学位論文

Autologous multilayered fibroblast sheets can reinforce bronchial stump in a rat model

（ラット動物モデルにおける自家積層線維芽細胞シートがもたらす気管支断端の補強効果）

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学位論文の関連論文の研究背景及び要旨

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〔題名〕

Autologous multilayered fibroblast sheets can reinforce bronchial stump in a rat model
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〔研究背景〕

肺切除術後の気管支断端瘻は重篤な術後合併症であり、肺葉切除の 0.5-3%、肺全摘の 2-20%に生じるとされる。そのリスク因子として低栄養、糖尿病、ステロイド療法、放射線療法、化学療法、気管支周囲のリンパ節郭清などが報告されていることから、その発生には気管支断端の血流低下による不十分な組織治癒が関係していると推察される。そのため、気管支断端瘻を予防するために肋間筋や大網、心膜などで気管支断端を被覆する手技が普及しているが、その効果は不明であり、真に有効な気管支断端瘻の予防法の開発は重要な課題である。

これまでに当科では、創傷治癒に重要な役割を果たす線維芽細胞を積層化させることで作製した積層線維芽細胞シートをマウス皮膚潰瘍モデルに移植することにより、移植された積層線維芽細胞シートから分泌される Vascular endothelial growth factor (VEGF）などがパラクライン効果により創傷治癒を促進させることを報告している。

そこで、積層線維芽細胞シートをラット気管支断端モデルに移植することによる気管支断端の補強効果を検証し、気管支断端瘻の新規予防法を開発することを目指すこととした。

〔要旨〕

【方法】

Wister/ST ラットに左肺全摘を行うことで気管支断端モデルを作製した。口腔粘膜組織から線維芽細胞を単離し、24well プレートに 5.0×10^5 個/well を播種して 72 時間培養することで積層線維芽細胞シートを作製した。積層線維芽細胞シートの移植による気管支断端の補強効果を検討するため、術後 7、14、28 日目に標本を摘出し、細胞シート移植の有無による 2 群間での気管支断端の変化を肉眼的、組織学的、力学的に比較した。

また、細胞シートによる組織修復のメカニズムを検証するために、創傷治癒に関わる成長因子、サイトカインを細胞シート作製時の培養液上清を用いた Enzyme-linked immunosorbent assay (ELIZA)法で in vitro に測定した。更に、Green fluorescent protein (GFP)遺伝子を導入した線維芽細胞で作製した細胞シートを移植し、移植した細胞の残存性を検証した。

【結果】

作製した積層線維芽細胞シートは直径 6 mm であった。線維芽細胞が 4-5 層に積層し、コラーゲン線維を含む細胞外マトリックスを保持した状態で回収された。

非移植群では、術後 7、14、28 日目のいずれのタイミングでも、気管支断端の閉鎖に用いた縫合糸が露出していた。一方で、移植群の気管支断端は新生組織で広く被覆されていた。なお、細胞シートの移植の有無に関わらず、標本摘出までの期間に気管支断端瘻が生じた個体はなかった。摘出した標本を組織学的に観察すると、非移植群の気管支断端周囲にはごく僅かな結合組織が形成されたのみであったが、移植群では気管支断端周囲に多量の結合組織が形成されていた。また、移植群に生じた気管支断端周囲の結合組織が徐々に成熟すること（Azan 染色）、その組織に多くの血管構造が含まれていること（抗 CD31 抗体による免疫染色）が観察された。新生組織を含む気管支壁の厚み、それに含まれる血管構造を定量化したところ、いずれのタイミングでも移植群の気管支壁が有意に厚く、より多くの血管構造を含んでいた。また、気管支断端の補強効果を力学的に検証するために、気管支断端の耐圧能を評価したところ、非移植群では全ての標本で気管支断端からエアリークが生じた。一方、移植群では 300 mmHg までの加圧によってエアリークが生じた標本はなく、その耐圧値は移植群で有意に高かった。

細胞シート作製時の培養液上清を用いた ELIZA 法では、VEGF、Hepatocyte growth factor (HGF)、C-X-C motif chemokine ligand 1 (CXCL1)、Angiopoietine-2、Monocyte chemoattractant protein-1 (MCP-1)、Transforming growth factor beta 1 (TGF-β)が細胞シートから分泌されていることが示された。

GFP 遺伝子導入積層線維芽細胞シートを移植したところ、術後 3 日目の時点では GFP 遺伝子導入線維芽細胞が気管支断端に残存していることが確認されたが、7、14 日目には GFP 遺伝子が発現した細胞は確認されなかった。

【考察】

気管支断端は僅かに新生された結合組織でのみ覆われることが報告されており、本研究においても、非移植群の気管支断端の周囲には僅かな結合組織が形成されたのみであった。一方、積層線維芽細胞シートを移植することにより、気管支断端の周囲に多くの血管構造を含んだ結合組織が形成され、力学的にも気管支断端の強度が増していることが示された。気管支断端瘻は、術後 1 週間から 3 ヶ月、特に 10 日目前後に多く発生するとされていることから、細胞シートの移植後 7 日目の時点で、気管支壁がより厚く、血管構造に富み、優れた耐圧性を獲得していることは、気管支断端瘻の予防にとって十分に効果的な可能性がある。

積層線維芽細胞シートにより気管支断端が補強される機序を解明するため、細胞シートが分泌する成長因子やサイトカインの測定、GFP 遺伝子導入細胞シートを用いた検証を行ったが、移植した GFP 遺伝子導入細胞は術後 3 日目には残存していたが、7 日目の時点では確認できなかった。このことは、移植した線維芽細胞が生存、増殖して結合組織を形成するのではなく、細胞シートの移植が宿主の組織治癒を促進させている可能性を示している。VEGF、HGF や TGF-β などの成長因子、サイトカインが細胞シートから分泌され、これらに宿主に働きかけることで、気管支断端周囲での組織形成や血管新生が促進されたと推察される。

積層線維芽細胞シートの移植後 7 日目の時点で、気管支断端周囲に血管新生を伴った結合組織が形成され、力学的な補強効果があることが示された。積層線維芽細胞シートの移植は、局所

の血流低下による組織治癒遅延が原因とされる気管支断端瘻に対して有効な予防法となる可能性がある。

【結語】積層線維芽細胞シートの移植による気管支断端の補強効果が示された。本法は、気管支断端瘻の有効な予防法となる可能性がある。

Autologous Multilayered Fibroblast Sheets Can Reinforce Bronchial Stump in a Rat Model

