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Full Length Research Paper

Synergic anti-tumor activity of gamma-irradiated exo-polysaccharide from submerged culture of *Grifola frondosa*

Keyong Ho Lee[1], Choa Hyung Cho[2] and Ki - Hyeong Rhee[3]*

[1]Kolon Life Science Inc., Yongin city, 446-797, Korea.
[2]College of Environmental and Biosciences, Chonbuk National University, Iksan 570-752, Korea.
[3]College of Industrial Sciences, Kongju National University, Yesan 340-702, Korea.

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Gamma-irradiated exo-polysaccharide can be obtained from submerged exo-polysaccharide from *Grifola frondosa* extracts. The low molecular exo-polysaccharide is obtained by irradiation showed potential anti-oxidant activity and then those effect showed synergic anti-tumor activity on adriamycin. Exo-polysaccharide modified by 10 kGy dose of irradiation showed strong radical scavenging activity by the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) reduction assay. The 50% rate inhibited the DPPH reduction concentration of gamma irradiated exo-polysaccharide were 350µg/ml. Gamma irradiated exo-polysaccharide showed stronger inhibitory effect on tumor growth of L1210 leukemia to compare with non-irradiated exo-polysaccharide at the same dose of 21 mg/kg. Modified exo-polysaccharide by irradiation reduced the tert-butylhydro peroxide (*t*BuOOH) induced cytotoxicity in various leukemic cell lines. In leukemic mouse model using L1210, the combination between adriamycin and gamma irradiated exo-polysaccharide decreased to about 60% compared to the treatment of adriamycin alone. These results indicate that gamma irradiated exo-polysaccharide has radical scavenging activity and prevents an oxidative stress and inhibit tumor growth by leukemia; therefore, gamma irradiated exo-polysaccharide is suggested as a food supplement having anti-oxidant properties exhibiting potent synergic effect against anti-leukemia in this study.

Key words: Gamma irradiated exo-polysaccharide, anti-oxidant, oxidative stress, anti-tumor activity.

INTRODUCTION

Grifola frondosa is one edible and medicine Basidiomycete fungus in Meripilaceae Polyporaceae family, and it has been reported to have functions for treating hepatopathy and diabetes, anti-hypertensive and antitumor (Dai et al., 2009). Fruit bodies and liquid-cultured mycelium of this mushroom have been reported to contain useful anti-tumor polysaccharides from various fractions. These polysaccharides have been identified as many types of glucans (Mizuno et al., 1986; Ohno et al., 1986; Shigesue et al., 2000). In general, the main components having anti-oxidant activity from natural products such as medicinal plants and Basidiomycetes are some kinds of phenolic compounds including various flavonoids. In recent research, anti-oxidant effect of some kinds of polymer, such as fucoidan, β-glucan and glycoprotein, have reported that those polymers have various anti-oxidant effect such as lipid peroxidation, ROS radical scavenging and anti-oxidant enzyme including SOD and GSH (Kim and Kim, 1999; Lee et al.,

*Corresponding author. E-mail: howard@kongju.ac.kr. Tel: + 82-41-330-1626. Fax: + 82-41-330-1629.

2003; Park and Han, 2008; Li et al., 2002; Yang et al., 2002). In a few kinds of studies, when the polysaccharides were degraded with the gamma irradiation, they could reduce the reactivity of radicals. DPPH radical scavenging activity of soybean was increased with the gamma irradiation (Variyar et al., 2004). After irradiation, a scavenging ability of phytic acid was found with no activity of non-irradiated phytic acid (Ahn et al., 2004). In recent, the hyaluronic acid showed the increased DPPH radical scavenging activity by the gamma irradiation (Kim et al., 2008).

In previous study, we found the anti-oxidant activity of chemically modified exo-polysaccharide which is obtained from *G. frondosa* extract (Lee and Yoon, 2009). Hence, the aim of this study is to test the anti-tumor activity of low molecular polysaccharide, which is modified by gamma irradiation from *G. frondosa* extract in order to improve their biological aspect for using irradiation, so that their molecular weights can be reduced by radiation technology. More specific, we investigated the change of molecular size, the anti-oxidant activity and synergic anti-tumor activity.

