Enhancement of Tumor Response by Wortmannin in C3H/HeJ Hepatocarcinoma

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1. Introduction

Phosphatidylinositol 3-kinase (PI3K) has been known as one of key molecules in survival signaling [1,2]. PI3K signaling is an important intracellular mediator which is involved in multiple cellular functions including proliferation, differentiation, anti-apoptosis, tumorigenesis, and angiogenesis [3]. Inhibition of this molecule might be used in cancer treatment. Wortmannin has been reported to be an efficient radiosensitizer [4]. The objective of this study was to explore whether PI3K inhibitor, wortmannin, could potentiate the antitumor effect of radiation in vivo, particularly on radioresistant murine tumor.

2. Methods and Results

C3H/HeJ mice bearing syngeneic hepatocarcinoma (HCa-I) were treated with wortmannin or 25 Gy radiation or both. Wortmannin was administered 1 mg/kg once daily intraperitoneally for 14 days. Tumor response to the treatment was determined by a tumor growth delay assay. To explore the mechanism underlying interaction between the drug and radiation, the level of apoptosis and regulating molecules were examined. The expression of regulating molecules was analyzed by Western blotting for p53, p21.

2.1 Tumor growth delay assay

The mice were randomly assigned to a treatment group when the tumors grew to 8 mm in diameter. The tumors were measured regularly for tumor growth delay after treatment. The effect of radiation on tumor growth was determined by measuring three orthogonal tumor diameters with caliper at 2-day intervals until tumors grew to at least 12 mm in diameter. The effect of the treatment on tumor growth delay (AGD), which was defined as the time in days for the tumors to reach 12 mm in treated group minus the mean time to reach 12 mm in the untreated control group. The enhancement factor (EF) was calculated by dividing the normalized tumor growth delay (NGD) by the AGD. The NGD was defined as the time in days for tumors to reach 12 mm in mice treated by the combination treatment minus the time in days for tumors to reach 12 mm in treated group by drug only.

The drug increased the effect of tumor radioresponse with an enhancement factor (EF) of 1.9.

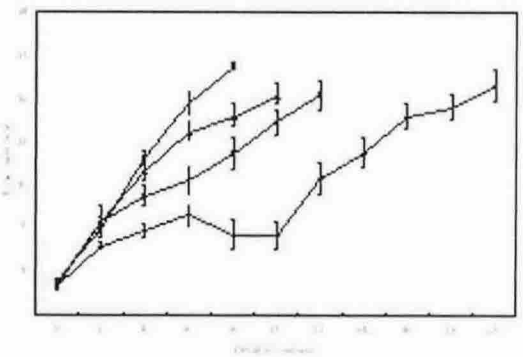

Figure 1. In tumor growth delay assay, wortmannin increased the antitumor effect of radiation with an enhancement factor (E.F.) of 1.9 (♦). ; control (■), radiation (●), wortmannin (▲), combination of radiation and wortmannin (♦).

2.2 Analysis of apoptosis

Apoptosis was assessed in tissue sections. The tumors were immediately excised and placed in neutral buffered formalin at 4, 8, 12, and 24 h after treatment. The tissues were embedded in paraffin blocks and four-micrometer sections were then cut and stained with the ApopTag staining kit (Oncor, Gaithersburg, MD.) [5]. Apoptotic cells were scored on coded slides at 100X magnification according to the morphological features. Ten fields of nonnecrotic areas were selected randomly across each tumor section, and in each field apoptotic bodies were expressed as a percentage based on the scoring of 1000 nuclei at each time interval after treatment. Combined treatment of 25 Gy radiation with wortmannin increased radiation induced apoptosis additively; peak apoptotic index was 11% in radiation alone, 13 % in drug alone and 19% in the combination treatment group.

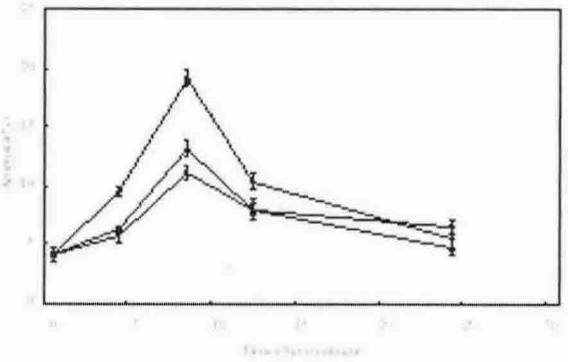

Figure 2. The level of induced apoptosis in radiation alone (●, 11%), wortmannin alone (▲, 13%), combination of radiation and wortmannin (♦, 19%).

