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Preliminary results of antiscarring therapy in the prevention of postendoscopic esophageal mucosectomy strictures

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Abstract

Background—Esophageal endoscopic submucosal dissection (ESD) is an effective minimally invasive therapy for early esophageal cancer and high-grade Barrett dysplasia. However, esophageal stricture formation after circumferential or large ESD has limited its wide adoption. Mitomycin C (MMC), halofuginone (Hal), and transforming growth factor β 3 (TGF- β 3) exhibits antiscarring effects that may prevent post-ESD stricture formation.

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Methods—Using endoscopic mucosectomy (EEM) technique, an 8- to 10-cm-long circumferential esophageal mucosal segment was excised in a porcine model. The site was either untreated (control, $n = 6$) or received 40 evenly distributed injections of antiscarring agent immediately and at weeks 1 and 2. High and low doses were used: MMC 5 mg ($n = 2$), 0.5 mg ($n = 2$); Hal 5 mg ($n = 2$), 1.5 mg ($n = 2$), 0.5 mg ($n = 2$); TGF- β 3 2 μ g ($n = 2$), 0.5 μ g ($n = 2$). The degree of stricture formation was determined by the percentage reduction of the esophageal lumen on weekly fluoroscopic examination. Animals were euthanized when strictures exceeded 80 % or the animals were unable to maintain weight.

Results—The control group had a luminal diameter reduction of 78.2 ± 10.9 % by 2 weeks and were euthanized by week 3. Compared at 2 weeks, the Hal group showed a decrease in mean stricture formation (68.4 % low dose, 57.7 % high dose), while both TGF- β 3 dosage groups showed no significant change (65.3 % low dose, 76.2 % high dose). MMC was most effective in stricture prevention (53.6 % low dose, 35 % high dose). Of concern, the esophageal wall treated with high-dose MMC appeared to be necrotic and eventually led to perforation. In contrast, low dose MMC, TGF- β 3 and Hal treated areas appeared re-epithelialized and healthy.

Conclusions—Preliminary data on MMC and Hal demonstrated promise in reducing esophageal stricture formation after EEM. More animal data are needed to perform adequate statistical analysis in order to determine overall efficacy of antiscarring therapy.

Keywords

Antifibrotic; Antiscarring; ESD; Esophageal stricture; Mucosectomy

The rate of adenocarcinoma of the esophagus and gastroesophageal junction is dramatically increasing [1, 2]. Traditionally, advanced high-grade Barrett dysplasia and early esophageal cancers have been treated with a highly morbid procedure: esophagectomy. With advancements in therapeutic endoscopy, less invasive techniques such as endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD) provide comparable survival benefits for these patients [3, 4]. Compared to mucosal ablative techniques or EMR, ESD resects the disease en bloc allowing more accurate histopathologic staging and decreases the risk of local recurrence or undertreatment [5]. Unfortunately, ESD has not gained wide clinical acceptance because of higher complication rates and the high rate of stricture formation (6–16 %) after large circumferential mucosectomies [6, 7]. Although several therapies are available for esophageal strictures, such as frequent dilations, stents, or surgery, ESD strictures are fairly refractory and affect quality of life. The ability to modify, prevent, or reduce post-ESD esophageal stricture formation will allow high-grade Barrett dysplasia and/or intramucosal cancer patients the option of receiving an effective one-time therapy with lower risks of local recurrence.

Strictures are the result of posttraumatic fibrosis, wound contracture, and scar formation during normal wound healing. The intricate process of adult wound healing consists of three overlapping well characterized phases, inflammation, proliferation, and remodeling [8, 9]. Natural modifications of these phases occurs during fetal and oral mucosal healing and result in accelerated, scarless or minimally scarred wounds [10, 11]. The ability to modify

the healing process with antiscarring agents may reduce or prevent post-ESD esophageal stricture formation.