MATERIALS AND METHODS

Materials

2,2-Diphenyl-1-picrylhidrazyl (DPPH), *tert*-butyl-hydroperoxide (*t*BuOOH), butylated hydroxy toluene (BHT), adriamycin and sulforhodamine B (SRB) were purchased from Sigma Chemical Company (St. Louis, USA). Dulbecco's modified Eagle Medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, L-glutamine, and antibiotics were purchased from Gibco BRL (Grand Island, NY, USA). Potato dextrose agar and potato dextrose broth were purchased from Difco and BBL (Texas, USA).

Cell lines

The human leukemia cell lines HL-60, AML193, K-562 and U937 and the murine leukemic cell lines KG-1, M1, MK-8057 and L1210 were used in this study. AML-193 was cultured in Iscove's Modified Dulbecco's Medium supplemented with 10% FBS and 40 ng/ml recombinant human GM-CSF (R & D Systems, Minneapolis, MN). U937, KG-1, M1, L1210 and HL60 were cultured in RPMI 1640 culture medium while the K562 cell line was cultured in Dulbecco's Modified Eagle Medium (Gibco, Invitrogen Corp., San Diego, CA, USA) with 10% FBS. All cells were maintained in the presence of 100 µg/ml penicillin and streptomycin.

Animals

Five-week-old DBA/2 mice (male) were purchased from Japan SLC, Inc. and acclimatized to the controlled standard conditions (temperature, 23±2°C; relative humidity, 50±5%; illumination Cycle, 12/12 h light/dark). They were housed in polycarbonate cages for a week prior to being used in experiments and were maintained by the accredited procedures in our facility. The mice were fed *ad libitum* with irradiated Samyang (Korea) chow and UV-sterilized water.

Production of exo-polysaccharide

G. frondosa HB0071 was maintained on potato dextrose agar (PDA) slants at 4°C and subcultured every three months. The seed culture was grown in a 250 ml flask containing 100 ml of potato dextrose broth (pH 5.0) at 25°C on a rotary shaker incubator at 120 rpm for 7 days. The fermentation was carried out under the conditions of temperature 25°C, aeration rate 2 vvm, agitation speed 250 rpm, pH 5.5, and working volume 10 L. The fermentation medium was inoculated with 3% (v/v) of the seed culture and then cultivated at 25°C in a 20 L stirred-tank fermenter (B Braun Korea Co Ltd).

Preparation of gamma-irradiated exo-polysaccharide

Crude exo-polysaccharide were irradiated in a cobalt-60 gamma-irradiator (IR-221, Nordion International Ltd., Ontario, Canada) equipped with a 11.1 PBq strength at 15±0.5°C and operated at a dose rate of 10 kGy/h. The applied dose levels were 10, 30 and 50 kGy, respectively. Dosimetry was performed with 5 mm diameter alanine dosimeters (Bruker Instruments, Rheinstetten, Germany), and after irradiation process the samples were stored at 4°C for further experiments.

Measurement of molecular weight

Gel permeation chromatography (GPC) was conducted to monitor the changes of the molecular weight distribution of the polysaccharides in dry form by the gamma-irradiation. GPC was performed by Waters system (Milford, MA) equipped with separation module (Waters 2690), refractive index detector (RI, Waters 2410) and PLaquagel-60, 40 and 30 column (300 7.5 and 8 mm, Polymer laboratories, Ltd., UK). The mobile phase was 0.1 M sodium nitrate at the flow rate of 1 ml/min, and the column was operated at 40°C. The injection volume was 200 µl and the calibration was carried out using pullulan standard (Showa Denko, Tokyo, Japan). The weight average molecular weight was calculated using Empower soft ware (System Software, Empower option GPC, Waters Co.).

Analysis of total phenolic content

The concentration of total phenolics in exo-polysaccharide was estimated by the Folin-Ciocalteu method (Singleton and Rossi, 1965). As a brief, aliquots of 0.1 ml of the extract residue dissolved in DMSO (0.1 mg/ml) was added in a test tube with 0.5 ml of Folin-Ciocalteu reagent and mixed thoroughly. After an interval of 3 min, 0.5 ml of 10% Na_2CO_3 solution was added, and the mixture was allowed to stand for 1 h at room temperature. The absorbance of the mixture was measured at 760 nm. A standard curve using gallic acid was also prepared. Results were expressed as mg/ml of extract of gallic acid equivalents.

