Published in final edited form as:

Cancer Lett. 2009 September 28; 283(1): 52–58. doi:10.1016/j.canlet.2009.03.024.

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.

Keywords

caspase-6; cell cycle; hepatocellular carcinoma; p53; proliferation; ZBP-89

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

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Conflict of interest statement: All authors do not have any financial and personal relationships with other people or organizations that could inappropriately influence this work.

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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 mechanism [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.

Materials and Methods

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.

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 hours. 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.

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 μ L of medium and incubated for 24 hours. The cells were then treated with 20 μ g/ml 5-FU or 0.125 μ M 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 hours at 37°C. The medium was then replaced with 200 μ L 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.

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 μ g/ml) and RNase A (200 μ g/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.

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.

Statistical analysis

Data were reported as the mean $\pm$ 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.

Results

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) (Figure 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. Collectively, there was not a definite pattern to explain the relationship between the different p53 statuses and the cell death induced by ZBP-89 in these HCC cells since

ZBP-89 alone significantly induced cell death in all cells tested. However, it appears that HepG2 with wild-type p53 is the most sensitive to ZBP-89 among all cells tested.

The induction of the cell death was correlated with an increase in caspase-6 activity

The death of HCC cells infected with Ad5-ZBP-89 was reduced by either 5-FU (Figure 2) or STS (data not shown) in a similar time-dependent manner between 24 and 72 hours (all $p < 0.01$). The highest level of the cell death was recorded at 72 hours after the treatment in all HCC cells except Hep3B in which 48-hour and 72-hour treatments showed a similar elevation of the cell death (Figure 2). The increase in the cell death was significant and correlated with the increase in caspase-6 activity in the HCC cells (Figure 3). The highest activity of caspase-6 was found at 72 hours in all HCC cells. However, similar to the cell death of Hep3B, the level of caspase-6 activity between 48-hour and 72-hour points did not differ significantly. In order to confirm the role of caspase-6, we treated HepG2 cells with z-VEID-FMK, a specific caspase-6 inhibitor. It was found that z-VEID-FMK significantly prevented the promoting effect of ZBP-89 on cell death but has little effect on 5-FU-mediated inhibition (Figure 4). Similar results were also obtained in the rest HCC cells (data not shown). This finding confirms that caspase-6 significantly contributes to the cell death induced by ZBP-89.

ZBP-89 abolishes the G2M phase but induces the arrest at the S phase

HCC cells infected with Ad5-ZBP-89 were analyzed for the cell cycle. The percentage of the cells treated with ZBP-89 in the G2M phase was markedly reduced compared to the control (infected with Ad5-vector) (Figure 5). In contrast, the percentage of the cells treated with ZBP-89 in the S phase was significantly higher compared with control. The percentage of the cells in the G0/G1 phase was not different between the cells with and without Ad5-ZBP-89 infection.

Discussion

Our previous study on HCC demonstrated that ZBP-89 can co-localize with p53 in the nucleus and that ZBP-89 binds not only wild-type p53 but also some of mutant forms [15]. The action of ZBP-89 was thought to be stabilization of p53 [13,15]. Theoretically, the stabilization of p53 in the nucleus will render the cells more responsive to the anti-tumor agents [25,26]. Our present study shows that the overexpression of ZBP-89 significantly promotes cell death in five different types of HCC cells and greatly enhances the sensitivity of these cells to 5-FU or STS treatment. One of the main differences in these five types of HCC cells tested is the status of p53. The p53 gene was deleted in Hep 3B, rearranged in SK-Hep-1, and mutated in PLC/PRF/5 and Huh-7 at codon 249 (G:C-->T:A) and 220 (A:T-->G:C) respectively [19-21]. HepG2 contains wild-type p53 [19]. Although ZBP-89 can significantly induce cell death of all HCC cells tested, wild-type p53 containing HepG2 appears to be most sensitive to ZBP-89 treatment. The combination treatment (ZBP-89 plus 5-FU or ZBP-89 plus STS) is much more effective in inducing the death of all HCC cells tested than 5-FU alone or STS alone. However, it has been noted that the combination treatment fails to induce more death in Hep3B and PLC/PRF/5, compared with ZBP-89 treatment alone. The difference appears due the fact that ZBP-89 alone has already induced the significant death of these two types of cells to the similar level caused by the combination treatment. Nevertheless, ZBP-89 can markedly induce the cell death of HCC cells containing different p53 statuses though HepG2 with wild-type p53 is most sensitive to the treatment. The finding also suggests that the sensitization of HCC cells to the anti-tumor agents by ZBP-89 may not totally determined by the p53 state alone. Factors other than p53 should also be involved in the action of ZBP-89. It has been previously reported that human colon cancer cells (HT29) with p53 mutant at codon 273 are resistant to ZBP-89 [17]. The

