Oral Administration of Conditioned Medium Obtained from Mesenchymal Stem

Cell Culture Prevents Subsequent Stricture Formation after Esophageal

Submucosal Dissection in Pigs

Takeshi Mizushima,1 Shunsuke Ohnishi,1, * Hidetaka Hosono,1 Kenichi Yamahara,2

Momoko Tsuda,1 Yuichi Shimizu,3 Mototsugu Kato,3 Masahiro Asaka,4

and Naoya Sakamoto1

1Department of Gastroenterology and Hepatology, Hokkaido University Graduate School of Medicine, Sapporo, Japan.

2Department of Transfusion Medicine and Cell Therapy, Hyogo College of Medicine, Nishinomiya, Japan.

3Division of Endoscopy, Hokkaido University Hospital, Sapporo, Japan.

4Department of Cancer Preventive Medicine, Hokkaido University Graduate School of Medicine, Sapporo, Japan.
Correspondence to: Dr. Shunsuke Ohnishi, Department of Gastroenterology and Hepatology, Hokkaido University Graduate School of Medicine, N15, W7, Kita-ku, Sapporo 060-8638, Japan. Phone: +81-11-716-1161; Fax: +81-11-706-7867; E-mail: sonishi@pop.med.hokudai.ac.jp
ABSTRACT

Background and Aims: Endoscopic submucosal dissection (ESD) for esophageal cancer often causes postoperative stricture when more than three-quarters of the circumference of the esophagus is dissected. Mesenchymal stem cells are a valuable cell source in regenerative medicine, and conditioned medium (CM) obtained from mesenchymal stem cells reportedly inhibits inflammation. In this study, we evaluated whether CM could prevent esophageal stricture after ESD.

Methods: We resected a semi-circumference of pig esophagus by ESD. We prepared CM gel by mixing with 5% carboxymethyl cellulose, and endoscopically applied it onto the wound bed immediately after ESD, and on day 8 and 15 (weekly CM group), or orally administered from day 1 through day 4 (daily CM group). We also injected triamcinolone acetonide into the remaining submucosa immediately after ESD (steroid group). We euthanized the pigs on day 8 or day 22 to measure the stricture rate and perform histological analysis.

Results: Stricture rate in weekly and daily CM groups and steroid groups were significantly lower than in the control group on day 22. Moreover, CM significantly
attenuated the number of activated myofibroblasts and fiber thickness on day 22. CM also significantly decreased the infiltration of neutrophils and macrophages compared with the control group on day 8.

**Conclusions:** CM gel prevents esophageal stricture formation by suppressing myofibroblast activation and fibrosis following the infiltration of neutrophils and macrophages. Oral administration of CM gel would be a promising treatment for the prevention of post-ESD stricture.

**Keywords:** Endoscopic submucosal dissection, Esophageal stricture, Mesenchymal stem cells, Conditioned medium, Amnion

**Abbreviations:**

AMSCs, amnion-derived mesenchymal stem cells; α-SMA, α-smooth muscle actin; CM, conditioned medium; ESD, endoscopic submucosal dissection; IL, interleukin; MEM, minimal essential medium; MPO, myeloperoxidase.
**INTRODUCTION**

Esophageal cancer is the eighth most common cancer and the sixth most common cause of cancer death worldwide.[1] Treatment options for esophageal cancer include endoscopic resection, chemotherapy, operation and radiotherapy; however, endoscopic resection has been particularly selected for early stage esophageal cancer.[2]

Endoscopic submucosal dissection (ESD) for gastrointestinal neoplasms has been widely accepted in past decades because ESD enables *en bloc* resection of any tumor size and location. ESD has low complication rates and excellent long-term outcome.[3-6] However, it often causes postoperative stricture when wide dissection is necessary.[7, 8] The stricture, especially in esophageal ESD cases, occurs when more than three-quarters of the circumference of the esophagus is dissected, and sometimes requires multiple balloon dilation sessions, thereby lowering quality of life for patients.[9] To prevent stricture after ESD, balloon dilation, local injection or oral administration of steroid are generally performed.[10-13] Although these methods are effective, complications such as perforation, mediastinum abscess and steroid-induced side effects are of concern.[14, 15]
Mesenchymal stem cells are multipotent cells that can differentiate into a variety of lineages, including bone, cartilage or fat, and are present in adult tissue.[16] At present, mesenchymal stem cells have been investigated in regenerative medicine because of their differentiation ability and their potential to improve damaged tissues by the secretion of a variety of growth factors and anti-inflammatory molecules.[17, 18]

The fetal membrane consists of amnion and chorion, which envelops the developing fetus.

