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The anticancer effect of plant enzymes on mouse breast cancer model

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ABSTRACT

Excessive nutrition and increasingly westernizing dietary choices may be contributing toward a recent trend in rising incidences of chronic lifestyle-related diseases. In this study, we like to evaluate the anti-cancer properties of plant enzyme derived *Validux* using a mouse model. The anti-cancer properties of Plant Enzyme *Validux* (PEV) was evaluated using a mouse model. 5 weeks old male BalB/c mice were randomly distributed into four experimental groups: Control, PEV only, 2Gy (gray) only, and PEV + 2Gy. Experimental units were administrated (oral administration by stomach sonde) with 500 mg/kg daily dosage of PEV for 14 days before being subjected to 2Gy of whole body irradiation. Flow cytometry was used to observe the changes in leukocyte and T-Lymphocyte numbers. The PEV treatment group showed statistically significant increase in cytotoxic CD8+ lymphocyte count versus the control group while increased leukocyte and lymphocyte recovery rate was observed in the PEV + 2Gy group. Ordinary trend was also observed in granulocytes. In order to observe PEV's effect on tumor growth, Experimental units were inoculated with SSC7 mouse carcinoma cell line following 14 days of daily PEV administration. PEV treatment groups showed decrease in tumor volume and mass vis-à-vis control. Thus, we would like to propose that PEV may be potentially used as a radioprotective supplement in radiotherapy for tumor suppression.

Keywords: Plant enzymes (*Validux*), radioprotection, T-lymphocytes, CD8, anti-tumor effects, immune system.

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INTRODUCTON

Excessive nutrition and increasingly westernizing dietary choices may be contributing toward a recent trend in rising incidences of chronic lifestyle-related diseases. Chief among them is the cancer, one of the leading cause of death (Gu et al., 2005; Kang et al., 2006; Terai et al., 2006). Current clinical regimen in cancer treatments involves surgery, radiation therapy, and chemotherapy but the accompanying side effects and the metastasis during recurrence often results in the degradation of Quality of Life (QOL) indexes for cancer patients (Gu et al., 2002, Gu et al., 2005, Choi et al., 2005; Nair et al., 2005; Gu et al., 2005). Thus, in recent years research in immunotherapy via alternative medicine derived from natural substances has made some in roads as it may have lesser side effects (Gu et al., 2017; Ukawa et al., 2005; Gu et al., 2008; Gu et al., 2007; Nakamura et al., 2007).

Fermented foods, developed as a measure of food preservation, seem to play a role in maintaining health and fitness. Antioxidant properties and immunomodulating functions of fermented foods and other natural products has been previously documented ((Liu et al., 1990; Liu and Mori, 1992; Kim et al., 1998). Plant fermentation enzymes has been found to have antitumor effect: upregulation of natural killer (NK) cell activity as well as increase in immunopotentiating action of Lymphokine activated killer (LAK) cells has been reported (Hwang et al., 1996; Gu et al., 1997; Gu et al., 2000; Gu et al., 2009).

In this study, we planned to evaluate the anti-cancer properties of plant enzyme derived *Validux* (PEV) using a mouse model.

MATERIALS AND METHODS

Experiment on anticancer effect (use animals · breeding conditions)

For the animals used, male BalB/c mice 5 weeks old were used. First of all, preliminary breeding was carried out for one week in order to get used to breeding conditions (adapt to the animal rearing environment of our university). The breeding conditions were kept constant at room temperature of 22 ± 3°C and humidity of 60%, and water and feed (Pellet-shaped bait of 1 cm^2 size) were allowed to be taken *ad libitum*.

Experiment group

The experimental group was divided into control, PEV only, 2Gy only, and PEV + 2Gy administration group.

Mouse breast cancer cells were transplanted on the right femoral region of a syngeneic BalB/c mouse using 4T1 (high grade) of mouse breast cancer cell line established from spontaneous breast cancer derived from BalB/c mouse.

