Enhancement of Umbilical Cord Blood Cell Hematopoiesis by Maitake Beta-Glucan Is Mediated by Granulocyte Colony-Stimulating Factor Production

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Maitake beta-glucan (MBG) is an extract from the fruit body of the Grifola frondosa mushroom that is being widely used to treat cancer in Asia. We have previously reported that MBG enhances mouse bone marrow cell (BMC) hematopoiesis in vitro and protects BMC from doxorubicin (DOX) toxicity. In the current study, we investigated the ability of MBG to enhance hematopoiesis and to reduce the toxic effects of DOX on fresh human umbilical cord blood (CB) cells. MBG treatment significantly enhanced the colony formation unit (CFU) response of granulocytes-macrophages (CFU-GM response) over the whole dose range of 12.5 to 100 μg/ml (P < 0.05). The addition of MBG to DOX-treated CB cells significantly protected granulocyte-macrophage colony formation from the toxicity of DOX, which otherwise produced strong hematopoietic repression. MBG also partially replaced recombinant human granulocyte colony-stimulating factor (rhG-CSF), as shown by a significant augmentation of the CFU-GM response in the absence of rhG-CSF. We found that MBG induces granulocyte colony-stimulating factor (G-CSF) production in CB CD33+ monocytes, as detected by intracellular cytokine flow cytometric assessment. In contrast, we found that adult peripheral blood monocytes did not produce a significant G-CSF response to MBG, whereas both adult and CB monocytes produced G-CSF in response to lipopolysaccharide. These studies provide the first evidence that MBG induces hematopoietic stem cell proliferation and differentiation of CFU-GM in umbilical CB cells and acts directly to induce G-CSF.

Maitake beta-glucan (MBG) is an extract from the fruit body of the Grifola frondosa mushroom that is widely used in Asia for the treatment of cancers, although the mechanism(s) of action is unclear (4). MBG contains glucan polysaccharide compounds that have a beta-1,3-linked glucose (24). The results of experimental studies with animals suggest that MBG administered orally activates the host antitumor response through effects on the immune system rather than by direct cytostatic or cytotoxic effects on tumor cells (19, 37). However, dose-response relationships have not been shown (15, 21). Since other beta-glucans have been found to reduce myelosuppression and to enhance hematopoiesis in vitro and the mobilization of stem cells in vivo in animal models (16, 27, 33), we initially tested the effects of MBG on mouse bone marrow cells. Those studies showed for the first time that MBG enhances murine bone marrow cell proliferation and differentiation into granulocytes-macrophages (GMs) in a dose-dependent manner (22). In the presence of the chemotherapy drug doxorubicin (DOX), MBG promoted bone marrow cell viability and protected the bone marrow stem cell colony formation unit response of GMs (CFU-GM response) from DOX-induced hematopoietic toxicity. On the basis of the results of our studies with the mouse, we are interested in the possible use of MBG for myelosuppression secondary to cancer chemotherapy and for ex vivo expansion of human umbilical cord blood (CB) cells prior to stem cell transplantation. CB cells are increasingly used for stem cell transplantation for the treatment of leukemia, and CB progenitor cells have distinctive immunological properties that enable ex vivo expansion (2, 3). The aims of this study were to assess the potential ability of MBG to enhance CB hematopoietic progenitor cell activity in a CFU-GM assay and to determine the mechanism of action. As reported here, we found that MBG enhanced human umbilical cord blood cell proliferation and differentiation into CFU-GM in vitro. MBG also protected the CB cell colony-forming potential from the damaging effects resulting from the toxicity of DOX. In the absence of granulocyte colony-stimulating factor (G-CSF), MBG enhanced the colony-forming abilities of CB cells over those of cells in control cultures. Examination of the effects of MBG on the cytokine response showed that MBG induced intracellular G-CSF production in CD33+ monocytes and that G-CSF was secreted. The studies show that MBG has specific dose-related effects on human hematopoietic progenitor cells, indicating the potential for the therapeutic use of MBG for myelosuppression. In addition, the results support the possibility that MBG could enhance the expansion of CB cells ex vivo to improve engraftment.

