Immune tolerance of epithelium-denuded-cryopreserved tracheal allograft

Ilir Hysi, Alain Wurtz, Christophe Zawadzki, Eric Kipnis, Ramadan Jashari, Thomas Hubert, Alexandre Ung, Marie-Christine Copin and Brigitte Jude

INTRODUCTION

Circumferential replacement of the trachea remains a major challenge, the main issues being the lack of an identifiable vascular pedicle, which makes tracheal allografts unsuitable for direct revascularization, and immune rejection. There have been an impressive number of investigations and experimental studies, thoroughly analysed, for the development of a reliable tracheal substitute [1, 2]. Focusing on the revascularization issue, Delaere et al. [3] conducted experiments demonstrating the feasibility of revascularizing airway segments with the rabbit lateral thoracic fascial flap, thus allowing tracheal allotransplantation with this transferable flap as a vascular blood supply [4]; recently, they reported their clinical experience with a similar procedure [5, 6]. Moreover, we confirmed the reliability of this technique for heterotopic revascularization of allogenic aortas and aortic graft-based tracheal substitutes in rabbit models [7, 8].

Although these experimental results have been very encouraging, they have failed to provide a reliable circumferential tracheal substitute in current clinical practice. Thus, allogenic aortas ensured circumferential tracheal replacement but have encountered a stiffness problem, with patients requiring long-term airway stents [9-11], while tracheal allografts have presented immune rejection problems, with patients requiring immunosuppressive...
MATERIALS AND METHODS

Our experimental protocol (# CEEA 09212) was approved by the regional ethical board on experimental use of animals (Comité d'éthique en expérimentation animale Nord-Pas-de-Calais). Experiments were performed according to the standard guidelines of the French Ministry of Agriculture, food-processing industry and forest (Ministère de l'Agriculture, de l'agroalimentaire et de la forêt), which regulates animal research in France.

Animals

Eighteen syngeneic adult New Zealand (NZ) white rabbits, weighing 3400–4450 g, were used [C.E.G.A.V. (SSC) La Passerie 61350 Saint-Mars-D’Egremn, France]. All animals were housed in our institution at the University Hospital Department of Experimental Research.

Epithelium-denuded-cryopreserved tracheal allografts

Five females served as donors. They were premedicated with an intramuscular injection of ketamine (50 mg/kg) and xylazine (2.5 mg/kg), and then euthanized using an intracardiac injection of embutramide, mebezonium and tetracaine (T61; Intervet, Beaucouzé, France). The entire trachea (from the cricoid cartilage to the carina) was harvested through a midline cervical and transmediastinal approach. The tracheal mucosa was mechanically peeled off the underlying lamina propria with a small sterile peanut gauze ball in order to obtain an epithelium-denuded trachea. Tracheal allografts were directly immersed in the ice-cold (4-8°C) incubation solution, an antibiotic cocktail containing vancomycin 50 µg/ml, lincomycin 120 µg/ml and polymyxin B 120 µg/ml in 50 ml of Hank’s medium 199, and then transferred to the European Homograft Bank (EHB). Subsequently, they were incubated in this solution (used routinely for preparation of cardiovascular allografts) for 40–48 h at a temperature between 4 and 8°C. Preparation of these cryopreserved tracheal allografts was performed in a Class A clean room under vertical laminar flow with Class C background. The tracheal allografts were prepared for cryopreservation after having been placed in a 10% dimethylsulphoxide (DMSO) in Hank’s solution 199 as cryoprotecting medium. Freezing was performed in a controlled-rate freezer (Kryo 560-16, Planner, Sunbury-on-Thames, UK). The cooling rate was 1°C per minute down to −40°C followed by 5°C per minute down to −100°C. The allografts were then placed in the storage tank, in liquid nitrogen vapour at −150 to −187°C. The lengths of storage ranged from 12 to 43 days. Prior to use, cryopreserved allografts were thawed in two steps. First, the graft was kept for 5–6 min at room temperature for slow temperature increase above the critical recrystalization point (−123°C), then in a warm water bath at 37–40°C during 8–10 min to increase the temperature to 4°C without major temperature fluctuations. Washing out of DMSO was performed by means of cold isotonic sterile saline (+4°C) in four steps, decreasing its concentration from 10% to 6.6%, to 3.3% and to 1%. Finally, the allograft was kept in 200 ml of pure isotonic sterile saline until implantation ensuring that the DMSO was completely washed away.