HCC cells tested in this study do not contain a p53 mutation at codon 273. However, p53 deletion or mutants at other locations (codons 220 and 249) do appear to impact the degree of cell death induced by ZBP-89 in HCC cells to a certain degree. The experiment on colon cancer cells also suggests that p53 is not the sole factor responsible for the inhibitory effect of the ZBP-89, since p53^{-/-} HCT 116 cells still respond to the ZBP-89 treatment [17].

We further analyzed the cell cycle and found that ZBP-89 significantly decreased HCC cells in the G2-M phase but increased them in S phase. It therefore appears that the cell death promoting effect of ZBP-89 is a result of blocking the cells in S phase, preventing progression to the G2 phase. The ability of ZBP-89 to abolish HCC cells in the G2-M phase may contribute to its ability to function as a tumor growth inhibitor and is highly comparable to other potent anti-tumor agents. It is known that tumor cells are dependent on the G2 checkpoint [27]. Therefore, cell cycle G2 checkpoint abrogation by ZBP-89 may impact on cell death or proliferation. Similar to the effect of ZBP-89, some other well-known anti-tumor agents such as cisplatin, irifolven, okadaic acid and topotecan also arrest cells in S phase thereby inhibiting tumor proliferation [28-31]. Two anti-tumor agents 5-FU and STS used in the present experiment may also target cell cycle. 5-FU works by disrupting DNA synthesis since it is a nucleotide analog. It therefore arrests tumor cells at the G1/S phase [32]. STS is a broad-spectrum inhibitor of protein kinases and it induces mainly G2-M arrest in tumor cells [33]. The anti-tumor mechanism of ZBP-89 is not completely known. However, its negative effect on gastrin and ODC but positive effect on p21^{Waf1} and p53 probably contributes to its inhibitory effect on tumors [13-18]. The finding that ZBP-89 abolishes HCC cells in the G2-M phase and arrests them in S phase provides a piece of novel information as to how it suppresses the growth of tumor cells. The anti-tumor effectiveness often benefits from the combination of agents that function with complementary inhibition and act at different phases of the cell cycle [27,34]. The anti-tumor mechanism of ZBP-89 may not follow the pathways of 5-FU or STS. Therefore, the combination of ZBP-89 with chemotherapeutic agents 5-FU or STS may be one approach to achieve the most tumor killing.

Although ZBP-89 is able to enhance the sensitivity of some cancer cells including HCC cells to cell death induced by chemotherapeutic agents or apoptotic inducers, the mechanism responsible has not been well evaluated. Caspase-6 appears to play an important role in cell death or apoptosis in HCC cells. The activity of caspase-6 has been found to be correlated with apoptosis of HCC cells treated with various anti-tumor agents [35]. The arrest of S phase and the enhancement of caspase-6 activity by ZBP-89 mimic the action of the anti-hepatocarcinogenesis agent resveratrol [36], which can cause arrest in the S phase and induce apoptosis in a caspase-6 dependent manner [37]. The induction of caspase-6 expression lowers the cell death threshold in response to apoptotic signals that activate caspase-6 [38]. Moreover, it is evident that the activity of caspase-6 is correlated with p53 in cancer cells, in which caspase-6 can be an essential initiator [39]. In our current experiment, it appears that HCC cells with a wild-type p53 are more sensitive to cell death inducers than those with a mutant p53. Therefore, we assumed that the caspase-6 might be involved in the cell death induced by ZBP-89. In this study, we found that parallel to the increase in cell death, the activity of caspase-6 was elevated by the treatment in a manner that was well correlated with the changes in cell death. The result appears to correlate with the growth inhibitory effect of ZBP-89, indicating a positive role of caspase-6 in the cell death induced by ZBP-89 in the HCC cells tested. The role of caspase-6 in ZBP-89-mediated growth inhibition is further confirmed by application of caspase-6 inhibitor, in which the suppression of caspase-6 activity significantly reduces the inhibitory effect of ZBP-89 in HCC cells. Taken together, the above data support the positive involvement of caspase-6 in ZBP-89-mediated growth inhibition. The increase of caspase-6 and the S arrest execute cell death of HCC cells treated with ZBP-89. The increase of caspase-6 activity by ZBP-89

