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Enhancement of Tumor Radioresponse by Wortmannin in C3H/HeJ Hepatocarcinoma

Wonwoo KIM, Jinsil SEONG*, Jung Hee AN and Hae Jin OH

Wortmannin/Radiation/Apoptosis/Hepatocarcinoma.

The objective of this study was to explore whether a specific inhibitor of PI3K, wortmannin, could potentiate the antitumor effect of radiation *in vivo*, particularly on radioresistant murine tumors. C3H/HeJ mice bearing syngeneic hepatocarcinoma (HCa-I) were treated with 25 Gy radiation, wortmannin, or both. Wortmannin was administered intraperitoneally (1 mg/kg) once daily for 14 days. Tumor response to treatment was determined by a tumor growth delay assay. Possible mechanisms of action were explored by examining the level of apoptosis and regulating molecules. The expression of regulating molecules was analyzed by Western blot for p53 and $p21^{WAF1/CIP1}$, and immunohistochemical staining for $p21^{WAF1/CIP1}$, CD31 and VEGF. In the tumor growth delay assay, wortmannin increased the effect of tumor radioresponse with an enhancement factor (EF) of 2.00. The level of apoptosis achieved by the combined treatments was shown to be no more than an additive effect; peak apoptotic index was 11% in radiation alone, 13% in wortmannin alone, and 19% in the combination group. Markedly increased areas of necrosis at 24 h in the combination group were noted. Western blotting showed upregulation of $p21^{WAF1/CIP1}$ in the combination treatment group, which correlated with low levels of VEGF. Microvascular density was evidently also reduced, based on low expression of CD31. In murine hepatocarcinoma, the antitumor effect of radiation was potentiated by wortmannin. The mechanism seems to involve not only the increase of induced apoptosis but also enhanced vascular injury. Wortmannin, in combination with radiation therapy, may have potential benefits in cancer treatment.

INTRODUCTION

Ionizing radiation, like a variety of other cellular stress factors, initiates apoptosis, or programmed cell death, in many cell systems.[1] The activation of the execution machinery results in efficient elimination of the cell, which has prompted the examination of this mechanism as a potential tool to kill cancer cells.[2] Radiotherapy efficiency, however, is also impacted by each tumor's radioresponse. Intrinsic radioresistance could limit radiation treatment,[3–9] and so recent efforts have been made to enhance radiosensitivity. The synergistic combination of radiotherapy and chemotherapy has been shown to enhance tumor response.[10]

Phosphatidylinositol 3-kinase (PI3K) plays a central role in cell growth regulation and possibly tumorigenesis.[11–14] P13K is an important intracellular mediator involved in multiple cellular functions, including proliferation, differentiation, anti-apoptosis, tumorigenesis and angiogenesis.[15]

Akt, a protein kinase B (PKB), is a downstream molecule of PI3K. Akt has also been shown to phosphorylate, and thereby inactivate, the proapoptotic proteins Bad and caspase-9.[16] Accordingly, inhibition of this molecule might serve as an effective cancer treatment.

Wortmannin, a PI3K inhibitor, has been reported to enhance radiation-induced apoptosis and cytotoxicity in endothelial cells.[15,17,18] In several *in vitro* studies, wortmannin enhanced radiation-induced growth inhibition of the tumor cell lines GL261, MCF-7, Saos-2 and TK6.[19–22] Wortmannin also inhibits other PtdIns-3-kinase family members, PtdIns-4-kinase and PKB/Akt phosphorylation[23].

The objective of this study was to explore whether the PI3K inhibitor, wortmannin, could potentiate the antitumor effects of radiation *in vivo*, particularly on radioresistant murine tumors.

