Circumferential esophageal replacement using a tube-shaped tissue-engineered substitute: An experimental study in minipigs

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Background. Esophageal replacement by the colon or the stomach for malignant and nonmalignant esophageal diseases exposes to significant morbidity and mortality. In this setting, tissue engineering seems to be a seductive alternative.

Methods. In a porcine model, we performed a 5-cm-long circumferential replacement of the cervical esophagus by a tubulized acellular matrix (small intestinal submucosa) cellularized with autologous skeletal myoblasts and covered by a human amniotic membrane seeded with autologous oral epithelial cells. The substitute was grown for 2 weeks in the great omentum before esophageal replacement. Eighteen minipigs (divided into 3 groups: group A [substitute with esophageal endoprosthesis; n = 6], group B [substitute alone; n = 6], and group C [endoprosthesis alone; n = 6]) were included. The esophageal endoprosthesis was removed at 6 months. Animals were killed sequentially over a 12 month-period. Clinical, endoscopic, radiologic and histologic outcomes were analyzed.

Results. All animals except 1 of in groups B and C died during the first 2 months owing to refractory esophageal stenosis or endoprosthesis extrusion. Nutritional autonomy without endoprosthesis was observed in all animals of group A with a follow-up of > 6 months (n = 3). A phenotype similar to that of native esophagus, consisting of a mature epithelium, submucosal glands, and a circular muscular layer, was observed after 9 months.

Conclusion. In this model, the circumferential replacement of the cervical esophagus by a tube-shaped tissue-engineered substitute under the temporary cover of an esophageal endoprosthesis allowed nutritional autonomy and tissue remodeling toward an esophageal phenotype. (Surgery 2015;:

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AFTER ESOPHAGECTOMY FOR CANCER, esophageal replacement is usually performed with gastric or colonic transplants. The treatment of benign diseases, such as long-gap esophageal atresia or caustic and peptic strictures refractory to endoscopic dilation, also requires replacement of the whole esophagus.2,3 Esophageal replacement results in substantial morbidity and mortality. In the long term, disabling symptoms related to late complications such as anastomotic strictures, reflux, and delayed conduit emptying impair the patient’s quality of life.4,5 Finally, failure of esophageal reconstruction leads to a critical situation in which further reconstruction cannot be performed owing to the lack of an appropriate
esophageal substitute. For all these reasons, finding alternative procedures for esophageal replacement would be of great benefit.

In animal models and more recently in humans, advances in tissue engineering make this concept a credible option for esophageal replacement in the near future. In addition to a less invasive and risky procedure, preservation of native intraabdominal conduits, and a replacement tailored to the exact length of the esophageal defect represent theoretical advantages over usual techniques of esophageal replacement.

Several experimental models have been used in search of the ideal approach for esophageal tissue engineering. The hybrid approach, which is based on the in vitro combination of different cell types and matrices, seems to be the most promising. Simultaneously, it seemed that maturation of the substitute into a natural bioreactor, such as the great omentum before organ replacement is beneficial, by promoting early vascularization of the substitute.

In a previous work, we analyzed the feasibility and the optimal conditions of cell seeding, as well as cell phenotypic behavior, of an acellular matrix cellularized with porcine skeletal myoblasts (PSM) secondary covered with a human amniotic membrane (HAM) seeded with porcine oral epithelial cells (OEC). With the purpose of analyzing its capabilities to differentiate toward an esophageal phenotype in vivo, this substitute was used to replace circumferential defect of cervical esophagus in a minipig model.

MATERIAL AND METHODS

Animals. Eighteen male minipigs aged 2 years and weighing 35–45 kg were used (La Ferme du Noyer, Bretoncelles, France). All animals received care in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute of Laboratory Animal Resources, National Research Councils, and published by the National Academy Press, revised 1996, and with French regulations and institutional ethical committee guidelines for animal research. The experimental protocol was approved by ethics committee of our institution.

