IER5 promotes irradiation- and cisplatin-induced apoptosis in human hepatocellular carcinoma cells

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Abstract: Purpose: To elucidate the mechanisms of the immediate-early response gene 5 (IER5) effect on the apoptosis induced by irradiation and cisplatin (CDDP) in human hepatocellular carcinoma (HepG2) cells. Methods: We generated IER5 overexpression stable cells (HepG2/IER5) using Lipofectamine 2000 transfection HepG2 cells. Cell apoptosis was induced by irradiation and cisplatin treatments, and cell proliferation (viability) and apoptosis were evaluated by MTT and flow cytometry assays. Protein expression was determined by Western blot. Results: The growth of the IER5 overexpression cells was significantly inhibited after six days of 60Co γ-irradiation exposure (p<0.01) compared with the cell growth of vector control cells. Furthermore, the HepG2/IER5 cells were arrested at the G2/M phases. We also found that the expression of phospho-Akt was reduced, and the levels of cleaved caspase-3 and PARP were increased after the treatment of HepG2/IER5 cells with γ-irradiation and cisplatin. Conclusion: Our results suggest that the overexpression of IER5 can inhibit cell growth and enhance the cell apoptosis induced by exposure to radiation or cisplatin. The overexpression of IER5 can be utilized as a targeting strategy to improve the outcomes of radiotherapy used for the treatment of patients with liver cancer.

Keywords: IER5, apoptosis, γ-irradiation, cisplatin, human hepatocellular carcinoma

Introduction

Primary hepatocellular carcinoma (HCC) is one of the most widespread malignancies in the world. As indicated in human cancer statistics, the disease is the third most common cause of cancer-related deaths worldwide [1]. HCC often develops in patients suffering from chronic liver diseases associated with hepatitis B (HBV) or hepatitis C (HCV) virus infections. The natural development of this disease (without treatment) is exceedingly severe and leads to death in a short period of time [2]. Currently, liver resection and transplantation are both well-established options for the curative treatment of HCC. However, surgical resection is often accompanied by a high recurrence rate, and transplantation is not universally applicable. Radiofrequency ablation therapy and the percutaneous ethanol injection therapy are also used as curative treatments for HCC [1]. So far, there have been only limited effective therapeutic modalities for HCC patients [3]. Moreover, some of these treatment options have not been successfully applied in the therapy of HCC patients.

Radiotherapy is now becoming the most important treatment strategy in the treatment of unresectable HCC patients [4]. However, irradiation can indirectly activate H2O and yield a large quantity of excessive free radicals, breaking the balance between the oxidant and antioxidant systems and leading to oxidative stress in the cells [5]. Recently, the more precise treatment by accurate delivery of radiation to the target tumor tissue using 3-dimensional conformal radiation therapy has improved tumor con-
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cells. Herein, we highlighted that the overexpression of IER5 protein enhanced irradiation-induced cell apoptosis. The findings of this study can contribute to understanding the influence of IER5 on tumor sensitivity to radiation and facilitate the development of a new cancer treatment strategy.

Materials and methods

Reagents, antibodies, and cell lines

The anti-Flag and anti-β-actin antibodies were purchased from Sigma Aldrich; antibodies anti PARP, caspase-3, Akt, p-Akt, and p73 were obtained from Cell Signaling Technology; antibodies anti Bcl-2, Bcl-x, and Bax were acquired from Santa Cruz Biotechnology. The antibodies anti-p21 and p53 were purchased from Calbiochem, whereas the antibody anti-IER5 was purchased from Abcam. All reagents, including fetal bovine serum (FBS), penicillin G, streptomycin, G418, dimethyl sulfoxide (DMSO), ribonuclease (RNase), and propidium iodide (PI) were purchased from Invitrogen.

Cell lines

The human hepatocellular carcinoma cell line, HepG2, was a generous gift from the Fourth Laboratory, Institute of Medical Radiology, the Academy of Military Science of China. The cells were cultured in DMEM (GIBCO) with 10% FBS (GIBCO), 2 mM L-glutamine, and 1% penicillin-streptomycin at an incubator maintaining 37°C and a humidified atmosphere containing 5% CO2.

