The Liquid Culture Filtrates of Entomogenous fungus *Paecilomyces tenuipes* and Its Glycoprotein Constituent Protects against Anemia in Mice Treated with 5-Fluorouracil

Takanobu TAKATA, a Tomoaki TANAKA, a Nobuo YAHAGI, a Remiko YAHAGI, a Hideyuki TSUCHIDA, b Yasuhiro ISHIGAKI, b Naohisa TOMOSUGI, c Shinji FUSHIYA, d Fumihide TAKANO, *, a and Tomihisa OHTA a

a Department of Pharmacognosy and Chemistry of Natural Products, Graduate School of Natural Science and Technology, Kanazawa University; Kakuma-machi, Kanazawa 920–1192, Japan; b Division of Core Facility Medical Research Institute, Division of Molecular Oncology and Virology, Kanazawa Medical University; c Division of Advanced Medicine, Medical Research Institute, Kanazawa Medical University; 1–1 Daigaoka, Uchinada-machi, Kahoku-gun, Ishikawa 920–0293, Japan; and d Department of Kampo Pharmaceutical Sciences, Nihon Pharmaceutical University; Komuro 10281, Ina-machi, Kita-Adachi-gun, Saitama 362–0800, Japan.

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The purpose of the present study was to investigate the efficacy of a liquid culture filtrates of the entomogenous fungus *Paecilomyces tenuipes* (PTCF) and its main active glycoprotein-enriched (PGF) fraction against hematotoxicity in mice treated with 5-fluorouracil (5-FU). Oral administration of PTCF (100 mg/kg/d) for 7 consecutive days after 5-FU injection significantly suppressed reductions in the red and white blood cell counts in peripheral blood, and accelerated their recoveries. From PTCF, glycoprotein-enriched fraction (PGF, >90% protein, approximately 15 kDa determined by SDS-PAGE) was separated as active ingredient that ameliorates 5-FU-induced anemia. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analysis of trypsinized-PGF showed 11 fragment ion peaks. Effective recoveries of erythrocytopenia and leukocytopenia were observed when PGF was co-administered with murine recombinant erythropoietin (mrEPO; 5 U/mouse). Oral administration of PGF also inhibited 5-FU-induced decreases in peripheral reticulocyte and bone marrow cell counts on day 12, and markedly hastened their recoveries on day 20, in dose-dependent manners. Reductions in erythroid progenitor colonies, such as colony-forming units (CFU)-erythroid and burst-forming units-erythroid mix, formed by bone marrow cells from 5-FU-treated mice were markedly improved by oral administration of PGF with subcutaneous mrEPO. Oral administration of PGF also increased the myeloid lineage progenitor, CFU-granulocyte-macrophages, in cultured bone marrow cells. These findings suggest that PGF isolated from *P. tenuipes* has the potential to protect against 5-FU-induced erythrocytopenia and leukopenia, especially in combination with mrEPO, and also has hematopoietic activity, through stimulation of immature erythroid as well as myeloid progenitor cell differentiation.

Key words *Paecilomyces tenuipes*; glycoprotein; 5-fluorouracil; anemia; leukopenia; hematopoietic progenitor cell

Severe anemia that is resistant to medical treatment is often observed in patients with malignancies who are undergoing chemotherapy, and the pathogenesis of this anemia and leukopenia is multifactorial.1) Low levels of circulating hematopoietic growth factors, such as erythropoietin (EPO) and some cytokines, that shorten the survival time of circulating red blood cells (RBCs) and decrease the number of immature erythroid cells in the bone marrow, probably due to chemotherapy or chronic inflammation, have been demonstrated as causes of anemia.2) To cope with chronic anemia, blood transfusions as well as EPO have been used clinically in various situations. Clinical trials of EPO in patients undergoing cancer chemotherapy have demonstrated prevention of anemia development, although complete recovery has not been found.3) Clibon et al.4) reported that EPO cannot sufficiently overcome the reduced hematopoiesis induced by the inflammatory cytokine tumor necrosis factor (TNF)-α. Myelopoietic growth factors, such as granulocyte-macrophage-colony-stimulating factor (GM-CSF), monocyte colony-stimulating factor (M-CSF) and granulocyte-colony-stimulating factor (G-CSF), have also been used clinically for leukopenia in cancer chemotherapy.5) In addition to hematopoietic growth factors and transfusion, Japanese herbal medicines, such as Juzen-taiho-to, have been used clinically to ameliorate erythrocytopenia, fatigue or anorexia in patients undergoing cancer chemotherapy.6,7) In one clinical trial, Juzen-taiho-to was found to enhance the peripheral blood counts in cancer patients receiving radiation therapy,6) and the active constituents were identified as oleic and linoleic acids.3) Furthermore, an anti-cancer polysaccharide, lentinan, has been shown to stimulate the proliferation of immature erythroid progenitors, burst forming units-erythroid (BFU-E), and to improve 5-fluorouracil (5-FU)-induced reductions in the number of BFU-E in mice.8) We previously reported that hot-water extracts from *Angelica acutiloba* KITAGAWA, which is used as a herbal medicine for postmenstrual blood loss and EPO-resistant anemia in chronic renal failure, and its main active polysaccharide constituent increased peripheral RBC and reticulocyte counts, as well as BFU-E mix and colony forming units-erythroid (CFU-E) in cultured bone marrow cells from mice with 5-FU-induced anemia, and that these mechanisms were, in part, due to inhibition of inflammatory cytokine production without EPO mRNA expression in the liver and kidney.9) These results indicate that herbal medicines and plant metabolites that can stimulate the proliferation of erythroid progenitors and have the potential to recover erythrocytopenia in animal models of anemia may be useful for ameliorating anemia in clinical trials.

