Protective Effects of Lentinan against T Lymphocytes Injury in Mice under Chronic Radiation Stress

WANG Yong, LI Ming-chun*, FU Qing-jie

Medical College of Qingdao University, Qingdao 266071, China

Abstract: **Objective** To study the effects of lentinan (LTN) on mice exposed to chronic radiation. **Methods** Animals were divided into three groups (n = 10), they were animals exposed to radiation (Rad), normal control animals (Ctr), and irradiated animals treated with LTN (Rad + LTN). Animal model of chronic radiation stress injury was induced by irradiating mice with ^{60}Co γ-ray for 6 weeks from Monday to Friday consecutively. Before radiation, the mice in Rad + LTN group were ip injected with 0.5 mL LTN (0.01 mg/mL), whereas mice in other groups were injected with 0.9% physiological saline. The effects of LTN treatment on irradiated mice were examined by histological analysis on the spleen. The cell numbers and viability of T lymphocytes, which were isolated from the spleen, were determined by Trypan blue staining. Nitric oxide (NO) production and interleukin-2 (IL-2) secretion in T lymphocytes were also measured. **Results** Chronic radiation significantly reduced the body weights and the spleen and thymus indexes, associated with reduced T lymphocytes viability and functions, and elevated NO production. Treatment with LTN significantly normalized the elevated NO production, and attenuated the negative outcomes resulting from radiation mentioned above. **Conclusion** The results suggest that radioprotective effect of LTN may be contributed by improved T lymphocytes viability and functions via regulating the NO and IL-2 production in T lymphocytes.

Key words: chronic radiation stress; interleukin-2; lentinan; nitric oxide; T lymphocytes
DOI: 10.7501/j.issn.1674-6384.2013.01.008

Introduction

With the advancement of science and technology, radiation (Rad) has permeated our life continuously. Lower levels of Rad could be beneficial, particularly for cancer patients, resulting in increased T regulatory cell population (Kachikwu *et al*, 2011). Moreover, ionizing Rad may serve to enhance cellular defense systems (Audette-Stuart *et al*, 2011). However, for healthy subjects, ionizing Rad may induce a negative outcome, including directly inflicting damage in DNA of human lymphocytes (Rao *et al*, 2011).

In order to mitigate the negative effect of ionizing Rad on the immune system, it is necessary to develop an anti-Rad medication with minimal side effects. It is generally appreciated that lentinan (LTN) is a natural medicine with low toxicity. It consists of β-1,3-glucose and β-1,6-glucose which is distributed around β-1,3-glucose randomly (Taguchi *et al*, 1983). It is being used as an ancillary drug in the treatments of recurrent ovarian cancer, unresectable gastric cancer, and advanced colorectal cancer, which has been shown to effectively improve the five-year survival rate and quality of life (Fujimoto, Tomonaga, and Goto, 2006; Oba *et al*, 2009; Hazama *et al*, 2009). Numerous works have extensively documented the pharmacological action of LTN to exert the anticancer and immuno-regulatory effects (Zhou *et al*, 1995). However, the mechanisms of how LTN protects T lymphocytes against chronic Rad-induced injury are poorly understood.

Numerous studies have shown that Rad is associated with interleukin-2 (IL-2) and nitric oxide (NO). No matter what style of Rad (X-Rad or UV-B Rad), IL-2 was

* Corresponding author: Li MC Address: 22# Min-jiang Road, Shinan District, Qingdao 266071, China
Tel: +86-532-5187 0086 Fax: +86-532-5187 2904 E-mail: Lmc401y@163.com
Frist author: Wang Y E-mail: wangyeruwangyong@126.com
Received: September 16, 2012; Revised: November 8, 2012; Accepted: December 2, 2012
Fund: Military Medicine and Health Science Foundation (06MA094)

reduced with ^{3}H-TdR method (Russell *et al*, 1998). Also, NO synthase raised under the conduction of Rad. Even more important, Roupas *et al* (2012) have summarized that LTN may exert many of its activities via NO-mediated mechanism.

The aim of the present study is to investigate the effects of LTN on murine T lymphocytes in a model of chronic Rad-induced injury. To demonstrate the underlying mechanism of the anti-Rad effect of LTN, we determined the contents of NO and IL-2 of T lymphocytes supernatant.

Materials and methods

Animals

Male Kunming mice, aged 6—8 weeks and weighing between 18 to 22 g, were purchased from Experimental Animal and Animal Studies Center of Qingdao (China) and kept under the specific pathogen free condition. The Production license number was SCXK(Lu)20090007.