Reducing power

The reducing power of polysaccharides was determined according to the method of Oyaizu (1986). A polysaccharide solution of 1 ml was mixed with 2.5 ml of 0.2 M sodium phosphate buffer (pH6.6) and 2.5 ml of 1% potassium ferricyanide ($K_3Fe(CN_6)$). The concentration of samples in the assay mixture was appropriately diluted to meet the calibration range of spectrophotometer. The reaction mixtures were incubated in a temperature-controlled water bath at

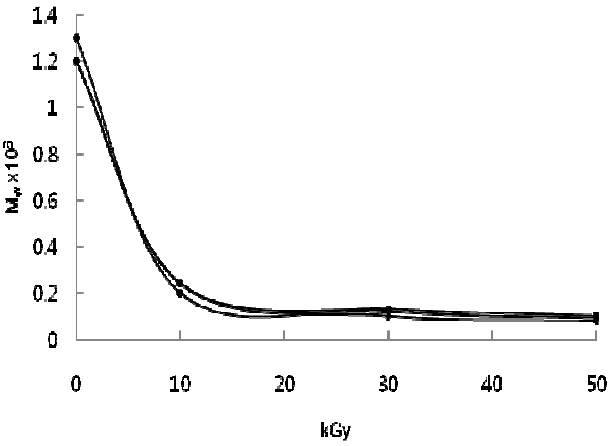

Figure 1. The changes in molecular size of exo-polysaccharide by irradiation.

50℃ for 20 min, followed by the addition of 2.5 ml of 10% trichloroacetic acid. The mixtures were then centrifuged at 750 x g using a centrifuge (VS-5500, Vision scientific Co. Ltd., Seoul, Republic of Korea) for 5 min at 25℃. The supernatant obtained (5 ml) was treated with 5 ml of distilled water and 1 ml of 1% ferricchloride. The absorbance of the reaction mixture was measured at 700 nm. An increase in absorbance was used as a measure of the reducing power.

DPPH assay

Free radical scavenging activity of gamma irradiated exo-polysaccharides and non-irradiated exo-polysaccharide was measured according to Brand-Williams et al. (1995) with some modification. A 0.1 mM solution of DPPH in methanol was prepared and 4 ml of this solution was added with 0.2 ml of the extract. The decrease in absorbance at 517 nm was measured at 60 min. A control was added with 0.2 ml of distilled water instead of the extract. Free radical scavenging activity of gamma irradiated exo-polysaccharides and non-irradiated exo-polysaccharide was compared with that of BHT. Free radical scavenging activity was expressed as the percentage of DPPH decrease.

*t*BuOOH treatment and SRB assay

To determine the ability of gamma irradiated exo-polysaccharide or non-irradiated exo-polysaccharide to ameliorate the cytotoxicity of *t*BuOOH, cells (1 x 10^5 cell/well) were plated in 96-well plates and incubated for 24 h, gamma irradiated exo-polysaccharide or non-irradiated exo-polysaccharide was added to leukemic cell cultures in 96-well plates at various concentrations (0.1, 1, 10 and 100 µM) for 1 h, followed by incubation with *t*BuOOH.100 µM for 24 h After treatment, the cell killing was determined by using the SRB assay (Skehan et al., 1990). First, the cells were fixed with 50% trichloroacetic acid and 0.4% SRB in 1% acetic acid was added to each well. After 15 min incubation, the plates were washed, the dyes were dissolved by 10 mM Tris buffer, and an enzyme-linked immunosorbent assay reader at 515 nm to determine the absorbance density values read the plate. All experiments were conducted in triplicate.

Effect of gamma irradiated exo-polysaccharide on the anti-tumor activity of adriamycin

The L1210 cell-inoculated and vehicle, gamma irradiated exo-polysaccharide and non-irradiated exo-polysaccharide-treated BDA/2 mice described above that developed s.c. nodules that were > 5 mm in diameter were given 2.1 and 15 mg/kg of adriamycin intravenously on days 5, 8 and 12. Doses of adriamycin were 1/10 and 1/2 of the evaluated LD_{50} concentration. Tumor size was determined by measuring the smallest and the largest tumor diameters with calipers and tumor volume was calculated according to 10 standard procedures by using the formula: volume (mm^3)= length x $width^2/2$ (Wang et al., 1997).

