Short Communication

The Inhibition of Cell Proliferation of Bile Duct Scar Fibroblasts in Miniature Pigs by 5-fluorouracil and its Mechanism

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ABSTRACT

The purpose of this study was to investigate the effect of 5-fluorouracil (5-FU) on the expression of TGF-β1, cyclinD1, and CDK4 in fibroblasts of miniature pig bile duct scar model cultured in vitro. The bile duct scar fibroblasts were replicated by incision and anastomosis in miniature pigs and bile duct scar fibroblasts were obtained. After cell identification, the bile duct scar fibroblasts were given different concentrations of 5-FU (0, 0.001, 0.01, and 0.1 mmol/L). After 48 h of intervention, CCK-8 method was used to detect the cell proliferation level of each group; Western blot was used to detect the expression of TGF-β1, cyclinD1, and CDK4 protein in each group of cells; and real-time polymerase chain reaction was used to detect the expression levels of TGF-β1, cyclinD1, and CDK4 mRNA in each group of cells. The results showed that after 5-FU intervention, the proliferation of bile duct scar fibroblasts was inhibited, and the expressions of TGF-β1, cyclinD1, CDK4 mRNA and protein in the cells were down-regulated (P<0.05), showing concentration-dependence. In conclusion, 5-FU may have a role in inhibiting bile duct scars. One of the mechanisms may be related to its ability to inhibit the expression of TGF-β1, cyclinD1, and CDK4 in bile duct scar fibroblasts.

Benign stenosis of the hilar bile duct mainly refers to the stenosis of the upper common hepatic duct and the left and right extrahepatic ducts (Maeda et al., 2019). Benign bile duct stenosis means the bile duct fibros tissue hyperplasia and scar contracture caused by biliary tract surgery, calculus, inflammation, and infection. The excessive proliferation of benign bile duct scar fibroblasts, which can lead to the excessive secretion of collagen, is a core link of benign bile duct scar stenosis (Geng et al., 2005; Xu et al., 2003).

Transforming growth factor-β1 (TGF-β1) is currently known as the closest and most representative growth factor to scar formation, and it is closely related to the occurrence and development of scars, and also affects scar contracture (Fang et al., 2019; Wang et al., 2020). TGF-β1 is a multifunctional cytokine with an autocrine mechanism and the ability to regulate cell proliferation, differentiation, and expression of the intercellular matrix (Chi et al., 2018; Ji et al., 2019). The continuous expression of TGF-β1 can lead to the subsequent secretion and overexpression of TGF-β1 through the inflammatory mediators produced during the healing process of bile duct injury and the autocrine mechanisms, thus stimulating the production and excessive deposition of extracellular matrix (ECM). ECM stimulates fibroblasts to secrete more TGF-β1, forming a positive feedback effect, and eventually resulting in hyperplasia of local tissue scars and the thickening of the bile duct wall fibrosis, scar contracture and stenosis.

One study has found that the transcriptional levels of cyclinD1 and CDK4 in hypertrophic scar fibroblasts are elevated (Gong et al., 2016). It is speculated that the abnormal expression of cyclinD1 and CDK4 is closely related to the occurrence of pathological scars.

Drug injection for the treatment of pathological scars is more and more accepted due to its simple operation, low cost and positive effects. Fluorouracil (5-FU) and other drugs are commonly used in clinical practice. 5-FU is a uracil metabolite. It is first converted into fluorouracil deoxynucleoside and then into uracil deoxynucleotide by enzymatic reaction in the body (Barsotti and Ipata, 2002). One study has shown that 5-FU has a certain effect in treating pathological scars (Hietanen et al., 2018).
fibroblasts (cell density 2 × 10⁴/well) in the logarithmic phase, E (0.1 mmol/L 5-FU).

Four healthy male experimental miniature pigs of clean grade were selected and randomized into a normal control group and a bile duct scar model group, with 2 pigs in each group. The pigs were put in fast for 12 h before the operation. They were intravenously anesthetized with 2.5% sodium pentobarbital solution (45 mg/kg body weight). A midline incision was made in the upper abdomen, the skin and subcutaneous tissue were cut, and the common bile duct was exposed. In the bile duct scar model group, the common bile duct with a length of about 1 cm was freed near the duodenum. It was cut transversely for about 1/3 of its circumference, and was sutured with Dexon thread. The abdomen was sutured layer by layer. In the normal control, the abdomen was sutured after opening, without opening the common bile duct. Both groups were fed as routines after 12-h fasting. The bile duct tissues of the bile duct scar model group and the normal control group were taken one week after the operation, washed with phosphate buffer solution (PBS) three times, and cut into 2 mm³ tissue pieces, transferred to the culture flask and placed in a 5% carbon dioxide incubator at 37°C. After 4-h incubation, the cells adhered well, and DMEM medium containing 20% inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate) was added to subculture the adherent cells. Fibroblasts were isolated and purified using differential adherence, and passaged to subculture the adherent cells. Fibroblasts were isolated for this experiment.

