Introduction

Benign esophageal stricture has major deleterious effects on a patient’s quality of life and may lead to severe complications, such as malformation, weight loss, vomiting, and aspiration. Benign esophageal stricture often follows severe inflammation of the esophageal wall, which is caused by chronic reflux of gastric acid, radiation injury, or accidental ingestion of alkali. According to recent advances in endoscopic therapy for early stage esophageal carcinoma or high-grade dysplasia, the incidence of esophageal stricture has been increasing as a complication after mucosal resection for widespread lesions. Patients often develop refractory esophageal stricture and need to undergo periodic balloon dilation. The fibrotic changes and scar formation that occur because of the healing process have been the most commonly recognized causes of esophageal stricture. However, few studies have investigated the etiology of esophageal stricture, and the details of the mechanism responsible for this condition have not been clarified.

The following series of hypotheses are set up, referring to scar formation of the skin after injuries or burns. First, severe inflammation caused by gastric acid, radiation, alkali, or iatrogenesis damages the deep layers of the esophageal wall, which is evaluated by the endoscopic observation and histological findings. Immunohistochemical staining was performed for collagen I or collagen III. In the biomechanical evaluation, rectangular-shaped specimens were obtained from the esophageal samples. The failure load and linear stiffness were measured using a tensile tester and compared healed tissue with normal.

Results: The dogs developed severe stricture. The macroscopic appearance showed a deep ulcer formation and severe mucosal constriction. No esophageal glands or muscularis mucosae were evident, and instead, they were replaced with condensed granulation tissue that were composed type I and III collagen fibers. The inner circular muscular layer of the muscularis propria showed partial atrophy and fibrosis in the center of the lesion. The failure load and linear stiffness were significantly decreased in the healed tissue.

Conclusion: The cause of benign esophageal stricture might have been the biomechanical deterioration that was caused by the change of the collagen components in the submucosal layers or atrophic changes of inner circular muscles.

Keywords: Esophageal stricture, Endoscopic resection, failure load, linear stiffness

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The mechanism of esophageal stricture is important for developing a suitable treatment for benign esophageal strictures.

**Materials and Methods**

**Animal Care and Anesthesia**

The beagle dogs that had been already used in other experiments and planned to sacrifice were enrolled in this study at their sacrifice. In the previous experiments, surgeries were performed for their peripheral nerve of inferior alveolar or extremities before more 3 months without a complication. They were premedicated by intramuscular administration of atropine sulfate at 0.05 mg/kg. They were then anesthetized with 15 mg/kg ketamine hydrochloride and 3 mg/kg xylazine hydrochloride, and incubated endotracheally. Sevoflurane and nitrous oxide gas were used for maintenance of anesthesia during the procedure, under mechanical ventilation. Animal care, housing, and surgery underwent in November 2009 to March 2011 in the institute for Frontier Medical Science, Kyoto University. And this study was in accordance with the Rules and Regulations of the Committee for Animal Research of Kyoto University, Japan.

**Study 1: Preparation of the stricture model**

**Circumferential ESD**

Three dogs were selected randomly and used in the following operation. With each dog under general anesthesia, a circumferential ESD was carried out in the esophagus between 25 and 30 cm from the dental arch using an endoscopic system (GIF-XQ240; Olympus Optical Co., Ltd., Tokyo, Japan). The injection solution consisted of 10% glycerin, 5% fructose, 0.9% saline (Glyceol; Chugai Pharmaceutical Co., Tokyo, Japan), and 0.005 mg/mL epinephrine. After marking dots were placed on the anal side and oral side with a hook-knife in a forced coagulation mode of 20 W, the mucosa was cut circumferentially with a hook-knife (Olympus) in 120 W endocut mode (ICC200; ERBE, Tübingen, Germany). To cut the submucosal layer, the setting was changed to 60 W forced coagulation (Fig. 1).

**Postoperative care**

The dogs were fasted on the day of EMR and on the day after, and then liquid food was given after postoperative day (POD) 2. After POD 5, the animals were fed with a solid diet. Endoscopic observation was performed every week and evaluated body weight and symptom of the dysphagia. If the dogs showed difficulty in taking the solid diet due to symptoms associated with esophageal stricture, they were fed a semi-solid or liquid diet depending on their condition.

**Histological evaluation**

In order to evaluate the macroscopic appearance and histology of the esophagus, each animal was killed 2 months after ESD. If the animals were unable to consume liquid at any point after the procedure, they were killed. The esophageal specimens were cut longitudinally and fixed in 10% formalin for 48 h in order to prepare paraffin blocks. Each section was examined after hematoxylin-eosin (H-E) and Masson’s trichrome staining.