Method of administration

After 1 week of preliminary breeding, oral administration on stomach sonde was performed every day until the end of the experiment. The administration concentration was 500 mg/kg each day, and the same amount of distilled water was administered to the control group. Plant fermented foods fermented black soybeans, wheat, rice bran, barley, rice germs with aspergillus oryzae. In addition, seaweed and black sesame are added as fermented products. This fermentation product was spontaneously fermented for 2 years without adding impurities (provided by *Validux* Corporation).

Tumor measurement item

The cancer cell (SCC-7) was inoculated two weeks after the start of administration, and the major axis and minor axis of the tumor were measured every two days from the 4th day after the inoculation, and the tumor volume was calculated by the formula (Equation 1). Inoculation of cancer cells (4T1 (high grade) of mouse breast cancer cell line) was transplanted to the right femoral region.

$$\text{Tumor volume (mm}^3\text{)} = (1/2) \times \text{major axis} \times (\text{minor axis})^2 \quad (1)$$

Experimental method for immunopotentiating and radioprotective effect

Male ICR mouse at 5 weeks of age was purchased from Japanese SLC Company and used. We have divided the mice into 4 groups; in control group, 2 Gy irradiation group, plant enzyme administration group, plant enzyme administration + 2 Gy irradiation group, namely 4 groups (n = 6-7).

Administration of plant enzymes was carried out daily by oral administration of in stomach sonde, and it was 500 mg/kg. Irradiation was performed 2 weeks after the start of administration, and the change in blood cell count was examined over time.

Statistical analysis

Experimental results are expressed as mean ± standard deviation. As the first test, the evaluation of each test sample administration group for one group (control) was performed by ANOVA test, and thereafter, as a second test Student T- test is performed to compare 1 group (control) with each test sample administration group. Measurement data are statistically processed using Stat View software VE 5.0 (HULINKS, Japan) and presented as mean ± standard error. Inter-group comparison of continuous variables uses variance analysis. P value is less than 0.05 is judged to be statistically significant difference. To see the reproducibility, the same experiment was conducted more than once.

This study has been approved by the Suzuka University of Medical Science Animal Research Ethics Committee (Ref: 17/721/32). In this research, the Ministry of Education, Culture, Sports, Science and Technology Notification No. 71 "Basic Guidelines on Implementation of Animal Experiments in Research Organizations" (June 1, 2006), Ministry of the Environment "Standards on breeding and storage of experimental animals and relief of pain "With reference to the reference.

RESULTS

Anti-cancer properties of PEV

The calculated tumor volume is shown in Table 1, Figures 1 and 2. As compared with the control group, for the plant enzyme group manufactured by Validux Co Ltd., significant growth suppression was observed from 11 days to 31 days later.

PEV's effect on T-lymphocytes

After determining the lymphocyte region in cytogram, we analyzed and found the number of CD3 positive, CD8

Table 1. Test materials for 4T1 (high grade) of mouse breast cancer cell line of antitumor effects by oral administration of *Validux Plant Enzyme.*

	5d	8d	11d	14d	17d
Control	201.6 ± 19.1	335.0 ± 47.8	410.8 ± 51.0	603.1 ± 129.2	937.9 ± 21.6
Validux Plant Enzyme	192.0 ± 7.2	271.9 ± 19.1	274.2 ± 42.1*	555.9 ± 78.6	865.4 ± 107.2
	20d	**23d**	**26d**	**29d**	**31d**
Control	1204.4 ± 127.7	1465.9 ± 290.7	2287.9 ± 323.5	3127.8 ± 421.1	3987.5 ± 501.7
Validux Plant Enzyme	1120.7 ± 112.3	1386.9 ± 244.4	2014.2 ± 298.4*	2968.7 ± 277.5*	3209.1 ± 514.4*

*Statistically significant (P < 0.05) from the control group.

