Targeting and Eradicating Hepatic Cancer Cells with a Cancer-Specific Vector Carrying the Buforin II Gene

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Abstract

Objective: The aim of this study was to investigate the suppressive effects of Buforin II on the growth of HepG2 cells. To accomplish this, we created a recombinant plasmid (pSUR-Buforin2) in which the survivin promoter was modified to drive the Buforin II gene.

Methods: The DNA fragment encoding the Buforin II gene was obtained by gene synthesis and cloned into the pSUR-Luc plasmid behind the survivin promoter. The vector was subsequently transfected into HepG2 and LO2 cells. Cell proliferation was measured by the MTT assay, cell cytotoxicity detected by the LDH assay, and cell apoptosis determined by flow cytometry, DNA ladder assays, and immunoblot analysis.

Results: The pSUR-Buforin2 vector effectively suppressed the proliferation of HepG2 cells. The MTT and LDH assay demonstrated that under control of the survivin promoter, Buforin II was not expressed in LO2 cells, but it was expressed in tumor cells where cell death was also observed. AnnexinV-PI staining, DNA ladder assays, and western blots showed massive apoptosis in HepG2 cells transfected with pSUR-Buforin2.

Conclusion: pSUR-Buforin2 can significantly inhibit the growth of HepG2 cells, resulting in increased cancer cell apoptosis. Thus, this newly designed plasmid might provide a potent and selective anticancer therapy.

Key words: Buforin II, hepatocellular carcinoma (HCC), survivin

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world and the most common primary liver cancer.¹ It represents a major public health burden and is especially difficult to manage in Asia.² According to GloboCan2008 data,³ liver cancer is the fifth most common cancer in men and the seventh most common in women. Most of the burden is in developing countries, where ~85% of cases occur, and it is more prevalent in men than in women. There were an estimated 694,000 deaths from liver cancer in 2008 (477,000 in men and 217,000 in women). Because of its high fatality (overall ratio of mortality to incidence of 0.93), liver cancer is the third most common cause of death from cancer worldwide. Once diagnosed, HCC has a dismal prognosis. Although small, localized tumors can be removed with surgery, less than 20% of HCC patients receive radical surgical resection because most have advanced disease at

diagnosis. Local regional therapy includes cryoablation,⁴ radiofrequency ablation,⁵ and transarterial embolization,⁶ which is largely palliative. HCC is notoriously resistant to chemotherapy and other systemic treatment modalities.^{7,8} The multitargeted kinase inhibitor Sorafenib⁹ improves survival by 2.3–2.8 months¹⁰ and is the only systemic agent that increases survival time in patients with advanced HCC, making it the current standard of care.¹¹ Overall, the median survival for patients with advanced stage, unresectable HCC is less than 1 year. To overcome the limits of current chemotherapeutic drugs, many researchers have labored to identify new anticancer therapies. Recently, gene therapy has received much attention as an alternative treatment that overcomes the limits of current drugs. The development of gene therapy for cancer began over a decade ago and although the treatment has great potential many problems must be resolved before cancer patients can be safely and effectively treated.¹² Those issues include specificity, efficient transfection and

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transduction, and avoidance of toxicity. The most difficult aspect of human cancer gene therapy is to correctly target the specific cells of concern. Random delivery of a therapeutic gene will damage normal cells in essential organs, and may subsequently cause death in treated subjects. Thus specific targeting to tumor cells should improve the therapeutic effect of gene transfer by preventing damage to normal cells.^{13–15} When systemic gene transfer is performed this issue becomes especially crucial. Using a cancer-specific promoter to specifically express genes in tumor cells has shown promise.¹⁶ Although several genes have been identified that are specifically expressed in hepatic cancer cells, the most extensively studied is the survivin gene. At 16.5 kDa, survivin is the smallest member of the inhibitors of apoptosis (IAP) protein family.^{17,18} Proteins of the IAP family are highly expressed in primary tumors and cancer cell lines, but undetectable in most somatic tissues and normal differentiated cells. Research recently demonstrated that the survivin promoter has high transcriptional activity in a variety of human cancer cell lines and is useful for targeted transgene expression in human cancer cells.^{19–21} Owing to its massive upregulation in human tumors, the survivin promoter has the potential to be used for cancer-specific expression of a therapeutic gene.