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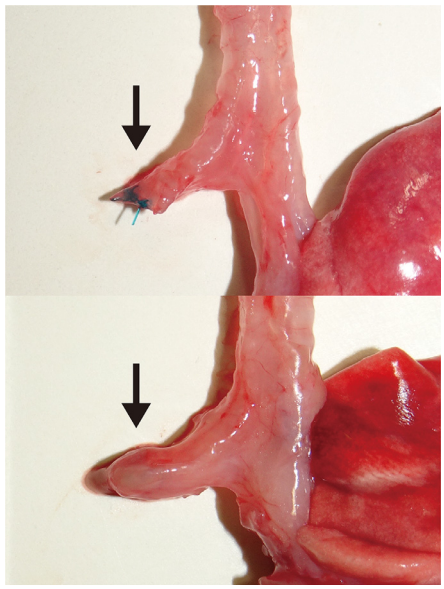
Bronchopleural fistula is one of the most serious postoperative complications caused by the incomplete healing of a bronchial stump. Fibroblasts play an important role in wound healing by facilitating connective tissue formation and inducing angiogenesis. We developed a method for production of multilayered fibroblast sheets that secreted some growth factors and promoted wound healing. The present study aimed to assess the treatment effect of multilayered fibroblast sheets on bronchial stump healing. In this rat model, left pneumonectomy was performed, and multilayered fibroblast sheets derived from autologous oral mucosal tissues were transplanted to the bronchial stump. The changes in the bronchial stump were examined macroscopically, histologically, and mechanically. The fibroblast sheets promoted the formation of thick connective tissues around the bronchial stump. The formed connective tissues were accompanied by new blood vessels, and fibrosis was observed over time. Then, 7 days after the transplantation of the fibroblast sheets, the bronchial wall became significantly thicker, and the area of the blood vessels for the bronchial wall tissues was significantly larger in the experimental group than in the control group. In addition, the burst pressure in the bronchial stump was significantly higher in the experimental group than in the control group. Bronchial stumps were reinforced by the transplantation of multilayered fibroblast sheets derived from autologous oral mucosal tissues.

Semin Thoracic Surg ■■:■■–■■

Keywords: Bronchopleural fistula, Bronchial stump, Fibroblast, Cell sheet, Tissue formation, Angiogenesis



The bronchial stump was coated with connective tissues by transplanting fibroblast sheets.

Central Message

Transplantation of multilayered fibroblast sheets derived from autologous oral mucosal tissues can reinforce bronchial stump with angiogenesis and tissue thickness.

Perspective Statement

Multilayered fibroblast sheets promoted connective tissue formation around the bronchial stump. From the perspective of bronchial stump healing, the transplantation of autologous fibroblast sheets might be a reasonable method that can prevent bronchopleural fistula, which is a serious complication of anatomical lung resection.

Abbreviations: BPF, Bronchopleural fistula; VEGF, Vascular endothelial growth factor; GFP, Green fluorescent protein; DMEM, Dulbecco's Modified Eagle's Medium; FBS, Fetal bovine serum; HGF, Hepatocyte growth factor; CXCL1, C-X-C motif chemokine ligand 1; MCP-1, Monocyte chemoattractant protein-1; TGF-β1, Transforming growth factor beta 1

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Conflicts of Interest: None.

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INTRODUCTION

Bronchopleural fistula (BPF) is one of the most serious postoperative complications and is sometimes fatal. The incidence rates of BPF are 0.5–3% in lobectomy and 2–20% in pneumonectomy, and the mortality rate is 18–50%.[1,2] Some retrospective studies demonstrated that the risk factors of BPF

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are malnutrition, diabetes, chronic obstructive lung disease, steroid therapy, radiation therapy, chemotherapy, right-sided surgery, and lymph node dissection.[2-5] Therefore, low blood flow to the bronchial stump may contribute to poor healing and BPF formation.

Currently, thoracic surgeons try to prevent BPF by covering the bronchial stump with autologous tissues, such as intercostal muscle,[6] omentum,[7] and pericardial flap.[8] Although these tissues may stimulate blood flow to the bronchial stump, their efficacy is unclear. Consequently, there is an urgent need to establish an effective procedure to enhance bronchial stump healing.

Recent studies have reported the effect of tissue repair via cell transplantation on various organs, and clinical trials have been conducted.[9,10] In some experiments, the local administration of bone marrow stromal cells or adipose-derived stem cells was effective in healing bronchial stumps in rat model.[11,12] A previous study showed that mixed sheets comprising fibroblasts and peripheral blood mononuclear cells secrete a large volume of vascular endothelial growth factor (VEGF) in vitro, and promoted angiogenesis and wound healing in a mouse skin ulcer model and rabbit lower limb ischemia model.[13,14] Furthermore, we developed a method of producing multilayered fibroblast sheets to increase the number of cells and improve ease of handling, and it proved to be effective in a mouse skin ulcer model.[15,16]

We hypothesized that transplantation of multilayered fibroblast sheets promotes bronchial stump healing by inducing angiogenesis at the bronchial stump. Fibroblasts play an important role in wound healing, and these cells have advantages over other cell sources as they can be easily obtained. The present study assessed the treatment effect of transplanting multilayered fibroblast sheets on bronchial stump healing in a rat model.

MATERIALS AND METHODS

Animals

A total of 52 male Wister/ST rats (age: 6 weeks, weight: 260−280 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and were divided into 3 groups (n = 20, control group; n = 20, cell sheet group; and n = 12, green fluorescent protein (GFP) cell sheet group). Surgery was performed at 8 weeks of age in the control group. Since the period from tissue collection to cell sheet preparation was 1 week, oral mucosal tissue was collected at 7 weeks of age, and surgery and transplantation were performed at 8 weeks of age in the cell sheet group. Since it took another week to introduce the GFP gene, oral mucosal tissue was collected at 6 weeks of age, and surgery and transplantation were performed at 8 weeks of age in the GFP cell sheet group. All animal experiments were approved by the Institutional Animal Care and Use Committee of Yamaguchi University (#31-003). The study was conducted in accordance with the relevant guidelines.

General Anesthesia

After induction of anesthesia with 5% isoflurane, tracheal intubation was performed with a 16-gauge ethylene-tetra fluoroethylene catheter. Then, the rats were connected to a ventilator (Model 683 small animal ventilator: Harvard, MA, USA). The tidal volume was set to 10 mL/kg; the respiratory rate was set to 70 breaths/min. Anesthesia was maintained with 2% isoflurane.

