This study aimed to evaluate the ability of the health food supplement *Cordyceps sinensis* (CS) to ameliorate suppressive effects of chemotherapy on bone marrow function as a model for cancer treatment. Mice were treated with Taxol (17 mg/kg body wt) one day before oral administration of a hot-water extract of CS (50 mg/kg daily) that was given daily for 3 weeks. White blood cell counts in peripheral blood of mice receiving Taxol were at 50% of normal levels on day 28 but had recovered completely in mice treated with CS. *In vitro* assays showed that CS enhanced the colony-forming ability of both granulocyte macrophage colony forming unit (GM-CFU) and osteogenic cells from bone marrow preparations and promoted the differentiation of bone marrow mesenchymal stromal cells into adipocytes, alkaline phosphatase–positive osteoblasts, and bone tissue. This result could be attributed to enhanced expression of Cbfa1 (core binding factor a) and BMP-2 (bone morphogenetic protein) with concurrent suppression of ODF (osteoclast differentiation factor/RANK [receptor activator of NF-κB]) ligand. In summary, CS enhances recovery of mice from leukopenia caused by Taxol treatment. It appears to do so by protecting both hematopoietic progenitor cells directly and the bone marrow stem cell niche through its effects on osteoblast differentiation. Exp Biol Med 233:447–455, 2008

**Key words:** *Cordyceps sinensis*, Taxol; leucopenia; osteoblast

### Introduction

Leukopenia is a common side effect of cancer therapy. The FDA has approved the use of the cytokines, granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), and interleukin-11 (IL-11) for the treatment of acute myelosuppression regardless of etiology. However, their clinical use is restricted to a narrow scenario, mainly because of their specificity, their intrinsic pleiotropism, and their cascade-like mechanism of action. Toxicity also limits their effectiveness (1). There is therefore a need to discover nontoxic agents that are applicable to a broader range of operational conditions and, in particular, to assist the recovery of bone marrow (BM) function compromised by cancer treatment.

Recovery of blood-forming stem cells requires not only the hematopoietic stem/progenitor cells (HSC/HPC) to be stimulated but also an appropriate microenvironment in which the cells can develop (see review in Ref. 2). Osteoblastic or vascular niches (3–5) can fulfill this function. Although the key molecular signals that trigger HSC survival and/or replication within a niche have not been well defined, a model based on HSC-osteoblast attachment has been proposed (2, 6, 7). Modulation of the HSC niche(s) through affecting mesenchymal stem cell (MSC) activities therefore offers opportunities for treating hematopoietic-related disorders, in addition to the use of hematopoietic growth factors.
In the past 20 years, as the result of the passage of the Dietary Supplement Health and Education Act of 1994 by the Congress for Food Safety and Applied Nutrition, there has been phenomenal growth in the interest in and use of complementary or alternative medicines. One of these, Cordyceps sinensis (CS), which is a fungal parasite of moth larvae (Lepidoptera spp.) in the genera Hepatitis and Thitarodes (8), has been widely used in traditional Chinese medicine. It has been promoted as a popular remedy that is devoid of toxicity for the side effects of cancer treatment (see reviews in [9–11]). A broad spectrum of pharmacologic actions including the modulation of hepatic, renal, cardiovascular, immune, nervous, endocrine, and steroid systems has also been described. At the cellular level, diverse biological effects of CS such as activating macrophages (12), modulating apoptosis (13, 14), and inhibiting tumor metastasis (15, 16) have been reported. Many of these effects can be attributed to production of cytokines such as interferon (INF)-γ, tumor necrosis factor-α (TNF-α), IL-1, IL-6, and GM-CSF (12, 17).

We have shown that CS could protect mice against radiation-induced BM failure. It accelerates recovery from radiation-induced leukopenia and enhances survival (18). The present study aimed to examine whether CS has similar effects after chemotherapy for cancer and to explore how CS affects the regeneration of HPCs. This preclinical study provides proof-of-principle that CS may be a potent remedy for leukopenia after cancer treatment and that it may act by enhancing the survival and differentiation of BM-HSCs and BM-MSCs.