MATERIALS AND METHODS

Animals and Tumors

Male C3H/HeJ mice, 8–10 weeks old, were used for this

*Corresponding author: Phone: +82-2-2228-8111,
Fax: +82-2-312-9033,
E-mail: jsseong@yumc.yonsei.ac.kr
Department of Radiation Oncology, Brain Korea 21 Project for Medicine, Yonsei University, Seoul, South Korea.
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study. The care and use of the animals were in accordance with the guidelines and regulations of Yonsei University. The murine hepatocarcinoma syngeneic to the C3H/HeJ and HCa-I cell lines is a highly radioresistant tumor with a median tissue culture dose of > 80 Gy. Tumors were generated by inoculating viable tumor cells into the right thigh muscles of the mice. Tumor cell suspensions were prepared as previously described.[24)]

Treatment and Tumor Growth Delay Analysis

For tumor growth delay analysis, four experimental groups were set: control, radiation alone, wortmannin alone, and wortmannin + radiation (RT). There were 10 mice in each group. Wortmannin (C23H24O8; molecular weight = 428.43) was obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). The radiation-alone group was irradiated when the tumors had grown to a mean 7.5–8 mm in diameter. The tumor-bearing legs were treated with a single dose of 25 Gy using a linear accelerator (Varian Co., Milpitas, CA, USA). The wortmannin-alone group was given 1 mg/kg once daily intraperitoneally for 14 days when the tumors had grown to a mean 6.5–6.8 mm in diameter.[20)] The described therapies were combined to treat the wortmannin + RT group. 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 calipers at 2-day intervals until the tumors grew to at least 12 mm in diameter.[25)] The effect of the treatment on tumor growth delay (Absolute growth delay: AGD) was defined as the time in days for the tumors to reach 12 mm in the treated group minus the mean time to reach 12 mm in the untreated control group. The enhancement factor of tumor radioresponse was obtained by dividing normalized tumor growth delay (NGD) with absolute tumor growth delay (AGD) caused by radiation. 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 the treated group by wortmannin only.[25,26)]

Animals were closely observed for any occurrence of toxicity until the last observation day.

Analysis of Apoptosis

For analysis of apoptosis, four experimental groups were set: control, radiation alone, wortmannin alone, and wortmannin + RT. There were 5 mice in each group and treatments were the same as described above. 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 4-μm sections were then cut and stained with the ApopTag staining kit (Oncogene, Cambridge, MA, USA).[27)] Apoptotic cells were scored on coded slides at 400X magnification according to the Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method. TUNEL-positive cells were considered apoptotic only when having apoptotic morphology. Ten fields of non-necrotic areas were selected randomly across each tumor section, and in each field, apoptotic bodies were expressed as a percentage of 1000 nuclei at each time interval after treatment.

Western Blot Analysis

Regulatory molecules of apoptosis were analyzed by Western blot. Tumor tissues were collected from tumor-bearing mice at different times from 4 to 24 h 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 μg/mL leupeptin and 2 μg/mL aprotinin for 1 hour. The samples were centrifuged at 4°C for 20 min, and supernatants were transferred into new tubes. The lysates were then denatured at 100°C for 5 min in the presence of 5% mercaptoethanol and loaded onto polyacrylamide gels. Proteins applied to each lane of the 13% polyacrylamide gel were adjusted to equal concentrations with a Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA). Proteins were fractionated using SDS-PAGE and transferred onto a nitrocellulose membrane (Milipore Corporation, Bedford, MA, USA) 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 targeted p53 and $p21^{WAF1/CIP1}$ (Ab-7, Ab-5, Oncogene). After washing in TBST, the membranes were subsequently incubated for 1 h at room temperature with either an anti-sheep or anti-mouse (Cell Signaling Technology, Beverly, Massachusetts, USA) immunoglobulin (IgG) antibody conjugate (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA). Detectable proteins were quantitated using densitometry (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) after chemiluminescence detection (Fuji photo film, Tokyo, Japan) using the ECL western blot detection system (Amersham Pharmacia Biotech).[28,29)]

Immunohistochemical Stain

Immunohistochemical staining was performed with 4-μm, formalin-fixed, paraffin-embedded tissue samples. After incubating the slide sections attached to a silane-coated slide overnight at 37°C, the tissue sections were deparaffinized in xylene (3 × 10 min) and rehydrated through a series of graded alcohols (100%, 95%, 90%, 80%, 70%) to diluted water. The deparaffinized sections were then heated and boiled (2 × 10 min) by microwaving in a 0.01 M citrate buffer (pH