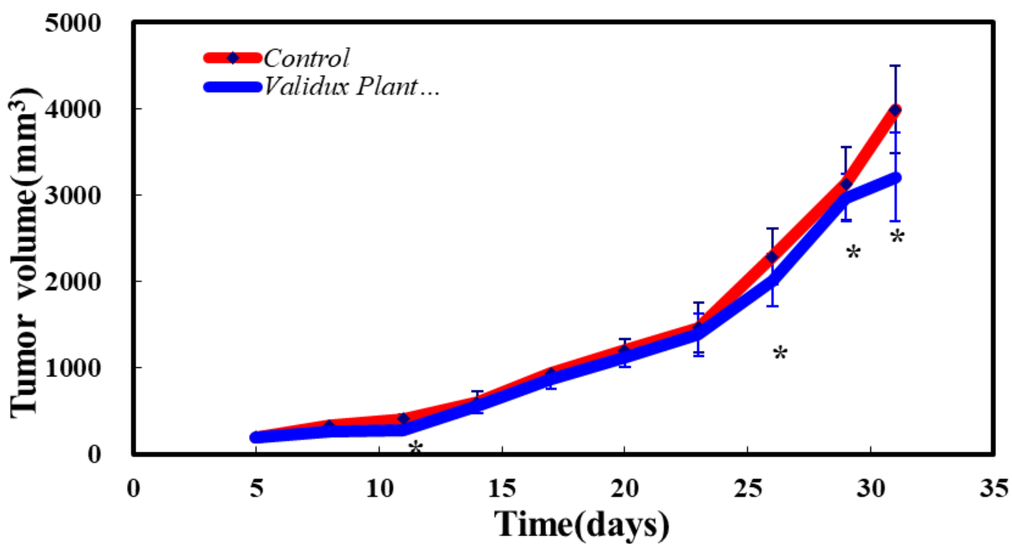

Figure 1. Effect of *Validux Plant Enzyme* on the tumor growth in mice inoculated with 4T1 (high grade) of mouse breast cancer cell line. Groups of ten mice each were subjected to each treatment. Results represent means ± S.D. * Statistically significant (P < 0.05) from the control.

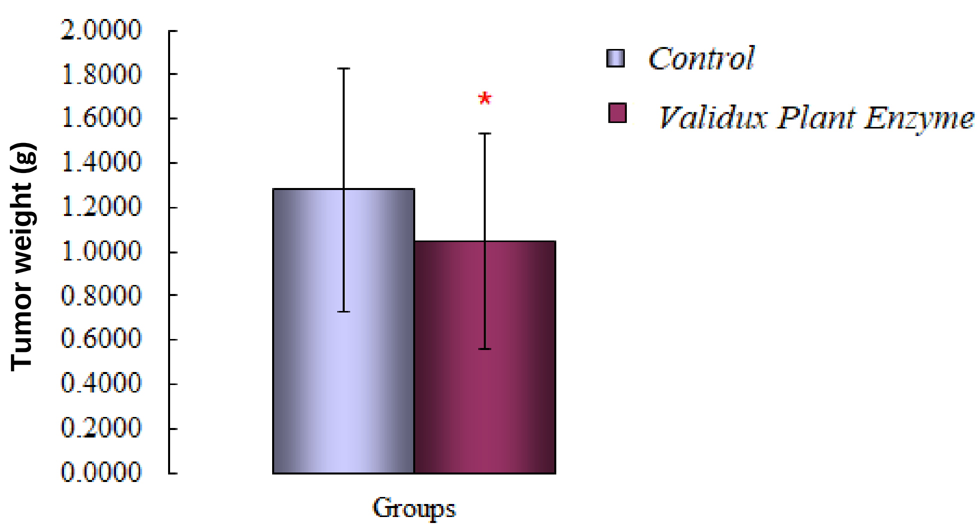

Figure 2. Effect of *Validux Plant Enzyme* on the tumor weight in mice inoculated with 4T1 (high grade) of mouse breast cancer cell line. Groups of ten mice each were subjected to each treatment. Results represent means ± S.D. * Statistically significant (P < 0.05) from the control group.

