Enhancement of tumor radioresponse by wortmannin in C3H/HeJ hepatocarcinoma

Wonwoo KIM

Department of Medical Science

The Graduate School, Yonsei University

Enhancement of tumor radioresponse by wortmannin in C3H/HeJ hepatocarcinoma

Directed by Professor Jinsil Seong

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Wonwoo Kim

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Thesis Supervisor: Jinsil Seong

Sun Young Rha

Jong Doo Lee

The Graduate School

Yonsei University

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LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Enhancement of tumor radioresponse by wortmannin in C3H/HeJ hepatocarcinoma

Wonwoo Kim

Department of Medical Science

The Graduate School, Yonsei University

(Directed by Professor Jinsil Seong)

Phosphatidylinositol 3-kinase (PI3K) has been known as one of the key molecules in survival signaling, involving proliferation, differentiation, anti-apoptosis, tumorigenesis, and angiogenesis. In this regard, inhibition of PI3K may be useful in improving the therapeutic efficacy of established anticancer agents. The objective of this study was to explore whether specific inhibitor of PI3K, wortmannin, could potentiate the antitumor effect of radiation in vivo, particularly on radioresistant murine tumor.

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 fundamental interaction between wortmannin and radiation, the level of apoptosis and regulating molecules were examined. The expression of regulating molecules was analyzed by Western blotting for p53, $p21^{WAF1/CIP1}$. Immunohistochemical stained for $p21^{WAF1/CIP1}$, CD31 and VEGF.

In tumor growth delay assay, the drug increased the effect of tumor radioresponse with an enhancement factor (EF) of 1.9. The level of apoptosis achieved by the combined treatments was shown to be no more than an additive effect; peak apoptotic index was 1.1% in radiation alone, 1.3% in drug alone and 1.9% in the combination group. The markedly increased area of necrosis at 24 h in the combination group was noted. Western blotting showed enhanced upregulation of $p21^{WAF1/CIP1}$ in the combination treatment group, which correlated with low level of VEGF. Microvascular density was also evidently decreased by a low expression of CD31.

In murine hepatocarcinoma, the antitumor effect of radiation could be potentiated by the use of wortmannin. The mechanism seems to involve not only the increase of induced apoptosis in an additive way but also enhanced vascular injury by wortmannin. Wortmannin in combination with radiation therapy may have potential benefits in cancer treatment.

Key words: wortmannin, radiation, apoptosis, hepatocarcinoma

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I. 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 the elimination of the cell. The efficiency of this mechanism has prompted the examination of the apoptotic execution machinery as a potential tool to kill cancer cells[2]. Efficiency of radiotherapy, however, is not only sufficient in

enhancing the antitumor efficacy but also determined with each tumor's radioresponse. Intrinsic radioresistance could be one of the limits in radiation treatment[3-9]. For conquest of this limitation, recent studies have made efforts to enhancement of radiosensitivity. The combination of radiotherapy and chemotherapy synergistically enhanced the tumor response[10].

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

A downstream molecule of PI3K is Akt, a protein kinase B (PKB). Akt has also been shown to phosphorylate and thereby inactivate the proapoptotic protein Bad, and, in a similar way, inactivate caspase-9, which is a crucial protein for execution of apoptosis[16]. Therefore, inhibition of this molecule might be used in cancer treatment.

Wortmannin, 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 tumor cell lines, including GL261, MCF-7, Saos-2 and TK6[18-22].

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

II. MATERIALS AND METHODS

1. Animals and tumors

Male C3H/HeJ mice, 8-10 weeks old, were used for this study. The care and use of the animals were in accordance with the guidelines and regulations of Yonsei University. The murine hepatocarcinoma syngeneic to C3H/HeJ, HCa-I, is a highly radioresistant tumor with a median tissue culture dose of >80 Gy. The tumors were generated by inoculating viable tumor cells into the muscles of the right thighs of the mice. Tumor cell suspensions were prepared as previously described[23].

2. Treatment and tumor growth delay analysis

For tumor growth delay analysis, four experimental groups were set: control, radiation alone, drug alone, and radiation plus drug; there were 10 mice in each group. Wortmannin ($C_{23}H_{24}O_8$; 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 7.5~8 mm mean 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 drug alone group was administered 1 mg/kg once daily intraperitoneally for 14 days when the tumors had grown to 6.5~6.8 mm mean in diameter[20]. The radiation plus drug group was treated as previously described. 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 the tumors grew to at least 12 mm in diameter[24]. 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 the 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 the treated group by drug only.