Semi-quantitative RT-PCR was used to analyze the expression of TGF-β1, cyclinD1, and CDK4 mRNA in each group of cells. The amplification conditions were pre-denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, extension at 72°C for 1 min, and cycling 30 times, and finally extension at 72°C for 8 min to terminate the reaction. The forward primer of TGF-β1 is 5'-GCCCTGG-ACACCAACTATGGC-3', and the reverse primer is 5'-GCACCTTGCGAGGCAGCA-3'. The forward primer of cyclinD1 is 5'-GAGACCATCCCCCTCGAGGC-3', and the reverse primer is 5'-TCTTCTCTCTCCTCGGC-3'. The forward primer of CDK4 is 5'-TGATGCGCCAGTTTCTAAGAGG-3', and the reverse primer is 5'-TTCCAATGCAGATTTTCACCA-3'. The forward primer of β-actin is 5'-GACACGGCTCC-GGATGTCAGGCA-3', and the reverse primer is 5'-TGAGG-ATGCTTCTCTTCTGCTG-3'. The ABI Stepone software v 2.3 qRTPCR system was used to collect data and analyze the results. The melting curve was a single peak type, indicating good primer specificity. With β-actin as an internal reference. The experiment was repeated three times to ensure the accuracy of the experimental results.

Data analysis was performed using SPSS 25.0 statistical software (SPSS Inc., Chicago, IL, USA). The
measurement data were expressed as mean ± standard deviation. Comparisons among multiple groups were analyzed by one-way variance with LSD-t test. The \( P<0.05 \) indicated that the difference was statistically significant.

**Results and discussion**

After 5-FU concentration gradient acted on bile duct scar fibroblasts, the OD value decreased significantly as the 5-FU concentration increased, indicating a significant increase in inhibition, and the difference was statistically significant (\( P<0.05 \); Fig. 1). It is suggested that 5-FU can inhibit the proliferation of bile duct scar fibroblasts in miniature pigs. This conclusion is basically consistent with the results reported in previous study (Ophir, 1991).

Compared with Group A, the expression levels of TGF-\( \beta \)-1, cyclinD1, CDK4 protein and mRNA in Groups B, C, D and E were increased (\( P<0.05 \); Figs. 2, 3). It is suggested that the production of TGF-\( \beta \)-1, cyclinD1 and CDK4 may play an important role in the process of bile duct scar formation in miniature pigs.

With the increase of 5-FU concentration, the expression levels of TGF-\( \beta \)-1, cyclinD1, CDK4 protein and mRNA in cells decreased significantly (\( P<0.05 \); Figs. 2, 3), showing concentration-dependence. It is speculated that the inhibition of the proliferation of bile duct scar fibroblasts in miniature pigs by 5-FU is related to the regulation of TGF-\( \beta \)-1, cyclinD1 and CDK4.

**Conclusion**

The results of this study indicate that 5-FU can inhibit the proliferation of bile duct scar fibroblasts in miniature pigs, and the mechanism may be related to the down-regulation of TGF-\( \beta \)-1, cyclinD1, and CDK4. However, whether 5-FU directly results in changes in TGF-\( \beta \)-1, cyclinD1, and CDK4, or down-regulates the expression of TGF-\( \beta \)-1 and then induces changes in cyclinD1 and CDK4 through TGF-\( \beta \)-1 requires further study in future.

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Statement of conflict of interest
Authors have declared no conflict of interest.

References
Barsotti, C. and Ipata, P.L., 2002. *Biochem. Pharmacol.*, **63**: 117-122. https://doi.org/10.1016/S0006-2952(01)00845-0

Chi, C., Liu, X., Hou, F., Yu, X., Li, C., Cui, L., Liu, R. and Yin, C., 2018. *BMC Complement. Altern. Med.*, **18**: 52-60. https://doi.org/10.1186/s12906-018-2119-7

Fang, X., Hu, X., Zheng, Z., Tao, K., Wang, H., Guan, H., Shi, J., Ji, P., Cai, W., Bai, X., Zhu, X., Han, J., Liu, J. and Hu, D., 2019. *J. mol. Histol.*, **50**: 503-514. https://doi.org/10.1007/s10735-019-09844-w

Geng, Z.M., Yao, Y.M., Liu, Q.G., Niu, X.J. and Liu, X.G., 2005. *World J. Gastroenterol.*, **11**: 293-295. https://doi.org/10.3748/wjg.v11.i2.293

Gong, Y.F., Zhang, X.M., Liu, F., Wang, Z.Z., Deng, X.F., Jiao, Y., Li, X.J. and Huang, X.Y., 2016. *Eur. J. Pharmacol.*, **791**: 647-654. https://doi.org/10.1016/j.ejphar.2016.09.034

Hietanen, K.E., Järvinen, T.A., Huhtala, H., Tolonen, T.T., Kuokkanen, H.O. and Kaartinen, I.S., 2019. *J. Plast. Reconstr. Aesthet. Surg.*, **2**: 4-11. https://doi.org/10.1016/j.bjps.2018.05.052

Ji, X., Tang, Z., Shuai, W., Zhang, Z., Li, J., Chen, L., Cao, J. and Yin, W., 2019. *Life Sci.*, **231**: 116674. https://doi.org/10.1016/j.lfs.2019.116674

Maeda, T., Ebata, T., Yokoyama, Y., Igami, T., Mizuno, T., Yamaguchi, J., Onoe, S., Watanabe, N. and Nagino, M., 2019. *Surg. Case Rep.*, **5**: 177. https://doi.org/10.1186/s40792-019-0745-z

Ophir, A., 1991. *Exp. Eye Res.*, **53**: 799-803. https://doi.org/10.1016/0014-4835(91)90116-V

Wang, L., Cao, Y., Guan, Z., Luo, G., Luo, L., Yang, X. and Chu, M., 2020. *Int. Urol. Nephrol.*, **52**: 97-106. https://doi.org/10.1007/s11255-019-02286-z

Xu, J., Geng, Z.M. and Ma, Q.Y., 2003. *Hepatobilia. Pancreat. Dis. Int.*, **2**: 295-299.