Materials and Methods

Mice and Treatments. C57BL/6J mice were purchased from the National Laboratory Animal Center, Taipai, Taiwan, and housed in National Tsing-Hua University Laboratory Animal Center, Hsinchu, Taiwan. Seven- to eight-week-old male mice were used for experiments. Mice were divided into two major groups: control and Taxol treatment groups. Taxol (pactaxetel; Bristol-Myers Squibb, Princeton, NJ) was given by the intraperitoneal (ip) route in a dose of 17 mg/kg body wt. Control mice were given injections of the carrier, which was a mixture of 50% Cremophor EL (Sigma-Aldrich, St. Louis, MO) and 50% dehydrated alcohol. One day after Taxol treatment, the mice were given an extract of CS (50 mg/kg daily), prepared as described (18) or saline through an orogastric tube once a day on week days for a total of 3 weeks. At the indicated times, blood samples were collected from the tail and analyzed by MICROSC ABC LC-152 animal blood counter (Horiba Co., Kyoto, Japan) according to the manufacturer’s protocol. Experiments were repeated three times with three mice from each group in every experiment. Data analyses and statistical tests were performed by using GraphPad Prism software version 3.03 (GraphPad Software, Inc., San Diego, CA). In all experiments, mice were killed via CO2 inhalation on day 30 after Taxol treatment. During the experiments, all mouse care followed the recommendations of the approved guide for the care and use of laboratory animals by the Institutional Animal Care and Use Committee (IACUC approval number: 09508) of National Tsing Hua University.

Cell and Colony Formation Assays. BM cells were harvested by flushing the medullary cavities of femur and tibia bones with Hanks’ balanced salt solution. Cells (1 × 10⁶ cells/ml) were cultured in 6-well plates in 2 ml RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% penicillin/streptomycin, and 50 μM 2-mercaptoethanol (Sigma-Aldrich). For the GM-CFU colony assay, nonadherent BM cells were collected after a 24-hr incubation and their concentration was adjusted to 2 × 10⁵ cells/ml in phosphate-buffered saline (PBS). These nonadherent BM cells were then plated (2 × 10⁵ cells/ml, 0.3 ml) in Petri dishes (BD Falcon, San Jose, CA) with 1.1 ml per dish in the presence or absence of CS (500 μg/ml) along with premixed methylcellulose culture medium (Methocult M3234, Stem Cell Technologies, Vancouver, Canada) as described by Lin et al. (19) with final concentrations of 1% methylcellulose, 15% FCS, 1% bovine serum albumin, 10 μg/ml insulin, 200 μg/ml transferrin, 10⁻⁴ M 2-mercaptoethanol, 2 mM L-glutamine, 10 ng/ml recombinant murine IL-3, and 500 ng/ml GM-CSF (Invitrogen Biosource, Carlsbad, CA). After 7 days of incubation, GM-CFU colonies consisting of 50 or more cells were scored by using an inverted microscope.

