• *LIVER CANCER* •

Effects of adenovirus-mediated human cyclooxygenase-2 antisense RNA on the growth of hepatocellular carcinoma

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Abstract

AIM: To investigate the relation between the expression of cyclooxygenase-2 (COX-2) and liver cancer, to construct the recombinant adenovirus encoding human COX-2 antisense RNA, and to explore its effects on liver cancer cell proliferation.

METHODS: We studied the expression of COX-2 in 34 cases of hepatocellular carcinoma (HCC) and SMMC7402 and SMMC7721 by immunohistochemical technique. Recombinant adenovirus Ad-AShcox-2 was constructed and transfected into human HCC cell lines SMMC7402 and SMMC7721, and its effects on COX-2 expression, cell apoptosis and cell cycle were analyzed by flow cytometry. Cell proliferation was determined by colony-forming efficiency.

RESULTS: We observed COX-2 expression in 82.4% of HCC and SMMC7402 cells, but no COX-2 expression in SMMC7721 cells. In addition, recombinant adenovirus encoding antisense COX-2 fragment Ad-AShcox-2 was obtained with the titer of 1.06×10^{12} PFU/mL. Ad-AShcox-2 could reduce the expression of COX-2 and enhance the percentage of cells in G_1/G_0 phase in SMMC7402 cell line. The difference of apoptotic index between the Ad-AShcox-2 group and control group was statistically significant ($t_{control\ group} = 32.62$ and $t_{Ad-LacZ} = 10.93$, $P<0.001$) in SMMC7402 but not in SMMC7721. Similarly, colony-forming rates of SMMC7402 and SMMC7721 cell lines, after the transfer of Ad-AShcox-2, were $(2.7\pm0.94)\%$ and $(33.6\pm4.24)\%$, respectively.

CONCLUSION: Reduction in the expression of COX-2 can inhibit COX-2 expressing HCC cells.

INTRODUCTION

Liver cancer is a kind of malignant tumor with a high mortality at present. Its morbidity is rather high in our country. Although a great number of researches on liver cancer as well as many achievements have been made, the exact molecular pathogenesis of liver cancer is not clear. Cyclooxygenase-2 (COX-2) has a high expression in quite a few tumors such as colorectal cancer[1,2], esophageal cancer[3], and other cancers[4]. It is likely to be related to the genesis and development of tumor, but the causing-carcinoma effect can be resisted by COX-2 selective inhibitors[5-7]. Our prophase researches showed that COX-2 antisense RNA obviously inhibits the growth of carcinoma of esophagus with a high expression of COX-2[8,9]. COX-2 is likely to become a new target of treating tumor. The present study aimed to investigate the relationship between the expression of COX-2 and liver cancer, to the construction of the recombinant adenovirus encoding human COX-2 anti-sense RNA, and to treat hepatocellular carcinoma (HCC) with or without the expression of COX-2 and to explore its mechanism.

MATERIALS AND METHODS

Intraremovelase

*Bam*HI, *Xba*I, and T_4 DNA ligase were purchased from Huamei Biological Engineering Company. RPMI 1640 and Lipofectamine were provided by GIBCO/BRL. CsCl was from Sigma Company. COX-2 cDNA and primer of adenovirus PCR were designed and synthesized by Saibaisheng Biology Company.

Plasmid, adenovirus, and cell line

Ad-LacZ was a gift from Dr. Jia-Ning Wang in our Molecular Biological Laboratory. COX-2 plasmid PCR™II vector was supplied by Dr. You-Fei Guan in Vanderbilt University, USA. pHCMVSP1A and pJM17 were donated by academician Wu Zuze in the Second Institute of Academy of Military Medical Sciences. DH_{5a} was donated by Dr. Peng Xu in Cardiology Internal Medicine Department of No. 1 Hospital Peking University. 293 cells and HCC cell lines SMMC7721 and SMMC7402 were purchased from Shanghai Cell Bank

of Chinese Academy of Medical Sciences.

