学位論文　（博士）

Allogenic multilayered fibroblast sheets promote anastomotic site healing in a rat model of esophageal reconstruction

（他家積層線維芽細胞シートによるラットの食道縫合部の補強効果）

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令和５年６月

学位論文の関連論文の研究背景及び要旨

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〔題名〕
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American Journal of Translational Research　（令和5年4月アクセプト済）

〔研究背景〕
消化管手術の術後合併症である「消化管の縫合不全」は、術中に縫合した消化管が術後に破綻する病態であり、腹腔内膿瘍や腹膜炎、敗血症を引き起こし、致命的な転帰を辿る。縫合不全は食道から直腸までの全消化管で起こる合併症であり、特に食道の縫合不全は致命的な転帰を辿ることが多い。本邦では食道癌に対して年間6000件の手術を行っており、その内、縫合不全は10-15%に発生しており、年間800件の食道の縫合不全が発生している計算になる。食道の縫合不全は他部位の縫合不全と同様、頚部膿瘍や縦隔洞炎、膿胸などを引き起こし、致命的な転帰を辿る。食道の縫合不全による術後合併症を救命できた場合でも、数ヶ月に及ぶ長期入院や再手術を要し、患者と医療者の負担が大きい。そのため、食道の縫合不全を予防することは極めて重要な課題である。

縫合不全のリスク因子には、低栄養や糖尿病などの非手術因子と、縫合部の血流低下や過度の緊張などの手術因子が挙げられる。中でも縫合部の血流低下は最大のリスク因子と考えられている。実臨床では、非手術因子に対して様々な治療を行い、細心の注意を払って手術を行っているが縫合不全の予防には至っていない。これまでに、他の研究チームによって、間葉系細胞の全身投与や局所投与による予防法の開発が試みられてきたが、満足の行く結果は得られていない。

私が所属している研究室では、世界に先駆けて線維芽細胞を用いた再生医療の研究を開始した。その研究の過程で、線維芽細胞を積層化させた積層線維芽細胞シートを独自に開発した(特許第6941858号)。そして、当研究室では、この積層線維芽細胞シートを用いて、実験動物の皮膚潰瘍や気管支断端瘻、膵液瘻モデルで血管新生及び創傷治癒の促進を報告してきた。そこで私は、消化管の縫合部、特に漿膜を欠くため脆弱である「食道の縫合部」でも血管新生及び創傷治癒の促進が得られ、致命的な転帰を辿る食道の縫合不全が予防できるのではないかと考えた。

〔要旨〕
ラットで食道縫合モデルを作製し、他家積層線維芽細胞シートを移植することで縫合部の創傷治癒が促進するかを検証した。

積層線維芽細胞シートは過去に報告済みの方法で作製した(S. Yoshimine et al., Autologous multilayered fibroblast sheets can reinforce bronchial stump in a rat model. Semin Thorac Cardiovasc Surg 2022; 34:349-358.)。以下に簡潔に記載する。ラット(SD、8週齢)の口腔内組織を採取し、コラゲナーゼ処理により線維芽細胞を単離した。6日間の培養後、24-well plateに線維芽細胞を5.0×10^5 cells/2mL/wellで播種した。翌日に培地交換を行い、更に2日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は組織学的に20-30μmの厚さで、4-5層の線維芽細胞で構成されていた。積層線維芽細胞シート作製時の培養上清の成長因子をELISA解析することで、積層線維芽細胞シートからVEGFやHGF、TGF-β1、TIMP-1が分泌されていることを確認した。

食道縫合モデルはラット(Wistar/ST、10週齢)の食道を切離し、4点結節縫合による再吻合を行うことで作製した。コントロール群(食道の切離/縫合のみ)と他家積層線維芽細胞シート移植群(食道の切離/縫合後、他家積層線維芽細胞シートを前壁/後壁に1枚ずつ計2枚移植)に分け、移植直後、移植後3日目、移植後5日目に犠牲死させ、縫合部の耐圧能や縫合不全率を評価した。また、縫合部の組織学的解析(Azan染色、CD31免疫染色、CD3免疫染色)および遺伝子発現解析(qPCR解析：collagen I型/III型、VEGF、MMP-2、TIMP-1)を行った。

移植後5日目において縫合部の耐圧能は、コントロール群と比較して、他家積層線維芽細胞シート移植群で有意に高値だった(251.3 mmHg vs. 164.6 mmHg, $p = 0.014$)。縫合不全率は、コントロール群と比較して、他家積層線維芽細胞シート群で低率であったが、有意差は認めなかった(1/12 (8.3%) vs. 5/12 (41.6%), $p = 0.15$)。組織学的解析では、コントロール群と比較して、他家積層線維芽細胞シート移植群でAzan染色陽性の範囲が広く、縫合部でのコラーゲン量の増加が示された($p = 0.018$)。また、他家積層線維芽細胞シート群では、CD31陽性の範囲が広く、縫合部での血管新生が示唆されたが、コントロール群と比較したところ、有意差は認めなかった($p = 0.08$)。縫合部の遺伝子発現解析では、移植後5日目において、コントロール群と比較したところ、他家積層線維芽細胞シート移植群でcollagen I型/III型のmRNA発現が増加していた($p = 0.049$, $p = 0.042$)。また、移植直後において、他家積層線維芽細胞シート移植群では、VEGF、MMP-2、TIMP-1のmRNA発現が増加していた($p = 0.013$, $p < 0.001$, $p < 0.001$)。

GFP発現ラット(SD-Tg、8週齢)から作製した他家積層線維芽細胞シートをラット食道縫合モデル(Wistar/ST)に移植したところ、移植後3日目から7日目までは、GFP陽性細胞が確認できたが、移植後10日目には確認できなかった。また、移植後5日目において、コントロール群と比較したところ、他家積層線維芽細胞シート移植群での縫合部におけるCD3陽性細胞の有意な増加は観察されなかったことから、移植後5日目までは過剰な免疫応答は誘導されていないと考えられた($p = 0.75$)。

ラット食道縫合モデルでは、他家積層線維芽細胞シート移植によって縫合部の耐圧能が上昇した。また、他家積層線維芽細胞シートは、統計学的有意差を認めなかったものの縫合不全の発生率を低下させた。他家積層線維芽細胞シートは、縫合部のコラーゲン量の増加や血管新生によって縫合部が補強される可能性が示唆された。

Am J Transl Res 2023;15(5):3217-3228
www.ajtr.org /ISSN:1943-8141/AJTR0147398

Original Article

Allogenic multilayered fibroblast sheets promote anastomotic site healing in a rat model of esophageal reconstruction

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Received October 31, 2022; Accepted April 13, 2023; Epub May 15, 2023; Published May 30, 2023