Anaesthesia and analgesia of recipient animals

Anaesthesia of recipient male NZ rabbits was induced with an intramuscular injection of ketamine (50 mg/kg) and xylazine (2.5 mg/kg), and maintained using inhaled isoflurane and oxygen through mask ventilation. Isoflurane discontinuation allowed animal awakening after approximately 25–30 min. Postoperative analgesia was provided with two intramuscular injections of buprenorphin (0.05 mg/kg) on Day 1.

Operative technique

Tracheal allografts were divided in segments measuring 20 mm in length (8–12 tracheal rings) and used without additional preparation in a first group of recipients. Nine animals were initially scheduled in this group, but according to preliminary results observed (progressive alteration of normal tubular morphology and longitudinal shrinking of tracheal allografts), their number was reduced from 9 to 4. A second group of nine recipients received a tracheal segment prepared with a 6-mm outer diameter polyethylene tube inserted into the lumen and stitched with 6/0 absorbable polydioxane monofilament (PDS II, Ethicon France, Issy Les Moulineaux, France) to both edges of the tracheal allograft to ensure stretching to its original length and maintain lumen patency during the revascularization period (Fig. 1).

Figure 1: Operative view showing the lateral thoracic fascial flap and the tracheal allograft segment with its luminal tube, before flap wrap. Arrow: Vascular pedicle of the fascial flap; stars: stitching of the luminal tube at both edges of the tracheal segment to ensure stretching to its original length.
With the animals in supine position, a vertical skin thoracic incision at the level of the left mammary line was performed. The left lateral thoracic skin was dissected laterally from the underlying lateral thoracic fascia, which was elevated and pedicled on the lateral thoracic vessels, as previously described [4] (Fig. 1). The edge of the fascial flap was wrapped around the tracheal segment and then sutured with 6/0 absorbable PDS II running suture. This composite allograft was implanted under the skin of the left lateral thoracic wall, and the skin was closed with 2/0 absorbable Optime R (Péters surgical, Bobigny, France) interrupted sutures.

Follow-up

Postoperatively, the rabbits were observed for ~3 h before being returned to their individual cages, where standard feed and water were available ad lib. Antibioprophylaxis (enrofloxacin 10 mg/kg) was administrated postoperatively over 5 days. Immunosuppressive therapy was not given at any time. Animal sacrifice was scheduled at regular intervals up to 91 days.

Macroscopic assessment

Each wrapped allograft was retrieved en bloc with its pedicled flap. Macroscopic analysis consisted in the evaluation of graft appearance and length, after polyethylene tube removal in the last nine rabbits. The graft consistency was assessed manually by compressing the graft with forceps but no formal biomechanical measurements were performed.

Histological examination

A graft sample was cryopreserved, and transverse and longitudinal sections were cut at the level of the wrapped allograft and pedicled flap following 2 days of formalin fixation. Specimens were embedded in paraffin, cut into 3 µm slides and stained with hematoxylin-eosin-saffron for microscopic examination, with special attention to lymphocyte infiltration, cartilage viability and calcification assessed as follows: +: mild, central deposits; ++: moderate, 10–50% of the cartilage surface; +++: intense, superior to 50% of the cartilage surface [8]. The findings were compared with the morphology of epithelium-denuded-cryopreserved tracheal allograft in a control rabbit.

