Construction of a tube-shaped tracheal substitute using fascial flap-wrapped revascularized allogenic aorta

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Received 10 May 2011; received in revised form 6 July 2011; accepted 19 July 2011

Abstract

OBJECTIVES: Animal studies have demonstrated the feasibility of tracheal replacement by silicone-stented allogenic aortas (AAs), showing mature cartilage regeneration into the grafts. In clinical trials, this graft did not prove stiff enough to allow long-term stent withdrawal. This graft insufficiency could be due to ischaemic phase prior to neoangiogenesis. To solve this issue, we investigated both the efficacy of the rabbit lateral thoracic fascial flap as a vehicle for revascularization of the AA and construction of a tube-shaped graft with transferable vascular pedicle, for more efficient replacement of the trachea.

METHODS: Thirty-four New Zealand rabbits were used. After harvesting of donors’ thoracic aortas, the fresh aortic allografts were transplanted within 1 h, and the others were cryopreserved. Fifteen male and four female rabbits were used as recipients for fresh (n = 9) or cryopreserved (n = 10) aortic allografts that were implanted under the skin of the chest wall, after graft wrap using a pedicled lateral thoracic fascial flap. Animal sacrifice was scheduled at regular intervals up to 61 days. Macroscopic and microscopic examinations and fluorescence in situ hybridization (FISH) were used to study the morphology, revascularization process and viability of the construct.

RESULTS: There was no operative death. Animals showed no graft rejection, despite the absence of immunosuppressive therapy. They all had a satisfactory tubular morphology of their construct. Of the 19 rabbits, 15 were found to have a generally preserved histological structure of the aorta and satisfactory neoangiogenesis. In the last four, a severe wound complication was associated with necrosis of the aortic graft. FISH on three aortic grafts with satisfactory neoangiogenesis showed migration of recipient cells into the aortic graft, decreasing from the adventitial to the luminal side, associated with the persistence of cells from the donor.

CONCLUSIONS: Our results showed that the chimeric construct transformed into a well-vascularized tube-shaped organ with transferable pedicle and some degree of stiffness. Persistence of donor’s cells of normal morphology into the aortic graft was suggestive of minimal ischaemia during the initial phase of revascularization. This construct might be investigated in the setting of tracheal replacement in the rabbit model.

Keywords: Animal model • Allograft • Aorta • Angiogenesis • Tracheal surgery • Transplantation

INTRODUCTION

Transplantation or replacement of hollow organs such as the trachea and the oesophagus poses major problems, mainly due to the lack of an identifiable vascular pedicle which makes grafts unsuitable for direct revascularization. In the setting of tracheal surgery, the search for reliable substitutes has been the object of an impressive number of investigations spanning several decades [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 and tracheal allotransplantation with this transferable flap as a vascular blood supply [4]. The clinical application of this procedure has been reported recently [5]. Focusing on alternatives to tracheal allografts, experimental studies in animal models, otherwise, showed that replacement of the trachea with fresh or cryopreserved allogenic aortas (AAs), without additional immunosuppression, produced a conduit that shared the elements of a native trachea [6–8]. Following these consistent results, we performed tracheal replacement with a silicone-stented AA in patients with extensive tracheal tumours [9, 10]. Despite successful transformation of the AA into a well-vascularized conduit, no
graft developed sufficient stiffness to allow definitive stent withdrawal [10]. A critical factor in the discrepancy between the successful AA graft cartilaginization in animal models and insufficient AA graft stiffness in patients could be the initial ischaemia prior to neoangiogenesis, which might be prolonged in patients due to multiple factors such as patients’ underlying microvasculature, perioperative macrocirculatory and microcirculatory haemodynamics in the context of such major surgery. Our hypothesis is that techniques that optimize graft revascularization should overcome shortcomings of these previous techniques in central airway replacement. Therefore, we investigated in rabbits both the efficacy of lateral thoracic fascial flap as a vehicle for revascularization of the AA and the construction of a stiffer tube-shaped graft with a transferable vascular pedicle (Fig. 1), as prerequisites for future reliable replacement of the trachea.

MATERIALS AND METHODS

The experimental protocol was approved by the Comité d’éthique en experimentation animale Nord-Pas-de-Calais (num: AF 10/2010). Experiments were performed according to the standard guidelines of the French Ministry of Agriculture et de la Pêche that regulates animal research in France.