6.0) to retrieve the antigens. Mouse monoclonal antibodies targeting the $p21^{WAF1/CIP1}$ protein (sc-6246; 1/100 dilution; Santa Cruz Biotechnology Inc.), VEGF protein (sc-7269; 1/100 dilution; Santa Cruz Biotechnology Inc.), Mouse monoclonal anti- proliferating cell nuclear antigen (PCNA) clone PC10 (M0879; 1/100 dilution; Dako A/S.) or CD31 (PECAM-1) protein (557355; 1/100 dilution; PharMingen, Fallbrook CA, USA) were applied and incubated at 4°C overnight. After washing three times with PBS, sections were incubated with the biotinylated link (LSAB2; Dako A/S, Glostrup, Denmark) for 20 min. They were then washed three times with PBS, treated with streptavidin-hrp (LSAB2; Dako A/S.) for 20 min, and washed again with PBS three times. The peroxidase binding sites were detected by staining with diaminobenzidine (DAB; DAKO A/S.), and the sections were finally counterstained with Mayer's hematoxylin and observed under a light microscope.

The expression of $p21^{WAF1/CIP1}$ and PCNA were assessed according to the mean ± standard error (SE) of $p21^{WAF1/CIP1}$ and PCNA –positive nuclei in a total of 1000 tumor cells. Microvascular density was assessed according to the mean ± standard deviation (SD) of immunoreactive vessels in three areas of highest intensity.

For VEGF, samples were given an additive immunoreactive score (IRS) composed of the signal intensity (expression grade 0 = very weak, 1 = weak, 2 = moderate, 3 = heavy) plus the number of VEGF-positive cells (0 = no staining, 1 = 1–10%, 2 = 10–50%, 3 = > 50%); total ≤ 4, IRS = 1+, ≥ 5 = 2+.

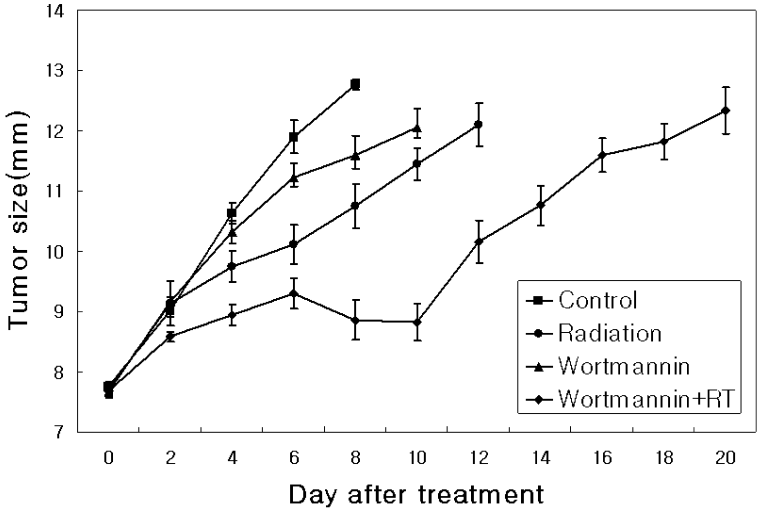

Fig 1. Tumor growth delay assay of HCa-I treated with radiation (●), wortmannin (▲) or wortmannin + RT (◆). Wortmannin increased the antitumor effect of radiation with an enhancement factor (E.F.) of 2.00.