Isolation of Fibroblasts

The oral mucosal tissue was minced and incubated in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), 5% collagenase, and 1% penicillin-streptomycin overnight. After centrifugation, the supernatant was removed, and tissues were cultured in DMEM supplemented with 10% FBS for 48 hours. The cells were collected with 0.05% trypsin-ethylenediaminetetraacetic acid, and the tissue components were removed using a 40-μm cell strainer. The collected cells were evaluated for composition by flow cytometry (NovoCyte; ACEA Biosciences, CA) using anti-CD90 antibody (ab225; Abcam, Cambridge, UK), anti-CD56 antibody (ab9272; Abcam), and anti-mouse IgG1 antibody (ab18443; Abcam) for isotype control. Goat anti-mouse IgG (H+L) highly cross-adsorbed secondary antibody Alexa Fluor 488 (A-11029, Thermo Fisher Scientific) was used as secondary antibody.

Preparation of Multilayered Fibroblast Sheet

The cell sheets were prepared using the previously reported method,[15,16] 5.0×10^5 fibroblasts were cultured in a 24-well culture dish with 2 mL of DMEM supplemented with 10% FBS. After incubation for 24 hours, the medium was replaced with 2 mL of CTS AIM V (Thermo Fisher Scientific) and HFDM-1 (+) (Cell Science & Technology Institute, Sendai, Japan) supplemented with 5% FBS. After additional incubation for 48 hours, the cell sheets were detached using dispase.

Preparation of Multilayered Fibroblasts Sheet Introduced GFP Gene

The GFP gene was introduced into the fibroblasts using lentivirus particles prepared using the method previously reported.[16] The fibroblasts were incubated for 72 hours in DMEM supplemented with 10% FBS containing the lentiviral particles with TransDux MAX Transduction Reagent (#LV860A-1; System Biosciences). The rate of GFP-positive cells was evaluated for composition by flow cytometry. The cell sheets were prepared using the method described above.

Left Pneumonectomy

Left thoracotomy was performed at the fourth intercostal space. The left pulmonary artery and vein were ligated with 8-0 polypropylene suture and dissected. The tissues surrounding the left bronchus between the tracheal bifurcation and first bifurcation of the left bronchus was thoroughly removed. The

central side of the left main bronchus was blocked with the clip (KN353; Natsume, Tokyo, Japan) and was cut off. Dissection of the left bronchus was performed at the center of one cartilage ring from the first bifurcation, and the bronchial stump was closed by suturing 2 cartilage rings away from the dissection with 2 interrupted 7-0 polypropylene sutures. After removing the clip, the chest cavity was filled with saline to confirm the absence of air leak in the bronchial stump (Video 1). All surgeries were performed by 1 surgeon (S.Y.).

Transplantation of Multilayered Fibroblast Sheets

Two multilayered fibroblast sheets were transplanted to the bronchial stump, one on the bronchial cartilage side and the other one on the membranous portion side. The sheets were transported from 24-well culture dish to the forceps' handle using the 1000 μL tip with the edge cut and transplanted by affixing to the bronchial wall for 5 minutes.

Histological Analysis

The cell sheet was placed on a raw ham to prepare tissue sections. At 7, 14, and 28 days postoperatively, 4 rats each from the control and cell sheet groups were sacrificed and histologically evaluated. At 3, 7, and 14 days postoperatively, 4 rats in the GFP sheet groups were sacrificed and the survival of the transplanted cells was evaluated. All specimens were fixed in 10% formalin neutral buffer solution and embedded in paraffin. Then, the tissue sections (3 μm thickness) were used. Hematoxylin and eosin staining and Azan staining, which stains the collagen fibers within fibrous connective tissue (blue), were performed. The area of the bronchial wall on the membranous side containing the formed tissue was evaluated within 3000 μm from the stump. In immunohistochemistry, deparaffinized sections were treated with anti-vimentin antibody (ab92547; Abcam), a marker of mesenchymal-derived cells or cells undergoing an epithelial-to-mesenchymal transition, and anti-CD31 antibody (ab182981; Abcam), an endothelial marker in paraffin sections that stains small and large vessels. Three fields (100 ×) were randomly extracted from each section, and the blood vessel area in the lumen surrounded by anti-CD31 antibody-positive cells and bronchial wall area were measured. The blood vessel area per 100 μm^2 of the bronchial wall area was calculated. In fluorescent immunostaining, deparaffinized sections were treated with anti-GFP antibody (#2956, Cell Signaling, Danvers, MA), anti-vimentin antibody (ab8069; Abcam), anti-rabbit IgG secondary antibody (ab97050; Abcam), and anti-mouse IgG secondary antibody (Alexa555, A-21422, Thermo Fisher Scientific). All histological images were captured using the BZ-X710 microscope and analyzed using the Image J software (National Institutes of Health). All histological analyses were performed in a blind manner and supervised by a pathologist (E.I.).

Evaluation of Pressure Resistance

Four rats each from the control and cell sheet groups were sacrificed 7 and 14 days postoperatively. After collecting the trachea-lung block and inserting a 16-gauge ethylene-tetra fluoroethylene catheter into the trachea, the trachea and the right main bronchus were ligated with 3-0 silk, connected to an aneroid sphygmomanometer (HT-1500; NISSEI, Gunma, Japan), and pressurized using a 10 mL syringe. The specimen was submerged in saline, and the burst pressure was defined as the pressure at which the bronchial stump failed, and air bubbles were observed. The measurement limit value was 300 mm Hg.

Enzyme-Linked Immunosorbent Assay

The concentrations of VEGF, hepatocyte growth factor (HGF), C-X-C motif chemokine ligand 1 (CXCL1), angiopoietine-2, monocyte chemotactic protein-1 (MCP-1), and transforming growth factor beta 1 (TGF-β1) in the cell culture supernatant of the cell sheet were measured using the enzyme-linked immunosorbent assay kits (R&D Systems, Inc., Minneapolis, Minnesota), according to the manufacturer's instructions. CTS AIM V and HFDM-1 (+) supplemented with 5% FBS were used as controls.

Statistical Analysis

The results are presented as mean ± standard deviation. All statistical analyses were performed using STATA/IC version 16 (StataCorp, College Station, TX,). Statistical significance between 2 groups was assessed using the2-tailed unpaired *t*-tests. Statistical significance among multiple groups was analyzed using one-way analysis of variance, followed by the Tukey-Kramer post-hoc test. A *P*-value of < 0.05 was considered significant.