Experimentally, transforming growth factor beta3 (TGF- β 3), mitomycin C (MMC), and halofuginone (Hal) have modified each of the three wound healing phases. Elevated levels of the cytokine TGF- β 3, an inhibitor of the scar inducing TGF- β 1, and TGF- β 2, is thought to contribute to scarless fetal wound healing, accelerated buccal mucosal healing, and improved scar appearance after adipocyte stem cell therapy [10–12]. MMC is an antimitotic chemotherapy agent that has been used for years. Its new role as an antiscarring agent shows promise by inhibiting fibroblast proliferation and activity [13, 14]. Lastly, because excess type I collagen deposition is associated with fibrosis, Hal (inhibits collagen α I expression and matrix metalloproteinase 2) can change the remodeling phase of wound healing and potentially decrease scar formation [15].

All three agents have shown promise as antiscarring agents, but to our knowledge, their use after ESD has never been investigated. Our laboratory has an established porcine model of esophageal stricture after endoscopic esophageal mucosectomy (EEM). Though the EEM technique is slightly different from ESD, the resulting wound is the same. The mucosal layer is removed from the underlying submucosal and muscularis layer of the esophagus. This study investigated the effects of TGF- β 3, Hal, and MMC on post-EEM stricture formation.

Materials and methods

Design

The study consisted of a total of 20 domestic swine. Each animal was subjected to EEM with concomitant application of one of three antiscarring agents at varying doses or no additional treatment (control). Animals were endoscopically and fluoroscopically surveyed at weekly intervals to monitor for stricture progression and for redosing of the antiscarring agent at weeks 1 and 2. When the esophageal lumen percentage decrease was >80 %, the animal was unable to maintain weight, or by 14 weeks, the animal was euthanized and tissue collected for histologic analysis.

Materials

MMC (Accord Healthcare, Durham, NC) was dissolved in normal saline, halofuginone hydrobromide (Hal) (Sigma-Aldrich, St. Louis, MO) was dissolved in DMSO, and TGF- β 3 (R&D Systems, Minneapolis, MN) was dissolved in 4 mM HCl + 0.1 % bovine albumin. All concentrated solutions were stored at –20 °C before use and were then diluted in normal saline just before injection.

Methods

All procedures were approved by the Case Western Reserve University Institutional Animal Care and Use Committee (IACUC). After 1-week acclimatization per IACUC standards, animals were sedated (Telazol, 10 mg/kg i.m., Fort Dodge Animal Health, Fort Dodge, IA), intubated, ventilated, monitored, and maintained with inhalational anesthetic (Isothesia, Butler Schein Animal Health, Dublin, OH). Preprocedural antibiotic (tulathromycin, 2.5

mg/kg i.m., Draxxin, Pfizer, New York, NY) was provided, and the animals were kept well hydrated with intravenous fluids. All endoscopic procedures were digitally recorded. EEM was performed as previously described [16]. Briefly, EEM was accomplished with a single-channel flexible device (GIF 1T160; Olympus America, Center Valley, PA) preloaded with a commercially available band ligating device. The band ligator and snare were used to complete a circumferential incision through the submucosal layer approximately 20 cm proximal to the lower esophageal sphincter. A column of mucosa (~8–10 cm) was dissected free and excised from the underlying submucosal and muscularis layers using electrocautery and a snare. The excised mucosal tissue was fixed in formalin and stained with hematoxylin and eosin (H&E) for analysis of tissue type.

Six animals received no further treatment (control group). The remaining 14 animals received antiscarring agents injected at multiple sites evenly distributed along the EEM site (0.25 mL injections $\times$ 40 sites = 10 mL total volume) using an endoscopic needle (U.S. Endoscopy, Mentor, OH). The doses for each antiscarring agent were derived from other published animal studies. Two animals were used for each dose of agent. For MMC, each animal received either a total of 0.5 mg ($n = 2$) or 5 mg ($n = 2$). For Hal, each animal received either a total of 0.5 mg ($n = 2$), 1.5 mg ($n = 2$), or 5 mg ($n = 2$). For TGF- β 3, each animal received either a total of 0.5 μ g ($n = 2$) or 2 μ g ($n = 2$).