**Immunohistochemical staining**

Immunohistochemical staining was performed using the standard enzyme-labeled antibody method. After deparaffinization and rehydration, three consecutive sections 4 μm thick were treated by immersion in proteinase K (1%) for 5 min at 37°C. These sections were incubated with 3% hydrogen peroxide for 10 min at room temperature, and incubated overnight at 4°C with a goat polyclonal antibody for alfa-smooth-muscle actin (α-SMA), collagen I or collagen III (Southern Biotechnology Associates; Birmingham, AL, USA) at a dilution of 1:100. On the following day, Histofine simple stain MAX-PO (G)-conjugated rat anti-goat IgG was used as a secondary antibody (Nichirei, Tokyo, Japan) for 30 min and then staining was performed with a 3,3'-diaminobenzidine hydrochloride (DAB+) substrate kit (Dako, USA). Negative controls were generated by incubating slides with immunoglobulin from the same species and at the same final concentration, but with no primary antibody.
Study 2: Assessment of the biomechanical properties
Preparation of the scar tissue of the esophagus

Six adult beagle dogs weighing 8.4–10.6 kg were enrolled in this study. After general anesthesia, a cap-assisted EMR was performed using the endoscopic system (GIF-XQ240; Olympus Optical Co., Ltd., Tokyo Japan). All EMR procedures were performed using a cap with an outer diameter of 12 mm and a high-frequency-wave snare in the esophagus between 20 and 40 cm from the dental arch. We prepared 4 mucosal defects with a fixed size in each dog (Fig. 2a). All dogs were killed after postoperative month 2, and their esophagus was cut longitudinally. Rectangular-shaped specimens were obtained from the esophageal samples using surgical scissors. Material specimens that were approximately 5 cm × 1 cm in the short axis and the long axis, respectively, were obtained for both normal and healing-wound portions of the esophagus (Fig. 2b). Specimens were carefully separated into 2 sheets: the mucosa plus submucosal and the muscularis propria layer.

Fig. 2 Measurement of biomechanical properties.
a. Preparation of the esophageal scar by the cap-assisted endoscopic mucosal resection.
b. The specimens were obtained from the extracted esophagus. The long axis (*) and short axis (***) of the scar tissue and the short axis of normal tissue (***) are shown.
c. The tensile tester. The specimen was elongated from zero length to failure at a rate of 20 mm/s.
d. The load-displacement curve. The peak of the load indicates the failure load. Linear stiffness was determined as the slope over the range from 50% to 80% of the failure displacement (black line).
**Tensile test**

The specimens were placed in a Tensile tester (Model 1356, Aikoh Engineering Co., Ltd., Osaka, Japan) and kept wet with a saline solution at 37°C. Once the specimen was aligned in order to ensure proper axial loading, both the upper and lower serrated soft tissue grips were completely tightened. Each specimen was initially mounted in a slightly slackened fashion in order to avoid axial loading before testing. The specimen was then elongated from zero length to failure (the breaking point) at a constant rate (20 mm/second) (Fig. 2c). The failure load, failure displacement, and linear stiffness measurements were obtained from the load-displacement curve. The failure load and displacement identify the ultimate strength and maximum extensibility of the material, respectively. The linear stiffness was determined as the slope of the load-displacement curve over the range from 50% to 80% of the failure displacement (Fig. 2d).

**Statistical analysis**

The data shown represent mean value (standard deviation). Difference between the groups was evaluated by using Student’s t-test. \( p \) values less than 0.05 were considered to be significant.

**Results**

**Study 1**

**Clinical and endoscopic findings**

All procedures were performed successfully in the study animals, and there were no perioperative complications, such as perforation or postoperative bleeding. Endoscopic findings revealed that all dogs had an esophageal ulcer on POD 7 (Fig. 3a). However, 2 of the dogs developed severe stricture on POD 21 (Fig. 3b). These animals were not able to consume liquid and therefore required euthanasia. The remaining dogs developed relatively mild strictures, were able to consume a semi-solid diet, and survived for 4 weeks until they were killed. The animals showed a decrease in weight of 1.7 kg (-18.7%).

**Macroscopic appearance and histological findings**

The esophageal specimens excised from the dogs that developed severe strictures are shown in Fig. 3c. The specimens showed a deep ulcer formation and severe mucosal constriction. The epithelial cells had proliferated and migrated from the border of the lesion to the center, as shown with histology. No esophageal glands or muscularis mucosae were evident, and instead, they were replaced with condensed granulation tissue (Fig. 3d). The inner circular muscular layer of the muscularis propria showed partial atrophy and fibrosis in the center of the lesion (Fig. 3e).

**Immunohistochemical staining**

For comparison, the staining patterns of type I and III collagen in the normal tissue are shown in Fig. 3f. Both types I and III of collagen, which appear as brown fibers, were evident in the submucosal layer, and the orientation of these collagen bundles was regularly arranged. However, the healed granulation bundles was disordered in all lesions (Fig. 3g).

**Study 2**

All procedures were performed successfully in the study animals, and there were no complications, including perforation, stricture, or weight loss. The failure loads of the mucosa were 5.51 (1.57) kg in the controls and 2.49 (1.03) kg in the scar tissue in the direction of the long axis \( (p = 0.045) \) and 2.45 (0.47) kg in the controls and 0.82 (0.29) kg in the scar tissue in the direction of the short axis \( (p = 0.001) \). The failure loads of the muscularis propria were 1.06 (0.41) kg in the controls and 1.13 (0.25) kg in the scar tissue in the direction of the long axis \( (p = 0.78) \) and 0.62 (0.20) kg in the controls and 0.39 (0.22) kg in the scar tissue in the direction of the short axis \( (p = 0.17) \) (Fig. 4a).

