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Report

Isolation and characterization of an active compound from black soybean [*Glycine max* (L.) Merr.] and its effect on proliferation and differentiation of human leukemic U937 cells

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Black soybean [*Glycine max* (L.) Merr.] has been used as a health food and herb in China for hundreds of years. In the present study, we purified a unique polysaccharide component from black soybean (PSBS) and found that it indirectly inhibits proliferation and induces differentiation of human leukemic U937 cells via activation of mononuclear cells (MNCs). We prepared conditioned media (MNC-CM) by incubating MNCs from human peripheral blood with or without PSBS (PSBS-MNC-CM and normal MNC-CM, respectively). Treatment of human leukemic U937 cells with PSBS-MNC-CM significantly inhibited proliferation of U937 cells, reducing their growth by 98.5%. Furthermore, PSBS-MNC-CM induced U937 cells to differentiate into mature monocytes/macrophages (83% by morphological examination and 90% by the nitroblue tetrazolium test). Neither PSBS alone nor normal MNC-CM had such effects. The molecular weight of PSBS was about 480 000 by gel filtration. Structural analysis of PSBS revealed that (1,6)-α-D-glucan might be its major active component. Our results suggest that the PSBS may inhibit proliferation and induce differentiation in human leukemic U937 cells by activating the immune response of MNCs. [© 2001 Lippincott Williams & Wilkins.]

Key words: α-Glucan, black soybean polysaccharide, immunomodulation, leukemic U937 cell, mononuclear cell conditioned media.

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Introduction

Black soybean [*Glycine max* (L.) Merr.] (BS), a soybean cultivar with a black seed coat, has been used for hundreds of years as a detoxifier, anti-inflammatory and blood nutrient in traditional Chinese medicine. It has been reported that soybean contains several important components with various physiological and biological activities. For example, high-dose soy daidzein enhances the macrophage phagocytic response and several other immune functions,[1] soybean isoflavones and phytochemicals inhibit the growth of transplanted human prostate carcinoma and tumor angiogenesis in mice,[2] soybean agglutinin (SBA) lectin induces a local inflammatory reaction but has an anti-inflammatory effect when present in circulating blood,[3] soybean saponins at 150–600 p.p.m. inhibit growth of human carcinoma cells HCT-15,[4] and soybean oil provides linoleic acid for maintaining immune responses.[5] Taken together, these reports indicate that soybean is capable of modulating immune responses, preventing carcinogenesis and inhibiting tumor growth.

Although BS was thought to be similar to soybean, for some reason soybeans with black seed coats were specifically selected for disease treatment and health maintenance in traditional Chinese medicine.[6] In fact, Yang *et al*. have proven that BS possesses higher antioxidative activity than regular soybean.[7] However, the active component and anti-tumor mechanism of BS have not been delineated.

In the present study, we used serial extraction and purification to isolate the active component of BS. Then, we treated the leukemic cell line U937 with BS

fractions alone or BS-treated mononuclear cell conditioned media (MNC-CMs) to look for anti-tumor activity. We determined the chemical structure of the active compound and analyzed the glycosidic linkages that might be responsible for its effects.

Materials and methods

Isolation of polysaccharide from BS (PSBS) and estimation of molecular weight

The procedures for isolating PSBS are outlined in Figure 1. BS (500 g) was shredded and extracted with dichloromethane (CH_2Cl_2), followed by ethanol (EtOH) and then by water at 70°C. U937 cells were treated by adding extracts alone or MNC-CM which were prepared with various fractions as described below. None of the pure extracts had a direct anti-tumor effect (data not shown). The water extract combined with MNC-CM did inhibit U937 cell growth, however. Therefore, we further separated the water extract by liquid chromatography with Sephadex LH-20 (Amersham Pharmacia Biotech, Little Chalfont, UK) and then Fractogel TSK HW-55F (Merck, Darmstad, Germany) columns. An active fraction, denoted pre-PSBS, was deproteinized with proteinase K (50 μg/ml; Sigma, St Louis, MO) at 37°C for 1 h. Finally, gel filtration using a Sephacryl S-400 column (Amersham Pharmacia Biotech) was performed to yield an active polysaccharide compound (PSBS). Its molecular weight was estimated using standard dextran (Amersham Pharmacia Biotech) for calibration.[8,9] Total carbohydrate determination was performed by a phenol–sulfuric acid colorimeric assay using glucose as a standard.[10]