Anesthesia. All operative procedures were performed under general anesthesia after administration of a 10-mg subcutaneous injection of nalbuphine (Nalbuphine, Merck, Paris, France), an intramuscular injection of 0.3 mg/kg of midazolam (Hypnovel, Roche, Neuilly-sur-Seine, France), and 5 mg/kg of ketamine (Ketamine Virbac, Virbac, Carros, France). Tracheal intubation was performed subsequently with 0.2 mL/kg/h of intravenous propofol (Diprivan 1%, Astrazeneca, Rueil-Malmaison, France). During surgery, all animals were perfused with a balanced crystalloid solution (10 mg/kg/h), which was suppressed at the end of the operative procedure.

Substitute construction. Preparation of the hybrid substitute has been described previously. Briefly, autologous PSM were isolated from a right quadriceps muscle sampling. Muscle biopsies were dissociated enzymatically and cells cultured for 2 weeks in proliferation medium promoting the expansion of CD56 myogenic cells. Before matrix seeding, myoblast phenotype of the cell population was controlled by anti-CD56 staining (1/50, Becton Dickinson, San Jose, CA); NCAM clone 16.2, mouse immunoglobulin Ig2b PE), and anti-Desmin staining primary antibodies (1/50, Becton Dickinson; IgG1) revealed by the secondary goat anti-mouse antibody (1/100, fluorescein isothiocyanate), using a 5-parameter flow cytometer (FACS-calibur, Becton Dickinson) with CellQuestPro software (Becton Dickinson). The Biodesign 4 Layer Tissue Graft (Cook Medical, Charenton Le Pont, France), 7 × 10 cm, extracted from porcine small intestinal submucosa, was seeded with 0.5 × 10⁶ PSM/cm² and incubated at 37°C, 5% CO₂ for 1 week. Culture media was changed every 48 hours.

Autologous OEC were isolated from a buccal mucosa sampling, according to Beckstead et al with modifications. Briefly, OEC were enzymatically dissociated from buccal epithelium and expanded for 2 weeks. Before cell seeding, the epithelial phenotype of the cell population was controlled by immunohistochemistry (cytospin) using an anti-pancytokeratin antibody: AE1/AE3 (AE1/AE3 pancytokeratin 1/50 Dako, France). HAM were provided for nontherapeutic use by the Tissue Bank of Assistance Publique–Hôpitaux de Paris (Hôpital Saint-Louis, Paris, France). HAM was decellularized from its native epithelium by enzymatic digestion and seeded by 10⁶ OEC/cm² before being incubated for 1 week at 37°C, 5% CO₂. At the time of in vivo implantation, the 2 matrices were fixed one to the other with nonabsorbable (Prolene 5/0) sutures.

In vivo maturation of the substitute. Before being used for esophageal replacement, the substitute was placed through a midline laparotomy into the great omentum for a 2-week maturation period. To create a tubular structure, it was wrapped either around a self-expanding removable polyester silicone stent (group A; Polyester tracheal stent 18 × 80 mm; Boston Scientific, Montigny le Bretonneux, France) or around a
silicone tube (group B) and stitched to itself with nonabsorbable sutures (Prolene 5/0; Fig 1, A, B). The great omentum was stitched to itself around the substitute, to avoid its future migration (Fig 1, C).

At the end of the maturation period, and as a first step of the esophageal replacement procedure, a sampling of the substitute was placed in formaldehyde solution (4%) for histologic analysis.

**Esophageal replacement. Study design.** Three experimental groups were defined. After circumferential resection of the cervical esophagus, animals in group A (n = 6) underwent the interposition of the substitute and the 2 anastomoses were protected by the polyester tracheal stent (18 × 80 mm; Boston Scientific) for a 6-month period. Animals in group B (n = 6) underwent the same procedure, but without stent insertion. Therefore, animals of these 2 groups underwent 3 surgical steps (sampling procurement, positioning of the substitute into the great omentum, and esophageal replacement). In group C (n = 6), the esophageal defect was bridged with the stent only; therefore, these animals underwent only 1 operation. Sequential killing of the animals was planned between 3 and 12 months for pathologic analysis.