Certain kinds of entomogenous fungi, genera *Cordyceps*
and *Paecilomyces* (anamorphic stage of *Cordyceps*) (Clavicipitaceae), which infect specific insect hosts, are traditionally used as time-honored tonics and for improvement of blood loss, fatigue and anorexia in Japan, Korea and China. In our previous report, liquid culture filtrates of an entomoparasitic fungus, *Paecilomyces tenuipes* (="Isaria japonica Yasuda") (PTCF), selectively stimulated intestinal T cell immune responses, and the active constituent was identified as a glycoprotein-rich fraction. Furthermore, the glycoprotein also enhanced myeloid growth factors, such as GM-CSF, in cultured Peyer's patch cells in response to conacavalin A.

In the present study, we investigated whether PTCF and its main ingredient glycoprotein from the PTCF recovers anemia in 5-FU-treated mice. 5-FU mainly exerts its cytotoxic effect by inhibiting thymidylate synthase activity, and has often been used in chemotherapeutic combinations for malignancy. Using a well-established experimental model of 5-FU-induced hematoxicity, we attempted to evaluate the therapeutic efficacy of PTCF and its glycoprotein, and then investigated the pharmacological mechanisms of their effective extracts leading to hematopoiesis.

**MATERIALS AND METHODS**

**Culture of *P. tenuipes*** An entomoparasitic fungus, *P. tenuipes* (=*I. japonica*), was harvested from infected moth chrysalises at Mogami-Gun (Yamagata Prefecture, Japan) in 2005, taxonomically identified and deposited in a database at the Graduate School of Natural Science and Technology (Kanazawa University, Ishikawa, Japan) under registration number PT2005-3. Conidiospores isolated from the conidia of this fungus were inoculated into autoclaved semi-solid culture medium composed of 2% (w/v) agar, 0.3% (w/v) beer dry yeast extract (Iwaki Pharmaceutical, Tokyo, Japan), 0.5% (w/v) glucose and 0.016% (w/v) inosine (PT medium) in 200-ml flasks, and incubated at 15 °C with an 8-16 h light:16-h dark rhythm. Typical conidiophores formed from the newly cultivated hyphae in the artificial medium by approximately 80 d after inoculation. Conidiospores formed from the new conidia were carefully collected, floated on PT medium without agar and further cultured for 200 d under the same conditions.

**Isolation of Glycoprotein-Enriched Fraction (PGF) from Culture Filtrates of *P. tenuipes* (PTCF)** Hyphae and conidia of *P. tenuipes* were carefully and completely removed from the surface of the culture medium by filtration (pore size: 0.45 μm) and the filtrates were centrifuged at 12000×g for 60 min. The supernatants were lyophilized to give a dark-brownish powder (PTCF) in a yield of 0.3% (w/v). Isolation of PGF from the PTCF was performed by the methods described in our previous report with minor modifications. Briefly, lyophilized PTCF powder was dissolved in distilled water (1 g/30 ml) and dialyzed (MW cut-off: 7000—8000 Da) against a 10-fold volume of distilled water at 4 °C for 18 h. The resulting three samples were combined and lyophilized to give a light-brownish powder (460 mg). Next, the lyophilized sample (230 mg) was subjected to column chromatography using Sepharose CL-6B (Sigma, St. Louis, MO, U.S.A.; bed volume: 100 ml). The column was subsequently eluted with H₂O (500 ml) to afford 100 fractions of 5 ml and the absorbance was monitored at 220 nm. After lyophilization, some of the eluted fractions (fractions 20—25) were combined and lyophilized to give Fraction II (PGF: 31.9 mg). This method effectively extracted PGF, which represented the active fraction isolated from PTCF.

**Analyses of PGF by Photodiode Array (PDA)-high Performance Liquid Chromatography (HPLC) and Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry (MS)** Lyophilized PGF (1 mg) was dissolved in 0.1 M phosphate buffer and filtered through a membrane filter (pore size, 0.45 μm). The resulting filtrates were analyzed by PDA-HPLC (Hitachi, Tokyo, Japan) equipped with L-7100 pumps, a D-7000 interface, an L-7455 PDA detector, an L-7200 auto sampler, an L-7300 column oven and a COSMOSIL 5 Diol-300-II gel filtration column (600×7.5 mm i.d.; Nakalai Tesque, Kyoto, Japan). The solvent used in this analysis was 0.1 M phosphate buffer (pH 7.2). Effluents were detected by UV at 200—600 nm.

PGF (5 mg) was partly subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using a 4% stacking gel and 10% separating gel. To visualize the mass shift of PGF, gels were stained with colloidal Coomassie G250.

For in-gel digestion, gel pieces were excised, transferred into vials and destained with 10% (v/v) acetic acid, 30% (v/v) ethanol and 60% (v/v) water. The gel pieces were then rinsed with 100 μl of 50 mM ammonium hydrogen carbonate buffer (pH 9.0), dehydrated four times with 100 μl of 100% acetonitrile and dried in a Speedvac for 15 min. Next, 50 μl of 50 mM ammonium hydrogen carbonate buffer and 2 μl of trypsin (MS grade; Promega, Madison, WI, U.S.A.) were added to each vial, and the gel pieces were digested for 12 h at 37 °C. After centrifugation, the supernatants were carefully removed and the pellets were reconstituted in 50 μl of 50 mM ammonium hydrogen carbonate buffer for 30 min and centrifuged. After removal of the supernatants, each pellet was extracted with 50 μl of acetonitrile with sonication. The supernatants were pooled and subjected to further analysis.