3. Analysis of apoptosis

For analysis of apoptosis, four experimental groups were set: control, radiation alone, drug alone, and radiation plus drug; there were 5 mice in each group. 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 treated as previously described. 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)[25]. Apoptotic cells were scored on coded slides at 400X magnification according to the Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL)-positive cells as apoptotic only when accompanied by apoptotic morphology. 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.

4. Western blot analysis

The regulating molecules of apoptosis were analyzed by Western blotting. 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 ㎍/ml leupeptin, 2 ㎍/ml aprotinin for 1 hour. The samples were centrifuged at 4℃ for 20 minutes, and supernatants were transferred into new tubes. The lysates were then denatured at 100℃ for 5 minutes in the presence of 5% 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, 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/λ Tris base, 20% methanol, 0.04% SDS, and 30 mM/λ 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, Cambridge, MA, USA). After washing in TBST, the membranes were subsequently incubated for 1 h at room temperature in either an antisheep or antimouse (Cell Signaling Technology, Beverly, MA, USA) immunoglobulin (Ig) G 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 blotting detection system (Amersham Pharmacia Biotech, Piscataway, New jersey, USA)[26].

5. Immunohistochemical stain

Immunohistochemical staining was performed with 4-㎛, formalin-fixed, paraffin-embedded tissue samples. After incubating the slide sections attached on a silane-coating slide, overnight at 37℃, 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. The antibodies used were: a mouse monoclonal antibody against $p21^{WAF1/CIP1}$ protein (sc-6246; 1/100 dilution; Santa Cruz Biotechnology Inc., Santa cruz, California, USA) at 4℃ for overnight; a mouse monoclonal antibody against VEGF protein (sc-7269; 1/100 dilution; Santa Cruz Biotechnology Inc., Santa cruz, California, USA) at 4℃ for overnight; a mouse monoclonal antibody against CD31(PECAM-1) protein (557355; 1/100 dilution; PharMingen, Fallbrook CA, USA) at 4℃ for overnight. After washing three times with PBS, sections were incubated with biotinylated link (LSAB2; Dako A/S, Glostrup, Denmark) for 20 minutes. They were then washed three times with PBS, treated with streptavidin-hrp (LSAB2; Dako A/S, Glostrup, Denmark) for 20 minutes, and again washed with PBS three times again. The peroxidase binding sites were detected by staining with diaminobenzidine (DAB; DAKO A/S, Glostrup, Denmark), and the sections finally counterstained with Mayer's hematoxylin and observed under a light microscope.

The expression of $p21^{WAF1/CIP1}$ was assessed according to the mean ±

standard error (SE) of $p21^{WAF1/CIP1}$-positive nuclei in a total of 1000 tumor cells.

Microvascular density was assessed the mean ± standard deviation (SD) of immunoreactive vessels in three areas of the highest intensity.

For VEGF, the immunhistochemical signals in tumor were evaluated using an additive immunoreactive score (IRS) composed of 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+.

6. Statistical analysis

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

III. RESULTS

1. Enhancement of tumor radioresponse by wortmannin

The time for tumor growth from 8 to 12 mm was 10.3 days and 8.5 days in radiation alone and in wortmannin alone group, respectively, which accords with 3.0 days and 4.8 days of the AGD, in wortmannin alone and radiation alone group. When radiation was combined with wortmannin, the time for growth from 8 to 12 mm was 18.1 days and NGD was 6.5 days. Enhancement factor was 1.9. This data suggests that wortmannin increased the antitumor effect of radiation (Fig. 1).

