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• *BRIEF REPORTS* •

Effect of *c-fos* antisense probe on prostaglandin E_2-induced upregulation of vascular endothelial growth factor mRNA in human liver cancer cells

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Abstract

AIM: To examine the effect of prostaglandin E_2 (PGE_2) on the expression of vascular endothelial growth factor (VEGF) mRNA in the human hepatocellular carcinoma (HCC) HepG2 cells and the possible involvement of c-fos protein in this process.

METHODS: Human HCC HepG2 cells were divided into three groups treated respectively with PGE_2, a combination of PGE_2 and *c-fos* antisense oligodeoxynucleotide (ASO), and PGE_2 plus *c-fos* sense oligodeoxynucleotide (SO). The expression of VEGF mRNA in HepG2 cells after different treatments was detected by reverse transcriptase-polymerase chain reaction (RT-PCR). The relative expression level of VEGF mRNA in HepG2 cells in each group was measured.

RESULTS: Administration of PGE_2 resulted in an increased expression of *c-fos* and VEGF mRNA in HepG2 cells. The relative expression level of *c-fos* mRNA reached the peak at 3 h (68.4±4.7%) after PGE_2 treatment, which was significantly higher than that at 0 h (20.6±1.7%, $P<0.01$). Whereas, the highest expression level of VEGF mRNA was observed at 6 h (100.5±6.1%) after PGE_2 treatment, which was significantly higher than that at 0 h (33.2±2.4%, $P<0.01$). *C-fos* ASO significantly reduced PGE_2-induced VEGF mRNA expression in HepG2 cells.

CONCLUSION: PGE_2 increases the expression and secretion of VEGF in HCC cells by activating the transcription factor *c-fos*, promotes the angiogenesis of HCC and plays an important role in the pathogenesis of liver cancer.

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Key words: Hepatocellular carcinoma; Prostaglandin E_2; *c-fos*; Vascular endothelial growth factor; Angiogenesis

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INTRODUCTION

PGE_2 is produced in various kinds of cancer cells and seems to be particularly important for carcinogenesis[1-3]. PGE2 activates multiple G-protein-linked receptor subtypes (EP1-EP4) in an autocrine or paracrine fashion, which may lead to tumor growth promotion via growth factors and oncogenes[4-6]. However, the mechanism of PGE_2 in promoting tumor growth still remains unclear. VEGF is a regulator of pathological angiogenesis and plays an important role in tumor growth. Studies have revealed that VEGF can be produced by liver cancer cells in a paracrine manner, thus promoting the angiogenesis of liver cancer[7,8]. Studies also indicate that many tumor growth factors stimulate the production of VEGF in tumor cells[3,9]. This study was undertaken to estimate if PGE_2 could affect the expression of VEGF in HCC HepG2 cells and the possible involvement of the oncogene *c-fos* in this process.

MATERIALS AND METHODS

Cell culture and PGE_2 administration

HepG2 cells were cultured in RPMI-1640 medium (Gibco) containing 10 mL/L fetal bovine serum, 100 kU/L penicillin and 0.1 g/L streptomycin at 37 ℃ in 50 mL/L CO_2/950 mL/L air for 4-6 d and then put into fresh 35 mm dishes. Twenty-four hours later, PGE_2 (Sigma) was added into each dish in a final concentration of 1 μmol/L. The dose of PGE_2 in the present study was chosen based on the previous reports and our preliminary experiments. The cells were then cultured for 0, 1, 3, 6, 12, and 24 h, respectively (n = 4/each time point) and collected for RNA extraction.

C-fos ASO administration

C-fos ASO (5'-GAACATCATCGTGGC-3') was synthesized according to reported human *c-fos* mRNA sequence (GenBank Accession No. M16287). C-*fos* SO (5'-GCCA-CGATGATGTTC-3') was also synthesized as a control. Both ASO and SO were modified phosphorothioate

oligodeoxynucleotide.

HepG2 cells were cultured as mentioned above and divided into: (1) control group in which 10 μL physical saline was added, (2) PGE_2-treated groups in which 1 μmol/L of PGE_2 was added, (3) SO-treated group in which 10 μL (50 μg) *c-fos* SO was added followed by addition of 1 μmol/L of PGE_2 after 30 min, (4) ASO-treated group in which 10 μL (50 mg) *c-fos* ASO was added followed by addition of 1 μmol/L of PGE_2 after 30 min. The cells were cultured for 6 h and then collected for RNA extraction.