Abstract: Objective: Anastomotic leakage is a common and severe complication of esophageal reconstruction. Accordingly, there is a clinical need for novel methods to prevent it. We developed multilayered, growth factor-secreting fibroblast sheets that promote wound healing and angiogenesis. The present study aimed to assess the utility of allogenic multilayered fibroblast sheets in preventing esophageal anastomotic leakage in a rat model of esophageal reconstruction. Methods: Allogenic multilayered fibroblast sheets prepared from oral mucosal tissues were implanted at esophageal anastomotic sites. Results: The allogenic multilayered fibroblast sheet group had significantly higher burst pressure and collagen deposition compared to a control group five days postoperatively. The expression levels of collagen type I and III mRNAs around esophageal suture sites were higher in the allogenic multilayered fibroblast sheet group compared to the control group on postoperative days 0, 3, and 5. There was a trend toward lower anastomotic leakage and lower abscess scores in the allogenic multilayered fibroblast sheet group compared to the control group; however, these differences did not reach statistical significance. Allogenic multilayered fibroblast sheets completely disappeared at ten days after implantation. Further, no inflammation was observed at suture sites with implanted allogenic multilayered fibroblast sheets at five days after surgery. Conclusion: Allogenic multilayered fibroblast sheets may represent a promising method of preventing esophageal anastomotic leakage.

Keywords: Regenerative medicine, allogenic multilayered fibroblast sheet, sheet implantation, anastomotic site healing, proliferation of granulation tissue

Introduction

Anastomotic leakage is a common and severe complication of gastrointestinal surgery. Esophageal anastomotic leakage reportedly occurs in 11.4-21.2% of cases after esophageal reconstruction. Leakage typically requires reoperation with associated increases in hospital stays and medical costs [1, 2]. Several studies have reported the prevention of esophageal anastomotic leakage by covering the anastomotic site with autologous tissues such as pleura, pericardial fat, and omentum [3]. However, current methods are unable to completely prevent the leakage.

We previously reported that autologous fibroblast sheets containing peripheral blood mononuclear cells have therapeutic efficacy in promoting the healing of refractory skin ulcers in animals and humans [4-7]. The therapeutic mechanism is presumed to involve paracrine effects mediated by factors secreted from implanted cells that promote tissue regeneration by inducing host cell proliferation [8-10]. We also previously reported that autologous multilayered fibroblast sheets prevent postoperative complications in rat models [11, 12]. Autologous multilayered fibroblast sheets have previously been shown to promote tissue growth, angiogenesis, and fibrosis at the implant site and prevent postoperative bronchial and pancreatic fistulation.

However, autologous cell implantation is an expensive and laborious treatment and there is often a lag in treatment as autologous cell implantation requires the expansion of a large number of cells in vitro. Further, autologous cells may not necessarily proliferate due to a lack of functionality [7]. Therefore, the clinical application of cell implantation therapy requires the development of allogenic cell implantation techniques allowing timely treatment at low cost. Several studies have reported that allogenic sheet implantation has comparable efficacy to autologous sheet implantation in animal models [13-16], which may be attributable to a similar amount of growth factor secretion from autologous and allogenic cells.

We hypothesized that allogenic multilayered fibroblast sheets promote tissue growth, angiogenesis, and fibrosis at gastrointestinal anastomotic sites and prevent anastomotic leakage by paracrine effects. The present study demonstrates the implantation of allogenic multilayered fibroblast sheets at esophageal anastomotic sites in a rat model, indicating their possible future clinical application.

Materials and methods

Animals

Male Wistar/ST, SD, and SD-Tg (CAG-EGFP, AG promoter with cytomegalovirus-immediate early enhancer (CAG), enhanced green fluorescent protein (EGFP)) rats (aged 7 weeks) were purchased from Japan SLC (Shizuoka, Japan) [17, 18]. The animals were housed in a temperature-, humidity-, and light-controlled room (22 ± 2°C, 70 ± 20%, and 12 h light/dark cycles, respectively). Food and water were provided *ad libitum*. The present study was conducted in accordance with all relevant guidelines and was approved by the Institutional Animal Care and Use Committee of Yamaguchi University (IACUC; No. 31-007).

Preparation of multilayered fibroblast sheets

SD and SD-Tg rats (aged 8 weeks) were anesthetized with 5% isoflurane (MSD Animal Health, Tokyo, Japan), intubated with 18-gauge ethylene-tetrafluoroethylene catheters (TERUMO, Tokyo, Japan) and ventilated using a small animal ventilator Model 683 (Harvard Apparatus, Massachusetts, USA). The tidal volume was set at 10 mL/kg and the respiratory rate was set at 70 breaths/min. Oral mucosal tissues were dissected using scissors. Oral mucosal tissues were minced and incubated in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Massachusetts, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), 5% collagenase (FUJIFILM Wako, Osaka, Japan), and 1% penicillin-streptomycin (Thermo Fisher Scientific). On the following day, culture medium with tissue fragments was centrifuged and the supernatant was removed. Residual tissue fragments were cultured in DMEM supplemented with 10% FBS for two days. Culture media containing tissue fragments and migrated cells were collected with 0.05% trypsin-ethylenediaminetetraacetic acid (Thermo Fisher Scientific) and passed through a 40 μm cell strainer to remove tissue fragments. Culture medium with migrated cells was centrifuged and the supernatant was removed. Residual cells were cultured in DMEM supplemented with 10% FBS for three days. Culture medium containing proliferating cells was collected with 0.05% trypsin-ethylenediaminetetraacetic acid and passed through a 40 μm cell strainer. A total of 5.0×10^5 cells was seeded in a 24-well culture dish with 2 mL of DMEM supplemented with 10% FBS. On the following day, culture medium was replaced with 2 mL of CTS™ AIM V™ SFM (Thermo Fisher Scientific) and HFDM-1 (+) (Cell Science & Technology Institute, Miyagi, Japan) supplemented with 5% FBS. After further incubation for two days, multilayered fibroblast sheets were detached using dispase (FUJIFILM Wako) (**Figure 1A**).

Enzyme-linked immunosorbent assay (ELISA)

Culture medium was collected prior to the detachment of cell sheets. The concentrations of vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), transforming growth factor beta 1 (TGF-β1), and tissue inhibitor of metalloproteinases 1 (TIMP-1) in the culture media were measured using enzyme-linked immunosorbent assay kits (R&D Systems, Minnesota, USA). CTS™ AIM V™ SFM and HFDM-1 (+) supplemented with 5% FBS were used as controls.