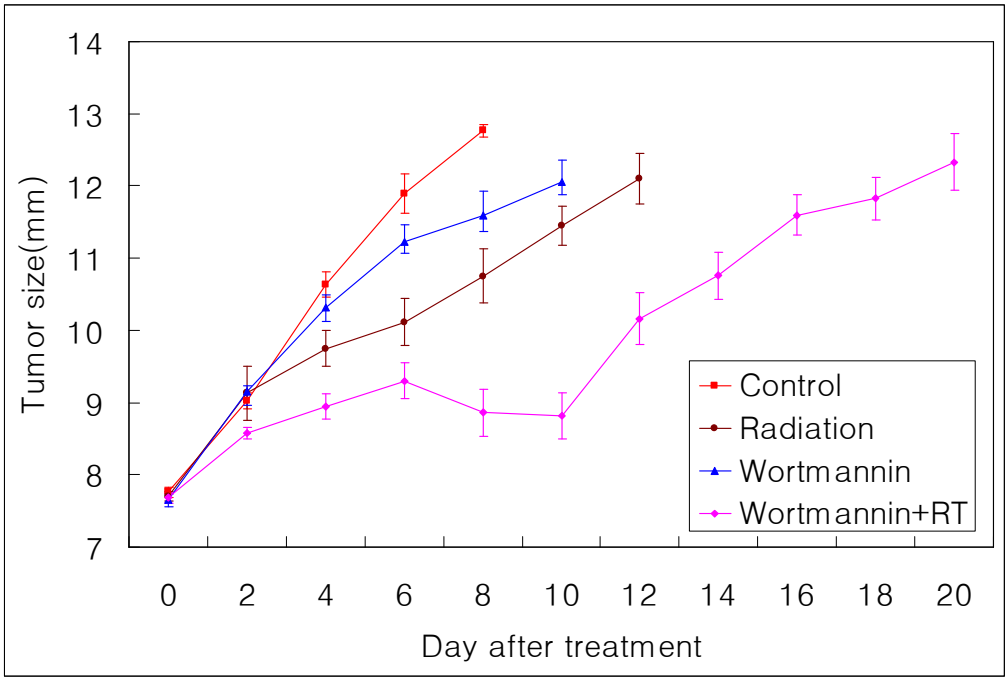

Fig. 1. Tumor growth delay assay of HCa-I treated with radiation (●), wortmannin (▲) or wortmannin + radiation (◆). Wortmannin increased the antitumor effect of radiation with an enhancement factor (E.F.) of 1.9.

2. Induced apoptosis by wortmannin and radiation

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

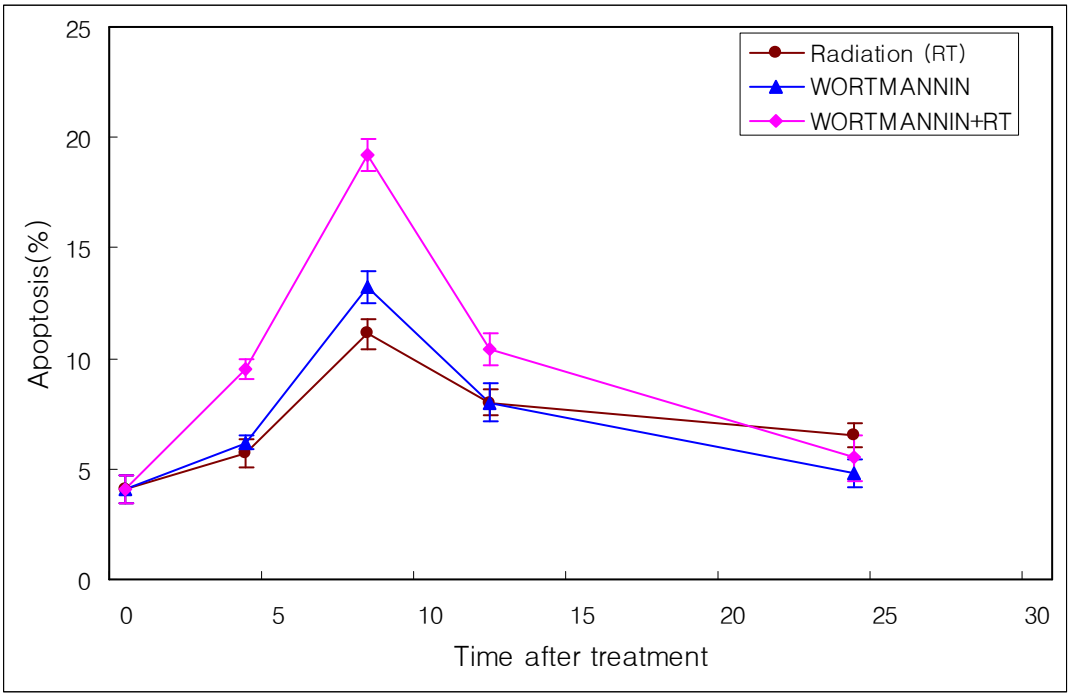

Fig. 2. The level of induced apoptosis in HCa-I. It was 11% in radiation alone (●), 13% in wortmannin alone (▲), and 19% in wortmannin + radiation (◆), suggesting no more than additive effect.

