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Apoptosis of Human Breast Cancer Cells (MCF-7) Induced by Polysacccharides 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 where 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 -P53, Bax, lesser extent to Bcl-xl, Caspase, PARP and β-Actin, where as 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 proteoglucans 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 ranged 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]. Breast cancer is the most frequent neoplasm in women from western countries. Cancer prevention by use of naturally occurring dietary science is considered as a potential approach to reduce the ever increasing incidence of cancer [3]. The intervention of multistage carcinogenesis by modulating intracellular signaling pathways may provide a molecular basis for chemoprevention with a wide variety of dietary phytochemicals [4]. An excellent approach to inhibit the promotion and progression of carcinogenesis and to remove genetically regulated, premalignant and malignant cells from the body is by inducing the cell cycle arrest or apoptosis using dietary chemo preventive. Compounds extracellular polysaccharide-peptide complex obtained from *Cordyceps sphecocephala* strongly inhibited the growth of human hepatocarcinoma [5].

Polysaccharides are composed of many monosaccharide units that are joined one another by a glycoside linkage to give a long chain .These polysaccharide are extracted from plant seeds, plant exudates, marine algae and animals. Over the past few decades the number of exopolysaccharide [EPS] produced by microbial fermentation has been gradually increasing. Basically polysaccharides are main components of dietary fibers. A lot of researches have been done to understand better the interactions of those polysaccharides with other macromolecules like proteins [6].

Polysaccharo peptides and Polysaccharo krestin possess anticancer activity and oral administration of PSP/PSK has controlled various carcinomas in experimental animals and human as reported [7]. In this 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 incubatory shakes. 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 RPM I-1640 supplemented with 10% FBS, Penicillin 100 U/ml in a humidified atmosphere of 50 µg/ml CO_2 at 37°C.

Morphological studies of MCF 7 cells were done using normal

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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

Tetrazolium salt assay: Tetrazolium salts are widely used to asses 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 microtitre 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 48 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

% cell viable = A_{570} of treated cells/A_{570} of control cells × 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 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 µg/ml 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). The anticancer activity exhibited by the sample on cells and its effect was also determined by protein extraction and western blot analysis. The anticancer activity exhibited by the sample on cells and its effect is also determined by protein extraction and western blot analysis.

Protein from treated cells was fractionated by electrophoresis on 15% SDS-PAGE and electro transferred to immune blot PVDF membrane. The PVDF membrane was treated with PBS containing 5% skimmed milk at room temperature for 1 hr followed by incubation with the primary antibodies anti PARP dil. 1:2000, mouse monoclonal primary antibodies against anti human caspase 3 (1:2000) anti human caspase 9 (1:2000), Anti–human Bax (1:2000) Bcl-xl (1:10000) Bim (1:5000) Anti A paf 1, anti Blm (1:5000) at 37°C for 1hr or at 4°C overnight.

After washing with PBS 3 times for 15 min, 10 mins and 1min respectively, the corresponding second antibody (dilution- 1:1000) was added and incubated at room temperature. After reaction with horseradish per oxidase-conjugated goat anti-mouse antibody (secondary antibody) the immune complexes were visualized using chemiluminescence ECL- PLUS detection reagents following the manufacturer's procedure, Santa cruz, CA, USA and Neo Markers, USA.

Detection of DNA fragmentation

The formation of ladder pattern by the DNA fragmentation indicating apoptosis was performed according to the protocol described [8]. Cells were seeded at 15×10^5 cells/well and treated with IC_{50} concentration of EPS and incubated for 48hr. The cells were trypsinized and the cell pellets were re suspended in lysis buffer (10 mm Tris- Hcl, pH 8.0, 25 mm EDTA, 0.5/SDS, 100 m 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 3M 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 cytotoxic property increases after the protein digestion (Table 2). According to the results of cytotoxicity measurement the IC_{50} of EPS-1 was less compared to EPS-2. Exopolysaccharide from (EPS-1) *Bacillus spp* and (EPS-2*) Pseudomonas sp* treated MCF-7 cell lines

Sample Con-centration	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 Con	Viable cells MCF-7(EPS-1)	Viable cells VEROCELLS (EPS-1)	Viable MCF-7(EPS-2)	Viable VERO CELLS (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

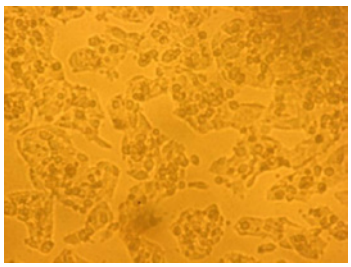

Figure 1: Apoptotic Bodies.

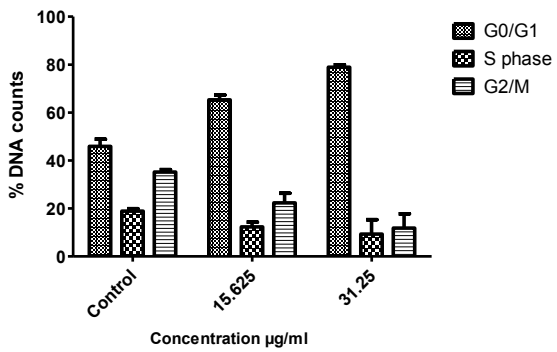

Figure 2: FACS Analysis.

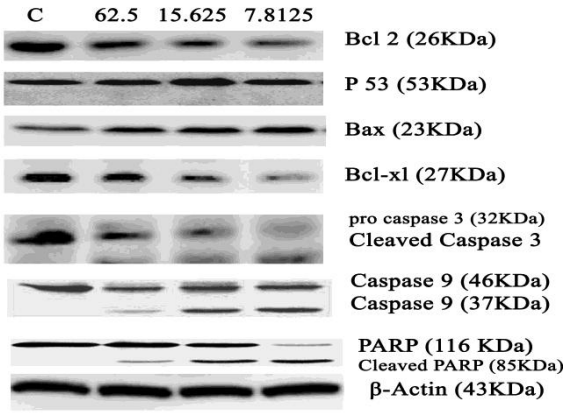

Figure 3: SDS CASPASE.

Compound	Concentration	Dna Fragmentation [bp]
EPS-1	7.8 µg/ml	500/700/900/1000
EPS-2	15.6 µg/ml	300/500/600/800/1000
Doxorubicin	6.25 µg/ml	300/500/600/800/900/1000

Table 3: DNA Fragmentation.

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 *Lectinus 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 *Coriolus 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 sphecocephala* 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 antitoxic property 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 verabacose 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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