Although human fetal membrane is usually discarded as medical waste after delivery, fetal tissues have been found to be rich sources of mesenchymal stem cells.[19, 20] We have demonstrated that systemic administration of fetal membrane- or amnion-derived mesenchymal stem cells (AMSCs) improved rats with hindlimb ischemia,[21] myocarditis,[22, 23] glomerulonephritis,[24] ischemia/reperfusion-induced acute kidney injury,[25] severe colitis,[26] radiation proctitis,[27] pancreatitis,[28] and liver fibrosis,[29] possibly through secretory factors from transplanted AMSCs.

Thus, the aim of this study was to examine the effect of conditioned medium (CM) obtained from AMSC culture on the prevention of esophageal stricture after ESD, and
to investigate the underlying mechanisms.
MATERIALS AND METHODS

Animals

The experimental protocol was approved by the Animal Care and Use Committees of Hokkaido University. Female domestic pigs (20–25 kg, Sankyo Labo Service, Tokyo, Japan) were used in this study.

Isolation and expansion of human AMSCs

The Medical Ethical Committee of Hokkaido University Graduate School of Medicine, Sapporo, Japan approved this examination, and all pregnant women gave written informed consent. The human fetal membrane was obtained during caesarean deliveries, and the amnion was separated from the chorion by peeling. AMSCs were isolated and expanded by digestion with collagenase (Nippi, Tokyo, Japan) and dispase I (Wako Pure Chemical Industries, Osaka, Japan), followed by seeding in uncoated plastic dishes with minimal essential medium (MEM) α (DS Pharma Biomedical, Osaka, Japan) supplemented with 10% fetal bovine serum (Moregate Biotech, Bulimba, Australia) and 40 μg/ml of gentamicin (MSD, Tokyo, Japan). The culture was maintained at 37°C in a
humidified atmosphere of 95% air and 5% CO₂. After 3–4 days in culture, the non-adherent cells were removed and the adherent cells were maintained in culture until they reached 80% confluence. The passage was performed using 1 mM of ethylenediaminetetraacetic acid (Invitrogen, Carlsbad, California, USA) and 0.1 mg/ml of trypsin (Roche Diagnostics, Basel, Switzerland).

**Differentiation of human AMSCs into adipocytes and osteocytes**

Human AMSCs were seeded onto six-well plates, and differentiation into adipocytes and osteocytes was induced when the AMSCs were 80%–90% confluent. To induce differentiation into adipocytes, AMSCs were cultured with hMSC Mesenchymal Stem Cell Adipogenic Differentiation Medium (Lonza, Basel, Switzerland), according to manufacturer’s instructions. After 3 weeks of differentiation, cells were stained with Oil Red O (Sigma-Aldrich, St. Louis, Missouri, USA). To induce differentiation into osteocytes, hAMSCs were cultured in hMSC Mesenchymal Stem Cell Osteogenic Differentiation Medium (Lonza), according to manufacturer's instructions. After 2 weeks of differentiation, cells were stained with Alizarin Red S (Sigma-Aldrich).
**Flow cytometry**

Cultured AMSCs were stained using the Human MSC Analysis Kit (Becton, Dickinson and Company [BD], Franklin Lakes, New Jersey, USA), which included mesenchymal markers such as fluorescein-isothiocyanate-conjugated antibody against CD90, PerCP-Cy5.5-conjugated antibody against CD105 and allophycocyanin-conjugated antibody against CD73 as well as a negative cocktail (phycoerythrin-conjugated CD11b, CD19, CD34, CD45 and HLA-DR), which are markers for hematopoietic cells and endothelial cells, according to manufacturer’s instructions. Cells were analysed by a flow cytometer (FACSCanto II, BD).

