Apoptosis of Human Breast Cancer Cells (MCF-7) Induced by Polysaccharides Produced by Bacteria

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Abstract

Two different polysaccharides produced by Bacillus species and Pseudomonas species were investigated for their anticancer activities against Human Breast cancer cell lines and colon cancer cell lines. Exopolysaccharide from Bacillus was purely polysaccharide whereas as Pseudomonas formed Polysaccharide-Peptide complex. Bacillus polysaccharide were highly active at its low concentration of 7.8 µg/ml by inducing Bax, a death promoting protein, Caspase-3 which induces the caspase cascade of apoptosis and PARP. The Polysaccharides of Bacillus species was active with -PS3, Bax, lesser extent to Bcl-xl, Caspase, PARP and β-Actin, whereas interestingly the inhibitory effect of EPS from Pseudomonas increased after protease digestion suggesting that the inhibitory effect is due to carbohydrate rather than protein. The result of DNA fragmentation was confirmed by DNA ladder assay, we conclude that exopolysaccharide from bacteria has high potential at its low concentration, as a novel therapeutic agent for the treatment of Breast cancer cells without any cytotoxicity against normal cells.

Keywords: Anti cancer agents; In vitro assay; Bax; Death promoting protein; Exopolysaccarides; Apoptosis

Introduction

In the past three decades an increasing number of reports describing the isolation and bioactivity of polysaccharide glucans and proteoglycans from plant and other sources highlight the potential use of this class of molecules in cancer therapy as a result of its immune stimulatory properties. Naturally derived polysaccharides including heteroglycans and proteoglycans of certain molecular weight and structure have specific broad range immune stimulatory properties which have been recognized for several decades [1]. Cancer is treated conventionally by radiotherapy surgery, chemotherapy, immunotherapy, molecular targeting or combination of these methods [2]. In our study we demonstrated that the exopolysaccharide from Bacillus and Pseudomonas inhibited the Breast cancer cell by promoting cell apoptosis, involving the expression of apoptosis related proteins. To the best of our knowledge this is the first report demonstrating the Biological activity of exopolysaccharide obtained from bacteria.

Materials and Methods

Exopolysaccharide extraction

The isolates were inoculated separately in Nutrient Broth supplemented with 1 gm of glucose and incubated at 35°C for 6 days at 180 rpm in incubator shaker. The inoculated broths were centrifuged after 6 days of incubation for 10 min at 10,000 rpm to remove the cell pellets. The cell supernatant was then treated with thrice its volume of ice cold ethanol and left at 4°C for overnight. EPS pellets were collected by centrifugation at 15,000 rpm for 20 min and collected pellets were purified by dialysis and lyophilized for future use.

Cell line and culture

Human Breast adenocarcinoma (MCF-7) GD0055, were obtained from National Centre for Cell Science (NCCS), Pune. The cells were maintained in RPMI-1640 supplemented with 10% FBS, Penicillin 100 U/ml and in a humidified atmosphere of 50 µg/ml CO2 at 37°C.

Morphological studies of MCF 7 cells were done using normal...
inverted microscope and out in order to observe the morphological changes of cell death in MCF-7 cells elicited by the exopolysaccharide sample. The concentration of IC$_{50}$ value was used for morphological studies.

**Cell cytotoxicity assay**

The tetrazolium salt assay: Tetrazolium salts are widely used to assess the anticancer activity or cytotoxicity of many compounds both from natural and synthetic origin on various tumor cells. The cytotoxicity of the samples on Breast cancer cell was determined by MTT assay. Cells (1 × 10$^5$/ well) were plated in 100 µl of medium/well in 96 well microtibre plates. After 48 hr incubation the cell reaches the confluence. Then, cells were incubated in the presence of various concentration of the sample solution and washing with phosphate buffered saline (pH-7.4) 20 µl/well of 0.5% (5 mg/ml) MTT buffered saline solution was added. After 4 hrs of incubation, 0.04 M Hcl isopropanol was added. Viable cells were determined by the absorbance at 570 nm with reference at 655 nm. Measurements were performed in triplicates and the concentration required for a 50% inhibition of viability of (IC$_{50}$) was determined graphically. The effect of samples on the proliferation of Human cancer cell is expressed as the % cell viability using the formula

\[
\% \text{ cell viable} = \frac{A_{570} \text{ of treated cells}}{A_{570} \text{ of control cells}} \times 100
\]

The IC$_{50}$ values against the Human cancer cell lines were calculated for the sample inhibiting at least 50% inhibition when tested at a concentration.

