Active Hexose Correlated Compound (AHCC) Alleviates Gemcitabine-Induced Hematological Toxicity in Non-Tumor-Bearing Mice*

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ABSTRACT

Active hexose correlated compound (AHCC) is known as a dietary supplement derived from an extract of a basidiomycete mushroom. The present study was conducted to evaluate the role of AHCC in alleviating the side effects, particularly hematological toxicity, in non-tumor-bearing mice receiving monotherapy with gemcitabine (GEM). The results from the GEM treatment groups with and without AHCC administration were compared to control group that received vehicle alone. The GEM alone treatment reduced peripheral leukocytes and hemoglobin, and bone marrow cell viability in spite of no influence on body weight, food consumption, and renal and hepatic parameters. Supplementation with AHCC significantly alleviated these side effects. The colony forming assay of bone marrow cells revealed that AHCC improved reduction of colony forming unit-granulocyte macrophage (CFU-GM) and burst forming unit-erythroid (BFU-E) related to GEM administration. However, when mRNA expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) and erythropoietin (EPO) was examined using a quantitative reverse transcription polymerase chain reaction (RT-PCR), AHCC showed no effect for the mRNA levels of their hematopoietic growth factors. These results support the concept that AHCC can be beneficial for cancer patients with GEM treatment through alleviating the hematotoxicity.

Keywords: Anticancer Drug; Bone Marrow Suppression; Colony Formation; Hemoglobin; Mushroom Extract; White Blood Cells

1. Introduction

Gemcitabine (2',2'-difluoro-2'-deoxycytidine, GEM), a pyrimidine based nucleoside analog, [1] is metabolized to gemcitabine diphosphate and triphosphate inside the cell by nucleoside kinases [2]. Gemcitabine diphosphate is a potent inhibitor of ribonucleotide reductase, which is associated with deoxyribonucleotide pools [3]. A reduction of deoxyribonucleotide concentration leads to the inhibition of DNA synthesis. Gemcitabine triphosphate competes with deoxycytidine triphosphate (dCTP) in binding to replicating DNA polymerases and then is incorporated into DNA to prevent further elongation of the replicating strand, resulting from increase in the ratio of cellular concentrations of gemcitabine triphosphate to dCTP [4]. Thus, the major mechanism of action of GEM is the direct or indirect inhibition of DNA synthesis.

In cancer therapy, GEM is commonly used as a component of adjuvant chemotherapy for advanced pancreatic cancer [5]. Additionally GEM is also used for the treatment of various other carcinomas such as non-small cell lung cancer [6], ovarian cancer [7], breast cancer [8], and biliary tract cancer [9]. The limited toxicity associated with GEM therapy compared to other cytotoxic anticancer drugs is one of the major reasons for the widespread use in chemotherapy. Although hematological toxicity and flu-like symptoms caused by GEM are the most common side effect, they are mild and short-lived [10]. However, these toxicities related to GEM can lower the quality of life in cancer patients and often trigger reductions in the dosage, frequency and duration of chemotherapy, ultimately decreasing potential for optimal therapeutic outcomes.

An approach to relieve the side effects of anticancer drugs including GEM leads to the use of complementary and alternative medicine (CAM) that has attracted great attention. Many cancer patients are currently using CAM in order to reduce the side effects and obtain additional chemotherapeutic effects through boosting the immune system [11]. In Japan, 44.6 percent of cancer patients...
reported using CAM with the most frequently used treatment being dietary supplements of mushrooms such as agaricus (Agaricus blazei Murill) and active hexose correlated compound (AHCC) [12].