Table 1. Effect of wortmannin on radioresponse of HCa-I tumor: Influence of time interval between wortmannin administration and radiation delivery

Treatment[a]	Time in days that tumors are required to grow from 8 to 12 mm[b]	AGD[c]	NGD[d]	EF[e]
Control	5.52 ± 0.23			
Radiation	10.28 ± 1.36	4.76		
Wortmannin	8.54 ± 0.83	3.02		
Wortmannin + RT	18.08 ± 1.55	12.56	9.54	2.00

[a]Mice bearing 8-mm tumors in the right thighs were giveni p. 1 mg/kg wortmannin or 25 Gy local tumor irradiation. Groups consisted of 10 mice each.
[b]Mean ± SE.
[c]AGD (Absolute growth delay) is defined as the time in days forutmors in the treated groups (wortmannin or radiation or wortmannin + RT) to grow from 8 to 12 mm minus the time in days for tumors in the untreated control group to reach the samesize.
[d]NGD (Normalized tumor growth delay) is defined as the time forumors in groups treated with wortmannin + RT to grow from 8 to 12 mm minus the time to reach the samesize in mice treated with wortmannin alone.
[e]EF was calculated as the ratio of NGD in mice treated with wortmannin + RT to AGD in mice treated by radiation alone.

Statistical Analysis

Results are expressed as mean ± SE, mean ± SD and IRS. For comparison of means, a t-test was used. All tests were two-sided, and a P-value less than 0.05 indicated statistical significance.

RESULTS

Enhancement of Tumor Radioresponse by Wortmannin

The time for tumor growth from 8 to 12 mm was 10.28 days and 8.54 days in the radiation-alone and the wortmannin-alone groups, respectively, which corresponds with an AGD of 4.76 days (radiation-alone) and 3.02 days (wortmannin-alone). When radiation was combined with wortmannin, the time for growth from 8 to 12 mm was 18.08 days and the NGD was 9.48 days with an enhancement factor of 2.00. These data suggest that wortmannin increased the antitumor effect of radiation (Fig. 1). During the entire observation time for tumor growth delay, no significant toxicity why seen including locomotor activity behavioral change.

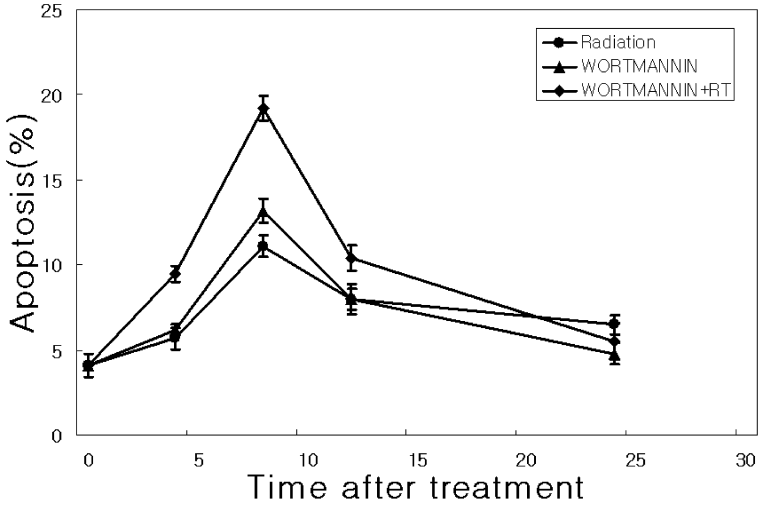

Fig 2. The level of induced apoptosis in HCa-I. The maximum was 11% in radiation alone (●), 13% in wortmannin alone (▲), and 19% in wortmannin + RT (◆), suggesting no more than additive effect.

