Stem-Cell Therapy for Esophageal Anastomotic Leakage by Autografting Stromal Cells in Fibrin Scaffold

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Key Words. Esophageal anastomotic leakage • Mesenchymal stromal cells • Fibrin scaffold • Autograft

ABSTRACT

Esophageal anastomotic leakage (EAL) is a devastating complication for esophagectomy but the available therapies are unsatisfactory. Due to the healing effects of mesenchymal stromal cells (MSCs) and supporting capability of fibrin scaffold (FS), we evaluated the efficacy of a stem-cell therapy for EAL by engrafting adult and autologous MSCs (AAMSCs) in FS and investigated the potential mechanism. Twenty-one rabbits were assigned to AAMSC/FS group (n = 12) and control group (n = 9). After harvested, AAMSCs were identified and then labeled with lenti.GFP. To construct EAL model, a polyethylene tube was indwelled through the anastomosis for 1 week. A total of 2 × 106 AAMSCs in 0.2 ml FS were engrafted onto the EAL for the AAMSC/FS group, whereas FS was injected for control. Magnetic Resonance Imaging (MRI) examination was performed after 5 weeks. Esophageal tissues were harvested for macroscopic, histological analyses, Western blot, and immunohistochemistry at 8 weeks. The animal model of EAL was established successfully. MRI scanning revealed a decreased inflammation reaction in AAMSC/FS group. Accordingly, AAMSC/FS group presented a higher closure rate (83.3% vs. 11.1%, p = .02) and lower infection rate (33.3% vs. 88.9%, p = .02). Histological analyses showed the autografted MSCs resided in the injection site. Furthermore, milder inflammation responses and less collagen deposition were observed in AAMSC/FS group. Western blot and immunohistochemistry studies suggested that the therapeutic effect might be related to the secretions of IL-10 and MMP-9. Engrafting AAMSCs in FS could be a promising therapeutic strategy for the treatment of EAL by suppressing inflammation response and alleviating fibrosis progression. STEM CELLS TRANSLATIONAL MEDICINE 2019;8:548–556

SIGNIFICANCE STATEMENT

For future applications in clinical practice, the autografting of mesenchymal stromal cells in fibrin scaffold could be used concomitantly with the esophagectomy to prevent the occurrence of esophageal anastomotic leakage, thus improving the surgical outcome for esophageal cancer. In addition, the proposed approach would be used to close or repair various leakages and fistulas, such as tracheoesophageal fistula, intestinal fistula, and even the incision of natural orifice transluminal endoscopic surgery (NOTES) through an endoscope. Therefore, the present approach provides a promising alternative for the treatment of several gastroenterological diseases in future clinical practice.

INTRODUCTION

Esophagectomy is the mainstay of curative treatment for locoregional esophageal cancer with or without chemo/radiotherapy [1]. Despite of improvements in surgical technique and postoperative care, esophageal anastomotic leakage (EAL) remains one of the most devastating complications after esophagectomy with a reported incidence up to 35% [2]. EAL could lead to mediastinitis and pyothorax during postoperative period and therefore significantly increases the postoperative morbidity and mortality. Recent research reported that EAL could also adversely impact the long-term survival and induce the recurrence of locoregional cancer [3]. Conservative strategies including drainage, anti-infection treatment, and even aggressive surgical repair are not yet satisfactory for the treatment of EAL.

Mesenchymal stromal cells (MSCs), defined as possessing the capacity for multilineage differentiation, are potential candidates for replacement...
of damaged tissues [4]. In recent years, there are increasing interests in manipulation of MSCs as a regenerative therapy for various diseases, such as myocardial infarction [5], spinal cord injury [6], liver cirrhosis [7], gastric perforation [8], bronchoplastic leakage [9], penetrating ulcers [10], and Colitis [11]. Furthermore, the manipulation of adult and autologous MSCs (AAMSCs) is particularly promising, which could improve the endogenous regenerative potential without risks of rejection or ethical issues related to heterologous or homologous stem cell transplantation [12]. These potential benefits of AAMSCs provide the possibility for the therapy of EAL. However, migration and death of the implanted MSCs has been widely reported to decrease the therapeutic effect in several studies [13, 14]. In recent years, fibrin has been demonstrated with the potential of supporting long-term survival and proliferation of MSCs through specific homing and in situ cell retention, and therefore enhances the therapy efficacy of MSC transplantation [15].

For these reasons, we hypothesized that engrafing AAMSCs in fibrin scaffold (FS) could provide a promising therapy for EAL. The efficacy and underlying mechanisms of this strategy were investigated in this work.

