Protective effects of a water-soluble extract from cultured medium of *Ganoderma lucidum* (Rei-shi) mycelia and *Agaricus blazei* murill against X-irradiation in B6C3F1 mice: Increased small intestinal crypt survival and prolongation of average time to animal death

NANAMI KUBO[1], YUKI MYOJIN[1], FUMIO SHIMAMOTO[1], NAOKI KASHIMOTO[2], EIKAI KYO[3], KENJI KAMIYA[2] and HIROMITSU WATANABE[2]

[1]Department of Health Science, Hiroshima Prefectural Women's University, 1-1-71 Ujina-higashi, Minami-ku, Hiroshima 734-8558; [2]Department of Experimental Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3, Kasumi, Minami-ku, Hiroshima 734-8553; [3]Health Care Institute, Wakunaga Pharmaceutical Co., Ltd., 1624, Shimokotachi, Koda-cho, Akitakata, Hiroshima 739-1195, Japan

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Abstract. Radioprotective effects of a water-soluble extracts from cultured medium of *Ganoderma lucidum* (Rei-shi) mycelia (designed as MAK) and *Agaricus blazei* (Agaricus) against the shortening of survival time or the injury of crypt by X-irradiation were investigated in male B6C3F1 mice. MAK and Agaricus at three different doses were mixed into basal diet into biscuits at 5, 2.5 and 1.25% and administered from 1 week before irradiation. MAK (5% group) significantly prolonged animal survival as compared with basal diet group (control group) after 7 Gy of X-ray irradiation at a dose rate of 2 Gy min^{-1}. At doses of 8, 10 and 12 Gy X-irradiation at a dose rate of 4 Gy min^{-1} MAK (5% group) significantly increased crypt survival as compared to other groups. These results suggest that MAK can act as a radioprotective agent.

Introduction

One major goal of radiobiology research is the development of drugs that can be used to provide protection against radiation injury, and numerous compounds have been developed and tested. The observed protective effects point to the possibility of improving the therapeutic index of cancer radiotherapy, or reducing the acute radiation effects in persons exposed in accidents. The strategy of reducing radiation injury to normal tissues might thus have significant benefit in terms of medical applications. Hsu *et al* (1-4) reported radioprotective effects of several kinds of Chinese traditional prescriptions and enhanced immunocompetence after irradiation was found. These results have encouraged us to search for other drugs that might exert radioprotective influence.

Various mushrooms have a long history of use in folk medicine, and become subjects of great interest, due to their multiple nutritional and pharmacological properties. Mushroom extracts are widely sold as nutritional supplements and touted as beneficial for health. However, only a few studies are available on the biological effects of mushroom consumption. *Ganoderma lucidum* (Fr.) Karst, belonging to the Basidiomycetes class of fungi, is colloquially known as 'Rei-shi' or 'Mannentake' in China and Japan, and it has been attributed with various medical virtues handed down in folklore. *Ganoderma lucidum* exhibits anti-hepatoxic and free radical scavenging activity (5), exerts influence on the cell cycle and cellular signal transduction (6), inhibits leukemic-cell growth (7), and induces differentiation of leukemic cells into mature monocytes/macrophages (8). In addition, it may inhibit platelet aggregation (9), impede complex interactions of viruses with cell plasma membranes (10), inhibit tumor growth (11) and decrease the incidence of mouse lung tumors (12). A water soluble extract from cultured medium *Ganoderma lucidum* (Rei-shi) mycelia (designed MAK) contains various kinds of high molecular constituents, i.e. polysaccharides with protein or water-soluble lignin, and low molecular constituents, i.e. triterpenes. Previously, we have reported that MAK prevented the development of azoxymethane induced aberrant crypt foci (ACF,13), development of N,N'-dimethylhydrazine-induced colon tumors in ICR mice (14) and colon tumors induced by azoxymethane in F344 rats (15).

Correspondence to: Dr Hiromitsu Watanabe, Department of Experimental Oncology, Research Institute for Radiation Biology and Medicine, Hiroshima University, 1-2-3, Kasumi, Minami-ku, Hiroshima 734-8553, Japan
E-mail: tonko@hiroshima-u.ac.jp

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Table I. Change of testis 28 days after 6 Gy X-irradiation.

