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ORIGINAL ARTICLE

Tissue factor pathway inhibitor-2 induced hepatocellular carcinoma cell differentiation

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Abstract To investigate the effect of over-expression of tissue factor pathway inhibitor-2 (TFPI-2) on the differentiation of hepatocellular carcinoma (HCC) cells (Hep3B and HepG2). The TFPI-2 recombinant adenovirus (pAd-TFPI-2) was constructed using the pAdeasy-1 vector system. Transfected by pAd-TFPI-2, the cell proliferation of HCC cells was evaluated by CCK-8 assay, flow cytometry was used to detect cell apoptosis and CD133 expression. Real-time PCR and Western blot were used to detect the expression levels of markers of hepatocellular cancer stem cells (CSC) and hepatocytes. The over-expression of TFPI-2 significantly suppressed cell proliferation, induced apoptosis, and dramatically decreased the percentage of CD133 cells, which was considered as CSC in HCC. Real-time PCR and Western blot showed that the expression of markers of CSC in Hep3B cells and HepG2 cells infected with pAd-TFPI-2 was markedly lower than those of the control group ($P < 0.05$), while the expression of markers of hepatocytes was significantly increased ($P < 0.05$). Hence, TFPI-2 could induce the differentiation of hepatocellular carcinoma cells into hepatocytes, and is expected to serve as a novel way for the treatment of HCC.

1. Introduction

Hepatocellular carcinoma is one of the malignancies with a high incidence in the world, and its incidence is increasing (Forner et al., 2012; Kim and Park, 2014). Hepatocellular carcinoma has a silent onset with no symptoms in the early stage, while it progresses rapidly. Currently the treatment methods for hepatocellular carcinoma are mainly hepatic resection, hepatic transplantation, and drug therapy. However, only 10–20% of the patients with HCC can be surgically excised, yet attended with a high frequency of recurrence (Liu et al., 2014). Further, HCC is chemoresistant and the current drug therapy is associated with limited efficacy. The prognosis of HCC patients is generally poor (Altekruse et al., 2014). Although new strategies have been applied for HCC treatment, efficacies are still beyond satisfactory (Kozyreva et al., 2011). Therefore, it is of immense importance to seek some new ways for the treatment of hepatocellular carcinoma.

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Tissue factor pathway inhibitor-2 (TFPI-2) is also known as matrix-associated serine protease inhibitor (MSPI) and placental protein 5 (PP5) (Rao et al., 1995; Kisiel et al., 1994). TFPI-2 is a kunitz-type serine proteinase inhibitor, which is abundantly expressed in a variety of human tissues such as liver, pancreas, skeletal and directionally secreted into the extracellular matrix (ECM) (Miyagi et al., 1994; Sugiyama et al., 2002; Herman et al., 2001). TFPI-2 is thought to negatively regulate the enzymatic activity including matrix metalloproteinase (MMP), plasmin, cathepsin G, trypsin, and plasma kallikrein (Stamenkovic, 2003; Kempaiah et al., 2007). Previous studies have suggested that the expression of TFPI-2 is down-regulated in many malignant tumors, including breast cancer, gastric stromal tumor, cervical cancer, gliomas and non-small-cell lung cancer, and low expression of TFPI-2 was associated with poor prognosis in cancer patients (Wang et al., 2014; Zhang et al., 2013; Rao et al., 2001; Rollin et al., 2005; Xu et al., 2013).

The therapy of induced differentiation of tumors is proposed in recent years, which indicates a new direction for the treatment of hepatocellular carcinoma. Commonly used differentiation-inducing agents are mostly substances that might work on other malignancies. But generally speaking, the therapy of induced differentiation of hepatocellular carcinoma has not yet yielded satisfactory results. It has been reported that transmembrane protease, serine 4 (TMPRSS4) is upregulated by the silencing of TFPI-2 through aberrant DNA methylation in non-small-cell lung cancer (Hamamoto et al., 2015). TMPRSS4 has been shown to be an important regulator during the epithelial-mesenchymal transition (EMT) in human epithelial cancer cells (Li et al., 2011). EMT is a physiological mechanism which is present during development, including mesoderm formation and neural tube formation (Kalluri and Weinberg, 2009). Previous studies showed that the EMT process may facilitate the generation of cancer cells with the mesenchymal traits needed for dissemination as well as the self-renewal properties needed for initiating secondary tumors (Hollier et al., 2009). Our previous studies indicated that TFPI-2 could not only inhibit the proliferation, invasion and metastasis of Hep3B and HepG2, but also significantly reduce the expression and secretion of alpha-fetal protein (AFP), a maker of HCC (Xu et al., 2011). Therefore, we hypothesize that TFPI-2 may show an effect on inducing the differentiation of hepatocellular carcinoma cells (HCC) into mature hepatocytes and serve as a novel way for the treatment of hepatocellular carcinoma.

