Stem Cell Transplantation Effectively Occludes Bronchopleural Fistula in an Animal Model

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Background. **Bronchopleural fistula after lung resection still represents a challenging life-threatening complication for thoracic surgeons. Considering its extremely high mortality rate, an effective treatment is urgently required. Our project investigated the hypothesis of experimental bronchopleural fistula closure by bronchoscopic injection of autologous bone marrow–derived mesenchymal stem cells into the cavity of the fistula, evaluating its feasibility and safety in a large animal model.**

Methods. **An experimental bronchopleural fistula was created in 9 goats after right upper tracheal lobectomy. The animals were randomly assigned to two groups: one received autologous bone marrow–derived mesenchymal stem cell bronchoscopic transplantation; the other received standard bronchoscopic fibrin glue injection.**

Results. **All animals receiving bronchoscopic stem cell transplantation presented fistula closure by extraluminal fibroblast proliferation and collagenous matrix development; none (0%) died during the study period. All animals receiving standard treatment still presented bronchopleural fistula; 2 of them (40%) died. Findings were confirmed by pathology examination, computed tomography, and magnetic resonance imaging.**

Conclusions. **Bronchoscopic transplantation of bone marrow–derived mesenchymal stem cells effectively closes experimental bronchopleural fistula by extraluminal fibroblast proliferation and collagenous matrix development. Stem cells may play a crucial role in the treatment of postresectional bronchopleural fistula after standard lung resection. Although these results provide a basis for the development of clinical therapeutic strategies, the exact mechanism by which they are obtained is not yet completely clear; further studies are required to understand exactly how stem cells work in this field.**

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Lung cancer is the leading cause of cancer death worldwide [1]. For patients with limited disease, surgical resection is the most effective method of controlling the primary tumor and provides the best opportunity for cure. Therefore every patient with non-small cell lung cancer is approached as a surgical candidate [2].

Postresectional bronchopleural fistula (BPF) is a pathological connection between the airway (bronchus) and the pleural space that may develop after lung resection. In lung cancer operations, the incidence of BPF ranges from 1% to 4%, but its mortality ranges from 12.5% to 71.2% [3]. Bronchopleural fistula may be caused by incomplete bronchial closure, impediment of bronchial stump wound healing, or stump destruction by residual neoplastic tissue [3].

The clinical effect of failure of the bronchial stump to heal after anatomic lung resection may culminate in a life-threatening septic and ventilatory catastrophe [4]. For many patients with empyema, the presence or absence of a fistula makes the difference among recovery, chronicity, or death [2].

We investigated experimental BPF closure by bronchoscopic injection of autologous bone marrow–derived mesenchymal stem cells (MSC) into the cavity of the fistula, evaluating its feasibility and safety in a large animal model.

Material and Methods

The choice of a goat model was based on the anatomical similarities of the pleural cavity and mediastinum with those of human beings to reproduce all the aspects of clinical BPF as closely as possible: infection, suture technique, vascular ligation, bronchial dissection, and bronchial dehiscence.

Technology

Animals were treated according the requirements of the European Union Directive 86/609 regarding the protection of animals used for experimental or other scientific

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purpose and the Council of Europe Convention for the protection of vertebrate animals used for experimental and other scientific purpose (ETS 123).

The present study was performed in nine 22- to 26-month-old female goats with a mean initial weight of 35 kg (±2.5 kg). Four animals entered the study group and 5 the control group. Each study group animal received a bone marrow biopsy of the iliac crest by a Snarecoil bone marrow biopsy needle (Ranfac Corp; Avon, MA). Autologous bone marrow–derived MSC were then isolated and expanded according to previous reports [5]. In detail, 10 mL bone marrow was aseptically collected in sterile heparinized tubes. The whole marrow washouts were layered onto Ficoll-Paque and centrifuged at 400*g* for 30 min at room temperature. A mononucleate cell ring was then removed and centrifuged then supplemented with ammonium chloride solution to eliminate red blood cells. Cells were cultured in Dulbecco's modified Eagle Medium with 20% fetal bovine serum with the addition of HEPES.