Equipments

High-speed refrigerated centrifuge (Jouan, Italy), carbon dioxide incubator (Sany D, Japan), ultrahigh refrigerated centrifuge (Hitachi, Japan), inversion difference microscope (Olympus IX-To, Japan), PCR amplifier (Amplitron II, USA), flow cytometer (EPICS XL, Beckman Coulter Company, USA), ultraviolet spectrophotometer (752, Shanghai), air bath, and water bath cradle were used in our study.

Expression of COX-2 in HCC tissue

Immunohistochemical transfection was conducted by streptomy-glair-in-biotin-horseradish peroxidase (SP) compound method. The tissue was fixed with paraffin and cut into 5-μm-thick sections. HCC tissue slides were fixed in 4% PFA in PBS for 10 min, washed twice with PBS, and incubated in 3% H_2O_2 to eliminate the endogenous peroxidase activities. The tissues were then incubated either at room temperature for 1 h or at 4 ℃ overnight with 1% goat serum in PBS to block the nonspecific binding of antibodies. The slides were further incubated sequentially with rabbit anti-man COX-2 polyclonal antibody. PBS was used as a control. Color development was performed by incubation with 3,3-diaminobenzidine tetrahydrochloride in 0.03% H_2O_2 and 50 mmol/L Tris-HCl, pH 7.4. The percentage of positive tumor cells was determined semiquantitatively by assessing the whole tumor section, and each sample was assigned to one of the following categories: 0 (0-4%), 1 (5-24%), 2 (25-49%), 3 (50-74%), 4 (75-100%). Transfection strength was indicated with 0 (negative), 1 (weak positive), and 2 (positive). The expression levels were indicated with positive rate of tumor cells of one tumor tissue showing the different transfection strength. Its expression level was the transfection strength fraction by positive rate fraction. For example, if 50 tumor cells of one tumor tissue showed positive staining, we could mark 3 by 2 equaling 6; if 25 showed weak positive staining, we could mark 2 by 1 equaling 2; if 25% showed negative staining, we could mark 2 by 0 equaling 0. Thus the total was 6 plus 2 plus 0 equaling 8. The specific result statistics were carried out as previously described[10].

Construction, identification, amplification, purification, and titer assay

First, we obtained a COX-2 cDNA fragment using *Xba*I and *Bam*HI to bi-mold-cut the plasmid PCR™IICOX-2, and cloned the COX-2 cDNA fragment in the reverse direction onto two spots of *Xba*I and *Bam*HI of the pHCMUSP1A, the connection product was pAd-Ashcox-2. Then the latter was plasmid-amplified by transformed bacillus coli DH_{5a} and identified as positive plasmid by mold-cutting. The shuttle plasmid pAd-Ashcox-2 mediated Lipofectamine and pJM17 were co-transferred into 293 cells. When 293 cells had pathologic lesions and exfoliative cells made up 20%, we collected the upper clear liquid. Then we extracted viral DNA from the upper clear liquid and identified it by PCR. COX-2 primer's upstream sequences are 5'-TTG GCT TCA AGA CTG AGA TA-3' and 5'-AGC CCA AAT TAT TGG TTC-3'. Adenovirus primer's upstream sequence is 5'-TCG TTT CTC AGC AGC TGT TG-3', downstream sequence

is 5'-CAT CTG AAC TCA AAG CGT GG-3'. PCR reaction system contained 10 μL viral DNA, 10 μL 20 μmol/L primer respectively (5 μL adenovirus), 5 μL 10× PCR buffer, 2 μL $MgCl_2$, 5 μL 2 mmol/L dNTP and 1 μL Taq enzyme. Water was added until the final volume of PCR reaction was 50 μL. Thirty cycles of PCR were carried out, each consisting of 30 s at 94 ℃, 30 s at 56 ℃, 1 min at 72 ℃, then a final extension at 72 ℃ for 10 min. Electrophoresis in 1.0% agarose gel was performed to amplify cDNA and adenovirus fragments as positive reconstructive cloning. Without adding mould, PCR amplification was used as a negative control. After positive reconstructive adenovirus was identified, we transferred 293 cells again. 293 cells were collected, refrigerated, melted, fragmented and ultracentrifuged twice with CsCl, viruses were collected and purified. At last the virus titer was assayed.

