INTRODUCTION

Benign biliary stricture (BBS) includes several clinical features based on different etiologies, such as biliary wound healing, repeated biliary infection, liver transplantation, and biliary abnormalities. Postsurgical BBS often occurs after orthotopic liver transplantation and laparoscopic cholecystectomy, with reported incidences ranging from 3 to 13% and 0.2 to 0.7%, respectively. BBS is mainly caused by over-healing and fibroblasts proliferate actively and differentiate into myofibroblasts. Fibroblasts and myofibroblasts are mainly scar contracture and stricture of the bile duct, while high expression of transforming growth factor-β1 (TGF-β1) and α-SMA are closely related to the active proliferation of fibroblasts and BBS. P311 (neuronal regeneration related protein) may be involved in the pathogenesis of hypertrophic scarring; however, its role in producing BBS fibroblasts is unknown. If BBS were left untreated, repeated cholangitis, biliary cirrhosis, hepatic failure and death can occur. Surgery and endoscopic treatments are the main interventions for BBS; however, they are associated with high morbidity and mortality. Pharmacological interventions for BBS keloids are available, such as 5-FU, interferon; however, they lack efficiency, reliability, and specificity.

Tetramethylpyrazine (TMP) has been reported to inhibit liver and kidney fibrosis. However, to date, the effect of TMP on BBS fibroblasts has not been elucidated. The aim of this study was to investigate the expression of scar-related genes in fibroblasts, and whether TMP could attenuate the expression of these genes in a rabbit model of BBS.

METHODOLOGY

This experimental study was conducted on rabbit fibroblasts at Guizhou Medical University, Guiyang, Guizhou, China, from April to December 2015. All animal treatments were performed in accordance with the international ethical guidelines and the National Institutes of Health Guidelines concerning the Care and Use of Laboratory Animals. This study was approved by the Animal Ethical and Welfare Committee of the Guizhou Provincial People's Hospital. Four rabbits (two rabbits for establishing the BBS model and two rabbits for acquiring normal bile ducts) were obtained and were raised in the Animal Experiment Center of Guizhou Medical University, China. Rabbits with an average weight of 2.0 - 2.5 kg were anesthetized with an intravenous injection of 2.5% (v/v) sodium pentobarbital (35-45 mg/kg).
An incision across the abdominal midline was made and the common bile duct was separated 1 cm away from the superior margin of the duodenum. A transverse incision on the anterior wall of the common bile duct was made with the length being one-third of its circumference between the vertical axis of both sides. The incision on the common bile duct was Anastomosed with non-invasive Dexon sutures, and then the abdomen was closed. Following surgery, animals were fasted for 8 hours then fed as normal until the end of the study. Intravenous injection of antibiotics (penicillin 1 x 10^6 U/kg was administered by intravenous injection) was performed for 3 days. Control animals underwent surgery without incision of the bile duct.

BBS specimens were obtained during the 1st week after the operation. Rabbits were anesthetized with an intravenous injection of 2.5% (v/v) sodium pentobarbital. Anastomotic stoma and tissue approximately 2 cm around the stoma were removed. Normal bile ducts were removed from control animals. Rabbits were sacrificed by deep narcosis (with sodium pentobarbital). Primary bile duct fibroblast cultures were established as previously described. Bile duct tissues were washed with phosphate-buffered saline (PBS) multiple times, and cut into 15 - 20 pieces. Tissues were plated in 25 cm^2 flasks and incubated for 4 hours at 37°C to allow sufficient attachment. BBS fibroblasts and normal fibroblasts were maintained in Dulbecco’s Modified Eagle’s Medium (Hyclone, USA), supplemented with 20% (v/v) fetal bovine serum (Gibco, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified incubator with 5% (v/v) CO₂ in air. The cells were passaged by Trypsin-EDTA (2.5 g/l) when over 90% confluent (every 7 - 10 days). Subsequent cultures were performed under the same conditions. The cell strains were maintained and stored in liquid nitrogen tanks, and only cells from passages 3 - 5 were used.

After gross inspection, the cultured cells were further identified by immunofluorescence staining. Cells were transferred to 6-well plates (at a density of 2 x 10⁵ cells/well) in culture medium for 24 hours. After rinsing with PBS, the cells were fixed with 4% (w/v) paraformaldehyde and 0.2% (v/v) Triton X-100 for 15 minutes. Cells were labelled with anti-vimentin and anti-cytokeratin primary antibodies (V2258, C-1801, Sigma, USA). Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) (Sigma). Cells were imaged using an inverted fluorescence microscope (Olympus, Japan).

Fibroblasts were seeded into 6-well plates at a density of 2x10⁵ cells/well and cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% (v/v) fetal bovine serum. After starving in serum-free medium overnight, the cells were incubated in medium for 48 hours containing 0.08, 0.4 or 2.0 mg/ml TMP. NF and MF cultures were not exposed to TMP. Fibroblasts were seeded in 96-well plates at a density of 2x10⁴ cells/well in Dulbecco’s Modified Eagle’s Medium supplemented with 10% (v/v) fetal bovine serum. After treatment for 48 hours with or without TMP, Cell Counting Kit-8 (Hyclone, USA) solution (10 µl) was added to each well (100 µl). The mixture was incubated at 37°C for 2.5 hours, followed by analysis using a microplate reader (Bio-Rad, USA) at 450 nm. All cell counting Kit-8 assays were repeated five times.