After therapy, fluoroscopic barium swallow was performed. The esophagus was intubated with a cuffed endotracheal tube (8.0 mm). With the balloon inflated, contrast suspension (60 mL, E-Z-Paque, E-Z-EM Canada Inc., Lake Success, NY) was infused to visualize the lumen of the esophagus. A radiopaque ruler was placed under the supine animal to provide scale. The animal was then permitted to recover and was returned to its housing. Animals were weighed weekly and sedated for fluoroscopic and endoscopic surveillance of stricture progression. In addition, during weeks 1 and 2, the same dosing treatment regimen of antiscarring agent provided at week 0 was repeated. The treatment regimen was deferred only if the stricture had progressed to a point that prevented passage of the endoscope. A liquid or soft diet was provided to animals that were unable to tolerate regular food. Animals were euthanized when the stricture appeared to reduce esophageal luminal diameter by ~80 % compared to week 0 (where 100 % indicates complete closure and 0 % indicates absence of any stricture formation), when the animal was unable to gain weight, or at the end of the study (14 weeks).

At the conclusion of the study, the animals were euthanized (>100 mg/kg i.v., Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI) and the esophagus excised. Tissue samples from the native esophagus and the stricture zone were collected, fixed in formalin, embedded, sectioned, and stained with H&E for histologic analysis (inflammation, fibrosis, and healing).

End points

The degree of stricture formation was the primary end point. Using digitized fluoroscopic contrast images, the degree of stricture was determined by taking the average diameter of the esophagus at three points along the stricture site and comparing it to the baseline diameter of the proximal esophagus at week 0. Other indirect indications of stricture formation, such as

proximal esophageal dilation and esophageal shortening were also recorded and compared. For our secondary end points, a surgical pathologist blinded to the treatment arms classified the degree of tissue inflammation, collagen formation and fibrosis as compared to native tissue using the histologic specimens, with 0 indicating normal, + indicating mild, ++ indicating moderate, and +++ indicating severe. Because this exploratory study was scaled to provide only preliminary data on the potential of these antiscarring agents, it was not powered adequately for robust statistical conclusions.

Results

Each of the 20 EEM procedures was conducted without complications (hemorrhage, perforation or excessive resection). Though the desired 40 injections per animal per session were completed, the drug delivery was inconsistent as a result of leakage from the injection sites. All animals; treated groups and controls, did not differ in their initial weight (range 29.0–36.5 kg) or in the amount of weight gain.

Degree of stricture

Without fail, all of the control animals progressively developed severe strictures as determined by a percentage decrease in esophageal luminal diameter (63.0 ± 11.7 % by week 1, 78.2 ± 10.9 % by week 2, and 90.1 ± 9.6 % by week 3) (Fig. 1). Correspondingly, a 79.8 ± 6.8 % increase in proximal esophageal dilation and 65.7 ± 1.4 % shortening of the esophagus was found at week 3.

By week 2, the TGF- β 3 treated animals (Fig. 2), had a mean esophageal luminal diameter reduction of 65.3 % with 0.5 μ g and 76.2 % with 2 μ g. One animal from the 2 μ g TGF- β 3 group was euthanized after week 1 as a result of the inability to pass the endoscope. Similar to controls, an increase in mean proximal esophageal dilation (50 % for 0.5 μ g, 35.7 % for 2 μ g, and 69.5 % for controls) and esophageal shortening (60.5 % for 0.5 μ g, 60.0 % for 2 μ g, and 59.3 % for controls) was seen at week 2.

Though small, Hal seems to have a mild dose-dependent effect on esophageal stricture formation (Fig. 3). Compared at 2 weeks, the low dose (0.5 mg) had a mean 68.4 %, mid dose (1.5 mg) had a mean 57.7 %, and the high dose (5 mg) had a mean 55.0 % esophageal luminal diameter reduction (Controls 78.2 %). In addition, both animals in the high dose group survived to week 3, which was 1 week more than the lower dose animals. But, they still eventually developed clinically significant strictures (mean 76.4 %) and were euthanized.

