Clinical Transplantation of a Tissue-Engineered Airway

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Abstract— An integrated approach has been developed toward the definition of an optimal strategy to bring functional tracheal substitutes to the clinic. In particular, our idea has exploited non-immunogenetic eterologous decellularised matrices, autologous cells (differentiated and stem cells), a newly developed bioreactor, and appropriate animal models (allograft and xenograft, heterotopical and orthotopical implants). Thanks to the promising positive results obtained throughout our preclinical studies, a first pioneer clinical case has been successfully performed. A donor tracheal matrix was treated via a detergent-enzymatic method to remove cells and MHC antigens. Autologous respiratory epithelial cells and mesenchymal stem cells (MSCs, then differentiated into chondrocytes) were isolated, characterized and expanded. A novel rotating bioreactor was developed to allow co-culture of the two cell types onto the tubular matrix, proper oxygenation of the culture milieu and enhanced mass transport to and from the adhering cells. MSCs-derived chondrocytes and bronchial epithelial cells were therefore seeded and cultured on either side of the acellular matrix and the re-personalised construct was implanted into the patient as a replacement for her left main bronchus. This case provides new evidences that autologous cells combined with appropriate biomaterials and cultured in bioreactor can be successfully used to provide solutions to serious clinical problems. Whilst focusing on tracheal regeneration, the developed approach could be rolled out for use in other tissues and whole organ regeneration settings.

I. INTRODUCTION

Extensive defects which compromise large airways seriously threaten duration and quality of life. Tracheal resection with primary reconstruction is the only curative treatment in patients suffering from a variety of benign or malignant tracheal lesions. Unfortunately, the resectable length of the diseased trachea is usually restricted to approximately 30% in children and around 6 cm in adults, and any further increase of this resection rate depends on the development of a safe tracheal replacement (Gaisser et al., 2004). This last is not yet clinically available since almost every attempt to provide an autologous, allogeneic or synthetic safe and reproducible tracheal graft has been disappointing so far because of stenosis, immunologic complication, bacterial infections and material failure (Grillo, 2002; Kalathur et al., 2010).

The advances in regenerative medicine during the past decades have provided a new approach toward the concept of functional substitutes and represent the most promising alternative to the shortage of suitable grafts for reconstructive airway surgery (Fuchs et al., 2001).

Although encouraging, previously reported experiences were however unaccomplished and several issues hampered their clinical applicability. Most of the approaches focused on the regeneration of a tube-like cartilage graft, but it is now clear that a functional respiratory epithelium at the time of implant is mandatory. The ideal scaffold had yet to be designed and only static culture approaches had been attempted. No consensus exists about optimal cell sources and culture techniques. Moreover, the adopted strategies were too lengthy and complex for routine clinical use (Asnaghi et al., 2009a).

Due to structural and functional complexity, the in vitro generation of a functional substitute for such an organ is thus a significant challenge. Complementary advances in materials science, cell biology, bioengineering and surgery were necessary to develop in vitro engineered substitutes suitable for clinical application. Here we describe our integrated approach to prepare a tissue-engineered autologous airway substitute subsequently implanted in a 30-year old woman with end-stage bronchomalacia (Macchiarini et al., 2008).

II. MATERIALS AND METHODS

A 7cm tracheal segment was retrieved from a 51 year old white female transplant donor who had died of cerebral haemorrhage and was treated via a detergent-enzymatic method over 6 weeks to remove cells and Major Histocompatibility Complex (MHC) antigens as previously described (Jungebluth, 2009). Briefly, tissue was extensively washed with distilled water supplemented with antibiotic and antimicotic for 72 h, then incubated in 4% sodium deoxycholate and 2000 kU deoxyribonuclease 1 in 1 M sodium chloride (Sigma Chemicals, Barcelona, Spain). To quantify the remaining cells after each detergent enzymatic cycle, we stained paraffin-embedded sections of the matrix with 4’-6-diamidino-2-phenylindole (DAPI,
Vector Laboratories, Burlingame, CA, USA), and counted the total number of nuclei with fluorescence microscopy (mean nuclear count x10⁶ per μm² [SD]). For morphological assessment, we stained adjacent, paraffin-embedded sections with haematoxylin and eosin (both Merck, Darmstadt, Germany). We measured MHC antigen expression by use of primary anti-human HLA-DR, HLA-DP, HLA-DQ antibodies (all three BD Biosciences, Oxford, UK) and HLA-ABC antibodies (Abcam, Cambridge, UK), secondary antibodies (Vectastain ABC kit, Vector Laboratories), and then a peroxidase substrate kit (DAB, Vector Laboratories). For negative controls, we omitted the primary antibody. Samples of the treated matrix were fixed with 3% glutaraldehyde (Merk, Darmstadt, Germany) in 0.1 M cacodylate buffer (Prolabo, Paris, France), subjected to critical point drying and gold sputtering, and examined by scanning electron microscope (JSM6490, JEOL, Japan).