**Cancer-prevention by exopolysaccharides**

Flow cytometer was used to evaluate the behavior of cells on treatment with extracted EPS. Cancer cell apoptosis is induced using test compound and a negative control is prepared without test compound. After 10 min incubation on ice, the cells were measured immediately. Then, cells were fixed, permeabilized. The treated and untreated MCF-7 cells were harvested and washed with cold PBS. An aliquot (10$^5$ cells/ 100 µl) of cell suspension was added with 1µl fluorescein Isothiocyanate (FITC)-conjugated annexin-V and 2.5 µl Propidium Iodide PI:250 µl [10 5 cells/ 100 mm Tris- Hcl, pH 8.0, 25 mm EDTA, 0.5/SDS, 100 m Nacl and 200 mg/ml Proteinase K] and incubated at 55°C for 2 hr. The cell lysate was extracted with phenol and precipitated with one tenth the volume of isopropanol and 3 volumes of 100% ethanol.

DNA samples obtained are dissolved in 1X TE buffer and Electrophoresis was carried out on 15% agarose gel at 6.0 V/cm for 60 min. DNA fragments were visualized on the gel stained with ethidium bromide under UV light.

**Result**

Prior to the tests for anticancer activity the total carbohydrate concentration of extracted EPS was estimated by phenol sulfuric acid assay and barfoads assay. The compound was found to be made of high percentage of sugar and noticeable amount of protein (nearly 20% of proteins). EPS-1 from Bacillus sp showed very high sugar concentration than proteins whereas EPS-2 from Pseudomonas showed high protein concentration. Both the EPS showed a dose dependent mortality of tumor cells. The author assessed anti proliferative effects of both the exopolysaccharide against cell lines using MTT assay as given in Table 1. The cytotoxicity measurement increases after the protein digestion (Table 2).

| Sample Concentration | Viable cells MCF-7(EPS-1) | Viable cells VEROCELLS (EPS-1) | Viable cells MCF-7(EPS-2) | Viable cells VEROCELLS (EPS-2) |
|-----------------------|---------------------------|--------------------------------|--------------------------|-----------------------------|
| 3.9 µg                | 90%                       | 96%                            | 99%                      | 99%                         |
| 7.8 µg                | 78%                       | 90%                            | 90%                      | 96%                         |
| 15.6 µg               | 64%                       | 82%                            | 84%                      | 90%                         |
| 31.2 µg               | 49%                       | 76.5%                          | 80%                      | 88%                         |
| 62.5 µg               | 40%                       | 66.5%                          | 60%                      | 80%                         |
| 125 µg                | 31%                       | 59.5%                          | 54%                      | 75%                         |

**Table 1:** Cell viability by MTT assay after treatment with Crude EPS.

| Sample Concentration | Viable cells MCF-7(EPS-1) | Viable cells VEROCELLS (EPS-1) | Viable cells MCF-7(EPS-2) | Viable cells VEROCELLS (EPS-2) |
|----------------------|---------------------------|--------------------------------|--------------------------|-----------------------------|
| 3.9 µg               | 84%                       | 90%                            | 91%                      | 985                         |
| 7.8 µg               | 76%                       | 84%                            | 85%                      | 94%                         |
| 15.6 µg              | 60%                       | 78%                            | 75%                      | 90%                         |
| 31.2 µg              | 46%                       | 69%                            | 68%                      | 85%                         |
| 62.5 µg              | 38%                       | 61%                            | 59%                      | 76%                         |
| 125 µg               | 30%                       | 50%                            | 50%                      | 69%                         |

**Table 2:** Cell viability by MTT assay after treatment with pure EPS.
showed typical apoptotic bodies (Figure 1). The slight change in EPS-2 may indicate heavy lyses at one point and uneven distribution of the sample even though diluted and mixed. This could be due to the gelling property of EPS from *Pseudomonas* observed in another experiment when dissolved in DMSO. This property seems to be reduced after removal of protein moiety. Eps-1 was tested for its apoptosis activity at its IC$_{50}$ 31.25 µg/ml.

As a direct evidence for induced apoptosis by EPS, Flow cytometric analysis with Annexin V-FITC conjugated to Propidium Iodine was done and presented here. Cells were fixed, permeabilized and stained with Propidium iodide to measure DNA content by fluorescence-activated cell sorting (FACS) analysis. The percentages of cells in each phase of the cell cycle (G1, S and G2) are indicated. This represents the fractions undergoing apoptotic DNA degradation. Values are expressed as mean ± SD (n=3). Each point represents the average of two independent experiments by one way ANOVA and student t test (Figure 2).