Efficiency of virus transfection

The recombinant adenovirus Ad-LacZ with genes encoding LacZ was used to infect liver cancer cell lines SMMC7721 and SMMC7402 according to multiplicity of infection-MOI 25, 50, 100, and 200. After 48 h, the cells were fixed and stained with X-gal. The number of cells stained blue was recorded under microscope. Then the percentage of cells with blue staining was calculated.

Transfection of recombinant adenovirus and expression of COX-2

Human liver cancer cell lines SMMC7721 and SMMC7402 were incubated in a 50 mL/L CO_2 at 37 ℃. When the density reached 40-50%, the medium was replaced by serum-free RPMI 1640 and incubated for 24 h. The experimental group infected the two cell lines with 100MOI, and was incubated for 2 h with serum. During the process, the culture fluid was shaken every 15 min. Two hours later, the medium was replaced by 10% FCS RPMI 1640. The control groups were Ad-LacZ group and non-virus group. Carcinoma cells were incubated for 24, 48, 72, 96 h respectively, fixed for 10 min in 4 ℃ cold acetone, and dried for 10 min at room temperature. Then the cells were stored at -85 ℃. The experiment was carried out with SP immunohistochemical method.

Flow cytometry

When the density of the two cell lines reached 40-50%, the medium was replaced by RPMI 1640 containing serum and incubated for 24 h. Forty-eight hours after the experimental group and control group finished virus transfection, the cells were collected (using trypsin digestion method) and centrifuged (1 000 r/min, 5 min). The upper clear fluid was discarded, PBS was added to adjust cell density to 10^6/mL. One hundred microliters of cell suspension was put into the tube, 200 μL DNA-PREP™-PR was added to it and mixed. Two microliters DNA-PREP™ stain reagent (PI stain) was added and mixed after 30 s. After 30 min, the cell cycle and apoptosis was detected with flow cytometry (FCM).

Colony-forming experiment

The two cell lines was digested into single-cell suspension

respectively, and inoculated into a six-well board. Each well was inoculated with 500 cells. Each group had three pang-punches. After being treated with culture fluid Ad-AShcox-2, they were incubated for 10 d and fixed with 1:3 acetic acid:methanol, stained with Rui stain fluid. Then the number of colonies with more than 50 cells was recorded.

Statistical analysis

Expression level of COX-2 in liver cancer tissue was expressed as mean±SD. t-Test was used to show the cell apoptosis rate and cell cycle distribution rate.

RESULTS

Expression of COX-2 in liver cancer tissue

COX-2 was positive in 28 of 34 cases of liver cancers and was negative in 6 of 34 cases of liver cancer. Positive rate was 82.4%. The positive staining of COX-2 was mainly in cytoplasm. High expression of COX-2 was correlated with clinical pathological features of liver cancer (Figure 1). COX-2 expression level was high, if the cancer cells were well differentiated. COX-2 expression was not related to AFP levels, source cells and transference in liver (Table 1).
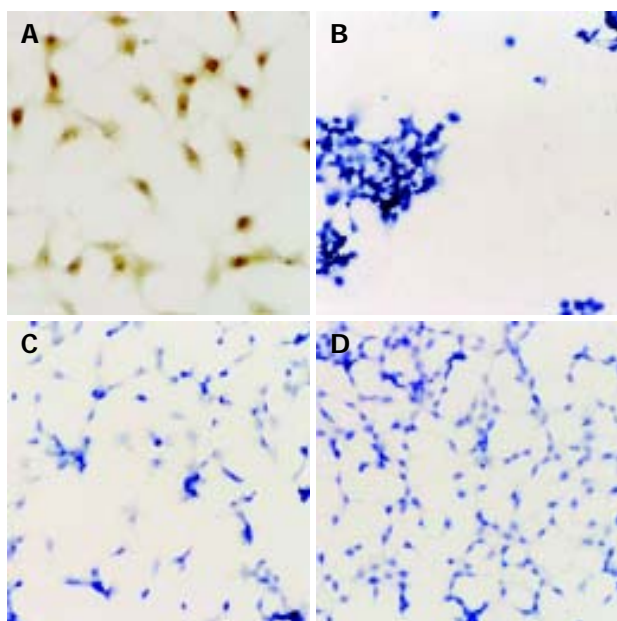

Figure 1 Expression of COX-2 in SMMC7402 and SMMC7721 before (**A** and **B**) and after (**C** and **D**) the transfer of Ad-AShcox-2.