Increased life span

Survival time of the remaining 8 rats in each group was recorded. The mean survival time of NS group was reckoned as control. Increased life span (%ILS) was calculated as %ILS = ((Mean survival of treated group / Mean survival of control group) -1) x 100.

Statistical analysis

All results are expressed as mean ± SD, and were analyzed by one-way analysis of variance (ANOVA), and means were compared by Tukey's or Dunnet's test, with $P < 0.05$ being considered as statistically significant.

RESUITS

Analysis of molecular weight and total phenolic contents

It is very well known that polysaccharides in dry form or in solution degrade when exposed to ionizing radiation. For the investigation of the effect of gamma irradiation on the molecule size of exo-polysaccharide was evaluated by Gel permeation chromatography (GPC) as shown in Fig.1. As the irradiation dose increased, average molecular weight of the exo-polysaccharide is shifted to lower molecular weight. Average molecular weights decrease rapidly at 10 kGy and then this decrease was slowed down at between 30 to 50 kGy. According to this result, optimum irradiation dose is fixed as 10 kGy for various biological tests. Total phenolic and flavonoid contents of non-irradiated exo-polysaccharide were 116 and 29 mg/g, respectively, and those of irradiated sample at the dose of 10 kGy were 110 and 20.6 mg/g, respectively. However, the phenolic compounds of irradiated sample were slightly decreased in this experiment (Table 1).

Free radical scavenging activity

To examine the scavenging activity between gamma

Table 1. Total phenolic and flavonoids contents of 10kGy irradiated and non-irradiated sample isolated from *G. frondosa*.

Sample	Total polyphenol (mg/g)	Total flavonoid (mg/g)
Non-irradiated	116±8.2*	29.0±0.4*
Irradiated	110±5.1*	20.6±0.3*

All expressed as mg of gallic acid equiv/g dry weight of residue. Each value is expressed as mean ± SD (n=3) (*$p < 0.05$).

irradiated exo-polysaccharide and non-irradiated exo-polysaccharide, DPPH assay is carried out. Relative to the radical scavenging activity of BHT, 10, 100 and 500 µg/ml of gamma irradiated exo-polysaccharide decreased the absorbance of DPPH at 517 nm by 15, 37 and 69%, respectively. Thus gamma irradiated exo-polysaccharide has strong radical scavenging activity that is dose-dependent and has an IC_{50} of 350 µg/ml (Figure 2a). We also tested non-irradiated exo-polysaccharide to determine how to change the anti-oxidant activity of non-irradiated exo-polysaccharide. Upon irradiation, gamma irradiated exo-polysaccharide showed much of its radical scavenging activity. In the other hand, the same concentrations of non-irradiated exo-polysaccharide resulted in DPPH radical scavenging ability of 0, 8 and 14%, respectively. In (Figure 2b), the change of reducing power in exo-polysaccharide was shown. In the experi ments for measuring reducing power, the presence of reducers causes the reduction of $Fe^{3+/}$ferri cyanide complex to the ferrous form. The ability of exo-polysaccharide to reduce iron cations in dry form was increased by the gamma irradiation. After the gamma irradiation, reducing power of exo-polysaccahride was increased in dose dependant manner. There have been several reports showing that the DPPH radical scavenging activity was proportionally increased with the increase in reducing power (Ahn et al., 2004; Kim et al., 2008) and the same result is obtained with this study.

Effect of gamma irradiated exo-polysaccharide on *t*BuOOH -induced cytotoxicity

tBuOOH is cytotoxic because it induces oxidative stress (Kanno et al., 2007). We used the SRB assay to examine whether this effect of *t*BuOOH on various leukemia cell lines would be mitigated by gamma irradiated exo-polysaccharide. 100 µM of *t*BuOOH efficiently killed all eight leukemia cell lines that were tested, and this effect was significantly reduced by gamma irradiated exo-polysaccharide in a dose-dependent manner. However, non-irradiated exo-polysaccharide did not protected *t*BuOOH-mediated cytotoxicity markedly at the tested dose (Table 2). Thus, the anti-oxidant effect of gamma irradiated exo-polysaccharide is more potential than non-irradiated exo-polysaccharide to prevent exogenous oxidative stress.