As MSC profiles vary among animals, the "stemness" of isolated cells was assessed by inducing osteogenic, chondrogenic, and adipogenic differentiation as described by Pittenger et al. [5], rather than immunogenic detection of antigens, differing from one animal to another. The same procedure was performed in the control group with medium and fibrin sealant delivery without MSC.

Cell growth in a fibrin substrate (Evicel; Ethicon; Somerville, NJ) was evaluated using MSC infected with a green fluorescent protein (GFP)-expressing lentiviral vector (Fig 1).

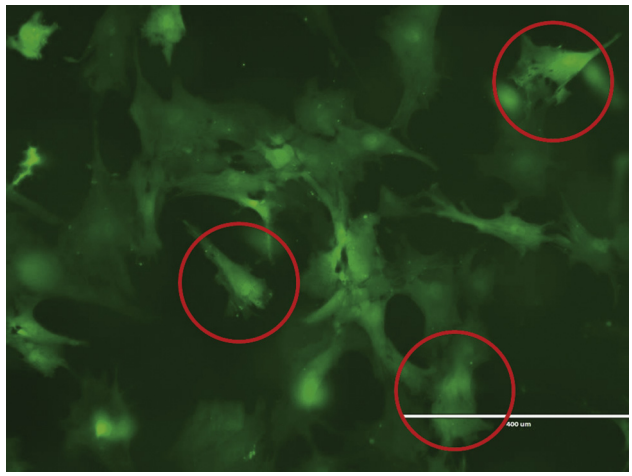

Fig 1. Mesenchymal stem cells infected with a green fluorescent protein (GFP)-expressing lentiviral vector (encircled) growing in a modified fibrin sealant substrate.

Technique

Standard right upper tracheal lobectomy was performed on a goat model under general anesthesia without the need for single lung ventilation.

Right lateral muscle-sparing thoracotomy was performed; mediastinal vessels for the upper lobe were isolated and then transected after double manual ligation. The right upper bronchus was then exposed, transected, and clamped close to the tracheal takeoff to avoid gas and oxygen leakage during intraoperative ventilation. Lobectomy was completed by fissure manual division similarly to human right upper fissureless lobectomy.

Bronchial stump closure was performed by single interrupted 3/0 polypropylene stitches (Prolene nonabsorbable monofilament; Ethicon; Somerville, NJ); the medial edge of the stump was left open and the caliber of the fistula homogenously created by a standard 4-mm caliber probe left inside the bronchial lumen at the time of last stitch application. Water submersion test under standard airway pressure of 15 cm H_2O was then performed to confirm the bronchial fistula and exclude other sites of air leakage.

Mean whole bronchial stump caliber was 10.1 ± 1 mm in the transplanted group and 9.9 ± 1 mm in the control group without any significant discrepancy among animals ($p > 0.05$), the created fistula thus accounting for almost one-third of the entire stump caliber. Then 24 Fr/Ch (8.0 mm) silicone tubular drainage (Kendall Argyle-Tyco Healthcare; Tullamore, Ireland) was placed and connected to a unidirectional Heimlich valve (Laboratoires Pharmaceutiques VYGON; Ecouen, France) before chest closure. The chest tube was routinely removed on postoperative day 1 because of the high risk of incidental removal during stable housing of the animals and consequent external infection. A "purse string" suture on the chest drain insertion orifice was then tied to work as a Heimlich valve in case of major air leaks to avoid tension pneumothorax.