Antimicrobial peptides (AMPs) constitute an evolutionarily conserved part of the innate immune defense system. In recent years a number of AMPs have been isolated from a wide range of animal, plant, and bacterial species. AMPs are active against bacteria, fungi, and viruses and possess anti-tumor activities.²² Buforin II is an AMP that is isolated from the stomach of the Asian toad *Bufo bufo* gargarizans²³ and displays strong, selective anticancer activity against a broad spectrum of cancer cells.^{24,25}

In this study, a recombinant vector (pSUR-Buforin2) containing the Buforin II gene driven by the survivin promoter was transfected into HepG2 and LO2 cells. We hypothesized that pSUR-Buforin2 would inhibit the proliferation of HepG2 cells *in vitro*.

Materials and Methods

Cell line and cell culture

A line of normal human liver cells (LO2) and the human HCC cell line HepG2 were bought from KeyGEN BioTECH. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal calf serum (Gibco) at 37°C with 5% CO₂ atmosphere.

Western blot analysis

At about 70% confluence, LO2 and HepG2 cells were harvested with trypsin/EDTA, and phosphate-buffered saline (PBS)-washed cell pellets were stored at –70°C until use. Whole-cell protein was extracted from the cell pellets. For survivin detection, 30 µg protein was separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to polyvinylidene fluoride membranes (Millipore). Membranes were subsequently incubated overnight at 4°C with an antibody against GAPDH or survivin (Santa Cruz), followed by incubation with a peroxidase-conjugated goat antirabbit second antibody (Sigma) at 37°C for 1 hour. The peroxidase activity was detected using electrochemiluminescence (ECL; GE healthcare).

Relative protein expression levels were quantified using GAPDH as a loading control.

Construction of plasmids

The plasmid pSUR-Luc that contained the survivin promoter was provided by professor Mien-Chie Hung (MD Anderson Cancer Center).²¹ Two plasmids were constructed (Fig. 1B) to make the GFP constructs. pSUR-GFP was generated by replacing the luciferase gene in pSUR-Luc with GFP using *Nco*I and *Xba*I digestion. To generate the Buforin II construct (pSUR-Buforin2), the luciferase gene of pSUR-Luc was replaced with Buforin II by *Nco*I and *Xba*I digestion. The fragment of GFP was previously preserved in our laboratory. Based on the amino acid sequence of Buforin II, a novel Buforin II gene was designed and the full-length gene synthesized. These plasmids were confirmed by restriction endonuclease digestion and sequence analysis (Invitrogen).

Transient transfection and regulation of GFP expression with the survivin promoter

To assess gene delivery efficiency, confirm tumor specificity, and confirm gene expression efficiency of the survivin promoter, we performed reporter gene assays using the X-tremeGENE HP DNA Transfection Reagent (Roche). HepG2 and LO2 cells were cultured in eight-well chamber slides (NUNC) and six-well plates. When cells reached 60% confluence, they were transfected with the pSUR-GFP vector and the X-tremeGENE HP DNA Transfection Reagent (Roche) according to the instructions. After 48 hours, the transfected cells in the eight-well chamber slides were observed under a fluorescence microscope (Leica), and the transfected cells in six-well plates were analyzed with flow cytometry (BD) to evaluate the transfection efficiency and GFP expression.

MTT assay on cell proliferation

Cells were seeded onto 96-well culture plates at a density of 2×10^4 cells/well in 200 µL DMEM and transfected with

FIG. 1. Survivin expression in LO2 and HepG2 cells and plasmid constructs. **(A)** Western blot analysis of Survivin (16.5 kDa) in LO2 and HepG2 cells. **(B)** The recombinant gene encoding Buforin2 was inserted into the pSUR vector under the control of the survivin promoter.

pSUR-Buforin2 and pSUR-Luc. After 48 hours, 50 μ L 5 mg/mL MTT (thiazolyl blue) solution was added to each well and incubated for 4 hours at 37°C in a humidified 5% CO₂ atmosphere. The medium was removed from each well and the residual MTT formazan was solubilized in 150 μ L dimethyl sulfoxide. The absorbance at 550 nm (A₅₅₀) was determined with a microplate reader (BIO-RAD).

