Protection against Radiation-Induced Bone Marrow and Intestinal Injuries by *Cordyceps sinensis*, a Chinese Herbal Medicine

Wei-Chung Liu,[a,b] Shu-Chi Wang,[a] Min-Lung Tsai,[a] Meng-Chi Chen.[a] Ya-Chen Wang,[a] Ji-Hong Hong,[c]
William H. McBride[d] and Chi-Shiun Chiang[a,1]

[a] *Department of Biomedical Engineering and Environmental Sciences, National Tsing Hua University, Hsinchu 30013, Taiwan;* [b] *Department of Radiological Technology, Tzu Chi College of Technology, Hualien 970, Taiwan;* [c] *Department of Radiation Oncology, Chang Gung Memorial Hospital, Tao-Yuan 30033, Taiwan; and* [d] *Department of Radiation Oncology, UCLA, Los Angles, California 90095*

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Bone marrow and intestinal damage limits the efficacy of radiotherapy for cancer and can result in death if the whole body is exposed to too high a dose, as might be the case in a nuclear accident or terrorist incident. Identification of an effective nontoxic biological radioprotector is therefore a matter of some urgency. In this study, we show that an orally administered hot-water extract from a Chinese herbal medicine, *Cordyceps sinensis* (CS), protects mice from bone marrow and intestinal injuries after total-body irradiation (TBI). CS increased the median time to death from 13 to 20 days after 8 Gy TBI and from 9 to 18 days after 10 Gy TBI. Although CS-treated mice receiving 10 Gy TBI survived intestinal injury, most died from bone marrow failure, as shown by severe marrow hypoplasia in mice dying between 18 and 24 days. At lower TBI doses of 5.5 and 6.5 Gy, CS protected against bone marrow death, an effect that was confirmed by the finding that white blood cell counts recovered more rapidly. *In vitro*, CS reduced the levels of free radical species (ROS) within cells, and this is one likely mechanism for the radioprotective effects of CS, although probably not the only one. © 2006 by Radiation Research Society

INTRODUCTION

Radiation injury to stem cells in bone marrow and in intestinal crypts can cause death after whole-body radiation exposure. In addition to their usefulness in controlled clinical situations, the increasing risk of a nuclear accident or "dirty" bomb accentuates the need for chemical or biological modifiers of radiation-induced damage that can prevent or enhance recovery from radiation tissue damage. Many

preclinical and clinical findings have shown the potential benefits of certain cytokines in mitigating the radiation responses of bone marrow and gastrointestinal tissues [reviewed in refs. (*1, 2*)]. The FDA has approved several cytokines including G-CSF, GM-CSF, pegylated G-CSF and IL11 for the treatment of acute myelosuppression regardless of etiology. However, their use is often limited to a narrow scenario, mainly because of their specificity, their intrinsic pleiotropism, and their cascadic mechanism of action. The complex biological interactions that result sometimes make prediction of response difficult, especially with regard to the toxic side effects that often limit dose (*3*). There is therefore a need to find nontoxic radioprotectors that are applicable to a broad variety of operational conditions.

Cordyceps sinensis (CS) is a fungal parasite of moth larvae (*Lepidoptera*) in the genera *Hepitalus* and *Thitarodes* (*4*) that has been widely used in traditional Chinese medicine as a tonic agent [see reviews in refs. (*5–7*)]. In the past 20 years, as the result of the "Dietary Supplement Health and Education Act of 1994" from the Center for Food Safety and Applied Nutrition, there has been a phenomenal growth of interest in and use of complementary or alternative medicines that has promoted CS as a popular remedy for cancer treatment in Chinese society. In addition to its anticancer activity, CS has been ascribed a broad spectrum of pharmacological actions, including modulating the hepatic, renal, cardiovascular, immune, nervous, endocrine and steroid systems [see reviews in refs. (*5–7*)]. At the cellular level, diverse biological effects of CS, such as activating macrophages (*8*), modulating apoptosis (*9, 10*), or inhibiting tumor metastasis (*11, 12*), have been described, many of which can be attributed to production of cytokines, such as IFNG, TNFA, IL1, IL6 or GM-CSF (*8, 13*). This study was undertaken to test in mice the hypothesis that CS would protect against radiation-induced gastrointestinal (GI) syndrome and bone marrow failure.

MATERIALS AND METHODS

Mice and Treatments

C57BL/6J mice were purchased from the National Laboratory Animal Center, Taiwan, and housed in National Tsing-Hua University Laboratory