AHCC is a mixture of polysaccharides, amino acids, lipids and minerals derived from mycelial culture of the basidiomycete, Lentinula edodes. The predominant component of AHCC is oligosaccharides, which contain α-1,4 glucans and partially acetylated α-1,4 glucans with a molecular weight of around 5000 Daltons. AHCC has been shown to increase the number and function of dendritic cells in healthy adult humans [13] and enhance both the activation and proliferation of CD4^+ and CD8^+ T cells in tumor-bearing mice [14]. AHCC also strengthened the chemotherapeutic effects of UFT (tegafur and uracil in a 4:1 molar concentration) for mammary adenocarcinoma SST-2 cells in rats [15] and cisplatin for Colon-26 tumor cells in mice [16]. Furthermore, two human clinical studies in liver cancer patients showed a significant increase in survival rate among those taking AHCC. [17,18] Several studies have explored the alleviating effects of AHCC for chemotherapy-related side effects. In cisplatin-treated tumor-bearing mice, AHCC improved food consumption, renal damage and myelosuppression [16]. The role of AHCC in attenuating various side effects was also explored in non-tumor-bearing mice receiving monotherapy with paclitaxel, or multidrug chemotherapy including cisplatin plus paclitaxel, cisplatin plus 5-fluorouracil, 5-fluorouracil plus irinotecan, cyclophosphamide plus doxorubicin, and 6-mercaptopurine plus methotrexate [19,20]. In newborn rats, the AHCC-treated group was protected from cytosine arabinoside-caused hair loss [20].

We investigated the influence of AHCC on some of the side effects associated with GEM as an initial study in preparation for a human clinical trial. Non-tumor-bearing mice, but not tumor-bearing mice, were chosen so that the intrinsic alterations related to the anticancer agent could be assessed independent of oncological variables. In the present study, we focused on GEM-induced hematotoxicity including bone marrow suppression, which is a dose-limiting toxicity.

2. Materials and Methods

2.1. Reagents

Active hexose correlated compound (AHCC; Amino Up Chemical Co., Ltd., Sapporo, Japan) was produced from the mycelia culture of Lentinula edodes in a manufacturing process according to Good Manufacturing Practice (GMP) standards for dietary supplements, and ISO9001: 2008 and ISO22000: 2005 criteria [16]. After pre-cultivation in flasks, the basidiomycete was cultured in 15-ton large tanks for 45 days, and then AHCC was obtained through filtration, sterilization, concentration and freeze-drying. Gemcitabine (GEM) is a commercially available anticancer drug as Gemzar Injection (Eli Lilly Japan K. K., Kobe, Japan), and the drug was obtained from JUNSEI CHEMICAL CO., LTD. (Tokyo, Japan).

2.2. Animals

Specific pathogen-free male ddY mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and studied at six weeks of age. Animals were maintained in a temperature- and humidity-controlled room at 23°C ± 1°C and 55% - 60%, respectively, under a 12-hour light-dark cycle (lights on 08:00 to 20:00), fed a standard pelleted rodent chow (CE-2; CLEA Japan Inc., Tokyo, Japan), and given water ad libitum. Mice were divided into three groups: control (untreated), GEM alone, and GEM plus AHCC. Each group consisted of ten mice.

2.3. Treatments

The GEM solution was injected intraperitoneally at a dose of 400 mg/kg (1200 mg/m^2) once a week for three weeks (days 7, 14 and 21). The treatment was similar to the regimen actually used in clinical practice (1000 mg/m^2 of weekly drip infusion three times followed by one week cessation of the drug). AHCC was prepared as a solution at a dosage of 1 g/kg and administered daily by gavage to mice seven days before the first injection of GEM and throughout the experiment (day 1 to day 28). The control group received a vehicle (saline) instead of GEM and AHCC. All animals were killed under anesthesia, and blood, bone marrow (BM) cells, spleen and kidney were harvested at day 28.

Since the effect of AHCC was assessed at a dosage range from 100 mg/kg to 1 g/kg in previous studies, [14-16,19,20] a dose of 1 g/kg of AHCC was chosen in the current study. The experimental protocol was approved by the Animal Care Committee of Amino Up Chemical Co., Ltd.

2.4. Evaluation of Parameters

The following parameters were assessed: body weight, food consumption, liver function (serum aspartate aminotransferase; AST), kidney function (blood nitrogen urea; BUN), hematological toxicities (peripheral total white blood cell count and hemoglobin content), and myelosuppression. Body weight and food consumption were measured twice a week. Serum AST and BUN were assessed using Transaminase CII-test WAKO and Urea Nitrogen B-test WAKO assay kits (Wako Pure Chemical Industries Limited, Osaka, Japan), respectively. Cardiac blood samples were diluted to 1:10 with Turk solution (Wako Pure Chemical Industries Limited) to determine...
the number of total white blood cells in accordance with the Nageotte chamber counting procedure, [21] and hemoglobin content in blood was measured using a Hemo globin B-test kit (Wako Pure Chemical Industries Limited). Myelosuppression was determined by measuring BM cell viability and by evaluating the responses to colony forming unit granulocyte-macrophage (CFU-GM) and burst forming unit erythroid (BFU-E).