**MATERIALS AND METHODS**

**Animals**

The healthy adult male New Zealand rabbits aged to 12 months were purchased from the animal center of Second Medical University. The animals were maintained under specific pathogen-free condition in accordance with ethical guidelines for the care of the Laboratory Animals of Changhai Hospital. This study was approved by the Committee of Changhai Hospital on the Use and Care of Animals (CHEC-2014-0015).

**AAMSCs Preparation**

A total of 21 rabbits were randomly assigned to the AAMSC/FS group (n = 12) and control group (n = 9). For the preparation of AAMSCs, 1 ml bone marrow was aspirated from the tibia of each animal after anesthetization. Then the AAMSCs were isolated by Ficoll-paque density gradient, plated in 6 well cell culture plates and incubated (37°C, 5% CO2) in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Grand Island, NY, https://www.thermofisher.com/) containing 1% penicillin-streptomycin (Sigma–Aldrich, St. Louis, MO, https://www.sigmaaldrich.com/) and 20% fetal bovine serum (FBS, Gibco) for 14 days as primary culture. The medium were exchanged every 2 days. MSC-specific cell surface markers were identified by flow cytometry for the cultured cells. Briefly, the cells were incubated with Mouse Mesenchymal Stromal Cell Marker antibodies (1:100, Abcam, Cambridge, U.K., http://www.abcam.com/) for CD29, CD44, CD90, and CD45 for 1 hour at room temperature. After being washed to remove unbound primary antibodies, the cells were incubated for 30 minutes with goat anti-chicken Alexa fluor-488 conjugated secondary antibody (1:100, Jackson Laboratory Bar Harbor, ME, https://www.jax.org/) and then analyzed on a flow cytometry (Miltenyi Biotec, Bergisch Gladbach, Germany, https://www.miltenyi-biotec.com/CN-en/) with the FlowJo software (Tree Star Inc., https://www.flowjo.com/).

The adipogenic and osteogenic differentiation was induced with adipogenesis medium (Gibco) for 14 days and osteogenesis medium (Gibco) for 28 days, respectively. The differentiation into adipocyte or osteocytes was respectively confirmed by staining with oil-red O or alizarin red. For in vivo tracing, the AAMSCs were transfected with lentil.GFP (MOI: 40 TU per cell) under normal growth condition for 6 hours. The third passage of spindle-shaped GFP+-MSCs were used for autograft.

**EAL Model Construction**

For each animal, the cervical esophagus was isolated, transplanted and Anastomosed with a 2 mm leakage left. Then a polyethylene tube (2.4 mm caliber) was put through the leakage to create EAL with its inlet left in the esophageal lumen and the outlet was left outside the cervical skin for 1 week (Fig. 1A, 1B). After the surgery, oral intake was stopped and enteral nutrition was fed via the indwelling polyethylene tube for all animals. Broad spectrum antibiotic treatment was administered intravenously. One week after the model construction, the sutures of cervical incisions were taken out and the polyethylene tube was removed. The EAL was carefully exposed and the caliber of EAL was measured (Fig. 1C).

**Engrafing AAMSCs in FS to EAL**

The FS was prepared prior to engrafment as following. In brief, the lyophilized fibrinogen was mixed with 2 ml dilution buffer. Then a total of 2 ml of 40 mmol/l CaCl2 solution was added to 500 IU/ml thrombin. The 2 solutions were mixed 1:1 to resemble the FS through a Y-shape syringe during the procedure of engrafment (Fig. 2D). A total of 2×10⁶ AAMSCs in 0.2 ml FS were injected onto EAL for each of the animals in AAMSC/FS group, whereas 0.2 ml FS alone was injected for animal in control group. Afterward, the cervical incisions were closed by running suturing. All animals were fed through a stomach tube after the procedure.

**Cervical Magnetic Resonance Imaging Evaluation**

Five weeks after the treatment, all the animals underwent cervical Magnetic Resonance Imaging (MRI) scanning to evaluate the focal status of EAL by using 3.0 T superconducting MRI scanner (Toshiba, Tokyo, Japan, http://www.toshiba.co.jp/worldwide/). All the images were reviewed and interpreted by 2 senior radiologists, and the analysis were performed under standard procedure, the radiologists were blinded for analysis. Horizontal T2-weighted images were obtained using following settings: spin-echo sequence: T2WI, time of repetition: 702 seconds, time of echo: 17 seconds, field of view: 320 mm × 320 mm and section thickness: 5 mm.