Esophageal anastomosis model

ST rats (aged 10 weeks) were anesthetized according to the method described above. Anesthetic concentrations were 3% from the

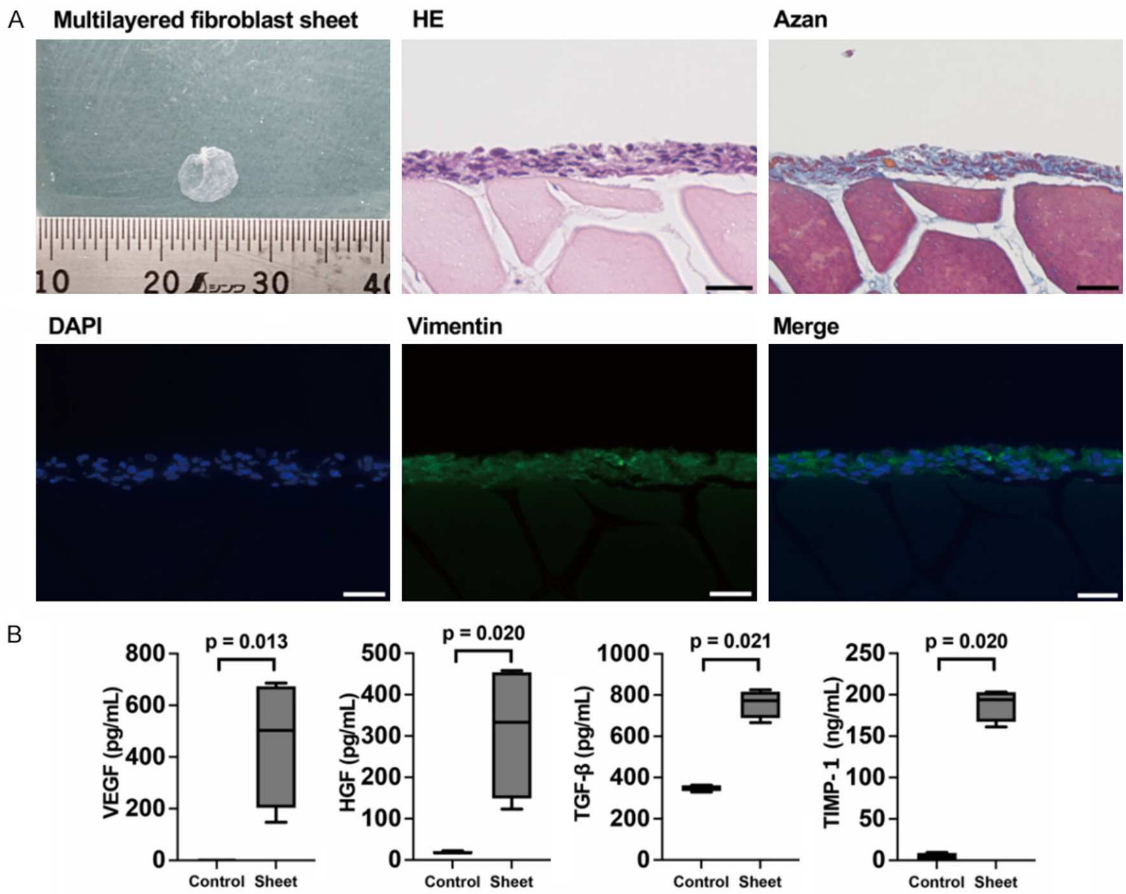


Figure 1. A. Multilayered fibroblast sheets. A multilayered fibroblast sheet consisting of fibroblasts with collagen fibers. Bars indicate 30 μm. HE, Hematoxylin Eosin; DAPI, 4',6-diamidino-2-phenylindole. B. Growth factors and enzyme concentrations in culture media. The culture media of multilayered fibroblast sheets contained greater concentrations of growth factors and enzymes compared with controls. VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; TGF-β1, transforming growth factor beta 1; TIMP-1, tissue inhibitor of metalloproteinase 1. Control indicates medium consisting of CTS™ AIM V™ SFM and HFDM-1 (+) supplemented with 5% FBS (n = 4 per group).

start of the procedure to 10 minutes, 2% from 10 to 20 minutes, and 1.5% from 20 minutes to the end of the procedure. Rats were immobilized in the supine position. A longitudinal incision was made in the neck to expose the trachea and esophagus. Subsequent procedures were performed under a microscope (TAKAGI SEIKO, Nagano, Japan). The trachea and esophagus were separated and the esophagus was taped. The esophagus was clipped in place and transected at the level of the 9th and 10th tracheal cartilage rings. The esophagus was sutured using four stitches of 7-0 polypropylene thread (Medtronic, Minnesota, USA). Esophageal lumen patency was confirmed before suturing to the posterior wall. The suture bite was 1 mm. The esophagus was returned to its original position and the incision was closed (**Figure 2** and Video S1). The rats were provided with water only until day five postoperatively to avoid injury to the anastomotic site. All surgeries were performed by the same surgeon (N.Y.).

Implantation of allogenic multilayered fibroblast sheets

After suturing the esophagus, two allogenic multilayered fibroblast sheets were implanted at the anterior and posterior esophageal anastomotic sites. Cell sheets were transferred with a micropipette and a spoon and implanted at the anastomotic site (Video S2). To adhere the cell sheets to the anastomotic site, the esophagus was returned to its original position five minutes after implantation.