Animals

Thirty-four syngenetic adult New Zealand white rabbits, weighing 3840–5010 g, were used [C.E.G.A.V (SSC), Saint-Mars-D’Egrenne, France]. All animals were housed in our institution at the University Hospital Department of Experimental Research.

Fresh and cryopreserved AAs

Eight females and seven males 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 thoracic aortas (average length: 57 mm) were harvested through a sternotomy. The others were transferred to the European Homograft Bank in ice-cold isotonic sterile saline within 24 h of harvesting. Surgical preparations of these cryopreserved AAs were performed in a clean room under vertical laminar flow Class A with background of Class C, following the GMP Standards and European requirements on Cardiovascular tissue banking. The AAs were incubated in the antibiotic cocktail containing lincomycin, vancomycin and polymixin B for 48 h and then cryopreserved after having been placed in 10% dimethylsulphoxide (DMSO) in Hank's solution 199 as a cryoprotecting medium. Freezing was performed in a controlled-rate freezer (Kryo 560-16, Planner, Sunbury-on-Thames, UK). The cooling rate was 1°C/min down to −40°C followed by 5°C/min down to −100°C. The allografts were then placed in the storage tank in liquid nitrogen vapour at −150 to −187°C.

Prior to use, cryopreserved allografts were thawed in a warm water bath at 37–40°C for 8–10 min. 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 pure isotonic sterile saline until implantation.

Figure 1: Drawing of the tube-shaped construct, juxtaposition of recipient’s fascial vascular carrier as an outer layer (including a unique transferable vascular pedicle) and revascularized donor’s aorta as an inner layer.

Figure 2: Operative view showing: (A) the aortic allograft with an inserted polyethylene tube and (B) the fascial flap wrapped around the aortic allograft.
Anaesthesia

The recipients were premedicated with an intramuscular injection of ketamine (50 mg/kg) and xylazine (2.5 mg/kg), and anaesthesia was maintained using inhalation of isoflurane and oxygen through mask ventilation.

Postoperative analgesia was provided with two intramuscular injections of Nalbuphine (1 mg/kg) on day 1.

Operative technique

In all recipients, the entire thoracic AAs (n = 11) or segments of AAs (n = 8) were prepared, with an inserted 4.2 mm outer diameter polyethylene tube to maintain a patent lumen [11], the AA being occluded with ligation at both extremities of the tube with 2/0 absorbable Polysorb (Covidien France, La Clef St Pierre, France) sutures (Fig. 2A). Fifteen male and four female rabbits were used as recipients for fresh (n = 9) or cryopreserved (n = 10) AAs (Table 1).

With the animals in the 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 described previously [3]. The edge of the fascial flap was rolled around the AA and then sutured with 6/0 absorbable PDS (Ethicon France, Issy Les Moulineaux, France) running suture (Fig. 2B). This composite graft 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 approximately 3 h before being returned to their individual cages, where standard feed and water were available ad lib. Neither anti-inflammatory therapy was given at any time. Animal sacrifice was scheduled at regular intervals up to 61 days (Table 1).

Macroscopic evaluation

After harvesting the construct with its pedicled flap, the polyethylene tube was removed. Macroscopic analysis consisted in the evaluation of graft appearance and consistency. Mechanical properties were evaluated manually by bending and then compressing the graft with forceps until the lumen was closed [11]. 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 graft and pedicled flap following 2 days of formalin fixation. Specimens were embedded in paraffin, cut into 3 µm slides and stained with haematoxylin–eosin–saffron for microscopic examination. The findings were compared to the normal morphology of both fresh and cryopreserved aortic allografts in control rabbits.