Cell transfections

HepG2 cells were transfected with Pcmv-3 × Flag or 3 × Flag-IER5 plasmids using Lipofectamine 2000TM (Invitrogen) according to manufacturer’s instructions. Stable positive cell clones (HepG2/IER5, HepG2/Vector) were selected in medium supplemented with G418.

Flow cytometry analysis

The HepG2/IER5 and HepG2/Vector cells were plated in 6-well plates (5 × 104 cells/well) in DMEM growth medium and were cultured overnight. Then, the cells were exposed to 4 Gy of γ-ray irradiation and collected after treatment durations of 12 h and 24 h. Next, they were fixed by 70% ethanol and washed with PBS.
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Further, the cell pellets were suspended in 200 μL of 1x propidium iodide (PI)+Rnase Staining Solution and incubated at 37°C for 30 min in the dark. The DNA histograms and cell cycle phase distributions of the 20,000 cells in the suspension were analyzed by flow cytometry (FACS Calibur instrument; Becton Dickinson), and the data were analyzed using the CELL-Quest software.

Cell viability assay (MTT)

The cells were seeded in 96-well plates at an initial density of 2000 cells per well and were cultured overnight. Then, the cells were exposed to 0 and 4.0 Gy of γ-ray irradiation at a dose rate of 5.0 cGy/min. After 24-h and 48-h exposure to radiation, the medium was removed. The MTT reagent (Sigma) was added, and the cells were incubated for an additional 4 h at 37°C. Afterwards, 10% sodium dodecyl sulfate was added to dissolve the blue formazan precipitate, and the absorbance at 492 nm was measured in an enzyme-linked immunosorbent assay (ELISA) reader. All experiments were run with at least five replicate cultures and repeated three times.

Cells and gamma-irradiation (γ-irradiation) treatment

The HepG2/IER5 and HepG2/Vector cells were plated in 6-well plates (5 × 10⁴ cells/well) and were exposed to 0 and 4.0 Gy of γ-irradiation from a radioactive telecobalt therapy source at a dose rate of 5.0 Gy/min. Then, the cells were kept at 37°C in an atmosphere with 5% of CO₂ for the later experiments.

Western blot (protein immunoblot)

Cells (0.3 × 10⁶) were collected at 0 h, 24 h, and 48 h after the irradiation treatment (irradiation dose of 4 Gy) and washed twice with ice-cold phosphate buffered saline (PBS). Further, they were resuspended and sonicated in lysis buffer (20 mM Hapes, pH 8.0, 150 mM KCl, 5% glycerol, 10 mM MgCl₂, 0.5 mM EDTA, 0.02% NP-40, supplemented with NaF, NaVO₄, PMSF, and protease inhibitors). The cell supernatants were collected after centrifugation for 30 min at 14,000 rpm. The protein concentrations were determined using the standard Bradford assay (Bradford reagent was supplied by Bio-Rad). Equal amounts of protein were separated on 10% and 12% polyacrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting was performed using the indicated antibodies. The protein was transferred to PVDF membranes in transfer buffer (50 mM Tris, 190 mM glycine, and 10% methanol) at 100 V for 2 h. The membranes were incubated with blocking buffer (5% non-fat milk, 0.1% Tween in PBS, pH 7.4) for 1 h at 4°C. The membranes were then washed three times (10 min each time) with the washing buffer, (50 mM Tris, 200 mM NaCl, and 0.2% Tween 20). The blots were incubated in an antibody solution containing the indicated primary antibodies at 4°C overnight. After that, the blots were incubated in a secondary antibody solution for 1 h at room temperature. The membranes were washed three times (10 min each time). The indicated proteins were detected by the enhanced chemiluminescence Western blotting detection system and autoradiography according to the manufacturer’s protocol. Each experiment was carried out in triplicate and performed at least three times.

