Myelophil, a mixture of Astragali Radix and Salviae Radix extract, moderates toxic side effects of fluorouracil in mice

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Abstract

AIM: To evaluate the efficacy of Myelophil, an extract containing Astragali Radix and Salviae Radix, for reducing complications induced by 5-fluorouracil (5-FU) in a gastrointestinal cancer model.

METHODS: We injected 5-FU into mice and then administered Myelophil to examine the ability of the drug to treat the side effects of 5-FU in mice. Peripheral blood counts, histological examinations, and colony-forming assays of bone marrow were conducted, followed by swimming tests and assessment of survival times.

RESULTS: Myelophil restored red and white blood cells and platelets in blood, and recovered cell density in bone marrow to levels comparable to those observed within the control group. In addition, Myelophil significantly increased colony-forming unit granulocyte-macrophage (CFU-GM) and CFU-erythroid (CFU-E) compared to the control group. We confirmed that interleukin-3 gene expression was upregulated by Myelophil in spleen cells. Myelophil administration also doubled the survival rate of mice that were severely myelosuppressed as a result of 5-FU injection at a lethal dose of 70%. Finally, the swimming performance of mice significantly improved as a result of Myelophil treatment.

CONCLUSION: These results provide experimental evidence in support of clinical applications of Myelophil to minimize 5-FU-induced myelosuppression and improve general post-chemotherapy health.

INTRODUCTION

The occurrence of undesired effects that result from conventional chemotherapy or irradiation for cancer is inevitable. Nevertheless, reducing adverse effects is a critical issue for patients and doctors, given the importance of quality of life, as well as survival gains. Accordingly, mitigation of chemotherapy-induced side effects and the development of novel chemotherapeutic agents with fewer toxic effects have been major focuses of recent medical investigations. In particular, cancer-therapy-related fatigue, diarrhea or myelosuppression-related symptoms are closely associated with failure of the therapy itself. Therefore, many therapeutic developments, including herb-derived remedies, have focused on treating these side effects.

Fluorouracil is one of the most commonly used drugs to treat gastrointestinal cancers, including those in the stomach, colon and liver, and it commonly causes fatigue, diarrhea and sometimes myelosuppression. Myelophil is a mixture of Astragali Radix and Salviae Radix extract representing hematopoietic medicine. Astragali Radix displays immunomodulating, hematopoietic, and anti-fibrotic properties. Salviae Radix exhibits antioxidant, antiatherosclerosis, and antiplatelet aggregation pharmaceutical effects. We have used this...
drug to treat mainly gastrointestinal cancer patients with post-therapeutic complications such as leukopenia, anemia or severe fatigue since 2002.

Here, we evaluated the therapeutic efficacy of an extract mixture that contained Astragali Radix and Salviae Radix for reducing complications from cancer chemotherapy, using a 5-fluorouracil (5-FU)-induced myelosuppression mouse model.

MATERIALS AND METHODS

Manufacturing and fingerprinting of Myelophil

*Astragalus membranaceus* (Leguminosae, VS No: AM-2006-02-Ra) and *Salvia miltiorrhiza* (Labiatae, VS No: SM-2006-01-Ra) were provided by Daejeon Oriental Medical College, Dunsan Oriental Hospital, of Daejeon University, identified by Professor SI Yim of Daejeon University and stored at our laboratory for future use. Samik Pharmaceutical Company (Seoul, Korea) manufactured a lyophilized aqueous extract of Myelophil (mixture of Astragali Radix and Salviae Radix; 1:1) according to over-the-counter Korean monographs. A final product with 20.52% (w/w) yield was stored for future use (VS No: MP-2006-01-WE). 5-FU was purchased from Choongwae Pharma Corporation (Seoul, Korea). Other chemicals were obtained from Sigma (St. Louis, MO, USA).

For the fingerprinting of Myelophil, the water extract of Astragali radix and Salviae radix, and their standard components, formononetin (Sigma) and rosmarinic acid (Carl Roth, Karlsruhe, Germany) were prepared for the high performance thin layer chromatography (HPTLC) system (CAMAG, Muttenz, Switzerland). They were dissolved in HPLC-grade methanol and applied to pre-washed HPTLC plate silica gel 60 F 254 (Merck, Darmstadt, Germany) with an automated applicator (Linomat IV; CAMAG). The samples were then separated and the migrated components were visualized prior to or after derivatization under UV radiation at 366 nm or white light using Reprostar 3 with a digital camera (CAMAG, Figure 1).

Induction of myelosuppression: Hematological and histopathological analysis

To examine the therapeutic effects of Myelophil on 5-FU-induced myelosuppression, 60 6-wk-old male ICR mice (Koatech, Gyeonggi-do, Korea) were divided into three groups (20 control, and 20 with low- and 20 with high-concentration Myelophil groups). Another five mice were sacrificed to record physiological standards for hematological parameters at time 0. All three groups were injected intraperitoneally with 0.3 g/kg 5-FU on d 0. Beginning on d 2, Myelophil (0.05 g/kg or 0.1 g/kg) or distilled water (control group) was administered orally once daily for 10 consecutive days. Five mice per group were serially sacrificed on d 0, 4, 7, 10 and 13, and complete blood counts were analyzed using a blood cell counter (HEMAVET; CDC Technologies, CT, USA). In addition, all left-side femoral bones from the mice on d 7 were prepared for general histopathological evaluation, including fixation, decalcification, sectioning (4 µm thickness), as well as hematoxylin and cosin (HE) staining.