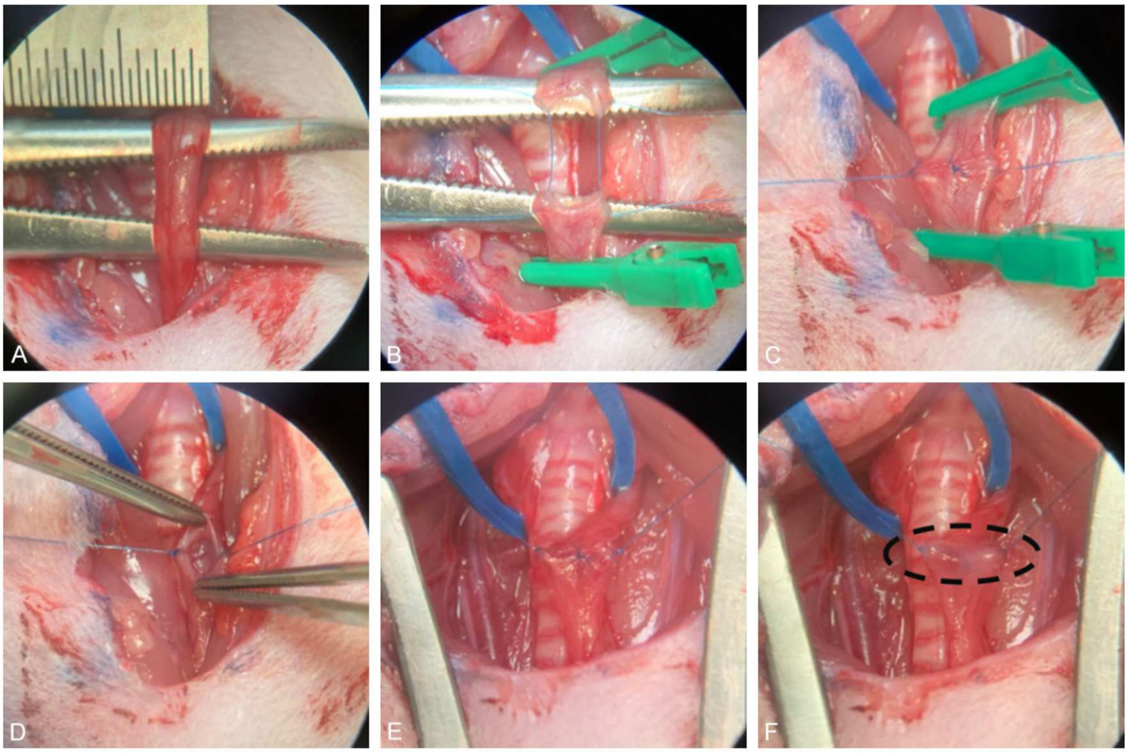


Figure 2. Esophageal anastomotic model. A. The esophagus is held in place. The esophagus is 3 mm in diameter. B. Both side walls were sutured using 7-0 polypropylene. C. The anterior wall was sutured and then ligated. D. The patency of the esophageal lumen was confirmed before suturing to the posterior wall. E. The posterior wall was sutured and then ligated. The esophagus was sutured using four stitches. F. In the cell sheet implantation group, cell sheets were implanted at the anastomotic site. Dotted lines demarcate cell sheets.

Evaluation of the anastomotic site and pressure resistance

Rats undergoing surgery were sacrificed on days 3 and 5. A median incision was made from the neck to the abdomen. As the anastomotic site was delicate, the esophagus was removed with the anterior trachea and posterior muscles adjacent to the anastomotic site. Anastomotic leakage was defined as the presence of abscess around the anastomotic site. The severity of abscesses was scored according to the previously described abscess score: 0 = no abscess, 0.5 = one small abscess (< 1 mm), 1 = several small abscesses, 2 = one medium abscess (1-3 mm), 3 = one large (3-5 mm) or several medium abscesses, 4 = one very large (> 5 mm) or several large abscesses [19, 20]. A 24-gauge ethylene-tetrafluoroethylene catheter was inserted into the esophagus to evaluate pressure resistance. The oral and anal ends of the esophagus were ligated with 4-0 nylon thread (NITCHO KOGYO, Tokyo, Japan). The catheter was connected to an aneroid sphygmomanometer HT-1500 (NISSEI, Gunma, Japan) and pressurized using a 10 mL syringe. The esophagus was submerged in saline. Burst pressure was defined as the pressure at which the anastomotic site failed and air bubbles were observed. The upper limit of the measuring device was 300 mmHg.

Histologic analysis

Multilayered fibroblast sheets were transferred onto raw ham and fixed in a 10% formalin neutral buffer solution (FUJIFILM Wako). Rats undergoing surgery were sacrificed on days 3 and 5 and the esophagus was removed according to the methods described above. All specimens were fixed in a 10% formalin neutral buffer solution and embedded in paraffin. Sections cut at 3 μm thickness were mounted on glass slides and stained with hematoxylin and eosin or azocarmine aniline blue (Azan). Since Azan staining specifically stains collagen fibers blue, the amount of collagen in the gastrointestinal wall could be measured by Azan staining. The

Table 1. Polymerase chain reaction primer sequences

	Forward	Reverse
Beta-actin	CTACCTCATGAAGATCCTGACCGAG	TTTCCCTCTCAGCTGTGGTGG
Collagen type I	ATGCTGCCTTTTCTGTTCCTTTCTC	TTTGGGGAGCAATGGAGGAGAG
Collagen type III	CACCCTGAACTCAAGAGCGGA	CATCCATCTTGCAGCCTTGGTTAG
Vascular endothelial growth factor A	TACTGCTGTACCTCCACCATGC	TTCTGCTCCCCTTCTGTCGTG
Matrix metalloproteinase 2	GAGCTCCCGGAAAAGATTGATGC	AATAGACCCAGTACTCATTCCCTGC
Tissue inhibitor of metalloproteinase 1	CAGTGTTTCCCTGTTCAGCCATC	ATCTGATCTGTCCACAAGCAATGAC

amount of collagen was measured in a 3 mm length of esophagus centered on the anastomotic site. Immunostaining was performed using the following primary antibodies: anti-vimentin (ab8069, 1:500, Abcam, Cambridge, UK), anti-CD31 (ab182981, 1:1000, Abcam), and anti-CD3 (ab16669, 1:150, Abcam). The following secondary antibodies were used: goat anti-rabbit IgG DyLight® 550 (ab96884, 1:200, Abcam) and goat anti-mouse IgG Alexa Fluor™ 488 (A11029, 1:500, Life Technologies, California, USA). Blood vessels in the gastrointestinal wall were stained using anti-CD31 antibodies. The total area of blood vessels over 300 μm^2 was measured in a 3 mm length of esophagus centered on the anastomotic site. To measure the amount of collagen and blood vessel areas, samples were standardized against a section of intestinal wall area to allow comparison of samples. T-lymphocytes in the gastrointestinal wall were stained using anti-CD3 antibodies. The CD3-positive area was measured in a 3 mm length of esophagus centered on the anastomotic site. The spleen was stained as a control. All histological images were captured using a BZ-X710 microscope (KEYENCE, Osaka, Japan) and analyzed using Image J software (National Institutes of Health, Maryland, USA). All histologic analyses were performed in a blind manner and supervised by a pathologist (A.O.).

Analysis of mRNA expression levels

Cell sheets prepared from SD rats were implanted at anterior and posterior esophageal anastomotic sites. Rats undergoing surgery were sacrificed on days 0, 3, and 5. A 3 mm length of esophagus centered on the anastomotic site was removed according to the methods described above. Total RNA was extracted using RNeasy Mini kits (Qiagen, Hilden, Germany). Total RNA was quantified using nanodrop (Thermo Fisher Scientific) followed by cDNA synthesis using PrimeScript Reverse Transcriptase (Takara Bio, Shiga, Japan). Quantitative real-time polymerase chain reaction (PCR) was conducted using StepOnePlus™ (Thermo Fisher Scientific) with SYBR™ Select Master Mix (Thermo Fisher Scientific). Quantitative real-time PCR parameters for cycling were as follows: 50°C for 2 min and 95°C for 2 min, 40 cycles at 95°C for 3 s, and 60°C for 30 s. All reactions were performed in a 10 μL reaction volume in triplicate. mRNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method. Primer sequences used in the present study are summarized in **Table 1**. The beta-actin gene was used as an endogenous control.

