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ZBP-89 reduces the cell death threshold in hepatocellular carcinoma cells by increasing caspase-6 and S phase cell cycle arrest

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ABSTRACT

ZBP-89 inhibits the some tumor cells but its role in HCC is unknown. We investigated effect of ZBP-89 on cell death of 5 HCC cell lines with different status of p53. We found that ZBP-89 significantly induced cell death of all HCC cells particularly those with wild-type p53. The inhibition was well correlated with the induction of caspase-6 activity. The inhibition of caspase-6 abolished the effect of ZBP-89. ZBP-89 reduced the cells in G2-M but increased them in S phase. With the changes in caspase-6 and cell cycle, ZBP-89 greatly enhanced the killing effectiveness of 5-fluorouracil or staurosporine in HCC cells.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers affecting millions of individuals worldwide, particularly in Africa, Eastern Asia, China and Hong Kong. Surgical resection of tumor is still the most effective treatment for HCC. However, about 70% of patients will die from recurrent tumor within 5 years after liver tumor resection. Furthermore, most patients with HCC are unresectable at the time of diagnosis because of widespread intra-hepatic spread, extra-hepatic metastasis or limited hepatic reserves resulting from coexisting advanced cirrhosis. The median survival for patients with unresectable tumor is less than 3 months. For these patients, chemotherapy is the only remaining choice of treatment. Many chemotherapy regimes have been tried in the effort to control advanced HCC but their effectiveness is in general unsatisfactory and the prognosis in these patients is extremely poor. Therefore, searching for novel treatments for HCC has been challenging for scientists and clinicians.

ZBP-89 (BFCOL1, BERF1, ZNF 148) is a Kruppel-type, zinc finger transcription factor that binds to GC-rich elements and activates or represses known target genes. For example, ZBP-89 activates the expression of genes such as cyclin-dependent inhibitor $p21^{Waf1}$, growth hormone receptor 1A, intestinal alkaline phosphatase gene, lymphocyte-specific protein tyrosine kinase, and matrix metalloproteinase-3 [1–6]. On the other hand, ZBP-89 may act as a repressor for gastrin, ornithine decarboxylase (ODC), beta-enolase, chemokine epithelial neutrophil-activating peptide-78, beta2 integrin CD11b and vimentin genes [7–12]. By regulating some of the above genes such as gastrin, ODC and $p21^{Waf1}$, ZBP-89 participates in the control of cell proliferation and growth. ZBP-89 may influence the cell growth through a p53-dependent or independent mecha-

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Abbreviations: MTT, 3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; 5-FU, 5-fluorouracil; pNA, chromophore p-nitroanilide; HCC, hepatocellular carcinoma; ODC, ornithine decarboxylase; SD, standard deviation; STS, staurosporine.

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nism [1,13–15]. We and others also demonstrated that ZBP-89 stabilizes wild-type p53 through direct protein contact and retention in the nucleus exclusive of Mdm2 and ARF, which is in agreement with the fact that ZBP-89 stabilizes p53 via its DNA binding and C-terminal domains [13,15].

There are reports showing that ZBP-89 inhibits the proliferation of gastric adenocarcinoma and colorectal cancer cells by inducing apoptosis [13,14,16,17]. The anti-tumor effect of ZBP-89 has been further confirmed in a ZBP-89 transgenic mouse experiment showing that the incidence of intestinal adenoma is reduced by 50% in the mice with ZBP-89 overexpression and that the overexpression of ZBP-89 is correlated with increased DNA fragmentation [18]. Our previous study has demonstrated that ZBP-89 can interact with p53 in HCC [15], suggesting that ZBP-89 may enhance the efficacy of anti-tumor agents by affecting cell death or cell proliferation in HCC cells. However, to our best knowledge, the effect of ZBP-89 on cell death and apoptosis in HCC cells has not been studied. In the present study, we investigated effect of anti-tumor agents, staurosporine (STS) and 5-fluorouracil (5-FU) on HCC cells with or without ZBP-89. Since p53 mutation is a frequent event in HCC and the potential relationship between p53 and ZBP-89, we employed HCC cells with different statuses of p53 to assess the inhibitory effect of ZBP-89.