Table 1: Clinical and pathological findings in 19 recipient rabbits

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Donor sex</th>
<th>Recipient sex</th>
<th>Fresh or cryo A aorta</th>
<th>Graft length (mm)</th>
<th>Complication</th>
<th>Sacrifice day</th>
<th>Neoangiogenesis</th>
<th>Inflammation</th>
<th>Graft necrosis</th>
</tr>
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<tbody>
<tr>
<td>1 (3950)</td>
<td>F</td>
<td>M</td>
<td>Cryo</td>
<td>65</td>
<td>—</td>
<td>5</td>
<td>+</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>2 (4885)</td>
<td>F</td>
<td>F</td>
<td>Fresh</td>
<td>30</td>
<td>—</td>
<td>6</td>
<td>+</td>
<td>+</td>
<td>+ (10%)</td>
</tr>
<tr>
<td>3 (4635)</td>
<td>M</td>
<td>F</td>
<td>Cryo</td>
<td>51</td>
<td>—</td>
<td>7</td>
<td>+</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>4 (4580)</td>
<td>F</td>
<td>M</td>
<td>Cryo</td>
<td>72</td>
<td>Wound dehiscence</td>
<td>9</td>
<td>+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5 (5100)</td>
<td>M</td>
<td>F</td>
<td>Cryo</td>
<td>49</td>
<td>—</td>
<td>10</td>
<td>+</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>6 (4215)</td>
<td>F</td>
<td>M</td>
<td>Fresh</td>
<td>35</td>
<td>Abscess</td>
<td>10</td>
<td>+</td>
<td>+</td>
<td>+ (10%)</td>
</tr>
<tr>
<td>7 (4035)</td>
<td>F</td>
<td>M</td>
<td>Fresh</td>
<td>76</td>
<td>Wound dehiscence</td>
<td>12</td>
<td>+</td>
<td>0</td>
<td>—</td>
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<tr>
<td>8 (4105)</td>
<td>M</td>
<td>M</td>
<td>Cryo</td>
<td>49</td>
<td>—</td>
<td>12</td>
<td>+</td>
<td>+</td>
<td>+ (10%)</td>
</tr>
<tr>
<td>9 (5060)</td>
<td>F</td>
<td>M</td>
<td>Cryo</td>
<td>66</td>
<td>Skin necrosis</td>
<td>12</td>
<td>—</td>
<td>+</td>
<td>+ (90%)</td>
</tr>
<tr>
<td>10 (4335)</td>
<td>F</td>
<td>M</td>
<td>Fresh</td>
<td>21</td>
<td>Skin necrosis</td>
<td>12</td>
<td>+</td>
<td>0</td>
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<td>11 (4785)</td>
<td>F</td>
<td>F</td>
<td>Fresh</td>
<td>26</td>
<td>—</td>
<td>13</td>
<td>+</td>
<td>++</td>
<td>+ (10%)</td>
</tr>
<tr>
<td>12 (4240)</td>
<td>M</td>
<td>M</td>
<td>Cryo</td>
<td>35</td>
<td>—</td>
<td>14</td>
<td>+</td>
<td>+++</td>
<td>+ (30%)</td>
</tr>
<tr>
<td>13 (4545)</td>
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<td>M</td>
<td>Cryo</td>
<td>70</td>
<td>—</td>
<td>14</td>
<td>+</td>
<td>++</td>
<td>—</td>
</tr>
<tr>
<td>14 (4765)</td>
<td>F</td>
<td>M</td>
<td>Fresh</td>
<td>29</td>
<td>—</td>
<td>14</td>
<td>+</td>
<td>+++</td>
<td>+ (50%)</td>
</tr>
<tr>
<td>15 (4125)</td>
<td>F</td>
<td>M</td>
<td>Fresh</td>
<td>20</td>
<td>Abscess</td>
<td>14</td>
<td>—</td>
<td>+++</td>
<td>+ (90%)</td>
</tr>
<tr>
<td>16 (4630)</td>
<td>M</td>
<td>M</td>
<td>Cryo</td>
<td>46</td>
<td>—</td>
<td>16</td>
<td>+</td>
<td>+++</td>
<td>—</td>
</tr>
<tr>
<td>17 (4075)</td>
<td>M</td>
<td>M</td>
<td>Fresh</td>
<td>56</td>
<td>Skin necrosis</td>
<td>20</td>
<td>—</td>
<td>++</td>
<td>+ (100%)</td>
</tr>
<tr>
<td>18 (3840)</td>
<td>F</td>
<td>M</td>
<td>Fresh</td>
<td>22</td>
<td>—</td>
<td>24</td>
<td>+</td>
<td>++</td>
<td>—</td>
</tr>
<tr>
<td>19 (4875)</td>
<td>M</td>
<td>M</td>
<td>Cryo</td>
<td>42</td>
<td>Abscess</td>
<td>61</td>
<td>—</td>
<td>++</td>
<td>+ (100%)</td>
</tr>
</tbody>
</table>