Osteogenic cultures were established from BM cells by culture in α-MEM (Sigma-Aldrich) supplemented with 10% FCS, 1% penicillin/streptomycin, and 50 μM 2-mercaptoethanol (Sigma-Aldrich) in the presence of 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate as described by Rickard et al. (20). Control, CS (500 μg/ml)-treated, and dexamethasone (10 nM)-treated cultures were established in 12-well plates (1.5 × 10⁵ cells/ml). One day later, nonadherent cells were removed by washing three times with 5 ml α-MEM medium, and the number of attached MSCs with pseudopodia processes were counted. Complete medium, with or without the appropriate supplements, was added and changed thereafter every 2 to 3 days. After 5 days, small osteoblast colonies that formed were counted in five fields under a microscope (magnification, ×100; see pictures in the insert of Fig. 2B). After 7, 10, 14, and 28 days of culture, adipocytes were identified by the presence of oil drops and counted in 30 fields by microscopy (magnification, ×100). After 7, 10, and 14 days of culture, preosteoblast colony-forming cells were identified by positive staining for alkaline phosphatase (ALP) using the FAST RED kit (Sigma-Aldrich) (20, 21) and scored by using microscopy (magnification, ×100) to count the number in five fields. After 28 and 60 days of culture, mineralized bone nodules were identified by staining with Alizarin red S reagent (Sigma-Aldrich).
The ability of BM cells to give rise to osteoclasts was tested by culturing them in 6-well plates in 2 ml (1 × 10^6/ml) RPMI 1640 medium (GIBCO) supplemented with 10% heat-inactivated FCS, 1% penicillin/streptomycin, and 50 μM 2-mercaptoethanol (Sigma-Aldrich) (22) with or without CS (500 μg/ml). After 7 days, nonadherent cells were removed by washing three times with PBS. The attached cells were then fixed with 10% formaldehyde in PBS, treated with ethanol-acetone (50:50), and stained for tartrate-resistant acid phosphatase (TRAP, a marker enzyme of osteoclasts) (22) by using a TRAP staining kit (acid phosphatase staining kit, Sigma-Aldrich). TRAP-positive multinucleated giant cells containing more than three nuclei were counted as osteoclasts.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Cbfa (core binding factor α), BMP (bone morphogenetic protein), and ODF (osteoclast differentiation factor/RANK [receptor activator of NFκB]) ligand mRNA expression was assessed by RT-PCR. Total cellular RNA was extracted from mesenchymal stromal cells after in vitro CS treatment for 3 or 5 days. Total cellular RNA was extracted with the RNA easy Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer’s instructions. Reverse transcription and PCR were conducted by using AMV reverse transcriptase (QIAGEN) and Taq DNA polymerase (QIAGEN), respectively. The primer pairs were picked by the Primer3 Web-based primer prediction program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi) and synthesized by Invitrogen Co. (Carlsbad, CA). They were ACCAGTGAC CAGGTTCG and TTCAGTGGGATTGAATG for the murine Cbfa1 gene (GenBank access number NM009820), TCGTTGACATTAGCATGGA and CCTCTCCCAATCTGGTTG for the murine ODF/RANKL gene (GenBank access number NM011613), TGGAAGTGCCCATTTAGAG and TGACGCTTTGTTG for the murine BMP2 gene (GenBank access number NM007553), and AACTTTGGGATG for the murine GAPDH gene (GenBank access number BC083149).

Figure 1. Taxol (17 mg/kg) treatment given by the ip route to mice caused a dramatic decrease in peripheral WBCs with 50% recovery by day 28. Oral administration of CS (50 mg/kg daily, 5 days per week for 3 weeks) reduced the WBC nadir and enhanced recovery to control levels within a month. Data are represented by the mean ± 1 SD for 9 mice from three repeated experiments, as analyzed by GraphPad Prism software version 3.03. *, P < 0.01 by ANOVA test (compared with control mice that received saline).

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Real-Time PCR. The expression level of BMP2 and RANKL mRNA expression was quantized by real-ime PCR. The primer pairs for BMP2 (GAGGTGTTCGATG and CACAGTTCAG and TCTCAGTGAGGGATGAAATG for the murine Cbfa1 gene (GenBank access number NM009820), TCGTTGACATTAGCATGGA and CCTCTCCCAATCTGGTTG for the murine ODF/RANKL gene (GenBank access number NM011613), TGGAAGTGCCCATTTAGAG and TGACGCTTTGTTG for the murine BMP2 gene (GenBank access number NM007553), and AACTTTGGGATG for the murine GAPDH gene (GenBank access number BC083149).
GACGTGAT and AGAACATGGTGCTGTAG), RANKL (ACTGGTCGGGCAATTCTGAA and GGGTTGGACACCTGAATGCT), and β-actin (CCACCA GACAAACTGCTGGTG and AGAGGTATCCCTGACCT GAAG) were designed by Primer express software v3.0 (Applied Biosystems). SYBR Green 1 dye (Applied Biosystems) was used as a reporter, and the reactions were performed by the ABI prism 7300 PCR machine. The quantitation analysis was performed by ABI 7300 systems sequence detection software v1.4 (Applied Biosystems).

Results

CS Prevents Taxol-Induced Leukopenia. Injection of a single dose of Taxol (17 mg/kg body wt given by the ip route; about 91 mg/m²) caused a severe loss of WBCs from the peripheral blood of mice (Fig. 1). The WBC number reached its nadir (mean, 6.2 \times 10^3/mm³; range, 0.6–12.4) 7 days after the start of Taxol treatment. This result is not dissimilar to the mean nadir of 1.3 \times 10^3/mm³ and range of 0 to 13.4 found in patients receiving an infusion course of 135 mg/m² over 3 hrs. Oral administration of CS water extracts (50 mg/kg daily, 5 days per week for 3 weeks) alone did not affect WBC counts, but it did significantly minimize the extent of Taxol-induced leukopenia (P < 0.01). Whereas Taxol-treated mice had only half the normal number of WBCs in the peripheral blood after 28 days, WBCs in those receiving CS had recovered completely (P < 0.01).