RESULTS

Multilayered Fibroblast Sheet

Particularly, 2.0×10^6 cells were obtained per rat (Fig. 1A), and the purity was confirmed by flow cytometry (Fig. 1B). The sheet was collected while keeping its circular shape (Fig. 1C). The sheet was 20−30-μm thick, the fibroblasts were laminated in 4−5 layers (Fig. 1D), and the extracellular matrix containing collagen fibers were retained (Fig. 1E). All cells comprising the sheet were positive for anti-vimentin antibody (Fig. 1F).

Macroscopic Evaluation

None of the rats died postoperatively, and there was no adhesion to the chest wall or pus in the thoracic cavity. At 7, 14, and 28 days postoperatively, the bronchial stump almost remained exposed in the control group. However, tissue formation was promoted mainly around the bronchial stump in the cell sheet group (Fig. 2).

Histopathologic Evaluation

Although only a small amount of tissue formed around the bronchial stump in the control group, more tissues formed in the cell sheet group (Fig. 3A). Azan staining revealed that the connective tissues matured with time after surgery in the cell

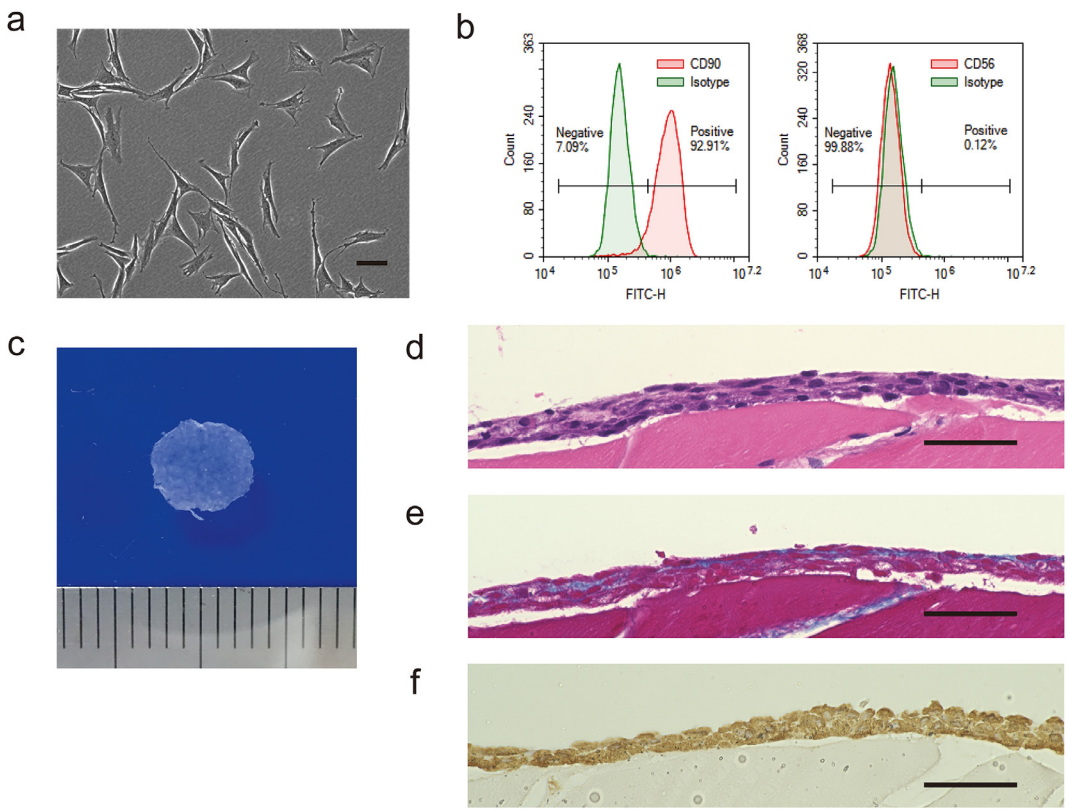

Figure 1. Multilayered fibroblast sheet. Flow cytometry confirmed that almost all cells collected from the oral mucosa were fibroblasts. The cell sheet had a diameter of approximately 6 mm and a thickness of approximately 20 μm, and fibroblasts were laminated. (A) Image of collected cells. (B) Flow cytometry. (C) Macroscopic image. (D) Hematoxylin and eosin staining. (E) Azan staining. (F) Immunostaining with anti-vimentin antibody. The bar indicates 50 μm.

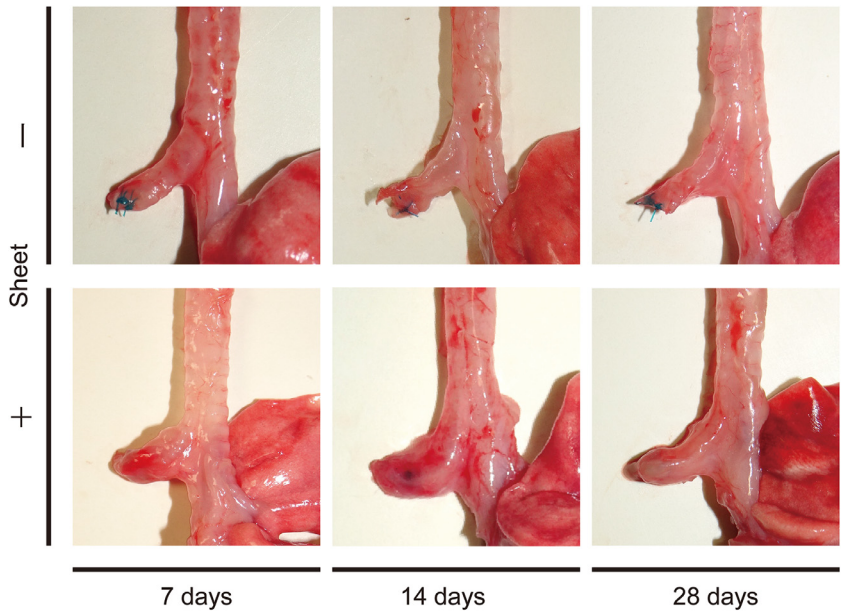

Figure 2. Macroscopic changes in the bronchial stump. The bronchial stumps almost remained exposed in the control group. However, tissue formation was promoted mainly around the bronchial stump in the cell sheet group. Since the membranous side was the front, the left side of the photograph was the left side of the specimen.