Tracking of cell sheets after implantation

The persistence of cell sheets after implantation was assessed using cells expressing green fluorescent protein (GFP). Cell sheets prepared from SD-Tg (CAG-EGFP) rats were implanted at anterior and posterior esophageal anastomotic sites in ST rats. Rats undergoing surgery were sacrificed on days 3, 5, 7, and 10, with the esophagus removed according to the methods described above. Immunostaining was performed using anti-GFP primary antibodies (#2956, 1:75, Cell Signaling Technology, Massachusetts, USA) and goat anti-rabbit IgG DyLight® 550 secondary antibodies. The GFP-positive area was measured in a 3 mm length of esophagus centered on the anastomotic site.

Statistical analysis

Continuous variables were presented as the mean and standard deviation for normally distributed data or median and interquartile range for non-normally distributed data. Differences between groups were analyzed using Student's t-test or the Mann-Whitney U test as appropriate. Categorical variables were presented as

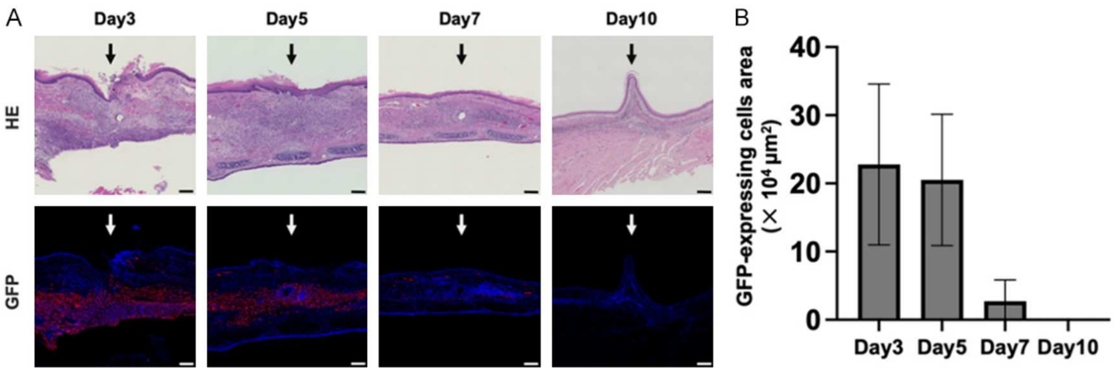


Figure 3. A. Persistence of cell sheets. Cell sheets prepared from SD-Tg rats were implanted at anastomotic sites. Green fluorescent protein (GFP)-expressing cells were visualized by immunostaining on postoperative days 3, 5, 7, and 10. Excised esophagus specimens were studied using primary antibodies against anti-GFP antibody and DyLight® 550-congugated antibody secondary antibodies. Bars indicate 200 μm. Arrows indicate anastomotic site. B. Analysis of GFP-expressing cell area. Areas of GFP-expressing cells were analyzed on days 3, 5, 7, and 10.

counts and percentages. Differences between groups were analyzed using Fisher's exact test. p-values < 0.05 were considered significant. All statistical analyses were performed using STATA/BE version 17 (Stata Corp, Texas, USA) and supervised by a biostatistician (M.S.).

Results

Histopathologic analysis and growth factor concentrations in multilayered fibroblast sheets

Multilayered fibroblast sheets were fabricated without defects and maintained in a circular shape. Cell sheets had a thickness of 20-30 μm with 4-5 fibroblast layers. Cell sheets expressed the fibroblast marker vimentin and contained extracellular matrix components such as collagen fibers (**Figure 1A**). Higher concentrations of growth factors and enzymes were observed in the culture media from cell sheets compared to the control. Cell sheet culture media contained 503.3 (259.5-660.6) pg/mL VEGF, 333.3 (174.5-449.5) pg/mL HGF, 773.1 (710.9-808.1) pg/mL TGF-β1, and 194.1 (173.5-202.8) ng/mL TIMP-1. The control had lower concentrations of VEGF, HGF, TGF-β1 (354.8 [340.1-360.8] pg/mL), and TIMP-1 (**Figure 1B**).

Esophageal anastomosis model and cell sheet implantation

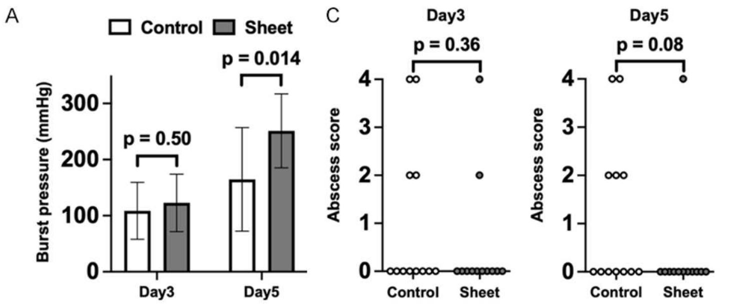
In our rat model, the esophagus was completely transected and four stitches were placed in the esophagus (**Figure 2** and Video S1). The patency of the esophageal lumen was confirmed before suturing to the posterior wall. Two cell sheets were implanted at the anterior and posterior wall outside the sutured region of esophagus (Video S2).

Persistence of cell sheets after implantation

Figure 3A shows representative images of allogenic multilayered GFP-expressing cell sheets after implantation. GFP expression decreased from postoperative day 3 to postoperative day 7. No GFP-expressing cells were observed at the anastomotic site on postoperative day 10 (**Figure 3B**).

Evaluation of the anastomotic site and pressure resistance

There was a trend toward a higher burst pressure in the cell sheet (allogenic multilayered fibroblast sheet implantation) group compared to the control (no cell sheet implantation) group on postoperative day 3; however, this difference did not reach statistical significance (122.8 ± 51.0 mmHg vs. 108.6 ± 50.8 mmHg; P = 0.50). In contrast, the cell sheet group had a significantly higher burst pressure than the control group on postoperative day 5 (251.3 ± 65.9 mmHg vs. 164.6 ± 92.3 mmHg; P = 0.014). The upper limit value of 300 mmHg was exceeded more frequently in the cell sheet group compared to the control group (6/12, 50% vs. 2/12, 16.6%; P = 0.019). Burst pressure values were higher on postoperative day 5



B

	Day3 Control (%)	Day3 Sheet (%)	Day3 p value	Day5 Control (%)	Day5 Sheet (%)	Day5 p value
Anastomotic leakage	4/12 (33.3)	2/12 (16.6)	0.64	5/12 (41.6)	1/12 (8.3)	0.15
Mortality	0/12	0/12	-	0/12	0/12	-

Figure 4. A. Evaluation of pressure resistance. Burst pressure was evaluated on postoperative days 3 and 5 (n = 12 per group). B. Anastomotic leakage and mortality. Anastomotic leakage and mortality were evaluated on postoperative days 3 and 5 (n = 12 per group). C. Abscess scores. Abscess scores were evaluated on postoperative days 3 and 5 (n = 12 per group).

than on postoperative day 3 in both groups (**Figure 4A**). There was a non-significant trend toward a lower rate of anastomotic leakage in the cell sheet group compared to the control group on postoperative day 5 (1/12, 8.3% vs. 5/12, 41.6%; *P* = 0.15). No mortality was observed in either group (**Figure 4B**). There was a non-significant trend toward a lower abscess score in the cell sheet group compared to the control group on postoperative day 5 (*P* = 0.08) (**Figure 4C**).