Effects of CS on BM Cells in Culture. The ability of CS to prevent Taxol-induced leukopenia suggested that it affected the survival and differentiation of HPCs and/or protected the stromal niche in which they grow. A series of in vitro colony-forming and differentiation assays were performed by using BM cells to examine these possibilities.

CS (500 µg/ml) increased granulocyte-macrophage colony formation from nonadherent BM cells cultured for 1 week in premixed methylcellulose culture medium containing IL-3 and GM-CSF (Fig. 2A). It also increased the number of attached MSCs with pseudopodia (insert of Fig. 2B) and the small preosteoblast colonies that formed from them after 5 days of culture (Fig. 2B). When the MSCs were further cultured in osteogenic differentiation medium, CS increased the numbers of ALP-positive osteoblast cells at day 10, oil-forming adipocytes at day 14, and bony tissue accumulation at day 28 (Fig. 3). CS (500 µg/ml) was only slightly less effective than the addition of 10 nM dexamethasone (20, 23) in causing osteoblastic differentiation (Fig. 3A).

These data indicate that CS supplement enhances the survival of both HSCs and MSCs. Furthermore, the scores for ALP-positive osteoblasts (Fig. 3B) and adipocytes (Fig. 3C) at different time points indicated that CS not only enhanced the survival of precursor cells but also accelerated osteogenic differentiation. For example, adipocyte numbers peaked at 14 days in the CS supplemented cultures instead of at 21 days in control cultures (Fig. 3B).
CS Affects the mRNA Levels of Cbfa1, BMP-2, and ODF/RANK Ligand Expressed by Osteoblasts.

To confirm that CS influences osteoblast differentiation, we examined the expression of the marker gene, Cbfa1, in MSC cultures by using RT-PCR (Fig. 4A). Cbfa1 is a transcription factor that drives osteoblast lineage determination at an early differentiation stage (24, 25). CS enhanced the expression of Cbfa1 mRNA after 3 days of osteoblastic culture, an event that occurred only at 5 days under normal conditions (Fig. 4A). In addition to Cbfa1, BMP-2 mRNA expression was enhanced by CS (Fig. 4A, B) BMP-2 plays a significant role in MSC differentiation (26, 27) and is required for Cbfa1-dependent induction of the osteoblast phenotype (28).

In contrast to increasing expression of osteogenic differentiation factors, CS decreased expression of the osteoclast differentiation factor ODF (RANKL) by osteoblasts (Fig. 4A, B). RANKL is a member of the TNF family, and osteoblasts express it in response to several osteotropic factors. It is critical for TRAP (tartrate-resistant acid phosphatase) production and differentiation of osteoclast precursor cells into multinucleated osteoclasts (29, 30) from their monocytic/macrophage precursors (see a review in [30]).

CS Affects Hematopoietic Differentiation. The finding that CS suppressed ODF (RANKL) mRNA expression in osteoblastic cells indicates that it may inhibit the differentiation of HSCs into osteoclasts (30–32). To determine whether this was the case, an osteoclast differentiation assay was performed. Osteoclasts develop through the interaction of HSCs with MSCs over 7 days of culture, can be identified by TRAP staining, and are characterized as multinucleated cells (22). The fact that this differentiation was inhibited by the addition of CS in a dose-dependent manner (Fig. 5) along with the suppressed ODF production by MSCs (Fig. 4) suggests that CS may increase the size of
the MSC niche to increase HSC production (3, 4). This is consistent with the finding of more hematopoietic cells in HSC and MSC coculture systems in the presence of CS supplementation (Fig. 6). CS may therefore act both directly and indirectly on HSCs to promote their survival and proliferation and counter chemotherapy-induced BM suppression.