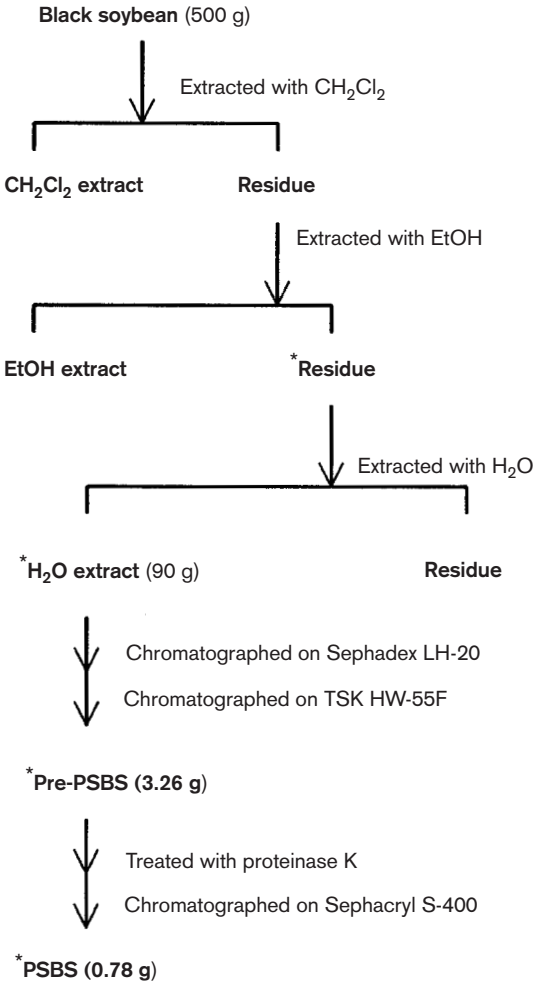

Figure 1. The procedure for BS extraction and purification. At each step, MNC-CM were prepared to determine fractions capable of anti-tumor activity. The active fractions are labeled with asterisks. The final yield of PSBS from BS was 0.16%.

Analysis of structural characteristics

The specific rotation of PSBS was detected on a JASCO DIP-370 digital polarimeter (JASCO, Tokyo, Japan) in water at 24°C with a sodium lamp at 589 nm. To determine the monosaccharide composition, PSBS was hydrolyzed with 2N trifluoroacetic acid (Merck) for 6 h at 100°C and subjected to anion-exchange chromatography using a Dionex DX-500 chromatography system with a CarboPac™ MA-1 column (Dionex, Sunnyvale, CA).[11] ^{1}H- and ^{13}C-NMR spectra were recorded by a Bruker AVANCE-400/500 spectrometer operated in a pulsed flourier transform mode. Gas liquid chromatography–mass spectroscopy (GLC-MS) was performed on an HP 5973 MSD with a HP 6890 series GC system (HP, Wilmington, DE) to analyze the sugar composition and linkage of PSBS.

Preparation of MNC-CM

Human peripheral blood from healthy donors 20–30 years old were collected after documentation with informed consent of each subject. The MNCs from blood samples were separated by centrifugation on a density gradient (Ficoll-Hypaque, 1.077 g/ml; Pharmacia Fine Chemicals, Wiksträmms, Sweden). Cells were cultured at a concentration of 1.5×10^6 cells/ml in 10% FCS-containing RPMI 1640 medium with various concentrations of PSBS (0, 25, 50, 100, 200 and 400 μg/ml) at 37°C in a fully humidified incubator with 5% CO_2. After 24 h, the MNC-CMs were collected, sterilized by filtration and then stored at −70°C until use.[12] Phytohemagglutinin P (PHA, 10 μg/ml; Difco, Detroit, MI) was also used to prepare MNC-CM (PHA-MNC-CM) for a positive control.