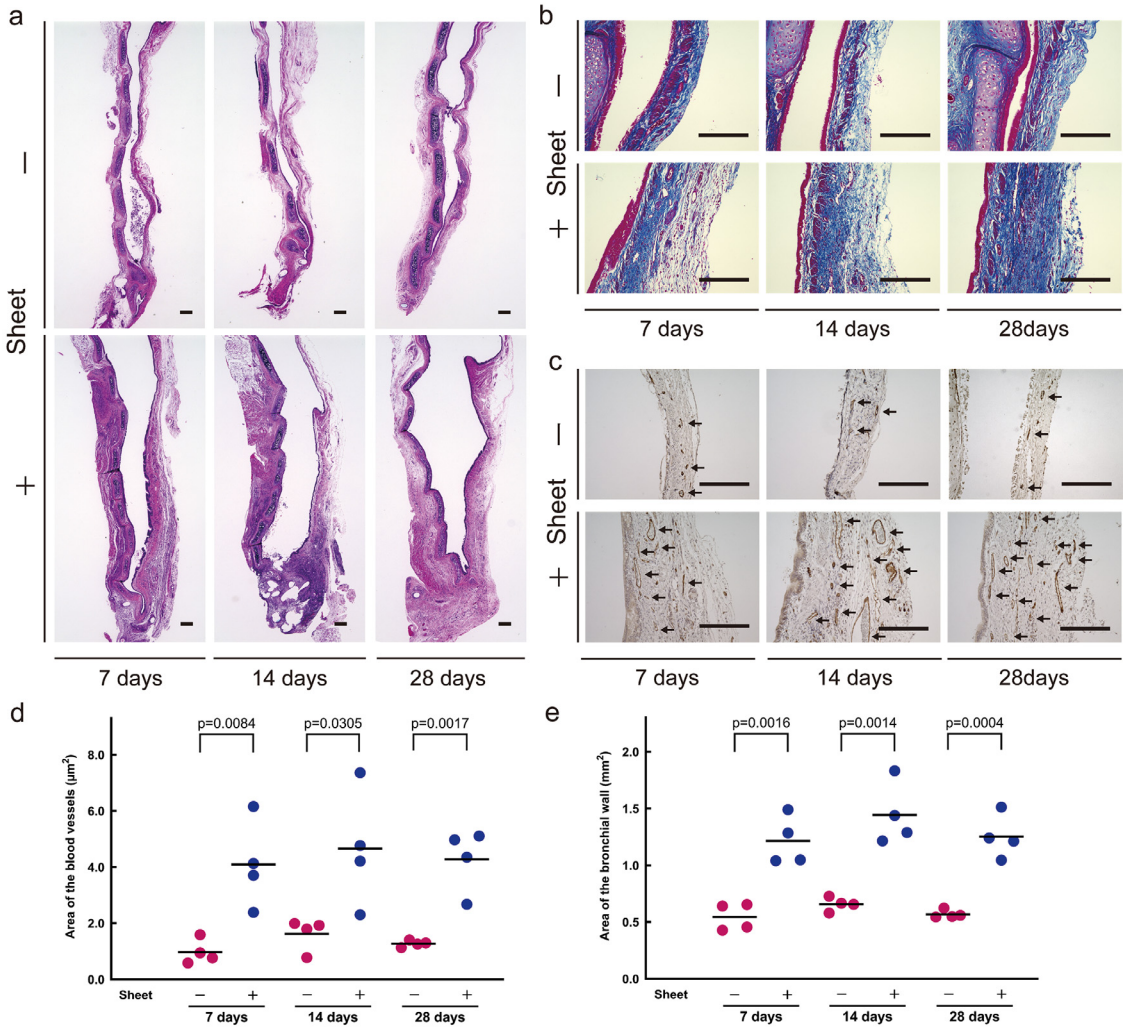

Figure 3. Histological changes in the bronchial wall. The bronchial wall on the membranous side was significantly thicker and had more abundant blood vessel area in the cell sheet group than in the control group. (A) Hematoxylin and eosin staining. (B) Azan staining and (C) Immunohistochemistry with anti-CD31 antibody of the bronchial wall on the membranous side. The arrow indicates blood vessels. The bar indicates 200 μm. (D) The thickness of the bronchial wall and (E) The blood vessel area in the bronchial wall on the membranous side. The horizontal lines indicate mean.

sheet group (Fig. 3B). Immunostaining with anti-CD31 antibody revealed that the connective tissues were accompanied by angiogenesis (Fig. 3C). The areas of the bronchial wall on the membranous side in the control and cell sheet groups were 0.54 ± 0.12 and 1.21 ± 0.21 mm^2 (P = 0.0016, 7 days), 0.65 ± 0.06 and 1.44 ± 0.27 mm^2 (P= 0.0014, 14 days), and 0.56 ± 0.03 and 1.25 ± 0.19 mm^2 (P = 0.0004, 28 days), respectively. The bronchial wall on the membranous side was significantly thicker in the cell sheet group than in the control group (Fig. 3D). The blood vessel areas per 100 μm^2 of the bronchial wall area in the control and cell sheet groups were 0.97 ± 0.43 and 4.09 ± 1.56 μm^2 (P = 0.0084, 7 days), 1.61 ± 0.56 and 4.66 ± 2.08 μm^2 (P= 0.0305, 14 days), and 1.27 ± 0.11 and 4.27 ± 1.11 μm^2 (P = 0.0017, 28 days), respectively. The area of the blood vessel for bronchial wall tissues was significantly larger in the cell sheet group than in the control group (Fig. 3E). There was no significant difference in terms of the bronchial wall thickness on the membranous side and the area of the blood vessel for bronchial wall tissues in each group with time after surgery.

Evaluation of Pressure Resistance

In the control group, air leak occurred with pressurization in all bronchial stumps. In the cell sheet group, no air leak occurred with to pressurization up to 300 mm Hg (Video 2). The burst pressures in the control and cell sheet groups were 165.5 ± 40.2 and >300 mm Hg (P = 0.0005, 7 days) and 167.5 ± 22.2 and >300 mm Hg (P< 0.0001, 14 days), respectively (Fig. 4). There was no significant difference in the burst pressure in each group with time after surgery.