Discussion

Leukopenia is a frequent side effect of many forms of cancer therapy, including chemotherapy and radiotherapy. In this study, we extended our previous findings on in vivo administration of a Chinese herbal medicine, Cordyceps sinensis, to show that it not only promotes the recovery from radiation-induced leukopenia (18) but also rescues mice from Taxol-induced leukopenia. This study also provides evidence that CS influences the survival and differentiation of HSCs both directly and indirectly by affecting the MSC niche.

We believe that this is the first report to show that CS affects the differentiation of BM-HSCs and BM-MSCs. GM-CFUs and preosteoblast colony numbers were increased by the addition of CS to nonadherent and adherent BM cells, respectively. Furthermore, an osteoblast differentiation assay demonstrated that CS could promote the differentiation of MSCs into bone tissue. Although the precise mechanism by which CS affects osteoblast differentiation is unclear, the enhanced expression of Cbfa1 and BMP-2 mRNA indicates that CS acts at early stage on osteoblastic progenitors. In contrast, we also found that CS retards the development of osteoclasts. This is probably due to its inhibitory effect on the expression of ODF (RANKL) in osteoblasts. RANKL, produced by marrow osteoblasts causes osteoclast precursors within the monocytic/macrophage precursor pool to express TRAT and form multinucleated osteoclasts (29, 30). The delicate balance between osteoblast and osteoclast differentiation is crucial during bone development, homeostasis, and repair (see a review in [30]) but may also determine the size of the niche in which HSCs develop. By suppressing HSC differentiation to osteoclast lineages, CS may also switch them to make other blood cell lineages by enhancing Cbfa1 and BMP-2 expression by MSCs.

The hematopoietic system and bone have an intimate relationship that is manifested in multiple ways. Osteoblasts produce hematopoietic growth factors such as GM-CSF and hepatocyte growth factor (33–35) that cooperatively permit the survival of hematopoietic progenitors. Taichman and Emerson (33, 36) first proposed that the osteoblast may have a role in hematopoietic recovery. This has been further supported by Zhang et al. (3) and Calvi et al. (4), who used genetic strategies to increase the size of the osteoblast population in specific regions of bone. They found that this caused parallel increases in the HSC population and that bone not only provides cavities in which HSC can differentiate into specialized blood cells but also that osteoblasts lining the trabecular bones provide “niches” for HSC development (3, 4). Calvi et al. (4) also demonstrated that osteoblastic cells were a regulatory component of the niche in vivo that influences HSC function through Notch activation. The ability of CS water extracts to promote the recovery of leukopenia after cancer therapy may therefore partially be through its effects on osteoblast clonogenicity, which includes inhibiting their differentiation into osteoclasts, as well as a direct effect in increasing the colony-forming ability of HSCs. However, this study does not exclude the possibility that CS directly protects blood leukocytes or BM stem cells from Taxol cytotoxicity.
A critical issue is which component of CS is responsible for its multiple effects and is the same component active in all cases. Polysaccharides seem to be responsible for many CS effects (37–39). The β-glucan polysaccharide extract from the fruit body of the Maitake mushroom (*Grifola frondosa*) also enhanced hematopoietic BM cell growth and differentiation into colony-forming cells (19), similar to what we found for CS in this study. We have been working to identify the CS ingredient(s) responsible for stimulating BM, but our preliminary data indicate that multiple components express activity (data not shown) and that these individual components are less

Figure 6. Mesenchymal stromal cells support the growth and differentiation of hematopoietic cells. (A) Hematopoietic cells are still detectable after 10 days in cultures containing FCS only, when they were cocultured with MSCs. (B) The number of hematopoietic cells was further increased in the culture medium containing CS (500 μg/ml). Values are means ± 1 SEM of six determinations. One represented data from three repeated experiments were shown. *, *P* < 0.01 by Student’s *t* test (compared with control).
effective than the whole extract and have a higher risk of side effects in vivo. This has been a major issue for many other traditional Chinese medicines. Multiple ingredients may be necessary, perhaps because they balance the side effects associated with a single compound (40) and stimulate multiple pathways and multiple cell types, as was seen in this study.

Nevertheless, this study demonstrates the potential of CS, a traditional Chinese medicine, as a remedy for leukopenia after cancer therapy and shows that it can promote the differentiation of HSCs both directly and indirectly through its action on osteoblast differentiation.

32. Taichman RS, Emerson SG. Human osteoblasts support hematopoiesis.