Group	Size (μm)	Total no.	PCNA negative	Ratio (negative/ total)
X-ray+ 5% MAK[a]	77.0±8.0	50.9±6.0	7.4±2.7[b]	14.0±5.0[c]
X-ray+ 5% Agaricus	70.0±8.0[b]	45.0±6.1	12.6±5.4[b]	28.0±11.0[c]
X-ray	75.0±8.0[b]	46.1±9.3	10.6±5.5	23.0±11.0
Control	175.0±14.0	29.0±3.8	0	0

[a]MAK, a water-soluble extract from cultured medium of *Ganoderma lucidum* (Rei-shi) mycelia. [b]$p<0.05$; [c]$p<0.01$.

The Basidiomycete mushroom *Agaricus blazei* Murrill, native to Brazil and popularly known in Japan as Himematsutake, has been largely produced and consumed as food and tea due to its medicinal effects, possibly including anti-carcinogenic activity (16,17). However, no experimental data exist regarding beneficial effects of this species of mushroom.

The present study was therefore conducted to assess the effects of MAK or *Agaricus blazei* extracts on crypt and animal survival after X-irradiation in mice.

Materials and methods

Animals. Six-week-old male B6C3F1 (Crj:B6C3F1) mice and our standard protocol for assessing radiation effects were employed in the present experiment. Animals were housed in polycarbonate cages, five per cage, and kept under constant conditions of temperature (24±2°C) and humidity (50±10%) with a 12 h light/12 h dark cycle, according to the Guide for Care and Use of Laboratory Animals established by Hiroshima University, and fed a commercial diet MF (Oriental Yeast Co. Ltd., Tokyo, Japan) alone or with a 5, 2.5 and 1.25% supplement of MAK and Agaricus in biscuits. Normal tap water was also provided *ad libitum*.

MAK and Agaricus. A water-soluble extract from culture medium of *Ganoderma lucidum* mycelia (designed as MAK) was prepared by Noda Shokkin-Kogyo Co., Ltd. (Chiba, Japan). In brief, *Ganoderma lucidum* (Rei-shi or Mannnentake) mycelia were cultured in a solid medium composed mainly of sugar-cane bagasse for 3 months, then the whole medium containing mycelia was extracted with hot water. The extract was filtered and spray-dried as MAK. Agaricus was purchased as a commercial powder of *Agaricus blazei* Murill.

Radiation. Groups of mice were whole body irradiated with 6 or 7 Gy of X-rays (each 10 animals) at a dose rate of 2 Gy/min for the animal survival study and 8, 10 or 12 Gy of X-rays once for crypt survival (each 5 animals) at a dose rate of

