Mechanism of benign biliary stricture: A morphological and immunohistochemical study

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Abstract

AIM: To explore the mechanism of benign biliary stricture.

METHODS: A model of trauma of bile duct was established in 28 dogs. The anastomosed tissues were resected and examined by light and electron microscopes on day 3, in wk 1, 3 and mo 3, 6 after operation. CD68, TGF-β1 and α-SMA were examined by immunohistochemical staining, respectively.

RESULTS: The mucosal epithelium of the bile duct was slowly recovered, chronic inflammation lasted for a long time, fibroblasts proliferated actively, extracellular matrix was over-deposited. Myofibroblasts functioned actively and lasted through the whole process. The expression of macrophages in lamina propria under mucosa, TGF-β1 and α-SMA in myofibroblasts were rather strong from the 1st wk to the 6th mo after operation.

CONCLUSION: The type of healing occurring in bile duct belongs to overhealing. Myofibroblasts are the main cause for scar contracture and stricture of bile duct. High expressions of CD68, TGF-β1 and α-SMA are closely related to the active proliferation of fibroblasts, extracellular matrix over-deposition and scar contracture of bile duct.

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Key words: Benign biliary stricture; Immunohistochemistry

Introduction

It is difficult to cope with benign biliary stricture in biliary surgery. Its postoperative manifestations are scar contracture and stenosis of bile duct, especially in hepatic porta or above\(^1,2\). We established an animal model of trauma-repair in bile duct in this experiment in order to explore the formation mechanism of benign biliary stricture. Changes of histology and ultrastructure during the process of trauma and repair in the bile duct were observed during tissue healing. Expression intensity, positive cells counts and distribution of macrophage, TGF-β1 and α-SMA were dynamically examined by immunohistochemical staining in different stages of healing.

Materials and Methods

Animal model

Twenty-eight hybrid dogs with an average weight of 15.3 kg were anesthetized with an intraperitoneal injection of 2.5% sodium thiopental (1 mL/kg). An incision across rectus of the right upper abdomen was made and common bile duct (CBD) was separated at the point of 2 cm away from the superior margin of duodenum (the range of separation was within 1 cm). Then an incision of the anterior wall of CBD was made transversally with the length of about one third of its circumference between the vertical axes of both sides. Then the incision of CBD was Anastomosed by microsurgical technology with non-invasive Dexion sutures, with the pinhole distance of 0.5-0.6 mm, and edge distance of 0.3-0.4 mm. After making sure that there was no bile leakage at the anastomotic stoma, an abdominal drainage-tube was placed at the site. Then the abdomen was closed. Postoperative anti-infection treatment was administered for 3 d, and the drainage-tube was then removed.

General condition of animals

General condition and behavior of the animals were observed, including diet, activities, reaction, drainage and postoperative complications.

Histological observation

Pathological examinations as follows were applied to specimens that were obtained on the 3rd d, in the 1st and 3rd wk, and the 3rd, 6th mo after operation, and 5 dogs were randomly selected each time.

Anastomotic stoma and tissues about 2 cm around the stoma were removed and fixed in 10% formaldehyde solution. Specimens were observed by a microscope with HE stain, Masson stain and Verhoeff stain respectively. Percentage of collagen area was calculated with VIDAS image analysis system to observe changes of collagen content. A piece of tissue around the stoma where the scar was obvious was selected and trimmed into a small piece of 1 mm\(^3\) at low temperature, followed by immediate fixation in 2.5% glutaraldehyde solution. It was observed with TEM (HITCH-600) and photographed. The piece of tissue was plated with gold by using an ion coater of EIKO-IB-3 type and photographed. To measure the content change of extracellular collagen, each photograph of TEM was analyzed with VIDAS image analysis system, and the change of relative extracellular volume density (ECVD) was calculated based on three-dimensional analysis principles.

Immunohistochemical observation

Rat anti-human macrophage (CD68) monoclonal antibody and rat anti-human smooth muscle actin (actin 1A4) monoclonal antibodies were purchased from Zhongshan Biotechnology Incorporation. Rabbit anti-human TGF-β1 polyclonal antibody was used for TGF-β1 detection.
was purchased from Santa Cruz Biotechnology Incorporation. Second antibodies, goat anti-rat IgG marked biotin and goat anti-rabbit IgG marked biotin were the product of ZYMED Incorporation. The expressions of CD68, TGF-β1 and α-SMA were detected by immunohistochemical SP method with positive and negative controls. The quantity of positive cells in unit area of the section in each group was analyzed by VIDAS image analysis system and positive cells were stained as brownish yellow granules in cytoplasm.