While the level of apoptosis was not significantly increased by combination treatments, tumor histology in each treatment group, showed a feature of necrosis; area of necrosis was negligible at 8 h in radiation alone and wortmannin alone, however in the combination group markedly increased area of necrosis was noted at 24 h (Fig. 3). This data suggests

that an enhancement of tumor growth delay in the combination group could be partly explained with increased tumor necrosis.

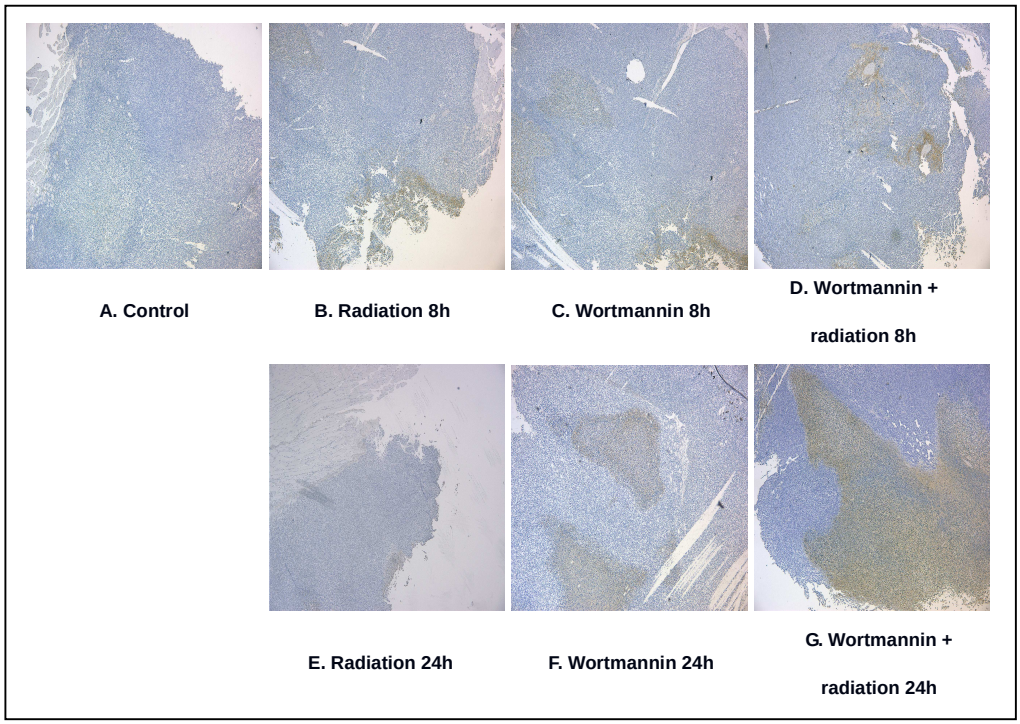

Fig. 3. TUNEL assay of HCa-I tissues. In the combination group markedly increased area of necrosis was noted at 24 h. A: Control, B: Radiation 8h, C: Wortmannin 8h, D: Wortmannin+radiation 8h, E: Radiation 24h, F: Wortmannin 24h, G: Wortmannin+radiation 24h.

3. Change in the expression of apoptosis-regulating molecules

Analysis of apoptosis regulating molecules with western blotting showed upregulation of p53 at 4 h, $p21^{WAF1/CIP1}$ at 8 h in the combination treatment group comparing to those in either radiation alone or drug alone group.

When radiation and wortmannin were combined, the most significant change was shown in $p21^{WAF1/CIP1}$, which reached a peak level to 2.1-fold at 8 h and still remained high at 24 h compared with radiation alone or drug 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 it started to gradually decrease.