Bone marrow stromal cells (BMSCs) were isolated from aspirates from the recipient, expanded in culture and characterized to assess the stem cell characteristics and the multi-lineage differentiation potential of the cell population. The multi-lineage differentiation potential of the passage 3 BMSCs was assessed by examining their osteogenic, adipogenic and chondrogenic capacities. BMSCs were grown in monolayer culture for 3 weeks in the presence of osteogenic differentiation medium, containing dexamethasone, ascorbic acid 2-phosphate and β-glycerolphosphate (R&D Systems, Abingdon, UK), and minerals deposited by stimulated cells were stained with 40 mM alizarin red (Fluka). BMSCs were also grown for 3 weeks in adipogenic differentiation medium, containing hydrocortisone, isobutylmethylxanthine and indomethacin (R&D Systems), and fat vacuoles in the stimulated cells were stained with fresh oil red-O solution. For chondrogenic differentiation, BMSCs were seeded onto fibronectin-coated polyglycolic acid (PGA) scaffolds and cultured on a gently rotating platform for 35 days in medium containing insulin-transferrin-selenium (Invitrogen), TGF-β3 (R&D Systems), dexamethasone and ascorbic acid 2-phosphate (Sigma), according to a previously published method (Kafienah et al., 2007). Biochemical assays were used to measure the amounts of various proteins in the tissue-engineered cartilage constructs: proteoglycan was measured by colourimetric assay, total collagen by amino acid analysis and collagen types I and II by specific ELISA assays. Having verified the stem cell characteristics of the BMSC population, passage 3 cells were induced to differentiate into chondrocytes to be used for the acellular matrix repopulation.

Respiratory epithelial cells were isolated from the recipient’s own tissue biopsies (right main bronchial mucosa and right inferior turbinate mucosa) and allowed to proliferate in culture as previously described (Rees et al., 2006). Only the bronchial epithelial cells were subsequently used for graft development since they grew far more readily than nasal cells did. Cytospins of cultured epithelial cells at first passage were subjected to dual-colour immunofluorescence histology for cytokeratins 5 and 8, counterstained with DAPI, to confirm epithelial phenotype before attachment to the matrix in the bioreactor. All cells in epithelial culture stained positive for cytokeratins immediately before seeding, and we did not detect any fibroblasts morphologically and immunohistologically.

A novel two-phase rotating bioreactor was developed to allow co-culture of the two cell types onto the tubular matrix, proper oxygenation of the culture milieu and enhanced mass transport to and from the adhering cells (Asnaghi et al., 2009b). Both cell seeding onto the scaffold and cellularized construct dynamic culture were performed inside the bioreactor, avoiding construct manipulation between the two operations and thus limiting the risk of cell construct contamination.

![Sequence of operations for trachea matrix preparation for cell seeding and culturing in the bioreactor.](image)

Chondrocytes were dropped longitudinally on the external surface of the matrix with a microsyringe, while epithelial cells were injected into the lumen. Every 30 min, the matrix was rotated and seeding repeated until all surfaces had been completely exposed to cells. After
completion of the seeding process, each chamber was filled up with their respective complete media to totally submerge the seeded matrix and the cellularized construct was maintained in static conditions for 24 h to promote cell adhesion (37°C, 5% CO2). Media volumes were then reduced so that nearly half of the matrix was exposed to the incubator atmosphere and dynamic culture was started at 1.5 revolutions per min (37°C, 5% CO2) for 72 h. At the end of the culture period, the bioreactor rotation was turned off, both chambers were emptied and completely refilled with fresh media and the bioreactor was delivered to the operating room.