**Preparation of CM gel**

CM was collected by culturing sub-confluent AMSCs with serum-free MEMα for 48 h after washing with phosphate-buffered saline (Invitrogen), and mixed with 5% carboxymethyl cellulose (Wako Pure Chemical Industries). Serum-free MEMα mixed with 5% carboxymethyl cellulose was used as a standard medium (SM) gel.
Animal model

Induction of anesthesia to the pigs (n=21) was performed with intramuscular injection of midazolam (20 mg, Astellas, Tokyo, Japan) and buprenorphine hydrochloride (0.2 mg, Otsuka Pharmaceutical, Tokyo, Japan), followed by inhalation of 5% sevoflurane (Maruishi Pharmaceutical, Osaka, Japan). Pigs were then intubated and connected to a mechanical ventilator under 3% sevoflurane in oxygen. ESD was performed under continuous monitoring of the heart rate, three-lead electrocardiography and oxygen saturation (Nihon Kohden, Tokyo, Japan), and a single-channel gastrointestinal endoscope (GIF-Q240, Olympus, Tokyo, Japan) with a transparent attachment hood fitted to the tip (Top, Tokyo, Japan) was used.[30] The markings for incision line were placed with a flush knife BT (Fujifilm, Tokyo, Japan) on the lower part of the esophagus as a semi-circumference and 5 cm of long axis. A glycerol solution was injected with 25-gauge needle (Top) into the submucosal layer before mucosal and submucosal cutting. After injection, a circumferential incision was made using a flush knife BT and IT nano knife (Olympus). Submucosal dissection was then performed using an IT nano knife (Fig. 1A). An electrosurgical generator (ESG-100, Olympus)
was set to the pulse cut slow mode (40W) or forced coagulation mode (50W) for incision of the mucosa and submucosa. Hemorrhage was controlled using hemostatic forceps, such as Coagrasper (Olympus) in the soft coagulation mode (40W). All ESD procedures were performed by one endoscopist (T. M.) who had performed more than 500 ESD in humans, including the esophagus, stomach and colon. For postoperative care, all pigs were given liquid from the next day after ESD, and then they were given solids the following days.

**Experimental design**

We conducted two experiments in this study. To evaluate the effect of CM gel for the prevention of esophageal stricture formation after ESD, we designed four groups: the first group was the weekly SM gel group (SM), the second group was weekly CM gel group (CM-W), the third group was daily CM gel group (CM-D) and the last group was steroid injection group (n=3 in each group, Fig. 1B). All pigs were sacrificed on day 22 in this experiment. In the second experiment, we designed three groups including SM gel group, CM gel group and steroid injection group to assess the effect of CM gel on
acute reaction after ESD (n=3 in each group, Fig. 1C). We euthanized the pigs on day 8 in this experiment. We performed ESD sequentially on a per-group basis in each experiment.

Administration of CM gel and triamcinolone

For the CM-W group, an 18-F tube (Terumo, Tokyo, Japan) was fixed along the endoscope with tape, and 20 mL of CM gel was endoscopically applied through the tube onto the wound bed of the esophagus immediately after ESD (day 1), day 8 and day 15 under general anesthesia (Fig. 1D). Twenty mL of SM gel was applied to the SM group.