Cell proliferation

The human myeloid leukemic cell line, U937, obtained from the ATCC (Rockville, MD) was used in this study. The cells were cultured in RPMI 1640 medium supplemented with 10% FCS and maintained at 37°C in a humidified 5% CO_2 incubator. We cultured 1×10^5 cells/ml in the presence or absence of 30% (v/v) of normal MNC-CM (N-MNC-CM), PSBS (25–400 μg/ml)-MNC-CMs or PSBS (25–400 μg/ml) alone. After 5 days of incubation, cells were collected by gently rubbing the dishes with a rubber policeman (Bellco Glass, Vineland, NJ) and the number of viable cells was counted using the Trypan blue dye exclusion test.

Maturation profile

After 5 days of the various treatments, the cells were collected and cytocentrifuged onto a microscope slide using a Cytospin[2] (Shandon Southern Instrument, Pittsburgh, PA), stained with Wright's stain and observed under an inverted microscope (Olympus, Melville, NY) with a magnification of × 1000. Based on morphology, the cells were classified as: (i) immature blasts, (ii) intermediates or (iii) mature monocytes or macrophages.[13]

Assay for superoxide

The production of cytoplasmic superoxide by differentiated myeloid cells was detected by the nitroblue tetrazolium (NBT) reduction test.[14] Cells collected from day 5 cultures were suspended in RPMI 1640 medium at a concentration of 1×10^6 cells/ml and incubated for 30 min at 37°C with an equal volume of NBT test stock solution (containing 2 mg NBT and 1 μM phorbol myristate acetate/ml PBS). Cytospin preparations were counter-stained with 0.5% Safranin O. The percentage of formazan-containing cells (out of 200 cells) was assessed microscopically.

Results

Yield of PSBS

As shown in Figure 1, 500 g of BS produced about 0.78 g of PSBS, for a final yield 0.16%. The active fraction 5, denoted as PSBS, had an estimated molecular weight of approximately 480 000 (Figure 2).

Structural analysis of PSBS

The monosaccharide composition of PSBS was 5% galactose, 6% mannose and 89% glucose. The specific rotation was $[\alpha]_D$+122.2°, c=0.45, in water. It dissolved in water to form a viscous solution. The ^{1}H- and ^{13}C-NMR spectra of PSBS are shown in Figure 3, with comparison with signals of known compounds. The spectra showed a characteristic major α-configuration composed of glucose. After methylation and acid hydrolysis of PSBS, the alditol acetate derivatives were analyzed by GLC-MS. As shown in Table 1, several methyl-hexose forms were detected, with 2,3,4-tri-*O*-methyl-glucose being the major component (about 76%). These results suggest that PSBS is essentially composed of a backbone of α-(1,6)-linked glucosidic residues with galactose or mannose residues substituted at C3 or C4 positions by branches with (1,3) or (1,4) linkages.

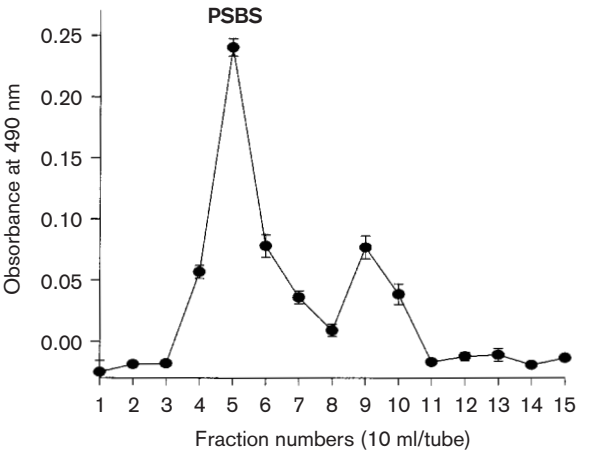

Figure 2. Optical density by phenol–sulfuric acid colorimetric assay (at 490 nm) of gel filtration fractions of pre-PSBS eluted by a Sephacryl S-400 column.