MS analysis was carried out using a MALDI-TOF instrument (AXIMA TOF; Shimadzu, Tokyo, Japan) in the reflector positive ion mode. A thin-layer sample preparation technique was applied using a saturated solution of un purified α-cyanohydroxy-cinnamic acid in 0.1% (v/v) trifluoroacetic acid in 50% (v/v) acetonitrile. An aliquot of each desalted sample was dissolved in 10 μl of water. A 0.5-μl aliquot of this solution was deposited on the thin crystallized matrix layer and air-dried at room temperature. A 0.5-μl aliquot of matrix solution was deposited on the stainless-steel target slide and the solvent was evaporated under atmospheric pressure at room temperature. For MS analysis, the 800—4000 m/z mass range was used with 1000 shots per spectrum. All analyses were performed using the default calibration standard peptides angiotensin III (931.11 Da) and substance P (1347.66 Da).

**Animals** Animals were treated according to the Guiding Principles for the Care and Use of Animals in the Field of Kanazawa University, and the experimental protocol was approved by the Institute for Experimental Animals (Kanazawa University Advanced Science Research Center, Ishikawa, Japan). Female C57BL/6J mice (8—9 weeks of age; Japan SLC, Shizuoka, Japan) were housed in groups of eight in an environmentally controlled facility.
plastic cages with a 12-h/12-h light/dark cycle and had free access to water and mouse chow ad libitum. The mice were allowed to adapt to these conditions for at least 1 week before the experiment commenced.

**Experimental Protocol for Anemic Mice** In the first experiment, 5-FU (Hoffman La Roche-Kyowa, Tokyo, Japan) at 150 mg/kg (0.01 ml/g body weight) was dissolved in saline and injected intravenously into the tail vein on day 0.9 PTCF or PGF samples dissolved in distilled water were injected orally at 1—30 mg/kg/d once a day for 6 consecutive days (days 0 to 5). In the second experiment, anemic mice treated with PGF were subcutaneously injected with 5 U of murine recombinant EPO (mEPO; Roche, Mannheim, Germany) dissolved in pyrogen-free phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA). The disease control group was treated with vehicle.

**Hematological Analysis** Peripheral blood samples were collected from an ocular sinus sampling method using an EDTA-coated capillary tube on days 0, 5, 8, 12, 15 and 20. White blood cells (WBCs), RBCs and platelets (PLTs) were counted using an automatic hemocytometer (MICROS abc, White blood cells (WBCs), RBCs and platelets (PLTs) were collected by an ocular sinus sampling method using an EDTA-coated capillary tube on days 0, 5, 8, 12, 15 and 20. White blood cells (WBCs), RBCs and platelets (PLTs) were counted using an automatic hemocytometer (MICROS abc, LC-152; Horiba, Kyoto, Japan).9,17) Peripheral reticulocytes were counted using an automatic hemocytometer (MICROS abc, White blood cells (WBCs), RBCs and platelets (PLTs) were counted using an automatic hemocytometer (MICROS abc, LC-152; Horiba, Kyoto, Japan).9,17) Peripheral reticulocytes were enumerated according to a previously described method.18) Briefly, freshly prepared methylene blue solution was mixed with an equal volume of peripheral blood and incubated at room temperature for 15 min. Blood smears were prepared on glass slides using a cytocentrifuge (Hitachi, Ibaraki, Japan) and stained with Wright solution (Muto Pure Chemicals, Tokyo, Japan). Microscopic observation was performed using an ocular micrometer disk as previously described.18)

**Bone Marrow Cell Preparation and CFU Assay** Bone marrow was obtained from tibiae and femurs as described previously.17) Briefly, a cell suspension collected by aspirating the bone with a syringe filled with PBS was washed twice with the same buffer. Bone marrow cells were counted individually using a hemocytometer, and stored in plastic tubes on ice before analysis by CFU assays. The bone marrow cell suspension was then washed twice with α-MEM (Invitrogen, Carlsbad, CA, U.S.A.) containing 10% fetal bovine serum (FBS), and resuspended in the same medium. CFU-E and BFU-E in the femoral bone marrow were analyzed using a methylcellulose-based colony assay.9,13,19) Cell suspensions were gently mixed with 0.8% methylcellulose in α-MEM containing 30% FBS, 1% BSA, 200 μM hemin and 100 μM 2-mercaptoethanol. For developing CFU-E (late erythroid progenitors), bone marrow cell suspensions (1×10^5 cells/ml) were plated in 35-mm Petri dishes, and stimulated with mEPO (2 U/ml) for 2 d. For developing BFU-E mix (myeloid-mixed early erythroid population), bone marrow cell suspensions (2×10^5 cells/ml) were plated in the same dishes, and stimulated with mEPO (2 U/ml) and interleukin (IL)-3 (5 ng/ml; Genzyme, Boston, MA, U.S.A.) for 9 d. All dishes were incubated at 37 °C in a humidified 5% CO2 incubator. After 2 d (for CFU-E) or 9 d (for BFU-E mix), the methylcellulose discs were transferred to glass slides and fixed with 2% (v/v) glutaraldehyde solution in PBS. For developing CFU-GM, bone marrow cell suspensions (1×10^5 cells/ml) were plated in 35-mm Petri dishes, and stimulated with mGM-CSF (25 ng/ml; Genzyme) for 7 d. The three colony types (BFU-E mix, CFU-E and CFU-GM) were enumerated under a microscope. Only colonies consisting of at least 10 cells were counted.

**Statistical Analysis** Results are expressed as the mean ± S.D. or S.E. of at least three independent experiments. Statistical significance was determined by Dunnett’s multiple test after one-way analysis of variance (ANOVA) for comparisons with physiologically normal and disease control groups. Values of p<0.05 were considered significant.