Cytotoxicity detection assay

Cells were seeded onto 96-well culture plates at a density of 2×10^4 cells/well in 200 μ L DMEM and transfected with pSUR-Buforin2 and pSUR-Luc. After 48 hours, 100 μ L of the cell-free culture supernatants was transferred into new 96-well plates. The LDH assay (Roche Molecular Diagnostics) was performed according to the manufacturer's protocol.

Quantification of apoptosis by AnnexinV-PI staining

The apoptotic cells were differentiated from viable or necrotic ones by the combined application of AnnexinV-FITC and propidium iodide (PI) (Becton, Dickinson and Company). HepG2 cells were treated with pSUR-Buforin2 and pSUR-Luc for 48 hours. After washing in cold PBS (PH7.4), 100 μ L solution (1×10^5 cells) was transferred to a 5-mL culture tube and 5 μ L Annexin V-FITC and 10 μ L PI were added. The cells were gently vortexed for 15 minutes at room temperature and analyzed on a flow cytometer FACS CantoII (Becton-Dickinson) after the addition of 400 μ L 1 $\times$ binding buffer to each tube. Apoptotic cells were defined as FITC

Annexin V-positive and PI-negative. Necrotic or dead cells were defined as FITC Annexin V-positive and PI-positive.

DNA fragmentation assay

HepG2 cells were harvested 48 hours after transfection with pSUR-Buforin2 and pSUR-Luc and centrifuged at 500 g for 5 minutes at room temperature. The cell pellet was re-suspended in 250 μ L PBS containing RNase A and proteinase K. Subsequently, the cells were lysed with 200 μ L lysis buffer and incubated for 10 minutes at 70°C. Next, 200 μ L ethanol was added and the samples were gently mixed by inversion. The mixture was transferred to a miniprep column and centrifuged at 8000 g for 1 minute at room temperature. The column was eluted with buffer twice and centrifuged to discard the remaining ethanol. Finally, the DNA fragments were eluted with 100 μ L elution buffer. The DNA fragments were separated by electrophoresis on a 1.5% agarose gel and visualized under ultraviolet light after being stained with Gloden View. The 1-kb DNA ladder served as a molecular marker (Tiangen).

Detection of apoptosis and proliferating cell nuclear antigen by western blot

Total protein from cells was extracted in lysis buffer and quantified using the BCA method. An equal amount of protein (30 μ g) of each sample was fractionated in 12% SDS-PAGE, transferred to polyvinylidene fluoride membranes (Millipore), and incubated overnight at 4°C with antibodies

FIG. 2. *NcoI/XbaI* double-enzyme digestion analysis of pSUR-Luc, pSUR-GFP, and pSUR-Buforin2. (A) M, DL15000 DNA marker; Lane 1, pSUR-Luc vector; Lane 2, pSUR-GFP vector; Lane 3, pSUR-Buforin2 vector; Lane 4, pSUR-Luc double-enzyme digestion; Lane 5, pSUR-GFP double-enzyme digestion, the GFP gene could be observed; Lane 6, pSUR-Buforin2 double-enzyme digestion, the Buforin2 gene (230 bp) was detected by 1.5% agarose gel electrophoresis. (B) Full-length Buforin2 gene (230 bp) sequence diagram.

against β -actin, caspase-3 (CST; #9662), caspase-8 (Abcam; ab32125), caspase-9 (Abcam; ab32539), PARP (Abcam; ab32138), and PCNA (Santa Cruz). After incubation with peroxidase-coupled antirabbit IgG (Sigma) at 37°C for 1 hour, protein bands were visualized with ECL (GE healthcare). Relative protein expression levels were quantified using β -actin and pro-caspase-3 as loading controls.

Statistical analysis

SPSS19.0 software was used and each assay was performed at least three times. The data were analyzed by using the Independent *t*-test to calculate the significance values, and analysis of variance was used to determine the significance of differences in multiple comparisons. A probability value (*p*) of <0.05 was considered to be statistically significant.