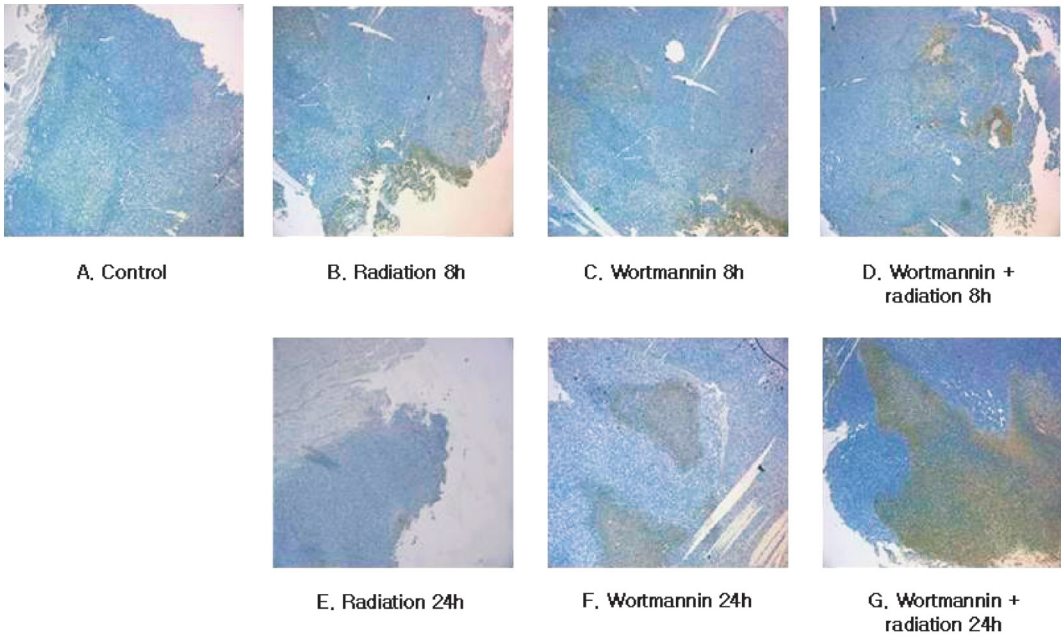

Fig 3. TUNEL assay of HCa-I tissues. In the combination group markedly increased areas of necrosis were noted at 24h. A. Control, B. Radiation 8h, C. Wortmannin 8h, D. Wortmannin + RT 8h, E. Radiation 24h, F. Wortmannin 24h, G. Wortmannin + RT 24h.