On postoperative day 7 cervical tracheostomy was performed under general anesthesia; a fiberoptic bronchoscope was inserted, the bronchial stump was inspected, and the bronchial fistula identified. A transbronchial aspiration needle (Olympus SmoothShot 19G × 13 mm; Olympus; Zoeterwoude, The Netherlands) was inserted through the bronchoscope operative channel; 5 mL medium with modified fibrin glue (Evicel; Ethicon; Somerville, NJ) containing 2×10^6/mL MSC were injected into the fistula and in the submucosal aspect of the bronchial stump. The same procedure was performed in the control group with medium and fibrin glue delivery without MSC.

On postoperative day 28, all animals were euthanized by intravenous injection of pentobarbital sodium. Re-do ipsilateral thoracotomy was performed and the right tracheobronchial system was harvested and then frozen. Autoptic evaluation and pathology examination together with computed tomography (CT) and magnetic resonance imaging (MRI) were also performed on the specimens.

Results

Clinical Experience

All animals in both groups still presented BPF on postoperative day 7, at the time of operative bronchoscopy and injection of MSC or fibrin sealant alone, proven by

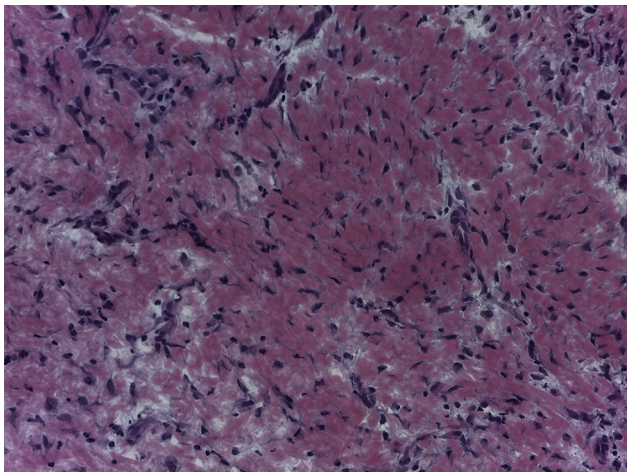

Fig 2. Hematoxylin-eosin staining (magnification 20×) of extraluminal fibroblast proliferation and collagenous matrix development, observed at the distal part of the bronchial stump in transplanted animals.

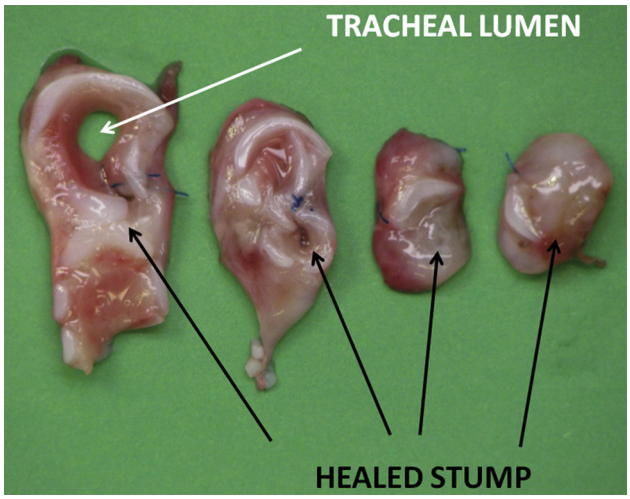

Fig 3. Gross images of specimens demonstrating healing of the bronchopleural fistula in animals receiving bronchoscopic stem cell transplantation.

standard endoscopic water test, confirming homogeneous BPF caliber persistence in both groups.

By postoperative day 28, all animals receiving autologous MSC bronchoscopic transplantation had BPF closure, which was evident by extraluminal fibroblast proliferation and collagenous matrix development (BPF closure rate: 100%) (Fig 2) and at necropsy (Fig 3); none of them (0%) died during the study period. Both MRI and CT scanning disclosed new peribronchial tissue occluding the bronchial stumps (Fig 4A). All control group animals still presented BPF after endoscopic treatment with fibrin sealant (BPF closure rate: 0%), and 2 of them (40%) died from pleural empyema. Magnetic resonance imaging and CT scanning (Fig 4B), as well as pathology examination (Fig 5) confirmed BPF persistence in control animals.