MATERIALS AND METHODS

Chemicals and reagents. MBG is an extract from the fruit body of the maitake mushroom (Grifola frondosa), which was made under patented methods (Japan patent no. 2659843/U.S. patent no. 5,854,404 [24a]) and provided by Yuikiguni Maitake Corp. through the Tradeworks group. The extract was stored in a
and nonactivated cells. FACSCalibur flow cytometer (BD Biosciences) and Cell Quest software. Three were resuspended in phosphate-buffered saline and analyzed immediately with a stimulating factor (GM-CSF) phycoerythrin (BD Biosciences) and anti-G-CSF cellular detection of cytokine included anti-granulocyte-macrophage colony-abilization solution and then incubated with monoclonal antibodies directed CD33 monoclonal antibodies used for cell surface staining were directly conju- bles, 30% fetal bovine serum, 1% bovine serum albumin, 10 mM KHCO₃ (pH 7.4 to 7.6). Buffered ammonium chloride solution was added to human umbilical cord blood at 10:1 (vol/vol). The cell suspension was vortexed gently and placed on ice for 20 min to allow the red blood cells to lyse. The cells were washed twice with Iscove’s modified Dulbecco’s medium (IMDM) and then resuspended in IMDM. The mononuclear cells were stained with trypsin and counted under a light microscope by using a hemocytometer, and the concentration of viable mononuclear cells was adjusted to 5 x 10⁵ cells/ml. Colony formation unit assay. The colony formation unit assay was carried out under defined conditions by standard techniques (StemCell Technologies Inc., Vancouver, British Columbia, Canada) (22). Brieﬂy, a complete culture medium was prepared by adding growth factors, rhG-CSF, rhIL-3, and IMDM into a premixed methylcellulose culture medium (Methocult H4230; StemCell Technologies Inc.), which contained methylcellulose, fetal bovine serum, and bovine serum albumin in IMDM. The ﬁnal adjusted concentrations were 1% methylcellulose, 30% fetal bovine serum, 1% bovine serum albumin, 10 mM M-2-mercaptoethanol, 2 mM 1-glutamine, 10 ng/ml rhIL-3, and 500 ng/ml rhG-CSF. For studies of determination of whether MBG could replace growth factors, either rhG-CSF or rhIL-3 was omitted. Aliquots of 0.3 ml of the cord blood cell suspension (5 x 10⁵ cells/ml) were added to each tube in 2.7 ml complete IMDM with methylcellulose, with the addition of MBG or DOX at the required concentration in a volume of 20 μl. The tubes were then vortexed, and the mixtures were plated in duplicate into sterile cell culture dishes (35 by 10 mm; Fisher Scientiﬁc International) at 1.1 ml/dish. Then, all cultures were incubated in a water-saturated incubator at 37°C with 5% CO₂. After 14 days of incubation, CFU-GM colonies consisting of 20 or more cells were identiﬁed and scored by using an inverted microscope. Intracellular cytokine assay. Intracellular cytokine production was assessed by standard techniques with fresh cord blood or whole blood from healthy adult donors. Blood was collected in sodium heparin Vacutainer tubes and was maintain- ed at room temperature. Cord blood or adult peripheral blood was diluted 1:2 with RPMI 1640 medium containing 25 mM HEPES buffer, 2 μg/ml glucose per ml, 40 IU penicillin, and 40 μg streptomycin per ml. Lipopolysaccharide (LPS) at 1 μg/ml, MBG at 100 μg/ml, or medium as a control was added in the presence of brefledin A (BFA; Sigma, St. Louis, MO) at a ﬁnal concentration of 10 μg/ml. After incubation in a water-saturated incubator at 37°C with 5% CO₂, the cells were removed from the culture and initially stained with monoclonal antibodies directed against lineage-speciﬁc surface antigens. The anti-human CD33 monoclonal antibodies used for cell surface staining were directly conju- gated to ﬂuorescein isothiocyanate (FITC; BD Biosciences, San Jose, CA). The cells were then permeabilized with a ﬂuorescence-activated cell sorter perme- abilization solution and then incubated with monoclonal antibodies directed toward the cytokines of interest. The monoclonal antibodies used for the intra- cellular detection of cytokine included anti-granulocyte-macrophage colony- stimulating factor (GM-CSF) phycoerythrin (BD Biosciences) and anti-G-CSF phycoerythrin (R&D Systems, Minneapolis, MN). After a ﬁnal wash, the cells were resuspended in phosphate-buffered saline and analyzed immediately with a FACScan Calibur flow cytometer (BD Biosciences) and Cell Quest software. Three hundred thousand total events were collected. Gating was established by using forward and side light scatter and ﬂuorescence backgating with CD33 to deﬁne the populations. Data were analyzed by use of a histogram overlay of activated and nonactivated cells. ELISA measurement of G-CSF. To test that the intracellular cytokine produc- tion detected by the intracellular assay tracked the level of secretion, parallel cultures were established as described above but were carried out in the absence of BFA. Blood was incubated with LPS at 1 μg/ml or MBG at 100 μg/ml for 18 h. After incubation, the supernatants were collected by centrifugation at 1,500 rpm for 10 min. The G-CSF levels in the supernatant were measured by the Quan- tikine high-sensitivity G-CSF immunoassay, obtained from R&D Systems. Brieﬂy, 100 μl of supernatant or each of the dilutions of the standard recombi- tant human interleukin-3 (rhIL-3) were obtained from Amgen (Thousand Oaks, CA) and Intergen (Purchase, NY), respectively. Human umbilical cord blood. Human umbilical cord blood samples from healthy full-term infants were obtained at the time of delivery and were trans- ferred into heparinized sterile blood collection tubes and studied while they were fresh. Peripheral blood samples from healthy adults were obtained by venipunc- ture and were studied while they were fresh. This study was approved by the institutional review board of the Cornell University Weill Medical College.