More encouraging results were seen with the MMC-treated animals (Fig. 4). At the lower dose, 0.5 mg, the esophageal diameter only narrowed by a mean of 53.6 % by week 2. The degree of stricture seems to plateau with esophageal diameter reduction remaining at 52.4 % (week 3) and 47.6 % (week 4) in one animal. The second animal was euthanized after week 2 with 61.9 % luminal reduction as a result of the inability to pass the scope. Interestingly, the amount of mean proximal esophageal dilation (57.2 %) and esophageal shortening (61 %) did not differ from controls at week 2. At the higher MMC dose, 5 mg, an even more dramatic effect was seen. Luminal diameter was only reduced by a mean of 35 % by week 2

and 48.9 % by week 3. Unfortunately, one high-dose MMC animal died as a result of esophageal perforations and the second was euthanized as a result of an unhealthy luminal appearance during endoscopic surveillance.

Pathologic and histologic examination

Histologic analysis of the mucosectomy samples showed no difference in the type or depth of the resected tissue from each animal. The resected mucosa consisted of normal squamous epithelium, lamina propria, muscularis mucosae, and occasionally superficial submucosa. A few polymorphonuclear leukocytes (PMNs), lymphocytes and eosinophils were present in about half the samples. Fibrosis and neovascularization were not noted in any of the mucosal resections. Similarly, the native uninjured esophageal tissue sampled at time of necropsy showed no differences between groups. H&E stains consistently demonstrated normal tissue, with the presence of squamous epithelium, lamina propria, muscularis mucosae, submucosa, muscularis propria, and adventitia. A few PMNs, lymphocytes, and eosinophils were present in about half the samples. Evidence of minimal fibrosis was occasionally noted. Submucosal glands appeared normal, and neovascularization was not present in any of the native tissue samples.

On gross inspection, the esophageal mucosal lining at the stricture site in the controls, TGF- β 3, Hal, and the lower-dose MMC group appeared normal and healthy (Fig. 5). Explanted esophageal tissue from the high-dose MMC-treated EEM site showed deterioration of the mucosa and the underlying muscularis layer. This was confirmed histologically by the presence of transmural infarction with chronic serositis, serosal exudate, abscess, and fibrosis (Fig. 6). Compared to the uninjured tissue, stricture sites from all groups had higher degrees of inflammation (more PMNs and lymphocytes), fibrosis (more fibroblasts and collagen), and neovascularization. Although re-epithelialization was not seen in the high dose of MMC, low-dose Hal, or low-dose TGF- β 3, an epithelial layer was present in the area of the stricture for the low dose of MMC, the high dose of Hal, and the high dose of TGF- β 3. The degree of inflammation, (PMNs, lymphocytes, and PCs) was severe (+++) in the control group. There was somewhat less inflammation among the high-dose TGF- β 3 group (++), the mid dose Hal group (0 to +++), and both MMC groups (+ to ++) (Table 1). The level of fibrosis was also lower among the mid dose Hal (+ to ++) group and both doses of MMC (+ to ++) compared to controls (+++). Consistent with its mechanism of action, the decrease in collagen seen with mid dose Hal was not associated with increased fibroblast cellularity. Because of the small number of animals, definitive conclusions concerning inflammation, fibroblast proliferation, and collagen deposition cannot be made.

Discussion

Attenuating fibrosis or scar formation is pertinent across many different disciplines. The complex process of wound healing has been extensively studied, and as a result, anti-scarring agents have been developed. From the benchtop to the bedside, all three antiscarring agents—TGF- β 3, Hal, and MMC—have been shown to improve the appearance of the scar, improve the quality of the healed tissue, or decrease stricture formation. The circumferential defect, the large surface area, and the technical difficulty of endoscopic

delivery makes the application of antiscarring agents more challenging in EEM. In this exploratory study, we evaluated the potential effect of three antiscarring agents in treating post-EEM esophageal stricture formation.