Histologic analysis

Figure 5A shows representative images of Azan staining on postoperative days 3 and 5. Azan-positive areas in the whole layer were significantly larger in the cell sheet group compared to the control group on postoperative day 5 (25.0 ± 4.8% vs. 17.1 ± 4.8%; *P* = 0.018; **Figure 5B**). In particular, Azan-positive areas in the muscle layer to outer membrane were significantly larger in the cell sheet group compared to the control group on postoperative day 5 (7.1 ± 3.9% vs. 2.3 ± 1.6%; *P* = 0.021). In contrast, Azan-positive areas in the muscle layer to outer membrane were significantly smaller in the cell sheet group compared to the control group on postoperative day 3 (3.4 ± 1.0% vs. 5.5 ± 1.7%; *P* = 0.030; **Figure 5B**). No significant differences in Azan-positive staining of the mucosa and submucosa were observed between the groups on postoperative days 3 and 5 (**Figure 5B**). **Figure 5C** shows representative images of CD31-positive staining. CD31 was used as an endothelial marker. There was a non-significant trend toward a larger area of blood vessels in the cell sheet group compared to the control group on postoperative day 3 (3.7 ± 1.1% vs. 2.4 ± 1.1%; *P* = 0.08) (**Figure 5D**). **Figure 5E** shows representative images of CD3-positive staining. CD3 was used as a T-lymphocyte marker. Since only a small number of CD3-positive cells were detected, no significant difference was observed between the two groups (**Figure 5F**).

mRNA expression levels at the anastomotic site

Expression levels of collagen type I and III mRNA were significantly higher in the cell sheet group compared to the control group on postoperative days 0, 3, and 5 (**Figure 6**). Collagen type I mRNA expression increased from postoperative day 0 to postoperative day 5. Collagen type III mRNA expression decreased on postoperative day 3 compared to postoperative day 0 and then increased on postoperative day 5. VEGF-A mRNA expression was significantly higher in the cell sheet group compared to the control group on postoperative day 0; however, no significant difference in VEGF-A mRNA expression was observed between the two groups on postoperative days 3 and 5. VEGF-A mRNA expression decreased from postoperative day 0 to postoperative day 5. Expression levels of MMP2 and TIMP-1 mRNA were significantly higher in the cell sheet group compared to the control group on postoperative day 0. Expression levels of MMP2 and TIMP-1 mRNA decreased from postoperative day 0 to postoperative day 5.

Discussion

The results of the present study demonstrate that allogenic multilayered fibroblast sheets