The linear stiffness of the mucosa was 5.56 (2.08) kg/mm in the controls and 4.51 (1.36) kg/mm in the scar tissue in the direction of the long axis \( (p = 0.433) \) and 30.25 (4.84) kg/mm in the controls and 16.07 (3.87) kg/mm in the scar tissue in the direction of the short axis \( (p = 0.007) \). The linear stiffness of the muscularis propria was 21.45 (6.17) kg/mm in the controls and 16.69 (4.49) kg/mm in the scar tissue in the direction of the long axis \( (p = 0.258) \) and 83.98 (14.88) kg/mm in the controls and 53.55 (6.58) kg/mm in the scar tissue in the direction of the short axis \( (p = 0.007) \) (Fig. 4b).

**Discussion**

Severe inflammation of the esophageal wall causes ulcer formation. During the healing process of the ulcer, granulation tissue gradually arises on the base of the ulcer and new epithelial cells proliferate and migrate from the border of the ulcer, as well as the skin wounds healing. Our present results exhibit the histological and biomechanical changes that occur in the healed part of the esophagus.

Study 1 showed that the granulation tissue contained dense collagen fibers. The healed esophageal wall lacked both the esophageal glands and muscularis mucosae that had been originally present in the submucosa, and the wall thickness was thinner than normal. In order
Fig. 3 Endoscopic, macroscopic, and histological findings.

a. The ulcer formation was evident on postoperative day (POD) 7 after circumferential ESD.
b. A severe esophageal stricture was evident on POD 21.
c. Macroscopic appearance. In the central portion of the ulcer, mucosal constriction is evident.
d. Histological findings from the black line on the section shown in figure 3C (hematoxylin-eosin stain, ×40). The proliferation and migration of new epithelium cells were evident. No esophageal glands or muscularis mucosae were evident in the healed submucosa.
e. The muscularis propria showed partial atrophy and fibrosis in the center of the ulcer (Masson's trichrome stain, ×100).
Fig. 3  Endoscopic, macroscopic, and histological findings.
f. Immunohistochemical staining of type I and III collagen fibers in the normal submucosal layer (×100).
g. Immunohistochemical staining of type I and III collagen fibers and α-smooth muscle actin (α-SMA) in the lesion (×100).
to investigate the components of the granulation tissue in more detail, immunohistochemical staining was performed. The orientation of both type I and III collagen fibers were disordered, and stromal cells that were α-SMA-positive were evident. Collagen type III fibers, which are abundantly present in the submucosal layer of the gastrointestinal tract, are reticular fibers with elastic properties. Collagen fibers are regularly assembled, and their orientation contributes to the mechanical characteristics and functions of these tissues. The stromal cells, which are α-SMA-positive, are thought to be myofibroblast cells that were differentiated from fibroblasts by the stimulation of inflammatory cytokines such as transforming growth factor β1 or fibronectin. These cells have a role in the production of the contractile force that results in wound closure and scar formation. In addition, the inner circular muscle developed atrophic changes, although the muscularis propria layer appeared not to have been damaged by the EMR/ESD procedures. Study 2 showed that the histological changes mentioned above influenced the biomechanical features of the esophagus. Originally, the esophagus is organized with a strong durability and high elasticity. However, the healed tissue, especially in the direction of the short axis, lost these characteristics, and the failure load and linear stiffness were decreased. It is thought that a decrease in the elasticity of the short axis causes dysfunctional esophageal dilation. In brief, benign esophageal stricture might have been caused by a combination of the above factors: the change of the collagen components and orientation in the submucosal layers, the differentiation to myofibroblasts, and atrophic changes of inner circular muscles.

The esophageal stricture is quite refractory. Because our present results showed that the failure load of the scar tissue was significantly decreased, a risk of perforation by the dilatation therapy should be considered. A preventive strategy for stricture should be a primary focus when severe esophagitis or ulcers are treated. There are 2 main approaches for the prevention of esophageal stricture: anti-inflammatory therapy and regenerative medicine. First, anti-inflammatory therapy includes the use of a regime in which there is a sufficient period of non-feeding, and the administration of antibiotics and proton-pump inhibitors reduces the effects of gastric acid. Cooling or washing might be effective after radiation, the accidental ingestion of alkali, or EMR/ESD. On the other hand, there is no clear evidence that steroid administration has an anti-inflammatory and protective effect on the esophagus after corrosive esophagitis or EMR/ESD. Second, as a regenerative approach, the use of an autologous keratinocyte sheet or xenogenic extracellular matrix that can prevent stricture after esophageal EMR has been reported. The purpose of this treatment was to closely approximate the native structure of the healing tissue for the prevention of esophageal stricture. Although these techniques, such as the preparation of a cell sheet or the insertion of the extracellular matrix, are quite difficult and have not been widely accepted yet, this strategy seems promising as an application of regenerative medicine. Further studies will be needed in order to develop methodology that is simple and able to be performed in every hospital.

Reference
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