Preparation of umbilical cord blood cells. To prepare human umbilical cord blood cells for the colony formation assay, red blood cells were removed by lysis with ammonium chloride buffer containing 155 mM NH₄Cl, 0.1 mM EDTA, and 10 mM KHCO₃ (pH 7.4 to 7.6). Buffered ammonium chloride solution was added to human umbilical cord blood at 10:1 (vol/vol). The cell suspension was vortexed gently and placed on ice for 20 min to allow the red blood cells to lyse. The cells were washed twice with Iscove’s modiﬁed Dulbecco’s medium (IMDM) and then resuspended in IMDM. The mononuclear cells were stained with trypsin and counted under a light microscope by using a hemocytometer, and the concentration of viable mononuclear cells was adjusted to 5 x 10⁵ cells/ml.

Statistical analysis and data presentation. Data are presented as the mean percentage ± standard deviation (SD) or mean ± SD. The CFU-GM responses from duplicate cultures were averaged and collected to compile the group data. As each cord blood sample came from a genetically distinct host, we also nor- malized the data for each separate cord blood sample by using the average of duplicate control cultures as 100% and then comparing the average of duplicate experimental cultures for each subject to this standard. The data were then compiled from the individual results collected to form the group results. Statistical analysis was performed by a one-way analysis of variance and multiple comparisons with SPSS (version 12.01).

RESULTS

Effect of MBG on CFU-GM responses of umbilical cord blood cells. Umbilical cord blood samples were collected from healthy full-term infants after delivery. The red blood cells were removed by ammonium chloride lysis, and the mononu- clear cells were counted with a hemocytometer. Viability was determined by trypan dye exclusion and exceeded 90% for all samples. To study the effects of MBG on cord blood cell hematopoietic activity, cord blood cells from eight neonates were cultured at a ﬁnal concentration of 5 x 10³ per dish in duplicate in the presence or the absence of multiple concentra- tions of MBG in the methylcellulose complete culture me- dium. The actual number of colonies in these cultures ranged from 38 to 97 per plate in individual experiments. The values for duplicate cultures were within 10 to 15% of each other. To normalize the genetic distinctiveness of an infant’s cord blood sample, for each CB sample, the mean colony counts in control cultures with no MBG were considered a 100% response and were used to calculate the percentage of the difference from the control CFU-GM response. As shown in Fig. 1, MBG enhanced the colony-forming abilities of cord blood cells in the group when MBG was added at doses ranging from 12.5 to 100 μg/ml. The differences in colony counts compared with those of the control cultures for the group were signiﬁcantly greater when MBG was present at 12.5 μg/ml (P = 0.015), 25 μg/ml (P = 0.003), 50 μg/ml (P < 0.001), and 100 μg/ml (P < 0.001). The addition of MBG at 100 μg/ml increased the colony counts up to 140% of those for the controls. When we evaluated this difference using the actual mean number of CFU-GM colonies, we obtained essentially the same results (data not shown).

When MBG was added at 200 μg/ml, a slight but not signif- icant increase was seen compared to the ﬁndings for cultures to which MBG was not added. This was not due to any cytotoxic or cytotoxic effects. The addition of MBG at 200 μg/ml had no cytotoxic effect on the viabilities of the cord blood cells, which was tested by the 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-
[(phenylamino)carbonyl]-2H-tetrazolium hydroxide cytotoxicity assay (data are not shown) and which we also showed in our previous studies with mice (22).

