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Antitumor effect of soybean lectin mediated through reactive oxygen species-dependent pathway

Prashanta Kumar Panda [a], Subhadip Mukhopadhyay [a], Birendra Behera [b], Chandra Sekhar Bhol [a], Sandeep Dey [a], Durgesh Nandini Das [a], Niharika Sinha [a], Akalabya Bissoyi [c], Krishna Pramanik [c], Tapas K. Maiti [b], Sujit K. Bhutia [a,*]

[a] Department of Life Science, National Institute of Technology, Rourkela, Odisha, India
[b] Department of Biotechnology, Indian Institute of Technology, Kharagpur, West Bengal, India
[c] Department of Biotechnology & Medical Engineering, National Institute of Technology, Rourkela, Odisha, India

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ABSTRACT

Aims: The present study evaluated the potential role of soybean lectin's (SBL) anticancer effect in vitro in different cancer cell lines and the therapeutic effectiveness in vivo in Dalton's lymphoma (DL) bearing mice model.
Main methods: The effect of SBL on cell growth and viability was measured using MTT assay in different cancer cells in vitro. Apoptosis, autophagic cell death, DNA-damaging potential and reactive oxygen species (ROS) were analyzed in HeLa cells. The in vivo efficacy of SBL was demonstrated in Dalton's lymphoma (DL) bearing mice.
Key findings: SBL demonstrated clear, strong antiproliferative activity without affecting normal cells; however, heat denaturation of SBL diminished the antiproliferative efficacy of molecule as demonstrated by MTT assay. A sharp 74.51 ± 3.5% and 82.95 ± 5.8% inhibition of tumor cell proliferation in DL mice occurred when SBL was administered at a dosage of 1 and 2 mg/kg body weight (i.p.), respectively, for ten days with the induction of autophagic and apoptotic cell death. An in vitro investigation revealed that SBL-mediated autophagy, apoptosis and DNA damage in HeLa cells were inflicted through the generation of ROS in a dose-dependent manner. Interestingly, pre-treating HeLa cells with N-acetylcysteine (NAC), a typical ROS scavenger, led to a noticeable reduction in SBL-induced autophagy, apoptosis and DNA-damaging activities, suggesting that SBL's antitumor potential was governed by ROS activation.
Significance: In this study, we evaluated the apoptotic, autophagic death, and DNA-damaging effects of SBL in cancer cells, which may have the potential to be used as a phyto-derived protein for cancer therapy.

Introduction

Cancer is known to comprise a deadly array of many diseases in which tissues grow and spread throughout the body, ultimately leading to death, and it is manifested by various hallmarks such as high proliferative signal, resistance to cell death, avoidance of growth suppressors, a high replication potential and sustained angiogenesis along with activation of tissue invasion and metastasis, as well as the avoidance of immune attacks (Hanahan and Weinberg, 2011). Although surgery, chemotherapy, and radiation are typically used for cancer treatment, they are often not effective and are associated with numerous side effects. According to the recommendations from the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR), a plant-based diet is necessary to reduce the risk of developing cancer (de Mejia et al., 2003). This association is based on the role of plant lectins to act as a patient-tailored cancer cell-directed therapeutic approach, a process that is thought to be more effective than current drug therapies or other available combination-based treatment regimens. Moreover, plant lectins could be the most promising potential candidates because they have few side effects and are widely available (de Mejía and Prisecaru, 2005; Liu et al., 2010; Fu et al., 2011).

Lectins are ubiquitous proteins, or glycoproteins, that are non-immunoglobulin in nature and contain at least one non-catalytic domain that binds reversibly to specific carbohydrates without altering their structure. Soybean lectin (SBL) is a legume lectin isolated from the seeds of soybeans (*Glycin max* L.). SBL shows specificity towards N-acetyl-D-galactosamine and, to a lesser extent, towards D-galactose (Pereira et al., 1974). SBL, one of the best-characterized lectin, is a tetramer consisting of four 30 kDa subunits with two saccharide binding sites per 120 kDa (Lis and Sharon, 1973; Lotan et al., 1974). Evidence indicates that this lectin has a role in recognition between different types

* Corresponding author at: Department of Life Science, National Institute of Technology Rourkela, Rourkela 769008, Odisha, India. Tel.: +91 6612462686; fax: +91 661 2472926.
E-mail addresses: sbhutia@nitrkl.ac.in, bhutiask@gmail.com (S.K. Bhutia).

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of cells and/or various carbohydrate-containing molecules involved in the regulation of physiological functions. SBL has been used as a diagnostic component in cancer treatment combined with anticancer drugs and in the removal of tumor cells from bone marrow, as well as in targeted drug delivery treatments (de Mejia et al., 2003; Rossi et al., 2003; Gupta et al., 2012; Bayraktar et al., 2013). Moreover, a dimeric 50 kDa melibiose-binding lectin from the seeds of the *Glycine max* soybean cultivar, commonly called the small glossy black soybean, demonstrated anticancer activity towards breast cancer (MCF7) and hepatoma (HepG2) with an IC_{50} of 2.6 μM and 4.1 μM, respectively (Lin et al., 2008). In this study, we evaluated the mechanistic aspects of SBL, highlighting its autophagic, apoptotic, and DNA-damaging effects in cancer cells in vitro and in vivo. In addition, we report that the ROS generated by SBL was found to modulate DNA damage, autophagic and apoptotic death.

