

Restenosis following balloon dilation of benign esophageal stenosis

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Abstract

AIM: To elucidate the mechanism of restenosis following balloon dilation of benign esophageal stenosis.

METHODS: A total of 49 rats with esophageal stenosis were induced in 70 rats using 5 ml of 50 % sodium hydroxide solution and the double-balloon method, and an esophageal restenosis (RS) model was developed by esophageal stenosis using dilation of a percutaneous transluminal coronary angioplasty (PTCA) balloon catheter. These 49 rats were divided into two groups: rats with benign esophageal stricture caused by chemical burn only (control group, $n=21$) and rats with their esophageal stricture treated with balloon catheter dilation (experimental group, $n=28$). Imaging analysis and immunohistochemistry were used for both quantitative and qualitative analyses of esophageal stenosis and RS formation in the rats, respectively.

RESULTS: Cross-sectional areas and perimeters of the esophageal mucosa layer, muscle layer, and the entire esophageal layers increased significantly in the experimental group compared with the control group. Proliferating cell nuclear antigen (PCNA) was expressed on the 5th day after dilation, and was still present at 1 month. Fibronectin (FN) was expressed on the 1st day after dilation, and was still present at 1 month.

CONCLUSION: Expression of PCNA and FN plays an important role in RS after balloon dilation of benign esophageal stenosis.

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INTRODUCTION

Balloon catheter dilation is a common nonsurgical treatment for benign esophageal stricture. Its short-term effect is good, but its long-term effect is not so good, because esophageal restenosis is a major complication. The underlying mechanism of esophageal restenosis has not been understood yet. To study this mechanism, we established a benign esophageal stricture model and restenosis model in Sprague-Dawley (SD) rats. We performed quantitative histopathological image analysis of sections of the rat esophagus, and qualitative immunohistochemical analysis of the related indicators of proliferation and restenosis of mucous and striated muscle layers of rat esophagus dilated by a balloon catheter at different time points. This provided us with an effective experimental model for investigation of the causes of restenosis.

MATERIALS AND METHODS

Materials

All protocols used for animal experiment and maintenance were approved by the Animal Ethics Committee in our university and conformed to the highest international standards of humane care.

70 male Sprague-Dawley (SD) rats weighing 305 ± 50 g were obtained from the Shanghai Experimental Animal Center (Shanghai, China). The animals were weighed on day 0 and then 1 day before sacrifice. After anesthesia with 10 % ketamine (80 mg/kg) by abdominal injection, the animals were placed in a supine position and stabilized on an operating table. A 3F segmental epidural catheter was inserted into the mouth, and 10 ml of edible vinegar was injected into the stomach. The epidural catheter was then replaced by two 3F percutaneous transluminal coronary angioplasty (PTCA) balloon catheters at the mid-to-lower segment of the esophagus. The two balloons were at least 2 cm apart. These balloons were simultaneously inflated with air until they expanded to cling to the wall of the esophagus. Then 5 ml of a freshly prepared 50 % sodium hydroxide solution was injected through the orifice of the balloon catheter. After three minutes the air was released from the balloon. Distilled water was injected repeatedly through the same orifice for rinsing for 1 minute. The balloon catheters were removed and the animals returned to their cages for feeding. An esophageal barium-contrast examination was performed 2 and 4 weeks later to ascertain whether benign esophageal strictures had formed. We achieved 49 animal models from the 70 rats. These 49 rats were divided into two groups: rats with benign esophageal stricture caused by chemical burn only (control group, $n=21$) and rats with their esophageal stricture treated with balloon catheter dilation (experimental group, $n=28$).

Samples were collected at different times for immunohistochemical assay. We divided rats in both groups into seven subgroups according to the time after the dilation procedure when the samples were collected on day 1, 3, 5, 7, 14, 21 and 30. At the time of sacrifice, samples were fixed in 4 % buffered formaldehyde solution.

Table 1 Source of antigens and their effective concentrations

First antibody			Second antibody		
Goat antihuman PDGF	Promega	1:40	Biotinylated house antigoat IgG	Vector	1:200
Mouse antihuman FN	Life	1:20	Biotinylated house antimouse IgG	Vector	1:200
Mouse antihuman PCNA	Maxim	1:20	Biotinylated house antimouse IgG	Maxim	1:200

Statistical analysis: Data were expressed as the mean \pm SD. Statistical analysis was performed using the unpaired or paired *t*-test. A probability value less than 0.05 was considered significant.