Growth inhibition of human leukemic U937 cells

As demonstrated in Figure 4(A), the proliferation of U937 cells was significantly inhibited by PSBS-MNC-CM. The growth inhibition increased (up to 98.5 ± 0.4%) with increasing concentrations of PSBS. The positive control PHA (10 μg/ml)-MNC-CM inhibited 53.8 ± 8.1% cell growth. However, no significant inhibition ($p > 0.05$ as compared with untreated control) was observed in normal MNC-CM or PSBS (up to 400 μg/ml) alone (Figure 4A and B).

Induction of differentiation

As demonstrated in Figure 5, PSBS-MNC-CM triggered differentiation of immature blast cells into mature monocytes and macrophages. The percentage of blast

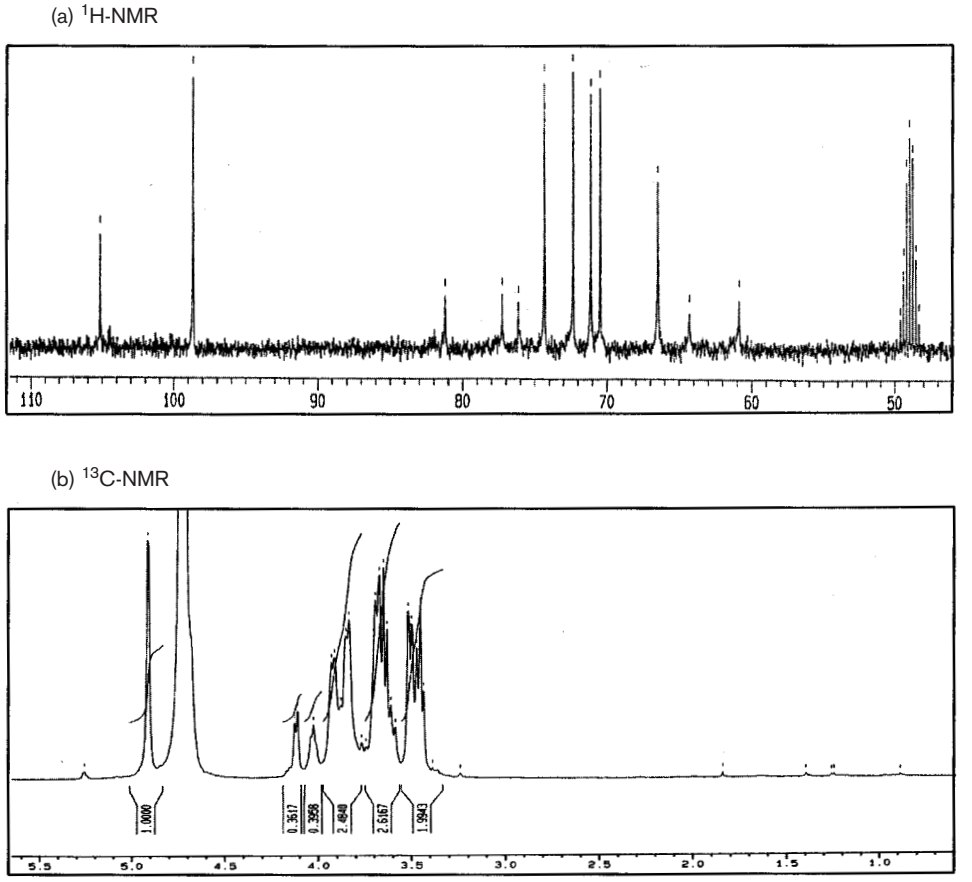

Figure 3. ^{1}H- and ^{13}C-NMR spectra of PSBS recorded on a Bruker ANOVANCE-400/500 fourier transform nuclear magnetic resonance spectrometer using HOD (4.8 p.p.m.) and methanol-d4 (49.15 p.p.m.) as the internal standard.