Identification of recombinant adenovirus by PCR co-amplification method

DNA was extracted and precipitated, then PCR amplification was performed on this DNA model. The product of PCR amplification was evaluated with 0.8% agarose electrophoresis, and 277-bp COX-2 cDNA fragments and 860-bp virus gene frame fragments were obtained. After pAd-AShcox-2 and pJM17 were co-transferred to 293 cells, we produced anti-sense RNA recombinant adenovirus which could express COX-2.

Table 1 Relationship between high expression of COX-2 and clinical pathological features of liver cancer (mean±SD)

Clinical pathological features	n	Stain fraction	t	P
AFP (ng/mL)				
<20	4	6.48±1.46	0.74	>0.05
≥20	24	6.36±1.15		
Cell category				
Liver cell category	27	6.79±1.60		
Bile duct cell category	1	6.26±1.25		
Transference in liver				
Yes	3	6.43±1.42	1.08	<0.05
No	25	7.15±1.80		
Pathological differentiation				
High	22	8.25±0.86	5.23	<0.05
Middle and low	6	6.11±1.02		

Titer and transfection rate of Ad-AShcox-2

After Ad-AShcox-2 was amplified, extracted, purified, and assayed with ultraviolet spectrophotometer, the virus titer was 1.06×10^{12} PFU/mL. When the MOI was 50 or greater, the transfection rate of SMMC7721 and SMMC7402 reached 100%.

Expression of COX-2 in HCC cell lines

Immunohistochemistry showed that SMMC7402 had a high expression of COX-2. But after Ad-AShcox-2 was transferred, the expression of COX-2 decreased obviously. Before and after Ad-AShcox-2 was transferred, SMMC7721 did not express COX-2 (Figures 1A-D).

Apoptosis of liver cancer cells

Forty-eight hours after SMMC7402 cells were treated with Ad-AShcox-2, FCM showed that apoptotic cells reached apoptosis peak prior to G_0/G_1 peak. The apoptosis rates of Ad-AShcox-2, Ad-LacZ, and the control group were (33.3±3.02)%, (0.42±0.14)%, and (0.25±0.08)%, respectively. The apoptosis rate of the experimental group increased obviously in comparison to the control group. However, after SMMC7721 was treated with Ad-AShcox-2, we could not detect the apoptosis peak with FCM. The apoptosis rates were (0.61±0.25)%, (0.48±0.09)%, and (0.56±0.11)%, respectively. The apoptosis rates of these two liver cancer cell lines, induced by Ad-AShcox-2, differed sharply when compared to each other (t = 32.05, P<0.001).

Effect of Ad-AShcox-2 on cell cycle

Cell cycle from FCM is listed in Table 2. After SMMC7402 cells were treated with Ad-LacZ and non-virus culture fluid, the cell proliferation rate decreased in G_0/G_1 phase, but it was high in S phase indicating that the proliferation of cells was active. After they were treated with Ad-AShcox-2, the number of cells in G_0/G_1 phase increased obviously, while in S phase, the number of cells decreased, suggesting that there was a significant difference compared to control group (P<0.05). The cells were blocked in G_1 phase. But after SMMC7721 was treated with Ad-AShcox-2, the cell proliferation rates in G_1/G_0 phase were low both in experimental group and in control group (Table 3); yet they were high in

Table 2 Cell apoptosis rate and cell cycle after SMMC7402 was treated in experimental group and control group ($n = 9$, mean±SD)

Groups	Apoptosis rate	Cell cycle (%)		
	(%)	G_0/G_1	S	G_2/M
Control group	0.25	40.39±3.86	49.61±4.27	13.10±1.03
Ad-LacZ	0.42	40.17±4.03	50.18±3.66	12.57±2.85
Ad-ASHcox-2	33.3	66.52±5.48	22.15±3.26	10.33±1.12

Table 3 Cell apoptosis rate and cell cycle after SMMC7721 was treated in experimental group and control group (mean±SD)

Groups	Apoptosis rate	Cell cycle (%)		
	(%)	G_0/G_1	S	G_2/M
Control group	0.56	40.39±3.86	49.61±4.27	13.10±1.03
Ad-LacZ	0.48	42.17±2.63	50.26±2.85	12.27±2.72
Ad-ASHcox-2	0.91	39.92±4.11	49.28±4.13	12.64±1.25

S phase. There was no difference ($P>0.05$) when compared to each other.