Animal Center, Taiwan. Seven- to eight-week-old male mice were used for experiments. The mice were given *Cordyceps sinensis* (CS) (50 mg/kg per day) once daily for 7 days through an orogastric tube. The control group of animals received saline only. One day after the last treatment with CS, groups of five unanesthetized mice were placed in a circular plexiglass rotating jig to receive total-body irradiation (TBI). Irradiation with 5.5, 6.5, 8, 10, 12 and 15 Gy was delivered at 2 Gy/min using a ^{137}Cs irradiator (model Gammacell®1000 Elite, Nordion International Inc., Canada). The number of surviving mice was recorded daily for 30 days after TBI. Data from two or three experiments were pooled, and survival curve analysis was performed using GraphPad Prism software version 3.03 (GraphPad Software, Inc., San Diego, CA). In all experiments, bone and intestinal tissues of mice were collected at the indicated times, at the time of death, when animals were euthanized after displaying labored breathing, or at the termination of the experiment at day 30 after TBI. In one experiment, groups of three mice were killed at 4 h, 3.5 days or 10 days and tissues from bone and intestine were prepared for histopathological examination. During the experiments, all mouse care followed the recommendations of the Guide for the Care and use of Laboratory Animals approved by the Institutional Animal Care and Use Committee (IACUC approval number: 09508) of National Tsing Hua University, Taiwan.

Hot-Water Extract of Cordyceps sinensis

CS was purchased from the Chinese Medicine Drug store, Taipei, and genetically identified (*14, 15*) by Dr. Ruey-Shyang Hseu (*16*) in the Institute of Microbiology and Biochemistry, National Taiwan University. The CS was first dried in a 45°C oven overnight and ground in a blender into a dry powder. The powder was dissolved in deionized water in a 1:20 (w/v) ratio (1 g in 20 ml) and then placed into a 90°C water bath for 2 h. After cooling to room temperature, it was spun at 3000 rpm for 10 min. The supernatants were collected and filtered through a 0.22-μm filter. To ensure sample quality, the filtered CS extracts were subjected to HPLC fingerprinting (*17*) and analysis of mineral elements [(*18*), M. L. Tsai, manuscript in preparation].

Histology of Intestines

After humane killing of the mice, the small intestines were collected and the intestinal contents were removed. The small intestines were fixed with 10% neutral-buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) for examination. The surviving crypt stem cells were assessed as described by Withers and Elkind (*19*). Three sections from each mouse were counted. The average for three mice was used for statistical analysis. Apoptotic cells stained with H&E were scored on cell the basis of position within the crypts of the small intestine according to the method of Ijiri and Potten (*20*). For every counting procedure, 40 half-crypts were counted from each individual mouse in every group. Apoptosis was assessed on the basis of morphological characteristics such as cell shrinkage, chromatin condensation and cellular fragmentation (*20*) and was confirmed in some sections by terminal deoxy transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining using the DeadEnd™ colorimetric system (Catalog no. G7130, Promega, WI).

Histology of Bone Tissues

After killing, femurs and tibias were removed and fixed in 10% paraformaldehyde solution for 5 h. The samples were decalcified in 12–18% sodium EDTA (pH 7.4–7.5) for 10 days. Bone specimens were then dehydrated through graded ethanol concentrations. Paraffin embedding was carried out under vacuum in low-melting-point medium (Leica Histomax) at 55°C for 4 h. Bone tissues were cut into 5-μm sections using a microtome (Leica RM2245), then stained with H&E.
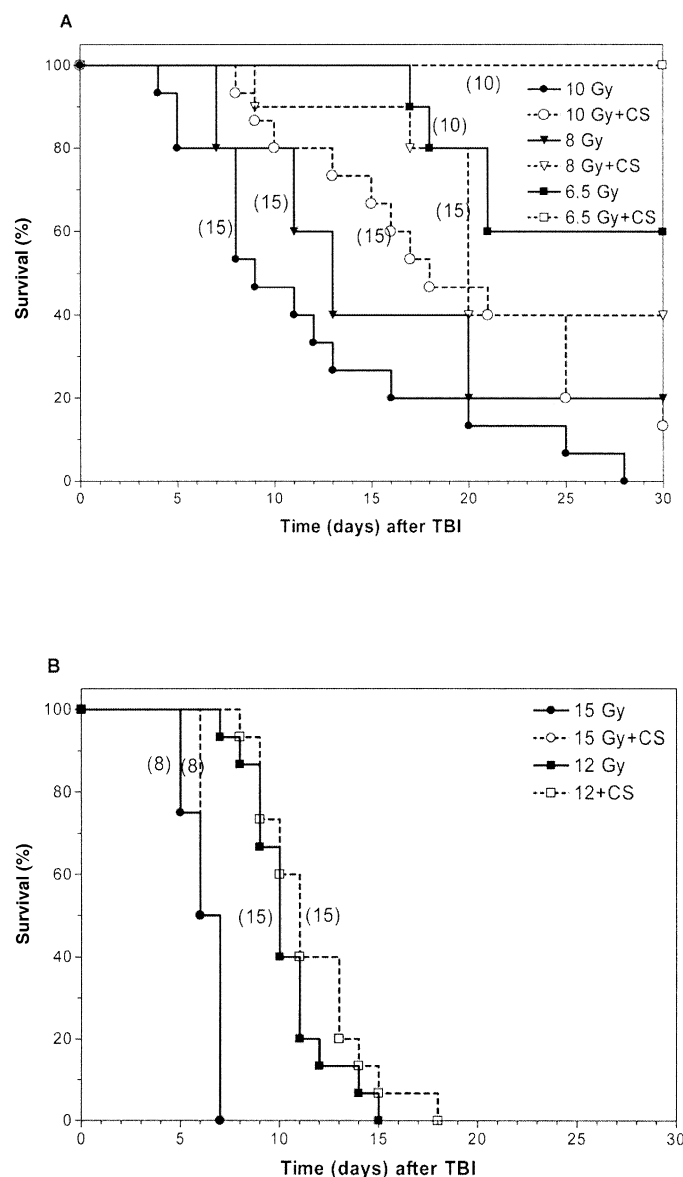