For the CM-D group, the same method as described above was performed on day 1, and 20 mL of CM gel was orally administered twice a day, in the morning and in the evening, from day 2 through day 4. For the steroid group, 0.5 mL of 20 mg/mL triamcinolone acetonide (Bristol-Myers Squibb, New York, New York, USA) in saline was directly injected into the ulcer bed submucosa immediately after ESD at 8 sites, using a 3 mm-length 25-gauge injection needle (Top).
Assessment of the degree of esophageal stricture after ESD

All pigs were sacrificed on day 22 by intravenous injection of 20 mL of 15% potassium chloride (Terumo) after general anesthesia. The anterior neck and abdomen were incised, and transhiatal esophagectomy was performed. The resected esophagus was immediately placed on a corkboard and fixed with pins. The degree of stricture at the lesion site was expressed as the lateral mucosal constriction rate calculated by following formula, as described previously:[31]

\[
\text{Mucosal constriction rate (\%) = } \left[ 1 - \frac{\text{length of short axis at site of maximal constriction}}{\text{length of short axis at a normal mucosal site on upper side + length of short axis at a normal mucosal site on lower side)/2}} \right] \times 100.
\]

Histological and immunohistochemical examination

The esophagus was fixed in 40 g/l of formaldehyde saline, embedded in paraffin and cut into 5 \( \mu \)m sections. Tissue sections were stained with Masson’s trichrome to examine the accumulation of collagen fibers. Three fields on a section from each pig were
photographed, and the thickness of the stained areas was measured (×200) with a digital image analyser (WinROOF, Mitani Co., Fukui, Japan and NDP. view2 software, Hamamatsu, Japan).

The tissue sections were stained with anti-α-smooth muscle actin (α-SMA) antibody (clone 1A4, dilution; 1:1,000, Sigma-Aldrich) for 60 min, anti-myeloperoxidase (MPO) antibody (dilution; 1:300, Thermo Scientific, Waltham, Massachusetts, USA) for 40 min, anti-CD107a antibody (clone 4E9/11, dilution; 1:300, AbD Serotec, Kidlington, UK) for 60 min, anti-CD31 antibody (clone M-20, dilution; 1:600, Santa Cruz Biotechnology, Dallas, Texas, USA) for 30 min and anti-Ki-67 antibody (clone MIB-1, dilution; 1:100, Dako, Glostrup, Denmark) for 60 min at room temperature. Nine random fields on a section from each esophagus were photographed, and stained areas were calculated from the entire cross-sectional area of the esophagus.

**Statistical Analysis**

Data were expressed as mean (SD). Outcomes in the four groups were compared using one-way ANOVA with post hoc comparisons made (upon rejecting the hypothesis that
all four group means were the same) by the Newman-Keuls procedure. For these tests statistical significance was taken as \( p < 0.05 \). Although there was multiple testing of outcome data arising from individual patients, correction by Bonferroni’s method would not have removed significance from any findings, so all \( p \)-values are presented uncorrected for multiple testing. Also, because an underlying assumption needed for ANOVA is homoscedasticity (equality of all variances in the groups compared), the Brown-Forsythe test was used to ensure there were not indications of violation of this assumption, requiring only \( p < 0.10 \) for this examination. All analyses were performed using GraphPad Prism version 6 (GraphPad Software, San Diego, California, USA).
RESULTS

Characterization of human AMSCs

To evaluate the multipotency of human AMSCs, we induced differentiation of cultured AMSCs into adipocytes and osteocytes. AMSCs differentiated into adipocytes and osteocytes, as demonstrated by Oil Red O and Alizarin Red S staining, respectively (Fig. 2A). Flow cytometry of cultured AMSCs demonstrated that they expressed CD44, CD73, CD90 and CD105, but not CD34, CD11b, CD19, CD45 and HLA-DR, which is characteristic of MSCs (Fig. 2B).[32]

Homoscedasticity

Although the standard deviations in each parameter apperred to be somewhat different (table1 and 2), they all were examined and found not to be significantly heteroscedastic, suggesting that there were no differences as homoscedasticity in each analysis by Brown-Forsythe test.
Effect of CM gel in the prevention of esophageal stricture formation after ESD

To investigate the effect of CM gel in the prevention of esophageal stricture formation after ESD, we resected a semi-circumference of pig esophagus by ESD, and endoscopically applied CM gel directly onto the wound bed weekly through the tube for three weeks (CM-W group), or orally every day for four days (CM-D group). The esophagus was removed at day 22, and the mucosal constriction rate was evaluated. Compared with the control (SM group), the esophageal stricture was significantly suppressed in the CM-W and CM-D group, and was comparable to the steroid group (80.0 (2.0) % vs. 56.3 (7.1) %, 52.7 (19.3) % and 49.3 (4.2) %, respectively) (Fig. 3, Table1).