Results

The overexpression of IER5 significantly inhibited the growth of HepG2 cells

We generated a stable IER5 overexpression cell line (HepG2/IER5) using IER5-3 × Flag vectors transfected in HepG2 cells according to the Lipofectamin 2000 transfection procedure. G418 was used for cell selection. Stable cells without IER5 expression, HepG2/Vector (lacking an IRE5 cDNA insert as a negative control), were also produced through the Pcmv-3 × Flag vector. To reduce the influence of endogenous IER5 gene expression, we knocked down the endogenous IER5 gene using specific IER5 siRNA. IER5 overexpression was verified by Western blot analysis using anti-IER5 or anti-Flag antibodies. High IER5 protein expression was manifested in HepG2/IER5, but not in HepG2/Vector cells (Figure 1A, 1B).

We first explored the effect of IER5 overexpression on the apoptosis induced by γ-ray irradiation. The stable cells were treated with γ-ray irradiation at a dose of 4 Gy for six days. We found that the number of HepG2/IER5 cells was significantly reduced compared with that of the vector control cells (P<0.05). Interestingly, the growth of the untreated (IER5-0Gy) cells was
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Also inhibited in comparison with that of the control cells (Vector-0Gy), which indicated that IER5 overexpression could suppress cell growth (Figure 1C). In confirmation of this finding, we also discovered that the knockdown of IER5 considerably promoted the cell growth in both the cells treated with γ-ray irradiation (SiRNA-4Gy) and those with no treatment (SiRNA-0Gy) (Figure 1D). Taken together, these results suggested that the γ-ray irradiation-induced IER5 overexpression inhibited cell growth and enhanced cell apoptosis.

**IER5 enhanced the irradiation-induced HepG₂ cell apoptosis by increasing the cell cycle arrest at the G2/M phase**

G2/M: To determine the influence of IER5 on the cell cycle distribution, we treated the HepG₂/IER5 cells with γ-ray irradiation and measured the cell cycle distribution using flow cytometric analysis. The results showed a significant increase of the cell population in G2/M phase compared with the control after both durations of the irradiation treatment, 12 h (p<0.01) and 24 h (p<0.05) (Figure 2A, 2B). We then investigated if the knockdown of IER5 still exerted an effect on cell cycle distribution, while the cell population showed no difference between the IER5 knockdown cells and the control cells (Figure 2C, 2D). These results indicated that the overexpression of IER5 increased the cell cycle arrest at the G2/M phase and enhanced the cell apoptosis induced by irradiation.

**IER5 enhanced the irradiation- and cisplatin-induced apoptosis by increasing the cleavage of caspase-3 and PARP**

To further explore the mechanism of the influence of the overexpression of IER5 on tumor apoptosis, we examined the expression of some factors involved in the apoptosis signaling pathway by Western blot analysis. The protein lysates from HepG₂/IER5 and control cells were subjected to the Western blotting after these cells had been exposed to 4 Gy of γ-ray irradiation for 24 h and 48 h. The results indicated that cleavage caspase-3 and cleavage PARP were dramatically increased in the HepG₂/IER5 cells after their treatment with...
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γ-ray irradiation compared with HepG2/Vector cells (Figure 3A). This suggested that the overexpression of IER5 could promote irradiation-induced tumor cell apoptosis by enhancing the cleavage of caspase-3 and PARP. Interestingly, the phospho-Akt level was reduced in the HepG2/IER5 cells, but not in the HepG2/Vector control cells (Figure 3B), which implied that the cell survival signaling pathway was blocked by IER5. Next, to evaluate the action of the overexpression of IRE5 on the cell apoptosis induced by the anti-malignancies chemotherapy drug cisplatin (CDDP), we treated the HepG2/IER5 and HepG2/Vector cells for 3 h, 6 h, and 9 h with 100 nM of cisplatin (CDDP). We found that the cleavage PARP and cleavage Caspase-3 were also dramatically increased in the HepG2/IER5 cells in comparison with the HepG2/Vector control cells (Figure 3E). Taken together, we confirmed that the overexpression of IER5 could enhance both the irradiation-induced and the cisplatin-induced apoptosis in human hepatocellular carcinoma cells. However, the results showed no changes in IER5 overexpression cells versus control cells treated with γ-ray irradiation for other tumor suppressor genes or oncogenes involved in the apoptosis signaling pathway, such as P53, P21, and P73, or the Bcl-2 family members, including the genes Bcl-2, Bcl-x, and Bax (Figure 3C, 3D).