FIG. 1. Influence of CS on animal survival in response to various doses of TBI. Oral administration of CS for 1 week prior to TBI protected mice from radiation damage in the dose range of 6.5 to 10 Gy (panel A), but it did not affect the time to death after 12 or 15 Gy TBI (panel B). Data from two or three repeated experiments were pooled and analyzed by GraphPad Prism software version 3.03. Numbers in parentheses are the number of mice used in each group.

White Blood Cell Counts

Peripheral blood (PB) was collected from tail veins and total blood cell counts were obtained using a blood counter. Nucleated PB cells were prepared by sedimenting erythrocytes through 3% dextran (T-500); residual red blood cells were further removed by erythrocyte lysing reagent kit (Partec, Germany). Differential counts were performed on cytocentrifuged (600 rpm) preparations stained by Leu's Stain (*21*).

Colony Formation Assay

Bone marrow cells (BMC) were harvested from the femur and tibial bones by flushing the medullary cavities with Hanks' balanced salt solution. Cells (1 $\times$ 10^6 cells/ml) were cultured in six-well plates in 2 ml
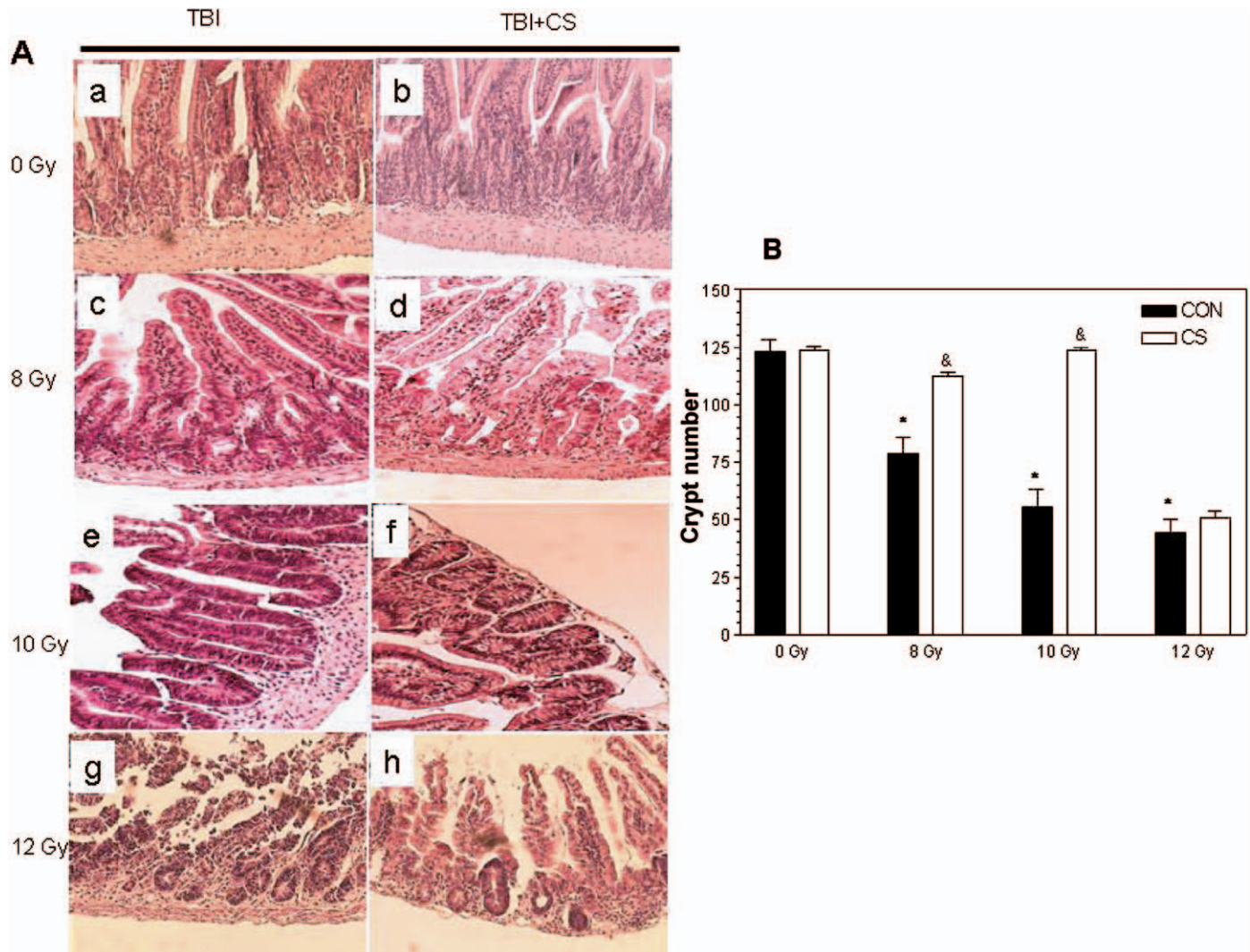
FIG. 2. CS protects mice against TBI-induced intestinal injuries. Panel A: H&E staining of intestine 10 days after various doses of TBI in mice receiving CS or saline. Panel B: Crypt stem cell survival assay scored 3.5 days after various doses of TBI quantifying the radioprotective effects of CS. CS increased crypt stem cell survival after 8 and 10 Gy but not after 12 Gy TBI. Data and error bars represent the average ± 1 SD of three mice from one of three representative experiments. *$P < 0.05$ by ANOVA compared with 0-Gy control mice that received saline. &$P < 0.05$ by Student's *t* test compared with same TBI dose for control animal.

of RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated FCS, 1% penicillin/streptomycin, and 50 μM 2-mercaptoethanol (Sigma-Aldrich). After 24 h incubation, nonadherent BMC were collected and adjusted to a concentration of 2×10^5 cells/ml in PBS. Cells were separated into two major groups [control and CS (500 μg/ml)-treated]. After 1 h at 37°C, cells were exposed to various doses of radiation using a cobalt source in the Nuclear Science and Technology Development Center, National Tsing Hua University, Taiwan, at a dose rate of 50 cGy/min. After irradiation, cells were washed three times with PBS to remove the CS and then plated in premixed methycellulose culture medium (Methocut M3234, Stem Cell Technologies; Vancouver, Cananda) as described by Lin *et al.* (*22*). Final adjusted concentrations were 1% methylcellulose, 15% FCS, 1% BSA, 10 μg/ml insulin, 200 μg/ml transferrin, 10^{-4} M 2-mercaptoethanol, and 2 mM L-glutamine. Recombinant murine IL3 and GM-CSF (Biosource) were added at 10 ng/ml and 500 ng/ml, respectively. BMC suspensions (2×10^5 cells/ml, 0.3 ml) were added to complete mixed culture medium (2.7 ml), vortexed and plated in petri dishes (Falcon, Becton Dickinson) at 1.1 ml/dish. After 7 days incubation, GM-CFU colonies consisting of 50 or more cells were scored using an inverted microscope.

Detection of ROS Formation

Murine osteoblastic cells (MC3T3-E1, subclone 4) obtained from ATCC (Catalog no. CRL-2593) were cultured in α-MEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FCS and antibiotics (100 μg/ml penicillin G, 50 μg/ml gentamycin, and 0.3 μg/ml fungizone). MC3T3-E1 cells at 80% confluence were further incubated in the presence or absence of CS (500 μg/ml) for 24 h. After 24 h incubation, a free radical responsive dye, 20 μM 2,7-dichlorodihydrofluorescein (DCF) diacetate (Catalog no. 35845, Biochemika), was added for 1 h at 37°C. After incubation and washing, samples were analyzed by flow cytometry (Cy-Flow®, Partec, GmbH) for green fluorescence in live cells.

RESULTS

Cordyceps sinensis Protects against Radiation Lethality

The ability of a hot-water extract of *Cordyceps sinensis* (CS) to protect mice against the lethal effects of total-body
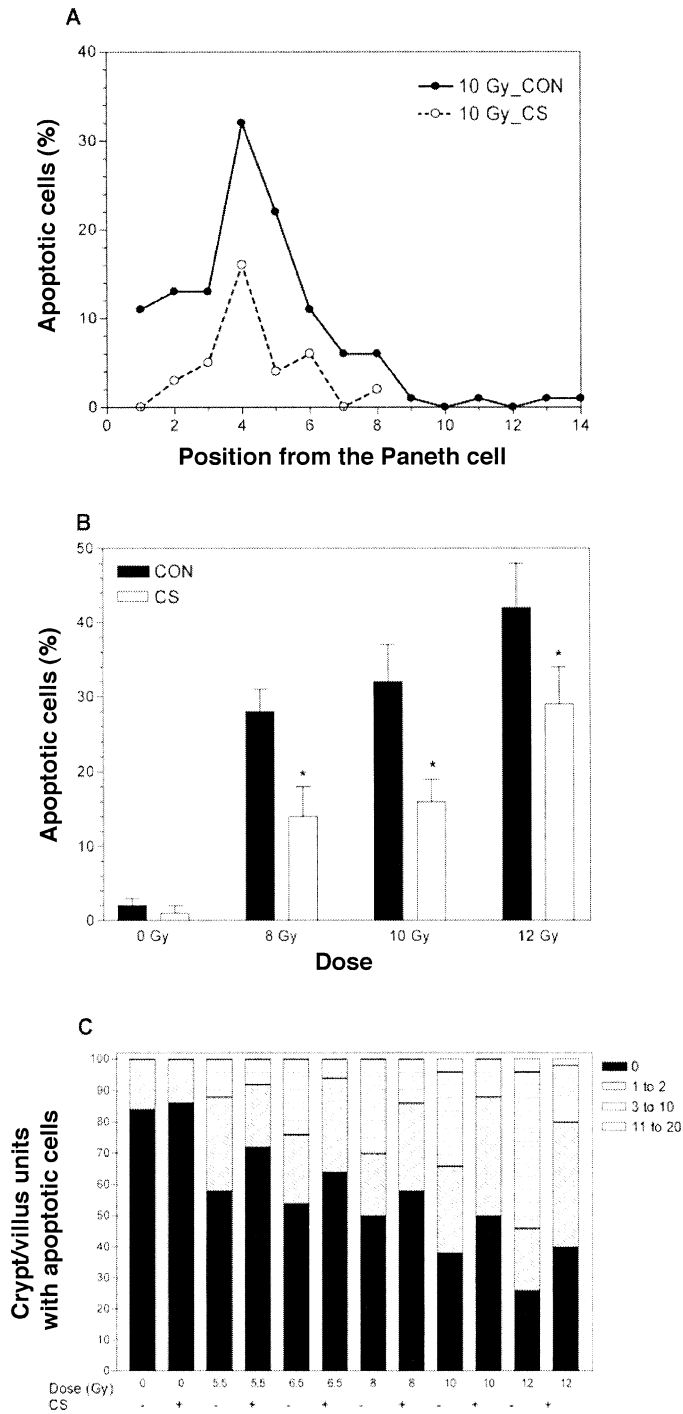
A