**Operative procedure.** The esophagus was dissected through a left cervical incision in all animals, with special attention to recurrent and vagus nerves and a circumferential 5-cm-long resection of the entire wall of the esophagus was performed. In animals of group C, a self-expanding stent was inserted into the upper and lower esophageal ends to which it was attached by absorbable sutures (Vicryl 3/0; Fig 2, A). Animals in groups A and B underwent a midline laparotomy and an epiploplasty was constructed, containing the substitute and vascularized by the right or left gastroepiploic vessels. Then, the skin incision was extended from laparotomy to cervicotomy, and the pedicled graft was brought up to the neck in a subcutaneous presternal position (Fig 2, B). In animals in group A, the self-expanding stent was inserted in the upper and lower ends of the cervical esophagus before performing 2 end-to-end anastomoses with running 4-0 Prolene sutures, between the esophagus and the substitute (Fig 2, C). The stent was attached to the upper esophagus with full-thickness stitches of absorbable sutures (Vicryl 3/0). Adequate positioning of the stent was controlled endoscopically. Animals in group B underwent the same procedure, without stent insertion, but with placement of a nasogastric tube. No external drainage was used.

**Postoperative care.** Cefazolin (1 g/d intramuscularly) for 10 days and omeprazole (20 mg/d orally) were administered for 15 days. Oral feeding
was initiated on postoperative day 2 in groups A and C. In group B, animals were fed from day 2 by nasogastric tube, which was removed at day 10 and then oral feeding was introduced. Liquid hypercaloric and hyperprotidic feeding 3,000 kcal/d (RealDiet, 1.3 kcal/mL) was administered during the first postoperative month. During the following 5 months, they had semiliquid food (flour mixed with water) with 2,200–2,500 kcal/d; after month 6, a normal diet was given.

Clinical, endoscopic, and radiologic evaluations.
Postoperative monitoring was performed daily throughout the animal survival, assessing respiratory and dietary conditions as well as the occurrence of surgical complications. The animals were weighed on day 7, then once a week until day 90. From month 4 onward, animals were examined and weighed monthly. In case of vomiting or weight lost suggestive of stent migration or stenosis occurrence, a new stent (Polyflex esophageal stent 16/20 × 90 mm; Boston Scientific) was inserted after balloon endoscopic dilatation (esophageal wire-guided balloon dilatation catheter; Boston Scientific). In case of complications, such as pneumonia, cervical abscess, or gastric outlet obstruction by stent migration, animals were killed if not cured within 1 week. In the absence of postoperative complications, the esophageal stent was removed endoscopically at 6 months. Endoscopy was performed systematically before killing to assess esophageal patency and evaluate the inner aspect of the graft area. A barium swallow was performed only in animals with the longest follow-up.

Graft area analysis. After killing by means of an intravenous injection of propofol followed by potassium chloride and propofol, the native cervical esophagus, the graft and the surrounding tissues were dissected en bloc. More specifically, resection was carried out, taking 6–8 cm of the esophagotracheal bloc. Specimens were fixed in a 4% formaldehyde solution. Macroscopic analysis consisted of the evaluation of graft appearance and consistency. Samplings taken at the end of the maturation period in the great omentum and the esophagotracheal bloc obtained after animals were killed underwent histologic analysis. Samples were included in paraffin. Histologic sections of 4 μm followed by hematoxylin erythrosine saffron (HES) staining was performed. For immunohistochemical analysis, antigen retrieval was performed by heating the tissue section in 0.1 mol/L citrate buffer (pH 6.0). Immunostaining was performed in a Ventana processor (Ventana, Illkirch, France). The antibodies used for immunostaining are listed in Table I.