IER5 inhibited the survival of tumor cells by enhancing the irradiation-induced apoptosis

Then, to examine the impact of IER5 on tumor cell survival, we measured the HepG2/IER5 cell viability using MTT assay, as described in the Materials and Methods. We found that the cell survival was significantly inhibited in the HepG2/IER5 cells treated with γ-ray irradiation versus the no-treatment control at either the 24-h (p<0.01) or the 48-h (p<0.05) time point, but the cell survival was not suppressed in the HepG2/Vector control cells (Figure 4A, 4B).
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However, the inhibition of cell survival was not detected when we knocked down the IER5 gene using IER5-specific siRNA (Figure 4C, 4D). These results indicated that IER5 was the major factor that inhibited the tumor cell survival and enhanced irradiation-induced apoptosis.

Discussion

Human hepatocellular carcinoma (HCC) is the most common primary malignant tumor and the second leading cause of cancer mortality in China. Surgical resection has been accepted as the major therapy for primary liver cancer. Unfortunately, most patients are surgically unresectable. Radiation therapy has also been commonly used in the treatment of unamenable human hepatoma. However, the main disadvantage of this treatment is the tolerance to radiation exposure. New approaches that reduce side effects and provide good quality of life are required. Thus, it is imperative to improve tumor sensitivity to radiation therapy.

Radiotherapy is an efficient and widely used method for the treatment of cancer if the optimal and effective for tumor control radiation dose is correctly determined, and minimal exposure to the surrounding normal tissue is ensured. Tumor sensitivity to radiation exposure that is higher than that of the surrounding normal tissues would be exceedingly beneficial to HCC patients [10]. Up to now, some effective methods for prediction of optimal radiosensitivity and the most relevant parameters for its achieving have been discovered [11, 12]. Findings of experimental studies and clinical trials have been reported that molecular diagnosis or gene therapy strategies can be used in advanced symptomatic HCC patients to obtain an effective treatment outcome [13]. In our study, we discovered that the overexpression of IER5 can enhance the tumor cells sensitivity to radiation exposure. Further, we found that the proliferation of HepG2/IER5 cells was significantly decreased compared with that of the HepG2/Vector control cells after the γ-ray irradiation treatment (Figure 4A, 4B). The results indicated that IER5 could successfully inhibit cell proliferation.

IER5 is an intronless gene which encodes a serum- and growth factor-inducible message of...
IER5 gene belongs to the slow-kinetics immediate-early gene family, which exhibits growth factor induction kinetics similar to that of pip92/IER2/ETR101 [9]. In our investigation, we evidenced that IER5 was an important factor that regulates cell cycle progression and apoptosis. The pretreatment of hepatocellular carcinoma cells with overexpressed IER5 protein enhanced cell killing induced by irradiation. The overexpression of IER5 caused a significant increase in tumor cell death when it was combined with a treatment with moderate doses of radiation (Figure 4A, 4B).

DNA is the major target for cell killing by radiation [10, 14]. Some rare hereditary conditions demonstrate a high cancer risk and hypersensitivity in response to exposures to agents, such as ultraviolet or ionizing radiation, and which are characterized by a defective processing of DNA damage [11]. Ionizing radiation exposure gives rise to a variety of lesions in DNA that result in genetic instability and potentially tumorigenesis or cell death. It has been confirmed that the individual risk of cancer may be related to the ability of a cell to identify and repair DNA damage. Moreover, individuals who were genetically susceptible to cancer manifested increased DNA radiosensitivity [12, 15]. This hypersensitivity is a response specific to G2-phase cells, and it is directly linked to the failure in the activation of the ataxia-telangiectasia mutated (ATM)-dependent early G2/M checkpoint after the application of low treatment doses [10, 16, 17]. The mechanisms of DNA damage-induced apoptosis are realized through the inactivation of DNA repair and eventually result in cell death [18].