**RESULTS**

**PTCF and the Main Glycoprotein Constituent PGF** In order to investigate anti-hematotoxic efficacy of *P. tenuipes*, we first prepared the liquid culture filtrates of this fungus. That is, conidiospores formed from the conidia of *P. tenuipes* grown in natural field were floated on liquid medium containing yeast and further cultured for 6—7 month. Then, the culture fluid (PTCF) was collected by centrifugation and filtration. We next tried to reveal chemical character of the constituent glycoprotein from the PTCF that protected against anemia. SDS-PAGE analysis of PTCF indicated that the molecular weight range was divided into three different molecules. Specifically, a major band was present in the proximity of 15 kDa, while three minor bands appeared at 10, 17 and 25 kDa, respectively (Fig. 1A). We further analyzed glycoprotein components by PDA-HPLC, SDS-PAGE and MALDI-TOF/MS. As shown in Fig. 1B, a glycoprotein-enriched fraction was fractionated by chromatography on a Sepharose CL6B column from a dialysate of PTCF to give three fractions (Fraction I, II and III, Fig. 1B). Then the main constituent, Fraction II (fraction 20—25: PGF), was collected, and the purity was checked by PDA-HPLC. Analysis
of PGF by PDA-HPLC (equipped with a gel filtration column), revealed that a sharp, but somewhat broad, peak of ingredients from PGF appeared at 24—25 min (Fig. 1C).

Next, we analyzed the mass spectra of the three different bands detected by SDS-PAGE using MALDI-TOF. As shown in Table 1, the trypsinized 15-kDa bands (Fraction II: PGF) obtained after electrophoresis of PGF individually showed 11 fragment ion peaks.

PGF was determined to consist of up to 90% protein and <10% carbohydrate by phenol-sulfuric acid and Lowry–Folin tests, respectively. Although no data are shown, PGF increased the production of hematopoietic growth factor such as GM-CSF in cultured Peyer’s patch cells, and PGF was sufficient material for investigating its anti-anemic activity.

### Effects of PTCF, PGF, EPO and PGF plus EPO on Hematotoxicity in 5-FU-Treated Mice

The protective effects of PTCF, an active fraction PGF and mrEPO (as a positive control) against 5-FU-induced hematotoxicity in mice were examined. To avoid interference by endotoxin contaminants in the test samples, we used endotoxin-non-responder C57BL/6J mice in this experiment. After intravenous injection of 5-FU, the numbers of circulating RBCs and WBCs were reduced by 28.0% and 50.2% on day 10, respectively, whereas PLTs were dramatically enhanced by 148.8% at this time point (Fig. 2). Oral administration of PTCF at 100 mg/kg/d for 6 consecutive days effectively protected against anemia and slightly improved peripheral leukocytopenia on day 10 (Figs. 2A, B). Administration of PGF isolated from PTCF at 30 mg/kg/d also protected against leukocytopenia on day 10 (Figs. 2A, B). In the present experiment, oral injection of culture medium alone could not protect against 5-FU-induced hematotoxicity, even with the same oral injection of culture medium alone could not protect against 5-FU-induced hematotoxicity, even with the same

Peripheral blood samples were collected at 10 d after injection with 5-FU (150 mg/kg) and the hematological parameters of the RBC (A), WBC (B) and PLT (C) counts were measured using an automatic hemocytometer on day 10. All tested samples were orally (PGF and PTCF) and/or subcutaneously (mrEPO) administered to mice once daily from days 0 to 5 after the 5-FU treatment. Values are presented as the mean ± S.E. (*p < 0.05 vs. normal mice; **p < 0.05 vs. 5-FU-treated mice.

### Kinetics of RBCs, WBCs, PLTs and Reticulocytes after 5-FU Administration and Anti-hematotoxic Effects of PGF, EPO and PGF plus mrEPO

In our previous report, the hematological parameters in 5-FU-treated mice varied from days 0 to 20 with typical patterns, such as the “damaged phase” (5—12 d after 5-FU injection), “recovery phase” (6—15 d) and “rebound phase” (15—20 d). Therefore, we next investigated the effects of the tested samples on 5-FU-induced hematotoxicity at these typical time points. As shown in Fig. 3, peripheral anemia and leukopenia appeared at 12 d after injection with 5-FU alone at a dose of 150 mg/kg, and thrombocytopenia was apparent at 8 d after the injection. A rebound reaction was observed in the case of PLTs on day 20 (Fig. 3C). Since the peripheral reticulocyte count reflects erythropoiesis, we also enumerated peripheral reticulocytes in 5-FU-treated mice. The number of reticulocytes in peripheral blood was dramatically reduced on day 5, and then quickly increased to 142% (relative to the normal level) by 16 d after 5-FU injection (Fig. 3D). On day 20, the number of reticulocytes had begun to recover to the normal level (Fig. 3D).

Oral administration of PGF at 30 mg/kg/d for 7 consecu-
tive days markedly improved the leukocytopenia (Fig. 3B) and slightly, but significantly, inhibited the reduction in erythrocytopenia on day 12 (Fig. 3A). In particular, the WBC counts of the PGF-treated group on day 20 were 1.2—1.3-fold higher than those of the 5-FU-alone-treated group (Fig. 3B), indicating that PGF effectively ameliorates leucopenia. PGF at 30 mg/kg/d also significantly increased the number of peripheral erythrocytes on day 12, although the number was the same as that for 5-FU-alone-treated mice on day 20 (Fig. 3). Oral administration of PGF did not affect the thrombocytopenia caused by 5-FU at the same regimen (Fig. 3C). Subcutaneous injection of mrEPO produced a dramatic improvement in the erythrocytopenia and thrombocytopenia as well as the reduction in reticulocytes, and showed slight improvement in the leucopenia at all time points (Figs. 3A—C). Furthermore, the leukocytopenia, anemia and thrombocytopenia caused by 5-FU were strongly recovered by co-injection of mrEPO and PGF compared to treatment with mrEPO or PGF alone, and these effects were more pronounced on day 12 than on day 20 (Figs. 3A—C). Co-treatment with PGF plus mrEPO increased peripheral reticulocytes on days 8, 12 and 20 in comparison with the efficacy of treatment with PGF alone (Fig. 3D). Furthermore, this PGF plus mrEPO regimen hastened the recovery of the thrombocytosis on day 20 (Fig. 3C).