Combination effect of gamma irradiated exo-polysaccharide with adriamycin

To test whether gamma irradiated exo-polysaccharide and non-irradiated exo-polysaccharide inhibit *in vivo* leukemic tumor growth, we used gamma irradiated exo-polysaccharide and non-irradiated exo-polysaccharide at each dose of 21 mg/kg. This dose was determined by preliminary studies examining the effect of gamma irradiated exo-polysaccharide, not a non-irradiated exo-polysaccharide dose on *in vivo* L1210 tumor growth; these studies showed that the ED_{50} of gamma irradiated exo-polysaccharide was 43 mg/kg (data not shown). Thus, half of this concentration was used to treat L1210-inoculated mice in the subsequent experiments. At the end of the month, gamma irradiated exo-polysaccharide treatment had reduced the growth of tumors by 23%; this inhibitory effect first became apparent after 18 days of treatment (data not shown). Upon non-irradiation, however, exo-polysaccharide was not showed its activity to inhibit L1210 tumor growth.

We then asked whether gamma irradiated exo-polysaccharide would enhance the anti-tumor effects of adriamycin 2.1 and 15 mg/kg, a well known to generate free radicals responsible for cardiotoxic side effects. Thus, mice bearing s.c L1210 tumors that were treated with vehicle, gamma irradiated exo-polysaccharide or non-irradiated exo-polysaccharide were given intravenous injections of adriamycin on days 6, 9 and 12 and tumor volumes were calculated on every 6 day during days 30 (Figure 3). When administered on its own, adriamycin 2.1 mg/kg markedly suppressed tumor growth as the tumor volumes remained static for 6 days after the third injection (see the vehicle-treated mice in Figure 3a). After day 18, tumor growth resumed at a rate that was maintained until the end of the experiment. However, the inhibitory effect of adriamycin 2.1 mg/kg observed between days 12 to 18 was not extended to day 36 by co-treatment with non-irradiated exo-polysaccharide.