Drugs

Lentinan (110103_3) was provided by 401 Hospital of Chinese People's Liberation Army and produced by Jinling Pharmaceutical Co., Ltd. (China). It was diluted to 1% with physiological saline before injection. This concentration of LTN (0.5 mL) injected to mice was found with optimum intervention effects against chronic Rad stress (Liu *et al*, 2012).

Chemical

RPMI 1640 tissue culture medium was purchased from Tianjin Haoyang Biological Manufacture Co., Ltd. (China). Fetal calf serum (FCS) was obtained from Zhejiang Tianhang Biological Technology Co., Ltd. (China). Trypan blue was purchased from Beijing Solarbio Science & Technology Co., Ltd. (China). Nitric oxide (NO) assay kit and total protein assay kit were purchased from Nanjing Jiancheng Bioengineering Institute (China). Interleukin-2 (IL-2) assay kit was purchased from American R & D systems.

Histological analysis

After Rad for 6 weeks, five mice were sacrificed by cervical dislocation, and the spleens were harvested and fixed in 10% formalin. Images were obtained with a Leica TCS SP5X by HE staining.

T lymphocyte culture

Mice were killed by cervical dislocation, and were soaked in 75% ethanol for 30 s. The spleen was immediately removed and put into ice-cold PBS, shred into fragments with a 10 mL syringe, followed by centrifugation at 1500 r/min at 4 ℃ for 10 min. The tissue pellets were then treated with erythrocyte lysis buffer, followed by centrifugation (1500 r/min, 4 ℃, 7 min). The procedure was repeated for another 5 min after washout of cells with PBS liquor. Cell pellets were re-suspended with 3 mL PBS buffer containing FCS, later poured into a Nylon Fiber Column T and incubated at 37 ℃, 5% CO_2 for 1 h. T lymphocytes were eluted by warm medium, and the purity of T lymphocytes was 85%—95%.

Cell counts and viability of T lymphocytes

T lymphocytes were cultured in an incubator at 37 ℃, 5% CO_2 for 2 h. T lymphocyte suspension (0.5 mL) was mixed with 0.5 mL 0.4% Trypan blue for 2—3 min and the cell numbers were determined using a hemocytometer. Total cell counts and viability of T lymphocytes were determined as follows:

Total cell number of T lymphocytes = cell counts of four bigger grids / $4 \times 10^4 \times 2$

Viability of T lymphocytes = counts of viable cells / counts of total cells

Evaluation of NO levels

NO levels were determined by measuring its stable metabolite, nitrate, using a NO assay kit according to kit instruction. The absorbance (*A*) of the samples was read on a microplate reader at 450 nm.

Determination of IL-2 levels

After incubation at 37 ℃, 5% CO_2 for 24 h, IL-2 levels in the T lymphocytes were detected by an Elisa kit according to the instruction provided. The *A* of the samples was read on a microplate reader at 450 nm.

Statistical analysis

All data were expressed as $\bar{x} \pm s$. One-way ANOVA with Tukey's post hoc test was used for the statistical analysis where appropriate using SPSS 19.0. $P < 0.05$ was considered statistically significant.

Results

Body weight

To investigate the effects of LTN on the mice exposed to chronic Rad, body weight of mice was monitored. As shown in Fig. 1, body weights of mice exposed to Rad were markedly reduced as compared to

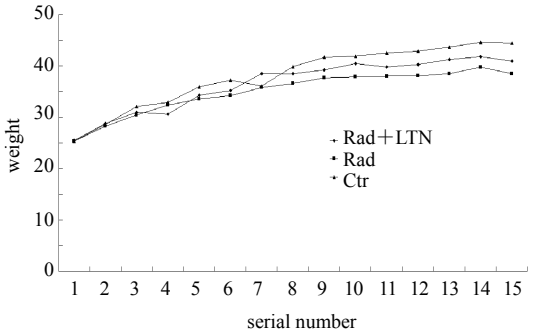


Fig. 1 Variation of weight of mice in different groups ($\bar{X} \pm s$, n = 10)

the control (Ctr) group, which was ameliorated by LTN treatment, suggesting that LTN was protective against Rad-induced weight loss.

Spleen and thymus indexes

Spleen and thymus indexes were also determined to confirm the protective effect of LTN. As a result of chronic Rad-induced injury, both of the indices were significantly lower in the radiated mice when compared to the control animals. Of interest, treatment with LTN significantly normalized both indices (Table 1), indicating that the natural medicine is therapeutically effective.