Effect of Ad-AShcox-2 on colony-forming ability of liver cancer cells

After Ad-AShcox-2 infected SMMC7721 and SMMC7402, the colony-forming rates were (2.7±0.94)% and (33.6±4.24)%. There were significant differences between the two groups ($P<0.05$, $n = 6$).

DISCUSSION

Cyclooxygenase is a kind of a rate-limiting enzyme, which catalyzes and synthesizes prostaglandin. It has two isomers -COX-1 and COX-2. COX-1 is constitutively expressed in most mammalian tissues and is thought to carry out "housekeeping" functions such as cytoprotection of the gastric mucosa, regulation of renal blood flow, and control of platelet aggregation. In contrast, COX-2 mRNA and protein are normally undetectable in most tissues, but can be rapidly induced by proinflammatory or mitogenic stimuli including cytokines, endotoxins, interleukins, and phorbolester. It has high expression levels in many cancer tissues. Researches showed that COX-2 can promote the formation and opening of tumor vessels[11,12], inhibit tumor immunity[13,14], stimulate transference of tumors and inhibit apoptosis[15-18].

Studies indicate that COX-2 selective inhibitor can inhibit proliferation of colon cancer cells[19,20], and induce their apoptosis[21,22]. In our prophase researches, by using the carrier of COX-2 antisense RNA adenovirus-transferred esophageal carcinoma cells, we successfully inhibited the expression of COX-2 in esophageal carcinoma cells[8,9]. The growth and proliferation of esophageal carcinoma cells were obviously inhibited, showing that reducing the expression of COX-2 or inhibiting the activity of COX-2 is likely to become a new way to treat tumors.

COX-2 is strongly positive in 28 of 34 cases of HCC. The positive rate reached 82.4%, suggesting that the high expression of COX-2 is related to the carcinogenesis and development of HCC. We also found that most HCCs with positive expression of COX-2 were well differentiated, but poorly differentiated HCC had a low expression of COX-2, indicating that COX-2 can promote the proliferation and differentiation of liver cancer cells, which is in line with the report of Koga *et al.*[23]. We can infer that HCC is related to COX-2 inhibiting cell apoptosis and increasing cytoplasmic matrix protein.

We successfully constructed the recombinant adenovirus expressing COX-2 anti-sense RNA. When the two liver cancer cells were treated with it, the expression of COX-2 decreased obviously. Meanwhile, the growth of cells was inhibited and the colony-forming rates of cells decreased apparently as well. But the colony-forming rates of SMMC7721 did not decrease, indicating that inhibiting the expression of COX-2 could inhibit the growth of liver cancer cells with positive expression of COX-2. However, cells with negative expression of COX-2 could not be inhibited. We further confirmed that the feasibility of COX-2 independent way in which NSAIDs could inhibit the colon cancer cells without the expression of COX-2[24].

After SMMC7402 was transferred in Ad-AShcox-2, the rate of cell apoptosis increased notably. There was a significant difference when control group, Ad-LacZ treatment group, and blank group were compared to each other ($P<0.001$), but the apoptosis rate of SMMC7721 did not increase, showing that by reducing the expression of COX-2, we can increase the apoptosis of cancer cells. This is in line with the result of a previous study[17].

Cell cycle test showed that Ad-AShcox-2 could reduce the expression of COX-2 in SMMC7402, the cell number increased evidently in G_0/G_1 phase. It could prevent cells passing from G_1 to S phase. It is likely to be linked with the expression of raised P_{27} and the inhibition of cyclin/CDK activity[25,26].

In conclusion, the adenovirus carrier with the expression of COX-2 anti-sense RNA is able to inhibit SMMC7402 and induce apoptosis.

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