Primer design and synthesis

Specific primers for human *c-fos* and VEGF were synthesized according to their reported mRNA sequences. The primer pair of *c-fos* were: sense: 5'- TGC TGA AGG AGA AGG AAA AA -3'; antisense: 5'- TGC ATA GAA GGA CCC AGA TA -3' (GenBank Accession No. M16287). The primer pair of VEGF were: sense: 5'-ACC CAT GGC AGA AGG AGG AG -3' antisense 5'-ACG CGA GTC TGT GTT TTT GC-3' (GenBank Accession No. M32977). The primers (sense: 5'- GGC ATC CAC GAA ACT ACC TT-3' antisense 5'-CGT CAT ACT CCT GCT TGC TG -3') for human β-actin (GenBank Accession No. M10277) were also synthesized as internal control in the PCR reaction. The length of PCR product for *c-fos*, VEGF and β-action was 344 bp, 433 bp and 274 bp, respectively.

RNA extraction

Total cellular RNA was extracted from $HepG_2$ cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The purity and integrity of the RNA samples were assessed by $A_{260/280}$ spectrophotometric measurement.

RT-PCR

After measurement of the concentration, cDNA was reversely transcribed in a 50 μL mixture containing 2 μg total RNA, 10 μL 5× RT buffer, 5 μL 10 mmol/L dNTPs, 0.5 μL RNase inhibitor (4×10^5 U/L, Invitrogen) 0.5 μL oligo $(dT)_{12\text{-}18}$ (500 g/L, Invitrogen) 1 μL SuperScript II reverse transcriptase (2×10^4 U/L, Invitrogen), 0.5 μL 0.1 mol/L DTT at 42 ℃ for 60 min followed by enzyme denaturation at 70 ℃ for 10 min. Thirty cycles of PCR were carried out in 25 μL reaction mixture containing 0.1 μg synthesized cDNA, 2.5 μL 10× PCR buffer, 2.5 μL dNTPs (2 mmol/L), 2.5 μL $MgCl_2$ (2.5 mmol/L), 1 μL of each primer (20 μmol/L), 2.5 u of Taq DNA polymerase (Takara) using a PTC-100 programmed thermal controller (MJ Research), each consisting of denaturation at 94 ℃ for 1 min, annealing at 56 ℃ for 30 s, extension at 72 ℃ for 1 min. Then, 10 μL of each PCR product was separated by electrophoresis on a 30 g/L agarose gel and visualized by ethidium bromide staining.

Statistical analysis

For each template, PCR amplification was performed 2-3 times. The electrophoresis results were observed through a gel imaging system (UVP) and the density of each positive band was analyzed by Labworks software. The relative expression level of *c-fos* and VEGF mRNA was expressed as a ratio of densitometric measurements (*c-fos*/β-actin or VEGF/β-actin). The data were expressed as mean±SE, and analyzed by analysis of variance and Dunnets test using SPSS10.1 software.

RESULTS

Effect of PGE_2 on expression of c-fos and VEGF mRNA in HepG2 cells

Addition of PGE_2 to the HepG2 cells resulted in a time-dependent increase in the expression of *c-fos* and VEGF mRNA (Figure 1A). Compared to the expression level at 0 h (20.6±1.7%), the expression of *c-fos* mRNA induced by PGE_2 treatment reached the highest level at 1 h (62.3±4.3%, $P<0.01$) and 3 h (68.4±4.7%, $P<0.01$), and slightly higher level at 6 h (55.3±3.8%, $P<0.05$; Figure 1B). The expression level of VEGF mRNA significantly increased at 3 h after PGE_2 administration (87.6±6.4%, $P<0.01$) when compared to the expression level at 0 h (33.2±2.4%). Its expression level reached a maximum at 6 h (100.5±6.1%, $P<0.01$). At 24 h, the expression level returned to its level at 0 h (35.2±2.8%, $P>0.05$; Figure 1B). The expression level of β-actin mRNA remained unchanged at each time-point, indicating the equal amount of the template used in PCR.

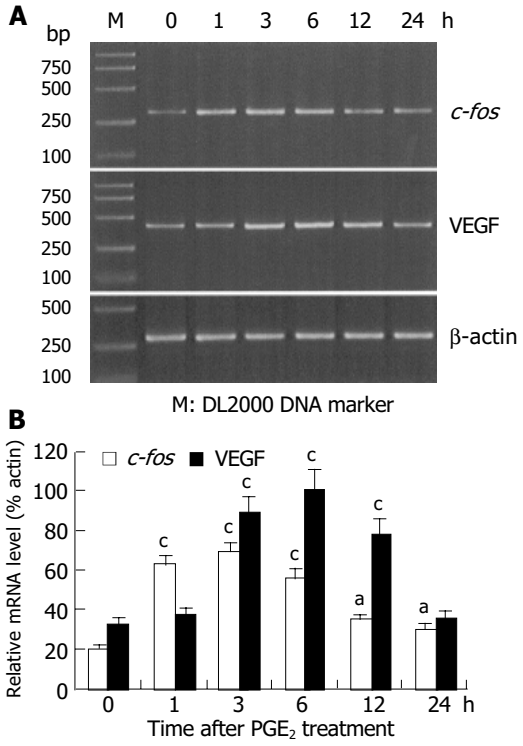

Figure 1 RT-PCR results (**A**) and histographs (**B**) showing effect of PGE_2 on expression of *c-fos* and VEGF mRNA in HepG2 cells. $^aP<0.05$, $^cP<0.05$ *vs* 0 h.