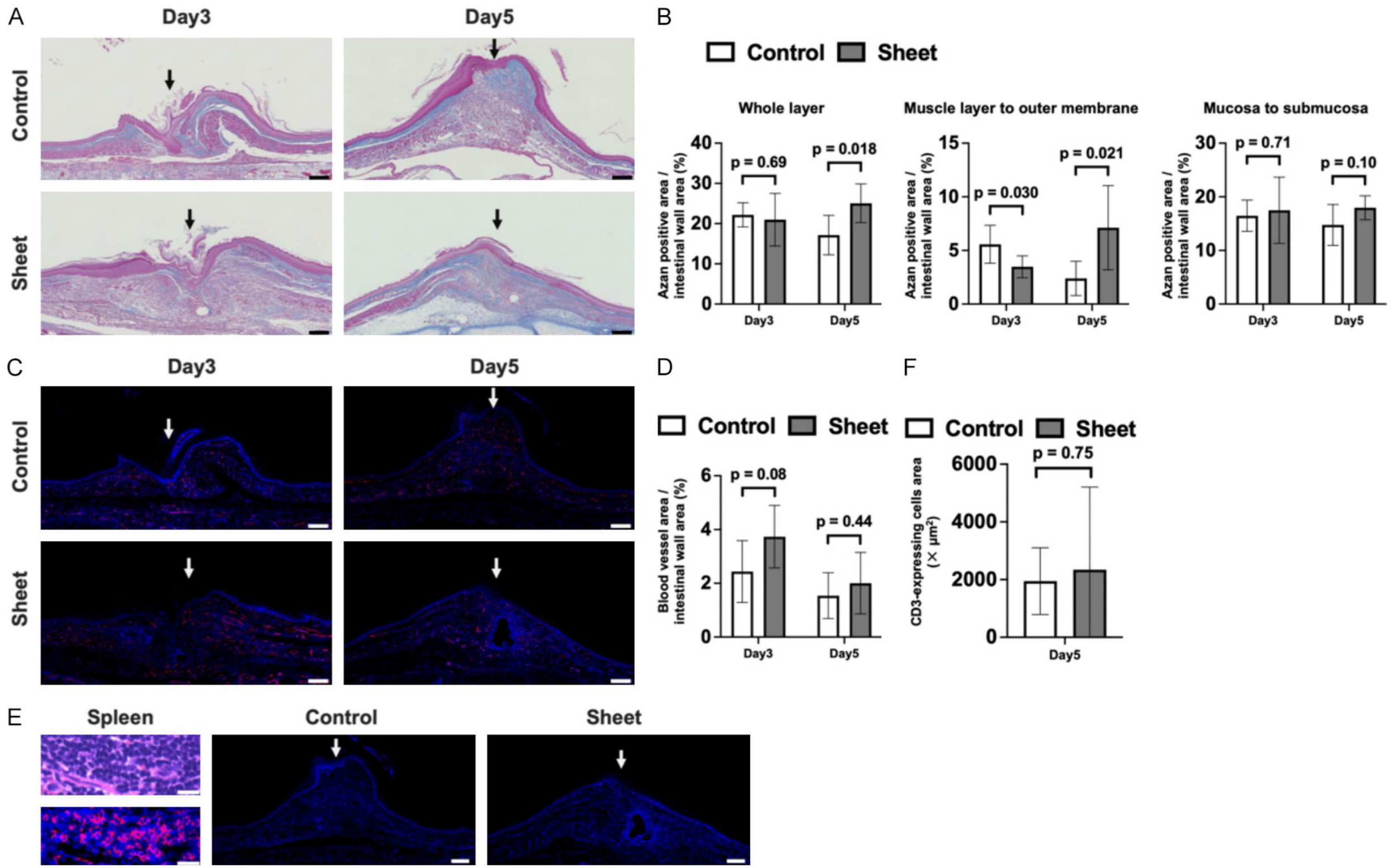


Figure 5. A. Excised esophagus specimens were stained with Azan on postoperative days 3 and 5. Bars indicate 200 μm. Arrows indicate anastomotic site. B. Analysis of Azan-positive area. Whole layer (left), muscle layer to outer membrane (middle), and mucosa to submucosa (right). Azan-positive area was normalized to intestinal wall area (n = 6 per group). C. Immunostaining of blood vessel at anastomotic sites. Endothelial cells were stained using anti-CD31 antibody on postoperative days 3 and 5. Bars indicate 200 μm. Arrows indicate anastomotic site. D. Analysis of blood vessel area. Vessel area was normalized to intestinal wall area (n = 6 per group). E. Immunostaining of T-lymphocytes at anastomotic site. T-lymphocytes were stained using anti-CD3 antibody on postoperative days 3 and 5. Bars indicate 200 μm. Arrows indicate anastomotic site. Rat spleen specimens were used as positive controls for anti-CD3 staining. Bars indicate 20 μm. F. Analysis of CD3-positive area (n = 6 per group).

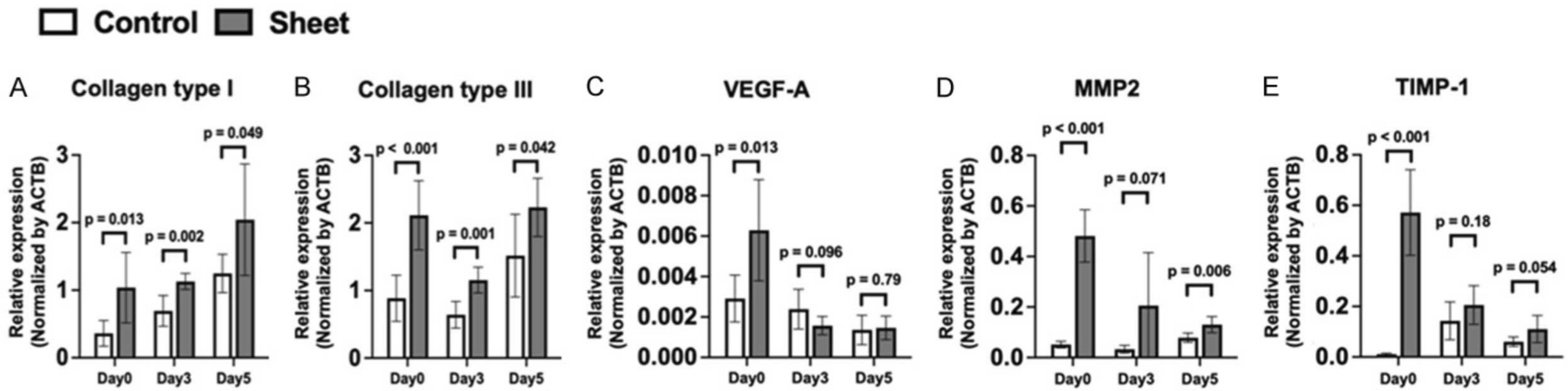


Figure 6. Analysis of mRNA expression levels using quantitative real-time polymerase chain reaction (PCR). Expression levels of mRNA were measured on postoperative days 0, 3, and 5. Target genes were: (A) collagen type I, (B) collagen type III, (C) vascular endothelial growth factor-A (VEGF-A), (D) matrix metalloproteinase 2 (MMP2), and (E) tissue inhibitor of metalloproteinases-1 (TIMP-1). Day 0 refers to expression levels in excised esophagus specimens immediately after cell sheet implantation in the multilayered fibroblast sheet group. Target gene expression levels were normalized to beta-actin (ACTB) (n = 6 per group).

promote anastomotic site healing by inducing proliferation of granulation tissue, collagen deposition, and angiogenesis (Figure S1). At the beginning of the experiment, autologous multilayered fibroblast sheet implantation was performed at the esophageal anastomotic site. The cell sheet group had significantly higher burst pressure compared to the control group, but no significant differences in anastomotic leakage or abscess score were observed between the cell sheet and control groups (Figure S2). No significant differences in anastomotic leakage or abscess score were observed between the allogenic cell sheet and control groups (**Figure 4B** and **4C**). However, rates of anastomotic leakage in the control group were 33.3% and 41.6% on postoperative days 3 and 5, respectively. Rates of anastomotic leakage in the allogenic sheet group were 16.6% and 8.3% on postoperative days 3 and 5, respectively (**Figure 4B**). This finding suggests allogenic cell sheets may have utility from a clinical perspective. Although significant differences were not observed in all comparisons between the cell sheet and control groups in the present study, we believe greater statistical significance would have been observed with a greater sample size; however, this would have required more animal sacrifices.

Autologous cell implantation is currently an expensive and laborious treatment which may have potential in future clinical applications. Previous animal studies have reported that allogenic sheet implantation has comparable efficacy to autologous sheet implantation, including allogeneic multilayered fibroblast sheets for skin regeneration [13, 14], allogenic chondrocyte sheets for cartilage regeneration [15], and periodontal ligament-derived multipotent mesenchymal stromal cell sheets for periodontal tissue regeneration [16]. Allogenic multilayered fibroblast sheets have been shown to have comparable pressure resistance, anatomic leakage, mortality, and abscess scores to autologous multilayered fibroblast sheets (**Figure 4** and Figure S2). These findings indicate that paracrine effects underlie the therapeutic value of cell sheets at esophageal anastomotic sites.

Despite concerns regarding aberrant immune responses to allogeneic cell implantation, no obvious immune responses such as T-lymphocyte accumulation were observed in the present study (**Figure 5E** and **5F**). We previously reported higher numbers of CD3-positive cells in allogenic fibroblast sheets compared to autologous fibroblast sheets in a mouse model of cutaneous wound healing on postoperative day 14; however, no significant difference was observed on postoperative day 5 [13]. The findings of our previous study indicate no accumulation of CD3-positive cells at esophageal anastomotic sites in rats at five days after implantation of allogenic fibroblast sheets. Strong immune responses may not have occurred in this model since allogenic fibroblast sheets were found to exert early paracrine effects and allogenic cell sheets were seen to be gradually disappeared during anastomotic healing. These results suggest that allogenic multilayered fibroblast sheets may have future clinical application as a therapeutic material to preventpostoperative complications of esophageal reconstruction.