Results

Survivin protein is expressed in HepG2 cells but not in LO2 cells

To determine the expression of survivin in HepG2 and LO2 cells, protein was extracted from cells and subjected to

western blot analysis. As shown in Figure 1A, survivin protein was detected as an intense band at 16.5 kDa in HepG2 cell lines, but not in the normal human liver cells, LO2.

Identification of recombinant plasmid pSUR-Buforin2

The Buforin II cDNA (230 bp) was successfully subcloned into the pSUR-Luc plasmid, as confirmed by Nco I and Xba I digestion (Fig. 2A). We also sequenced the plasmid to test its fidelity (Fig. 2B).

Cancer-specific activation of the survivin promoter in vitro

To assess whether the activation of the survivin promoter is cancer-specific we transfected both cancer (HepG2) and normal (LO2) cells with the pSUR-GFP plasmid, that contains the GFP gene under control of the survivin promoter. Our data (Fig. 3) demonstrated that the detected fluorescent signal was more intense in HepG2 cells (40.9%) than in LO2 cells (3.2%), suggesting that the survivin promoter is selectively active in cancer cells compared with normal cells.

FIG. 3. GFP expression in HepG2 and LO2 cells transfected with pSUR-GFP. **(A)** GFP expression was easily detectable in HepG2 cells using a fluorescent microscope. (original magnification, $\times 100$). **(B)** Flow cytometry for GFP expression in HepG2 cell lines (40.9%) and in LO2 cells (3.2%). These results suggest that the survivin promoter is selectively active in cancer cells.

Effect of Buforin II driven by the survivin promoter on proliferation and cytotoxicity in HepG2 and LO2 cells in vitro

To determine whether pSUR-Buforin2 had inhibitory effects on the growth of HepG2 cells, a MTT assay was conducted to determine cell proliferation. In LO2 cells, plasmid expression failed to produce a measurable difference between the control group, pSUR-Luc group, and pSUR-Buforin2 group after 48 hours ($p > 0.05$, Fig. 4A). In the

FIG. 4. Effect of Buforin II driven by the survivin promoter on cell proliferation and cytotoxicity in HepG2 and LO2 cells *in vitro* (** $p < 0.01$). The data shown are performed in histogram. (A) LO2 cells failed to produce a measurable difference among control group, pSUR-Luc group and pSUR-Buforin2 group ($p > 0.05$). The HepG2 cells failed to produce a measurable difference between control group and pSUR-Luc group ($p = 0.119$). However, the proliferation of HepG2 transfected with pSUR-Buforin2 was significantly inhibited ($p = 0.001$). (B) No differences in cell cytotoxicity were observed between control LO2 cells and those transfected with either pSUR-Luc or pSUR-Buforin2 ($p > 0.05$). No differences in cell cytotoxicity were observed between control transfected HepG2 cells and those transfected with pSUR-Luc group ($p > 0.05$). Cytotoxicity of HepG2 cells transfected with pSUR-Buforin2 was significantly released ($p < 0.05$).

HepG2 cells, transfection with pSUR-Luc also failed to produce a measurable difference from the control group after 48 hours ($p = 0.119$). However, HepG2 cells transfected with pSUR-Buforin2 displayed significantly decreased proliferation 48 hours after transfection (48 hours, $p = 0.001$).

The cytotoxicity detection assay measures cell lysis by detecting LDH activity released from damaged cells. LDH release was increased in the HepG2 cells transfected with pSUR-Buforin2. As shown in Figure 4B, no differences were observed between all LO2 cells and HepG2 cells treated with pSUR-Luc.