F, female; M, male; cryo, cryopreserved; A, allogenic.
Fluorescence in situ hybridization

Transplantation with gender mismatch (Table 1) enabled us to analyse the presence of cells of female and male origin into the graft tissue, by using the fluorescence in situ hybridization (FISH) method for detection of both X and Y chromosomes. The slides were fixed, treated with mild pepsin solution and dehydrated (Histology FISH accessory kit, Dako France SAS, Trappes, France). After denaturation, detection of the X and Y chromosomes was performed by overnight probe hybridization at 37°C, according to the manufacturer’s protocol (Whole Chromosome Paints, Rabbit X Cy3-labelled and Rabbit Y FITC-labelled, STARFISH, Adgenix, Voisins le Bretonneux, France). After washing, DNA was counterstained with 4',6'-diamidino-2-phenylindole (DAPI blue). Cells of female origin were labelled with double-red fluorescence and cells of male origin with both red and green fluorescence.

RESULTS

All animals survived the experiments. Eight animals, however, experienced wound complications (42%): skin necrosis (n = 2), wound dehiscence (n = 3) or local sepsis (n = 3).

Macroscopic evaluation

Sequential sacrifice of the animals was scheduled from day 5 to day 61 (Table 1). Macroscopically, all the rabbits with no wound complication invariably developed seroma in the operative field, which facilitated dissection and harvesting of the construct in the early postoperative period (before days 10–12). All rabbits were found to have a satisfactory tubular morphology of their construct with the whitish pearly internal face of the aortic graft surrounded by a thick wall (Fig. 3). In the rabbit euthanized on day 61, the whitish internal aspect had disappeared. The grafts showed some degree of rigidity, but lacked flexibility in comparison with native tracheas from rabbits.

Pathological findings

Microscopic examination of both fresh and cryopreserved aortic allografts in control rabbits showed similar structure of the aortic wall composed of elastic tissue with a small number of smooth cells. Microscopic examination of all specimens showed the aortic graft surrounded by the recipient’s fascia of an average thickness of 0.5 mm, which was composed of a muscular layer between two layers of the adipose tissue (Fig. 4). In all rabbits, the endothelium had disappeared. In 14 rabbits, the normal histological structure of the aortic wall (Fig. 5A) was intact with preservation of the elastic tissue or was minimally involved by necrosis restricted to the luminal side and limited to 10% (n = 4).
to 30% (n = 1) of the graft circumference. Neoangiogenesis appeared at the adventitial side of the aortic graft, composed of small capillaries extending to the external part of the aortic elastic tissue (Fig. 5B). The neocapillaries increased in number from day 5 to day 24. On day 5, a mild inflammatory infiltrate was observed in the graft. The inflammatory reaction, mainly composed of polymorphonuclear leukocytes and macrophages, became more intense from day 10 to day 24 in the majority of animals. In these animals with minimal histological changes, the wound dehiscence observed in rabbits 4, 7 and 10 and the sepsis in rabbit 6 had no impact on both structure and revascularization of the aortic graft. In contrast, the four rabbits that experienced severe local complications with macroscopic changes such as sepsis with abscess formation (rabbits 15 and 19) or large skin necrosis (rabbits 9 and 17) were found to have an extensive graft necrosis. Finally, in rabbit 14, the necrosis involving 50% of the graft circumference could not be explained by a local complication.

Pathological findings are summarized in Table 1.

Fluorescence in situ hybridization

The FISH analysis was performed in three rabbits (5, 10 and 18) and transplanted with gender mismatch (Table 1), which were euthanized on days 10, 12 and 24, respectively.

In all samples, a migration into the male aorta of cells originating from the female recipient (rabbit 5) or a migration into the female aorta of cells originating from the male recipient was observed (rabbits 10 and 18). The number of cells decreased along a gradient from the peripheral to the luminal layer of the aorta, which was found to be composed only of smooth cells originating from the donor (Fig. 6).