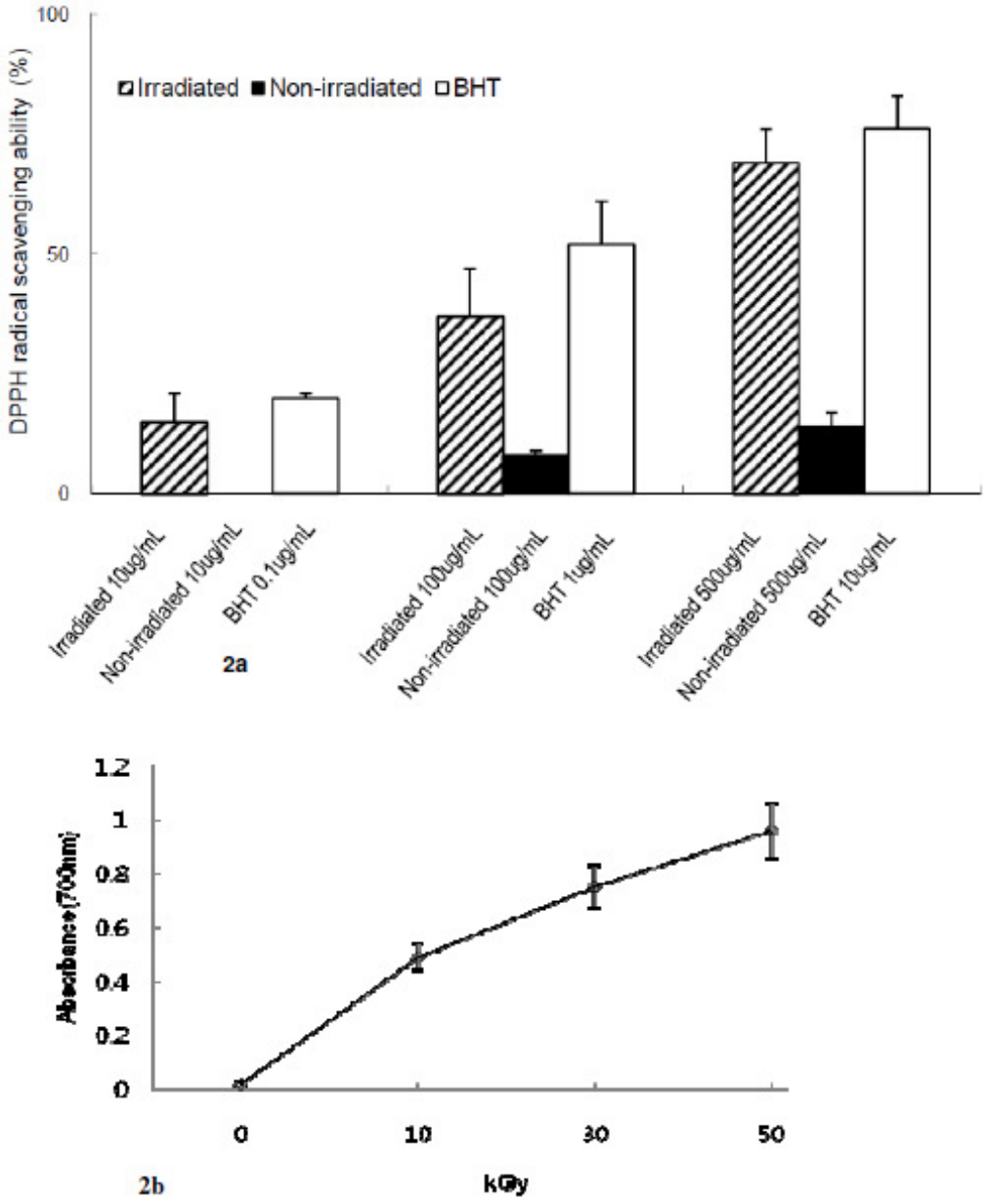

Figure 2. Radical scavenging activity (a) and reducing power (b) of 10 kGy irradiated and non-irradiated exo-polysaccharide ($p < 0.05$).

Significantly, adriamycin + gamma irradiated exo-polysaccharide co-treatment actually reduced the tumor volumes by about half during days 18 to 36. Thereafter, tumor growth resumed at the same rate observed in the adriamycin-alone group after day 18. The augmenting effect of gamma irradiated exo-polysaccharide was not observed when non-irradiated exo-polysaccharide was used instead. Those effects are markedly exhibited at the treatment of adriamycin 15 mg/kg (Figure 3b). Thus, gamma irradiated exo-polysaccharide acts synergistically with adriamycin to inhibit tumor growth, probably by acting as a radical scavenger.

Increased life span

Compared with the survival time (38.2 day) in control group, the tumor-bearing survival time was greatly prolonged in animals that received adriamycin 2.1 mg/kg with irradiated exo-polysaccharide (50.8 day), or

Table 2. Ability of 10 kGy irradiated exo-polysaccharide and non-irradiated exo-polysaccharide to prevent *t*BuOOH-induced killing of leukemia cell lines.

Cell lines	*t*BuOOH	Sample	Survival (%) in the presence of sample concentrations (μg/ml)			
			10	100	500	1000
AML-193	-	Irradiated	100	100	100	96
	-	Non-irraddiated	100	100	100	95
	+	Irradiated	37	45	78	95
	+	Non-irraddiated	31	39	33	41
HL-60	-	Irradiated	100	100	100	100
	-	Non-irraddiated	100	100	100	97
	+	Irradiated	30	45	59	72
	+	Non-irraddiated	28	34	43	49
K-562	-	Irradiated	100	100	98	97
	-	Non-irraddiated	97	97	96	95
	+	Irradiated	39	51	65	70
	+	Non-irraddiated	20	39	44	55
KG-1	-	Irradiated	100	100	98	94
	-	Non-irraddiated	100	100	96	95
	+	Irradiated	36	42	65	70
	+	Non-irraddiated	27	30	31	40
L1210	-	Irradiated	100	100	100	100
	-	Non-irraddiated	100	100	100	100
	+	Irradiated	54	63	75	80
	+	Non-irraddiated	24	34	43	46
M1	-	Irradiated	100	100	100	100
	-	Non-irraddiated	100	100	97	96
	+	Irradiated	29	37	51	58
	+	Non-irraddiated	34	37	42	47
U-937	-	Irradiated	100	100	100	95
	-	Non-irraddiated	100	100	100	96
	+	Irradiated	41	53	72	79
	+	Non-irraddiated	34	40	46	50