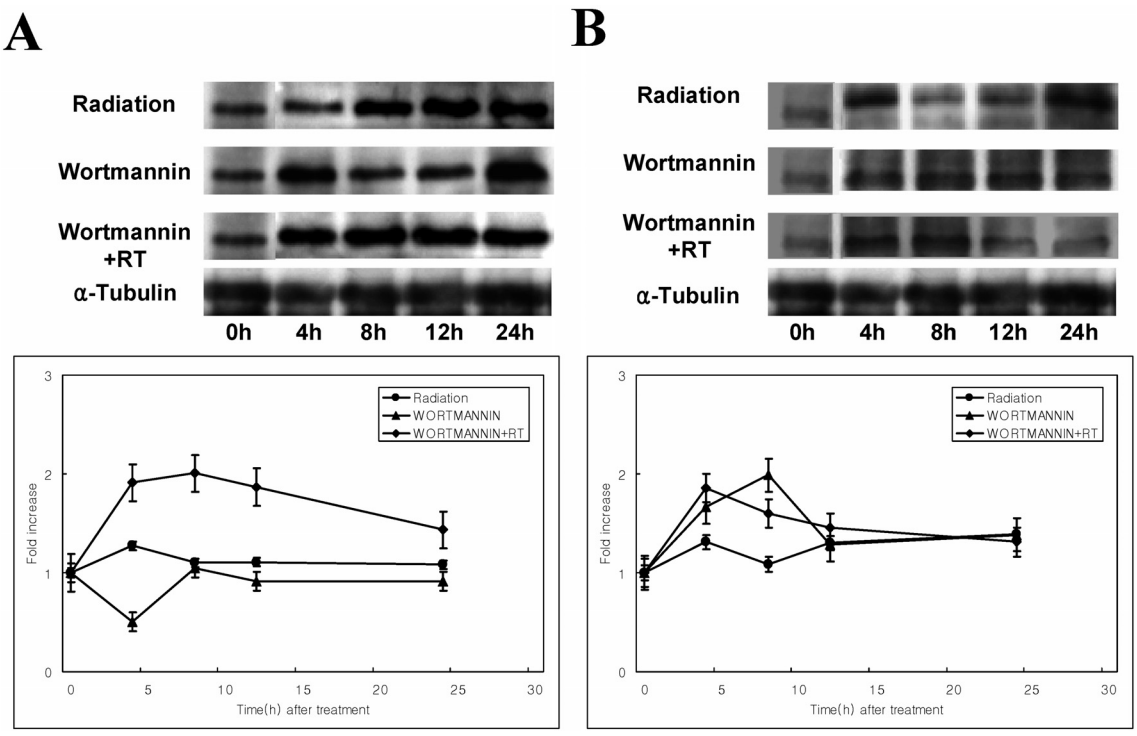

Fig 4. Western blot analysis of apoptosis regulating molecules $p21^{WAF1/CIP1}$ (A) and p53 (B). Densitometric analyses showed a significant increase of $p21^{WAF1/CIP1}$ expression in the wortmannin + RT group (◆) in comparison to radiation alone (●), or wortmannin alone (▲).

Table 2. Comparison of $p21^{WAF1/CIP1}$, CD31, VEGF and PCNA expression by immunohistochemical staining

Group	* p21 mean ± SE	†CD31 mean ± SE	•‡VEGF grade	• PCNA mean ± SE
Control	9.3 ± 1.38	23.33 ± 3.21	2+	55.3 ± 1.09
Radiation 4h	14.2 ± 0.95 p*	21.33 ± 2.08 NS	1+	43.2 ± 1.27
Radiation 8h	12.1 ± 1.03 NS	20.33 ± 3.21 NS	1+	38.1 ± 1.02
Radiation 12h	12.4 ± 1.60 NS	18.67 ± 2.08 NS	2+	35.6 ± 1.20
Radiation 24h	12.5 ± 1.38 NS	15.67 ± 0.58 p*	2+	32.5 ± 1.37
Wortmannin 4h	6.6 ± 0.93 NS	21.67 ± 2.52 NS	2+	40.8 ± 1.10
Wortmannin 8h	11.2 ± 1.06 NS	21.33 ± 2.08 NS	1+	37.2 ± 0.98
Wortmannin 12h	9.0 ± 1.34 NS	20.67 ± 2.08 NS	1+	35.2 ± 1.60
Wortmannin 24h	9.1 ± 1.24 NS	18.67 ± 3.21 NS	1+	32.9 ± 0.59
Wortmannin + radiation 4h	17.9 ± 2.39 p*	15.33 ± 2.08 p*	1+	32.4 ± 0.60
Wortmannin + radiation 8h	21.2 ± 1.43 p*	13.00 ± 2.65 p*	1+	29.1 ± 0.69
Wortmannin + radiation 12h	16.5 ± 1.48 p*	9.67 ± 2.08 p*	1+	26.5 ± 0.67
Wortmannin + radiation 24h	15.9 ± 1.08 p*	6.67 ± 1.15 p*	1+	23.9 ± 0.74

* $p21^{WAF1/CIP1}$ mean ± SE: The number of p21-positive nuclei, The mean ± standard error (SE) of $p21^{WAF1/CIP1}$-positive nuclei in a total of 1000 tumor cells,; †CD31 mean ± SD: The number of CD31-positive vessels, The mean ± standard deviation (SD) of immunoreactive vessels in three areas of highest intensity,; ‡VEGF: immunoreactive score (IRS) = The signal intensity (expression grade 0 = very weak, 1 = weak, 2 = moderate, 3 = heavy) + the number of VEGF-positive cells (0 = no staining, 1 = 1–10%, 2 = 10–50%, 3 = >50%) = ≤ 4: 1 +, ≥ 5: 2+)• PCNA mean ± SE: The number of PCNA-positive nuclei, The mean ± standard error (SE) of PCNA-positive nuclei in a total of 1000 tumor cells, ; NS: not significant.

Induced Apoptosis by Wortmannin and Radiation

With radiation alone, the peak level of induced apoptosis was 11.1% at 8 h, decreasing to 6.5% at 24 h. The level of wortmannin-induced apoptosis gradually increased to a peak level of 13.2% at 8 h, and then declined to 4.8% at 24 h. The apoptosis induced by the combination of radiotherapy and wortmannin increased gradually to 9.5% at 4 h and 19.2% at 8 h, but then decreased to 5.5% at 24 h. The level of apoptosis achieved by the combined treatments was shown to be no more than an additive effect (Fig. 2).