DISCUSSION

Contrary to solid organ transplantation, replacement of hollow organs with no identifiable vascular pedicle in humans remains a major surgical challenge. New experiments are ongoing to provide a standardized solution to the so-far unsolved problem of tracheal transplantation [12]. In this field, Delaere et al. [5] elegantly demonstrated the feasibility of indirect revascularization of a tracheal allograft by means of wrap in the recipient’s left forearm fascial flap. Successful tracheal allotransplantation for severe tracheal stenosis was achieved by transferring the wrapped graft to the orthotopic position with an intact blood supply including the radial artery and two radial veins, which were sutured to the neck vessels. Despite excellent mid-term results, this method was found to have two limitations: (i) immunosuppressive drugs were required during the revascularization step and (ii) tracheal replacement had been performed in an essentially patchy fashion repair. Therefore, this procedure could not be considered for extensive circumferential replacement in patients with tracheal tumour such as adenoid cystic carcinoma that exhibits extensive submucosal and perineural spread [13]. Recently, Shadmehr et al. [14] underlined the need for novel approaches in this field. Our choice to investigate revascularization of AAs in the heterotopic position was supported by our previous clinical experience with six patients who had undergone extensive circumferential tracheal replacement for tumour using either fresh or cryopreserved silicone-stented AA, without immunosuppressive drugs [9, 10], enabling currently a local control of the disease with a mean 51-month follow-up. However, shortcomings were shown in the long-term. After stent removal, the graft consistently showed centripetal shrinking, and no patient had a graft stiff enough to allow definitive stent withdrawal. We therefore investigated indirect revascularization of either fresh or cryopreserved AA in the rabbit model to construct a more reliable tube-shaped organ for future tracheal replacement.

Our results showed that the composite construct, juxtaposition of recipient’s fascial vascular carrier [4] as an outer layer and donor’s aorta as an inner layer, had transformed into a chimeric tube-shaped organ (Fig. 4) including a unique transferable vascular pedicle (Figs 1 and 3), with early neoangiogenesis of the aortic component. The grafts, however, showed no biomechanical properties allowing a potential tracheal replacement without the need for postoperative silicone stenting. Both entire and segment of the thoracic aorta gave similar results. A severe wound complication such as abscess, however, led to a complete necrosis of the aortic component, which was observed in four animals. Therefore, an additional antibioprophylaxis might be useful in future experiments.

In the present investigation, using FISH analysis for both X and Y chromosome identification, we demonstrated that the migrating cells present in the aortic component of the construct were derived from the recipient. The precise nature of these cells has to be determined. Interestingly, both persistence of normal smooth cells originating from the donor into fresh and cryopreserved aortic grafts and preservation of the normal histological aortic structure, from day 10 to day 24 after the procedure (Fig. 6), were suggestive of minimal ischaemia, during the initial phase of the revascularization process. Despite the absence of immunosuppressive therapy, none of the animals showed evidence of graft rejection, the observed inflammatory reaction being composed mainly of polymorphonuclear leucocytes and macrophages with no significant number of lymphocytes. This was consistent with prior results of orthotopic transplantation of AA in humans [15] and heterotopic transplantation of AA in animal models [6–8, 16, 17]. This low-grade immunogenicity is most likely a consequence of the small number of smooth cells into aortic media of both rabbits and humans [15].

This tube-shaped construct, with a fascial flap transferable to the neck, might be investigated in tracheal replacement in the
rabbit model, before being considered in humans [4, 5]. Given the similar results with both fresh and cryopreserved AAs, the latter could be preferentially used for graft construction, being readily available in tissue banks and bacteriologically safe [8]. Future issues of particular interest will be to investigate whether the efficient neoangiogenesis, with no significant ischaemia during the initial phase of revascularization, that we observed: (i) prevents the graft from axial contraction [18, 19] and (ii) facilitates regeneration into the graft, which was previously observed in other mammals [6–8].

With regard to the versatility of this model, it might be otherwise investigated to overcome the pitfalls of oesophageal replacement in other surgical areas, for organ tissue repair in tubular or patch fashion.

ACKNOWLEDGEMENTS

We are grateful to Eric Kipnis for editorial assistance and Arnold Dive and Michel Pottier, Département Hospitalo-Universitaire de Recherche Expérimentale, Université de Lille 2, and Valérie Gomanne, Institut d’Hematologie-Transfusion, Centre de Biologie Pathologie, CHU de Lille, for laboratory work.

Funding

This work was supported by the Région Nord-Pas-de-Calais, Fond Régional à l’Innovation (FRI OSEO).

Conflict of interest: none declared.

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