B

C

FIG. 3. CS protects crypt stem cells and lamina propria cells from radiation-induced apoptosis. Panel A shows the distribution of apoptotic cells in the crypt 4 h after 10 Gy irradiation as assessed by TUNEL staining. The frequency of apoptotic cells peaks at position 4. CS treatment does not change the distribution but reduces the incidence of apoptosis at all positions. Panel B shows the dose response of apoptosis at the fourth cell position. CS treatment reduces the incidence of crypt stem cell apoptosis at all doses. Panel C shows the dose response of irradiated crypt-villus units in the lamina propria. CS treatment decreases the number of apoptotic cells. To obtain these data, the apoptotic cells in the crypt were counted in 40 half-crypts from each individual mouse in every group. Data points and error bars represent the averages ± 1 SD of three mice from one of three representative experiments. The apoptotic cells in the lamina propria were scored in 40 units per sample. *$P < 0.05$ by Student's *t* test compared with the same TBI dose to control animals.
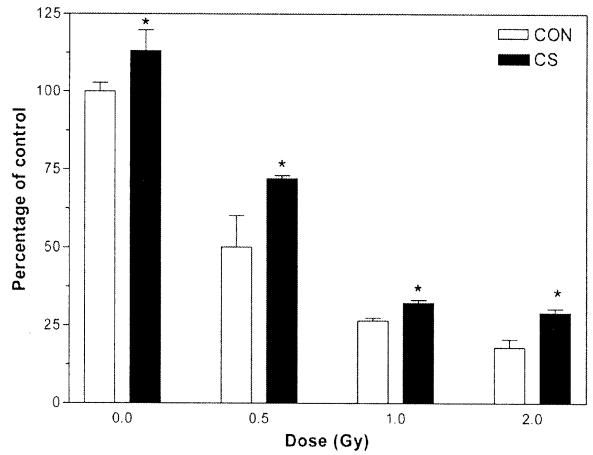

FIG. 4. Influence of *Cordyceps sinensis* on survival of bone marrow stem cells. The *in vitro* presence of CS enhanced the survival of non-adherent BMC GM-CFU after various doses of radiation as assessed by colony formation. For 0 Gy, the percentage was calculated by the ratio of the number of colonies in the presence or absence of CS. For other radiation doses, the percentage was calculated by the ratio to 0 Gy of each group.

irradiation (TBI) was tested by giving C57BL/6J mice CS by orogastric tube once daily for 7 days, followed 1 day later by TBI doses ranging from 5.5 to 15 Gy (Fig. 1). In preliminary studies, the effect of the timing of administration of CS relative to 10 Gy TBI was examined. The same degree of protection was found whether CS was given before or after TBI (see Supplementary Fig. 1); however, there were some unexpected deaths of mice receiving orogastric saline or CS after TBI that we suspect were due to damage caused by the use of the orogastric tube in a previously irradiated site. To avoid this complication, the agent was therefore given before irradiation.