Total RNA was extracted from treated fibroblasts using RNAiso Reagent (TaKaRa, Japan) and reverse transcribed to cDNA, followed by amplification using the SYBR® Premix Ex Taq TM Reverse Transcription-PCR kit.

Relative quantitative real time PCR was performed using the ABI StepOne System (Life Technologies, USA). The expression levels of the target genes were normalized to β-actin and expressed as a fold-change compared with the control. Melting curves were generated after each run to confirm amplification of specific transcripts.

The amount of protein isolated from treated fibroblasts was detected using the Micro BCA assay (Pierce Perbio Science, Germany). Protein (30 µg) was electrophoresed on 4 - 12% gradient bis-tris polyacrylamide gels (Life Technologies, USA) under reducing conditions and transferred onto polyvinylidene fluoride (PVDF) membrane (Sigma). Membranes were probed for TGF-β1, α-SMA and β-actin using monoclonal anti-rabbit primary antibodies (ab99562, ab7817, ab6276) from Abcam (UK), followed by incubation with the goat anti-mouse IgG-HRP secondary antibody (ab6789, Abcam). Protein bands were detected using the ECL kit (Amer sham, Germany). Changes in protein expression were normalized to β-actin.

Fibroblasts were seeded into 6-well plates at a density of 2x10⁵ cells/well and cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% (v/v) fetal bovine serum. After starving in serum-free medium overnight, the cells were incubated in medium for 48 hours containing 0.08, 0.4 or 2.0 mg/ml TMP. NF and MF cultures were not exposed to TMP.

Fibroblasts were seeded in 96-well plates at a density of 2x10⁴ cells/well in Dulbecco’s Modified Eagle’s Medium supplemented with 10% (v/v) fetal bovine serum. After treatment for 48 hours with or without TMP, Cell Counting Kit-8 (Hyclone, USA) solution (10 µl) was added to each well (100 µl). The mixture was incubated at 37°C for 2.5 hours, followed by analysis using a microplate reader (Bio-Rad, USA) at 450 nm. All cell counting Kit-8 assays were repeated five times.

Total RNA was extracted from treated fibroblasts using RNAiso Reagent (TaKaRa, Japan) and reverse transcribed to cDNA, followed by amplification using the SYBR® Premix Ex Taq TM Reverse Transcription-PCR kit.

Relative quantitative real time PCR was performed using the ABI StepOne System (Life Technologies, USA). The expression levels of the target genes were normalized to β-actin and expressed as a fold-change compared with the control. Melting curves were generated after each run to confirm amplification of specific transcripts.

The amount of protein isolated from treated fibroblasts was detected using the Micro BCA assay (Pierce Perbio Science, Germany). Protein (30 µg) was electrophoresed on 4 - 12% gradient bis-tris polyacrylamide gels (Life Technologies, USA) under reducing conditions and transferred onto polyvinylidene fluoride (PVDF) membrane (Sigma). Membranes were probed for TGF-β1, α-SMA and β-actin using monoclonal anti-rabbit primary antibodies (ab99562, ab7817, ab6276) from Abcam (UK), followed by incubation with the goat anti-mouse IgG-HRP secondary antibody (ab6789, Abcam). Protein bands were detected using the ECL kit (Amersham, Germany). Changes in protein expression were normalized to β-actin.

Fibroblasts were seeded into 6-well plates at a density of 2x10⁵ cells/well and cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% (v/v) fetal bovine serum. After starving in serum-free medium overnight, the cells were incubated in medium for 48 hours containing 0.08, 0.4 or 2.0 mg/ml TMP. NF and MF cultures were not exposed to TMP.
All cellular experiments were performed at least three times. The data of cells proliferation (optical density) and mRNA/protein expressions of α-SMA, TGF-β1 and P311 were numerical variable data. Data were expressed as the mean (χ) ± standard deviation (SD).

Statistical analyses were performed using SPSS version 16.0 for Windows. These data in the experimental and control groups were compared using One-way Analysis of Variance (Bonferroni post-hoc test). P < 0.05 was considered statistically significant.

RESULTS

Phase-contrast microscopy revealed that cultured rabbit bile duct cells appeared morphologically similarly to fibroblasts. Cells were further characterized by immunofluorescence staining against the cell-specific markers vimentin and cytokeratin, where fibroblasts stained positive for vimentin and negative for cytokeratin (Figure 1).

MF fibroblasts had a significantly higher optical density than NF group cells (0.533 ±0.011 vs. 0.331 ±0.005, p<0.0001), while BBS fibroblast in the TMP (0.08, 0.4 or 2.0 mg/ml) treated groups had a significantly lower optical density than those in the MF group (0.488 ±0.005 vs. 0.533 ±0.011, p<0.001; 0.399 ±0.011 vs. 0.533 ±0.011, p < 0.001, Table I). These results suggested that TMP can inhibit the proliferation of BBS fibroblasts.