Histological analysis of esophagus after applying CM gel

We next performed a histological analysis of the esophagus removed on day 22. Masson’s trichrome staining demonstrated that ESD caused severe fibrotic change, and the fiber accumulation reached too deep into the muscularis propria; however, fiber thickness was significantly reduced by applying CM gel or steroid (Fig. 4A, Table1).
The number of α-SMA-positive myofibroblasts was also significantly decreased by CM gel or steroid (Fig. 4B, Table1). However, the numbers of infiltrated MPO-positive neutrophils (Fig. 4C, Table1), CD107a-positive macrophages (Fig. 4D, Table1), capillary density (Suppl. Fig. S1A, Table1) and Ki-67-positive proliferating cells (Suppl. Fig. S1B, Table1) were not attenuated by CM gel and steroid.

**Effect of CM gel on acute reaction after ESD**

Because oral administration of CM gel from day 1 through day 4 was effective for the prevention of esophageal stricture formation after ESD, we hypothesized that CM affects the acute phase of wound healing after ESD. Therefore, we performed further experiment to observe the acute phase. We orally administered CM gel twice a day for one week after ESD, and performed the histological analysis on day 8. The numbers of infiltrated neutrophils and macrophages were significantly decreased in the CM gel group and steroid group compared with the SM gel group (Fig. 5A and 5B, Table2). The number of activated myofibroblasts tended to be decreased in the CM gel group and
steroid group (Fig. 5C, Table2).


**DISCUSSION**

In this study, we investigated the effect of CM obtained from AMSCs on esophageal stricture formation after ESD, and we found that (1) esophageal stricture is linked to the fiber thickness in the esophageal wall; (2) oral administration of CM gel prevented esophageal stricture formation after ESD; (3) the effect of CM gel was comparable with steroid injection; and (4) CM gel suppressed the fiber accumulation, activation of myofibroblasts, and the infiltration of neutrophils and macrophages in the esophagus.

Since stricture formation after large-scale esophageal ESD deteriorates the quality of life, prevention of postoperative stricture has recently been an issue of interest. Inflammation and fibrosis of the esophageal wall after ESD reduce the elasticity and compliance, which causes the postoperative stricture.[31] Fibrosis is the excessive accumulation of extracellular matrix such as collagen produced by the activated myofibroblasts during the wound healing process.[33] In addition, neutrophils and macrophages at the site of tissue damage release a variety of cytokines such as tumor necrosis factor-α, transforming growth factor-β and interleukin (IL)-1β, which activate resident fibroblasts into myofibroblasts. On the other hand, CM could decrease these
cytokines in several animal models.[34, 35] Therefore, we hypothesized that it is important to control inflammatory cells including neutrophils, macrophages and subsequent fibrosis for the prevention of postoperative esophageal ESD stricture. To prevent esophageal stricture, several animal experiments have been reported, and they are divided into two types: cell sheet type and cell injection type. Kanai et al. transplanted a fabricated autologous epidermal cell sheet to prevent stricture after circumferential ESD in pigs.[36] They reported that a lower stricture rate, early re-epithelialization and mild fibrosis were observed in the transplanted group compared with the control group. Perrod et al. performed transplantation of an adipose tissue-derived MSC sheet after semi-circumferential ESD in pigs.[37] They also reported a lower stricture rate and lower fibrosis development in comparison with the control group. Barret et al. tried to prevent stricture after circumferential ESD using human amniotic membrane graft in pigs.[38] They showed delayed development of stricture, but they could not prevent the stricture. Honda et al. injected autologous adipose tissue-derived cells into the residual submucosa after circumferential endoscopic mucosal resection in dogs.[31] They showed that the mucosal contraction
rate in the adipose tissue-derived cells group was significantly lower than in the control group, and adipose tissue-derived cells significantly upregulated microvessel formation. Although they did not investigate the infiltration of inflammatory cells, adipose tissue-derived cells have been shown to have an anti-inflammatory effect. In the present study, CM could decrease the infiltration of neutrophils and macrophages in the acute phase, followed by suppression of myofibroblast activation and fiber accumulation as the key mechanisms of prevention of stricture formation during the wound healing process. Although several factors such as prostaglandin E2 and IL-10 in CM have been suggested to be attributable for anti-inflammatory effects, further studies to clarify the underlying mechanisms are required.