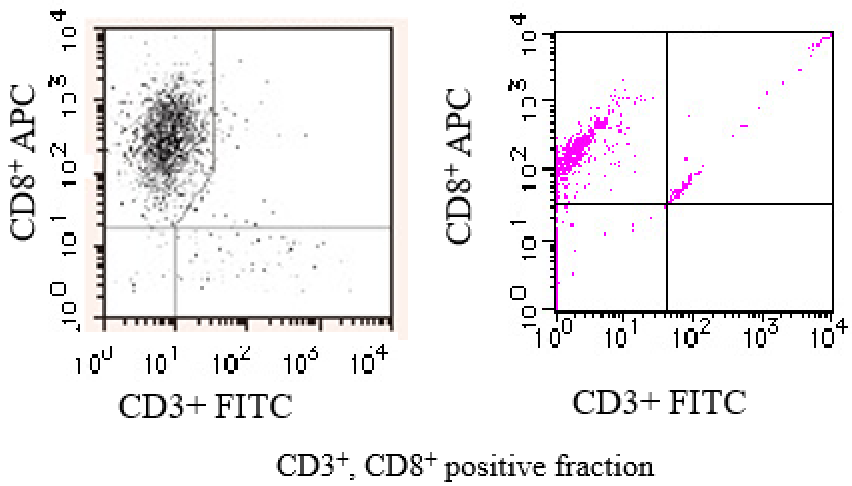

Figure 3. The change of CD8+ cells control and *Validux Plant Enzyme* administration.

positive cells and the number of killer T cells in T lymphocyte. The Cytogram at that time is shown in Figure 3, and the CD8 + analysis table (Dot plot) is shown in Figures 3 and 4, respectively.

As a result of measuring the number of T cells in T lymphocytes in each group by Flow cytometry meter, as can be seen in Figure 4, CD8 + in the plant enzymes (*Validux*) group, that is, T cells increased by 109% compared to the control group. In plant enzymes (*Validux*) administration + whole body 2 Gy irradiation group as compared with the radiation alone irradiation group (whole body 2 Gy) group showed a 50% increase from the 7th day of the investigation, reduction suppression and early recovery were observed.

The change of CD8+ cells after control and *Validux Plant Enzyme* administration group. The change of CD8+ cells after 2Gy of radiation and *Validux Plant Enzyme* administration group. Statistically significantly different *(* $P < 0.05$)* from the control group.

Radiation protection by PEV

Changes over time of each blood cell count of each group obtained by measurement are shown in Figure 5 to 7. Figure 5 shows the change in white blood cell count. For the control group, the PEV group showed a significant increase in global white blood cell count ($p < 0.01$).

Figure 5 shows the change in white blood cell count for investigation. In the 2 Gy group, the white blood cell count decreased markedly, whereas in the PEV + 2 Gy group, suppression of the decrease in white blood cell count and early recovery were observed ($p < 0.01$).

Figure 6 shows the change in lymphocyte count. For the Control group, the PEV group showed a significant increase in global white blood cell count ($p < 0.01$).

The change in lymphocyte number was shown for the investigation in Figure 4. While the number of lymphocytes in the 2Gy group decreased markedly, the PEV + 2Gy group showed suppression of lymphocyte reduction and early recovery ($p < 0.01$).

Figure 7 shows the change in granulocyte count. For the PEV group for the Control group, it showed a significant decrease in the overall granulocyte count ($p < 0.01$).

Figure 7 shows the change in the number of granulocytes recorded in the survey. In the 2 Gy group, the number of granulocytes remarkably decreased while the PEV + 2Gy group showed suppression of the decrease in granulocyte count and early recovery ($p < 0.01$).