After general anaesthesia and double-lumen endotracheal intubation, we did a left posterolateral thoracotomy and carefully dissected and fully mobilised the distal trachea, left main bronchus, and the left recurrent and phrenic nerves of the recipient. We then resected the left main bronchus, recreating its take-off on the lateral aspect of the distal trachea via a 2x2 cm orifice, and preserving distally the upper lobe take-off and the lobar carina. We then cut the graft to shape, and anastomosed it end-to-end proximally and distally. Fitting to the two lumens of different sizes was helped by the retained elasticity of the trachealis segment of the graft. Bilateral ventilation was restored and the recipient’s left lung immediately ventilated well. After we checked for leaks, the chest was closed, and the patient was extubated. We monitored the recipient for 2 days in the intensive care unit, when she was well enough to return to a general ward and was discharged on the tenth postoperative day. Ethical permission was obtained from the Spanish Transplantation Authority and the Ethics Committee of the Hospital Clinic, Barcelona.

III. RESULTS

25 cycles of detergent-enzymatic decellularisation treatment were able to efficiently remove cells and MHC antigens from a human donor tracheal matrix, providing a non-immunogenic biological scaffold, free from the risks of rejection, with preserved native architecture (Fig. 1).

BMSCs from both early and late passage were successfully differentiated into the three mesenchymal lineages of cartilage, fat and bone and expressed several cell surface markers associated with multipotential stem cell populations (Fig. 2 A–C).

Respiratory epithelial cells were also positively isolated from biopsies from the recipient and efficiently expanded in culture with no modifications in cell phenotype (Fig. 2 D).

Cell seeding of both cell types on either side of the tubular tracheal matrix in the developed bioreactor was easy and highly efficient. The bioreactor worked properly and no contamination was observed during the whole culture period (Fig. 3). Viable chondrocytes and epithelial cells were observed on the graft surfaces prior to implantation.

The bioreactor-cultured construct was implanted and immediately provided a regenerated, fully functional airway, with no immunosuppressive drugs (Table I). The recipient had no complications from the operation and was discharged from hospital on the tenth postoperative day. Histological studies confirmed angiogenesis and the persistence of viable cells at two months post-surgery.

![Fig. 2](image_url)

**Fig. 2.** Multilineage differentiation potential of BMSCs and epithelial cells characterization. Minerals characteristic of osteogenic differentiation stained with alizarin red (A) and fat vacuoles characteristic of adipocytes stained with oil red-O (B) (10x magnification). Chondrogenic differentiation: biochemical analysis of protein content (C) (PG=proteoglycan). Colour immunocytochemistry of bronchial cells before seeding onto the trachea matrix (D) (red=cytokeratins 5 and 8, epithelial).

<table>
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<th>Sept, 2008 (3m post-op)</th>
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<td>FVC [l]</td>
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<td>0.058</td>
<td>0.122</td>
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Although 3-D computed tomography reconstruction showed a significant improvement of the airway appearance from a near-total collapse (preoperatively) to wide patency (postoperatively) (Fig. 4), at 8 months a ventral collapse of proximal 1 cm of the graft occurred, and related to the pulsatile compression from the aortic arch superiorly and to the migration of the stem-cell-derived chondrocytes into the endoluminal surface of the graft. Although she was asymptomatic, a temporary endoluminal stent was placed as a precaution (Baiguera et al., 2010).

Fig. 4. Volume rendering CT before and 2 month after surgery: terminal long segment narrowing is completely reversed.

At 18 months postoperatively, serologic analysis revealed the complete absence of antidonor human leukocyte antigen antibodies, effectively ruling out any phenomenon of acute or chronic rejection. The patient is well and fully active at two years after the transplantation.

IV. CONCLUSION

Our results show that it is possible to in vitro engineer a functional airway substitute suitable for clinical application. The procedure described allowed to engineer a graft free from the risks of immune rejection, avoiding the need for any immunosuppressive therapies. This case provides new evidence that autologous cells combined with appropriate biomaterials and cultured in bioreactor systems can be successfully used to provide solutions to serious clinical problems. Whilst focussing on tracheal regeneration, the developed approach could be rolled out for use in other tissues and whole organ regeneration settings.

REFERENCES

– Kalathur M, Baiguera S, Macchiariini P, Translating tissue-engineered tracheal replacement from bench to bedside, Cell Mol Life Sci, published online at DOI: 10.1007/s00018-010-0499-z, 2010