Protective effect of MBG on cord blood CFU-GM responses in the presence of DOX. Experiments were then carried out to determine whether MBG was capable of protecting the proliferation and differentiation of progenitor CFU-GMs in cord blood cell samples from hematopoietic toxicity caused by DOX treatment. According to the findings presented in our previous report (22) and the findings of others (34), we used an appropriate dose range of doxorubicin (7.5 to 120 ng/ml) to detect suppressive effects. After the removal of red blood cells, cord blood cells from four infants were cultured as described above in methylcellulose medium containing rhIL-3 at 10 ng/ml and rhG-CSF at 500 ng/ml in the presence of serial dilutions of DOX (7.5 to 120 ng/ml) with or without MBG at 100 μg/ml. As shown in Fig. 2A, in the presence of a low dose of DOX (7.5 ng/ml), MBG was still able to significantly enhance the CFU-GM responses of CB cells over those of the cells in control cultures with no DOX present. The dose of MBG that elicited the maximum effect varied somewhat among individual cord blood samples, but overall, the effect was greatest at doses of 50 μg/ml (P = 0.018) and 100 μg/ml (P = 0.003). MBG was significantly protective against the toxicity of DOX for each CB sample. With high-dose DOX (120 ng/ml), the growth enhancement brought about by MBG fell to 33.3% of the growth for the control cultures. However, in the absence of MBG, treatment with 120 ng/ml DOX alone decreased the CFU-GM response to a nadir of 16% of the response for the controls. Thus, although the enhancing effect of the optimal concentration of MBG (100 μg/ml) on the CFU-GM response declined with increasing doses of DOX, the CFU-GM responses in the presence of MBG remained significantly higher than those with DOX alone across the entire range of DOX concentrations tested (P = 0.001). When we calculated the CFU-GM response as a percentage of the control response by using the CFU-GM responses to MBG at 100 μg per ml as the 100% control, we found essentially similar levels of protection and significance, as shown in Fig. 2B.

Effect of MBG on CFU-GM formation in the absence of rhG-CSF. Since in the previous experiments MBG appeared to induce the CFU-GM response, we next sought to determine if this effect was dependent upon the presence of G-CSF. To test this, four cord blood samples were collected and cultured in methylcellulose medium containing rhIL-3 at 10 ng/ml but no rhG-CSF. The colony counts obtained with various doses of MBG were compared with those of control cultures supplemented with both rhIL-3 (10 ng/ml) and rhG-CSF (500 ng/ml). As shown in Fig. 3, in the absence of rhG-CSF, the colony counts were reduced to 51.6% of those for the controls, while in the presence of MBG over the dose range of 25 μg/ml to 100 μg/ml, the CFU-GM response exceeded 80% of that for the controls. The results showed that statistically equivalent levels
of CFU-GM activity were observed when MBG was present and when control cells were cultured in medium with rhG-CSF. We separately carried out titration studies and determined that the optimal concentration of rhG-CSF for CFU-GM activity is 250 to 500 ng/ml. In contrast, MBG could not be used as a substitute for rhIL-3. In the absence of rhIL-3 with only rhG-CSF present, the colony counts decreased to 41.6% of those for the controls, and the addition of MBG over the same dose range did not improve the CFU-GM response (data not shown).

**MBG induction of intracellular G-CSF in CD33+ cord blood cells.** Experiments were undertaken to determine whether MBG induced granulocyte- or granulocyte-macrophage-stimulating growth factors in cord blood. Umbilical CB samples from 14 healthy full-term infants were collected and studied. Mononuclear cells were cultured with MBG for 4 h or 18 h in the presence of BFA, which disrupts the structure and function of the Golgi apparatus, inhibiting intracellular transport so that the cytokines produced during the culture period are not secreted. After 4 h of incubation, as shown in Fig. 4, the intracellular level of production of G-CSF in CD33+ cord blood cells was significantly higher than that in cultures without MBG ($P = 0.04$). At 4 h LPS did not show a significant effect on cord blood G-CSF production. To determine if this effect was specific to CB monocytes, we also obtained fresh peripheral blood samples from six adults. As shown in Fig. 5, after 18 h of culture, about 20% of the cord blood monocytes were producing G-CSF, whereas 10% of the monocytes in parallel control cultures were producing G-CSF ($P = 0.04$). In contrast, the level of induction of G-CSF in monocytes from adults at 18 h was not different from that of unstimulated cultures, although samples from a few adults showed a slight response. As shown in Fig. 5, in contrast to the results obtained at 4 h, the effect of LPS on the cord blood G-CSF response was stronger than that of MBG at 18 h. The CB monocyte response to LPS was greater than the adult monocyte response ($P = 0.04$). MBG did not induce intracellular GM-CSF production in CD33+ cord blood cells, although LPS did significantly induce GM-CSF production (data not shown).