DISCUSSION

Anti-cancer properties of PEV

In our mouse model, the Plant Enzyme *Validux* (PEV) treatment group exhibited statistically significant reduction in tumor volume and mass, suggesting PEV's potential use as an anti-tumor agent. However, the exact mechanism behind PEV's anti-tumor properties has yet to be elucidated. The fact that the use of PEV is commensurate with the elevated levels of cytotoxic T-lymphocytes (CD8+ lymphocytes) and leukocytes may suggest possible correlation between PEV and the activation of immune response. Previously, Hwang et al. (1996) have reported the activation of natural killer (NK) cell and lymphokine activated killer (LAK) cell via fermented plant enzymes (Hwang et al., 1996). Further elucidation by Riley (1994) and Gu (2013) determined

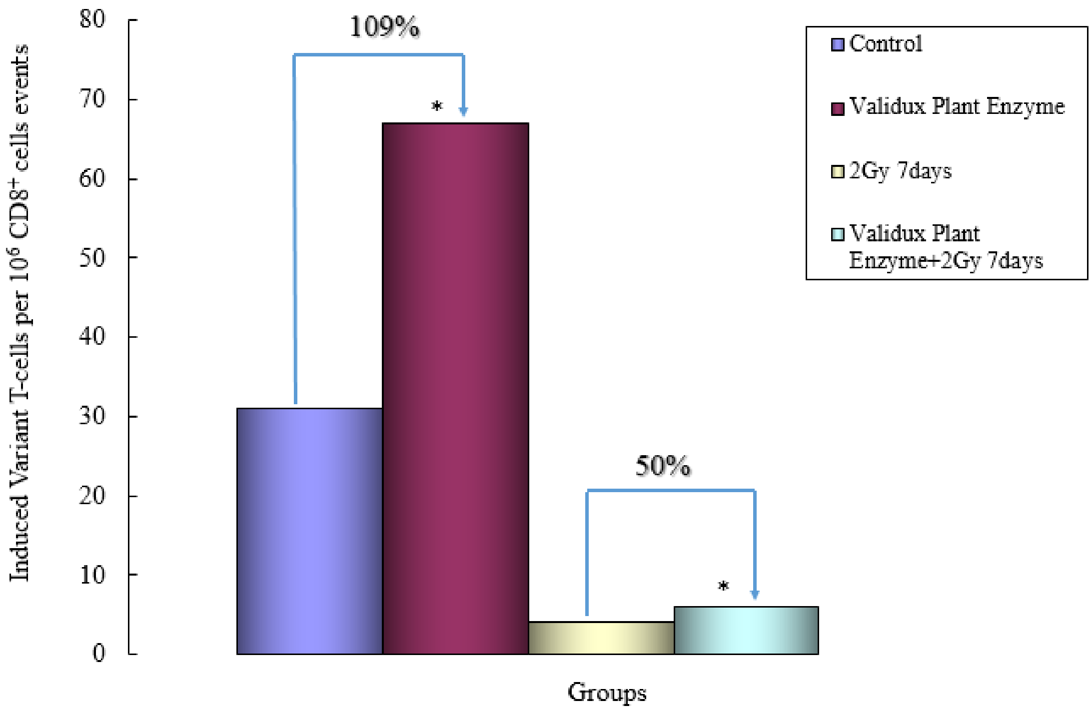

Figure 4. Lymphocytes were analyzed for CD8+ in C57BL/6crSlc mice at various time after 2Gy irradiation.