No tension pneumothorax was observed in either group after chest drain removal, probably because low-grade air leakage from the fistula was balanced by a high-volume residual cavity after lobectomy. In addition, a "purse string" suture on the chest drain insertion orifice after tube removal was tied to work as a Heimlich valve in case of major air leaks and thereby avoid tension pneumothorax. Further, the residual lung volume reexpansion, in conjunction with the characteristic hyperinosis of the animal species, could have limited the extent of the pneumothorax, but not the evolution of the fistula.

Two sample proportion tests disclosed a statistically significant difference between BPF closure rates (100% versus 0%; $p = 0.003$), whereas no difference was observed in mortality rates (0% versus 40%; $p = 0.15$), but this lack of difference in mortality rates may have been due to the small number of animals.

Comment

Bronchial stump dehiscence is still the most feared complication after curative lung resection [6]. For this reason the healing effects promoted by stem cells—by transformation into mature cells with a specialized function or by enhancing intrinsic repair mechanisms—may represent an effective and only partially explored therapeutic option [7, 8]. The mechanisms by which MSC induce tissue recovery are still widely debated, with cellular differentiation and paracrine effects being the two leading possibilities [9].

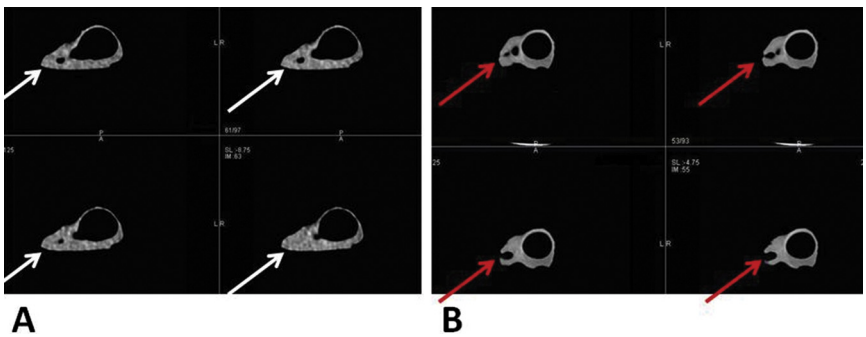

Fig 4. Computed tomography disclosed (A) peribronchial tissue occluding the bronchial stumps (white arrows) in animals receiving bronchoscopic stem cell transplantation and (B) persistent bronchopleural fistula (red arrows) in control animals.

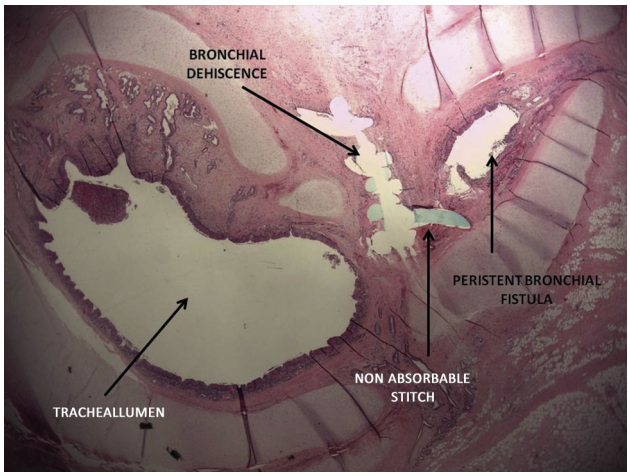

Fig 5. Persistent bronchopleural fistula in a control animal at pathology examination. Note bronchial dehiscence close to a nonabsorbable stitch and the typical tracheal takeoff of the right upper lobe of the goat (hematoxylin-eosin stain, magnification 10×).