RESULTS

Substitute construction. Twelve animals (groups A and B) underwent buccal (average weight 2.5 g) and right quadriceps biopsies (average weight 3.5 g). All cell isolations proceeded successfully. Phenotype analysis of PSM and OEC performed after 2 weeks of cell expansion showed stable myoblast and epithelial phenotypes, consistent with previous results.14

In vivo maturation of the substitute. Macroscopic finding. In groups A and B, after a 14-day in vivo maturation period, the 12 substitutes showed a rich vascular network, originating from the great omentum, from which they could not be divided. After silicone tube removal (group B), substitutes showed a flexible, 3-dimensional structure (Fig 1, D, E).

Pathologic analysis after in vivo maturation. HES staining of substitute samplings showed into the matrix a multilayered cell population (Fig 3, A, B) strongly positive for desmin staining (Fig 3, C, D). These cells were often organized in bundles. Some multinucleate fibers were observed. Pancytokeratin (anti-AE1/AE3) staining was negative in all samplings, suggesting that epithelial cells did not survive.
Clinical, endoscopic, and radiologic outcomes after esophageal replacement. All animals survived the operative procedure. Mean operative times were 105 minutes (±36), 100 minutes (±24), and 60 minutes (±6) for groups A, B, and C, respectively. There was no intraoperative incident. Data concerning weight changes of animals are reported Table II.

Group A (n = 6). In this group, 4 animals experienced stent complications during the first 6 months. Early stent folding was treated successfully by endoscopic repositioning in 1 animal. In the other 3 animals, stent migration at 1, 2, and 4 months lead to early stricture formation, endoscopic dilation, and new stenting. Two animals were killed at 3 months. One animal died after esophageal perforation owing to dilation at 4 months. The last 3 animals underwent stent removal at 6 months. At that time, endoscopy showed an ulcerated area in all animals (Fig 4, A). One animal developed a stricture at 7 months and was killed. The last 2 animals had an uneventful course and were killed at 9 and 12 months. At 12 months, there was no evidence of stricture and a macroscopically healthy mucosa was present (Fig 4, C). A barium swallow performed at that time showed a slight reduction of the esophageal lumen at the graft site, but no stricture (Fig 4, C). This animal gained 36 kg, an increase of 102% of its initial weight.

Group B (n = 6). All animals in this group developed a stricture 10–21 days after esophageal

### Table I. Antibodies used for immunostaining

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Provider</th>
<th>Dilution</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-desmin (Clone D33)</td>
<td>Dako</td>
<td>1/100</td>
<td>Myoblast, striated muscle and some smooth muscle cells</td>
</tr>
<tr>
<td>Anti-smooth muscle actin, α-isoform (Clone 1A4)</td>
<td>Dako</td>
<td>1/100</td>
<td>Myofibroblast and smooth muscle cells</td>
</tr>
<tr>
<td>Anti-cytokeratin (Clone AE1/AE3)</td>
<td>Dako</td>
<td>1/100</td>
<td>Epithelial cells</td>
</tr>
<tr>
<td>Proliferating Cell Nuclear Antigen (Clone PC10)</td>
<td>Dako</td>
<td>1/100</td>
<td>Proliferating epithelial cells</td>
</tr>
<tr>
<td>Polyclonal anti S 100</td>
<td>Dako</td>
<td>1/100</td>
<td>Axons, Schwann cells</td>
</tr>
<tr>
<td>Polyclonal Anti-von Willebrand factor</td>
<td>Dako</td>
<td>1/100</td>
<td>Endothelial cells</td>
</tr>
</tbody>
</table>

![Fig 3. Hematoxylin erythrosine saffron (HES) and immunohistochemistry (IHC) analysis of the substitute after a 2-weeks maturation period into the great omentum. A (original magnification, ×5), B (original magnification, ×10), HES. Multilayered cellular structure integrated into the small intestinal submucosa (SIS) matrix. C, (original magnification, ×2.5) D (original magnification, ×10), Desmin-positive cells organized in bundles (IHC).](image-url)
replacement. They all died within 2 months. Four animals were killed because of esophageal obstruction or stenosis refractory to dilation that occurred either initially or after 1 or 2 sessions of dilation. Two other animals were killed because of esophageal perforation during dilation.