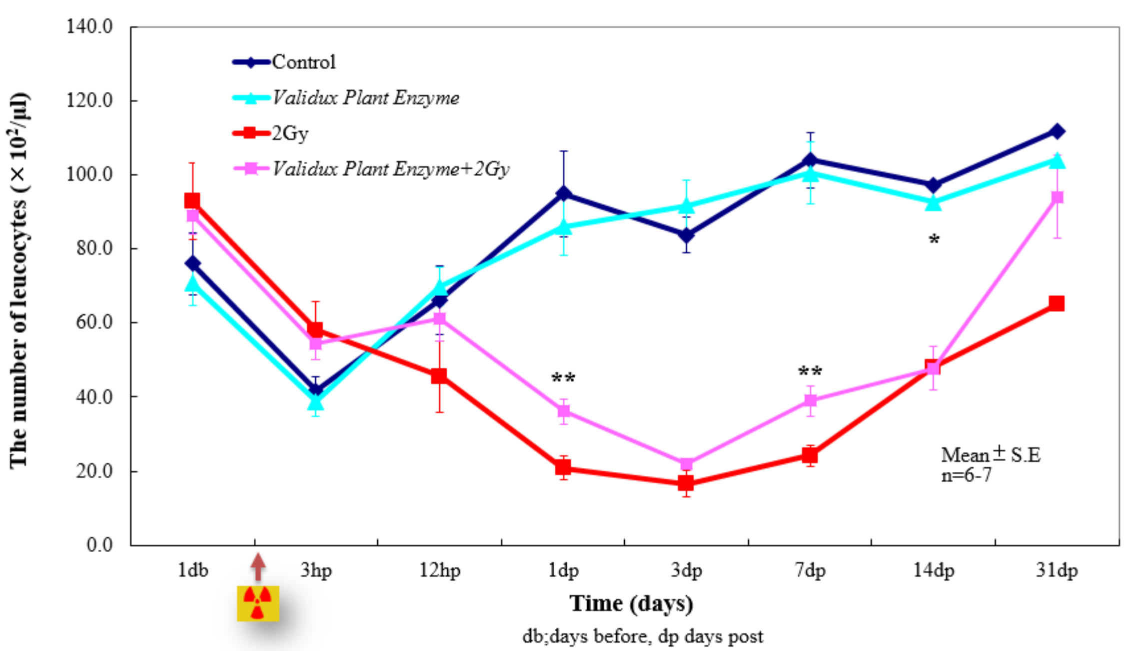

Figure 5. Single-dose effect of *Validux Plant Enzyme* on blood leukocyte counts in mice. There were 6-7 animals in each experimental group. Data are mean ± standard deviation values. Statistically significantly different (* $P < 0.05$, ** $P < 0.01$) from the control group. Statistically significantly different (* $P < 0.05$) from the 2Gy group.

that the oxidative burst caused by plant enzymes results in upregulation of interferon-γ (IFN-γ) which in turn activates the NK cells (Gu et al., 2013; Riley, 1994). Alternatively, β-glucan, a common carbohydrate in the