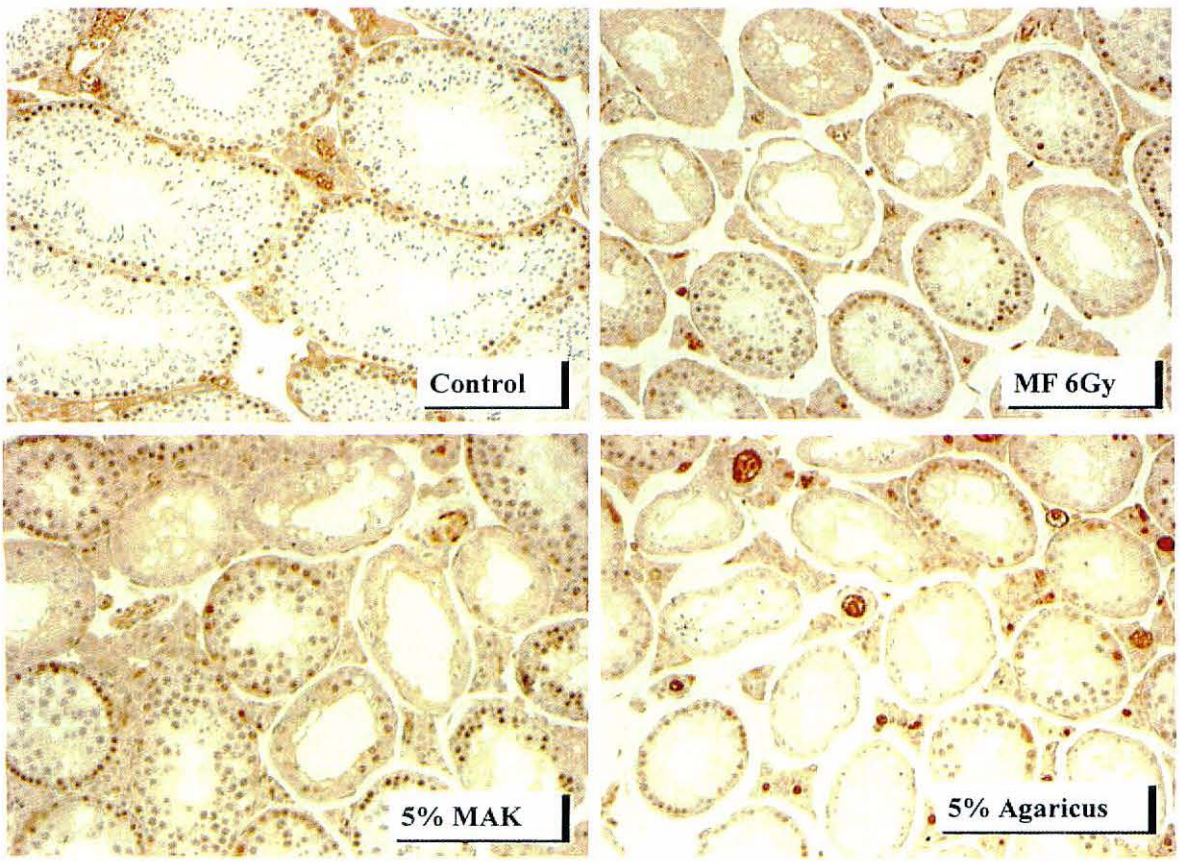

Figure 1. PCNA staining in seminiferous tubules.

Table II. Size of seminiferous tubules 4 weeks after 6 Gy whole body irradiation.

Group	Size (μm)
MF	75.0±7.8[a]
5% MAK	76.9±8.0
5% Agaricus	70.3±8.5[a]

MAK, a water-soluble extract from cultured medium of *Ganoderma lucidum* (Rei-shi) mycelia. [a]Significantly different from 5% MAK.

4 Gy/min as measured with a Radocon 555 dosimeter. The mice were not anaesthetized during the irradiation. Exposure factors were as follows: 200 kVp and a half-value layer 1.18 mm Cu. The X-ray air dose (in R) was then converted to the absorbed dose (in cGy) using a factor of 0.95 cGy/R.

One week before irradiation, the mice were given a diet supplemented with MAK and Agaricus and kept for 28 days on the same diet after X-irradiation with 6 and 7 Gy. The animals were observed every day at 8:00, 12:00 and 18:00, and deaths were recorded for the animal survival experiment. In the other groups, the animals were kept for 3.5 days after irradiation then sacrificed for determination of crypt survival.

Autopsy. Immediately after sacrifice, segments of the jejunum from the ileocecal junction (30 to 40 cm) were removed and fixed in Carnoy's solution. They were cut into several pieces, bundled together, embedded in paraffin, sectioned at a thickness of 3 μm and stained with hematoxylin-eosin. To quantitative regenerating crypts, number of crypts per circumference was determined in cross-section (18). In each mouse the number of surviving crypts in 10 gut cross-sections was scored.

Animals were sacrificed after cumulative irradiation for 28 days. Testes were fixed in FSA solution (37% formalin 5 ml, 5% sucrose solution 15 ml and acetic acid 0.8 ml) for 5 days, then embedded, sectioned and stained routinely. Sizes of seminiferous tubules were measured. For immunohistochemistry, paraffin-embedded sections were deparaffinized in xylene, and rehydrated through graded alcohols. A 0.05 M PBS buffer was used to prepare solutions and for washes between the various steps. Incubations were performed in a humidified chamber. Three μm-thick sections were treated for 30 min at room temperature with 2% BSA and incubated with primary antibodies against monocolonal mouse anti-proliferating cell nuclear antigen antibody (Dako-PCNA, PC 10, code No. M 879) for 1 h at room temperature. For each case, negative controls were performed on serial sections whereby incubation with the primary antibody was omitted. All slides were then exposed to the secondary antibody, biotinylated horse anti-universal-monkey IgG (Vectastain Universal Quick Kit, Vector Laboratories, Ca, Catalog No. PK-8800) and peroxidase conjugated streptoavidin complexes. Peroxidase activity was visualized by treatment with H_2O_2 and diaminobenzidine for 5 min. At the last step, the sections were counterstained with hematoxylin for 1 min. PCNA-positive cells in seminiferous tubules were counted.