- ; without induction by *t*BuOOH, +; with induction by *t*BuOOH.

adriamycin 15 mg/kg with irradiated exo-polysaccharide (39.6 day), or adriamycin with 2.1 mg/kg with non-irradiated exo-polysaccharide (47.2 day), or adriamycin 15 mg/kg with non-irradiated exo-polysaccharide (33.1 day) (Table 3).

In contrast, in case of adriamycin 15 mg/kg alone, mean survival time is decreased to 25.3 day by its chemical toxicitiy.

However, combination with irradiated exo-polysaccharide is prolonged by the survival time from 25.3 to 39.6 day, which was not longer than the control (38.2 day). In this result, the treatment of exo-polysaccharide has potentially significant chemical protector, and more irradiation was showed higher activity to increase the increased life span.

Conclusions

Here we showed that gamma irradiated

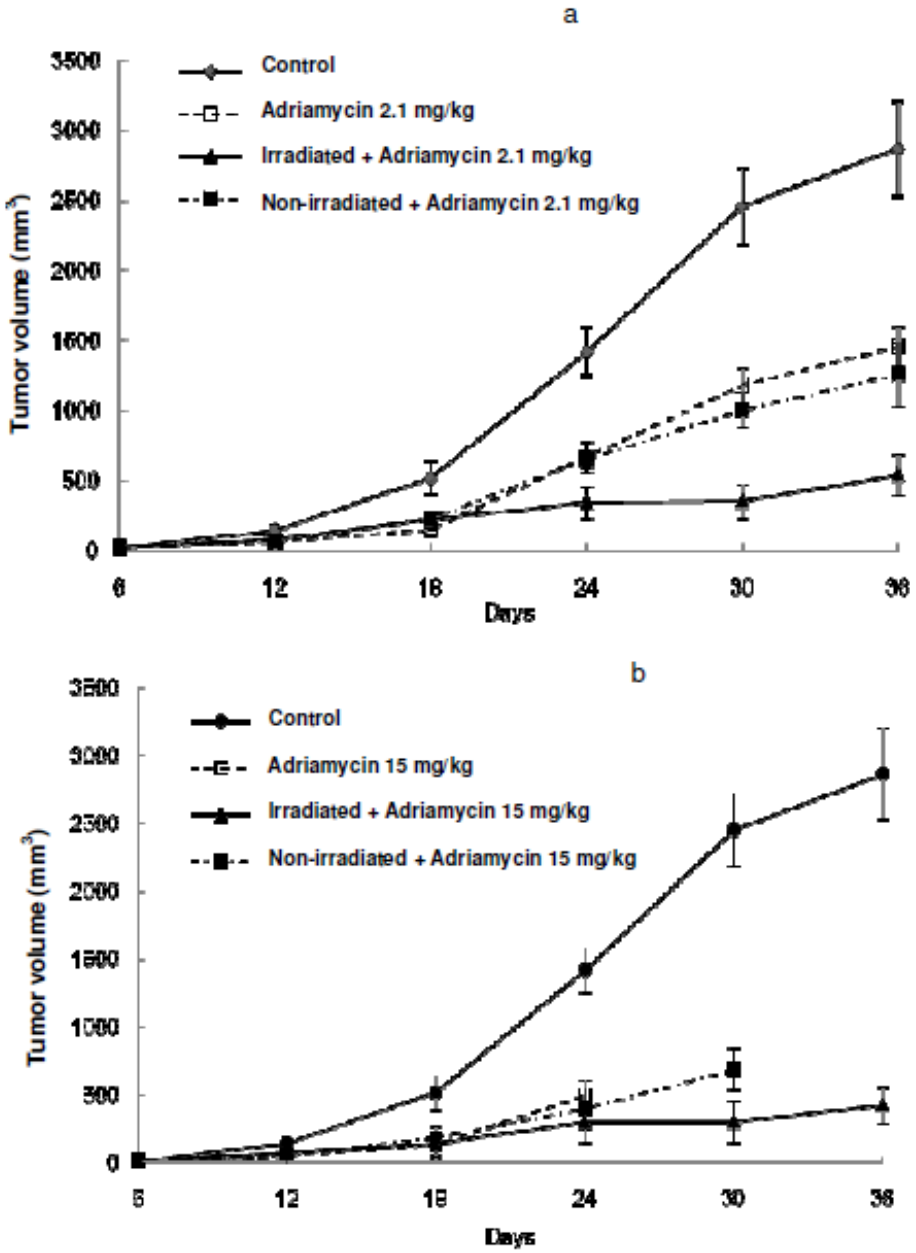

Figure 3. Synergic anti-tumor effect of 10 kGy irradiated and non-irradiated exo-polysaccharide in treatment of combination with adriamycin on mice bearing L1210 leukemia cell. The treatment; Adriamycin 2.1 mg/kg(a) and 15mg/kg(b) by intravenous at 6, 9 and 12 days; irradiated and non-irradiated exo-polysaccharide 21mg/kg by oral every 2 day for 30 days after 24 hour later when L1210 inoculated. Control group is treated by distilled water as the same schedule with exo-polysaccharide groups.

exo-polysaccharide acts synergistically with the chemotherapeutic agent adriamycin, and that its anti-tumor effects are due to its anti-oxidant activity. This is consistent with previous studies that have showed anti-oxidant activity from polysaccharide-enriched fractions extracted from pulp tissue of *Litchi chinensis* (Kong et al., 2010), and DPPH radical scavenging activity and reducing power of the gamma irradiated polysaccharides were significantly higher than those non-irradiated. These effects relates to the reduction of molecular weight due to structural change by irradiation (Choi et al., 2009).

In general, irradiation using radiation and UV induces chemical and physical transformation of polysaccharides;

Table 3. Mean survival time and increased life span (%ILS) after treatment (mean±SD).