Material and methods

Reagents

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), dimethylsulfoxide (DMSO), propidium iodide (PI), annexin V, N-acetyl-L-cysteine (NAC), and agarose were purchased from Sigma Aldrich, USA. Fetal bovine serum (sterile-filtered, South American origin), minimal essential medium (MEM), Dulbecco's minimal essential medium (DMEM), antibiotic-antimycotic (100×) solution, and Lipofectamine 2000® were purchased from Invitrogen, USA. Cyclophosphamide (Ledoxans) was obtained from Dabur Pharma Limited, New Delhi. Typed human blood cells were collected from healthy volunteer donors. Sephadex-G-100® and GeneRuler 1 kb DNA Ladder were obtained from GE healthcare, USA and Thermo Scientific, USA, respectively. Frosted-end microscope slides were purchased from Fisher Scientific, USA. All other reagents used in the experiments were of analytical grade or the highest quality available.

Isolation and characterization of soybean lectin

SBL isolation was carried out according to a previously described method (Hegde et al., 1991). In brief, the *G. max* seed kernel was extracted with 20% to 90% ammonium sulfate precipitation followed by affinity chromatography performed in a GE AKTA Prime Plus liquid chromatography system using a lactamyl Sephadex-G-100 affinity column. A 1 M solution of lactose was used to elute the protein of interest from the column. The eluted fraction was dialyzed overnight against 10 mM PBS (pH-7.4) at 4 ºC, and the samples were then lyophilized. The yield of isolated SBL was found to be 30 ± 4 mg from 100 g of seed kernel. Protein characterization was carried out using native and SDS PAGE followed by silver staining. We then analyzed the lectin activity using a hemagglutination assay (HA), which was performed to measure the lectin activity with the help of various human blood groups. The hemagglutination titer was measured after 2 h from the end point dilution (Hegde et al., 1991).

Cell lines

HeLa (cervical cancer), Hep2 (oral cancer), U373 MG (glioblastoma), and HepG2 (liver cancer) cells were cultured in Modified Eagle medium (MEM) and supplemented with antibiotic-antimycotic and 10% fetal bovine serum. MDA MB 231 (breast cancer) and HaCaT (human keratinocyte cell line) were cultured in Dulbecco's modified Eagle medium (DMEM) using similar supplements. All the cell lines were procured from the National Centre for Cell Science, Pune, India.

Antiproliferative activity determination on tumor cell lines

Cells from the logarithmic phase were maintained in culture after they were counted in a hemocytometer using trypan blue solution. The cell concentration was adjusted to 5×10^4 cells/ml, and the cells were then incubated with various concentrations of SBL in a 96-well plate. The efficacy of SBL on the viability of various cancer cell lines was determined using MTT dye reduction assay by measuring the optical density at 595 nm using a micro-plate reader spectrophotometer (Perkin-Elmer) (Bhutia et al., 2008). The control group was treated with PBS that was used to dissolve SBL.

Heat denaturation of SBL

A heat denaturation process of a 1 mg/ml solution of SBL in 10 mM PBS (pH 7.4) was carried out at 50 °C for 30 min followed by boiling at 100 °C in a water bath for 2 min as previously reported (Ghosh and Maiti, 2007). Heat-denatured SBL was made to flow through lactamyl Sephadex-G-100 affinity chromatography. The column-unbound denatured proteins were then collected and assayed for any anticancer activity.

Assay for clonogenic survival in HeLa cells

The effect of SBL action on the multiplicative potential of HeLa cells was assessed using colony formation assay. Cells were exposed to different concentrations of SBL for 24 h and were then collected using trypsinization. After being counted, the cells were replated in triplicates on a 6-well tissue culture plate containing 3000 cells/well followed by 14 days of incubation during which the growth medium was changed every 3 days. The cells were stained with 0.5% crystal violet (in methanol/water, 1:1), and the colonies were counted (Bhutia et al., 2008).

Reactive oxygen species (ROS) measurement

To detect reactive oxygen species (ROS), HeLa cells were treated with SBL for 24 h and incubated with 2.5 μg/ml Dihydrorhodamine 123 (Dhr123) in PBS for 30 min in a CO_2 incubator. Dhr123 is rapidly taken up by cells and is converted to rhodamine 123 in the presence of ROS. The cells were harvested and suspended in PBS, and ROS generation was measured by the fluorescence intensity (FL-1, 530 nm) of 50,000 cells (Bhutia et al., 2008).

4′6-Diamidino-2-phenylindole dihydrochloride (DAPI) staining both in vitro and in vivo cells

DAPI staining was performed to observe the morphological changes in nuclei both in the control and treated groups as previously reported (Bhutia et al., 2009). In brief, cancer cells for both the untreated and treated groups were smeared on a clean glass slide, and then the cells were fixed with 3.7% formaldehyde for 15 min, permeabilized with 0.1% Triton X-100 and stained with 1 μg/ml DAPI for 5 min at 37 °C. The cells were then washed with PBS two times and examined by fluorescence microscopy (Olympus IX 71) using Cell Sens Standard software.

DNA fragmentation assay in SBL-treated cells

DNA fragmentation was monitored by following an improved non-enzymatic DNA ladder assay in accordance with the procedure described by Suman et al. (2012). In short, HeLa cells (2×10^6) were seeded in a 60 mm petri-plate and treated with different doses of SBL. After a 24 h of exposure, cells were dislodged and pelleted down followed by the addition of 100 μl DMSO and mixed well via the combination of equal volumes of Tris-EDTA buffer (pH 7.4) and 2% SDS. The mixture was mixed and centrifuged at 12,000 ×*g* at 4 °C to collect 40 μl from