While apoptosis was not significantly increased by the combination treatment, tumor necrosis was. The area of necrosis was negligible at 8 h when treated with radiation alone and wortmannin alone, but markedly increased areas of necrosis were noted at 24 h in the wortmannin + RT group (Fig. 3).

These data suggest that an enhancement of tumor growth delay in the combination group could be partly explained by increased tumor necrosis.

Change in the Expression of Apoptosis-Regulating Molecules

Analysis of apoptosis regulating molecules with western blot showed upregulation of p53 at 4 h and $p21^{WAF1/CIP1}$ at 8 h in the combination treatment group compared to those treated with radiation alone or wortmannin alone. When radiation and wortmannin were combined, the most significant change was seen in $p21^{WAF1/CIP1}$, which reached a peak level of 2.1-fold at 8 h and still remained high at 24 h compared with radiation alone or wortmannin alone (Fig. 4.A). The level of p53 in the combined group increased to 1.86-fold at 4 h after irradiation compared to other groups (Fig. 4.B), then started to gradually decrease.

Although p53 expression peaked at different times for each group, all groups had a similar level of expression 24h after treatment.

Immunohistochemical Staining for $p21^{WAF1/CIP1}$, CD31, VEGF and PCNA

When radiation and wortmannin were combined, the expression of $p21^{WAF1/CIP1}$ was greater than with radiation alone or wortmannin alone (Table 2).

CD31 was overexpressed in the control group. Its expression decreased with radiation alone and wortmannin alone in comparison to the control group, and was the lowest in the combination group (Fig. 5).

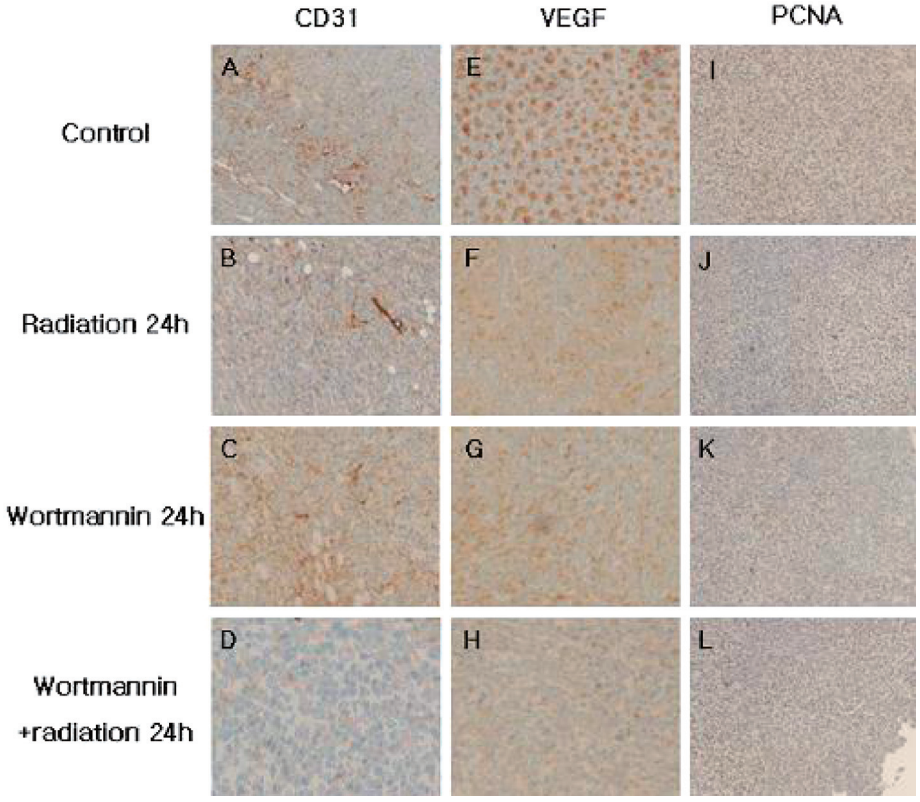

Fig 5. Immunohistochemical staining of CD31, VEGF and PCNA in HCa-I tissues. The expression of CD31 and VEGF in the combination group was significantly lower than with radiation or wortmannin alone. CD31: A. Control, B. Radiation 24h, C. Wortmannin 24h, D. Wortmannin + RT 24h, VEGF: E. Control, F. Radiation 24h, G. Wortmannin 24h, H. Wortmannin + RT 24h, PCNA: I. Control, J. Radiation 24h, K. Wortmannin 24h, L. Wortmannin + RT 24h.