Typically, death of mice within 10 days of TBI can be ascribed to gastrointestinal (GI) injury and occurs after higher radiation doses than does bone marrow insufficiency, which manifests itself as death within 10–30 days after more moderate doses (*23*). In our experiments, the dose of 10 Gy seemed on the borderline between these two forms of injury since 60% of mice died within 10 days and 40% within 10–30 days. CS administration spared mice from this dose so that only 20% died within 10 days and 67% died between 10 and 30 days, while 13% survived more than 30 days (Fig. 1A). Median survival times were increased by CS treatment from 13 to 20 days after 8 Gy TBI and 9 to 18 days after 10 Gy. The protective effect of CS was even more dramatic for the lower dose of 6.5 Gy TBI. All mice were fully protected compared to 40% of controls that died within 30 days. CS did not protect mice against supralethal doses of 12 or 15 Gy (Fig. 1B). No animal died from 5.5 Gy TBI with or without CS in 30 days (Supplementary Table 1). The $LD_{50/30}$ for control group of 6.6 ± 0.2 Gy was increased to 7.8 ± 0.2 Gy in the group given orally administered CS, giving a dose-modifying factor of 1.18.
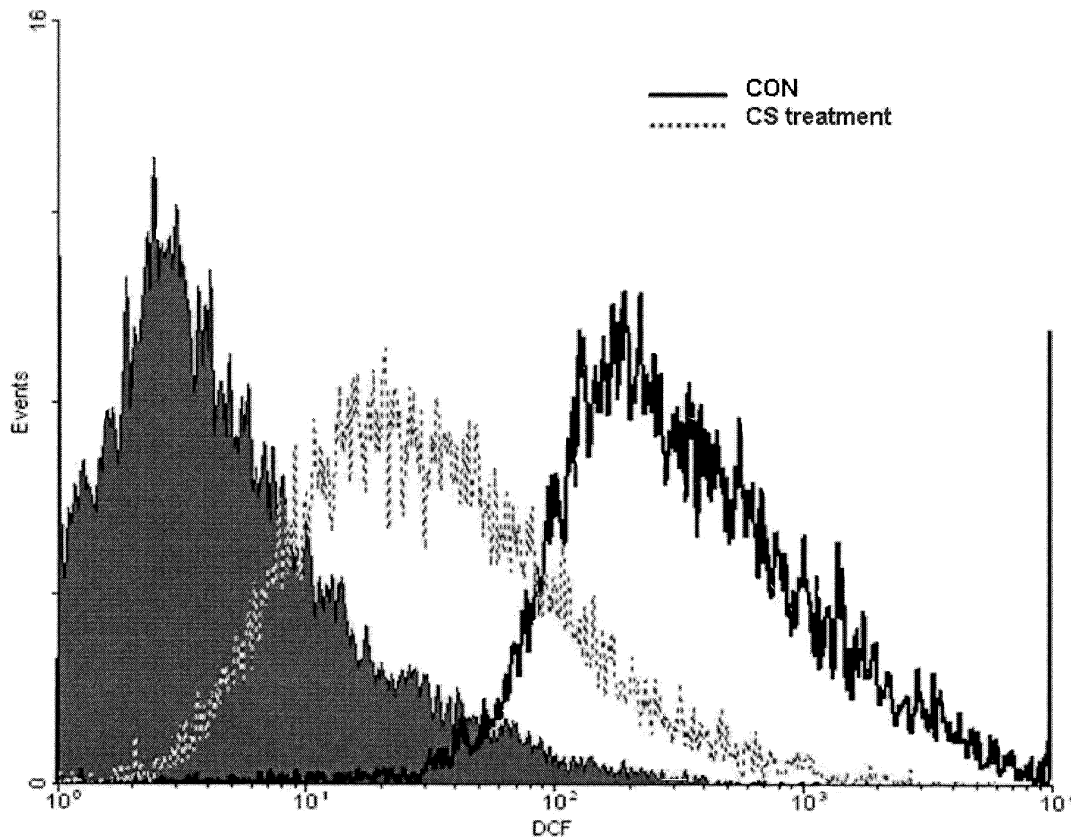
FIG. 5. Free radicals within MC3T3-E1 cells are decreased in cells grown in the presence of CS. The DCF free radical probe was added 24 h after incubation of cells with 500 μg/ml CS, and flow cytometry was performed 1 h later.

Cordyceps sinensis Protects Mice against Radiation-Induced Intestinal Injury

The 10-Gy survival data indicate that CS could protect mice from both intestinal injury and bone marrow failure after TBI. To confirm this, histological sections of small intestine and bone tissue were taken at various times after TBI. The most obvious damage to the small intestine 10 days after ≥10 Gy (Fig. 2A) was severe crypt cell loss. CS treatment allowed crypt regeneration after this dose. In mice receiving the TBI dose of 8 Gy, crypts regenerated irrespective of treatment, in keeping with the fact that this dose does not cause mortality from intestinal damage. Responses were quantified using the crypt stem cell assay (*19*) at 3.5 days after TBI. Figure 2B shows that CS could prevent crypt stem cell loss after doses of 8 or 10 Gy TBI but not 12 Gy. Recovery ratios of 1.42 and 2.22, calculated by dividing the surviving fraction of CS-treated mice by that of control mice, were found at 8 and 10 Gy, respectively.