**Group C (n = 6).** In all these animals except one, the stent was expelled either by mouth or by the cervicotony within 2 months after surgery. All were killed because of cervical cellulitis. One animal survived long term (12 months), thanks to 5 sessions of dilation and permanent stenting, but never recovered nutritional autonomy without a stent. This animal gained 6 kg, an increase of 14.2% of its initial weight.

**Graft area analysis.** **Macroscopic findings.** Macroscopic examination of the specimens in group A at all time points showed a patent lumen in the graft area. At 12 months, its macroscopic aspect was similar to that of the native esophagus (Fig 5, A, B).

**Table II. Surgical outcomes after circumferential esophageal replacement**

<table>
<thead>
<tr>
<th>Animals</th>
<th>Weight (kg)</th>
<th>Survival time (mo)</th>
<th>Status</th>
<th>Body weight change, kg (%)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>36</td>
<td>3</td>
<td>Planned death</td>
<td>+7 (+19.4)</td>
<td>Stricture*</td>
</tr>
<tr>
<td>A2</td>
<td>35</td>
<td>3</td>
<td>Planned death</td>
<td>+2 (+5.7)</td>
<td>Stricture*</td>
</tr>
<tr>
<td>A3</td>
<td>32</td>
<td>4</td>
<td>Killed</td>
<td>+7 (22)</td>
<td>Stricture* (perforation after dilation)</td>
</tr>
<tr>
<td>A4</td>
<td>31</td>
<td>7</td>
<td>Planned death</td>
<td>+11.5 (+37)</td>
<td>Remodeled</td>
</tr>
<tr>
<td>A5</td>
<td>38</td>
<td>9</td>
<td>Planned death</td>
<td>+11.5 (+50)</td>
<td>Remodeled</td>
</tr>
<tr>
<td>A6</td>
<td>36</td>
<td>12</td>
<td>Planned death</td>
<td>+56 (+102)</td>
<td>Remodeled</td>
</tr>
<tr>
<td>B1</td>
<td>36</td>
<td>1</td>
<td>Killed</td>
<td>−6 (−16.6)</td>
<td>Refractory stricture</td>
</tr>
<tr>
<td>B2</td>
<td>41</td>
<td>1</td>
<td>Killed</td>
<td>−5 (−12)</td>
<td>Refractory stricture</td>
</tr>
<tr>
<td>B3</td>
<td>40</td>
<td>2</td>
<td>Killed</td>
<td>−3 (−7.5)</td>
<td>Refractory stricture</td>
</tr>
<tr>
<td>B4</td>
<td>36</td>
<td>2</td>
<td>Killed</td>
<td>−6 (−16.6)</td>
<td>Refractory stricture</td>
</tr>
<tr>
<td>B5</td>
<td>43</td>
<td>2</td>
<td>Killed</td>
<td>−5 (−11.6)</td>
<td>Refractory stricture</td>
</tr>
<tr>
<td>B6</td>
<td>45</td>
<td>2</td>
<td>Killed</td>
<td>−6 (−13.3)</td>
<td>Refractory stricture</td>
</tr>
<tr>
<td>C1</td>
<td>40</td>
<td>1</td>
<td>Killed</td>
<td>−4 (−10)</td>
<td>Anastomotic leakage</td>
</tr>
<tr>
<td>C2</td>
<td>42</td>
<td>1</td>
<td>Killed</td>
<td>−5 (−12)</td>
<td>Anastomotic leakage</td>
</tr>
<tr>
<td>C3</td>
<td>45</td>
<td>1</td>
<td>Killed</td>
<td>−6 (−15.3)</td>
<td>Anastomotic leakage</td>
</tr>
<tr>
<td>C4</td>
<td>37</td>
<td>1</td>
<td>Killed</td>
<td>−5 (−13.5)</td>
<td>Anastomotic leakage</td>
</tr>
<tr>
<td>C5</td>
<td>39</td>
<td>1</td>
<td>Killed</td>
<td>−4 (−10.2)</td>
<td>Anastomotic leakage</td>
</tr>
<tr>
<td>C6</td>
<td>42</td>
<td>12</td>
<td>Planned death</td>
<td>+6 (+14.2)</td>
<td>Refractory stricture</td>
</tr>
</tbody>
</table>