Table 1. GLC and GLC-MS data for partially methylated alditol acetates

Methylated sugar	Retention time (min)	Primary mass fragments (*m/z*)	Mode of linkage
2,3,4,6-tetra-*O*-methyl-hexose	15.15/15.45	205,118,161,162	(terminal hexose)1→
2,3,4-tri*O*-methyl-hexose/Glc	16.77	233,118,189,162	→6(hexose/Glc)1→
2,3,6-tri-*O*-methyl-hexose	16.50	233,118	→4(hexose)1→
2,3-di-*O*-methyl-hexose	17.77	261,118	→4,6(hexose)1→
2,4-di-*O*-methyl-hexose	17.92	305,118,189,234	→3,6(hexose)1→
2-*O*-methyl-hexose	18.61	333,118	→3,4,6(hexose)1→

cells decreased dramatically from $98.7 \pm 0.9\%$ (untreated control) to $2.3 \pm 1.5\%$ (MNC-CM prepared with 400 μg/ml of PSBS). In contrast, the percentage of monocytes and macrophages increased from $0 \pm 0\%$ (untreated control) to $83.0 \pm 4.0\%$ (PSBS-MNC-CM).

Changes in superoxide production

Immature blast cells had little superoxide production. Treatment with PSBS-MNC-CM resulted in a marked increase in the percentage of superoxide-producing cells (up to $97.0 \pm 3.0\%$) (Figure 6). This occurred in a dose-dependent manner. However, neither PSBS alone nor normal MNC-CM had this effect.

Discussion

In the present study we purified a unique polysaccharide (PSBS) from BS and demonstrated that this compound markedly inhibits growth of human leukemic U937 cells inhibition and induces differentiation.

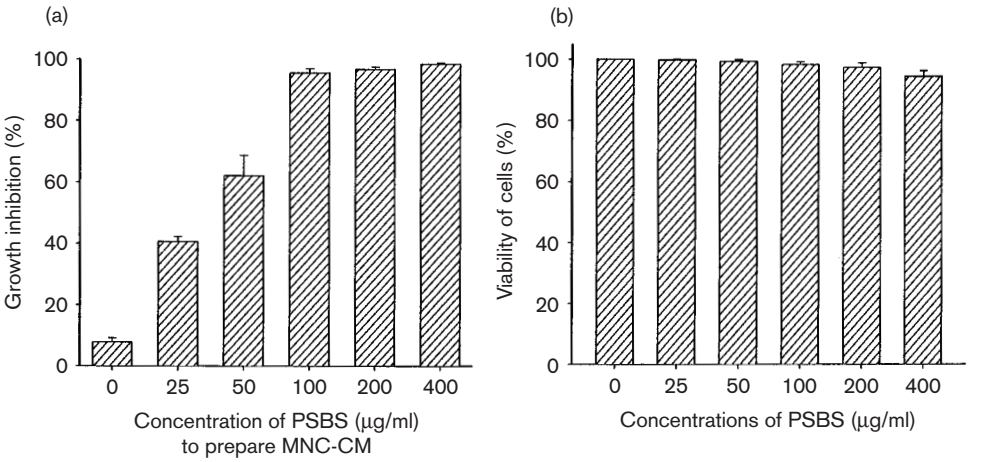

Figure 4. Changes in U937 cell growth after 5 days of incubation with 30% PSBS-MNC-CM or PSBS alone. (a) Growth inhibition by MNC-CM. (b) Viability of cells with PSBS treatment.

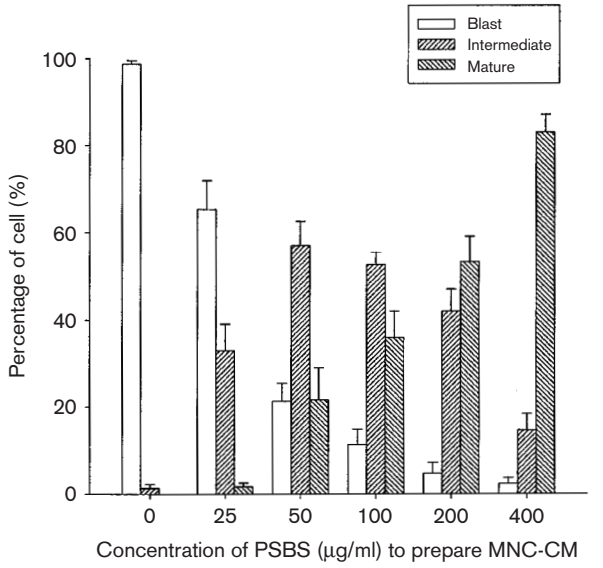

Figure 5. Change of morphology of U937 cells induced by PSBS-MNC-CM in day 5 cultures.

