ORIGINAL ARTICLES

In Vitro Study Of A Bacterial Polysaccharides Anti Cancer

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ABSTRACT

The aim of this study was to evaluate in vitro anti-cancerous and immunomodulatory potential of the purified bacterial polysaccharide (Bacillus subtilis sulphated levan; BSL). The antipromotion and anti-progression activities of BSL were assayed through a series of tests using cell line (Hep-G2 cells) and RAW macrophage 264.7 cells. Evaluation of different macrophage functions which include macrophage proliferation rate and levels of nitrous oxide (NO), Tumor necrosis factor-α (TNF-α) and Cyclooxygenase-2 (COX-2) generated from lipopolysaccharide (LPS) stimulated RAW macrophage 264.7 cells. Estimation of DNA fragmentation and cytotoxicity against human tumor cell line (Hep-G2 cells) illustrated its anti-progression activity. Statistical results showed that BSL inhibited NO production from LPS-stimulated macrophages and increased macrophages proliferation rate. BSL could be recognized as a significant COX-2 and TNF-α inhibitor. So it is evident that BSL is a potent anti-inflammatory agent. Furthermore, BSL was a significant DNA fragmentation inhibitor and its cytotoxicity assessed against Hep-G2 cells demonstrated its anti-progression activity. Conclusion: BSL proved to be a potent antipromotion and antiprogession drug against cancerous cells.

Key words: Macrophage, immunomodulatory properties, hepatocellular carcinoma, carcinogenesis

Introduction

Liver cancer is the fifth most common cancer in men and the eighth most common in women (American Cancer Society, 2007). Carcinogenesis can be broken down into three stages: initiation, promotion, and progression. The initiation phase involves exposure to a mutagen and often requires its subsequent metabolic transformation into a biologically active form. Promotion phase involves stimulation of cell division and result in the formation of small, benign tumors. Progression to malignancy occurs when the tight controls that normally govern cell cycle progression break down, resulting in the uncontrolled proliferation of cancerous cells. It also involves the ability of these cells to invade surrounding tissue and to eventually metastasize (Lee et al., 2005). The modulation of the host immune system attributed to polysaccharides, is likely to affect primarily the promotion and progression stages (Chakraborty, 2007).

Bacillus subtilis is a rod-shaped, Gram-positive, non pathogenic bacterium that forms spores (Piggot, 2009). It is one of the most investigated microbial groups, because they can produce a varieties of biotechnological interesting substances (Schallmey et al., 2004). It is known to secrete several proteases during the fermentation process (Rao et al., 1998). It produces levan from fermented soybean mucilage (Han, 1990). The polysaccharide levan (polyfructose) has previously been shown to exert an inhibitory effect on the growth of several murine tumors. This activity is mediated by a host reaction, involving mainly macrophages but also other elements of the immune system (Leibovici et al., 1986; Meng et al., 2003), so Bacillus subtilis sulphated levan (BSL) was chosen. Commercial interest in the production of levan has intensified in recent years (Vina et al., 1998). Levan has some potential pharmaceutical applications owing to its anticarcinogenic and hypocholesterolemic properties (Kim et al., 1998). The aim of this study was to evaluate potential anti-cancer and immunomodulatory properties of BSL.

2.Materials and Methods

2.1.Materials:

2.1.1.Levan Sedimentation from Culture Liquid:
Levan was isolated from culture filtrate of *Bacillus subtilis* after the stage of fermentation using sedimentation by ethanol. Ethanol (96%) was added to the culture filtrate 2:1 mixing was performed for 24 hr at room temperature. Sediment was then separated from the culture filtrate by decantation and used for investigations as levan. The production of levan was indicated after acid hydrolysis by chromatography (Tanaka *et al.*, 1978). The chromatography was sprayed with aniline phthalate (Block *et al.*, 1955). Sulphation of levan was carried out with chlorosulfonic acid (Hussein, 1994).

2.1.2. Cell Lines:

2.1.2.1. Hep-G2 cells:

Hep-G2 cells (ATCC, USA.) were cultured in tissue culture flasks and maintained in RPMI medium (Cambrex Bioscience, Copenhagen, Denmark.) with the required additives.