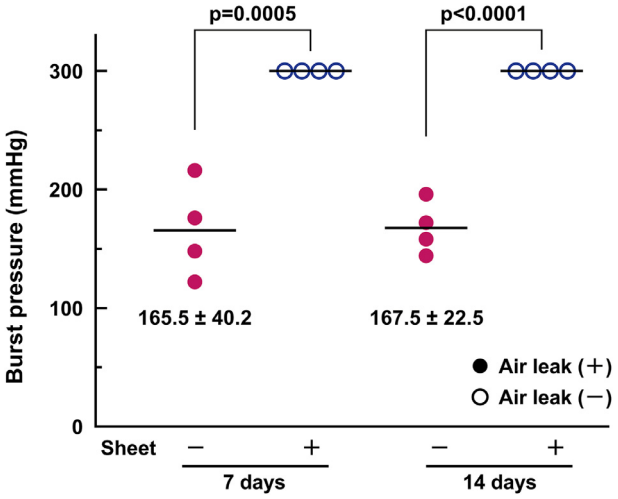

Figure 4. Comparison of burst pressure values in the bronchial stump. In the cell sheet group, no air leak occurred due to pressurization up to 300 mm Hg. The burst pressure was significantly higher in the cell sheet group. The horizontal lines indicate mean.

Secretion of Growth Factors in Vitro

The condition medium incubated for 48 hours after medium change contained 518.8 ± 28.0 pg/mL of VEGF, 1455.6 ± 42.8 pg/mL of HGF, 321.5 ± 19.9 pg/mL of CXCL1, 158.6 ± 20.7 pg/mL of angiopoietin-2, 87.5 ± 9.7 pg/mL of MCP-1, and 387.1 ± 16.7 pg/mL of TGF-β1 (Fig. 5). The fresh medium did not contain much VEGF, HGF, CXCL1, angiopoietin-2, MCP-1, and TGF-β1 (171.2 ± 6.6 pg/mL).

Analysis of Whether Transplanted Fibroblasts Remain on the Host

Expression of the GFP gene was confirmed in most fibroblasts, but a few fibroblasts did not express GFP (Fig. 6A and 6B). GFP-positive cells were detected 3 days postoperatively. However, they were not observed 7 and 14 days postoperatively (Fig. 6C).

DISCUSSION

The bronchial stumps were reinforced only by a small amount of newly formed connective tissue in addition to mechanical closure with sutures[17]. In the control group, air leakage occurred due to pressurization in all bronchial stumps, and there was no difference between the pressure values at 7 and 14 days postoperatively, whereas air leakage did not occur in the cell sheet group. Clinically, the intrathoracic pressure reaches 300 mm Hg due to coughing,[18] therefore, the mechanical reinforcement with tissues formed by transplantation of the cell sheets may be effective in preventing air leakage from a bronchial stump. In addition to mechanical reinforcement,

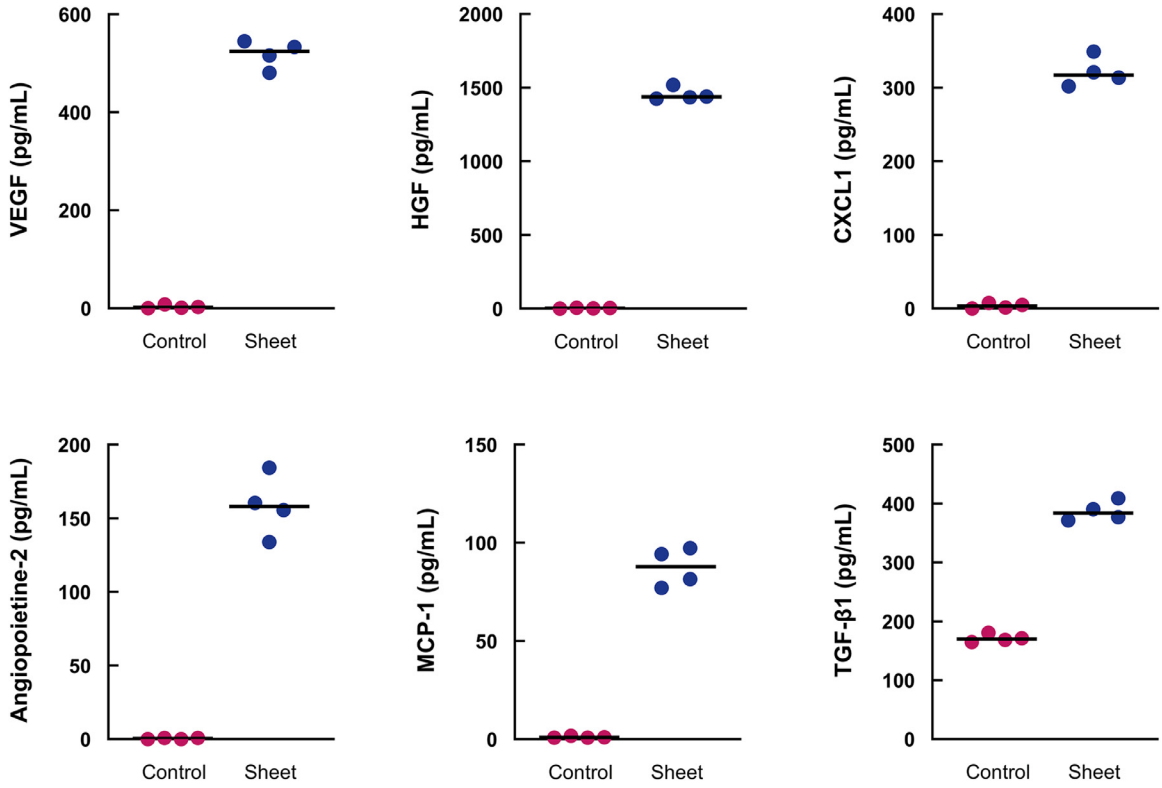

Figure 5. The concentration of growth factors and cytokines secreted by the multilayered fibroblast sheets in vitro. The condition medium incubated for 48 hours after medium change contained a great volume of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), C-X-C motif chemokine ligand 1 (CXCL1), angiopoietin-2, monocyte chemoattractant protein-1 (MCP-1), and transforming growth factor beta 1 (TGF-β1). The horizontal lines indicate mean.