Methods

Image analysis: Esophageal sections were stained with hematoxylin and eosin, and images were taken by a CCD camera (JVC, Osaka, Japan) and analyzed by a VIDAS imaging system (Carl Zeiss, Germany). The indicators used comprised the cross-sectional areas and perimeters of esophageal mucous layer, esophageal muscle layer, and the entire esophageal layers.

Immunohistochemical staining: ABC methods and SP methods were performed following the manufacturer's instructions using an ABC kit (Vector, USA) and an SP immunochemistry kit (Zymed Maxim, USA). The source of antibodies and their effective concentrations are listed in Table 1. The presence of platelet-derived growth factor (PDGF) and fibronectin (FN) was tested by the ABC method, and proliferating cell nuclear antigen (PCNA) was tested by the SP method.

RESULTS

Models of benign esophageal stricture and esophageal restenosis

Of the 49 model rats with benign esophageal stricture, 28 rats with esophageal restenosis were established.

Morphologic changes in benign esophageal stricture and esophageal restenosis

The control group showed chemical-burn lesions with an inflammatory reaction on the mucous layer of the esophagus, and comparatively slight thickening on the muscle layer of the esophagus. No broken regions were found in the muscle layer of the esophagus, and the esophageal wall was intact. Besides chemical-burn lesions, the experimental group showed mechanical damage in the mucosa of the esophagus. The muscle layer of the esophagus was thickened and broken, with accompanying inflammatory reactions (Figure 1). On the 5th day after the procedure, the broken section of the muscle layer of the esophagus became thickening, and 14 days later the degree of thickening was obvious. The changes in the cross-sectional areas and perimeters of mucosa, muscle layers, and the entire esophagus wall are listed in Table 2.

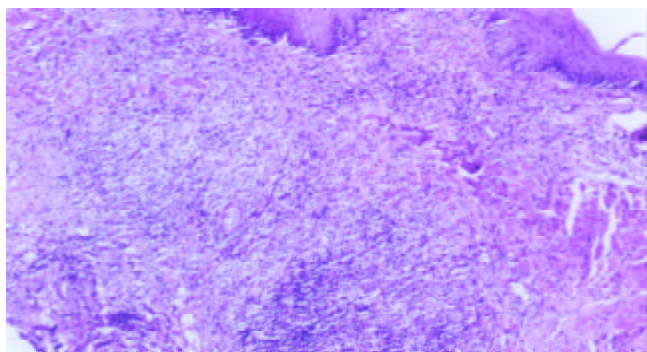


Figure 1 In the experimental group, on the 5th day after the procedure, muscle layers of the rat esophagus exhibited an inflammatory reaction. H&E stain, $\times 4$.

Table 2 Morphologic changes in benign esophageal stricture and esophageal restenosis (area, mm²; perimeter, mm)

	Control group	Experimental group
A1	0.49 \pm 0.14	0.75 \pm 0.18 ^b
A2	1.70 \pm 0.42	1.97 \pm 0.33 ^a
A3	2.20 \pm 0.45	2.72 \pm 0.46 ^a
P1	4.83 \pm 1.52	6.65 \pm 1.22 ^b
P2	6.89 \pm 1.96	8.80 \pm 1.67 ^b
P3	9.86 \pm 2.25	14.19 \pm 2.89 ^b

^b*P*<0.01, ^a*P*<0.05 vs control group and experimental group. Abbreviations: A1: cross-sectional area of mucosa of esophagus, A2: cross-sectional area of muscle layer of esophagus, A3: cross-sectional area of entire esophagus wall, P1: perimeter of mucosa of esophagus, P2: perimeter of muscle layer of esophagus, P3: perimeter of entire esophagus wall.

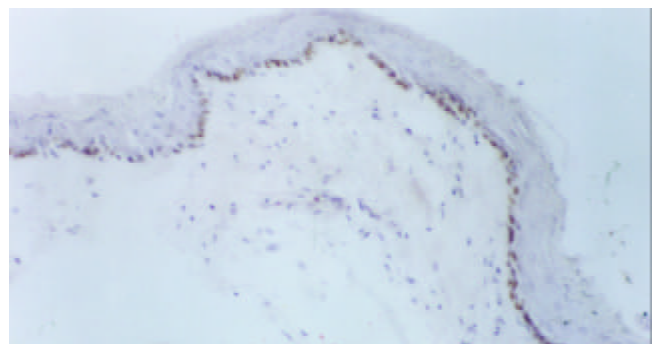


Figure 2 In the experimental group, on the 5th day after the procedure, the basal cells of the squamous epithelium in rat esophagus exhibited strong PCNA expression. Immunostaining, $\times 4$.