**Statistical analysis**

All data were expressed as mean±SD. Analysis of variance was used to analyze the difference, and analysis linear correlation was used to analyze the expression of CD68, TGF-β1 and α-SMA.

**RESULTS**

**General condition of animals after operation**

One dog died of bile fistula 12 d after operation, 2 dogs died of obstructive jaundice 4 and 5 mo after operation and 25 dogs survived. Appetite, activity and reaction of the dogs in the early stage were normal, but in the later stage, 4 of 8 dogs developed obstructive jaundice with declined appetite, weight loss, dark urine and Koaln stool 3 mo after operation. An average of 30-40 mL blood-bile mixture was drained from abdominal cavity.

**Histological changes**

Mucosa in anastomotic stoma of the bile duct became necrotic, and was exfoliated 3 d after operation and had acute inflammatory reactions. Surface exudation from stoma deceased one week after operation, and proliferation of granulation tissue could be observed under mucosa. Wall of the bile duct became thicker. Collagenous fibers were disorderly arranged, elastic fibers under mucosa ruptured into segments, and irregularly arranged capillary-like collagens could be found with Masson stain and Verhoeff stain. Mucosa of the stoma had chronic inflammatory reactions 3 wk after operation and mucosa partly recovered. Wall of the stoma that was proliferative with cicatricial tissue was over-thickened, the part of fibrous tissue had hyaline degeneration and capillaries were proliferated, dilated, and engorged. In Masson and Verhoeff stains neocollagenous fibers were massively proliferated and arranged densely in nodule or annual ring. Neocollagenous nodes that were circled with microvessels contained high-dense fibroblasts, and radiated neocollagenous fibers were arranged densely. Region of neocollagenous nodes was short of elastic fibers. During the 3rd-6th mo after operation mucosa of the stoma was infiltrated with chronic inflammatory cells and had a small quantity of mucous gland in lamina propria. Though the mucosa was almost completely repaired, it was arranged in disorder and became thinner. Proliferated blood capillaries here were degenerated more than before. Wall of the stoma was thinner, collagenous fibers were arranged more disorderly and densely than before. Specimens of the liver demonstrated hepatic congestion and bile stasis.

**Ultrastructural changes**

TEM One week after operation, the proliferation of fibroblasts could be observed in the scar tissue at stoma. The cells were active and in synthesis condition. Many collagenous fibers outside the cells could be observed, with a diameter of 40-80 nm. They were thinner than normal cells, and arranged densely in bundle, and scattered around without directivity. Meanwhile, a certain amount of larger, flat and spindle-shaped myofibroblasts could be observed. Well-developed microfilaments and dense body that paralleled to the long axis existed near the cytomembrane, besides the rich roughly surfaced endoplasmic reticulum and developed Golgi body. In addition, infiltration of inflammatory cells and transudatory erythrocytes could be observed in scar tissue. Three week after operation, the functions of fibroblasts and myofibroblasts in the scar tissue were more active. The percentage of myofibroblasts was increased. Microfilament and dense body could be observed more easily. Roughly surfaced endoplasmic reticulum was expanded, and the number of neutrophilic granulocytes and erythrocytes was reduced. Macrophagocytes and lymphocytes became the main inflammatory cells at this stage. Extracellular collagenous fibers were over-sedimentated in whirlpool or annual ring shape and arranged densely in a scattering manner. Three months after operation, the cells in scar tissue still kept active, and the number of myofibroblasts reached the highest. The extracellular collagenous fibers were still arranged without directivity, part of which melted into irregular lumps. Six mo after operation, fibrocytes appeared though they were still comparatively quiet in function. The number of myofibroblasts was decreased. Collagenous fibers began to show certain directivity and were arranged in a wave-shape manner.

SEM One week after operation, necrosis and exfoliation of mucosa epithelia in the stoma and non-repair could be observed. Three weeks after operation, mucosa was partly repaired, but thinner than normal one and its villus was low and flat. Three mo after operation, repair of the mucosa was nearly complete, but mucosa epithelia were arranged disorderly, papilla was low and flat and interspace was increased.