*Stricture after early stent migration.

Fig 4. Endoscopic and radiologic examinations at 6 (A) and 12 months (B, C). Arrow indicates the graft area.
In group B, all specimens presented a stenosis or an obstruction of the graft area. In group C, absence of continuity was observed between the 2 ends of the esophagus, except in the animal that survived 12 months (data not shown).

**Histologic findings.** *Group A.* Figure 6 shows histologic findings at each time point, compared with the native esophagus (Fig 6, A–P). Development of a mature epithelium was the earliest event observed, as soon as 3 months after esophageal replacement (Fig 6, E–G). In parallel, the extent of fibrosis and of mononuclear infiltrate, as attested by HES and α-actin smooth muscle staining, decreased with time (Fig 6, A–C, M–O). At 7 months, structures resembling esophageal submucosal glands and desmin-positive cells were present (Fig 6, B, E, J). Histologic sections taken at different levels of graft area showed no connection between muscle cells and native muscle tissue (data not shown). An increase in the number of desmin-positive cells was observed with time, testifying the development of a muscular layer that adopted at 12 months a circular pattern (Fig 6, I–K). The number of nervous filaments increased during 12 months, whereas the von Willebrand factor staining was maximal at 7 months (Fig 7). Thus, 12 months after esophageal replacement, all the components of the native esophagus were present and organized, although not perfectly matched to the native architecture.

*Group B.* Analysis of all specimens of group B showed a strong inflammatory reaction, without any desmin-positive cells. An immature layer of epithelial cells was seen (data not shown).

**DISCUSSION**

In the last decade, tissue engineering has seen considerable progress and now allows the achievement of tracheal, bladder, urethra, vaginal, and skin replacements in humans.8-10,18,19 Regarding esophageal replacement, 4 patients recently underwent a successful patch esophagoplasty with a xenogeneic acellular scaffold.20 However, owing to unsatisfactory results of experimental studies, a circumferential replacement of the esophagus has not yet been successfully performed in humans.

In this model, circumferential replacement of the cervical esophagus by a hybrid substitute, composed of 2 different matrix and cell types, allowed under the temporary cover of an esophageal endoprothesis, recovery of nutritional autonomy and tissue remodeling towards an esophageal phenotype.

All animals except one in group C (endoprosthesis alone) experienced stent extrusion and early death. Although previous works have reported this issue, reflecting the poor biocompatibility of such material,21 this control group was mandatory to ensure the beneficial role of our substitute. Surprisingly, 1 animal in group C experienced long-term survival at the cost of permanent stenting. However, the poor functional condition of this animal was reflected by its meager weight gain at the end of the 12-month follow-up compared with its counterparts in group A (experimental group). In group B (substitute without stenting), the inability of the substitute to maintain a 3-dimensional tubular form autonomously resulted in collapse, stricture formation, and death of all the animals. An explanation for systematic stricture in this group could be the absence of a protective effect of the endoprosthesis against infection. However, the absence of a quantitative difference in terms of mononuclear cells infiltrate in early sacrifices between animals with (group A) and without stenting (group B; data not shown), does not support this hypothesis. Most probably, the stent serves as a calibration tool against a high propensity for stricture formation owing to an...
inflammatory reaction. We previously demonstrated that, during circumferential esophageal replacement by an aortic allograft, temporary esophageal stenting was essential for stricture prevention.\textsuperscript{22,23} Stenting was also mandatory for a leak-free healing of the 2 anastomosis between the aorta and the native esophagus. In the present study, anastomosis leakage in the absence of
Endoprothesis was anecdotal and this seems to be a step forward compared with the use of native tissue, such as the aorta, in that setting.