**MBG induces secretion of G-CSF by cord blood CD33+ cells.** To determine whether intracellular cytokine detection...
DISCUSSION

The studies described here are the first to show that MBG induces the proliferation and differentiation of CFU-GM colonies from human umbilical cord blood progenitor cells in vitro and to demonstrate that MBG stimulates the intracellular production and secretion of G-CSF in cord blood CD33+ monocytes. We previously found that MBG stimulated hematopoiesis by murine bone marrow cells and enhanced the recovery of the CFU-GM response from DOX-induced hematopoietic suppression (22). In the present investigation we found a similar response over the same dose range, suggesting a fundamental similarity between mouse and human progenitor cell CFU-GM responses to MBG in vitro. As observed with murine bone marrow cells, the addition of MBG above the optimal range at 200 μg/ml showed only a modest effect on the colony-forming abilities of CB cells. This was not due to cytostatic or cytotoxic effects. Although a related maltite beta-glucan preparation, grifron-D, has been reported to have cytotoxic effects on PC-3 human prostate cells at a dose level of 480 μg/ml or greater (13), we did not find this effect using various tumor cell lines, including PC-3, with the MBG extract described here (H. Lin, unpublished data). Other studies of the antitumor effects of glucan extracts in the mouse appear to indicate the involvement of the host immune system (1, 12, 15, 42) rather than direct antitumor effects.

MBG is one of the fractions of the extract from the fruit body of Grifola frondosa which has shown the strongest antitumor effect (1, 17, 25). The MBG used in these studies was prepared under standardized conditions, has a molecular weight of 1,000,000, is highly soluble, and does not contain endotoxin. Membrane receptors that mediate the beta-glucan response include the murine dectin-1 receptor (5, 6), the human beta-glucan receptor (20, 40, 41), CR3 (39), the scavenger receptors (35), and lactosylceramide (43). Activation of both dectin-1 and beta-glucan receptor may lead to inflammatory cytokine production (10, 11). Dectin-1 expression has been shown to enhance Toll-like receptor-mediated activation of nuclear factor kappa B by beta-glucan-containing particles. In macrophages and dendritic cells, dectin-1 and Toll-like receptors are synergistic for the production of interleukin-12 and tumor necrosis factor alpha (14, 26). For orally active glucans, the mechanism of beta-glucan action has been shown to involve monocyte degradation of glucan into smaller fragments in the bone marrow prior to uptake by effector cells (18). A soluble barley CR3-specific beta-glucan has been shown to induce a primed state of CR3 that triggered the killing of iC3b target cells that were otherwise resistant to cytotoxicity (36) to enhance antitumor immunity (8, 23).

Other glucans have previously been shown to enhance hematopoiesis in vitro and the mobilization of hematopoietic cells in vivo. By using a partially purified glucan derived from zymosan, the authors of the first report showed that injection caused increased CFU-GM responses after 10 days in the spleen and to a much lesser extent in the bone marrow (7). Patchen and Lotzova studied glucan from the yeast Saccharomyces cerevisiae and reported that glucan mobilized progenitor cells to the spleen and periphery (28), increasing hematopoiesis in the spleen and increasing pluripotent stem cell activity in the bone marrow (30). The effects on splenic hematopoiesis were macrophage dependent, while the mobilization effect was not (29). Intravenously administered glucan was also found to improve the survival of sublethally irradiated recipient mice when it was given 1 day before or after radiotherapy (31), and the effect of giving both glucan and G-CSF was synergistic (32).

Recent studies have shown that after hematopoietic injury or G-CSF mobilization, stroma cells express iC3b and tether CR3+ hematopoietic progenitor cells to the spleen and periphery (28), increasing hematopoiesis in the spleen and increasing pluripotent stem cell activity in the bone marrow (30). The effects on splenic hematopoiesis were macrophage dependent, while the mobilization effect was not (29). Intravenously administered glucan was also found to improve the survival of sublethally irradiated recipient mice when it was given 1 day before or after radiotherapy (31), and the effect of giving both glucan and G-CSF was synergistic (32).

In the present study we show that MBG has significant hematopoietic potential to promote the proliferation and differentiation of progenitor stem cells in cord blood. MBG has
been shown to activate the production of G-CSF in CB CD34+ monocytes for the first time. Early induction of G-CSF in cord blood by MBG in CB monocytes appears to be the mechanism of CFU-GM enhancement and may reflect the unique hematopoietic potential of this compartment. Further investigation of enhancement of MBG on other lines, such as the CFU-megakaryocyte, burst-forming unit-erythroid, and long-term culture-initiating cell lines, as well as the signaling pathway involved in the induction of G-CSF elicited by MBG, may reveal fundamental information concerning the regulation of the accessory cord blood cells important for ex vivo expansion of CB progenitor and pluripotent stem cells for transplantation and the role of beta-glucan in the enhancement of hematopoiesis.

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