**Percentages of collagenous fiber area and ECVD**

The percentages of collagenous fiber area and ECVD of the stoma in different stages after operation are shown in Table 1.

| Group   | Percentage of collagenous fiber area (%) (n = 5, mean±SD) | EVCD   |
|---------|----------------------------------------------------------|--------|
| 1st wk  | 54.38±3.86                                               | 58.23±4.56 |
| 3rd wk  | 69.26±5.24                                               | 70.67±3.32 |
| 3rd mo  | 78.06±1.3                                                | 81.42±3.74 |
| 6th mo  | 72.48±4.52                                               | 75.48±4.36 |

Significant difference between all groups (P<0.05).

**Immunohistochemical observation**

CD68 was expressed in mucosa lamina propria of the bile duct but weak in submucosa. In normal control the expression of CD68 was negative. TGF-β1 was expressed in granulation tissue, fibroblasts, macrophages, cytoplasm and cytomembrane of endothelial cells of blood vessel. The expression of TGF-β1 was weak in fibrous tissue of normal bile duct wall. α-SMA was expressed in cytoplasm of myofibroblasts and smooth muscle tissue. In normal wall of bile duct, α-SMA was just expressed in a small amount of smooth muscle tissue (Table 2).

| Group   | CD68 Positive (%) | TGF-β1 Positive (%) | α-SMA Positive (%) |
|---------|-------------------|---------------------|--------------------|
| Normal  | -                 | 3.7±0.6             | 12.3±3.5           |
| 1st wk  | ++                | 65.3±5.3            | 68.3±5.22          |
| 3rd wk  | ++                | 42.2±4.2            | 65.3±4.2           |
| 3rd mo  | ++                | 45.1±6.2            | 59.4±5.0           |
| 6th mo  | ++                | 39.3±4.4            | 55.1±4.4           |

αP<0.05, vs 1st wk, bP<0.01, vs Normal.

**Table 2** Expression of CD68, TGF-β1, α-SMA in healing of bile duct (n = 5, mean±SD)
DIFFERENTIATION, healing process and are the important cause of cicatrical contracture.

Hyperplastic contraction of granulation tissue were almost and maintained it for a longer period. Cyclogenesis of the cells and appeared and three week after operation, they reached the peak experiment, a large number of myofibroblasts were observed fibroblasts and smooth muscle cells in ultrastructure. In this atypical fibroblasts, which have the characteristics of both region could lead to over-proliferation of cicatrix over-deposition of collagens in lesion degrees. Over-deposition of collagens were over-deposited in submucosa, and reconstruction was poor after the healing. All these result in proliferation of cicatrix and high incidence rate of stenosis of anastomotic stoma.

In recent years, it has gradually become clear that myofibroblasts are closely related to scar contracture[24]. Myofibroblasts are atypical fibroblasts, which have the characteristics of both fibroblasts and smooth muscle cells in ultrastructure. In this experiment, a large number of myofibroblasts were observed in the scar tissue. One week after operation, myofibroblasts appeared and three week after operation, they reached the peak and maintained it for a longer period. Cycogeny of the cells and hyperplastic contraction of granulation tissue were almost consistent, suggesting that myofibroblasts are significant in the healing process and are the important cause of cicatrical contracture in bile duct and biliary stenosis after operation. As a marker of differentiation, α-SMA could differentiate myofibroblasts from fibroblasts[9,10]. Our study has confirmed that myofibroblasts were the important cause of benign biliary stenosis.

The formation of wound healing is a series of interactions between inflammatory cells and repairing cells. As one kind of major inflammatory cells and immunologic cells, macrophages have been regarded as the “instructor” of tissue repair, which not only takes part in tissue inflammatory and immunologic reactions, but also influences tissue angiogenesis and fibrosis by releasing a variety of media in a direct or indirect, sole or synergic manner[11,12]. TGF-β is the most reproducible growth factor closely related to the formation of scar and is a kind of strong mitogens that play an important role in cell division, multiplication and migration. This is why TGF-β1 induces formation of granulation tissue. If this function became too strong, TGF-β1 would cause formation of scar[13,14].