Detection of apoptotic cells

Immunohistochemical staining (Apostain) was used to assess the immunohistochemical rejection of tracheal allografts. The number of apoptotic cells in cartilage and lamina propria/pericartilaginous tissue was semi-quantitatively assessed with a murine monoclonal antibody (clone F7–26, Abcam, Cambridge, MA, USA). This antibody is specific of small DNA fragments, which are specific markers of the apoptotic process. Briefly, the slides were permeabilized with phosphate buffered saline 0.02% saponin buffer (Sigma-Aldrich, St Louis, MO, USA) and incubated in formamide at 56°C. They were then blocked with 3% milk. Endogenous peroxidase was neutralized with a 3% hydrogen peroxide solution. Slides were then incubated overnight at 4°C with the primary antibody (1:500). The second antibody was a horse biotinylated antimouse (1:200) (Vector Laboratories, Burlingam, CA, USA) incubated for 1 h at room temperature. The slides were finally incubated with reagents of the avidin-biotin-peroxidase kit (ABC Kit; Vector Laboratories) and diaminobenzidine (Sigma-Aldrich). The slides were counterstained with hematoxylin.

RESULTS

All animals survived the experiments and none experienced any medical or surgical complications.

Macroscopic evaluation

Sequential sacrifice of the animals was scheduled from Days 7 to 91 (Table 1). Macroscopically, all wrapped allografts contained a clear fluid. Rabbits from the first group (allograft without endoluminal tube) were found to have progressive longitudinal shrinking leading to overlap of some tracheal rings; and an additional centripetal shrinking up to 50% of the initial luminal diameter in rabbit 11 sacrificed on Day 61 (Fig. 2A). In contrast, the nine rabbits from the second group had composite allografts displaying a satisfactory tubular morphology, no change in the initial length and normal tracheal structure with well-recognizable tracheal rings (Fig. 2B); and satisfactory strain ability.

Pathology findings

Despite the alteration of normal tubular morphology of tracheas in the first group, microscopic examination of specimens showed quite similar findings in all animals. The grafts surrounded by the recipient’s fascia (Fig. 3) displayed neoangiogenesis, composed of small capillaries, appearing mainly into the lamina propria from Day 7 (Fig. 4). A mild non-specific inflammatory infiltrate mainly composed of eosinophils, neutrophils and macrophages, stable over time, was observed into the graft from Day 19. Lymphocyte infiltrate was insignificant, apart from minimal areas in the lamina propria, in which a massive lymphocyte infiltrate was shown surrounding a remnant epithelial islet in Rabbits 2 and 4 (Fig. 5) or close to a few number of remnant basal cells in Rabbit 7. Finally, 10 rabbits (77%) were found to have a complete denudation of their epithelium (Table 1).

Cartilage tracheal rings displayed early central calcification deposit, which increased over time, while the remaining cartilage surface showed progressive ischaemia, characterized by the evanescence of cartilage cells and loss of stained affinity of extracellular matrix proteoglycans, as previously described [8]. This phenomenon accelerated after the first month. In parallel, the lamina propria was thickened by a progressively organized fibrosis (Fig. 6). Thorough examinations of the tracheal allograft lumen did not reveal any fibroplastic proliferation.

Apoptosis events

Sparse apoptotic cartilage cells were present only in the first week (Rabbit 1) and thereafter absent with negative Apostain staining. On the other hand, cell staining of the lamina propria and pericartilaginous tissue was more intense with a bell-curve distribution.
Table 1: Clinical and pathological findings in 13 male recipient rabbits

<table>
<thead>
<tr>
<th>Rabbit (weight, g)</th>
<th>Luminal tube</th>
<th>Sacrifice day</th>
<th>Remnant tracheal epithelium</th>
<th>Cartilage ischaemia</th>
<th>Inflammation</th>
<th>Lymphocyte infiltrate</th>
<th>Neoangiogenesis</th>
<th>Apoptosis lamina propria and PCT</th>
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<tr>
<td>1 (4450)</td>
<td>+</td>
<td>7</td>
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<td>2 (4040)</td>
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<td>14</td>
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<td>+</td>
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<td>3 (3825)</td>
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<td>15</td>
<td>50%</td>
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<td>4 (4115)</td>
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<td>7 (3620)</td>
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<td>29</td>
<td>-</td>
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PCT: pericartilaginous tissue.

aLymphocyte infiltrate surrounding islet of remnant tracheal epithelium.

