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Effect of cell seeding on neotissue formation in a tissue engineered trachea☆

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ABSTRACT

Background: Surgical management of long segment tracheal disease is limited by a paucity of donor tissue and poor performance of synthetic materials. A potential solution is the development of a tissue-engineered tracheal graft (TETG) which promises an autologous airway conduit with growth capacity.
Methods: We created a TETG by vacuum seeding bone marrow-derived mononuclear cells (BM-MNCs) on a polymeric nanofiber scaffold. First, we evaluated the role of scaffold porosity on cell seeding efficiency *in vitro*. We then determined the effect of cell seeding on graft performance *in vivo* using an ovine model.
Results: Seeding efficiency of normal porosity (NP) grafts was significantly increased when compared to high porosity (HP) grafts (NP: $360.3 \pm 69.19 \times 10^3$ cells/mm^2; HP: $133.7 \pm 22.73 \times 10^3$ cells/mm^2; $p < 0.004$). Lambs received unseeded ($n = 2$) or seeded ($n = 3$) NP scaffolds as tracheal interposition grafts for 6 weeks. Three animals were terminated early owing to respiratory complications ($n = 2$ unseeded, $n = 1$ seeded). Seeded TETG explants demonstrated wound healing, epithelial migration, and delayed stenosis when compared to their unseeded counterparts.
Conclusion: Vacuum seeding BM-MNCs on nanofiber scaffolds for immediate implantation as tracheal interposition grafts is a viable approach to generate TETGs, but further preclinical research is warranted before advocating this technology for clinical application.

Current options for the management of diseases involving large segments of the upper respiratory tract such as tracheal agenesis, tracheal clefts, and tracheal stenosis are limited [1,2]. While the current standard of care involves resection of diseased airway segments with primary anastomosis, removal of more than half the tracheal length in adults or more than 1/3 in children requires use of prosthetic materials. These materials lack growth potential and are fraught with complications such as stenosis, infection, rejection, and graft migration [3–5]. Donor allografts are an alternative to tracheal prostheses, but are limited by a paucity of suitable tissue, size mismatch, and the requirement for life-long immunosuppression. The application of tissue engineering technology for treatment of tracheal disease has the potential to create a fully functional tracheal conduit with growth capacity, however, mixed clinical results currently limit widespread adoption of this approach [6–9]. Early attempts with cell seeding of decellularized allografts have been recently applied with modest clinical success. Macchiarini et al. created the first tissue engineered tracheal graft (TETG) by seeding autologous mesenchymal stem cells and nasal epithelial cells onto a decellularized allograft followed by *in vitro* culture and implantation into the left mainstem bronchus of a 30 year old woman suffering from severe bronchomalacia [10,11]. Five-year follow-up described recurrent stenosis of the proximal anastomosis requiring multiple stents, but the remaining graft was patent and lined by a pseudostratified, ciliated columnar respiratory epithelium [12]. Similarly, Elliott et al. implanted a decellularized tracheal allograft into a 10 year old child as an urgent compassionate-use procedure [13],

☆ Level of Evidence: Level II – Therapeutic Study, Prospective Cohort Study.

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bypassing *in vitro* culture by saturating the graft in a suspension of bone marrow mononuclear cells (BM-MNCs) at the time of surgery. Lack of epithelialization and graft laxity required stenting for two years following implantation; however, the creation of a TETG without *in vitro* culture was a notable accomplishment in the field.

While the progress in translation of decellularized allografts is promising, this approach is inherently limited by the availability of suitable donor tissue and the long length of time required for graft decellularization, with some cycles taking up to 20 days [9]. Active research is therefore underway to study alternative source material for TETGs, including degradable and nondegradable polymeric scaffolds, which provide a three-dimensional environment that mimics native extracellular matrix (ECM) in structure and function, allowing for cell attachment, differentiation, and proliferation [6,14]. Recently, an electrospun polyethylene terephthalate/polyurethane (PET/PU) TETG scaffold manufactured by Nanofiber Solutions, Inc. has been seeded with autologous bone marrow-derived mononuclear cells and successfully implanted in four patients requiring tracheal replacement [7,15]. However, the clinical utility of this approach is limited by the requirement for cell culture in a bioreactor before implantation. Development of a seeding methodology for a TETG that bypasses *in vitro* culture has the potential to significantly advance this field. In the present study, we compared the efficiency of vacuum seeding BM-MNCs on this clinically utilized PET/PU TETG scaffold (defined as normal porosity, NP) to that of a high-porosity (HP) variant in order to identify a design that would provide the greatest cell density upon implantation. Next, we evaluated the effect of BM-MNC seeding on TETG performance following orthotopic implantation in a juvenile sheep model for 6 weeks.

1. Materials and methods

1.1. Animal care/ethics statement

The Institutional Animal Care and Use Committee of the Research Institute at Nationwide Children's Hospital (Columbus, OH) approved and monitored all animal procedures described in the present report. All animals received humane care in compliance for the Care and Use of Laboratory Animals (2011), from the Public Health Service, National Institutes of Health (Bethesda, MD).