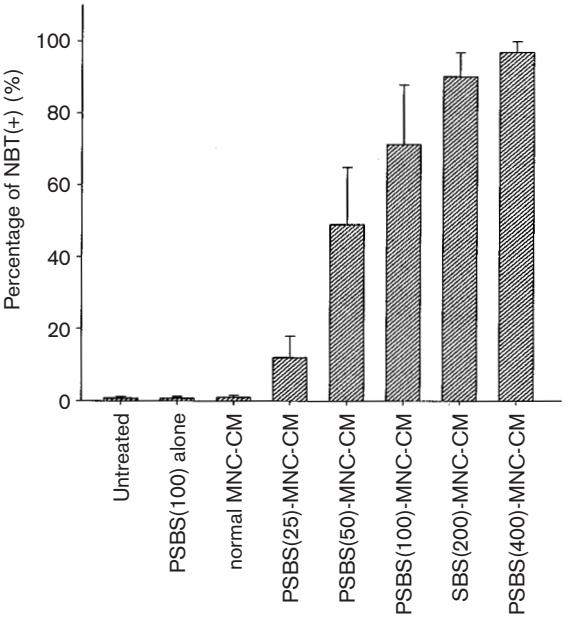

Figure 6. Analysis of NBT reduction for the production of superoxide by U937 cells induced by PSBS-MNC-CM after 5 days of incubation.

Our results showed that PSBS is safe for cultured spleen cells because there was no cytotoxicity observed even at higher concentrations. This anti-tumor and immunomodulating effect without cytotoxicity means that PSBS may be more suitable for clinical application in cancer patients than traditional cytotoxic chemotherapy.[15,16]

There are two ways for a natural product to act on leukemic cells, either by direct inhibition of cell proliferation or by stimulating the secretion of differentiation-inducing factors from immunocompetent cells.[17] In this study we demonstrated that the anti-tumor activity induced by BSPS was due to stimulation of immunomodulating response rather than direct inhibition of proliferation in leukemic U937 cells. Treatment of U937 cells with PSBS-MNC-CM resulted in a marked inhibition of proliferation and increase of mature monocytic functions, suggesting there may be mediators produced by MNC capable of triggering U937 cells to differentiate into mature, functional cells.

The carbohydrate characteristics of PSBS, including water solubility and heat stability, may make BS

particularly suitable as a health-promoting food. Previous studies of polysaccharides from *Ganoderma lucidum*,[18] *Grifola umbellata*[19] and *Cordyceps ophioglossides*[20] have shown that (1,3)- and (1,6)-β-D-glucan are responsible for their immunomodulating activities. However, the (1,6)-α-linked glucan isolated from rice bran has been also demonstrated to stimulate antitumor immunity.[21,22] PSBS has a large proportion of (1,6)-α-D-glucan, which may be responsible for its antitumor activity. In addition, these polysaccharides were known to have a strong stimulatory effect both on macrophages and T lymphocytes in promoting the release of various cytokines, including interleukin (IL)-1β, IL-6, tumor necrosis factor-α and interferon-γ.[12,13] We suggest that PSBS may stimulate MNC to secrete cytokines capable of affecting growth and differentiation.

In summary, (1,6)-α-D-glucan-rich PSBS stimulation of MNC both inhibits proliferation and induces differentiation of human leukemic U937 cells. We are currently conducting an *in vivo* study using a tumor implantation model to observe the effects of PSBS on tumor growth and host immune response after myelosuppressive chemotherapy.

Conclusion

The α-(1,6)-rich-glucan PSBS, as a biological response modifier, can inhibit proliferation and induce differentiation in human leukemic U937 cells through immunopotentiating effect.

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