Application of TGF- β 3 in our study showed little to no effect on reducing stricture formation after EEM. Before concluding that TGF- β 3 is ineffective, some technical aspects need to be further addressed. Because TGF- β 3's mechanism of action is through its inhibition of TGF- β 1 and TGF- β 2, some investigators feel TGF- β 3 should be administered before injury [17, 18] and sustained through the remodeling phase of healing [19]. The rationale is that the initial cascade of cytokines involved in wound healing and scarring are triggered immediately after injury and present until the healing process is complete [10, 18]. In contrast, other treatment regimens (one-time injection immediately after and 24 h after injury [20], immediately after as a sustained release formation [19], or after the appearance of scar [12]) achieved similar scar improvements. This suggests that timing may not be important. A more relevant factor may be the degree of involved surface area. This model creates a larger injured surface area than in other studies on dermal scars, vocal cord injury, and buccal mucosal injury [17, 18, 21]. Larger areas of injury lead to prolonged exposure of fibrotic cytokines. We injected TGF- β 3 immediately after injury and repeated it 1 week later. Though high-dose TGF- β 3 did show a slight decrease in inflammatory cells, suggesting that it had some effect, it is difficult to draw definitive conclusions. Further studies investigating higher dosages, sustaining TGF- β 3 presence, and timing of drug delivery are needed to find its true effect.

Another technical limitation is the accuracy of drug delivery. Though we attempted to inject the agent in quadrants an equal distance apart, the agent may not be as evenly distributed as we intended. In addition, we observed that all three drugs leaked out of the tissue during and after injection. The precise amount of drug delivered, therefore, was hard to establish. This may explain the small but partial antistricture (luminal diameter and histologic decrease in the amount of collagen) effect of Hal. One major advantage of Hal is its ability to affect only the cells that are stimulated to produce collagen [22, 23]. It can therefore work equally well systemically and locally [24]. Two separate groups evaluated the effectiveness of Hal on subglottic stenosis after trauma. One investigator used a one-time topical 5-min application of Hal immediately after subglottic trauma in rats, and the other gave Hal orally 3 days before injury and continued daily for 21 days in dogs. No adverse effects were seen in either study, and both studies showed significant improvements in fibrosis (stricture reduction from 72 to 0 % compared to controls) [25, 26]. With esophageal strictures, two rat studies showed mixed results. Arbell et al. [23] looked at caustic burn injury and Dabak et al. [27] looked at radiation-induced injury. Both gave Hal systemically as intraperitoneal injection (immediately after injury followed by daily injection $\times$ 1 month) or oral administration (3 days before injury followed by daily administration for 21 days). Hal significantly increased luminal patency from 11 to 73 % in the caustic injury rats, but there was no significant histologic difference in fibrosis and luminal diameter in the radiation-induced injured rats. Hal is still an experimental drug, but systemic administration or evenly disturbed topical application may be a more consistent and easier method of administering Hal for post-EEM esophageal stricture prevention.

MMC demonstrated the most encouraging antistricture effect with luminal reduction of only 53.6 % (low dose) and 35 % (high dose) compared to controls (78.2 %) at 2 weeks. Histologically, the degree of inflammation, collagen, and fibroblast cellularity was also the lowest. This is consistent with previously published data on radiation- or caustic injury-induced esophageal strictures treated with MMC [28–32]. Though the potential uses for MMC are encouraging, MMC can be dangerous, as demonstrated by one pig dying from an esophageal perforation. Clinically, McCurdy Hueman and Simpson [33] reported airway obstructions and death as a result of the accumulation of sloughed-off fibrinous debris after using MMC for laryngotracheal stenosis. Inhibition of fibroblast proliferation by MMC is dose dependent and becomes irreversible and cytotoxic at higher doses or longer treatments [34, 35]. From cell culture studies, animal studies, and clinical use, the ideal MMC dose ranges from 0.001 to 1 mg/mL, with the most commonly utilized dose being 0.4 mg/mL [28, 36]. Both our doses were within the published range, suggesting that either there was a different sensitivity range for MMC applied for treatment purposes (already scarred tissue) compared to prophylactic prevention, or that repeat injections resulted in too much drug effect.

The most commonly described technique of administering MMC for strictures is direct topical application of soaked cotton pledgets to the treatment site for a few minutes [37]. This regimen is derived from cell culture studies showing a single 5-min exposure of MMC stops cultured fibroblast proliferation and DNA synthesis for 3 weeks [34, 36]. Repeat applications were only for patients who had little or no success with the initial treatment [28, 29]. There is one case report of injecting MMC for a benign recalcitrant esophageal stricture [38]. Unfortunately, direct topical application of MMC soak pledgets or instilling and washing out MMC in an isolated esophagus (as in the rat caustic esophageal injury model) [31, 39] are not possible when using an endoscope. A safe and effective endoscopic delivery method needs to be developed in order to avoid toxic injury to surrounding normal tissue, especially when a highly potent and harmful drug such as MMC is used.