2.1.2.2. Murine RAW macrophage:

Murine RAW macrophage 264.7 (ATCC, USA.) were cultured in tissue culture flasks and maintained in RPMI medium supplemented with 10% fetal bovine serum, 2 µmol/ml L-glutamine, 250 ng/ml fungizone, 100 units/ml penicillin, and 100 units/ml streptomycin at 37 ºC in a humidified 5% CO₂ atmosphere. The cultures were passaged every 3 days by scrapping using rubber scrappers.

2.2. Methods:

2.2.1. Assays to evaluate activity of BSL as cancer antipromotion:

2.2.1.1. Macrophage Proliferation Index:

Proliferation index of macrophage was measured using the MTT Cell Viability Assay. MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) assay is based on the ability of active mitochondrial dehydrogenase enzyme of living cells to cleave the tetrazolium rings of the yellow MTT and form dark blue insoluble formazan crystals which are largely impermeable to cell membranes, resulting in its accumulation within healthy cells. Solubilization of the cells results in the liberation of the crystals, which are then solubilized. The number of viable cells is directly proportional to the level of soluble formazan dark blue color. The extent of the reduction of MTT was quantified by measuring the absorbance at 570 nm (Hansen, 1989). This conversion does not take place in dead cells.

2.2.1.2. LPS-stimulated NO generation from RAW macrophage 264.7 cells:

Nitrite accumulation was used as an indicator of NO production in the culture medium using a microplate assay based on the Griess reaction (Gerhäuser *et al.*, 2003).

2.2.1.3. LPS-stimulated TNF-α generation from RAW macrophage 264.7 cells:

The level of TNF-α was determined by using a standard sandwich enzyme linked immunosorbent assay (ELISA) using a monoclonal antibody to mice TNF-α.

Assay was performed in duplicates in an ELISA plate. The TNF-α level in the animal’s serum was determined using the standard curve equation

2.2.1.4. LPS-stimulated COX-2 generation from RAW macrophage 264.7 cells:

RAW 264.7 macrophages were stimulated with LPS as in the TNF-α experiment then, generated COX-2 was measured by Dot Blot technique.

2.2.2. Assays to evaluate activity of BSL as cancer antiproliferation:

2.2.2.1. Quantitative DNA fragmentation analysis:

This method is based on the notion that extensively fragmented double-stranded DNA can be separated from chromosomal DNA upon centrifugal sedimentation. The protocol includes the lysis of cells and the release
of nuclear DNA, a centrifugation step with the generation of two fractions (corresponding to intact and fragmented DNA, respectively), precipitation of DNA, hydrolysis and colorimetrical quantitation upon staining with diphenylamine (DPA), which binds to deoxyribose (McConkey et al., 1989). The percentage of fragmented DNA was calculated using the formula:

\[ \% \text{ Fragmented DNA} = \frac{S+T}{S+T+B} \times 100 \]

Where, S, T and B are the OD at 600 nm of fragmented DNA in the S, T (fragmented) and B fractions, respectively.

The fragmented DNA released by cells undergoing apoptosis and lysis during the experiment was recovered in the fraction S so should therefore be taken into consideration in particular circumstances.

2.2.2.2. Anti-tumor activity against human tumor cell line:

According to macrophage proliferation index experiment cytotoxicity of Hep-G2 cells was measured using the MTT Cell Viability Assay.

Percentage of relative viability was calculated using the following equation:

\[ \left[ 1 - \frac{\text{Absorbance of BSL}}{\text{Absorbance of control}} \right] \times 100 \]

2.2.3. Statistical analysis:

The results were expressed as the mean ± SD from n=3. Statistical analysis was performed with Student's t-test using inстаtе software (Version 3.05). Statistical significance was accepted at the level of \( P<0.05 \).

3. Results:

3.1. Evaluation of BSL as cancer anti-promotor:

3.1.1. Effect of BSL on RAW macrophage proliferation:

Using MTT assay, the effect of BSL on the growth of RAW macrophage cells was studied (Fig. 1). The cell growth enhanced starting from the lowest doses of BSL, and this enhanced growth was significantly changed (\( P<0.05 \)) at the dose of 100 µg/ml compared with untreated cells (Fig. 2). The following figure shows the percentage of relative viability of RAW macrophage cell compared with control.

![Fig. 1: Determination of the macrophage proliferation index (Mean±S.D.), using MTT assay, after treatment with gradual doses BSL, where the 100% represents no change in the cell growth.](image)

* Significantly different from control values at (\( P<0.05 \)).