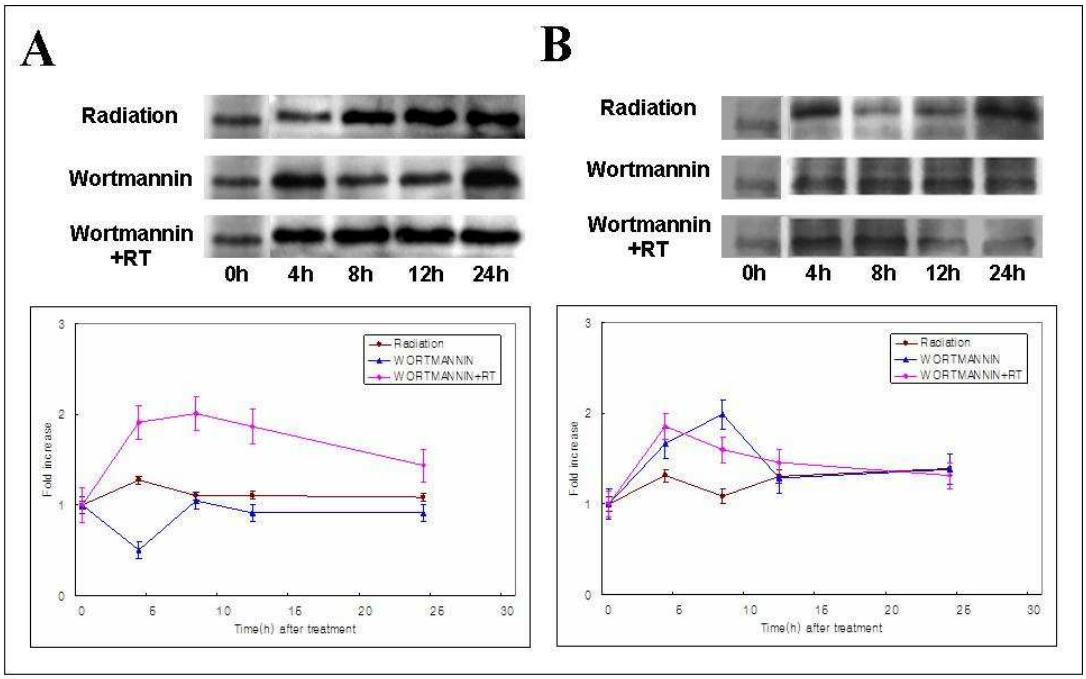

Fig. 4. Analysis of apoptosis regulating molecules for $p21^{WAF1/CIP1}$ (A) and p53(B). Western blotting and its densitometric analyses are plotted, showing significant increase of $p21^{WAF1/CIP1}$ expression in the combination group (◆) in comparison to radiation alone (●), or wortmannin alone (▲).

4. Immunohistochemical staining for $p21^{WAF1/CIP1}$, CD31 and VEGF.

When radiation and wortmannin were combined, the expression of $p21^{WAF1/CIP1}$ was increased in comparison to radiation alone or wortmannin alone (Table. 1.).

CD31 was overexpressed in the control group. Its expression was decreased in radiation alone and wortmannin alone group in comparison to the control group. The expression of CD31 was significantly decreased in the combination group compared to radiation alone or wortmannin alone (Fig. 5.).

VEGF was also overexpressed in the control group. Its expression was decreased 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 of enhancement of antitumor effect in the combined group.

Table 1. Comparison of p21$^{WAF1/CIP1}$, VEGF and CD31 expression by immunohistochemical staining.

Group	p21 mean+SE	CD31 mean+SD	VEGF grade
Control	9.3+1.38	23.33+3.21	2+
Radiation 4h	14.2+0.95 p*	21.33+2.08 NS	1+
Radiation 8h	12.1+1.03 NS	20.33+3.21 NS	1+
Radiation 12h	12.4+1.60 NS	18.67+2.08 NS	2+
Radiation 24h	12.5+1.38 NS	15.67+0.58 p*	2+
Wortmannin 4h	6.6+0.93 NS	21.67+2.52 NS	2+
Wortmannin 8h	11.2+1.06 NS	21.33+2.08 NS	1+
Wortmannin 12h	9.0+1.34 NS	20.67+2.08 NS	1+
Wortmannin 24h	9.1+1.24 NS	18.67+3.21 NS	1+
Wortmannin +radiation 4h	17.9+2.39 p*	15.33+2.08 p*	1+
Wortmannin +radiation 8h	21.2+1.43 p*	13.00+2.65 p*	1+
Wortmannin +radiation 12h	16.5+1.48 p*	9.67+2.08 p*	1+
Wortmannin +radiation 24h	15.9+1.08 p*	6.67+1.15 p*	1+

p21$^{WAF1/CIP1}$: The number of p21-positive nuclei, mean+SE; CD31: The number of CD31-positive vessels, mean+SD; VEGF: 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+; NS: not significant.