To prevent stricture, the duration of stent insertion has to be for a 6-month period at least. Nevertheless, 1 out of 3 animals under long-term follow-up developed a stricture after this delay. This high rate of stricture formation is an important drawback of the procedure, considering the frequency of stent migration in such a model. Moreover, it highlights the importance of inflammatory and fibrotic reactions, whose determinants are probably numerous.

Several studies have emphasized the beneficial effect of matrix cell seeding to reduce the inflammatory reaction and avoid systematic stenosis. Indeed, circumferential replacement of the esophagus by acellular scaffolds without cell seeding invariably leads to stricture and lack of function. In patch esophagoplasty models, the addition of muscle cells to the matrix lowers the inflammatory process, accelerates tissue regeneration and restores a better function. Badylak et al showed that covering an acellular scaffold with autologous muscle tissue avoids stricture formation after circumferential esophageal replacement. In our opinion, the use of myoblasts has several advantages over smooth and striated muscle cells, including a capacity to differentiate into striated muscle and to promote regeneration of damaged muscle fibers, autologous origin, defined ex vivo amplification, and lack of tumorigenicity owing to myogenic lineage restriction. We have demonstrated previously that autologous skeletal myoblast isolation, expansion, and transplantation was a feasible and straightforward procedure, and that myoblasts cultured on small intestinal submucosa preserve their ability to differentiate into striated muscle fibers. Although at the end of cell culture on small intestinal submucosa, myoblast did not enter the matrix, a large number of desmin-positive cells was observed into the matrix at the end of the maturation period into the great omentum. This testifies to the benefits brought by the use of this natural bioreactor in terms of cell survival, proliferation and organization. Surprisingly, it has not been possible to identify desmin-positive cells in the graft area early, regardless of the group of animals. However, in group A, such cells organized in multidirectional muscles fibers were observed after 7 months. At 12 months, a circumferential, unidirectional muscle layer was organized. This kinetic of desmin-positive cells may be explained by an early and massive muscle cell death occurring soon after esophageal replacement, owing to hypoxia, microenvironmental modifications between abdominal and cervical locations, or septic necrosis that likely played an important role in early cell loss at the cervical level. These results question the utility of myoblast seeding. However, Yamamoto et al evaluated whether omental pedicle wrapping could promote muscle cell regeneration after circumferential esophageal replacement with a nonseeded acellular collagen matrix, calibrated by a silicone tube in a dog model. All dogs died from esophageal stricture after tube removal. Compared with our results, this work argues in favor of a beneficial effect of myoblast seeding, even if their outcome remains unclear.

One hypothesis to muscle layer generation could be muscle cell migration from the edges of the native esophagus. However, no connection between desmin-positive cells into the graft area and the muscular layer of the native esophagus was observed at early time points. Survival of a small number of myoblasts seeded on scaffold giving rise to muscle fibers could be suggested. The tracing of

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**Fig 7.** Graft area explanted at 12 months. A, Proliferative basal epithelial layer (arrows). Proliferating cell nuclear antigen (immunohistochemistry [IHC]; original magnification, ×20). B, Multiple nerve filaments (arrows; PS100; IHC; original magnification, ×20). C, Multiple vessels (arrows; von Willebrand factor; IHC; original magnification, ×5).
seeded myoblasts using different cell markers, such as green fluorescent protein, was not performed in this study, but should be considered in future studies to investigate the contribution of the transplanted cells in esophageal tissue remodeling. Finally, transmutation of fibroblast progenitor cells and action of marrow derived mesenchymal stem cells, suggested by some authors, are still worth exploring.