Figure (2) demonstrated the induction in the growth of RAW macrophage cells to 150% that occurred after treatment with BSL compared with control untreated RAW macrophage cells.
3.1.2. Effect of BSL on NO generation from stimulated macrophage:

As a mimic *in vitro* model of inflammation, macrophages were stimulated by incubation with the bacterial LPS, which activates the macrophage functions including the generation of NO, the secretion of the pro-inflammatory cytokines and the induction of COX-2 synthesis.

Tracing the NO generation by LPS-stimulated cells was performed through tracing the nitrite content in cell supernatant, since most of the cellular generated NO is converted immediately into nitrites.

A standard curve of sodium nitrite was plotted in each experiment to calculate the nitrite content in the cell supernatant (Fig. 3).

Using Griess reaction, this trial indicated that BSL exhibited a remarkable dose-dependant inhibitory property of the NO generation from LPS-stimulated macrophages, BSL doses of 12.5, 25, 50 and 100µg/ml gradually decreased NO generation to 442, 395, 273 and 220 n mole nitrite/mg protein, respectively, where the highest inhibition is noticed after treatment with the highest examined dose (100µg/ml), as indicated at figure (4).
Fig. 4: Determination of the nitrite level (nmole nitrite / mg protein) Mean±S.E. as an index of NO generation from LPS-stimulated macrophage. After incubation with and without BSL, as measured by Griess assay. * Significantly different from control values at \( P<0.05 \).

IC\(_{50}\) of NO by BSL was calculated from the curve equation of the inhibition percentage and valued as 65.85µg/ml (Fig. 5). BSL can be recognized as NO inhibitor \( (P<0.05) \) starting from the dose 25 µg/ml, and subsequently can be recognized as a potent anti-inflammatory agent.

Fig. 5: The inhibition percentage of the stimulated NO level generated from LPS-treated macrophage after treatment with BSL as compared with LPS-treated cells. * Significantly different from control values at \( P<0.05 \).

3.1.3. Effect of BSL on TNF-\( \alpha \) generation from LPS-stimulated macrophages:

TNF-\( \alpha \) is one of the important pro-inflammatory cytokines that play a dangerous role in cancer promotion. Subsequently, the inhibition of the stimulated TNF-\( \alpha \) is one of the important cancer chemopreventive strategies. The standard curve for TNF-\( \alpha \) was plotted using ELISA assay readings at O.D of 450 nm versus log scale of TNF-\( \alpha \) (ng/ml) (Fig. 6).
LPS is an aggressive inducer for TNF-α generation by macrophage cells (10.2 ng TNF-α/mg protein), while control showed a minute amount of TNF-α (0.20 ng TNF-α/mg protein). BSL showed a significant dose dependent inhibition to TNF-α generation as 12.5, 25, 50 and 100 μg/ml BSL doses, respectively caused TNF-α generation inhibition to 9, 5.9, 5 and 4.6 ng/mg protein, data presented in figure (7).

IC₅₀ of TNF-α by BSL was calculated from the curve equation of the inhibition percentage and valued as 86.54 μg/ml (Fig. 8).

BSL could be recognized as TNF-α inhibitor (P<0.05) starting from the dose 12.5 μg/ml, and subsequently supported the results of NO as a potent anti-inflammatory agent.
Fig. 8: The inhibition percentage of the stimulated TNF-α secreted from LPS-treated macrophage after treatment with BSL as compared with LPS-treated cells. * Significantly different from control values at \((P<0.05)\).

3.1.4. Effect of BSL on COX-2 generation from LPS-stimulated macrophages:

It is known that the supernatants of LPS-stimulated RAW 264.7 cells is containing a high COX-2 concentration. After treatment of these stimulated cells with or without BSL, the cell supernatants were collected after 20 hr for COX-2 determination. COX-2 was determined immunochemically using Dot Blot system.

Figure (9) and (10), showed the treatment of the stimulated cells with gradual doses of BSL (12.5, 25 and 50 µg/ml) resulting in a gradual inhibition (19.5, 27 and 57%, respectively) in COX-2 concentration.

Fig. 9: Dot blot analysis for evaluation of COX-2 in untreated control cells, LPS-treated cells, and BSL- and LPS-treated cells.