2. Materials and methods

2.1. Construction of adenoviral vectors

The sequence of TFPI-2 gene coding sequence was amplified by PCR. The shuttle plasmid and the TFPI-2 DNA fragment were bound using the T4DNA ligase (TaKaRa, Japan) after the restriction enzyme digestion. The sequence was identified via DNA sequencing and restriction enzyme digestion. The pAdtrack-cmv-TFPI-2 and the pAdEasy-1 were co-transformed into *Escherichia coli* BJ5183 with backbone vector AdEasy-1 for homologous recombination. The recombinant plasmid pAd-TFPI-2 digested by Pac I (Fermentas, USA) was used to transfect Hek293 cells (Cellbank, China) by Lipofectamine™ 2000 (Invitrogen, USA) for further packaging and amplification of the virus.

2.2. Cell culture and transfection

Hepatoma cell lines HepG2 and Hep3B were obtained from the American Type Culture Collection (ATCC, USA). The cells were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (Gibico, USA), 1.0% glutamine, 100 μg/ml streptomycin and 100 μg/ml penicillin in a humidified atmosphere containing 5% CO_2 at 37 °C. The virus was added to the cell monolayers. Cells were then incubated for 2 h to complete the transfection of virus into the cells. The serum-free medium was replaced with serum-containing medium and cells were cultured for 48 h.

2.3. RT-PCR

Cells were harvested in Trizol (TaKaRa, Japan), and total RNA was isolated according to the manufacturer's instructions. After the RNA was reversely transcribed into cDNA, the change in the expression of TFPI-2 was detected using PCR. The cDNA was synthesized from 1 μg RNA as the template using RT-PCR kit (Takara, Japan). The original amount of TFPI-2 and β-actin was detected via PCR with Premix Taq (Takara, Japan). The primers were synthesized by The Beijing Genomics Institute (BGI, China) as follows: TFPI-2 sense 5′-ATAGGATCCACATGGACCCGCTCGC-3′ and antisense 5′-GGCCTCGAGAAATTGCTTCTTCCGAATTTCC-3′, β-actin sense 5′-GAGTCAACGGATTTGGTCGT-3′ and antisense 5′-GACAAGCTTCCCGTTCTCAG-3′. To study TFPI-2 gene expression, the PCR was initiated by a decontamination (95 °C for 5 min) and denaturation step (95 °C, 30 min), followed by 30 cycles at 60 °C for 30 s and at 72 °C for 40 s. The level of TFPI-2 mRNA was evaluated by the ratio of density of TFPI-2 to β-actin.

2.4. Western blot

The cells were collected at 72 h after infection. The cultures were washed several times with phosphate-buffered saline (PBS). Total proteins were harvested in cell lysates supplemented with PMSF (1 mmol/l) to inhibit the proteases. The samples were boiled for 5 min and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) on 12% polyacrylamide gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes and blocked with 5% non-fat milk for 2 h at 37 °C. After blocking, the membranes were incubated for 12 h at 4 °C with anti TFPI-2 antibody (Santa Cruz, USA) diluted by TBST. After several washes, the membranes were incubated horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG secondary antibody (Santa Cruz, USA). After washing, the blots were detected by Odyssey Infrared Imaging System (LI-COR).

2.5. Flow cytometry analysis

Flow cytometry was used to detect the cell apoptosis and CD133 expression. Briefly, cells (3×10^5/well) were seeded in a six-well plate, and infected with adenovirus. After 72 h, cells