Isolation of bone marrow cells and colony-forming assay

To directly examine the effects of Myelophil on bone marrow stem cells, C57BL/6 mice (three for each of the control, and low- and high-concentration Myelophil groups) were injected intraperitoneally with 5-FU (0.2 g/kg). Mice were given Myelophil (0.05 g/kg or 0.1 g/kg) or distilled water (for naïve and control groups) for 5 consecutive days beginning 2 d after 5-FU injection. Bone marrow cells were isolated from femurs, and nucleated cells were counted using a blood cell counter (HEMAVEC; CDC Technologies). After thoroughly mixing the nucleated cells (400 µL 2 × 10^5) with 4 mL MethoCult methylcellulose-based medium (Stem Cell Technologies, Seattle, WA, USA), media (1 mL per dish in triplicate) were cultured in a 5% CO₂ incubator for 7 d. According to the morphological characteristics, the number of colonies assessed by CFU-GM or CFU-E was counted under an inverted microscope.

Interleukin-3 (IL-3) gene expression analysis using real-time PCR

Splenocytes isolated from BALB/c male mice were seeded in a six-well culture plate (2.8 × 10^5 cells per well), and treated with or without Myelophil (0.001, 0.01 or 0.1 g/L) for 18 h. After purification of total RNA using an RNA mini kit (Qiagen, Valencia, CA, USA) and cDNA synthesis, quantitative real-time PCR was performed using SYBR Green Supermix reagent (Bio-Rad, CA, USA) according to the manufacturer's protocol. The primer sequences (forward and reverse, respectively) were:

A: Rosmarinic acid (RMA, 2 µg), Salviae Radix water extract (SRE, 200 µg) and Myelophil (MP, 400 µg) were separated using chloroform/ethyl acetate/benzene/formic acid/methanol (15:10:10:1:1), and then visualized by white light after derivatization with p-anisaldehyde sulfuric acid;

B: Formononetin (FMN, 0.1 µg), Astragali Radix water extract (ARE, 0.8 mg) and Myelophil (MP, 1.6 mg) were separated using dichloromethane/methanol/water (45:10:1), and then visualized under UV at 366 nm.
as follows: β-actin, GTGGGGCGCCCCAGGCACCA and CTCCTTATACTGTCAGCACGATTTCC; IL-3, TACATCTGCAAATGCCTCGACGTAGGTT.

Monitoring survival rates with or without swimming-forced stress
To examine whether Myelophil affected the survival time of mice with severe or moderate myelosuppression induced by 5-FU, we conducted two tests. First, severe myelosuppression was induced in 30 male ICR mice (10 in each of the control, and low- and high-concentration Myelophil groups) by 5-FU treatment (0.5 g/kg, intraperitoneally). Two days later, Myelophil (0.05 or 0.1 g/kg) or distilled water (for the control group) was given orally once daily for 10 consecutive days. The number of surviving mice was monitored for the next 20 d.

For the second test, moderate myelosuppression was induced in 30 male ICR mice (10 in each of the control, and low- and high-concentration Myelophil groups) by 5-FU treatment (0.3 g/kg, intraperitoneally). Beginning 2 d following 5-FU injection, Myelophil (0.05 or 0.1 g/kg) or distilled water (for the naive and control groups) was given orally once daily for five consecutive days. On the final day, all mice were forced to swim in a pool with 22°C water for 30 min. Mice were monitored for survival time and swimming performance.

Statistical analysis
The results were expressed as mean ± SD. Statistical analysis of the data was conducted using Student's t test with significance levels of \( P < 0.05 \).

RESULTS
Hematological parameters
First, we examined changes in hematological parameters (leukocyte, erythrocyte and platelet counts) in 5-FU-induced (0.3 g/kg) myelosuppressed mice every 3 d. As shown in Table 1, peripheral blood white blood cell, platelet, and red blood cell levels drastically decreased, with the lowest numbers recorded on d 7. However, the observed pancytopenia was ameliorated by Myelophil administration, and the number of leukocytes rapidly recovered compared to that in untreated control mice (0.05 g/kg Myelophil, \( P = 0.0387 \); 0.1 g/kg Myelophil, \( P = 0.0014 \)).

Histological examination of bone marrow
We examined the cellular density of femoral bone marrow from d 7 mice. Similar to the peripheral blood counts, 5-FU injection radically reduced the cellular component in bone marrow by vacuolation, and this was moderately improved by Myelophil treatment (Figure 2).