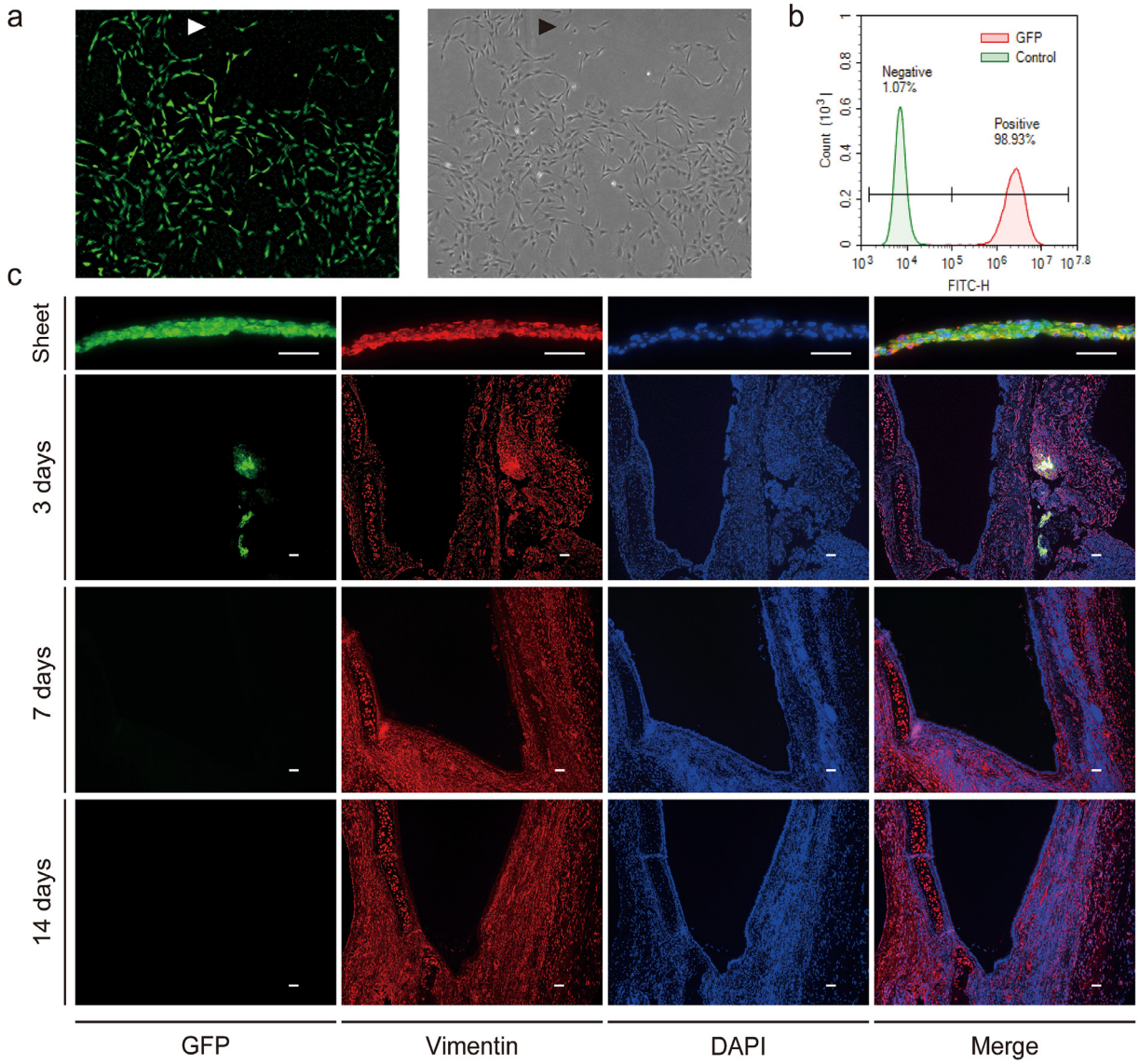

Figure 6. Tracking for the transplanted green fluorescent protein (GFP)-expressing cells. Anti-GFP antibody was used for detecting transplanted GFP-expressing cells and anti-vimentin antibody was used for detecting fibroblast. GFP-expressing cells were detected 3 days postoperatively. However, they were not observed 7 and 14 days postoperatively. (A) Image of fibroblasts into which the GFP gene has been introduced. Arrowhead indicates GFP-negative cell. (B) Flow cytometry in GFP-expressing fibroblasts. Red shows fibroblasts introducing GFP gene. Green shows normal fibroblasts. (C) Images of GFP cell sheet and bronchial stumps. The bar indicates 50 μm.

extensive angiogenesis occurred at the bronchial stump in the cell sheet group, therefore, transplantation of the cell sheets may avoid ischemia (Fig. 7). BPF often occurs 1 week to 3 months postoperatively, particularly 10 days.[1,3] Since the effects of transplantation of the cell sheets have been confirmed at 7 days postoperatively, sufficient reinforcement of the bronchial stump had already been achieved by the time when BPF is likely to occur Fig. 8.

In the present study, transplanted autologous cells could not be confirmed at least 7 days postoperatively. The mechanism of tissue healing by cell transplantation is controversial, but it is unlikely that tissue formation had been performed solely by the transplanted cells. The effect of the transplanted fibroblast, sheet-origin fibroblast proliferation or paracrine effect, was evaluated using the GFP sheets. The results indicated that the transplanted fibroblasts disappeared by 7 days postoperatively, which was similar to findings of previous studies on mouse skin ulcer model.[14,16] Transplantation of fibroblast sheets may promote cell proliferation in the host as a paracrine effect and result in tissue thickness. In addition to VEGF, which is one of the most important factors for angiogenesis, some growth factors and cytokines that promote angiogenesis and tissue healing[19,20] were secreted from the cell sheets in

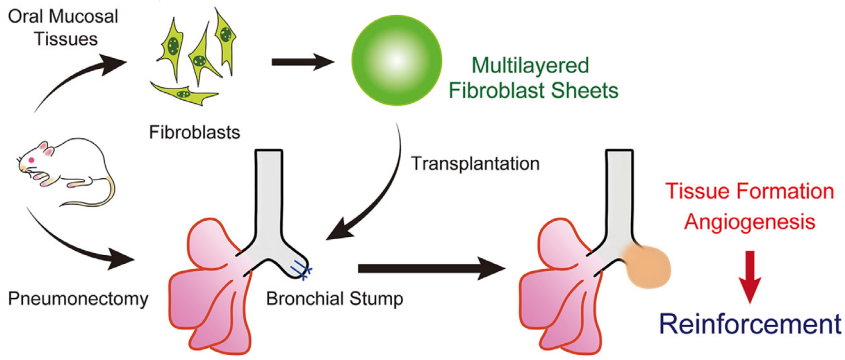

Figure 7. Multilayered fibroblast sheets derived from autologous oral mucosal tissues can be effective in the reinforcement of bronchial stump.