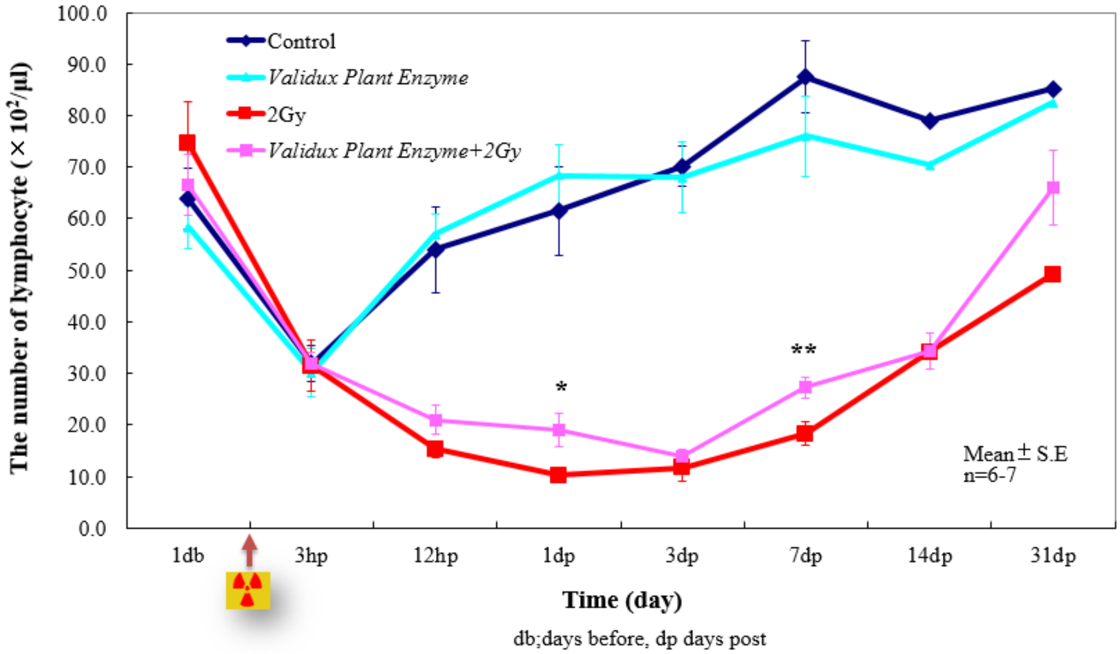

Figure 6. Single-dose effect of *Validux Plant Enzyme* on blood leukocyte counts in mice. There were 6-7 animals in each experimental group. Data are mean ± standard deviation values. Statistically significantly different (* $P < 0.05$, ** $P < 0.01$) from the 2Gy group.

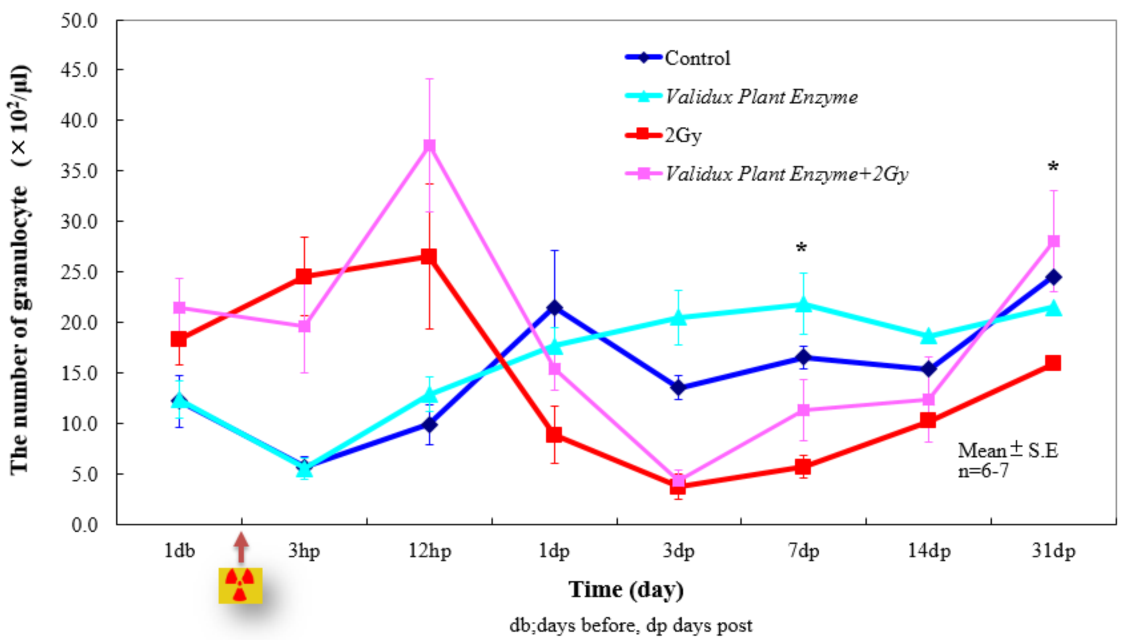

Figure 7. Single-dose effect of *Validux Plant Enzyme* on blood granurocyte counts in mice. There were 6-7 animals in each experimental group. Data are mean ± standard deviation values. Statistically significantly different (* $P < 0.05$) from the 2Gy group.