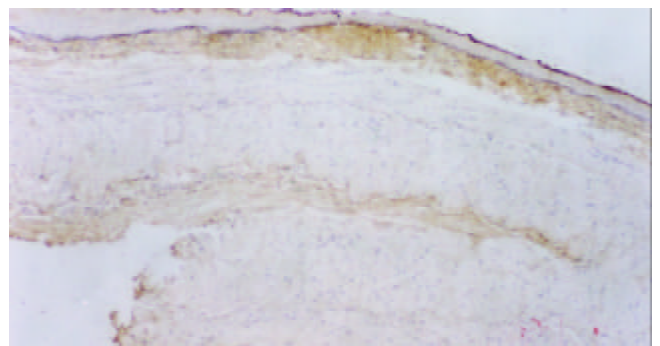


Figure 3 In the experimental group, on the 30th day after the procedure, the substratum of the rat esophageal mucosa and muscle layers exhibited strong FN expression. H&E stain, $\times 4$.

Immunohistochemical staining of benign esophageal stricture and esophageal restenosis: In the control group, basal cells of the squamous epithelium and striated-muscle cells of the esophagus exhibited no PCNA expression. Five days after the dilation procedure, PCNA expression became obvious in

basal cells of the squamous epithelium, and this positive expression lasted for 30 days (Figure 2). In the control group, 3-7 days after the dilation procedure, the basal layer of the esophagus exhibited weak positive expression. Fourteen days later there was no FN expression in the basal layer. In the experimental group, on the 1st day after the procedure, the collagen fibers in submucosa and in the striated-muscle layer of esophagus were positive for FN, and this was still the case on the 14th day. After 1 month, FN positive expression was still reasonably strong (Figure 3). PDGF was not expressed at all in striated-muscle cells from the 1st to the 30th day in both control and experimental groups.

DISCUSSION

Models of benign esophageal stricture and esophageal restenosis

The causes of benign esophageal stricture are numerous and complicated, and hence the models thereof are difficult to reproduce consistently. However, benign esophageal stricture eventually manifests as thickened scars and reduced luminal sizes. We used chemical burns to develop the model of benign esophageal stricture because it allowed timing to be controlled and exhibited a high rate of success. Early in the 1970s, Przymanowski *et al*^[1] used sodium hydroxide to establish a model of benign esophageal stricture. Their method was to perform an abdominal midsection on rats, thereby exposing the lower segment of the esophagus. They used surgical thread to tightly tie the region 2-cm either side of the lower segment of the esophagus. They then inserted a stomach tube via the mouth until it reached the tied point. Sodium hydroxide solution was injected, and then rinsed out three times for 3 minutes with distilled water later. Then they withdrew the tube, cut the threads, and closed the abdomen. Based on their procedure, we developed a nonsurgical method to establish a model of benign esophageal stricture. Since our method did not involve surgery, it was simpler and faster. Our experimental observations demonstrated that the model was satisfactorily established. Our use of two balloon catheters made manipulation somewhat difficult. We intended to make a single catheter with two balloons, but this was found to be too difficult since the rats had a narrow esophagus that demanded fine catheters and balloons. In contrast, a double-balloon catheter with a larger caliber was easy to be constructed. Therefore, the double-balloon method was used to establish the model of benign esophageal stricture.

The technique used to establish the model of esophageal restenosis is easier. After ascertaining the stricture position by esophageal visualization, we performed balloon catheter dilation under X-ray. In this way, the esophageal restenosis model was established. In a very few cases of severe stricture, the restenosis model could not be produced due to the catheters being unable to pass through.

Morphological changes in benign esophageal stricture and esophageal restenosis

After the benign esophageal stricture formed, its morphology was relatively stable. It manifested as thickened muscle layers, reduced luminal sizes, and inelastic lumens. Thus it caused dysphagia. The esophageal morphology was altered by balloon catheter dilation. The esophageal mucosa exhibited not only chemical-burn lesions, but also lesions caused by mechanical damage. The thickened muscle layer of the esophagus was torn or broken, causing the areas of mucosal and muscle layers of the esophagus to increase significantly in the experimental group. Significant differences were also observed in the perimeters of the mucosal and muscle layers of the esophagus and in the perimeter of the entire esophagus wall. Within the

same group, after the dilation procedure the areas of each layer increased rather than decreased, whilst the perimeters also increased. This indicated that dysphagia improvement was due to an enlargement of the lumen of the esophagus after dilation. Up to a certain time, these new scar tissues would further contract and cicatrize. As a result, the duct lumen was further reduced and lacked elasticity. This was one of the key factors in esophageal restenosis. This also illustrated that if there was no treatment plan after balloon dilation in benign esophageal stricture, esophageal restenosis could not be resolved^[2-28].