Figure 2: Macroscopic aspect after longitudinal section of fascial flap-wrapped tracheal allografts: (A) without endoluminal tube on Day 61 (Rabbit 7): longitudinal and centripetal graft shrinking leading to overlap of tracheal rings and reduction in the luminal diameter; (B) with endoluminal tube on Day 91 (Rabbit 13): normal tracheal structure with well-recognizable tracheal rings. FF: fascial flap; star: tracheal rings.

Figure 3: Histological examination of a fascial flap-wrapped tracheal allograft on Day 14 (Rabbit 2). (A) Longitudinal section. Arrowhead: neocapillary into the lamina propria; arrows: viable cartilage tracheal rings with central calcification; star: lateral thoracic fascial flap; (B) Transversal section showing similar findings. (hematoxylin-eosin-saffron stain ×25).
through the time, from Days 7 to 91 (Table 1). Apoptosis events (Fig. 7) reached a peak on Day 20.

DISCUSSION

The development of a reliable tracheal substitute is of utmost importance in both malignant and benign tracheal pathologies. Despite the extended experimental research in the field, the clinical applications of tracheal substitutes remain poor. With respect to this issue, often in the literature, there is a lack of distinction between partial and circumferential reconstructions, the latter involving the most difficult technical issues [2].

The most intuitive solution to perform circumferential central airway replacement is the tracheal allograft itself. Despite an initial short-term clinical success [13], tracheal transplantation has been progressively regarded as a failure due to necrosis, stenosis or malacic grafts [14] due to the consequences of interactions between immune rejection and ischaemia. In fact, tracheal replacement raises two major issues: (i) the lack of an individualized vascular pedicle, which impedes immediate revascularization of the graft, as in solid organ transplantation, and (ii) the need for immunosuppressive therapy, strictly contraindicated in malignancies. Due to anatomical reasons, direct arterial and venous revascularization of the thyroid-tracheal block is particularly complex and has led to only a single clinical report [15]. Another well-established approach is the indirect revascularization of the tracheal allograft in heterotopic position by wrap with omentum, muscle or fascia flaps, allowing secondary orthotopic implantation of a viable allograft with satisfactory clinical results [5, 6, 13]. As shown in our previous work on allogenic aorta [7], we found in this study that the heterotopic revascularization achieved by the fascial-flap wrap was a reliable technique, avoiding critical ischaemia of the graft during the initial phase of the revascularization process.

Another important aspect is the immune rejection of tracheal allografts. It is characterized by lymphocytic infiltration and oedema of the lamina propria, followed by destruction of the respiratory epithelium and cartilage architecture with a malacic graft [16]. Delaere et al. [4] observed an acute immune rejection with necrosis of their fascial flap-wrapped tracheal allografts within 14 days of revascularization in heterotopy without immunosuppressive treatment, while an immunosuppressive treatment with
ciclosporine (10 mg/kg) was well tolerated and ensured tolerance and viability of the allografts. With this technique, Delaere et al. [5, 6] reported some recent successful, albeit non-circumferential, clinical tracheal replacements. However, they also found a recurrent problem related to mucus production by the respiratory epithelium and its accumulation inside the lumen due to mucosal overgrowth and consecutive obstruction at both ends of the graft after 6 days. This led to infection of stagnating mucus and purulent collection responsible for progressive graft disintegration [4, 17].

To solve this issue in the clinical setting, after omentum wrap in the abdominal position of a tracheal allograft, Klepetko et al. [18] established an abdominal tracheostoma, allowing mucus clearance and graft checking. In our present study, denudation of the tracheal epithelium prevented these problems related to mucus secretion, thus reducing overall inflammatory response and preserving the tracheal allograft architecture.

