first 6 weeks after implantation, resulting in a significant increased matrix rigidity.⁶

The high sensitivity of the airway epithelium to ischemic injury is well described because it is experimentally known that an intact epithelial lining is essential for tracheal function to prevent bacterial colonization, infection, and consecutive graft degeneration and occlusion.^{3,9} Therefore, a functional epithelium is the cornerstone for the long-term functionality of a long-segment airway substitute. In our series we were able to cultivate respiratory epithelial cells over a period of 4 weeks. That the cells showed ciliar activity in vitro does not mean per se that they will keep their function in vivo because physiologic factors like air stream might induce shear stress affecting cellular adherence, interfere with mucous production, or both.

In conclusion, this study provides evidence that 10- to 15-cm-long vascular scaffolds harboring viable chondrocytes, SMCs, and RE might be generated by using tissue-engineering techniques. However, and frankly speaking, the bioartificial tissue-engineered trachea is far from clinical application because fundamental issues regarding functionality and biomechanics of the bioartificial vascular network and the viability of the in vitro-generated tissue components in the in vivo environment have to be addressed.

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Discussion

Dr Yolonda Colson (Boston, Mass). Could you help us better understand exactly how the layers are orientated? Do you place one layer of the collagen and then a layer over the top of it and then another layer, or is it that they are all plated at the same time and they distribute within layers themselves? Second, for the collagen and cartilage, is it laid out as actual rings, or is there a complete layer running the whole length of the trachea?

Dr Walles. Those are important questions. We seeded all cell types isolated and currently do not have grafts consisting of muscle cells and cartilage and endothelial cells. Thus far, we are able to coculture endothelial cells and SMCs to generate a muscular tube with a vascular network, but we have not succeeded in seeding cartilage on the same graft at this time. Very recently, we developed a bioreactor system that allows us to seed cartilage on a matrix, as we have shown here, but I am not able to show any data thus far. The chondrocytes are seeded in a liquid matrix that we developed and that was recently published in the *Annals*. The

chondrocytes seeded in that liquid matrix form their own cartilage, produce collagen, and can be transferred onto a scaffold by using the liquid matrix to generate a complex, 3-dimensional cartilaginous structure.

Dr Walter Weder (Zurich, Switzerland). As to the vascularized matrix, thus far you have only reperfused ex vivo through this recirculating system. You have been doing research with this vascularized matrix for several years. Have you ever used it in vivo? How are the coagulation problems in these small vessels? Thus far everybody has failed to produce a bioengineered construct with small vessels that does not immediately lead to coagulation of these small vessels. Do you have any information on this?

Dr Walles. Thank you for the question, Dr Weder. Yes, we have been working on this concept for several years already. We first started off by seeding the matrix with primary endothelial cells that we obtained, for example, from saphenous vein segments. Matrices that were seeded with these cells were implanted for up to 3 hours into animals to check for the problem of thrombosis. After explantation, we did not see thrombosis in these grafts. However, in the clinical setting the differentiation and use of autologous bone marrow-derived cells into SMCs, chondrocytes, and endothelial cells would make additional biopsy procedures to obtain vascular endothelium superfluous. Therefore, bone marrow-derived endothelial precursor cells are now our preferred cell type for bioartificial graft re-endothelialization. Apart from this, it has been shown that endothelial cells of different vascular regions show different function by expression, for example, of endothelial nitric oxide synthase. We hypothesized that the only way to create a functional bioartificial capillary system would require endothelial precursor cells that have the potential to differentiate into the endothelial subtypes that you need in your graft.

Dr Dao M. Nguyen (Bethesda, Md). This is a very interesting concept. I have 2 questions for you. First, how do you address the issues of the structural rigidity of your graft? Second, how about the geometric form of your graft? Is it going to be straight, like a trachea?

Dr Walles. I will start with your second question first. From clinical experience, where you take a jejunal segment for esophageal reconstruction, you do not have a geometric problem. It is a tube then, and I think we can generate the same in vitro.

As to the second question, the matrix itself withstands pressures of about 200 cm H₂O. If seeded, we can increase this to 300 cm H₂O. The limiting factor for generating tracheal substitutes thus far is the lack of sufficient graft rigidity, so that it does not collapse when you implant it orthotopically, for example, into an animal. This rigidity is provided by the cartilage that is synthesized by the chondrocytes. Thus far, our cartilage does not have the biomechanical properties to prevent collapse of the matrix. We are currently trying to improve the biomechanical properties of our bioartificial cartilage. The presence of sufficient airway rigidity is definitely something that one later has to document in an animal model after extensive in vitro graft evaluation.