Effect of c-fos ASO on PGE_2-induced upregulation of VEGF mRNA in HepG2 cells

Since the maximal expression level of VEGF mRNA was at 6 h after PGE_2 treatment, this time-point was selected to observe the effect of *c-fos* ASO. The results showed that the expression level of VEGF mRNA significantly decreased in *c-fos* ASO-treated group (39.6±3.2%) when compared to that in PGE_2-treated group (98.6±6.4%, $P<0.01$, Figure 2A

and B). In contrast, no such change in *c-fos* SO-treated group was observed (95.2±6.3%, $P>0.05$).

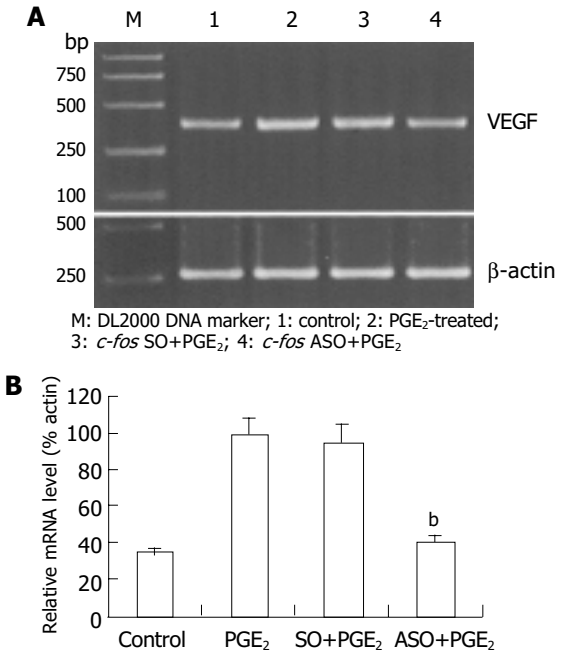

Figure 2 RT-PCR results (**A**) and histographs (**B**) showing effect of *c-fos* ASO on PGE_2-induced VEGF mRNA expression in HepG2 cells. $^bP<0.01$ *vs* PGE_2 group.

DISCUSSION

At present, the exact pathological function and mechanism of PGE_2 in tumors are not fully known. Previous studies indicate that PGE_2 can be produced by tumor cells and plays an important role in tumor immune inhibition[10-12]. Some studies revealed that the PGE_2 level in patients with cancer is higher than that in normal people, and that tumor tissues also contain higher concentration of PGE_2 than normal tissues[13]. Animal experiments indicate that PGE_2 produced by tumor cells, can promote the growth and development of tumors through its immune inhibitory function[10]. Further studies have proved that PGE_2 promotes the growth of liver cancer through its receptor EP3[14]. In the present study, we observed that PGE_2 could stimulate the expression of VEGF mRNA in HepG2 cells in a time-dependent manner, suggesting that PGE_2 may promote the angiogenesis of HCC by increasing the secretion of VEGF from liver cancer cells. This might be one of the mechanisms of PGE_2 in facilitating the growth of liver cancer.

It is well known that the oncogene *c-fos* can function as a third intracellular messenger. Its product Fos protein can form a homo-dimer itself or hetero-dimer with c-Jun protein and then binds to the AP-1 site in the target gene, thus promoting the transcription of target gene. It has been reported that the promoter region for the VEGF gene contains several AP-1 binding motifs[15] and the expression of VEGF gene is controlled by transcription factors AP-1 and AP-2[16-18]. In the present study, we observed that PGE_2 increased the expression of *c-fos* mRNA, the maximal level was at 1 and 3 h after PGE_2 administration, earlier than the PGE_2-induced highest expression of VEGF mRNA. Furthermore, *c-fos* ASO significantly reversed PGE_2-induced VEGF mRNA expression. These results indicate that Fos protein is involved in the PGE_2-induced VEGF expression in HepG2 cells.

The intracellular signaling pathway coupled to PGE_2 is complicated. As a third intracellular messenger, *c-fos* is just located in the downstream of the signaling pathway. Many other molecules should also be involved in the modulation of VEGF expression by PGE_2. In addition, several PGE_2 receptors are present in HCC[6,19]. Which receptors mediate the role of PGE_2 in tumor growth needs to be investigated.

In conclusion, PGE_2 stimulates VEGF induction in HepG2 cells by activating the transcription factor Fos protein.

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