should reduce the cell death threshold in HCC cells and thus sensitize the cells to anti-tumor agents.

In conclusion, our experiments demonstrate that ZBP-89 can significantly enhance the sensitivity of HCC cells to 5-FU or STS treatment by increasing caspase-6 activity, the G2M phase abrogation and the S phase arrest. It appears that p53 plays a limited role in the cell death of HCC cells induced by ZBP-89 though the cells with wild-type p53 are most sensitive to ZBP-89. ZBP-89 is a potential anti-tumor agent for HCC.

Acknowledgments

This work was supported by the Research Grants Council of the Hong Kong SAR. No: CUHK4551/05M to PBSL and GGC, and Public Health Service Grant R01 DK-55732 to JLM.

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Abbreviations used

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

Figure 1.

ZBP-89 sensitizes HCC cells to cell death induced by 5-FU or STS. The cells transfected with ZBP-89 were treated with 5-FU (20 $\mu\text{g/ml}$) or STS (0.125 μM) for 48 hours and the proliferation of the cells, reflected by the number of the living cells, was measured by MTT assay. The experiment was repeated at least three times. Compared with control cells (without ZBP-89, 5-FU or STS), ^aP<0.05, ^bP<0.01.

Figure 2.

ZBP-89 induces the death of HCC cells in a time-dependent manner. The cells infected with Ad5-ZBP-89 were treated with 5-FU (20 $\mu\text{g}/\text{ml}$) for 24, 48 and 72 hours and the death of the cells, reflected by the number of the living cells, was measured by MTT assay. The experiment was repeated at least three times. Comparison between the control and ZBP-89 plus 5-FU: ^a $P < 0.05$, ^b $P < 0.01$; between the control and ZBP-89: ^c $P < 0.05$, ^d $P < 0.01$; between the control and 5-FU: ^e $P < 0.05$, ^f $P < 0.01$.

Figure 3.

ZBP-89 increases the activity of caspase-6 in HCC cells in a time-dependent manner. The cells infected with Ad5-ZBP-89 were treated with 5-FU (20 $\mu\text{g}/\text{ml}$) for 24, 48 and 72 hours. After the treatment, the cell lysate was collected and assayed for the activity of caspase-6 by a colorimetric protease assay kit as described in the Materials and Methods. Comparison between the control and ZBP-89 plus 5-FU: ^a $P < 0.05$, ^b $P < 0.01$; between the control and ZBP-89: ^c $P < 0.05$, ^d $P < 0.01$; between the control and 5-FU: ^e $P < 0.05$, ^f $P < 0.01$.

Figure 4.

Influence of z-VEID-FMK on caspase-6 activity in HCC cells treated with ZBP-89 and 5-FU. HepG2 cells were treated with ZBP-89 or/and 5-FU in the presence or absence of 100 nM z-VEID-FMK for 48 hours. After the treatment, cell proliferation was measured by MTT assay. The experiment was repeated at least three times.

Figure 5.

The effect of ZBP-89 on the cell cycle. HepG2 cells were infected with Ad5-ZBP-89 and the cell phase distribution was assayed by determination of the DNA content. The experiment was repeated at least three times. Compared with control cells (infected with Ad5-vector), ** $P < 0.01$.