2.3 Western blot analysis

Tumor tissues were collected from tumor-bearing mice at different times from 4 to 24h after treatment. Small pieces of tumors were washed three times in ice-cold phosphate-buffered saline (PBS), and lysed in a cold buffer containing 100 mM HEPES, 200 mM NaCl, 20% glycerol, 2% NP40, 2 mM EDTA, 40 mM β-glyceraldehyde-phosphate, 2 mM sodium fluoride, 1 mM DTT, 1 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, 5 ㎍/ml leupeptin, 2 ㎍ /ml aprotinin for 1 hour. The samples were centrifuged at 4 ℃ for 20 min, and supernatants were transferred into new tubes. The lysates were then denatured at 100 ℃ for 5 min in the presence of 5% β mercaptoethanol and loaded onto polyacrylamide gels. Proteins applied to each lane of the polyacrylamide gel were adjusted to equal concentrations with a Bio-Rad protein assay (Bio-Rad, Hercules, CA). Proteins were fractionated using SDS-PAGE and transferred onto a nitrocellulose membrane (Milipore Corporation, Bedford, MA) in a transfer buffer, consisting of 48 mM/*l* Tris base, 20% methanol, 0.04% SDS, and 30 mM/*l* glycine. The membranes were incubated for 2 h at room temperature with each primary antibody at the appropriate dilution, as recommended by the supplier.

Antibodies included p53 and $p21^{WAF1/CIP1}$ (Ab-7, Ab-5, Oncogene Science, NY). After washing in TBST, the membranes were subsequently incubated for 1h at room temperature in either an antisheep or antimouse (Cell Signaling Technology) immunoglobulin (Ig) G antibody conjugate (Santa Cruz Biotechnology Inc.). Detectable proteins were quantitated using densitometry (Amersham Pharmacia Biotech) after chemiluminescence detection (Fuji film, Japan) using ECL western blotting detection system (Amersham Pharmacia Biotech) [6].

Analysis of apoptosis regulating molecules with Western blotting showed upregulation of p53, $p21^{WAF1/CIP1}$ in the combination treatment group comparing to those in either radiation alone or drug alone group.

To explore the effect of wortmannin on the radiation-induced changes, the regulating molecules were investigated. Expression of p53, $p21^{WAF1/CIP1}$ by radiation alone, drug alone, or a combination with wortmannin was analyzed. When radiation and the drug therapy were combined, the most significant change was shown in $p21^{WAF1/CIP1}$. It reached a peak level to 2.01-fold compared to radiation or drug alone at 8 h (figure 3). p53 of combined group increased to 1.86-fold at 4 h after irradiation compared to other groups (Data not shown).

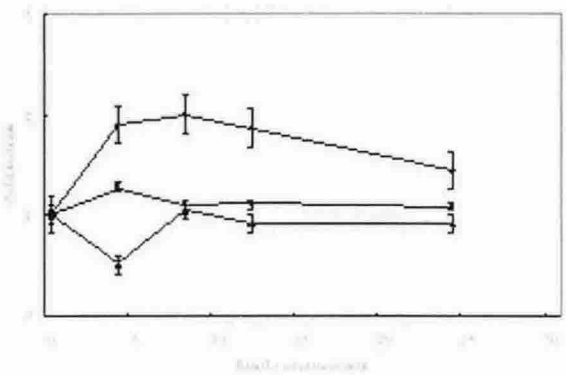

Figure 3. Analysis of apoptosis regulating molecules with western blotting showed upregulation of $p21^{WAF1/CIP1}$ in the combination group comparing to those in either radiation alone or wortmannin alone group. Densitometric analyses are plotted for radiation (●), wortmannin (▲), combination of radiation and wortmannin (♦).

3. Conclusion

In murine hepatocarcinoma, the antitumor effect of radiation could be potentiated by use of wortmannin. The mechanism seems to involve other than the increase of induced apoptosis. Wortmannin in combination with radiation therapy may have potential benefit in cancer treatment.

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