Our study not only confirmed autocrine of TGF-β1 but also demonstrated high expression of CD68 and TGF-β1 which is closely related to proliferation of biliary cicatrix. As an important substance of signal conduction, macrophages and TGF-β1 solely or synergically, directly or indirectly play an important role in the interaction between cells and extracellular matrix, and cause dysfunction of inflammatory cells and repairing cells as well as disorder of collagen metabolism, which might cause prolonged healing of bile duct trauma, over-deposition of extracellular matrix, cicatrix contracture, stenosis of anastomotic stoma.

Our study indicates that high expression of CD68 and TGF-β1 might be related to chronic inflammation of bile duct wall, which is caused by stimulation of bile. Continuous inflammatory reaction results in massive gathering of macrophages, which synthesize and secrete polypeptide growth factors such as MDGF, TGF-β1, etc. Polypeptide growth factor could cause high proliferation of fibroblasts, over-synthesis of collagen, and cicatrix stenosis of bile duct. Thus reducing bile-stimulated inflammation, shortening time of healing and inhibiting over-infiltration and over-function of macrophages can reduce proliferation of cicatrix of bile duct.

REFERENCES

1. Huang ZQ, Huang XQ. Changing patterns of traumatic bile duct injuries: a review of forty years experience. World J Gastroenterol 2002; 8: 5-12
2. Geng ZM, Xiang GA, Han Q, Liu XG, Su BS, Liu QG, Pan CE. An experimental study on mechanism of benign biliary stricture. Zhonghua Candun Waike Za Zhi 2001; 7: 618-619
3. Alster TS, Tanzi EL. Hypertrophic scars and keloids: etiology and management. Am J Clin Dermatol 2003; 4: 235-243
4. Urioste SS, Arndt KA, Dover JS. Keloids and hypertrophic scars: review and treatment strategies. Semin Cutan Med Surg 1999; 18: 159-171
5. Haverstock BD. Hypertrophic scars and keloids. Clin Podiatr Med Surg 2001; 18: 147-159
6. Brissett AE, Sherris DA. Scar contractures, hypertrophic scars, and keloids. Facial Plast Surg 2001; 17: 263-272
7. Nedelec B, Ghahary A, Scott PG, Tredget EE. Control of wound contraction. Basic and clinical features. Hand Clin 2000; 16: 289-302
8. Ramtani S, Fernandez-Morin E, Geiger D. Remodeled-matrix contraction by fibroblasts: numerical investigations. Comput Biol Med 2002; 32: 283-296
9. Chipev CC, Simran R, Hatch G, Katz AE, Siegel DM, Simon M. Myofibroblast phenotype and apoptosis in keloid and palmar fibroblasts in vitro. Cell Death Differ 2000; 7: 166-176
10. Badid C, Mounier N, Costa AM, Desmouliere A. Role of myofibroblasts during normal tissue repair and excessive scarring: interest of their assessment in nephropathies. Histol Histopathol 2000; 15: 269-280
11. Ashcroft GS, Mills SJ, Lei K, Gibbons L, Jeong MJ, Taniguchi M, Burow M, Horan MA, Wahl SM, Nakayama T. Estrogen modulates cutaneous wound healing by downregulating macrophage migration inhibitory factor. J Clin Invest 2003; 111: 1309-1318
12. Eroglu E, Sari A, Altuntas I, Delibas N, Candir C, Agalar F. The effect of GM-CSF (granulocyte macrophage colony stimulating factor) on doxorubicin induced tissue necrosis and wound healing. Indian J Cancer 2000; 37: 153-157
13. Tian Y, Tang S, Luo S. A study on the expressions and the correlation of TGF-beta and alpha-SMA in scars. Zhonghua Zhengxing Waike Za Zhi 2000; 16: 75-77
14. Tang S, Pang S, Cao Y. Changes in TGF-beta 1 and type I, III procollagen gene expression in keloid and hypertrophic scar. Zhonghua Zhengxing Shaoshang Waike Za Zhi 1999; 15: 283-285
15. Chen W, Fu X, Sun T, Sun X, Zhao Z, Sheng Z. Change of gene expression of transforming growth factor-beta1, Smad 2 and Smad 3 in hypertrophic scars skins. Zhonghua Waike Za Zhi 2002; 40: 17-19
16. Lu Y, Luo S, Liu J. The influence of transforming growth factor beta 1 (TGF beta 1) on fibroblast proliferation and collagen synthesis. Zhonghua Shaoshang Za Zhi 2001; 17: 345-347

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