**Histological Analyses**

The animals were sacrificed at the end time of the study (8 weeks after the treatment). The gross specimens of esophagus at EALs were harvested and inspected carefully for macroscopic investigation. The calibers of unclosed EALs were measured. Then the specimens were fixed with 10% buffered formaldehyde, dehydrated with a series of graded ethanol and embedded in paraffin. Specimens were sliced into 4 μm thick sections for histological analyses with hematoxylin-eosin (H&E) and Van Gieson (VG) staining. Microscopic examinations were performed under light microscope (Olympus, Japan, https://www.olympus-global.com/) and photographed by microscopy imaging system (Olympus).
Immunofluorescence Study

Immunofluorescence staining of GFP and α-SMA was carried out for tracing the biological behavior of engrafted GFP+-AAMSCs in vivo. Briefly, after dewaxing, rehydration, antigen retrieval and blocking, the sections were incubated with mouse anti α-SMA and GFP antibodies (1:50, Abcam), respectively, overnight at 4°C. Sections were then incubated with the goat anti-mouse Alexa-594 conjugated secondary antibody (1:500, Jackson Laboratory) and the goat anti-chicken Alexa-488 conjugated secondary antibody (1:500, Jackson Laboratory) for 1 hour. Finally, the nuclei were counterstained with DAPI (1:10,000, Beyotime, Shanghai, China, http://www.beyotime.com/index.html).

Cytokine Expression Assays

The primary antibodies used were anti TNF-α, TGF-β, IL-6, IL-10, MMP-2, MMP-9, HSP47, VEGF, Collagen-I (Abcam). All primary antibodies were used at dilution 1:100 for Western blot (WB) analyses and 1:50 for the immunohistochemistry study.

For WB analyses, total proteins were extracted from the tissues in EALs and the concentrations were measured using the Enhanced Bicinchoninic Acid Protein Assay Kit (Beyotime, China). Thirty micrograms of the proteins were loaded into each well on 10% SDS-polyacrylamide gels and then transferred to PVDF membranes (Millipore, Billerica, MA). The membranes were blocked with 5% skim milk solution for 1 hour at room temperature and incubated with primary antibody and anti-β-actin antibody (1:10,000, Protein Tech, https://www.ptlab.com/) overnight at 4°C. After being washed three times in 0.2% TBS-Tween, the membranes were incubated for 1 hour with HRP-conjugated secondary antibody. Finally, the blot was developed using the substrate (Thermo Fisher, Massachusetts, https://www.thermofisher.com/). The protein bands were quantified using Image J software (National Institutes of Health, Bethesda, Maryland, https://imagej.nih.gov/).

For the immunohistochemistry studies, the sections were firstly deparaffinized and rehydrated. Endogenous peroxidase was then quenched using 10% H2O2 for 10 minutes at room temperature. Subsequently, excess proteins were blocked with 10% goat serum for 1 hour. The sections were respectively incubated with the primary antibodies as mentioned above overnight at 4°C. Afterward, the sections were rinsed and incubated with biotin-labeled goat anti-mouse IgG. The DAB Horseradish Peroxidase Color Development Kit (Beyotime) was used for color development. Finally, the sections were counterstained with hematoxylin and mounted.

Statistics

All statistical analyses were performed with GraphPad Prism 6 (GraphPad Software, Inc., CA, https://www.graphpad.com/). A p value of less than .05 was considered significant. All parametric
statistical tests met the assumptions of the tests (normal distribution or equal variance). Quantitative data were represented as mean \( \pm \) SD. Two-tailed Student’s t test was performed to analyze the results of quantitative data and Fisher exact test was used to compare differences of rates. The survival curve was analyzed with the methods of Kaplan–Meier and the comparisons between 2 groups were made with log-rank test.

RESULTS

Characteristics of AAMSCs

The schematic diagram of the current work was illustrated in Figure 1A. The spindle-shaped third passage AAMSCs were plated at \( 5 \times 10^5 \) densities in 20% DMEM on cell culture plates. MSC-specific cell surface markers CD29, CD44, and CD90 strongly expressed in AAMSCs, whereas hematopoietic marker CD45 was negative (Fig. 2B). Furthermore, differentiation assays showed the positive staining of oil red O and alizarin red (Fig. 2C, 2D), indicating the differentiation of AAMSCs toward adipocytes and osteoblasts, respectively. Ten days after the transfection of lenti.GFP, the spindle shaped third passage AAMSCs approached 80% confluence on 6 well cell culture plates and the green fluorescence could be observed in \( \approx 60\% \) of the cells with inverted fluorescence microscopy (Fig. 2E, 2F).