Statistics. Statistical significance was determined with Dunnett's method and the Cox proportional hazard model for multiple comparisons using logarithmic transformation and the Student's t-test.

Results

Survival was not significantly affected with 6 Gy irradiation. Testes of surviving animals after 28 days of irradiation demonstrated significantly smaller seminiferous tubules in the 5% Agaricus group than with X-rays alone (Table I). The

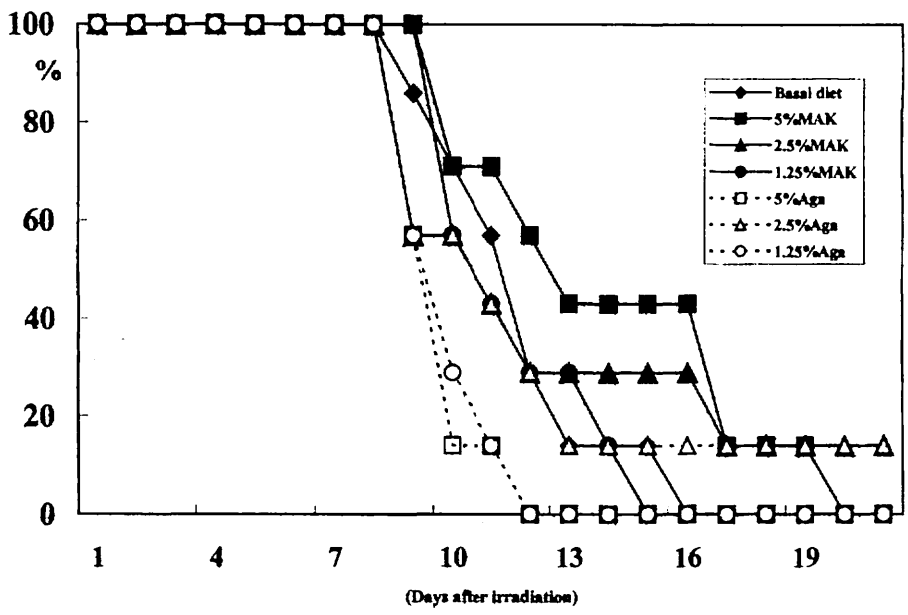

Figure 2. Survival after 7 Gy irradiation. MAK, a water-soluble extract at 5% from cultured medium of *Ganoderma lucidum* (Rei-shi) mycelia. MAK vs. Basal diet $p<0.02$, 5% MAK vs. 1.25% MAK $p<0.02$, 5% MAK vs. 5% or 1.25% AGA $p<0.007$.