Proapoptotic effects of Buforin II in HepG2 cells

To study the cellular and molecular events that occur after pSUR-Buforin2 treatment, we utilized flow cytometry to analyze HepG2 cells transfected with pSUR-Luc and pSUR-Buforin2. Flow cytometry revealed that the apoptotic rate of pSUR-Buforin2-transfected HepG2 cells (29.9%) was significantly higher than that of control HepG2 cells (1.3%) and pSUR-Luc-transfected HepG2 cells (3.4%; $p < 0.05$; Fig. 5A). Nevertheless, there was no difference in the apoptotic rate between control HepG2 cells and HepG2/pSUR-Luc cells. These results revealed that pSUR-Buforin2 could induce apoptosis in HepG2 cells. One of the biochemical reactions that occur during apoptosis is the activation of endonucleases. Nucleases act on the site connecting two nucleosomes of DNA, excising DNA into 180–200 bp fragments. After the DNA is extracted, a DNA ladder can be observed by agarose gel electrophoresis. In contrast, necrotic cells show continuous membranous bands, due to random DNA disintegration, that cannot be checked by agarose gel electrophoresis. The presence of a DNA ladder was clearly observed in HepG2/pSUR-Buforin2 cells, but was undetectable in the control groups (HepG2 and HepG2/pSUR-Luc) (Fig. 5B).

pSUR-Buforin2 modifies cell proliferation and apoptotic signaling

The Buforin II-induced apoptotic signaling pathway was further examined by analyzing the expression of caspases, key effectors of apoptosis. We used antibodies to determine the expression and activity of caspase-3, caspase-8, caspase-9, PARP, and PCNA in HepG2/pSUR-Buforin2 cells and control cells (HepG2 and HepG2/pSUR-GFP) 48 hours after transfection. The results demonstrated that the levels of cleaved caspase-3 were markedly higher in HepG2/pSUR-Buforin2 cells than in the control groups. Interestingly, PCNA, PARP, and caspase-9 levels were lower in HepG2/pSUR-Buforin2 cells than in the other groups (Fig. 6), whereas the expression of caspase-8 of HepG2/pSUR-Buforin2 group was not significantly more than that in the other groups. Together, our data suggest that Buforin II may induce HepG2 cells apoptosis through mitochondrial pathway by activation of caspase-3 (a key mediator of apoptosis of mammalian cells) and decrease the expression of PCNA and PARP.

Discussion

HCC is a common high-risk malignancy for which the current main therapeutic treatment is surgery. However, surgical therapy is not efficient¹⁵ and the general outcome is poor. For this reason, there is a considerable interest in

FIG. 5. The apoptotic rate of HepG2 cells transfected with pSUR-Luc, pSUR-Buforin2, respectively. **(A)** The apoptosis rate of pSUR-Buforin2-transfected HepG2 cells (29.9%) was significantly higher than that of control HepG2 cells (1.3%) and pSUR-Luc-transfected HepG2 cells (3.4%). **(B)** The presence of a DNA ladder was clearly detected in HepG2/pSUR-Buforin2 cells compared with control groups (HepG2 and HepG2/pSUR-Luc). M, 1 kDa DNA marker; Lane 1, HepG2 cells (control); Lane 2, pSUR-Luc vector/HepG2 cells; Lane 3, pSUR-Buforin2 vector/HepG2 cells.

developing novel antitumoral agents. Ideally, these agents would have different modes of action than the classic antitumoral drugs, for which cancer cells have acquired resistance. Gene therapy holds great promise because it aims to eradicate the cause rather than the symptoms of disease. Thus, this type of therapy has emerged as a promising strategy to treat a wide range of inherited and acquired diseases. Recently, many studies on Buforin II and its potential role in cancer research and therapy have been conducted.²⁶ Buforin II, which has selective cytotoxicity in 62 different tumor cell lines,²⁶ has a unique mechanism of action; it rapidly crosses over cell membranes without lysing cells,^{27,28} combines with the nucleic acid of tumor cells, and triggers mitochondrial-dependent apoptosis.²⁵ In most *in vitro* studies of Buforin II investigators use synthetic peptides of Buforin II.^{26,29} Unfortunately, the costs of synthetic AMPs are high and many are cytotoxic and may negatively affect host cells or symbiotic bacteria if they remain active *in vivo*. In addition, gene therapy with a "suicide" gene that can accurately target cancer cells is imperative. To address this issue, we cloned the Buforin II gene into the eukaryotic expression vector pSUR-Luc, generating

pSUR-Buforin2, and studied the effect of Buforin II in cancer cells.

