DCN prevent metastasis of HepG2 cells by elevating the expression of TGF-β and P15

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Abstract. Research shows that DCN prevent the prolifration of human hepatoma HepG2 cells and the involvement of transforming growth TGF-β signaling pathway. The results indicated that, cell proliferation was significantly decreased in HepG2 cells transfected with DCN. In addition, DCN transfection significantly increased the phosphorylation level of TGF-βRI in HepG2 cells. The expression of the downstream factor p15 was also significantly elevated in the DCN-transfected HepG2 cells. In conclusion, DCN elevated the expression level of TGF-βRII, increased the phosphorylation level of TGF-βRI, enhanced the expression of p15. These findings may contribute to the understanding of the role of DCN in the pathogenesis of hepatic carcinoma and assist in the disease treatment.

Introduction

DCN is one of the important members of the small leucine-rich proteoglycan family, which is mainly composed of the 44-kD core proteins and the dermatan sulfate side chains[1]. DCN is the main component of the extra cellular matrix (ECM), serving an important role in maintaining the biological activity of the ECM protein in general, regulating the cell proliferation and differentiation, and preventing tissue fibrosis [2]. Furthermore, DCN has been found to have significant antitumor effects [3]. Since DCN is widely expressed in the microenvironment of normal and tumor tissues, it may influence the biological activity of the tumor cells by affecting the matrix structure and regulating various receptors associated with cell proliferation and survival. However, the role of DCN in hepatic carcinoma has not yet been fully established.

The signaling pathways involved in the action of DCN include the following: The regulation of the epidermal growth factor receptor (EGFR) and other ErbB family members, and the subsequent activation of the MAPK signaling pathway [4]; the regulation of the transforming growth TGF-β pathway, including TGF-β1, TGF-β2, and TGF-β3[5]. In particular, the TGF-β signaling pathway serves different roles at different stages in the development of hepatic carcinoma. At the early stage, TGF-β acts as a tumor suppressor, inhibiting cell proliferation and enhancing cellular differentiation or apoptosis [6]. However, at the later stage, TGF-β gradually loses its antiproliferative effects, and subsequently stimulates angiogenesis, inhibits immune responses and promotes ECM formation, which facilitates the cell proliferation and tumor metastasis[7].

In the present study, the effects of DCN on the proliferation of human hepatoma HepG2 cells and the involvement of the TGF-β signaling pathway were investigated. The results demonstrated that DCN may elevate the expression of TGF-βRII, enhance the phosphorylation of TGF-βRI and then induce the overexpression of p15 protein, thus inhibiting the proliferation of HepG2 cells.

Materials and methods

Cell transfection. The pcDNA5/FRT vector (Invitrogen; Thermo Fisher Scientific, Inc.), harboring an FRT site and a cDNA fragment containing DCN, was transfected into the HepG2 cells using Lipofectamine 2000 transfection reagent (cat. no. 11668072; Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Enhanced green fluorescent protein

was used as the positive control. In order to detect the transfection efficiency, the cells were transfected with an enhanced green fluorescent protein-containing plasmid, and then stained with DAPI for 5 min. Fluorescence was detected with the IX83 microscope (Olympus Corp., Tokyo, Japan). The percentage of cells with green fluorescence was calculated to express the transfection efficiency.

Cell line and culture. Human hepatoma HepG2 cells were obtained from Thermo Fisher Scientific, Inc. (Gaithersburg, MD, USA). These cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA). The cells were divided into the four following groups: i) Control group, in which the cells were untreated; ii) DCN group, in which the cells were transfected with DCN; iii) siRNA group, in which the cells were transfected with mismatch-siRNA; and iv) DCN+siRNA, in which the cells were transfected with DCN+siRNA. siRNA (30 nM) was used to silence DCN, and the mismatch-siRNA, i.e. the non-sense RNS sequence, was used as the control. The transfection was performed with Lipofectamine 2000, according to the manufacturer's instructions. After 48 h, the cells were collected and subjected to analysis.

MTT assay. Cellular proliferation was assessed by the MTT assay. Briefly, HepG2 cells were seeded onto the 96-well plates at the density of 5,000 cells/well. After 48 h, the culture medium was discarded, and 200 µl MCDB 131 (cat. no. L-1202-500; Thermo Fisher Scientific, Inc., Gaithersburg, MD, USA) and 20 µl MTT (Cell Proliferation kit I; cat. no. 11465007001; Roche, Indianapolis, IN, USA) were added into each well. After 3.5-h incubation, the solution was discarded and 100 µl dimethyl sulfoxide (DMSO; cat. no. D2650; Sigma-Aldrich, St. Louis, MO, USA) was added. After 10 min, the DMSO solution was transferred into a new 96-well plate, and the absorbance at 490 nm was read on a microplate reader (iMark; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Western blot analysis. The protein expression levels of TGF-βRI, phosphorylated (p)TGF-βRI, TGF-βRII and p15 were detected by western blot analysis. Briefly, cells were cultured on 6-well plates at the density of 20,000 cells/well. After transfection, cells from all the control and transfection groups were collected and lysed on ice with the lysis buffer (cat. no. 74255; Sigma-Aldrich). A total of 30 µg protein sample was subjected to 10% SDS-PAGE, and electronically transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 50 g/l skimmed milk at room temperature for 1 h, and then incubated at 4˚C overnight with the following polyclonal rabbit anti-human primary antibodies: Anti-TGF-βRI antibody (dilution, 1:5,000; cat. no. ab31013), anti-pTGF-βRI antibody (dilution, 1:5,000; cat. no. ab112095), anti-p15 antibody (dilution, 1:5,000; cat. no. ab53034), and anti-TGF-βRII (dilution, 1:2,000; cat. no. ab61213; all from Abcam, Cambridge, MA, USA). Next, the membrane was incubated with goat anti-rabbit horseradish peroxidase-conjugated IgG secondary antibody (dilution, 1:1,000; cat. no. sc-2030; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. The protein bands were visualized using an enhanced chemiluminescence detection kit (Sigma-Aldrich). Image Lab software (version 3; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to acquire and analyze the images. GAPDH was used as the control.