A "target switching" model (*24*) has recently been proposed to refine the model of the response of the intestine to radiation. The hypothesis is that endothelial and crypt stem cells have different dose thresholds and time scales to radiation-induced apoptosis. To determine if CS was target cell specific, intestines were examined 4 h after TBI for the presence of apoptotic cells, which were scored by H&E staining (Supplementary Fig. 2A) and confirmed by the TUNEL assay (Supplementary Fig. 2B). Apoptotic cells were present in both the lamina propria and the crypts with similar responses at 4 h after even TBI doses as low as 5.5 Gy. Using a standard scoring system to quantify the apoptotic cells in the crypts (*20*), we confirmed that the maximal apoptosis index is at crypt position 4 (Fig. 3A). When the percentage of apoptotic cells at the fourth position was scored and plotted as a function of radiation dose (Fig. 3B), the response correlated inversely with crypt stem cell survival 3.5 days after TBI (Fig. 2B). CS treatment significantly reduced the number of radiation-induced apoptotic cells in both the crypts and lamina propria after 8 or 10 Gy TBI (Fig. 3B and C), in parallel with an increased number of surviving crypt stem cells. This indicates that CS protects both crypt stem cells and lamina propria cells against radiation-induced apoptosis. The latter are most likely endothelial cells, although marker analysis would need to be performed to confirm this. We did not observe different dose thresholds for radiation-induced apoptosis between these cell types proposed by the "target switching" model.

Cordyceps sinensis Protects Mice against Radiation-Induced Bone Marrow Damage

The data show that CS protected mice against bone marrow death when the TBI dose was 8 Gy. This was supported
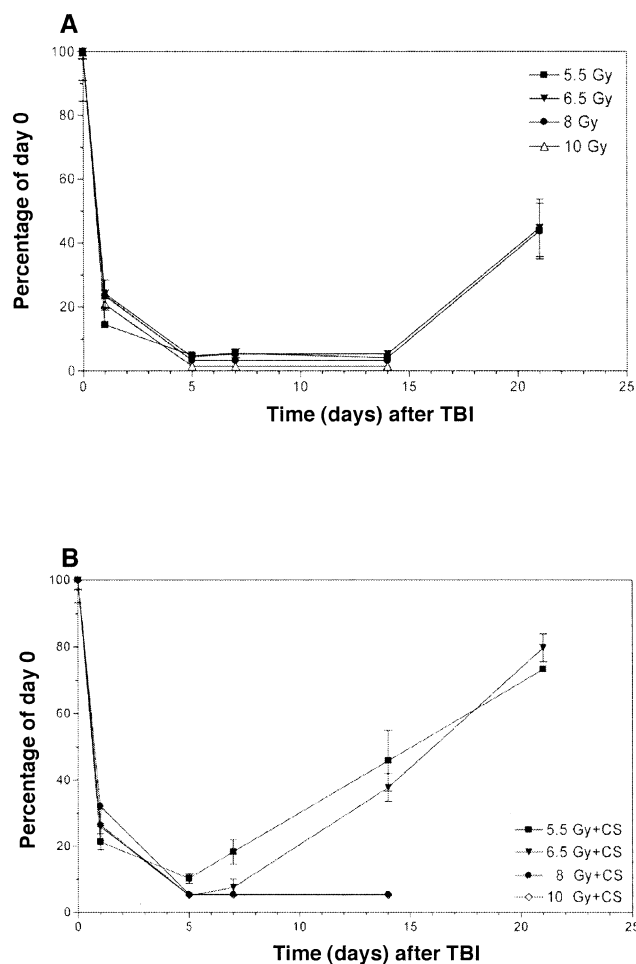
A

B

FIG. 6. CS enhances the recovery of peripheral WBC after TBI. Panel A: TBI mice receiving a dose ≥8 Gy died prior to recovery of WBC in the peripheral blood. After 6.5 or 5.5 Gy TBI, recovery began at 14 days. Panel B: CS did not influence recovery of WBC after TBI doses ≥8 Gy but accelerated recovery to start as early as 6 days after 6.5 or 5.5 Gy TBI. Data points and error bars represent the averages ± 1 SD for three mice from one of three representative experiments.

by the finding of more nucleated cells in the bone marrow of CS-treated mice 30 days after 8 Gy TBI, although this was not true at 3.5 days (Supplementary Fig. 3). Since it was harder to judge cell depletion in the bone marrow from histology after lower TBI doses, an *in vitro* colony survival assay was performed using bone marrow cells (BMC) from femurs and tibiae. Nonadherent BMC were collected after 24 h incubation, irradiated in the absence or presence of CS, washed and cultured in methylcelluose medium for the detection of granulocyte-macrophage colonies (GM-CFU). Figure 4A shows that CS protected GM-CFU during *in vitro* irradiation ($P < 0.01$; Student's paired *t* test). CS also increased the numbers of GM-CFU in the absence of radiation treatment (Fig. 4, 0 Gy). To further explore the protective effects of CS, the free radical content was measured by flow cytometry. Because of the heterogeneity in bone marrow cells, the osteoblast cell line MC3T3-E1 was used;

CS was shown to be a free radical scavenger (Fig. 5), confirming earlier data (*25, 26*).