The intensity analysis of the dots revealed that LPS increased the COX-2 level to 2.84-fold of the control baseline concentration. On the other hand, treatment of LPS-stimulated cells with BSL led to a progressive inhibition of COX-2. The most effective inhibition was noticed at the dose of 50µg/ml (Fig. 10).
Fig. 10: Determination of COX-2 concentration in the supernatants of LPS-stimulated macrophage, and after incubation with BSL, in addition to untreated cells. COX-2 was assayed by dot blot analysis.

* Significantly different from control values at ($P<0.05$)

IC$_{50}$ of COX-2 by BSL was calculated from the curve equation of the inhibition percentage and valued as 45.41µg/ml (Fig. 11).

BSL could be recognized as significant COX-2 inhibitor ($P<0.05$) only at the dose of 50 µg/ml, and subsequently supported the results of NO and TNF-α as a potent anti-inflammatory agent.

Fig. 11: The inhibition percentage of the stimulated COX-2 production from LPS-treated macrophage after treatment with BSL as compared with LPS-treated cells.

* Significantly different from control values at ($P<0.05$).

3.2. Evaluation of BSL as cancer anti-progresion:

3.2.1. Effect of BSL on stimulated DNA fragmentation:

Measurement of DNA fragmentation with DPA colorimetric assay is preferentially used for late apoptosis. The fragmentation was induced in Hep-G2 cells by H$_2$O$_2$ for 6 hr after the cells were treated with different doses of BSL for 24 hr to explore its effect on the fragmentation incidence.

The spectrophomeric results indicated that the treatment of cells with H$_2$O$_2$ in absence of BSL resulted in a strong induction of the fragmentation to 2.18-fold of the untreated cells (Fig 12). 12.5, 25, 50 and 100µg/ml BSL doses resulted in 9.8, 27.2, 41.6 and 46.8% of DNA fragmentation inhibition, respectively (figure 13).
Fig. 12: The determination of DNA fragmentation in H2O2-stimulated Hep-G2 cells, and after incubation with BSL, in addition to untreated cells.
* Significantly different from control values at \( P<0.05 \).

IC50 of DNA fragmentation by BSL was calculated from the curve equation of the inhibition percentage and valued as 97.72\( \mu \)g/ml (Fig. 13).

BSL could be recognized as significant \((P<0.05)\) inhibitor of DNA fragmentation starting from the dose of 25\( \mu \)g/ml. These results suggested that a preventive role of BSL for late apoptosis was evident.

Fig. 13: The inhibition percentage of the H2O2-stimulated DNA fragmentation in Hep-G2 cells after treatment with BSL as compared with H2O2 treated cells.
* Significantly different from control values at \( P<0.05 \).

3.2.2. Cell proliferation assay:

**Effect of BSL on Hep-G2 proliferation:**

MTT assay was used to follow up the effect of different doses of BSL (12.5, 25, 50 and 100\( \mu \)g/ml) on the growth of Hep G2 cells it resulted in 95, 76, 84 and 63\%, respectively inhibition on the cell growth. This means that, the inhibition was initiated by the lowest dose of BSL, but this inhibited proliferation was significantly changed \((P<0.05)\) starting from the dose of 50\( \mu \)g/ml compared with untreated cells and the IC50 value was found to be 87.36 \( \mu \)g/ml, which is a promising anti-cancer effect (Fig. 14).
Fig. 14: The cell proliferative activity: The viability percentage of Hep-G2 cells (square) after treatment with different concentrations of BSL for 24 hr, as followed by MTT assay.
* Significantly different from control values at \((P<0.05)\).

Discussion:

HCC is one of the most frequent human cancers, with~1 million of newly diagnosed cases each year (Thorgeirsson and Grisham, 2002; Bruix et al., 2004; Farazi and DePinho, 2006). Therefore, chemoprevention of HCC is of great importance because of its high incidence of in viral-associated cirrhotic patients.

A rational use of chemopreventive agents is based not only on the assessment of their efficacy and safety but also on understanding of their mechanisms of action. Several mechanisms, such as inhibition of genotoxic effects, antioxidant activity and scavenging of free radicals, inhibition of cell proliferation and signal transduction modulation can be involved (DeFlora et al., 1999; Miadokov et al., 2000; Miadokov et al., 2004). In the recent years, much evidence has been collected indicating that microbial polysaccharides play a role in signaling molecules for innate immune system (Medzhitov et al., 2000).