Immunohistochemical observation of benign esophageal stricture and esophageal restenosis

PCNA is a type of nuclear protein equivalent to the binding protein of DNA polymerase. It coordinates the synthesis of DNA up and down strands. The quantity of PCNA is minimal in normal cells at the G₀, whereas at the M stage the quantity of PCNA in transforming cells changes dramatically. The quantity of PCNA mostly declines at stage G₀/G₁. This quantitative change coincides with DNA synthesis. Therefore, PCNA is used as an indicator to assess cell proliferation. There were a number of reports on the application of immunohistochemical methods to the study of tumor-cell proliferation^[29]. In our study, we used the new method involving PCNA to investigate the basal-cell proliferation of the squamous epithelium in benign esophageal stricture by the procedure of balloon dilation. We found that there was no PCNA expression in the control group in basal cells of the squamous epithelium of the esophagus. However, in the experimental group, PCNA was expressed strongly from day 5 onwards 30 days later, PCNA expression was still positive. This consistently high proliferation of basal cells illustrated their importance in the development of esophageal restenosis.

FN was a glucoprotein with multiple functions^[30]. As a noncollagenous substance in the extracellular matrix, it participates in various reactions between cells as well as between cells and the extracellular matrix, including adhesion, migration, injury, restoration, and tumor metastasis. FN has two forms: a soluble dimerization in humor and a barely soluble polymerization in the extracellular matrix. After combining with its receptor through a tripeptide sequence Arg-Gug-(RGD), FN transmits cellular signals and facilitates cells' interfacing and kinetics. The study of FN expression in the lesion of benign esophageal stricture caused by balloon dilation is therefore helpful to elucidate the mechanism of proliferation and migration of cells. In the control group, we noticed that the expression of FN in the basal mucosa of the esophagus was weak, which indicates that FN expression after a chemical burn is related to the esophageal stricture. In the experimental group, soon after the procedure the squamous epithelium and striated-muscle cells expressed a large amount of FN. This reaction might be related to regulated cellular proliferation and chemotaxis. Previous studies have shown that FN has the similar function to growth factor in fibroblast cells. Even in small doses it can accelerate proliferation. An *in vitro* study has also shown that fibroblasts could adhere directly to the FN matrix or adhere to collagen through FN. FN can also facilitate unfolding of cells that adhere to the matrix. We also noticed that in the experimental group, FN was strongly expressed at both early and later stages after the procedure. This illustrates that FN is one of the key factors in the production of esophageal restenosis, especially at the late stage.

PDGF could stimulate the proliferation of fibroblasts *in vitro*^[31-38]. Initially it was found in platelet granules, and afterwards its secretion was also found in normal cells and transformed cells. It exists in three biologically active isoforms: PDGF-AB, PDGF-AA, and PDGF-BB; comprising PDGF-A and PDGF-B polypeptide chains. It acts on target cells through receptors consisting of two subunits, α and β . PDGF-AB

combines $\alpha\alpha$ and $\beta\beta$ functions. In our experiment, PDGF was not expressed in striated muscle cells of the esophagus, which indicates that PDGF is not a key factor in esophageal restenosis produced by balloon dilated esophageal stricture. However, the enhanced expression of PDGF was involved in the proliferation of smooth-muscle cells. In the study of restenosis, PDGF was regarded as a strong split promoter and chemotactic factor, playing an important role in the formation of blood vessel restenosis. The full length of the esophagus in SD rats (as used in our experiments) comprised striated muscle, and hence PDGF and its function could not be shown in esophageal restenosis in these rats. Besides, in clinical settings, relatively severe chemical burns of the esophagus are usually located at the middle and lower segments of the esophagus, while the upper segment is rarely involved. The middle and lower segments of the esophagus comprise smooth muscle, while the upper segment is striated muscle. This indirectly demonstrates that PDGF expressed in smooth-muscle cells plays a greater role than that in striated-muscle cells in the formation of benign esophageal stricture and restenosis.

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