Taken together with the histological findings at day 30 (Supplementary Fig. 3), these data indicate that CS can protect bone marrow stem cells from radiation cytotoxicity. Part of its action may be through free radical scavenging, but it also appears to enhance the subsequent proliferation and recovery of stem/precursor cells. This was supported by the more rapid, dose-dependent recovery of white blood cell (WBC) counts in the peripheral blood (Fig. 6 of CS-treated mice after TBI. After 5.5 or 6.5 Gy, WBC counts began to recover by day 6 in mice receiving CS compared to day 14 (Fig. 6A) in control mice (Fig. 6B).

DISCUSSION

This study shows that *in vivo* administration of hot-water extracts of CS increased survival of mice receiving lethal TBI by protecting both bone marrow and intestine. While the data are compelling, the mechanism of radioprotection is unclear and is probably multifactorial. The radioprotective effects of CS on bone marrow and intestine may even be linked since it has recently been reported that bone marrow stem cells may play a role in the regeneration of intestinal epithelium (*27–29*).

Our *in vitro* GM-CFU colony assay data and free radical measurements show that CS can directly protect cells from radiation cytotoxicity. Several studies have shown that CS can inhibit apoptosis by acting as a free radical scavenger (*25, 26*) or an antioxidant (*30*) or by down-regulating apoptosis genes such as those encoding FAS, Fas ligand, TNFA and caspase 3 (*31*). On the other hand, CS also seems to stimulate the proliferation and differentiation of bone marrow stem cells, but only after irradiation, as indicated by the presence of more nucleated cells in bone marrow taken from mice 30 days after TBI but not from unirradiated mice (Supplementary Fig. 3). We have also found that CS could stimulate the proliferation and differentiation of stromal cells *in vitro* (e.g. mesenchymal cells, adipocytes, osteoblasts and bone tissues) (manuscript in preparation). Such effects may provide a better microenvironment for the regrowth of hematopoietic stem cells (*32, 33*). The concept that the radioprotective effect of CS is due to more than simply a free radical scavenger action is further supported by the finding that CS treatment could accelerate the recovery of WBC after TBI.

While the ability of CS to spare bone marrow was not obvious with doses higher than 8 Gy, it was able to protect intestinal crypt stem cells from damage at 10 Gy. Jejunal crypt and cells in the lamina propria were both protected. The ability of cytokines such as IL1 and IL11 [(*34*) and reviewed in refs. (*1, 2*)] to both prevent apoptosis and accelerate recovery of intestinal cells has been reported and CS can stimulate production of cytokines, such as IFNG, TNFA, IL1, IL6 and GM-CSF (*8, 13*), suggesting that some

effects of CS may be indirect. Although we did not find significant increases of cytokines in the serum of mice treated with CS prior to TBI by RayBio® mouse cytokine antibody array (data not shown), local increases in these cytokines in the microenvironment of stem cells cannot be excluded.

In conclusion, our results indicate that CS administration can minimize damage induced by TBI by preventing radiation-induced death of bone marrow and intestinal crypt, as well as promoting the proliferation and differentiation of bone marrow stem cells. CS might be a useful radioprotector because it is a nontoxic natural product that has been administered to many thousands of individuals. Further studies are needed to better characterize the ingredients of CS extract responsible for the radioprotection effects and to examine whether CS has similar effects on the recovery of intestinal and bone marrow injuries regardless of etiology.

SUPPLEMENTARY INFORMATION

Supplementary Fig. 1: Effects of the sequence of CS administration on 10 Gy TBI-induced animal death: Oral administration of CS for a week prior to or after 12 Gy TBI protected mice from radiation damage with the same degree of protection. Five mice in each group were used in this preliminary study. Available online at http://dx.doi.org//10.1667/RR0670.1.s1.

Supplementary Table 1: *In vivo* radioprotective effects of CS. Available online at http://dx.doi.org//10.1667/RR0670.1.s2.

Supplementary Fig. 2: CS protects crypt stem cells from radiation-induced apoptosis. Panel A: Apoptosis was assessed by morphological characteristics, such as cell shrinkage, chromatin condensation and cellular fragmentation (arrow in b and f). Confirmation was obtained by terminal deoxy transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining using the DeadEnd™ colorimetric TUNEL system (panel B). Available online at http://dx.doi.org//10.1667/RR0670.1.s3 and http://dx.doi.org//10.1667/RR0670.1.s4, respectively.

Supplementary Fig. 3: CS decreased bone marrow failure in mice receiving TBI doses 8 Gy as shown by more nucleated cells at 30 days after TBI. Bone tissues at 3.5 or 30 days from mice receiving various TBI doses and saline or CS oral administration were examined by H&E staining. Severe loss of nucleated cells was found in 3.5-day samples (e, f, i, j, m, n). Regeneration of nucleated cells in the bone marrow was found at 30 days after 5.5 Gy TBI (g, h) but not after 8 Gy (p). However, more nucleated cells were found in CS-treated mice 30 days after 8 Gy TBI (q). Available online at http://dx.doi.org//10.1667/RR0670.1.s5.

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