Anti-hematotoxic Effects of PGF and PGF Plus mrEPO in Dose–Response Experiments On the basis of the time–course experiments, we next examined the dose–response properties of the protective activities of PGF and co-injection of mrEPO with PGF against hematotoxicity on days 12 and 20 in 5-FU-treated mice. Oral administration of PGF at a dose of 30 mg/kg/d effectively suppressed leucopenia on day 12 in a dose-dependent manner (Table 2). On day 20, the recovery of peripheral leukocytes was still higher in mice treated with PGF than in mice treated with 5-FU alone (Table 2). Peripheral anemia and thrombocytopenia were significantly suppressed on day 12 after administration of PGF alone at a higher dose (30 mg/kg/d). Administration of mrEPO (5 U/mouse) alone also significantly improved the 5-FU-induced leucopenia, but its effect was weaker than that of PGF at 30 mg/kg/d (Table 2; WBC). In contrast, mrEPO effectively ameliorated peripheral anemia on day 12, and the anti-anemic effects of mrEPO were increased on days 12 and 20 in 5-FU-treated mice. Oral administration of PGF with mrEPO and PGF compared to treatment with mrEPO or PGF alone, and these effects were more pronounced on day 12 than on day 20 (Figs. 3A—C). Co-treatment with PGF plus mrEPO increased peripheral reticulocytes on days 8, 12 and 20 in comparison with the efficacy of treatment with PGF alone (Fig. 3D). Furthermore, this PGF plus mrEPO regimen hastened the recovery of the thrombocytosis on day 20 (Fig. 3C).

Table 2. Effects of Various Doses of PGF and PGF Plus mrEPO on the WBC, RBC, PLT and Reticulocyte Counts in Peripheral Blood after 5-FU Administration

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>Dose mg/kg/d</th>
<th>WBC (×10³/μl)</th>
<th>RBC (×10⁶/μl)</th>
<th>PLT (×10⁴/μl)</th>
<th>Reticulocyte (×10⁵/μl)</th>
<th>n</th>
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<tbody>
<tr>
<td>12</td>
<td>Normal</td>
<td>—</td>
<td>3.8±0.1</td>
<td>9.9±0.2</td>
<td>411±18</td>
<td>392±10</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>—</td>
<td>1.1±0.1*</td>
<td>5.7±0.1*</td>
<td>138±22*</td>
<td>247±29*</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>PGF</td>
<td>3</td>
<td>1.1±0.3</td>
<td>6.2±0.1</td>
<td>150±16</td>
<td>261±30</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>PGF</td>
<td>10</td>
<td>1.3±0.1</td>
<td>6.1±0.3</td>
<td>137±29</td>
<td>239±18</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>PGF</td>
<td>30</td>
<td>2.5±0.3*</td>
<td>6.8±0.2*</td>
<td>281±40*</td>
<td>309±11*</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>EPO</td>
<td>5 U</td>
<td>2.3±0.1*</td>
<td>6.8±0.2*</td>
<td>305±10*</td>
<td>334±14*</td>
<td>10</td>
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<tr>
<td></td>
<td>EPO/PGF</td>
<td>5 U+3</td>
<td>2.0±0.1</td>
<td>6.3±0.2</td>
<td>305±10*</td>
<td>334±14*</td>
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<td>EPO/PGF</td>
<td>5 U+10</td>
<td>3.3±0.4*</td>
<td>6.9±1.1</td>
<td>337±11*</td>
<td>320±23*</td>
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<td></td>
<td>EPO/PGF</td>
<td>5 U+30</td>
<td>3.0±0.2*</td>
<td>7.3±0.5*</td>
<td>341±28*</td>
<td>359±7*</td>
<td>11</td>
</tr>
<tr>
<td>20</td>
<td>Control</td>
<td>—</td>
<td>3.3±0.5*</td>
<td>5.7±0.4*</td>
<td>723±54*</td>
<td>439±24*</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>PGF</td>
<td>3</td>
<td>2.7±0.1</td>
<td>5.9±0.2</td>
<td>634±66</td>
<td>399±21</td>
<td>12</td>
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<td></td>
<td>PGF</td>
<td>10</td>
<td>4.1±0.9</td>
<td>6.2±0.3</td>
<td>730±77</td>
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<td>PGF</td>
<td>30</td>
<td>4.9±0.4*</td>
<td>6.5±0.1*</td>
<td>607±45</td>
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<td>EPO</td>
<td>5 U</td>
<td>3.8±0.8</td>
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<td>553±39</td>
<td>551±22*</td>
<td>10</td>
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<td>9.3±0.2*</td>
<td>565±165</td>
<td>530±35</td>
<td>11</td>
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<td>EPO/PGF</td>
<td>5 U+10</td>
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<td>9.8±0.1*</td>
<td>501±103*</td>
<td>565±28*</td>
<td>10</td>
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<td></td>
<td>EPO/PGF</td>
<td>5 U+30</td>
<td>5.0±1.5*</td>
<td>10.7±0.8*</td>
<td>378±67*</td>
<td>612±13*</td>
<td>11</td>
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</table>

Mice were injected orally with various doses of PGF and/or subcutaneously with mrEPO (5 U/mouse) once daily for 6 consecutive days from days 0 to 5 after 5-FU injection. Peripheral blood samples were collected on days 12 and 20, and hematologic parameters were measured using a hemocytometer. Peripheral reticulocytes were also enumerated at the indicated time points. Data are expressed as the mean±S.E. The data represent one experiment repeated twice with similar results. *p<0.05 vs. normal mice. p<0.05 vs. 5-FU-treated mice.