1.2. Scaffold design and fabrication

Tracheas from juvenile sheep (45–60 kg) were donated from a local abattoir (Columbus, OH) and organ morphometry was used to inform scaffold design. Design criteria called for a D-shaped trachea a flexible dorsal ligament and C-shaped rings that mimic the native tracheal hyaline cartilage in gross morphology and mechanical stiffness. A custom mandrel was machined from stainless steel to match the dimensional measurements obtained from native juvenile sheep specimens (lumen diameter: 17 mm). Polymer nanofiber precursor solutions were prepared by: 1) dissolving 8% polyethylene terephthalate (PET) in 1,1,1,3,3,3-hexafluoroisopropanol (wt/wt) and heating the solution to 60 °C followed by continuous stirring until the PET was completely dissolved, and by 2) dissolving 3% polyurethane (PU) in 1,1,1,3,3,3-hexafluoroisopropanol (wt/wt) with continuous stirring at room temperature until the PU was completely dissolved. Once cooled, the solutions were combined to create a final ratio of 70% PET and 30% PU (wt/wt). The PET/PU solution was then electrospun in a custom designed electrospinning apparatus utilizing 20 gauge blunt tip needles, a high voltage DC power supply set to +14 kV, and a 15 cm tip-to-substrate distance. C-rings were fabricated from a medical grade polycarbonate sheet cut to the desired shape and thermo-molded around the electrospinning mandrel. The rings were then manually embedded into the graft during the process of electrospinnning and integrated within the scaffold matrix by completion of graft fabrication. All tracheal scaffolds were removed from the mandrel and placed in a vacuum overnight to ensure removal of residual solvent (typically less than 10 ppm) [16] To create the HP scaffold, salt crystals (0.5 mm particle size) were deposited onto the mandrel throughout the entire electrospinning process, followed by five 30 min washes in deionized water to completely dissolve the salt crystals dispersed through the construct as previously described [17]. The scaffolds were packaged in Tyvek pouches and terminally sterilized with 30 kGy of gamma irradiation. Scaffold manufacturing methods to produce the NP scaffold replicated those used clinically and characterized by Jungebluth et al. [15]. Both NP and HP scaffold types were developed and manufactured by Nanofiber Solutions, Inc. (Columbus, OH).

1.3. Scanning electron microscopy (SEM)

Samples for SEM were cut from the tracheal scaffold and mounted on aluminum pin mounts with carbon double-sided tape. The samples were then sputter coated with a 5 nm thick layer of gold for observation in an FEI Nova NanoSEM 400 with an accelerating voltage of 5 kV.

1.4. BM-MNC isolation and scaffold seeding

Bone marrow was aspirated from the iliac crests of juvenile sheep (n = 6, 25–45 kg). Animals were sedated with ketamine (IV, 4 mg/kg, Patterson Veterinary), diazepam (IV, 0.5 mg/kg, Patterson Veterinary), and buprenorphine (IM, 0.015 mg/kg, Patterson Veterinary), anesthetized with inhaled isoflurane (Patterson Veterinary) at 0–5%, vaporized with 100% oxygen at 30–60 mL/kg/min and prepared in sterile fashion. A small skin incision was made over the iliac crest, then a 15 gauge Illinois Sternal/Iliac Bone Marrow Needle (Care Express) was inserted to aspirate a maximum of 2 mL/kg of bone marrow per animal. Syringes were heparinized with 100 IU/mL in 0.9% saline. Aspirated bone marrow was then filtered through a 100-μm cell strainer (Falcon) and collected in sterile 50 mL conical tubes. The BM-MNC fraction was obtained from the filtered bone marrow aspirate with the Purecell Select System for Whole Blood MNC Enrichment (Pall Medical), following the manufacturer's protocol as previously described [18,19]. The Purecell Select System for Whole Blood MNC Enrichment (Pall Medical) has been validated by our group and is understood to yield a bone marrow mononuclear cell seeding suspension that is phenotypically comparable to that obtained by traditional density gradient centrifugation [18–20]. The cell suspension was subsequently vacuum seeded onto the polymeric TETG scaffolds using a previously described vacuum seeding technique (Fig. 2A) [19,21]. After seeding, TETGs were immersed in the post-seeding cell suspension (~140 mL) and immediately delivered to the surgical suite or sampled for further analysis.

1.5. Cell seeding efficiency

Pre-seeding and post-seeding cell suspensions were collected for in-process determination of cell seeding efficiency and cell viability using a manual hemocytometer and manual Trypan blue exclusion (Life Technologies). Seeded scaffolds were sampled (n = 5 samples per scaffold, 5 × 5 mm sample) for quantitative analysis of cell attachment within the scaffold using the QuantiT^{TM} PicoGreen® dsDNA assay (Life Technologies) following the manufacturer's protocol. One post-seeding sample from each scaffold type was fixed in neutral buffered 10% formalin (NBF) for 24 hours, routinely processed, paraffin embedded, and stained with hematoxylin and eosin (H&E) for qualitative assessment of cell seeding efficiency.

1.6. Surgical implantation

A 3.0 cm TETG was implanted into the mid-cervical trachea of juvenile sheep (25–45 kg, n = 2 unseeded, n = 3 seeded). Under general anesthesia, a midline cervical incision was made, exposing the trachea. A 3.0 cm long segment of the trachea was excised. The first endotracheal