BM cell viability was determined by collecting BM cells from the femur, which were first suspended in 0.83% NH4Cl solution and incubated at 37°C for ten minutes to hemolyze red blood cells. After centrifugation, the cells were prepared at a concentration of 1 × 10⁷ cells/mL in DMEM supplemented with 10% FBS. A 100-µL aliquot of the suspension was cultured in a 96-well plate for three days, and the viability (percent of control group) of BM cells was estimated by a MTT assay. The detection of CFU-GM and BFU-E was performed using a colony forming cell assay kit, MethoCult (StemCell Technologies, Vancouver, Canada). Briefly, BM cells were suspended in Iscove’s MDM (IMDM) with 2% FBS and the suspension (2 × 10⁶ cells/mL) was mixed with methylcellulose medium containing rmSCF, rmIL-3 and rhIL-6 (MethoCult 3534) at a 1:9 ratio. The prepared BM cells (2 × 10⁵ cells/mL) were plated onto a 35-mm dish and incubated at 37°C for eight days to form CFU-GM colony. For a mature BFU-E assay, after IMDM with 2% FBS was added at a 1:9 ratio to methylcellulose medium containing rhEpo (MethoCult 3334), BM cells (2 × 10⁶ cells/mL) in IMDM with 2% FBS were mixed with the diluted methylcellulose medium at a 1:9 ratio. The prepared BM cells (2 × 10⁵ cells/mL) were plated onto a 35-mm dish and incubated at 37°C for four days. Following the individual incubation time, CFU-GM and mature BFU-E colonies were counted under a microscope to quantify murine hematopoietic progenitor cells.

2.5. Reverse Transcription Polymerase Chain Reaction

Expression of granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoietin (EPO) and beta-2-microglobulin (B2M) mRNA was determined using a quantitative reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from 100 mg of spleen and kidney with TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer’s protocol. First-strand cDNA was obtained by incubation of 1.6 µg of total RNA with PrimeScript 2 1st strand cDNA Synthesis kit (Takara Bio Inc., Otsu, Japan), and the RT product was then diluted to 10 µg/µL and subjected to PCR using TaKaRa ExTaq (Takara Bio Inc.). Forty cycles of amplification were carried out for GM-CSF mRNA, and EPO mRNA and B2M mRNA were 37 and 22 cycles, respectively. The condition of each cycle was denaturing at 94°C for 30 seconds, annealing at 59°C (GM-CSF and B2M) and 65°C (EPO) for 45 seconds, and extension at 72°C for 30 seconds. The primers are described as follows; GM-CSF: 5'-GCCCCTTGAAGCAGATGAGAG-3' (sense) and 5'-ATGAAATCCGGCATGTTGGA-3' (antisense); EPO: 5'-CCACCCCTGCTGTCTTTACTC-3' (sense) and 5'-GGCCCTTGCAAACCTTATAGT-3' (antisense); B2M: 5'-TAGCTGTGCTGCGCCGTACT-3' (sense) and 5'-AGTGGGGGAATTGACTGTA-3' (antisense). The gene bands in each sample were normalized to the corresponding B2M band using Alpha Innotech red™ (Alpha Innotech Corp., San Leandro, CA, USA).

2.6. Statistical Analysis

Experimental data are shown as mean ± standard error of the mean (SEM). Data were analyzed by one-way analysis of variance (ANOVA). Fisher’s Protected Least Significance Difference (PLSD) was used as a post hoc test, and values of p less than 0.05 were determined to be statistically significant.