Group (mg/kg)	Mean survival time (d)	%ILS
Control	38.2	-
Adriamycin 2.1	43.8	14.6
Adriamycin 21	25.3	-33.8
Irradiated + Adriamycin 2.1	50.8	33.0
Irradiated + Adriamycin 21	39.6	3.7
Non-irradiated + Adriamycin 2.1	47.2	23.6
Non-irradiated + Adriamycin 21	33.1	-13.4

these effects affect their functional and physical properties including solubility, viscosity and anti-oxidant activity (Li et al., 2006; Wang et al., 2008; Zhao et al., 2005). The correlation between the sulfate content and scavenging superoxide radical ability was positive, the ratio of sulfate content/fucose was an effective indicator to anti-oxidant activity of the samples (Wang et al., 2008). In case of exo-polysaccharide from *Grifola frondosa*, irradiation induced the reduction of molecular size, the increase of phenolic contents and anti-oxidant activity, and then showed the synergic effect on anti-tumor activity. In this *in vitro* study using various leukemia cell lines, exo-polysacchride regardless of irradiation its own cytotoxicity is not shown any tested leukemia cell lines. However, *tBuOOH*-induced cell death is decreased by the treatment of exo-polysaccharide. Therefore, exo-polysaccharide acts synergistically with anti-leukemic agents like adriamycin, and that these activities correlate with its radical scavenging activity since protecting the cell death by tBuOOH-induced cytotoxicity, although it does not affects markedly to reduce the tumor volume (data not shown).

However, how the radical scavenging activity of gamma irradiated exo-polysaccharide synergistically enhances the anti-leukemic effects of adriamycin is still unclear. One of our authors had found the similar results that antioxidant ability have been affect to enhance to antitumor activity (Lee, 2008), so we also tried to have detected some clear understanding factors from exo-polysaccharide obtained by irradiation. Unfortunately, major clue, which affects the synergic effect to increase the antitumor activity, was not exhibited in this study. This question will be the subject of future studies.

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