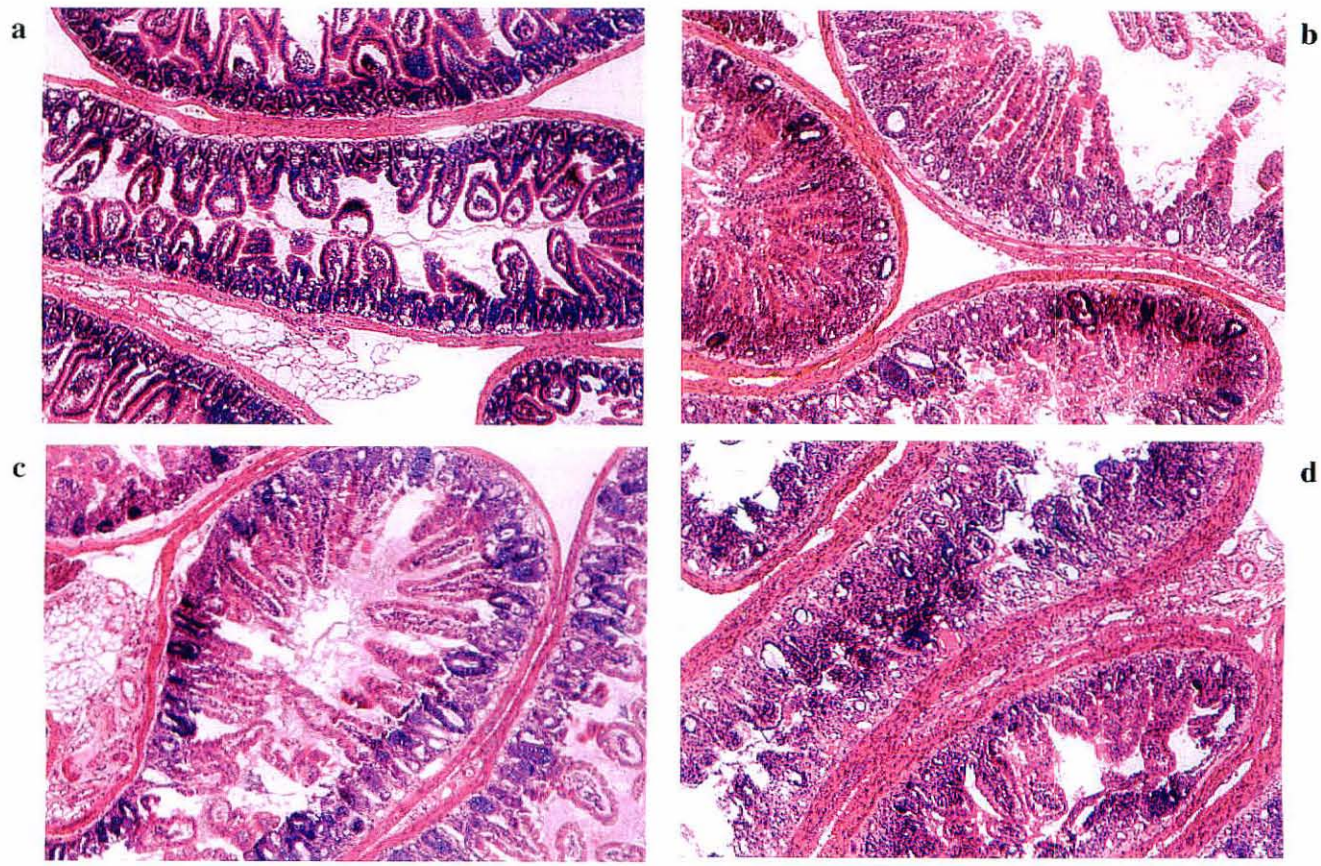

Figure 3. (a) Normal small intestine; (b) 10 Gy-irradiated small intestine in MF diet group. A few regenerated crypts were observed; (c) 10 Gy-irradiated small intestine in 5% MAK group. Many regenerated crypts were observed; (d) 10 Gy-irradiated small intestine in 5% Agaricus group. A few regenerated crypts were observed.

Table III. Crypt survival.

	0 Gy	8 Gy	10 Gy	12 Gy
MF	116.53±13.39	84.54±11.74	43.74±8.42	24.76±5.62
5% MAK		117.00±12.47	68.06±9.63	43.77±7.64
2.5% MAK		81.38±10.41	52.52±8.90[b]	28.08±5.49[b]
1.25% MAK		87.88±11.21	51.72±7.59[b]	29.78±4.44[b]
5% Agaricus		81.06±10.06	42.50±6.60	27.52±4.68[a]
2.5% Agaricus		83.52±10.18	49.98±7.30[b]	26.38±3.85
1.25% Agaricus		82.60±10.47	51.78±8.29[b]	27.02±4.98

MAK, a water-soluble extract from cultured medium of *Ganoderma lucidum* (Rei-shi) mycelia. 5% MAK was significantly different from other groups; ($p<0.01$). [a]Significantly different from MF group ($p<0.05$). [b]Significantly different from MF group ($p<0.01$).

number of PCNA-negative seminiferous tubules was zero in control animals (Fig. 1). Ration of PCNA negative vs. total seminiferous tubules in 5% MAK values was significantly smaller than that in 5% Agaricus values (Table II). Animals in Agaricus groups started to die 9 days after irradiation and survival is shown in Fig. 2. Delay in mortality was evident in 5% MAK group, with significantly increased survival in the MF ($p=0.02$), 1.25% MAK ($p=0.02$) and 1.25% Agaricus ($p=0.007$) by the Cox model.