Animal Model of EAL

One week after the construction for the animal model, the EALs could be observed in all the animals. The rabbits were identified as suffering with localized infection, if they had obvious signs of redness, swelling, purulent drainage around the EAL, or abnormal temperature. The calibers of EALs were 2.3 mm \( \pm \) 0.15 mm (\( n = 12 \)) for AAMSC/FS group and 2.2 mm \( \pm \) 0.17 mm (\( n = 9 \)) for control group, respectively (\( p > .05 \)). Meanwhile, there was no significant difference between groups in localized infection around EALs (15.7\% vs. 11.1\%, \( p > .05 \)).

Clinical Outcomes

Five weeks after the treatment, narrowed and localized high-intensity signals were observed by cervical MRI evaluation in the animals of AAMSC/FS group. In contrast, mass of high-intensity signals and tracheal deviation could be found in control animals, indicating severe focal abscess formed (Fig. 3A). In the control group, 88.9\% of the rabbits showed high signal intensity, whereas only 25\% in the AAMSC/FS group (\( p = .008 \)). The gross specimens of esophagi at EALs were harvested at
the end time of the study (8 weeks after the treatment). The closure of ELA was regarded as the mucosal layer occlusion and no signs of leakage such presence of abscess and formation of sinus. Macroscopic investigation suggested that EALs were occluded in 10 of 12 animals in AAMSC/FS group (83.3%) and 1 of 9 animals in control group (11.1%; \( p = .02 \); Fig. 3B). In addition, the infection rate was 33.3% (4/12) for AAMSC/FS group compared with 88.9% (8/9) for control with a significant difference (\( p = .02 \); Fig. 3C). The representative photographs of esophageal specimens at EALs were shown in Figure 3D for both groups.

In AAMSC/FS group, 3 of 12 rabbits died of sepsis caused by uncontrolled infection. Meanwhile, of the total 9 rabbits in control group, 5 died during the follow-up period. The causes of death were severe sepsis in 4 rabbits and respiratory failure induced by esophago-tracheal leakage in 1 rabbit. However, there was no significant difference for the mortality between the 2 groups (25.0% vs. 55.9%, \( p = 0.17 \); Fig. 3E).

**Focal Inflammation Response and Fibrosis Progression**

For animals in AAMSC/FS group with closed EALs, the H&E staining of esophagi at EALs showed dispersive inflammatory cells infiltration and VG staining revealed that the red-stained collagen fibers were sparse and regularly organized. In contrast, severe signs of the inflammation response could be observed for control group, such as diffuse infiltration of inflammatory cells, and aggravated proliferation of the adjacent tissues (Fig. 4A). Furthermore, collagen deposition between submucosa layer and muscular layer was heavier and the collagen fibers distributed compactly and disorderly.

**Fate of Engrafted AAMSCs**

In AAMSC/FS group, GFP+-AAMSCs could be identified from the submucosa of esophageal wall at EALs by immunofluorescence study, indicated that engrafted AAMSCs resided in the site of injection after 8 weeks of engraftment, as shown in Figure 4B. Interestingly, the expression of \( \alpha \)-SMA (red), which was a reliable biomarker of myofibroblasts, could be simultaneously observed with the engrafted AAMSCs (green) at EALs.

**Autografted MSCs Mediate Paracrine Action**

Our results of WB analyses and immunohistochemistry study revealed that compared with control group, the expressions...
of IL-10 and MMP-9 were significantly increased, whereas the expressions of TNF-α and TGF-β were significantly decreased after the engraftment of AAMSCs in FS, as shown in Figure 5A. The representative images were shown in Figure 5B, 5C. These evidences strongly suggest that the suppressing effects on inflammatory response and fibrosis progression might be mediated through the paracrine action of the engrafted AAMSCs.

**DISCUSSION**

EAL is one of the most frequent and troublesome postoperative complications after esophagectomy for patients with esophageal cancer [16, 17]. It may cause mediastinitis or pyothorax in early postoperative period and prolong the hospital stay. Conservative strategies including adequate drainage and infection control or even aggressive surgical repair for the treatment of EAL are not yet satisfactory [18]. In recent years, MSC transplantation has proved to be a promising strategy in cell therapy and regenerative medicine for various diseases and numerous clinical applications are under study in ischemic heart disease, atherosclerosis, stroke, diabetes, and organ’s reconstruction [19]. In the present study, we for the first time explored the application of adult and autologous MSCs (AAMSCs) for the treatment of EAL. To evaluate the therapeutic effect of this approach, the animal model of EAL should be established. However, to the best of our knowledge, it has not been proposed and reported as of now. Hence, we developed a method in the current study to establish the animal model of EAL with the adult rabbit by indwelling a polyethylene tube through the anastomosis to create a predictable EAL. At the time for engraftment, EALs could be identified in all the animals and no significant difference was detected in the calibers of leakages and infection rate between 2 groups. In this regard, we consider that the proposed method provides an effective and reliable way for the construction of the animal model of EAL and the established model is suitable for the evaluation of therapeutic effect on EAL.