Statistical analysis. Data are expressed as the mean ± standard deviation. SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA) software was used for the statistical analysis. The Student's t-test was used for pairwise comparison, while analysis of variance was performed for multiple comparison. $P<0.05$ was considered to indicate statistically significant differences.

Results

DCN induces TGF-βRI phosphorylation and elevates p15 expression. To investigate the underlying mechanisms through which DCN inhibited the proliferation of HepG2 cells, the expression of cell proliferation-associated signaling pathways was detected by the western blot analysis. The results showed that, compared with the control group, no statistically significant differences were observed in the total TGF-βRI protein expression level in the HepG2 cells transfected with DCN ($P>0.05$). By contrast, the phosphorylation of TGF-βRI was significantly

increased in HepG2 cells following DCN transfection (P<0.05). In addition, compared with the control group, the expression of the downstream factor p15 was significantly elevated in the DCN-transfected HepG2 cells (P<0.05). These results suggest that DCN transfection is able to activate TGF-βRI and elevate the expression of p15 in HepG2 cells, implying the involvement of the TGF-β signaling pathway in the inhibitory effects of DCN on HepG2 cell proliferation.

TGF-βRII silencing abolishes the effects of DCN on TGF-β signaling and HepG2 cell proliferation. In order to further investigate the role of TGF-βRII in DCN-induced phosphorylation of TGF-βRI in HepG2 cells, TGF-βRII was knocked down with siRNA silencing. RT-qPCR demonstrated that the mRNA expression level of TGF-βRII was not significantly altered by the mismatch-siRNA treatment, while the TGF-βRII mRNA expression level was significantly decreased in the siRNA silencing group (P<0.05; Fig. 1A). In addition, the results from western blot analysis showed that the siRNA silencing of TGF-βRII alone did not induce significant alteration in TGF-βRI phosphorylation in the HepG2 cells (P>0.05; Fig. 1B). However, TGF-βRII silencing significantly decreased the phosphorylation of TGF-βRI in DCN+siRNA-transfected HepG2 cells, when compared with the DCN group (P<0.05; Fig. 1B). Furthermore, the MTT assay revealed that TGF-βRII silencing abolished the inhibitory effects of DCN on the proliferation of HepG2 cells (P<0.05; Fig. 1C). These results suggest that TGF-βRII is a key player in the DCN-induced pTGF-βRI enhancement and HepG2 cell proliferation inhibition.

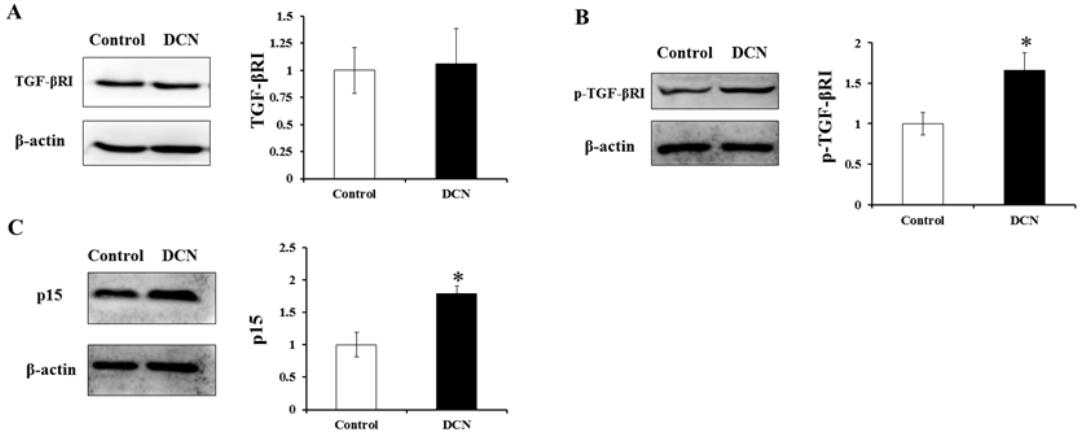

Figure. 1 DCN affected the expression of proteins associated with the TGF- β and p15 signaling pathway

Discussion

The results demonstrated that DCN transfection significantly inhibited the proliferation of HepG2 cells, in which the TGF-β signaling pathway was found to serve an important role. The DCN transfection elevated the phosphorylation level of TGF-βRI in HepG2 cells, without affecting the total TGF-βRI expression. The p15 protein is one of the key downstream factors of TGF-βRI, whose activation may influence cell cycle-associated proteins, further inhibiting the cell proliferation [8]. The current results showed that DCN transfection was able to significantly elevate the expression level of p15. Within cells, TGF-β binds with TGF-βRII to further phosphorylate TGF-βRI.The present study results showed that DCN transfection increased the expression level of TGF-βRII. In addition, when TGF-βRII was silenced with siRNA, the phosphorylated TGF-βRI in DCN-transfected HepG2 cells was significantly decreased, and the cell proliferation was also significantly inhibited.

In conclusion, the results of the present study showed that DCN transfection significantly elevated the expression of TGF-βRII, increased the phosphorylation of TGF-βRI, enhanced the expression of p15, and finally inhibited the proliferation of HepG2 cells. Upon silencing TGF-βRII with siRNA, the effects of DCN on the TGF-β signaling pathway and the HepG2 cell proliferation were abolished. The current findings may contribute to the understanding of the role of DCN in the pathogenesis of hepatic carcinoma and the disease treatment.

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