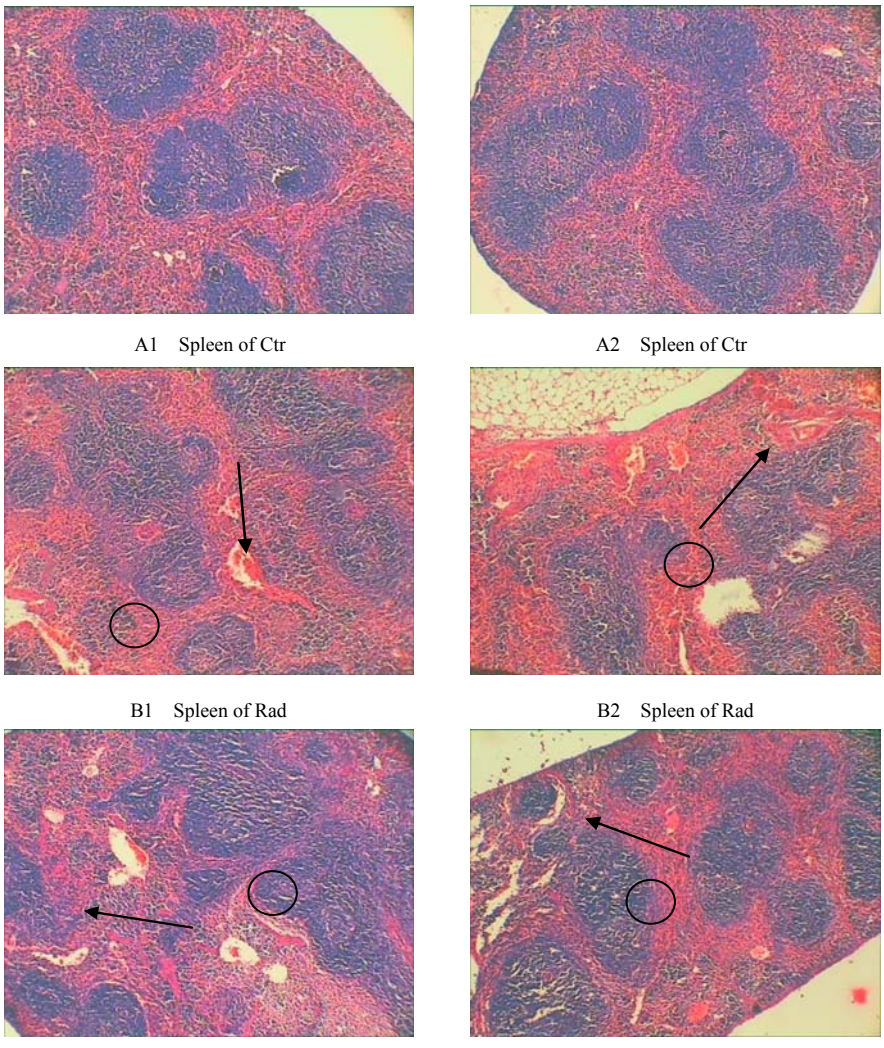
Table 1 Spleen and thymus indexes of mice ($\bar{X} \pm s$, n = 10)

Groups	Spleen index / ($mg \cdot g^{-1}$)	Thymus index / ($mg \cdot g^{-1}$)
Ctr	5.56 ± 0.54	1.54 ± 0.36
Rad	3.12 ± 1.03*	0.84 ± 0.21*
Rad + LTN	4.78 ± 0.69#	1.42 ± 0.34#

*P < 0.05 *vs* Ctr; #P < 0.05 *vs* Rad

Histological analysis

Tissue sections of the spleen were examined to determine the effect of LTN on chronic Rad stress injury in mice. As displayed in Fig. 2, the spleens of mice



A1 Spleen of Ctr A2 Spleen of Ctr

B1 Spleen of Rad B2 Spleen of Rad

C1 Spleen of Rad+LTN C2 Spleen of Rad+LTN

Fig. 2 Photographs of histological sections

exposed to Rad were damaged seriously. The serious bleeding phenomenon was found in Figs. 2B1 and 2B2. Additionally, the structure of the cell membrane is indistinct. However, spleens from mice treated with LTN had less bleeding and the cell membrane was clearly visible as shown in Figs. 2C1 and 2C2.