Our experimental model showed that extraluminal fibroblast proliferation and collagenous matrix development in the animals receiving MSC transplantation effectively occluded BPF by tissue regeneration, thereby preventing an almost-always fatal pleural empyema. Computed tomography scanning and MRI of specimens confirmed extrabronchial tissue proliferation in transplanted animals, suggesting peribronchial tissue regeneration as the stem cell–induced reparative mechanism.

Assuming that this pilot study on animals might be extended to a larger cohort of animals and eventually to a clinical setting in the future, both MRI and CT scans were acquired. The aim was to establish which examination may be more helpful for an in vivo analysis, to show the internal and external aspect of the bronchus, in a similar way to the pathological specimen, without requiring animal sacrifice. Moreover, our specimen imaging results may offer a starting point for future in vivo stem cell engraftment assessment both by CT and MRI [10].

Clinical observation showed a clearly better outcome in the transplanted group, all animals being alive and without infection at the time of suppression. By contrast, 2 of the 5 control group animals died from pleural empyema, as disclosed at necropsy, thus confirming the clinical relevance of persistent BPF.

In conclusion, our data suggest that MSC targeted to BPF through submucosal bronchoscopic injection can promote tissue regeneration, thereby occluding bronchial stump dehiscence and preventing pleural empyema. If proven effective in human beings, the technique may serve as an effective mini-invasive approach to BPF treatment, thus representing a potential alternative to both early reoperation when surgical procedures are not feasible and "open window" thoracostomy.

Our study has several limitations. First, the population of this preliminary study is very small and more animals or a third control arm—where systemic-derived blood is mixed with the modified fibrin glue—are needed to provide a stronger statistical evaluation. Second, our animal model mainly represents an acute BPF scenario that may differ from a case of late chronic BPF presenting significantly more inflammatory symptoms. As far as we know, MSC may work effectively and even be boosted by a greater inflammatory response, but further detailed models need to be developed. Third, longer-term follow-up and additional air leakage monitoring are needed to establish the exact timing of successful air leakage cessation by stem cell differentiation.

Although these results provide a basis for the development of clinical therapeutic strategies, the exact mechanism by which they were obtained is not yet completely clear and further studies are required to understand exactly how stem cells work in this field.

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References

1. Wakelee HA, Chang ET, Gomez SL, et al. Lung cancer incidence in never smokers. J Clin Oncol 2007;25:472–8.
2. Patterson GA, Cooper JD, Deslauires J, Lerut AEM, Luketich JD, Rice TW. Pearsons's Thoracic and Esophageal Surgery. 3rd ed. London, UK: Churchill Livingstone.
3. Sonobe M, Nakagawa M, Ichinose M, Ikegami N, Nagasawa M, Shindo T. Analysis of risk factors in bronchopleural fistula after pulmonary resection for primary lung cancer. Eur J Cardiothorac Surg 2000;18:519–23.
4. Ponn RB. Complications of pulmonary resection. In: Shields TW, LoCicero TW J III, Ponn RB, Rusch VW, eds. General Thoracic Surgery. 6th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2004:554–86.
5. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143–7.
6. Gomez-de-Antonio D, Zurita M, Santos M, Salas I, Vaquero J, Varela A. Stem cells and bronchial stump healing. J Thorac Cardiovasc Surg 2010;140:1397–401.
7. Wu Y, Chen L, Scott PG, Tredget EE. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. Stem Cells 2007;25:2648–59.
8. Mellough CB, Sernagor E, Moreno-Gimeno I, Steel DH, Lako M. Efficient stage-specific differentiation of human pluripotent stem cells toward retinal photoreceptor cells. Stem Cells 2012;30:673–86.
9. Fujimoto Y, Abematsu M, Falk A, et al. Treatment of a mouse model of spinal cord injury by transplantation of human induced pluripotent stem cell-derived long-term self-renewing neuroepithelial-like stem cells. Stem Cells 2012;30:1163–73.
10. Bulte JW. Science to practice: can stem cells be labeled inside the body instead of outside? Radiology 2013;269:1–3.