3. Results

3.1. Peripheral Hematological Toxicity

To determine whether AHCC is capable of protecting against GEM-related hematotoxicity, peripheral total white blood cell count and hemoglobin content in blood were monitored. As shown in Figures 1(a) and (b), GEM treatment was significantly associated with reductions of leukocyte count and hemoglobin content (p < 0.01), and supplementation with AHCC completely ameliorated both hematological toxicities (p < 0.01). The values of white blood cells (×10⁶ cells/mL) in the control, GEM, and GEM + AHCC groups were 3.05 ± 0.13, 2.01 ± 0.12, and 3.03 ± 0.13, respectively. Hemoglobin content (g/dL) was 14.4 ± 0.2 (control), 13.1 ± 0.3 (GEM), and 14.7 ± 0.3 (GEM plus AHCC).

3.2. Bone Marrow (BM) Suppression

To elucidate the alleviating effect of AHCC for GEM-induced myelosuppression, BM damage was assessed by BM cell viability and the colony forming ability of hematopoietic progenitor cells. The viability of BM cells isolated from GEM-treated mice was lower than that of the control group (p < 0.01; Figure 2), and AHCC administration significantly reversed the decline although it did not achieve complete recovery (p < 0.01 vs GEM, control). Treatment with GEM alone significantly lowered...
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3.3. Expression of GM-CSF and EPO mRNA

Expression of GM-CSF and EPO mRNA in spleen and kidney, respectively, was compared among control, GEM alone, and GEM plus AHCC groups. The expression level was calculated as a percent of control after each band of GM-CSF and EPO was normalized to the corresponding B2M band (Table 2). The mRNA levels of both GM-CSF and EPO in the GEM alone group were significantly higher than those of the control and the GEM plus AHCC groups ($p < 0.05$). In contrast, the expression levels in AHCC-treated mice were identical to control.

3.4. Other Toxicities

No changes in body weight, food consumption, and liver and renal functions were noted at the completion of the study. The average of body weight (g) was 35.4 ± 1.0, 36.3 ± 0.7, and 35.9 ± 0.6 in the control, GEM alone, and GEM+AHCC groups, respectively. Serum AST and BUN values were also normal and did not change during the course of the study (data not shown), which was consistent with previous data [10].

4. Discussion

Gemcitabine (GEM) has shown activity in a variety of solid tumors [22]. The drug has been approved for the treatment of non-small cell lung cancer, pancreatic can-

### Table 1. Colony forming responses of CFU-GM and BFU-E.

<table>
<thead>
<tr>
<th>Group</th>
<th>CFU-GM</th>
<th>BFU-E</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>88.3 ± 1.7</td>
<td>119.3 ± 6.1</td>
</tr>
<tr>
<td>GEM</td>
<td>64.3 ± 8.6*</td>
<td>29.0 ± 0.6**</td>
</tr>
<tr>
<td>GEM+AHCC</td>
<td>104.0 ± 3.0</td>
<td>109.0 ± 8.1</td>
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All values (colony counts) represent the mean ± SEM. *$p < 0.05$ vs control, **$p < 0.01$ vs control, GEM + AHCC. CFU-GM: colony forming unit-granulocyte macrophage, BFU-E: burst forming unit-erythroid.

### Table 2. mRNA levels of GM-CSF and EPO.

<table>
<thead>
<tr>
<th>Group</th>
<th>GM-CSF</th>
<th>EPO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 27.4</td>
<td>100.0 ± 6.6</td>
</tr>
<tr>
<td>GEM</td>
<td>323.3 ± 74.1*</td>
<td>161.9 ± 19.4*</td>
</tr>
<tr>
<td>GEM+AHCC</td>
<td>92.9 ± 20.0</td>
<td>99.6 ± 3.5</td>
</tr>
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</table>

All values (% of control) show the mean ± SEM. *$p < 0.05$ vs control, GEM + AHCC. GM-CSF: granulocyte-macrophage colony-stimulating factor, EPO: erythropoietin.
ner and biliary tract cancer in Japan, and non-small cell lung, pancreatic, ovarian and breast cancers in the United States. Although GEM is generally well tolerated and has a good toxicity profile, myelosuppression is the most common side effect, which can limit dose and thus potentially its therapeutic efficacy. This study was designed to investigate the impact of AHCC in terms of side effects, particularly hematological toxicity attributable to GEM injection, in non-tumor-bearing mice.