We selected CM gel for delivery because it can be easily applied to the wound bed. The sheet method is relatively time-consuming and costly, and the injection method requires skill to inject into the remaining very thin submucosa. We believe that oral administration of gel would be easy and widely acceptable as a delivery method. In addition, we performed oral administration of non-gel CM (i.e. without carboxymethyl cellulose) from day 1 through day 4; however, it was not effective for the prevention of
esophageal stricture three weeks after ESD (69.4 (3.4) %, n = 2). Therefore, it appears that gel formation would be imperative for the in situ anti-inflammatory effect, and for the subsequent inhibition of myofibroblast activation and fiber formation. The viscosity of the CM gel used in this study was 50,000–60,000 cP, and this viscosity appeared suitable for the retention of CM on the wound surface; although the vast majority of the CM passed through the wound area by esophageal peristalsis, a small amount of CM retained on the wound surface by making a coating.

For clinical application, steroid treatment has gradually been accepted to prevent postoperative stricture by its expected anti-inflammatory actions.[11, 12, 42] Although it is essential to compare steroid treatment with other preclinical methods on the effectiveness of prevention, there is no report on that so far. To investigate the effectiveness of CM gel for prevention in comparison with steroid treatment, we designed a steroid treatment group in this study, and we revealed that CM gel is as effective as steroid treatment to prevent stricture formation. Although systemic administration and local injection of steroid have been reported to be effective to prevent stricture after ESD, complications and side effects should be considered.
Systemic steroid was administered at 30 mg/day as an initial dose with tapering for 8
weeks, and then the cumulative dose of steroid was approximately 1,000 mg.[12]

Therefore, the potential risk of steroid-induced side effects such as immune suppression,
peptic ulceration, psychiatric disturbance, optical damage and diabetes are of
concern.[43] Since deep steroid injection into esophageal wall developed esophageal
abscess [14] or delayed perforation,[44] steroid has to be carefully injected into the
remaining submucosa after ESD.

This study has several limitations. (1) Our study was conducted in a small sample size.
(2) Since we used small pigs (20kg) in this study, it is not clear whether our results can
be extrapolated simply to human adults. (3) Although we used CM obtained from a
single donor, it should be clarified whether there is an individual variability of CM.

Furthermore, although FBS has mostly been used in order to culture and propagate
mesenchymal stem cells,[45] there is an urgent need for suitable human alternatives for
clinical use to avoid the risks of transmission of pathogens and xeno-immunization
against bovine antigens.

In conclusion, stricture after ESD is linked to the fiber thickness in the esophageal
wall, and oral administration of CM obtained from AMSCs prevented esophageal stricture formation by suppressing the infiltration of neutrophils and macrophages, followed by activation of myofibroblasts and collagen synthesis.

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COMPETING INTERESTS

The authors disclose no competing interests.

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AUTHOR CONTRIBUTION:

TM, MT and SOh performed the experiments and analyses and drafted the manuscript, TM performed ESD. KY and HH performed in vitro experiments. YS, MK, MA and NS supervised the entire project.
REFERENCE LIST


FIGURE LEGENDS

Figure 1. Experimental protocol for esophageal endoscopic submucosal dissection (ESD) model.

(A) Procedure of ESD. The markings for incision line were placed with a flush knife BT on the lower part of the esophagus as a semi-circumference and 5 cm of long axis. A circumferential incision was made using a flush knife BT and IT nano knife. Submucosal dissection was then performed using an IT nano knife.