Fig. 3. Time–Course Kinetics of RBCs, WBCs, PLTs and Reticulocytes in Peripheral Blood after 5-FU Injection

5-FU (150 mg/kg) was injected intravenously into mice on day 0. Peripheral blood samples were collected at the indicated times, and the hematologic parameters of the RBC (A), WBC (B) and PLT (C) counts were measured using an automatic hemocytometer. Peripheral reticulocytes (D) were also measured at the indicated times. All the tested samples were orally (PGF 30 mg/kg and PTCF 100 mg/kg) and/or subcutaneously (mrEPO 5 U/mouse) administered to mice once daily from days 0 to 6 after the 5-FU treatment. Values are presented as the mean±S.E. (n=9—12). *p<0.05 vs. day 0.
**PGF Promotes Immature Erythroid and Leukocyte Progenitor Cells in Cultured Bone Marrow Cells**

Since oral PGF ameliorated anemia and leukopenia, and hastened the recovery of reticulocytes (Figs. 1, 3; Table 2), the effects of PGF on the expressions of immature erythroid and leukocyte progenitor cells obtained from hematotoxic mice were examined. The number of myelocytes in the bone marrow was dramatically reduced at 10 d after 5-FU injection (Fig. 4A). On day 16, the number of bone marrow cells was still reduced (Fig. 4A), whereas the reticulocyte count had recovered (Fig. 3D). Bone marrow seems to be more sensitive to 5-FU than peripheral reticulocytes (Figs. 3, 4). PGF at 30 mg/kg/d significantly protected against the 5-FU-induced cytotoxicity toward myelocytes (Fig. 4A). Subcutaneous injection of mrEPO also inhibited the reduction in myelotoxicity, and hastened the recovery of the number of these cells (Fig. 4A). The number of myelocytes obtained from PGF-treated mice injected with mrEPO was significantly higher than the number obtained from mice treated with mrEPO alone (Fig. 4D).

The developing erythroid colonies of BFU-E mix and CFU-E in cultured bone marrow cells from normal mice were ca. 3000 and ca. 45000 per femur, respectively (Figs. 4B, C). Intravenous 5-FU dramatically reduced their formation by up to 90% on day 5, and only CFU-E formation had completely recovered on day 10 (Fig. 4B, Table 3). On the other hand, the expression of leukocyte progenitor colonies, such as CFU-GM, in cultured bone marrow cells began to decrease at 5—10 d after 5-FU administration, and the expression of colony formation surpassed that of normal mice by 1.6-fold at 20 d after 5-FU injection (Fig. 4D).

Although administration of PGF (30 mg/kg/d) or mrEPO (5 U/mouse) could not protect against the reductions in the development of CFU-E and BFU-E colonies on day 5, the recoveries of both colonies were enhanced by these regimens (Figs. 4B, C). On day 15, the reductions in BFU-E mix and CFU-E were dramatically recovered by PGF co-injected with mrEPO. The recovery activity of PGF plus mrEPO was greater for BFU-E mix expression than for CFU-E expression (Figs. 4B, C). CFU-GM expression in cultured bone marrow cells was enhanced on day 10 by oral PGF alone, and higher than that of control cells (Fig. 4D). Interestingly, the erythroid lineage-specific stimulating factor mrEPO significantly (p<0.05) ameliorated the reduction in CFU-GM, while the reduction and recovery of CFU-GM were markedly enhanced by PGF co-administered with EPO (Fig. 4D).

**Effects of PGF and PGF plus mrEPO on the Expressions of Erythroid and Leukocyte Progenitors in Dose–Response Experiments**

Oral administration of PGF alone effectively ameliorated the reduction in CFU-GM colonies at a dose of 30 mg/kg/d (Table 3; day 10), and hastened the recovery of colony formation in a dose-dependent manner (Table 3; day 15). A tendency for the colonies to increase was observed in 5-FU-treated mice after PGF administration even at a low dose (Table 3; 3 mg/kg/d). The reductions in BFU-E mix and CFU-E on day 10 were moderately suppressed by oral PGF, and these activities required a higher dose of PGF (Table 3; 30 mg/kg/d). The reductions in these erythroid progenitors were almost complete by day 15, and increased by oral administration of PGF. Furthermore, subcutaneous mrEPO (5 U/mouse) markedly stimulated colony formation of BFU-E mix and CFU-E on day 10 as well as on day 15, and mrEPO with PGF at a lower dose (3 mg/kg/d) remarkably enhanced the numbers of not only erythroid colonies but also myelocytes, especially on day 15 (Table 3).

**DISCUSSION**

Oral administration of PTCF at 100 mg/kg/d significantly protected against 5-FU-induced hematotoxicity, including anemia and leukopenia, in the toxic phase, and strongly hastened their recoveries in the recovery phase. Identical results were obtained for 5-FU-treated mice administered oral PGF.
isolated from PTCF at 3—30 mg/kg/d, suggesting that PTCF itself has the potential to induce recovery from erythrocytopenia and leukocytopenia in anemic mice and that PGF is one of the active glycoprotein components responsible for the effects of PTCF. Previous studies demonstrated that a hot-water extract of cultured mycelia of *Cordyceps sinensis* (200 mg/kg/d), a traditional Chinese medicine mainly used as a time-honored tonic, increased the blood flow rate and hepatic energy metabolism in dietary hypoferric anemic mice. To the best of our knowledge, there are no previous reports regarding the effects of the genus *Paecilomyces* (amorphic stage of *Cordyceps* fungi) on experimental hematotoxicity. Therefore, our present study is the first report that culture metabolites of *Paecilomyces* can reduce the hematotoxicity and hasten the recoveries of anemia and leukopenia in 5-FU-treated mice.