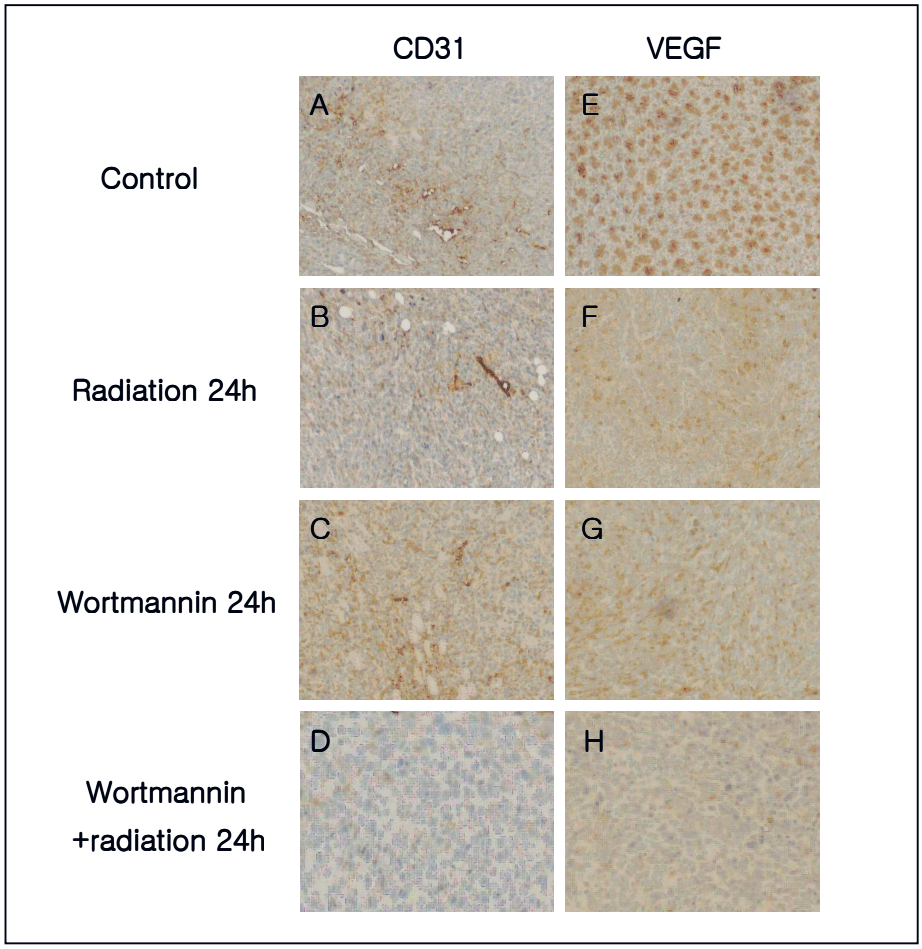

Fig. 5. Immunohistochemical staining of CD31 and VEGF in HCa-I tissues. The expression of CD31, VEGF in the combination group was significantly decreased in comparison to radiation or wortmannin. (CD31; A: Control, B: Radiation 24h, C: Wortmannin 24h, D: Wortmannin+radiation 24h, VEGF; E: Control, F: Radiation 24h, G: Wortmannin 24h, H: Wortmannin+radiation 24h.

IV. 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 the tumor response to cancer treatment[27]. For that reason, inhibition of this molecule might be used in cancer treatment.

Inhibition of PI3K has been attempted to enhance radiation-induced apoptosis and inhibition of tumor growth[17, 18]. In this study, we showed that wortmannin enhanced tumor growth delay in HCa-I, a well-known radioresistant tumor; wortmannin showed an EF of 1.9, which means enhancement effect in the tumor radioresponse compared with either treatment alone. It suggests that there might be a beneficial interaction between wortmannin and radiation.

To further investigate the mechanism underlying enhancement of tumor radioresponse by wortmannin, we first observed the level of apoptosis induced by wortmannin alone, radiation alone and the combination group.

In our study, the level of apoptosis in the combination group was

shown to be no more than an additive effect. The level of apoptosis was not significantly increased by combination treatment. In *in vitro* system, Shi *et al.* showed that wortmannin enhanced radiation-induced apoptosis[15]. They used TK6 human lymphoblastoid line and irradiated 2 Gy X-rays. Since we used an in vivo tumor HCa-I, which was highly radioresistant, action mechanism cannot be directly comparable to other reports. This part needs further investigation. However, the markedly increased area of necrosis at 24 h in the combination group suggests that a mechanism other than the apoptosis induction of tumor cells may work.