The treatment with GEM caused reduction of white blood cell count and hemoglobin content, respectively leading to leukopenia and anemia. Occurrence of leukopenia often induces infectious complications, which may compromise treatment efficacy. Opportunistic infections are a major cause of morbidity and mortality in cancer patients receiving myelotoxic chemotherapy, resulting from invasive fungal infections, particularly invasive aspergillosis, and an increasing spread of Gram-positive pathogens such as methicillin-resistant Staphylococcus aureus and vancomycin-resistant enterococci [23]. In current clinical practice, colony-stimulating factors such as granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) are increasingly used to recover white blood cell counts or increase dose-density [24]. In addition, hemoglobin reduction results in anemia, which is associated with a significant decrease in the quality of life and may limit the applicability and efficacy of anticancer drugs [25]. The treatment with recombinant human erythropoietin (rHu Epo) has been shown to improve anticancer drug-induced anemia in rats [26], and alleviating anemia with rHu Epo in humans has improved the quality of life of cancer patients [27].

Although G-CSF and GM-CSF are generally safe, well tolerated and have favorable outcomes, several reports of serious G-CSF and GM-CSF associated side effects exist, [28,29] including enhanced bone tumor growth by G-CSF in mice in an osteoclast-dependent manner [30]. Treatment with rHu EPO also has risks such as the potential to promote cellular proliferation and migration in melanoma and breast cancer cells expressing the Epo receptor [31,32]. AHCC exerted no influence on mRNA levels of GM-CSF and EPO in our study when the mRNA levels were measured. However, given the ameliorating effects of AHCC for GEM-associated BM cell viability, AHCC might be useful to complement the properties of G-CSF and GM-CSF as well as Epo. The beneficial effects of AHCC on hematoxicities caused by other anticancer drugs, such as cisplatin, paclitaxel, 5-fluorouracil and irinotecan, have been reported [16,19], although the mechanism of action is not yet clear.

AHCC supplementation was significantly associated with an improvement in the levels of colony forming unit granulocyte-macrophage (CFU-GM) and burst forming unit erythroid (BFU-E), which were severely depressed as a result of GEM treatment. AHCC might therefore alleviate chemotherapy-related hematological toxicity through protecting hematopoietic progenitor cells. This result is consistent with other studies demonstrating that Maitake β-glucans promoted bone marrow cell viability and protected the bone marrow stem cell colony formation unit from doxorubicin-induced hematological toxicity, [33] as well as induced hematopoietic stem cell proliferation and differentiation [34].

Despite the side effects, GEM may be a useful agent for tumor immunotherapy since it possesses significant immunomodulatory activity independent of its cytotoxic effects as shown in murine tumor models [35]. Other agents with potentially harsh side effects, such as cisplatin, have also been to increase the susceptibility of tumor cells to tumor-infiltrating lymphocytes or natural killer cells [36]. Therefore, AHCC may offer promise when used in conjunction with chemotherapy since AHCC may help reduce side effects of drugs like GEM or cisplatin and enable a full chemotherapeutic regimen to be administered. Furthermore, the previous study demonstrated that AHCC enhanced chemotherapeutic effect of cisplatin in tumor-bearing mice [16], suggesting an adjuvant action of AHCC.

The safety of AHCC in cancer patients and healthy volunteers has been previously reported [13,17,18,37]. The current and previous studies suggest that AHCC consumption may be safe in combination with GEM and perhaps other chemotherapy agents that are not metabolized via the CYP450 2D6 pathway [38] and clinical studies are warranted.

The present study was conducted to assess whether AHCC reduces GEM-induced side effects, particularly hematological toxicity that is a dose limiting factor for GEM, in non-tumor-bearing mice. As a consequence, AHCC significantly ameliorated reduction of peripheral total white blood cell count and hemoglobin content, and further resulted in recovering CFU-GM and BFU-E forming abilities. If these results are extended to humans, AHCC might contribute to improved quality of life and well-being of cancer patients undergoing chemotherapy including GEM treatment.

5. Acknowledgements

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