2. Materials and methods

2.1. Cell culture

All the cell lines used in the present study were obtained from American Type Culture Collection (Rockville, MD). Five human liver cancer cell lines were used including Hep3B, HepG2, SK-Hep-1, PLC/PRF/5 and Huh-7. The cells were cultured in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) at 37 °C. All media were supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml of penicillin and 0.1 mg/ml of streptomycin. p53 gene is deleted in Hep 3B, and mutated in PLC/PRF/5 and Huh-7 at codon 249 (G:C → T:A) and 220 (A:T → G:C), respectively [19,20]. p53 gene is rearranged in SK-Hep-1 [21]. HepG2 contains wild-type (wt) p53 [19]. We have confirmed the above mutations before further experiments.

2.2. Adenoviral infection

The rat ZBP-89 cDNA was cloned into replication-deficient recombinant adenoviral vector to form Ad5-ZBP-89 [1] and the infection procedure was carried out as previous publications [13,17]. Briefly, the cells were grown until 60% confluent and then infected with Ad5-ZBP-89 at an MOI of 10 for 8 h. The cells infected with the empty adenoviral vector (Ad5-vector) were set up as the control for each experiment. After the infection, the cells were treated with STS or 5-FU for different periods of time as indicated in the figures or figure legends before proceeding to other experiments detailed elsewhere in this report.

2.3. Determination of cell death

Cell death, reflected by the viability of cells, was measured by MTT (3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay [22,23]. Briefly, 10^4 cells were seeded into a 96-well plate in 100 μL of medium and incubated for 24 h. The cells were then treated with 20 μg/ml 5-FU or 0.125 μM STS (Sigma, St Louis, MO) for different periods of time. At the end of treatment, the cells were incubated with MTT solution (Sigma, St Louis, MO) for another 3 h at 37 °C. The medium was then replaced with 200 μL dimethyl sulfoxide. Reduced MTT was measured spectrophotometrically at 570 nm with a 630 nm reference. The result was expressed as the percentage of viable cells in relation to non-treated controls.

2.4. Cell cycle analysis

The cell phase distribution was assayed by determination of the DNA content [24]. The cells were fixed overnight in 5 ml of 70% ethanol at −20 °C. They were then washed with PBS and incubated with propidium iodide (20 μg/ml) and RNase A (200 μg/ml) in the dark for 30 min. The stained cell samples were subjected to flow cytometry. A DNA content frequency histogram using deconvolution software was employed to analyze the data.

2.5. Measurement of caspase-6 activity

Caspase-6 activity in cell lysates was measured with a colorimetric protease assay kit (Chemicon, Temecula, CA). The assay is based on spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labeled substrate VEID-pNA. The pNA light emission can be quantified using a microtiter plate reader at 405 nm. The activity of caspase-6 was positively correlated with the reading at 405 nm and expressed as absorbance (at 405 nm) of pNA.

2.6. Statistical analysis

Data were reported as the mean ± standard deviation (SD). Differences between groups were analyzed using one-way ANOVA or/and Student's *t*-test. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. ZBP-89 sensitizes HCC cells to cell death induced by 5-FU or STS

The cell death of Hep3B, HepG2, SK-Hep-1, PLC/PRF/5 and Huh-7 cells, as reflected by the number of living cells, was significantly increased in the cells infected with Ad5-ZBP-89, compared with the control cells (infected with Ad5-vector) (Fig. 1). The death of these cells infected with Ad5-ZBP-89 was further increased in all cells except Hep3B when they were co-treated with either 5-FU or STS. Therefore, compared with 5-FU or STS alone, the combination treatment (ZBP-89 plus 5-FU or ZBP-89 plus STS) was much more effective in killing all HCC cells tested (all $P < 0.01$ except 5-FU for SK-Hep-1). However, compared with ZBP-89 alone, the combination treatment (ZBP-89 plus 5-FU or ZBP-89 plus STS) was only more significant in HepG2, SK-Hep-1 and Huh-7 (all $P < 0.01$) but not in Hep3B and PLC/PRF/5. The difference was probably due the fact that ZBP-89 alone already induced cell death of Hep3B and PLC/PRF/5 to the similar level caused by the combination treatment. Col-