Western blot analysis of the apoptosis related gene expression further ensured the level of apoptotic proteins induced (Figure 3). The compound acts by inducing Bax (23 KDa) protein which is a death promoting protein and Caspase-9 an initiator protein. Presence of band for cleaved PARP (85 KDa) protein in the test sample clearly indicate that PARP (116 KDa) a protein that involve in DNA repair is cleaved leading the cells for apoptosis.

The DNA Fragmentation activity of extracted exopolysaccharide after comparing with marker of 1500 bp denotes the activity of test compound. The apoptotic activity is dose dependent when treated with various concentrations of samples (0,0.1,0.3 mcg/ml) which confirms the EPS mediated apoptosis. This work emphasizes on the most active ingredient responsible for the cytotoxicity and apoptotic effects against the cancer cell. Polysaccharide is inferred to be the active compound rather than protein moiety as the anti cancer activity increases on protein removal (Tables 2 and 3).

Earlier reports of HPLC of this compound showed the presence of rare combination of glucose and galactose in verbacose form an unusual product of bacterial origin and presence of mannose.

**Discussion**

In the present investigation we found that EPS-1 and EPS-2 markedly reduced the cell viability of MCF-7 cells in a dose dependent manner. Glucose and mannose are known to have receptors on macrophages that are highly specific according to tumor immunology [9]. The suppression of cell proliferation induced by EPS may be due to the induction of apoptosis [10-13].

The academic researcher receives fewer funds or none, in vitro cell based assays are the preferred screening techniques which are economically good enough to establish the anticancer activity of a compound and drugs. In vitro cytotoxicity assay is based on the concept of basal cytotoxicity of the compound that affects the basic functions of the cells and thus this in vitro assay would help the researchers to follow with in vivo trials.

Increase in the inhibitory effects of EPS obtained from *Pseudomonas* after Protein digestion correlates to the earlier findings [14]. But in contrast to the anti tumor activity of a protein rich fractions from *Tricholoma* sp [15] protein bound polysaccharides from the fruiting body of *Lentinus edodes* showed that protein bound polysaccharide seems to be highly active [16]. This created interest in the author to work further on the exact component or active ingredient responsible for the cytotoxicity and apoptosis against the cancer cells.

The protein moiety of PSK, a protein bound polysaccharide obtained...
from the *Curulis versicolor* strain CM-101 plays an important role in the exertion of the anticancer activity [8]. In contrast to the above reports [17] galacto oligosaccharides from Entomopathogenic fungus *Cordyceps sphenoecephala* may be the superior anticancer compound of their polypeptide complex. In this present study we found that the activity of extract increases after protein removal. Macrophages carry receptors for glucose and Mannose which are highly specific and presence of glucose and mannose in the polysaccharides extracted increases its importance in tumor immunology. This binding is known to trigger the immune enhancing and anticancer activities according to tumor immunology. Evidences suggest that apoptosis is a tightly regulated process that involves changes in the gene make up. Proto oncogene-Bcl-2 is one of the major genes that regulate apoptosis [18]. Bax a death promoting protein counteracts the anti apoptotic effect of Bcl-2 by formation of heterodimer with Bcl-2 [9]. The ratio of Bcl-2 to Bax rather than the levels of individual protein are considered to be critical in determining the survival or death of cells [19].

Figure 2 shows the strong bands of Bax and comparatively a light band at Bcl-2 which is an indirect evidence for the presence of high ratio of death promoting protein. Apoptotic cells often produce a unique ladder of nucleotide fragments at an interval of 180-200 bp visualized by DNA-AGE.

Fragmented DNA ladder formation is observed only when the extent of oligonucleosomal cleavage is prominent which is usually seen in later phase of apoptosis [5] and thus the DNA ladder DNA assay are less sensitive.

To conclude this study demonstrated that the EPS obtained from both the bacteria strongly inhibited (MCF-7) cells without any cytotoxic property. The extracted EPS also showed antitoxicity on HEP-G2 cells, HT-29 cells and HEP-2 cells whose cytological analysis are yet to be done. The present compound along with its advantage of having verbacose an un digestible disaccharide in human and susceptible only for plant and animal enzymes, may find its way as a new source with potential value for health food and therapeutics. This compound can find its way as an alternate drug for cancer and the findings in this study appear useful for further research aiming to identify the *in vivo* activity of this exopolysaccharide [20].

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