In our model, another issue might be an intraluminal fibroblastic proliferation due to epithelium denudation. Indeed, in small rodents (rats and mice), the immune response presents a particular aspect. In heterotopic position, the tracheal allograft is obliterated due to intraluminal fibroblastic proliferation, a phenomenon exacerbated by prior epithelial denudation of the graft [12]. Consequently, our study was designed to determine the need for an endoluminal tube to avoid such an issue during the revascularization period in heterotopy. In contrast to small rodents, our grafts displayed only moderate thickening of the lamina propria. We observed, however, significant reduction of the lumen patency because of the retraction of the graft in the first group of recipients (without endoluminal tube). This problem was not observed in the second group of animals that maintained normal tubular morphology of their grafts thanks to the endoluminal tube.

Tracheal epithelium seems to be a crucial parameter modulating immune response to tracheal allografts [19]. Other Japanese teams have demonstrated the feasibility of tracheal replacement with cryopreserved allografts wrapped in the omentum, and without immunosuppressive treatment [20, 21]. These works have demonstrated the low immunogenicity of the tracheal cartilage itself and have confirmed the determinant role of the respiratory epithelium in the immune rejection of tracheal allografts. Thus, Mukaida et al. [21] and Murakawa et al. [22] confirmed the role of secondary epithelial depletion following cryopreservation in allograft tolerance. Notably, Murakawa et al. conducted an immunological study in primates. They alternatively compared outcomes in fresh and cryopreserved non-circumferential tracheal allografts transplanted in baboons and demonstrated by immunohistochemical staining the presence of major histocompatibility complex (MHC) class II antigen in the respiratory epithelium of fresh tracheas, while it was not expressed in cryopreserved tracheal allografts. The cryopreservation process caused a depletion of the epithelium with loss of expression of MHC class II antigen, which is normally highly expressed [23], thus inducing immunological tolerance of the graft. In fact, it appeared to us that cryopreservation alone was insufficient to ensure a satisfactory immunological tolerance in our rabbits, since we observed a massive lymphocytic infiltrate surrounding a remnant epithelial islet in three animals. These findings demonstrate the usefulness of concomitant epithelial denudation/cryopreservation in preventing rejection.

Mild cartilage cell apoptosis events only observed in the first week suggested initial ischaemia and the absence of immune reaction of the cartilage tissue [24], while the more intense apoptosis events observed in the lamina propria and pericartilaginous tissue could be consecutive to minimal immune chronic rejection of other components of the epithelium-denuded allograft [25]. This hypothesis is supported by the fact that apoptosis had a bell-curve distribution and was always found on Day 91, when the revascularization by the fascial flap was well established (Table 1).

Finally, the reason why tracheal cartilages undergo progressive calcification deposits despite evidence of satisfactory neoangiogenesis of grafts remains elusive. As hypothesized by Tanaka et al. [24], this transformation might be a delayed consequence of initial ischaemia resulting in cartilage cell apoptosis and dystrophic calcification. However, this transformation did not alter the morphology and stiffness of tracheal rings, and the strain abilities of allografts. Investigations of cervical tracheal transplantation of such epithelium-denuded-cryopreserved tracheal allografts are ongoing in our lab, the rabbits being followed up by serial bronchoscopy. Intermediate results are promising with grafts displaying epithelial regeneration from the recipient. Similar studies need, however, to be conducted in bigger mammals before considering clinical applications.

In humans, heterotopic revascularization of allograft and additional buttressing of the tracheal membrane could be achieved by means of a flap wrap into the fascia lata and subsequent orthotopic transposition as a free flap to replace the cervical/mediastinal trachea. The rotational thoracodorsal artery perforator flap, which has the ability to reach the tracheal region, could be another option. After tracheal transplantation, lack of ciliated epithelium at the level of the epithelium-denuded allograft might potentially reduce the mucus clearance of the central airway. This issue might be solved thanks to on-demand bronchoscopy allowing suction of secretions in the early postoperative period. Following this, graft epithelial cell ingrowths coming from the edges of native trachea should allow normal mucus clearance.

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