We recently reported that PTCF and their main active fraction rich in glycoproteins stimulated the production of hematopoietic cytokines, such as GM-CSF, in cultured murine Peyer’s patch cells co-stimulated with the non-specific T cell activator concanavalin A. In the present study, we isolated the same glycoprotein-enriched fraction examined in the previous study, and partly determined its components by conventional analyses. MALDI-TOF analyses revealed that the trypsinized-PGF (Fr. II) represented a cluster of differently sized molecules, and the molecular weight range of PGF was determined as ca. 15 kDa. Further purification and structural elucidation of the sugar moieties and amino acid sequences of the PGF glycoproteins remain to be completed.

To further elucidate the therapeutic potential of the anti-hematotoxic activities of PTCF and PGF, we performed a chemical characterization of PGF and investigated the abilities of PTCF and PGF to decrease the myelotoxic effect of 5-FU.

*P. tenaipes* is well known to be an anamorphic stage of *Cordyceps takaomontana*, which infects moth chrysalides. In our previous study, aqueous extracts of the genera *Paecilomyces* (including *P. tenaipes*) and *Cordyceps* (*C. sinensis* and *Cordyceps militaris*) showed individually distinct protein profiles, as evaluated by capillary electrophoresis. Therefore, PGF is considered to be a specific anti-anemic and immunopotentiating metabolite of *P. tenaipes*. Moreover, some kinds of *Cordyceps* and *Paecilomyces* fungi have traditionally been used as health foods for preventing infectious diseases and tonic in Japan, Korea and China. If more data for distribution of glycoprotein like PGF in these species are accumulated, entomogenous fungi might be promising sources for anti-anemic agents. Further investigations in line with this idea are to be performed.

Oral administration of PGF partly enhanced the total number of late erythrocyt progenitor BFU-E mix, but not CFU-E, in the bone marrow. These results suggest that PGF stimulates late differentiation of erythroid lineage cells in bone marrow after 5-FU toxicity, thereby promoting hematopoiesis. Takatsu et al. reported that lentinan, an antitumor polysaccharide mainly composed of β (1→3) glucan and isolated from medicinal and edible mushrooms, augmented the level of BFU-E and accelerated the recovery of the reduced number of BFU-E in mice treated with 5-FU, but did not influence peripheral RBC and CFU-E numbers in the femoral bone marrow. Moreover, we revealed that a water-soluble polysaccharide-rich fraction isolated from the hematopoietic herb Angelica root increased peripheral RBC counts and BFU-E mix as well as CFU-E numbers in cultured bone marrow cells. Hence, the details of the anti-hematotoxic activity of PGF, which predominantly protected against leukopenia and hastened leukocyte recovery, differed somewhat from the activities of lentinan and the Angelica polysaccharide extract. Although it remains unclear why these high-molecular mass constituents (PGF, lentinan and Angelica polysaccharide) had opposite effects on erythrocytopenia and leukocytopenia, it is possible that the polysaccharide structure (lentinan and Angelica polysaccharide) is

### Table 3. Effects of Various Doses of PGF and PGF Plus mrEPO on the Formation of CFU-E Mix, BFU-E and CFU-GM Colonies in Cultured Bone Marrow Cells from Mice Treated with 5-FU

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>Dose (mg/kg/d)</th>
<th>Marrow cells (×10^3/femur)</th>
<th>CFU-E (×10^3/femur)</th>
<th>BFU-E mix (×10^3/femur)</th>
<th>CFU-GM (×10^3/femur)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Control</td>
<td>—</td>
<td>15.2±2.1</td>
<td>44.4±1.3</td>
<td>32.0±0.6</td>
<td>18.1±0.6</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>PGE</td>
<td>3</td>
<td>1.4±0.1*</td>
<td>41.7±0.3*</td>
<td>2.9±0.1*</td>
<td>5.3±1.8*</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>PGF</td>
<td>10</td>
<td>2.5±0.2</td>
<td>53.0±0.5*</td>
<td>9.9±0.2*</td>
<td>6.0±1.2*</td>
<td>9</td>
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<tr>
<td></td>
<td>EPO</td>
<td>5U</td>
<td>4.0±0.1*</td>
<td>53.9±0.8*</td>
<td>11.1±0.8*</td>
<td>12.6±0.7*</td>
<td>10</td>
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<td></td>
<td>EPO/PGF</td>
<td>5U+3</td>
<td>3.3±0.2</td>
<td>53.3±1.0*</td>
<td>15.5±0.7*</td>
<td>12.5±1.3*</td>
<td>9</td>
</tr>
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<td></td>
<td>EPO/PGF</td>
<td>5U+10</td>
<td>5.1±0.1*</td>
<td>59.6±1.0*</td>
<td>18.2±1.1*</td>
<td>18.0±1.1*</td>
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<td>EPO/PGF</td>
<td>5U+30</td>
<td>6.3±0.2*</td>
<td>69.2±1.5*</td>
<td>20.0±1.1*</td>
<td>23.7±1.0*</td>
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<tr>
<td>15</td>
<td>Control</td>
<td>—</td>
<td>7.2±0.8</td>
<td>37.1±3.6</td>
<td>33.8±0.5</td>
<td>27.0±0.6</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>PGF</td>
<td>3</td>
<td>7.9±0.2</td>
<td>38.0±1.8</td>
<td>32.7±0.1</td>
<td>28.1±1.5*</td>
<td>9</td>
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<tr>
<td></td>
<td>PGF</td>
<td>10</td>
<td>9.0±0.8*</td>
<td>37.2±0.7</td>
<td>38.1±1.1*</td>
<td>33.0±1.0*</td>
<td>9</td>
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<td>PGF</td>
<td>30</td>
<td>8.9±1.1*</td>
<td>41.2±0.6*</td>
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<td>EPO</td>
<td>5U</td>
<td>9.2±0.5*</td>
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<td>41.7±0.5*</td>
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<td>43.1±3.4*</td>
<td>34.3±2.5*</td>
<td>9</td>
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<td>44.6±1.3*</td>
<td>48.0±3.2*</td>
<td>36.3±3.0*</td>
<td>9</td>
</tr>
<tr>
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<td>EPO/PGF</td>
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<td>10.3±1.6*</td>
<td>51.0±1.0*</td>
<td>53.0±2.5*</td>
<td>45.2±2.0*</td>
<td>10</td>
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</tbody>
</table>