VEGF was also overexpressed in the control group. Its expression was lower in the combination group compared with radiation alone or wortmannin alone (Fig. 5). These data suggest that vascular injury might be involved in the mechanism enhancing the antitumor effect in the combined group.

DISCUSSION

Phosphatidylinositol 3-kinase (PI3K) plays a central role in the control of metabolism, cell growth, proliferation, survival and migration, as well as membrane transport and secretion. This molecule plays a major role not only in tumor development but also in tumor response to cancer treatment.[14] The PI3K family of enzymes is well characterized with respect to promotion of cellular growth, survival and suppression of apoptosis in cancer cells. These kinases can be activated from a cell surface growth factor receptor and are known to play a critical role in regulating the balance between cell survival and apoptosis. For these reasons, we asked whether a pharmacological inhibitor of PI3K might be used as a treatment for cancer. Wortmannin has antitumor activity against a variety of tumor xenografts in animal models.[30] Wortmannin also inhibits other PtdIns-3-kinase family members such as DNA-dependent protein kinase, ataxia telangiectasia mutated, ataxia telangiectasia related, the mammalian target of rapamycin and PtdIns-4-kinase.[31] Among them, inhibition of PI3K has been used to try to enhance radiation-induced apoptosis and inhibition of tumor growth.[17,18]

In this study, we showed that wortmannin delayed tumor growth in HCa-I, a well-known radioresistant tumor cell line. Wortmannin induced an enhanced tumor radioresponse (EF of 2.00) compared with either treatment alone, suggesting a possible beneficial interaction between radiation and wortmannin.

To further investigate the mechanism underlying enhancement of tumor radioresponse by wortmannin, we observed the level of apoptosis induced by radiation alone, wortmannin alone and the combined treatment. We found that the level of apoptosis in the combination group was no more than an additive effect, and that the level of apoptosis was not significantly increased by combination treatment. Shi *et al*. showed *in vitro* that wortmannin enhanced radiation-induced apoptosis[15] using the TK6 human lymphoblastoid line irradiated with 2 Gy X-rays. Tomita *et al*. showed the same result[32] using human leukemia MOLT-4 cells irradiated with X-rays. Because we used the *in vivo* tumor HCa-I, which is highly radioresistant, the mechanisms of action cannot be directly compared to other reports. However, the marked increase of necrosis at 24 h in the combination group suggests that a mechanism other than apoptosis induction of tumor cells may be at work. This part needs further investigation.

To examine the potential mechanism of interaction between radiation and wortmannin, we investigated the impact of treatment on tumor blood vessels using immunohistochemical staining. Kim *et al*. reported that wortmannin markedly inhibited tube formation of the tumor.[33] Edward *et al*. demonstrated that PI3K antagonists enhanced radiation-induced destruction of tumor blood vessels.[19] We used CD31 as a marker for microvessel density, and found high expression in the tumor control group. CD31 expression was lower at 24 h in the combination group in comparison to radiation alone or wortmannin alone. Since VEGF is a potent inducer of new vessel growth, we also examined its expression. Gounis *et al*. showed that the expression of CD31-positive nuclei parallels VEGF concentration.[34] In our study, when radiation and wortmannin were combined, VEGF expression was also lower than with radiation alone or wortmannin alone. These data suggest that vascular injury might be involved in enhancing the antitumor effect observed in the combined group.

Taken together, these results suggest that wortmannin, in combination with radiation therapy, could be useful in treating tumors. Wortmannin appears to act by a complex mechanism of action involving enhancement of radiation-induced apoptosis and vascular injury.

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