Cell counts and viability of T lymphocytes

Since the protective effect of LTN against chronic Rad stress was significant, we further examined whether it had any effect on the cell number and viability of T lymphocytes. As shown in Fig. 3, there was no significant difference in cell counts of T lymphocytes among the Ctr, Rad, and treated mice, however, the viability of T lymphocytes was markedly reduced in the irradiated mice compared with normal Ctr mice, which was significantly mitigated by LTN treatment (Fig. 3).

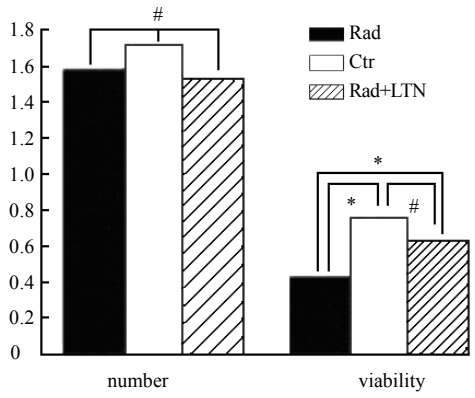


Fig. 3 Cell numbers and viability of T lymphocytes
$^{*}P < 0.05$ *vs* Ctr and Rad groups; $P > 0.05$ *vs* Ctr group

NO and IL-2 production in T lymphocytes

As shown in Table 2, NO production in T lymphocytes was significantly augmented in the mice exposed to chronic Rad when compared to Ctr animals. The treatment with LTN effectively normalized the NO production in T lymphocytes, suggesting that LTN might protect T lymphocytes from Rad injury via regulating NO production in the cells. The secretion of IL-2 was markedly impaired in the T lymphocytes isolated from irradiated mice, whereas LTN treatment effectively normalized this impairment.

Discussion

It has been documented that LTN injection protected mice against chronic Rad-induced injury (Liu *et al*, 2012), which is consistent with our observations, in terms of body weights, spleen and thymus indices, and histological analysis. Throughout past and current investigation, LTN at above doses has been proved to exert optimum protective effects against Rad by indexes of animal experiments. Based on these results, we further investigated the protective effect of LTN on T lymphocytes function in mice exposed to chronic Rad.

Table 2 Effects of LTN on NO and IL-2 levels in mice under chronic Rad stress

Groups	NO / (μmol·L^{-1})	IL-2 / (μmol·L^{-1})
Ctr	17.54 ± 0.21	64.26 ± 4.21
Rad	43.21 ± 0.44*	38.52 ± 3.16*
Rad + LTN	23.51 ± 0.18$^{*\#}$	56.62 ± 3.54$^{*\#}$

$^{*}P < 0.05$ vs Ctr group; $^{\#}P < 0.05$ *vs* Rad group

Although low levels of Rad have been shown to boost the immune system by altering the cytokine profiles (Shin *et al*, 2010), it is generally accepted that Rad is detrimental to T lymphocytes. T lymphocytes play an important role in the immune system, participating in various types and processes of immune response. Of importance, we demonstrated that LTN treatment ameliorated the viability and functions of T lymphocytes in the irradiated mice, which might in turn improve the immune competence.

In the present study, we demonstrated that LTN effectively normalized NO production in T lymphocytes up-regulated by Rad. NO is an important signaling molecule in the immune system and central nervous system. In addition, NO has been shown to play a significant role in mediating proliferation, DNA double-strand breaks, and thus mutagenesis in bystander cells exposed to Rad (Han *et al*, 2010). Moreover, de Ridder *et al* (2008) reported that the clinical immunoadjuvant OM-174 could increase the radiosensitivity of tumor cells by elevating NO synthesis in $CD8^{+}$ T lymphocytes. At the same time, increased NO production in T lymphocytes may evoke slow repair of DNA, which lead to the differential radiosensitivity of T lymphocytes (Sharma *et al*, 2010).

IL-2 is an important cytokine which produces significant effects to many immune cells. It was reported that IL-2 showed anti-Rad effects through inhibiting apoptosis and necrosis of cells (Fu *et al*, 2007). These data are corroborated with our observation that the viability of T lymphocytes was

improved by LTN, which normalized the IL-2 secretion impaired in the irradiated mice.

Conclusion

In conclusion, we demonstrated that LTN injection could attenuate the effect of chronic Rad on body weight, spleen index, and thymus index in mice. The LTN treatment could also significantly improve the viability and function of T lymphocytes impaired by radiation. However, more experiments are warranted to determine whether the anti-Rad effect of LTN is directly correlated with its effect on T lymphocytes function and viability.