The relative mRNA expressions of TGF-β1, P311 and α-SMA in the MF group were significantly up-regulated compared with the NF group (2.648 ±0.097 vs. 1.000 ±0.069, p < 0.001; 4.146 ±0.074 vs. 1.000 ±0.050, p<0.001; 4.026 ±0.052 vs. 1.000 ±0.112, p<0.001, Table II). The relative mRNA expressions of TGF-β1 (2.334 ±0.196 vs. 2.648 ±0.097, p=0.203; 1.913 ±0.251 vs. 2.648 ±0.097, p=0.001; 1.713 ±0.199 vs. 2.648 ±0.097, p<0.001), and α-SMA (2.959 ±0.031 vs. 4.026 ±0.052, p<0.001; 2.863 ±0.201 vs. 4.026 ±0.052, p<0.001; 2.574 ±0.383 vs. 4.026 ±0.052, p<0.001) in BBS fibroblasts following TMP (0.08, 0.4 or 2.0 mg/ml) treatment were significantly down-regulated in a dose-dependent manner compared with the MF group (p<0.01; 0.08-2.0 mg/ml; Table II).

Tetramethylpyrazine attenuates biliary stricture
Protein expressions of TGF-β1 and α-SMA were significantly up-regulated in the MF group compared with the NF group (2.096 ±0.059 vs. 1.000 ±0.093, p<0.001; 1.425 ±0.070 vs. 2.096 ±0.059, p<0.001) and α-SMA (2.535 ±0.118 vs. 3.231 ±0.232, p<0.001; 2.411±0.154 vs. 3.231 ±0.232, p<0.001; 2.172 ±0.152 vs. 3.231 ±0.232, p<0.001) in BBS fibroblasts of TMP (0.08, 0.4 or 2.0 mg/ml) treatment were significantly down-regulated in a dose-dependent manner compared with the MF group (p<0.01; Table III and Figures 2).

DISCUSSION

The formation of a hypertrophic scar is an inevitable result of wound healing, which is subsequently accompanied by the formation of a keloid scar of different degrees. A hypertrophic scar in the bile duct results in high morbidity for a benign condition.

Fibroblasts are one of the most abundant cell types in connective tissues and are responsible for tissue homeostasis under normal physiological conditions. When tissues are injured, fibroblasts are activated and differentiated into myofibroblasts, which generate large contractions and actively produce extracellular matrix proteins to facilitate both wound closure and scar contracture and actively produce extracellular matrix proteins to facilitate both wound closure and scar progression. During reconstruction of the bile duct, epithelial cells of the bile duct recover poorly, but fibroblasts proliferate actively and play an important role in the formation of the cicatrix and the high morbidity of BBS in animals.

A key feature of myofibroblasts is the expression of α-SMA. Moreover, α-SMA can induce the differentiation of fibroblasts into myofibroblasts. Most myofibroblasts are derived from resident fibroblasts and are atypical, having ultrastructural characteristics of both fibroblasts and smooth muscle cells. In addition, fibroblasts and myofibroblasts are involved in scar contracture and BBS.

TGF-β1 is a major growth factor involved in the formation of scars and is a mitogen that plays an important role in cell division, multiplication and migration. In addition, over expression of TGF-β1 plays an important role in the pathogenesis of BBS. In TGF-β signalling components, including TGF-β, TGFβRI, TGFβRII, Smad4 and Smad7 are highly expressed during stenosis of the bile duct. TGF-β signalling components, including TGF-β, TGFβRI, TGFβRII, Smad2/Smad3, Smad4, Smad6/Smad7 and endoglin, and the TGF-β-Smad transduction pathway can be regulated by positive and negative feedback loops. The intracellular effectors of TGF-β signalling, the Smad proteins are activated by receptors and translocate to the nucleus where they regulate transcription.

P311, also called neuronal regeneration related protein (NREP), is an 8-kDa protein with several PEST domains (including much Pro, Glu, Ser, and Thr) that is found in fibroblasts and myofibroblasts, and may be involved in the pathogenesis of hypertrophic scars. Transfection of fibroblasts with the P311 gene has been shown to stimulate the expressions of α-SMA and TGF-β1. TMP is a purified and chemically identified component of the Chinese Herb Ligusticum Wallichii, and has been reported to exert inhibitory effects against liver and kidney fibrosis. This antibiotic effect of TMP has been shown to be associated with the reduction of TGF-β1 expression in liver and kidney fibrosis.

CONCLUSION

This study demonstrated that TMP can significantly reduce the proliferation of fibroblasts in a rabbit model of BBS, and significantly down-regulate the mRNA and protein expressions of TGF-β1, α-SMA and P311. Further, it was suggested that TMP may be of therapeutic benefit for the treatment of BBS.

REFERENCES