On the basis of this exploratory study, TGF- β 3 did not seem to have an antiscarring effect, while Hal and MMC demonstrated evidence of antiscarring effect in preventing post-EEM stricture. These results are encouraging, and continued investigation with more animals should be performed. In addition, this study also revealed difficulties specifically to preventing post-EEM strictures. Currently we lack an accurate, consistent, and safe way of delivering these agents through an endoscope. Sustained-release drug-eluting stents or an adherent semisolid substance containing the antiscarring agents may allow consistent, prolonged (or one-time dose), and endoscopically feasible way of drug delivery. Further studies on this technical aspect would not only benefit this disease process but also advance the field of therapeutic endoscopy.

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Fig. 1.
Endoscopic and fluoroscopic views of control animals from weeks 0 to 2. Progressive esophageal stricturing is seen

Fig. 2.

Effect of TGF- β 3 on esophageal stricture formation as determined by the percentage reduction of the esophageal diameter compared to control animals

Fig. 3.
Effect of Hal on esophageal stricture formation as determined by the percentage reduction of the esophageal diameter compared to control animals

Fig. 4. Effect of MMC on esophageal stricture formation as determined by the percentage reduction of the esophageal diameter compared to control animals

Fig. 5.

Gross pathologic appearance of esophageal stricture for representative animals: control at 2 weeks, TGF- β 3 (0.5 μ g) at 2 weeks, TGF- β 3 (2 μ g) at 2 weeks, Hal (0.5 mg) at 2 weeks, Hal (1.5 μ g) at 2 weeks, Hal (5 mg) at 3 weeks, MMC (0.5 mg) at 4 weeks, and MMC (5 mg) at 3 weeks

Fig. 6. Representative H&E stain of the esophageal stricture site for control animal at 3 weeks, TGF- β 3 (0.5 μ g) at 2 weeks, TGF- β 3 (2 μ g) at 2 weeks, Hal (0.5 mg) at 2 weeks, Hal (1.5 μ g) at 2 weeks, MMC (0.5 mg) at 4 weeks, and MMC (5 mg) at 2 weeks

Table 1

Inflammation, fibrosis, and healing among the study groups

Pig group	Inflammation		Collagen	Fibrosis		Healing Neovascularity	Additional comments
	PMN	Lymph/PC		Fibroblast	cellularity		
Control	+++ S	+++ S	+++ S	++		Present	
TGF- β 3 (0.5 μ g)							
1	+++ S	++ SM	+++ SM to MM	+++		Present	Foreign body giant cell reaction
2	+++ S	++ SM	+++ SM to MM	+++		Present	
TGF- β 3 (2 μ g)							
1	++ S	++ SM	++ SM to MM	+++		Present	
2	++ S	+ SM	+ SM to MM	+++		Present	
Hal (0.5 mg)							
1	+++ S	+++ SM	+++ SM to MM	+++		Present	
2	++ S	++ SM	++ SM	++		–	
Hal (1.5 mg)							
1	–	++ SM	+ SM	++		–	
2	++ S	+++ SM	++ SM	+++		Present	
MMC (0.5 mg)							
1	–	+ SM	+ SM	+		–	Foreign body giant cell reaction
2	++ S	+ SM	++ SM	+++		Present	
MMC (5 mg)							
1	++ S	+ S	++ SM to MM	++		Present	Chronic serositis
2	Unable to assess as a result of transmural infarction with exudate and abscess						

Inflammation and fibrosis parameters were graded as negative (–), mild (+), moderate (++) and severe (+++). The extended area or areas of the parameter on the specimen are represented as S surface, SM submucosa, and MM muscularis mucosae

PMN polymorphonuclear leukocyte, lymph lymphocytes, PC plasma cell, TGF transforming growth factor, Hal halofuginone, MMC mitomycin C