Acknowledgment

Thank Miss LIU Ying-hua and Mr LI Qiang for their help with experiments. We also appreciate No. 401 Hospital of Chinese People's Liberation Army for their assistance.

References

Audette-Stuart M, Kim SB, Mchullin D, Festarini A, Yankovich TL, Carr J, Mulpuru S, 2011. Adaptive response in frogs chronically exposed to low doses of ionizing radiation in the environment. *J Environ Radioact* 102: 566-573.

de Ridder M, Jiang H, van Esch G, Law K, Monsaert C, van den Berge DL, Verellen D, Verovski VN, Storme GA, 2008. IFN-γ+ $CD8^+$ T lymphocytes: Possible link between immune and radiation response in tumor-relative hypoxia. *Int J Radiat Oncol Biol Phys* 71(3): 647-651.

Fu KF, Peng RY, Gao YB, Wang DW, Luo QL, Dong B, Ma JJ, 2007. Effect of IL-2 on the growth and apoptosis of intestinal epithelial cells radiated by neutron and mechanisms of IL-2 on the injured IEC-6. *Chin J Cell Mol Immunol* 23(8): 723-727.

Fujimoto K, Tomonaga M, Goto S, 2006. A case of recurrent ovarian cancer successfully treated with adoptive immunotherapy and lentinan. *Anticancer Res* 26: 4015-4018.

Han W, Chen SP, Yu KN, Wu LJ, 2010. Nitric oxide mediated DNA double strand breaks induced in proliferating bystander cells after-particle irradiation. *Mutat Res* 684: 81-89.

Hazama S, Watanabe S, Ohashi M, Yagi M, Suzuki M, Matsuda K, Yamamoto T, Suga Y, Suga T, Nakazawa S, Oka M, 2009. Efficacy of orally administered superfine dispersed lentinan (β-1,3-glucan) for the treatment of advanced colorectal cancer. *Anticancer Res* 29: 2611-2618.

Liu YH, Li MC, Fu QJ, 2012. Effects of protection of lentinan injection on chronic low-dose radiation injury mouse. *J Chin People's Liberat Army* 28(3): 125-129.

Kachikwu EL, Iwamoto KS, Liao YP, DeMarco JJ, Agazaryan N, Economou JS, McBride WH, Schaue D, 2011. Radiation enhances regulatory T cell representation. *J Biol Contribut* 81(4): 1128-1135.

Oba K, Kobayashi M, Matsui T, Kodera Y, Sakamoto J, 2009. Individual patient based meta-analysis of lentinan for unresectable/ recurrent gastric cancer. *Anticancer Res* 29: 2739-2746.

Rao BN, Archana PR, Aithal BK, Rao BS, 2011. Protective effect of Zingerone, a dietary compound against radiation induced genetic damage and apoptosis in human lymphocytes. *Eur J Pharm* 657: 59-66.

Roupas P, Keogh J, Noakes M, Margetts C, Taylor P, 2012. The role of edible mushrooms in health: Evaluation of the evidence. *J Funct Foods* 4(4): 687-709.

Russell CA, Wulf HC, Sorensen PB, Vindelov LL, 1998. Evaluation of X-radiation and UV-B radiation for elimination of ^{3}H-thymidine incorporation into responder cells in the IL-2 producing helper T lymphocyte precursor assay. *J Immunol Methods* 220: 161-168.

Sharma D, Sandur SK, Rashmi R, Maurya DK, Suryavanshi S, Checker R, Krishnan S, Sainis KB, 2010. Differential activation of NF-B and nitric oxide in lymphocytes regulates *in vitro* and *in vivo* radiosensitivity. *Mutat Res* 703: 149-157.

Shin SC, Lee KM, Kang YM, Kim K, Kim CS, Yang KH, Jin YW, Kim CS, Kim HS, 2010. Alteration of cytokine profiles in mice exposed to chronic low-dose ionizing radiation. *Biochem Biophys Res Commun* 397: 644-649.

Taguchi T, Furue H, Kimura T, Kondo T, Hattori T, Ogawa N, 1983. Clinical efficacy of lentinan on neoplastic diseases. *J Med Biol* 166: 181-187.

Zhou AW, Ge DZ, Liang JS, Wang B, Zheng FL, Li CY, Chen MZ, 1995. Immunomodulatory effects of lentinan. *J Chin Pharmacol Bull* 11(2): 157-159.