Survivin is strongly and diffusely expressed in embryonic and fetal organs,³⁰ but it is undetectable in most terminally differentiated normal tissues.³¹ Thus, it is hypothesized that the survivin promoter might be a cancer-specific promoter with utility in gene therapy. We used fluorescence microscopy and flow cytometry to reveal that the survivin promoter could induce pSUR-GFP expression in HCC cells (HepG2) but not in normal cells (LO2). Thus, Buforin II driven by the cancer-specific survivin promoter could be selectively expressed in and kill tumor cells, while leaving normal tissue unharmed. Consistent with this, MTT assay and LDH assay demonstrated that pSUR-Buforin2 could significantly inhibit the proliferation of HepG2 cells but had no effect on normal hepatic cell lines such as LO2. Our data further confirmed that Buforin II driven by the survivin promoter could effectively and specifically kill tumor cells *in vitro*.

Next, we studied the cellular and molecular events that occurred with pSUR-Buforin2 transfection. The flow cytometry and DNA ladder assay indicated that apoptosis was

FIG. 6. pSUR-Buforin2 modifies the cell proliferation and apoptotic signaling. **(A)** The protein levels of PCNA and active caspase-3 were detected. The expression of PCNA in HepG2/pSUR-Buforin2 was lower than the HepG2 and HepG2/pSUR-Luc groups. Cleaved Caspase 3 levels were higher in the HepG2/pSUR-Buforin2 group than the other groups. Lane 1, HepG2 cells (control); Lane 2, pSUR-GFP vector/HepG2 cells; Lane 3, pSUR-Buforin2 vector/HepG2 cells. **(B)** PARP, caspase-9 levels were lower in HepG2/pSUR-Buforin2 than the other groups, whereas the expression of caspase-8 of HepG2/pSUR-Buforin2 group was not significantly more than that in the other groups. Lane 1, HepG2 cells (control); Lane 2, pSUR-GFP vector/HepG2 cells; Lane 3, pSUR-Buforin2 vector/HepG2 cells.

induced in the pSUR-Buforin2/HepG2 group. These findings reveal that the plasmid carrying Buforin II can exert a potent antitumor effect *in vitro* by suppressing proliferation and promoting apoptosis of cancer cells. Further, our results showed that the expression of pro-apoptotic factors like cleaved-caspase-3 were markedly upregulated in the pSUR-Buforin2/HepG2 group. It is generally believed that caspase-3 is the effector and most important terminal shear enzyme in the process of apoptosis. Based on these data and previous data that Buforin II exhibits very strong DNA and RNA

binding activity,²⁴ pSUR-Buforin2 transfection-induced death of cancer cells may be ascribed at least in part to activation of apoptosis pathways.³² Two major pathways for caspase activation in mammalian cells are presented, the extrinsic and intrinsic.^{33,34} The extrinsic pathway is triggered by members of the TNF-family of cytokine receptors, these proteins recruit adapter proteins to their cytosolic Death Domains, which then bind pro-caspases, particularly pro-caspase-8. This pathway is under suppression by pro-caspase-8. The intrinsic pathway is triggered by release of cytochrome c from mitochondria, in the cytosol; cytochrome c binds and activates Apaf1, allowing it to bind and activate pro-caspase-9. Active caspase-9 (intrinsic) and caspase-8 (extrinsic) have been shown to directly cleave and activate the effector protease, caspase-3. Our data suggest that Buforin II may induce HepG2 cells apoptosis through intrinsic pathway; future studies are necessary to identify the exact mechanism of activation. Further, it will be exciting to determine the effects of pSUR-Buforin2 in murine models of HCC.

The pathogenesis of HCC is complex, involving many genes and various other factors. Therefore, the effect of tumor gene therapy could be improved by using a variety of gene therapies or combining gene therapy with chemotherapy or other treatments. In conclusion, we have successfully generated a pSUR-Buforin2 recombinant plasmid that can achieve selective cytotoxicity in tumor cells by activating apoptotic pathways and inhibiting cell proliferation. Our study suggests that the pSUR-Buforin2 plasmid should be further investigated for its use in treating cancer.

Grants Support

This work was supported by the National Natural Science Foundation of China (No. 30972913), the Project of Science and Education for Developing Health of Jiangsu Province (No. RC2011081), and the Natural Science Foundation of Jiangsu Province (No. BK2009451).

Disclosure Statement

No competing financial interests exist.

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