(B) ESD was performed on day 1 to all pigs. Twenty mL of standard medium (SM) gel or conditioned medium (CM) gel was endoscopically applied to the dissected surface weekly for 3 times (SM and CM-W group, respectively). For the CM-D group, 20 mL of CM gel was orally administered twice a day until day 4. For the steroid group, 0.5 mL of 20 mg/mL triamcinolone was directly injected at 8 sites in the dissected surface on day 1. All pigs were sacrificed on day 22 (n=3 in each group).

(C) ESD was performed to the pigs on day 1 for all pigs. Twenty mL of SM gel or CM gel was orally administered twice a day for 7 days. For the steroid group, 0.5 mL of 20 mg/mL triamcinolone was directly injected at 8 sites in the dissected surface on day 1.
All pigs were sacrificed on day 8 (n=3 in each group).

(D) CM gel was endoscopically applied to the dissected surface.

Figure 2. Characterization of cultured amnion-derived mesenchymal stem cells (AMSCs).

(A) Multipotency of AMSCs. Differentiation into adipocytes was confirmed by the existence of lipid vesicles stained with Oil red O (left). Differentiation into osteocytes was confirmed by the existence of mineral nodule deposition stained with Alizarin Red S (right). Scale bars, 50 μm.

(B) Flow cytometry of AMSCs. The negative cocktail contained antibodies against CD11b, CD19, CD34, CD45 and HLA-DR. Closed areas indicate staining with a specific antibody, whereas open areas represent staining with isotype control antibodies.

Figure 3. Effect of amnion-derived mesenchymal stem cell-conditioned medium (CM) gel in the prevention of esophageal stricture three weeks after endoscopic submucosal dissection (ESD).
Macroscopic finding of the esophagus (upper panels) and the degree of stricture of the esophagus (lower panel). Scale bars, 10 mm. The values were the mean (SD) of three animals/group. *p < 0.05 vs. SM group.

SM; standard medium gel, CM-W; weekly administration of CM gel (day 1, 8 and 15), CM-D; daily administration of CM gel (day 1 through day 4).

Figure 4. Effect of amnion-derived mesenchymal stem cell-conditioned medium (CM) gel on the histological findings three weeks after endoscopic submucosal dissection (ESD).

(A) Masson’s trichrome staining and the fiber thickness.

(B) α-SMA expression.

(C) Myeloperoxidase (MPO) staining.

(D) CD107a expression.

Scale bars of A, 1 mm. Scale bars of B-D, 50µm. The values were the mean (SD) of three animals/group. *p < 0.05, **p < 0.01 vs. SM group.

SM; standard medium gel, CM-W; weekly administration of CM gel, CM-D; daily
administration of CM gel.

**Figure 5.** Effect of amnion-derived mesenchymal stem cell-conditioned medium (CM) gel on the histological findings one week after endoscopic submucosal dissection (ESD).

(A) Myeloperoxidase (MPO) staining.

(B) CD107a expression.

(C) α-SMA expression.

Scale bars, 50 µm. The values were the mean (SD) of three animals/group. **p < 0.01 vs. SM group, ††p < 0.01 vs. steroid group.

SM; standard medium gel, CM; conditioned medium gel.
SUPPLEMENTARY FIGURES

Figure S1. Effect of amnion-derived mesenchymal stem cell-conditioned medium (CM) gel on the histological findings three weeks after endoscopic submucosal dissection (ESD).

(A) CD31 staining.

(B) Ki-67 staining.

Scale bars of A, 200 µm. Scale bars of B, 1mm. The values were the mean (SD) of three animals/group. †p < 0.05 vs. steroid group.