To examine the potential action mechanism between wortmannin and radiation, we investigated whether it would decrease the tumor vessels by immunohistochemical staining. Kim *et al.* reported that wortmannin markedly inhibited tube formation of the tumor[28]. Edward *et al.* demonstrated that PI3K antagonists enhanced radiation-induced destruction of tumor blood vessels[19]. In our study, microvessel density was analyzed through CD31 immunohistochemical stain, which high expression in tumor control group. Its expression was decreased at 24 h in the combination group in comparison to radiation alone or wortmannin alone group. 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[29]. In our study, when radiation and wortmannin were combined, the expression of VEGF was also decreased compared with radiation alone or wortmannin alone group. These data suggest that vascular injury might be involved in the mechanism of enhancement of antitumor effect in the combined group.

Taken together, these results suggest that wortmannin, in combination with radiation therapy, may work in a complex way involving enhancement of radiation-induced apoptosis and vascular injury. Therefore wortmannin could be useful in terms of antitumor efficacy by radiation.

V. CONCLUSION

In murine hepatocarcinoma, the antitumor effect of radiation could be potentiated by the use of wortmannin. The mechanism seems to involve not only the increase of induced apoptosis in an additive way but also enhanced vascular injury by wortmannin. Wortmannin in combination with radiation therapy may have potential benefit in cancer treatment.

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ABSTRACT(IN KOREAN)

마우스 간암에서 WORTMANNIN 에 의한 방사선 감수성 향상 효과

<지도교수 성 진 실>

연세대학교 대학원 의과학과

김 원 우

Phosphatidylinositol 3-kinase (PI3K)는 생존 신호 중의 하나로 알려져 있으며, PI3K신호 기작은 증식, 분화, apoptosis 억제, 종양 형성과 신생 혈관 생성에 관여한다. 이 분자의 억제가 항암 약제로 치료 효율을 높이는 데 사용될 수 있을 것이다. 본 연구에서는 방사선 치료에 내성이 높은 마우스 간암에서 PI3K 억제제인 wortmannin이 방사선에 의한 항암 작용을 증진시키는지 알아보고자 하였다.

C3H/HeJ 웅성 마우스에 HCa-I를 단측 대퇴부 근육 내에 이식하여, 종양의 평균 직경 6 mm일 때부터 wortmannin 1 mg/kg을 14일 동안 하루에 한 번 복강 내 주사하였고, 종양 평균 직경 8 mm일 때 대퇴부만을 고정하여 25 Gy를 조사

하여, 종양 성장 양상을 관찰하였다. wortmannin과 방사선과의 상호 기작을 조사하기 위해 apoptosis를 계수하였고, 관련 분자들을 western blotting으로 p53, p21의 발현을 조사하였고, 면역화학염색법으로 $p21^{WAF1/CIP1}$, CD31과 VEGF의 발현양상을 조사하였다.

종양 성장 지연 분석에서 증강지수가 1.9로 wortmannin이 종양의 방사선 감수성을 증가시키는 것으로 나타났다. 복합 치료시의 apoptosis 지수는 부가적 효과보다 낮은 효과를 보였다; 방사선 치료 시 apoptosis 지수는 1.1%, 약물 치료 시 1.3%, 복합 치료 시 1.9%로 조사되었다. 복합 치료 시 24시간에서 괴사부분이 증가하였다. Western blotting에서 복합 치료 시 $p21^{WAF1/CIP1}$은 VEGF의 낮은 발현과 관여되어 복합 치료 시 높은 발현 양상을 보였다. 미세혈관밀도는 CD31의 낮은 발현으로 인하여 감소하였다.

마우스 간암에서 wortmannin의 사용으로 인하여 방사선 치료의 항암 효과를 증진 시킬 수 있을 것이며, 이 기작은 방사선으로 유도되는 apoptosis의 증진뿐만 아니라 wortmannin으로 인한 혈관 상해를 증진시켰다. wortmannin과 방사선 치료의 복합치료는 항암 치료에 있어서 치료 효율의 상승을 유도할 수 있을 것으로 사료된다.

핵심되는 말: wortmannin, 방사선, apoptosis, 간암.