cell walls of bacteria, fungi, and some plants, was found to modulate immune response via upregulation of inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, and IL-8 (Gu et al., 2007; Ljungman et al., 1998; Engstad et al., 2002). It was also found to be involved in the activation of macrophages and T-lymphocytes via receptor Dectin-1 and complement receptor 3 (CR3) (Herre et al., 2004; Hong et al., 2004). Thus it may be possible to hypothesize that the anti-tumor properties of PEV may involve promotion of both innate and adaptive immune responses (Nakaya et al., 2006; Shibata et al., 2012; Shibata et al., 2013).

PEV's effect on T-lymphocytes

Throughout the study, the PEV treatment group displayed statistically significant upregulation of CD8+ lymphocytes vis-à-vis control. This was accompanied by improvement in CD8+ lymphocyte recovery rate following whole body irradiation as demonstrated by PEV + 2Gy group. Therefore, it may be possible to suggest that PEV may have effect on hematopoiesis of CD8+ lymphocytes. Hiroshikichi and Kamide (2001) suggested that the PEV may be involved in the upregulation of macrophages (Hoffman et al., 1993). Since antigen presentation by macrophages results in proliferation of T helper lymphocytes (CD4+ lymphocytes) via secretion of IL-1 and IL-12, we believe upregulation of macrophages by PEV may cascade into the upregulation of CD8+ lymphocytes and regulatory T cells (TREG) via IL-2 secreted by CD4+ lymphocytes (Kikuchi et al., 2001).

Radiation protection by PEV

Irradiation is a known source of oxygen free radicals which may cause tissue damage via oxidative stress (Riley, 1994; Gu et al., 2013; Gu et al., 2003; Hoffman et al., 1993; Kikuchiet al., 2001). Recent studies have implicated oxidative stress as causative agent of various diseases and cellular aging, leading to an increased interest in natural and synthetic antioxidant research (Otomatu et al., 2015). Irradiation during radiation therapy is particularly problematic as lymphocytes and other mature peripheral blood cells are found to be highly sensitive to radiation. Even a low dosage radiation exposure of 0.25 Gy resulted in rapid apoptosis of lymphocytes leading to significant decrease in cell count (Sies et al., 1991). Davis and Lamson (1999) suggested antioxidant properties of PEV may be beneficial in reducing the side effects of radiation therapy and chemotherapy (Davis et al., 1999). Our findings may be in line with suggestion by Lamson and Brignall as experimental units of PEV treatment group following 2Gy of whole body irradiation (PEV + 2Gy group) exhibited statistically significant increase in the recovery rate of lymphocytes and leukocytes (in contrast to the 2Gy only group). The role of sulfhydryl (-SH) functional group as a radical scavenger has been previously demonstrated by the use of aminothiol cysteamine as a radioprotective agent (Davis et al., 1999). We believe antioxidant properties of PEV may also be attributed to the extensive presence of SH groups provided by amino acid cysteine (Nakamura et al. 2006). Compounds with disulfide (S-S) functional group such as cystamine may also serve similar role but we believe their clinical role is limited due to short duration of antioxidant properties and toxicity.30 In addition to the lymphocytes and leukocytes, we have also observed transient increase in granulocyte recovery, but we believe it may be due to its release from granulocyte preserved organs (such as spleen and blood vessels) rather than effect of PEV (Herve and Bacq, 1949).

CONCLUSION

PEV was found to upregulate CD8+ lymphocytes and leukocytes as well as reducing tumor volume and mass. We believe two phenomena may be correlated but further research is required to elucidate exact role of PEV. Nonetheless it may be possible to suggest that PEV could be used as potential anti-cancer agent. PEV was also found to improve recovery rate of lymphocytes and leukocytes following irradiation. We believe this may be due to antioxidant properties of PEV suggesting its possible use as alternative radioprotective agent with reduced side effects.

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