SM; standard medium gel, CM-W; weekly administration of CM gel, CM-D; daily administration of CM gel.
### TABLE 1. Histological analysis of esophagus three weeks after applying CM gel

<table>
<thead>
<tr>
<th></th>
<th>SM</th>
<th>CM-W</th>
<th>CM-D</th>
<th>Steroid</th>
<th>ANOVA p-value</th>
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<tr>
<td><strong>Mucosal stricture</strong></td>
<td>80.0</td>
<td>56.3</td>
<td>52.3</td>
<td>49.3</td>
<td>0.03</td>
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<tr>
<td>%, mean (SD)</td>
<td>(2.0)</td>
<td>(7.1)</td>
<td>(19.3)</td>
<td>(4.0)</td>
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<tr>
<td><strong>Fiber thickness</strong></td>
<td>1609</td>
<td>833</td>
<td>987</td>
<td>944</td>
<td>0.02</td>
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<tr>
<td>µm, mean (SD)</td>
<td>(418)</td>
<td>(26)</td>
<td>(145)</td>
<td>(251)</td>
<td></td>
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<tr>
<td><strong>Activated myofibroblasts</strong></td>
<td>68.3</td>
<td>26.8</td>
<td>21.5</td>
<td>20.6</td>
<td>&lt; 0.01</td>
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<tr>
<td>cells/HPF, mean (SD)</td>
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<td>(8.6)</td>
<td>(4.9)</td>
<td>(2.3)</td>
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<tr>
<td><strong>MPO-positive neutrophils</strong></td>
<td>16.8</td>
<td>7.2</td>
<td>26.3</td>
<td>24.9</td>
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<td>(7.1)</td>
<td>(3.8)</td>
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<td><strong>CD107a-positive macrophages</strong></td>
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<td>28.2</td>
<td>23.0</td>
<td>24.6</td>
<td>0.21</td>
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<td>cells/HPF, mean (SD)</td>
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<td>(13.6)</td>
<td>(6.2)</td>
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<td><strong>Capillary density</strong></td>
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<td>27.7</td>
<td>28.9</td>
<td>19.4</td>
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<td>(4.3)</td>
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<td><strong>Ki-67 positive cells</strong></td>
<td>741.3</td>
<td>516.8</td>
<td>1170.0</td>
<td>989.2</td>
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<td>cells/LPF, mean (SD)</td>
<td>(298.7)</td>
<td>(88)</td>
<td>(188.9)</td>
<td>(738.7)</td>
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### TABLE 2. Histological analysis of esophagus one week after applying CM gel

<table>
<thead>
<tr>
<th></th>
<th>SM</th>
<th>CM</th>
<th>Steroid</th>
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<tr>
<td><strong>MPO-positive neutrophils</strong></td>
<td>68.1</td>
<td>31.7</td>
<td>22.7</td>
<td>&lt; 0.01</td>
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<tr>
<td>cells/HPF, mean (SD)</td>
<td>(14.2)</td>
<td>(5.9)</td>
<td>(4.5)</td>
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<tr>
<td><strong>CD107a-positive macrophages</strong></td>
<td>33.9</td>
<td>13.2</td>
<td>22.6</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>cells/HPF, mean (SD)</td>
<td>(2.8)</td>
<td>(1.7)</td>
<td>(2.5)</td>
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</tr>
<tr>
<td><strong>Activated myofibroblasts</strong></td>
<td>13.6</td>
<td>6.8</td>
<td>10.9</td>
<td>0.14</td>
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<tr>
<td>cells/HPF, mean (SD)</td>
<td>(3.9)</td>
<td>(4.0)</td>
<td>(2.6)</td>
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</tbody>
</table>
Fig. 1B
After applying CM gel
Fig. 3
Fig. 4A

Graph showing fiber thickness (µm) for different treatments:
- SM
- CM-W
- CM-D
- Steroid

The graph indicates a decrease in fiber thickness for CM-W, CM-D, and Steroid compared to SM.
Fig. 4C
Fig. 5C

**SM**

**CM**

**Steroid**

**Activated myofibroblasts**

<table>
<thead>
<tr>
<th></th>
<th>SM</th>
<th>CM</th>
<th>Steroid</th>
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</thead>
<tbody>
<tr>
<td>(cells/HPF)</td>
<td>15±2</td>
<td>7±1</td>
<td>18±3</td>
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Supplementary Figure 1B