Macrophages are the first line of defense in innate immunity against microbial infection. Macrophage defense against pathogens includes cytokine secretions like TNF-\(\alpha\), and inflammation mediators like NO. In phagocytes, NO is produced in large quantities by the action of iNOS, and COX-2 (MacMicking et al., 1997). The present study demonstrated that BSL have a significant enhancing effect on the proliferation rate of the macrophage in vitro. The induction of macrophage proliferation by BSL might be due to sulphation. This proliferative effect on macrophage may point to its possible immunomodulatory effect which plays an important role in the elimination of tumor cells.

The present results revealed that, BSL was a good inhibitor of NO production from LPS stimulated RAW 264.7 macrophage. This suggesting that, BSL prevents NO mediated genotoxicity thus having an anti-promotion and immunomodulatory (anti-inflammatory) activities towards cancer cells. The inhibitory property of BSL toward NO may be due to a direct scavenging capacity to NO, an inhibition of the iNOS pathway, and/or a modulation of other factors in the NO cascade, e.g. transcriptional factors. In accordance to our work, Xu et al. (2008) found that polysaccharides from \(L.\) edodes significantly decreased the level of serum NO\(_2\) in high-fat-diet rats. Where, Han et al (2006) reported that, the acidic polysaccharide from Phellinus linteus (PL) was an immunostimulator that has therapeutic activity against cancers. PL also increased macrophage NO production. Lentinan treatment also enhances production of NO and the cytotoxic activity of macrophage (Ohno et al., 2001; Hou and Chen, 2008).

In addition, this study demonstrated that BSL inhibited TNF-\(\alpha\) release after LPS stimulation of cultured cells. TNF-\(\alpha\) has been found to be required in chemical carcinogen-elicited skin carcinogenesis (Huang, et al., 1999). In addition, TNF-\(\alpha\) can induce DNA damage, inhibit DNA repair (Jaiswal et al., 2000b), and act as a growth factor for tumor cells (Wu et al., 1993). Accordingly, the inhibition of stimulated TNF-\(\alpha\) is an important target in prevention of cancer and in countering of inflammatory diseases,using the Raw 264.7 macrophage model. this study demonstrated that BSL inhibits TNF-\(\alpha\) release after LPS stimulation of cultured cells. And this suggesting that, BSL has anti-promotion and immunomodulatory (anti-inflammatory) activities towards cancer cells.
Aberrant overexpression of COX-2 was implicated in inhibition of apoptosis and induction of proliferation (Ishiko et al., 2001). Inhibition of COX-2 is hence recognized as one of the most feasible strategies for cancer chemoprevention and treatment. Using immunoblotting analysis, COX-2 enzyme was assessed in macrophage lysates and we found that BSL showed significant inhibition to macrophages COX-2 production in vitro. This inhibition might be due to blocking of the NF-κB signaling pathway. In agreement with our results, Gamal-Eldeen et al., (2009) found that different fractions of water-soluble polysaccharide extract derived from S. latifolium possessed potential anti-promoting properties as indicated by their anti-inflammatory activity through enhancement of the macrophage proliferation; however they dramatically inhibited the stimulated NO, TNF-α and COX-2.

It is widely accepted that the interaction of DNA with carcinogens and reactive oxygen species (ROS), generated during carcinogen metabolism and inflammation accompanying early stages of hepatocarcinogenesis, results in genomic instability which renders the genome prone to the accumulation of severe DNA defects during the clonal expansion of initiated cells (Kawanishi et al., 2006). Our results revealed that, BSL could be recognized as significant inhibitor of DNA fragmentation that induced in Hep G2 cells by H2O2. These results were suggesting the preventive role of BSL to DNA damage. Since, NO clearly induces DNA damage inhibition of NO level by BSL might be responsible for reducing DNA damage when estimated using DNA fragmentation assay.

Our findings demonstrate that BSL showed anti-proliferative property against Hep G2. This inhibitory effect might be due to inducing cancer cell death by apoptosis or due to induction of cell cycle arrest. These data are supported by the fact that different fractions of water-soluble polysaccharide extract derived from S. latifolium showed a selective cytotoxicity against lymphoblastic leukemia (Gamal-Eldeen et al., 2009). Also, Bae et al. (2005) demonstrated that polysaccharides isolated from Phellinus gilvus had antiproliferative effects to inhibit tumor-induced proliferation in melanoma.

Conclusion:

BSL proved to be a potent antipromotion and anti progression drug

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References