CFUs in bone marrow
To investigate how Myelophil affected the hematopoietic stem cells, leukocyte or erythrocyte-lineage colonies were determined using colony forming assay. Myelophil treatment significantly increased the colony numbers of both leukocyte (0.05 g/kg Myelophil, \( P = 0.0087 \); 0.1 g/kg Myelophil, \( P = 0.0029 \)) and erythrocyte lineages (0.05 g/kg Myelophil, 0.1 g/kg Myelophil, \( P = 0.0014 \)).
IL-3 gene expression in vitro
We examined changes in IL-3 gene expression in splenocytes following co-culturing with Myelophil using RT-PCR. IL-3 expression was increased in a dose-dependent manner. At a concentration of 0.1 g/L Myelophil, this gene was up-regulated four-fold (Figure 3B).

Survival rate of myelosuppressed mice
Following injection with 0.5 g/kg 5-FU (LD70, determined in our experiments), the survival rate of the control group was 30% by 14 d, whereas in the Myelophil-treated groups it was 70%-80% (Figure 4A). Myelophil treatment significantly protected mice from loss of body weight after 5-FU injection (data not shown). In addition, Myelophil treatment extended the survival time of mice that were forced to swim in a pool after injection with 0.3 g/kg of 5-FU (Figure 4B).

DISCUSSION
We used a 5-FU-induced myelosuppression mouse model to evaluate the experimental efficacy of Myelophil, with relevance to the clinical application of reducing chemotherapy-induced side effects. 5-FU is one of the most commonly used anti-metabolitic chemotherapeutic drugs for colon, stomach, liver, and head and neck cancer. It has also been applied in anti-myelosuppressive studies due to its observed toxicity toward bone marrow [18-20]. We observed that a single injection of 0.5 g/kg 5-FU caused >70% mortality within 20 d, whereas 0.3 g/kg 5-FU induced mild myelosuppression with no incidence of death.

Peripheral white blood cell, platelet and red blood cell levels drastically decreased in 5-FU-induced (0.3 g/kg) myelosuppressed mice. On d 7, these levels were the lowest and were in accordance with those of severe leukopenia, moderate thrombocytopenia and mild anemia. However, the observed pancytopenia was ameliorated by Myelophil administration, and the number of leukocytes rapidly recovered compared to that in untreated control mice. Next, we examined the cellular density of femoral bone marrow from d 7 mice. Similar to the peripheral blood results, 5-FU injection radically reduced the cellular component in bone marrow induced large vacuole formation, and this was moderately improved
by Myelophil treatment (Figure 2). These results suggest that Myelophil might be beneficial for the alleviation of chemotherapy-associated high susceptibility to pathogenic microorganisms, which is a major problem post-treatment[20,21].

Consequently, we investigated how Myelophil affected the hematopoietic stem cells via differential examination of leukocyte- or erythrocyte-lineage colonies. The number and lineage of a colony was decided mainly by quantity and quality of stem cells in different groups. Our results showed Myelophil treatment significantly increased the colony numbers of both leukocyte and erythrocyte lineages (Figure 3A). Processes of hematopoiesis are under the control of various hematopoietic growth factors, such as IL-3, erythropoietin (EPO), thrombopoietin (TPO), granulocyte colony-stimulating factor (G-CSF), or granulocyte/macrophage CSF (GM-CSF)[22]. These growth factors have lineage-specific hematopoietic functions and different cellular excretion sources[23]. Specifically, IL-3 supports proliferation and differentiation of hematopoietic stem cells, as well as various cell lineages in hematopoiesis[24], and is secreted mainly from natural killer T cells[25]. Therefore, we examined changes in IL-3 gene expression in splenocytes following co-culturing with Myelophil. The result showed that IL-3 expression was increased four-fold (Figure 3B).

Given the above results, we observed how Myelophil could restore myelosuppression via IL-3 up-regulation. Generally, myelosuppression is linked strongly to other common side effects caused by conventional cancer therapy, such as fatigue or low energy, as well as low immunity.

Myelophil-treatment significantly protected mice from loss of body weight after 5-FU (0.5 g/kg) injection (data not shown). In addition, Myelophil treatment extended the survival times of mice that were forced to swim in a pool after injection of 0.3 g/kg 5-FU (Figure 4B).

5-FU-induced myelosuppression is the dose-limiting toxicity associated with substantial life-threatening risk and life span of cancer patients[26,27]. Many herbal medicines are currently being investigated as good candidates for improving quality of life and reducing toxic side effects such as myelosuppression[28,29]. One group has reported the efficacy of one of the two medicinal plant extracts in Myelophil on hematopoiesis induction in mice[30]. We found that a mixture of Astragal Radix and Salviae Radix was more effective than a single herb administered alone in our model system (data not shown). We have prescribed Myelophil to treat post-therapeutic complications such as anemia, leukopenia or severe fatigue, mainly for gastrointestinal cancer patients, according to its oriental pharmaceutical theory since 2002.

Herein, we have provided experimental evidence relevant to clinical applications of Myelophil for minimizing cancer chemotherapy-induced side-effects, using a fluorouracil-induced myelosuppression mouse model.

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