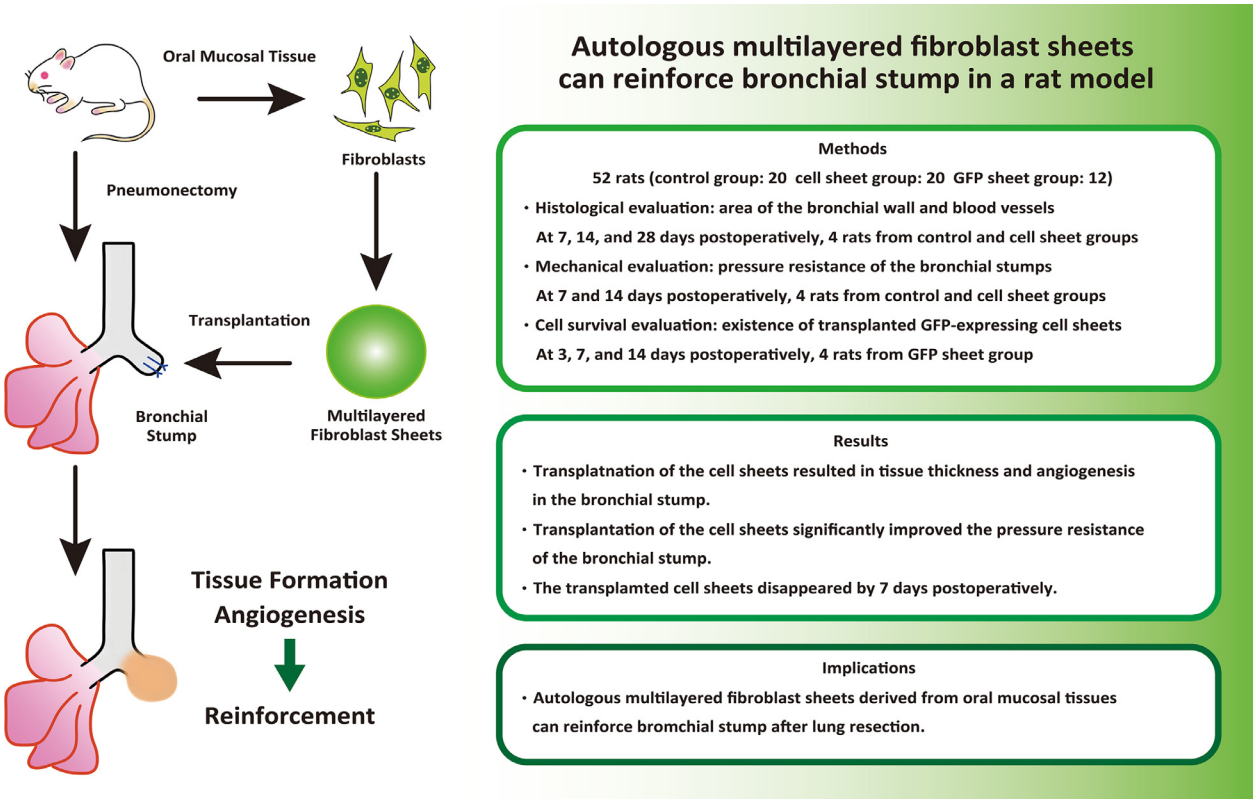

Figure 8. Autologous multilayered fibroblast sheets can reinforce bronchial stump. The experiment was performed in 52 rats. Multilayered fibroblast sheets derived from autologous oral mucosal tissues were transplanted to the bronchial stump. Histological and mechanical change in bronchial stumps, and survival of transplanted cells were evaluated. Transplantation of cell sheets resulted in tissue thickness and angiogenesis in the bronchial wall, and improved the pressure resistance of the bronchial stump. The transplanted cells disappeared 7 days postoperatively. Bronchial stumps were reinforced by the transplantation of multilayered fibroblast sheets.

vitro, and these growth factors and cytokines might promote tissue healing in the host.

Although omentum or pericardial fat also secreted growth factors and cytokines in vitro,[21,22] and the coverage with intercostal muscle or omentum had the effect of enhancing blood flow to the bronchial stump in large animal models,[23,24] the clinical effect of covering with autologous tissue to prevent BPF is not fully validated. In the clinical application of the cell sheet technique in the future, it is necessary to compare the preventive effect of BPF between the coverage with autologous tissue and transplantation of cell sheets.

The coverage with autologous tissue requires additional invasive procedure, such as collecting tissue and suturing the bronchial stump. In contrast, cell therapy can be considered a new simple and minimally invasive method. The local administration of bone marrow stromal cells or adipose-derived stem

cells to the bronchial stump increased fibrous connective tissues and the incidence of ulcers and necrosis was reduced in rat model,[11,12] and these cells have also been studied in large animal BPF models and clinically applied in humans.[25,26] Compared with these cells, fibroblasts can be obtained via minimally invasive procedures. Fibroblasts are heterogeneous cells whose gene expression differs depending on the tissue of origin,[27] and fibroblasts collected from the oral tissue are more effective in wound healing than those collected from the skin.[28,29] Therefore, the multilayered fibroblast sheets derived from autologous oral mucosal tissues can be a more convenient biomaterial.

The present study has several limitations. This study on rat t was an experiment with a model in which BPF occurs and did not prove that transplantation of the sheets prevents BPF. Additionally, the model in this experiment differed from the clinical situation of BPF. Specifically, due to the anatomical characteristics of rat and technical convenience, left pneumonectomy was performed, the bronchial stump was closed using a nonabsorbable suture instead of stapler, and the coverage with intercostal muscle or omentum was not compared. Considering clinical application, further research should be performed in larger animals that reflect human BPF formation and assess the bronchial stump from the endobronchial side with endoscopy.

Transplantation of multilayered fibroblast sheets derived from autologous oral mucosal tissues can reinforce the bronchial stump. This procedure may be effective in the prevention of BPF and may also be applied to the reinforcement of bronchial anastomosis at the sleeve resection and lung transplantation.

CONCLUSIONS

The multilayered fibroblast sheets derived from autologous oral mucosal tissues can be effective in the reinforcement of bronchial stump.

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SUPPLEMENTARY MATERIAL

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