In a canine model, Ohki et al showed that application of autologous epithelial cell sheet after extensive esophageal mucosectomy reduces local inflammation and enhances the regeneration of a mature epithelium. Epithelial cells also accelerate muscle regeneration in a patch esophageoplasty model and, when seeded on HAM, protect against stricture after circumferential esophageal replacement by a substitute composed of polyglycolic acid and smooth muscle cells. In our experience, despite the demonstration that epithelial cells survived on HAM in vitro, cytokeratin AE1/AE3–positive staining was undetectable at the end of the maturation period. Therefore, the theoretical early benefit brought by the presence of this cell component has been lost. However, a pluristratified epithelial layer was observed from month 2, partially covering the surface of the graft, probably reflecting cell migration to the graft area from the native esophagus, as observed after esophageal replacement by an allogeneic aorta.

The use of the bioreactor with the aim to create a tissue-engineered esophagus has been reported with varying success. Its theoretical role is to provide optimal physiologic conditions to avoid premature cell death and to stimulate tissue remodeling by inducing neovascularization. This is of particular importance in the case of the esophagus because of its location between avascular fascias, which compromises the revascularization of the substitute. Complex systems (ex situ bioreactor) mimicking the normal conditions of the organ function using hydro-aerodynamic stimuli were used in tracheal and lung regeneration in humans with conclusive results. However, the use of such systems is expensive and time consuming, major drawbacks for use in tissue engineering. Another strategy to address this issue is to cover the construct with a well-vascularized structure (eg, omentum) to provide timely and efficient vascularization. Hayashi et al showed that maturation of a complex substitute made of different cell types and collagen type I in rat muscle allowed the development of a mature and structured substitute, mimicking esophageal architecture, whereas no such development was seen after in vitro incubation for the same period of time. Such natural bioreactor has been used with success for urinary bladder replacement in humans.

The only limit to the use of the great omentum is the realization of an omentectomy during a previous surgery. In our preliminary experiments, the pedicled transplant was brought up to the neck retrosternally (data not shown). Significant operative morbidity (pneumothorax) and mortality (cardiac arrest and massive hemorrhage owing to innominate vein wound) led us to give preference to the subcutaneous route, in our model.

The small number of long-term surviving animals does not allow us to draw conclusions concerning the risks of the procedure versus the use of classical methods of esophageal replacement. The low long-term survival rate in the experimental group (substitute with endoprosthesis) was related to the design of the study, which necessitated sequential killing of experimental animals, to analyze the remodeling process toward an esophageal substitute. The actual goal of our study was not to immediately compete with the usual techniques of esophageal replacement in terms of clinical results, but to explore new alternatives. Thus, 5 out of 6 animals in the experimental group were killed as planned and there was only 1 unplanned death. We intend to repeat our experiments with a larger number of animals and a longer follow-up, to provide a reliable survival analysis of the technique, which was not the purpose of the present study.

Functional explorations of the substitute could have been beneficial in understanding the functionality of the new tissue. Elastometry and manometry could have been a source of information on such functional issue. These were not performed presuming that weight gain under classical diet suggested the presence of a functional substitute.

After esophageal replacement, 1, 2, or 3 additional procedures per animal were performed mostly for dilation after stent migration, with no distinction between groups. According to some authors, the use of an absorbable stent is associated with less migration and could be used in future experiments. On the other hand, absorbable stents present with inconveniences such as a short time to absorption (3 months) that could require a second stent insertion, and tissue hyperplasia that would increase the deleterious effect of the preexisting inflammatory reaction.

In this study, we demonstrated the functionality of a short circumferential replacement of the
esophagus by a tissue engineering approach. In parallel, the appearance of a histologic phenotype similar to that of the native esophagus was observed. Still, the results obtained to date are probably not transferrable to longer replacement of the esophagus. Development in scaffold designs and use of other cell types, such as multipotent or pluripotent cells, could help to reduce the inflammatory reaction and reach the expected results.

REFERENCES