Mice were injected orally with various doses of PGF and/or subcutaneously with mrEPO (5 IU/mouse) for 6 consecutive days after 5-FU injection. Bone marrow cells were collected at 10 and 15 d after the 5-FU injection, and cultured with 2 U/ml of mrEPO with (for BFU-E) or without (for CFU-E) 5 ng/ml of IL-3 or with 25 ng/ml of GM-CSF (for CFU-GM). The developing colonies were enumerated after 2 (CFU-E), 7 (CFU-GM) or 9 (BFU-E mix) d of cultivation. Data are expressed as the mean±S.E. The data represent one experiment repeated twice with similar results. *p<0.05 vs. normal mice. **p<0.05 vs. 5-FU-treated mice.
required for eliciting anti-anemic effects, while the peptide constituent is important for elucidating anti-leukopenia effects. In fact, the carbohydrate content of PGF was less than 10%. Further investigations in line with this hypothesis are underway.

Since CFU-E are less mature than peripheral reticulocytes, the maximum recovery of CFU-E was observed on day 10, while the recovery of reticulocytes was detected on day 20. Furthermore, since BFU-E are known to be more immature than CFU-E, BFU-E recovery was expected to occur earlier than that of CFU-E. However, the maximum recovery of BFU-E mix, which contains immature myeloid cells and megakaryocytes, was observed on day 15 in a CFU assay. Our results are consistent with those of Rich,13 who demonstrated erythropoiesis in mice after 5-FU injection. Subcutaneous mrEPO (5 U/mouse for 3 consecutive days) not only strongly recovered the peripheral anemia but also reduced erythroid precursor lineages, such as bone marrow cells, CFU-E, BFU-E and reticulocytes, suggesting that this hematopoietic cytokine predominantly stimulated early differentiation of erythroid lineage cells in bone marrow after 5-FU toxicity. Interestingly, we found that in vivo administration of mrEPO significantly improved the reduction in leukocyte precursor colonies, CFU-GM, without any increment or recovery of BFU-E, BFU-GM, with any increment or recovery of the number of peripheral leukocytes, and this effect was weaker than that of PGF. These findings indicate that mrEPO had the potential to decrease leukopenia, especially during early proliferation of myeloid progenitors in the bone marrow. Indeed, Sperschneider et al.24 previously reported that no increases in the total leukocyte numbers of polymorphonuclear granulocytes, monocytes and lymphocytes in the peripheral blood during human EPO therapy were observed in patients on maintenance hemodialysis.

Oral administration of PGF at 30 mg/kg/d remarkably increased the numbers of bone marrow cells as well as leukocytes on days 10 and 20. However, PGF showed a weak anti-anemic effect for the same regimen. Thus, these results indicate that the effect of PGF on 5-FU-induced hematotoxicity was more pronounced for leukopenia than for erythrocytopenia in contrast to the efficacy of mrEPO. PGF isolated from PTCF and PGF was reported to induce the production of GM-CSF by cultured murine intestinal lymphoid tissue.15 Since GM-CSF has been reported to stimulate immature myeloid lineages to differentiate into leukocyte progenitors,25 the mechanisms by which PGF protects against 5-FU-induced leukopenia may be partly due to the induction of GM-CSF. This hypothesis is supported by the finding that CFU-GM numbers in cultured bone marrow cells from mice after administration of PGF were significantly high at all time points.

In the present study, we found significantly higher peripheral RBC, WBC and reticulocyte counts after administration of subcutaneous mrEPO and oral administration of PGF in 5-FU-treated mice. It is also noteworthy that the combination of mrEPO and PGF improved both thrombocytopenia and thrombocytosis, whereas administration of PGF or mrEPO alone never improved these aspects with the same regimen. Furthermore, the mrEPO plus PGF regimen increased the femoral bone marrow cell counts and recoveries of BFU-E mix and CFU-E on days 12 and 16 in contrast to the efficacies of administration of mrEPO or PGF alone. Hence, it is clear that PGF augments the effects of EPO on erythropoiesis. A recent report indicated that subcutaneous mrEPO and IL-3 administration completely abolished 5-FU-induced peripheral anemia and leukopenia, and hastened the recovery of erythroid progenitor cells.21 It is therefore possible that the mechanism by which PGF potentiates the erythropoietic effects of mrEPO is partly due to PGF-induced endogenous hematopoietic growth factors, including GM-CSF.12 In fact, EPO mRNA expression was increased in the mouse kidney and liver after 5-FU injection,26 and both serum G-CSF and IL-6 levels were elevated in cancer patients during chemotherapy.27 We are now planning to investigate the effects of PGF plus mrEPO administration on the expression levels of hematopoietic cytokines in myelosuppressive mice induced by 5-FU.

In summary, the present study has demonstrated that oral administration of PTCF and PGF can improve 5-FU-induced anemia and leukopenia, especially in combination with EPO, and hasten the recoveries of erythrocytopenia and leukopenia due to their stimulatory activities on erythroid and myeloid progenitor cells. Since anemia and leukopenia are serious side effects of clinical treatments, especially cancer chemotherapy, the entomoparasitic P. tenuipes metabolite PGF (and also PTCF) have therapeutic and prophylactic potential for erythrocytopenia and leukopenia, especially when caused by anti-cancer agents.

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