We found that the overexpression of IER5 caused an apparent accumulation of cells in the G2/M phase at both 12 h (p<0.01) and 24
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IER5 promotes apoptosis in hepatocellular carcinoma cells (Figure 2A, 2B). However, the cell population showed no increase in G2/M phase when we knocked down IER5 gene using IER5-specific siRNA (Figure 2C, 2D). These results suggested that the overexpression of IER5 increased cell cycle arrest at the G2/M phase, promoted apoptosis, and enhanced the tumor sensitivity to radiation treatment. Our results were consistent with the findings of other studies revealing that the suppression of IER5 by RNA interference technology dramatically increased the radioresistance of HeLa cells to radiation treatment [7].

HCC has been reported to be exceedingly resistant to radio- or chemotherapy [13]. Our results indicated that the upregulation of IER5 protein could be used to promote tumor cell responses to the treatment with radiation and might become a new strategy for hepatocellular carcinomas therapy. The findings of the present examination might also contribute to understanding better tumor radiosensitivity, which will exert favorable effects on the therapeutic outcomes in liver cancer patients.

Previous studies have shown that cell proliferation and apoptosis are critical steps in tumor metastasis. In an investigation, the imbalance between cell proliferation and apoptosis enhanced the growth of tumor cells [19]. Apoptosis is essential for the maintenance of inherited genomic integrity and is a fundamental cellular process which orchestrates a series of controlled events and ultimately leads to cell death [18]. Caspases are major proteases involved in apoptosis. The caspase family members contribute to cellular disintegration via the action of cleavage proteins involved in many processes in the cell, such as DNA repair and checkpoint activation [20]. Caspase-3 is a key protease that controls other caspase members in the programmed cell death (PCD) [21]. Impaired apoptosis is a crucial step in the process of cancer development [22]. Of the members of the caspase family, caspase-3, caspase-6, and caspase-7 have been shown to be the major effectors in apoptosis [18, 23]. Many anticancer agents induce apoptosis, which trigger the apoptosis via caspase activation [3, 24-26]. Our results indicated that the overexpression of IER5 substantially increased the cleavage of caspase-3 and PARP after either γ-ray irradiation treatment or cisplatin treatment compared with the control cells with no IER5 expression (Figure 3A, 3E). Interestingly, we also found that phospho-Akt (p-Akt) was inhibited in the HepG2/IER5 cells investigated (Figure 3B). Therefore, it is possible that the overexpression of IER5 not only activates the DNA damage signaling pathway, but also inactivates the MAP kinase signaling pathway, as speculated in previous reports [27]. Our results are in agreement with those of Dr. Luo, who found that Akt deactivation was associated with both caspase-independent and caspase-dependent cell death in multiple cellular systems [28]. Herein, our research data indicated that IER5 could induce apoptosis by inhibiting p-Akt.

As reported earlier, similarly to p53, p73 could also be involved in cell apoptosis induced by irradiation [29]. There was also evidence that the radiation-induced bystander effect caused the activation of p21, nuclear factor-κB (NF-κB), Bax, Bcl-2, and the cleavage of poly(ADP-ribose) polymerase, which indicated that there were signals transmitted from the target cells to bystander cells in a paracrine manner [30]. However, in our IER5 overexpression models, we did not discover any evidence that those tumors suppressed the expression of genes or oncogenes, such as P53, P21, P73, or the Bcl-2 family members, including Bcl-2, Bcl-x, and Bax genes which are involved in the apoptosis signaling pathway (Figure 3C, 3D).

Our results provide experimental evidence that the overexpression of IER5 activated and promoted both irradiation-induced and cisplatin-induced apoptosis and inhibited the growth of hepatocellular carcinoma cells. Our findings might facilitate the better understanding of tumor radiosensitivity and promote the development of new strategies for liver cancer treatment targeting IER5 gene.

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Disclosure of conflict of interest

None.

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