The results of the present study demonstrate that the cell sheet group had significantly higher burst pressure, lower anastomotic leakage, and lower abscess scores compared to the control group. These findings corroborate prior studies reporting that cell sheet implantation is associated with higher burst pressure, lower anastomotic leakage, and lower abscess scores compared to controls [20-23]. However, these studies evaluated the use of cell sheets at the small and large intestine with the serosa. To date, no studies have evaluated the implantation of cell sheets at the esophagus without the serosa. Multilayered fibroblast sheets, which we have developed independently, may promote anastomotic site healing with similar efficacy to previously reported cell sheets.

In the present study, the allogenic sheet group had significantly higher collagen deposition and significantly greater vessel area at the anastomotic site (**Figure 5**). Quantitative real-time PCR demonstrated higher expression of collagen type I and III on postoperative days 0, 3 and 5, and VEGF-A on day 0 in the cell sheet group compared to the control group (**Figure 6**). Histology analyses demonstrated greater collagen deposition on postoperative day 5 and a larger vessel area on postoperative day 3 in the cell sheet group compared to the control group (**Figure 5B**). Previous studies including our own previous study have demonstrated tissues adjacent to implanted cell sheets have higher expression of collagen type I and III mRNA, greater collagen deposition, and larger vessel area compared to controls [12, 21, 22]. Since fibroblasts are known to promote collagen deposition and angiogenesis, multilayered fibroblast sheets are also posited to promote collagen deposition and angiogenesis. Anastomotic strength is reportedly determined by the balance between the amounts of collagen degradation and collagen synthesis [24]. Accordingly, anastomotic strength decreases in the first three days postoperatively as collagen degradation exceeds collagen synthesis and anastomotic strength increases with increased collagen synthesis. In the present study, the allogenic sheet group had significantly higher expression of collagen type I and III on postoperative days 0, 3, and 5 (**Figure 6**), indicating allogenic sheets may alter the balance between collagen degradation and collagen synthesis.

In conclusion, allogenic multilayered fibroblast sheets may have utility as a therapeutic material for decreasing anastomotic leakage after esophageal reconstruction.

Acknowledgements

We thank Yukari Hironaka for their technical assistance. This work was supported by a JSPS Grant-in-Aid for Early-Career Scientists (22-K16464 to Y.T.) and R&D Promotion Subsidy System (Yamaguchi Prefecture Government to K.H.).

Disclosure of conflict of interest

None.

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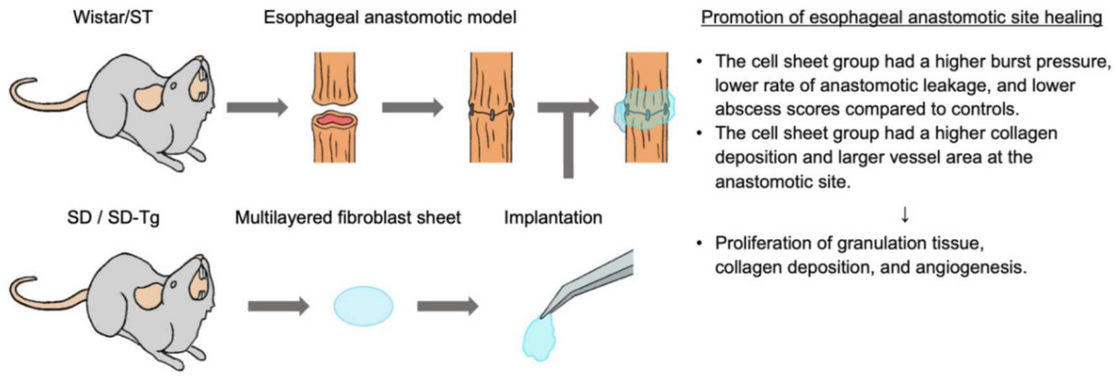
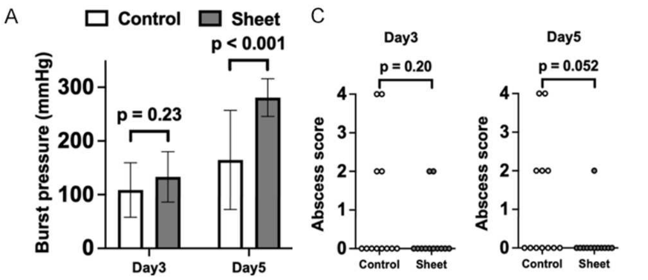


Figure S1. Summary of the experiment. Multilayered fibroblast sheets promote esophageal anastomotic site healing by inducing proliferation of granulation tissue.



B

	Day3 Control (%)	Day3 Sheet (%)	p value	Day5 Control (%)	Day5 Sheet (%)	p value
Anastomotic leakage	4/12 (33.3)	2/12 (16.6)	0.64	5/12 (41.6)	1/12 (8.3)	0.15
Mortality	0/12	0/12	-	0/12	0/12	-

Figure S2. There was a non-significant trend toward higher burst pressure in the autologous multilayered fibroblast sheet (autologous sheet) group compared to the control group on postoperative day 3 (133.1 ± 47.0 mmHg vs. 108.6 ± 50.8 mmHg; $P = 0.23$). In contrast, the autologous sheet group had significantly higher burst pressure than the control group on postoperative day 5 (280.8 ± 35.2 mmHg vs. 164.6 ± 92.3 mmHg; $P < 0.001$). There was a non-significant trend toward a lower rate of anastomotic leakage in the autologous sheet group compared to the control group on postoperative day 5 (1/12 (8.3%) vs. 5/12 (41.6%), $P = 0.15$). No mortality was observed in either group. There was a non-significant trend toward lower abscess scores in the autologous sheet group compared to the control group on postoperative day 5 ($P = 0.052$).

Video S1. Esophageal anastomosis model. The trachea and esophagus were separated. The esophagus was clipped and held in place then transected at the level of the 9th and 10th tracheal cartilage rings. The esophagus was sutured using four stitches of 7-0 polypropylene. The patency of the esophageal lumen was confirmed before suturing to the posterior wall. The suture bite was 1 mm. The esophagus was returned to its original position and the incision was then closed.

Video S2. After suturing the esophagus, two multilayered fibroblast sheets were implanted at the anterior and posterior esophageal anastomotic sites. Cell sheets were transferred with a micropipette to a spoon and then implanted at the anastomotic site.

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CERTIFICATE

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Approval date：December 10.2020

Receipt Number：20-088

Research Number：31-007

Title of Research : Study of cell sheet transplantation for the prevention of esophageal anastomotic leakage

Period of Research：November 1.2020 ～ October 31.2022

This is to certify that the above mentioned research has been approved by the Institutional Animal Care and Use Committee of Yamaguchi University.

Chairperson
Institutional Animal Care and Use Committee
of Yamaguchi University, "Ube" area