The most common routes for MSC transplantation are systemic (intravenous) and local injection. Local injection offers more cells in targets site than systemic injection, but the migration and death of implanted MSCs decreased the viable cells at the target site [20]. Fibrin has been demonstrated to be
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and chemokines on cells in their vicinity [26, 27]. To investigate

by synthesizing and secreting a broad spectrum of cytokines
studies implicated that MSCs exerted the tissue repair function

EAL by immunofluorescence study, suggesting that the autografted MSCs in FS could attenuate the

response and inflammation response and

inflammation response and fibrosis progression. Many previous studies implicated that MSCs exerted the tissue repair function by synthesizing and secreting a broad spectrum of cytokines and chemokines on cells in their vicinity [26, 27]. To investigate

the underlying mechanism for the healing effects of autografting MSCs in FS on EAL, immunohistochemistry studies and WB analyses were performed in the present study. Our results revealed the upregulation of IL-10 and MMP-9, whereas down-regulation of TNF-α and TGF-β after autograft of MSCs in FS. The 4 cytokines have been widely demonstrated to be involved in the paracrine function of MSCs [28–32]. Specifically, IL-10 is a well-known anti-inflammatory cytokine that can inhibit the secretion of proinflammatory cytokines, such as IL-2, IL6, TNF-α, and TGF-β [31, 32]. MMP-9, are one family of enzymes which are capable to digest collagen and promote extracellular matrix (ECM) degradation in tissue repair process [33]. In contrast, TNF-α is considered to be a strong proinflammatory cytokine which could initiate the cascade of proinflammatory cytokines through enhancing activation of the NF-κB signaling pathway [30]. TGF-β a role in all phases of tissue repair process and one of the most important functions of TGF-β is chemotactic availability, enabling it to recruit fibroblasts and immune cells such as macrophages, monocytes, and T-cells [34, 35]. The downregulation of TNF-α and TGF-β might be related with the inhibiting function of IL-10 which has been verified in several studies. Taken together, these evidences strongly suggest that the suppressing effects on inflammatory response and fibrosis progression around EAL might be mediated through the paracrine pathway of the autografted MSCs in FS.

In addition, a clearly higher closure rate of EAL was achieved in AAMSC/FS group than control group (83.3% vs. 11.1%).

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Moreover, the infection rate of AAMSC/FS group was lower than control (33.3% vs. 88.9%). We refer that the alleviated inflammation and fibrosis improved the tissue regeneration around EAL and promoted the closure of EAL in AAMSC/FS group. The decreased infection rate might be also contributed to the closure of EAL. It has been reported that MSCs could attenuate infection via inhibiting the overproduction of prostaglandin and recruitment of macrophages [36].

Furthermore, the survival rate of AAMSC/FS group was more than twice higher than control group (55.9% vs. 25%), although no statistic difference in mortality was detected between the 2 groups. All these results suggested that autografting of MSCs in FS provide a superior healing effect on the therapy of EAL.

**CONCLUSION**

With the supporting of FS, adult and autologous MSCs could promote the healing effect of EAL by attenuating the inflammatory response and fibrosis through their paracrine pathway (Fig. 6), which can be pursued as a novel and efficient.

**CLINICAL PROSPECTIVE**

For future application in clinical practice, the autografting of MSCs in FS could be used concomitantly with the esophagectomy to prevent the occurrence of EAL, thus improve the surgical outcome for esophageal cancer. In addition, the proposed approach would be used to close or repair various leakages and fistulas, such as tracheoesophageal fistula, intestinal fistula and even the incision of natural orifice transluminal endoscopic surgery (NOTES) through an endoscope. Therefore, the present approach provides a promising alternative for the treatment of several gastroenterological diseases in future clinical practice.

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**AUTHOR CONTRIBUTIONS**

X.X., Y.Y., Y.M.: contributed equally to this work as first authors; X.X., Y.Y., Y.M.: performed the experiments, drafted the manuscript; Y.Y., C.G.L.: provided critical research resources and consultation; X.L.L.: collected, analyzed and interpreted the data; H.Z.C., Z.Y.X.: developed the study hypothesis and designed the experiments; H.Z.: supported and supervised the study; X.X., Y.Y., Y.M., Y.Y., C.L., X.L., Z.X., H.C., H.Z.: read and approved the final manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicated no potential conflicts of interest.

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