The number of crypts in one circumference in the non-irradiated group was 116.5±13.4 (Fig. 3a). A dose-dependent decrease was evident with 8-12 Gy (Table III and Fig. 3b) and surviving crypts in 5% MAK (Fig. 3c) were significantly increased, compared to other groups in every dose. Crypt survival was evident with a significant difference in 2.5 and 1.25% MAK and Agaricus (Fig. 3d) ($p<0.01$) as compared with MF group in 10 Gy irradiation and in 2.5%, 1.25% MAK ($p<0.01$) and 5% Agaricus ($p<0.05$).

Discussion

The present paper documents a significant increase in the survival of crypts in animals receiving 5% MAK associated with a prolongation of average time to animal death after X-irradiation. Hsu *et al* (4,19) earlier reported that intraperioneal injections of the extract from *Ganoderma lucidum* before irradiation of 5 or 6.5 Gy X-rays improved the 30-day survival of ICR mice and increased recovery as assessed by hemograms, the 10-day blood forming stem cells (CFU) also being significantly higher for the *Ganoderma lucidum* treated group than for the untreated group. Chen *et al* (20,21) reported that administration of an extract of *Ganoderma lucidum* was able to enhance the recovery of cellular immunocompetence after 4 Gy-ray irradiation of ICR mice. It is well documented that radiation is a potent immunosuppressive agent, and moderate doses exert clear inhibitory effects on the counts of total leukocytes, lymphocytes and neutrophils. Radiation also has destructive effects on the leukocytepoietic organs such as the spleen, thymus and bone marrow, and protection effects of traditional Chinese medicines (1-4,19), ginseng (12,22,23) and garlic (24) have been investigated. They were able to enhance the recovery from decreased cellular immunocompetence, with protection or stimulation of the reticuloendothelial system, and induction of free radical scavenger. Recently, we reported that a water-soluble extract from culture medium of *Ganoderma lucidum* mycelia (designed as MAK) may stimulate the natural immune system or the aquire immune system in tumor-bearing mice (25). Therefore MAK might be a potent immunomodulater that up-regulates against immunosuppression by X-irradiation.

Houchen *et al* (26) have reported that expression of FGF-2 is induced by radiation injury and that recombinant human FGF-2 markedly enhanced crypt survival. Takahama *et al* (27) found that a replication-deficient adenovirus containing the HST-1 gene acts as a potent protector against lethal irradiation associated with injury to the intestinal tract as well as myelosuppression in the bone marrow and spleen. Farrell *et al* (28) have presented findings that recombinant human keratinocyte growth factor can protect mice from chemotherapy- and radiation-induced gastrointestinal injury and mortality, at least in terms of death from intestinal and marrow toxicity. We also found VEGF to have a protective influence (29). Cytokine-like substances in MAK may thus play an important role in the protection and/or the recovery and repopulation of critical tissue elements when given prior to and during radiation exposure. However, to our knowledge, there are no reports regarding cytokines in MAK.

MAK contains various kinds of high molecular constituents, i.e. polysaccharides with protein or water-soluble lignin, and low molecular constituents, i.e. triterpenes. *Ganoderma lucidum* mycelia were cultured in a solid medium composed mainly of sugar-cane bagasse for 3 months. Lignin is processed or converted to water-soluble lignin with enzymes during growth of mycelia. A water-soluble lignin is seemed to be the characteristic constituent, and does not contribute to every function of MAK, but is closely related to some function of MAK.

The present experiment provided evidence of recovery of seminiferous tubule size and DNA synthesis with MAK, but not Agaricus treatment. The testis is the most sensitive organ for radiation injury (30,31) and the turnover time from primary spermatogonia to sperm is 60 days (32). Thus, both the smaller testis size and number of PCNA-positive cells of seminiferous tubes in the Agaricus group as compared with the MAK group, are compelling evidence of less radiation protection.

Studies are now in progress in our laboratory to further elucidate the active compounds of the water-soluble extract, the mode of their action included immune parameter and further clinical study for pharmaceutical effects.

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