Antiproliferative effects of porins extracted from *Klebsiella pneumoniae* on HL-60, NIH/3T3 and Human foreskin fibroblast cell (HFFC) lines measured by ELISA method

تاثير البورينات المستخلصه من بكتريا *Klebsiella peumoniae* المضاد لتكاثر خلايا -HL 60, NIH/3T3, (HFFC) والمقاس بطريقة ELISA

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Abstract

Approximately, 50% of the dry mass of the outer membrane of gram-negative bacteria consists of proteins, and more than 20 immunochemically distinct proteins (termed outer membrane proteins [OMPs]) have been identified. An identified local strain of *Klebsiella pneumoniae* was used as a primary source for the isolation and purification of porins. Multiple concentrations of purified porins (5, 10, 15, 20, 25) µg/ml were incubated with three different cell lines for (24, 72 , 120) hrs, after the end of the incubation periods, the cells were treated with *Cell proliferation ELISA, BrdU (colorimetric) kit* to evaluate the antiproliferative effects of porins. The results revealed that porins are potent antiproliferative agent in a time and concentration dependent manner and thus could greatly affect prokaryote-eukaryote interaction as well as the whole inflammatory process resulted after infection with gram negative bacteria.

المستخلص

تشكل البروتينات حوالي 50% من الكتله الجافه للغشاء الخارجي للبكتريا السالبه لصبغة الكرام . ويوجد اكثر من 20 نوع مميزه مناعيا من بروتينات الغشاء الخارجي . استعملت عزله محليه من بكتريا *Klebsiella pneumoniae* كمصدر لعزل وتنقية بروتينات الغشاء الخارجي وقد استعملت عدة تراكيز من بروتينات الغشاء الخارجي (5, 10, 15, 20, 25) مايكروغرام/مليليتر وحضنت لثلاث مدد (24, 72, 120) ساعه مع ثلاثة انواع من الخلايا وبعد انتهاء مدة الحضن تم معاملة الخلايا مع عدة Cell proliferation ELISA, BrdU (colorimetric) . اظهرت نتائج الدراسه ان بروتينات الغشاء الخارجي عامل مثبط لتكاثر الخلايا بطريقه تعتمد على التركيز وفترة الحضن وهذا بدوره يؤثر على طريقة التاثر بين خلايا كاذبة النواة وخلايا حقيقية النواة وكذلك على العمليه الالتهابيه الناتجه عن الاصابه بالبكتريا السالبه لصبغة الكرام .

Introduction

The outer membrane of gram-negative bacteria plays a significant role in a variety of functions; it serves as a diffusion barrier to extracellular solutes and interacts with the bacterial environment. This membrane is composed of a bilayer containing phospholipids, lipopolysaccharide, and outer membrane proteins (OMPs) [1].

Since *Escherichia coli* major porins OmpC and OmpF were defined, a large number of OmpC- or OmpF-type porins have been described in other enterobacterial species. Two major porins, OmpK36 and OmpK35 have been previously described in *Klebsiella*

pneumoniae. They are homologous to OmpC and OmpF, and they are expressed in large amounts in most *Klebsiella pneumoniae* clinical isolates independently of the isolation source [2]. Apart from their structural role, OMPs have also been shown to have other functions, particularly with regard to transport, and have been classified as permeases and porins. Furthermore, several OMPs have been shown to be potent inducers of cytokine synthesis [3]. This study aims to evaluate the antiproliferative effects of porins on three different cell lines (HL-60, NIH/3T3 and HFFC) by using ELISA method.

Materials and methods

1. Identification of *Klebsiella pneumoniae*
   Isolated bacteria were identified biochemically depending on previous researches [4] and according to methods described by others [5]. Identification was potentiated further using API 20E test which was carried out according to the manufacturer's (bio-Merieux) instructions.
2. Extraction and purification of outer membrane proteins (porins).
   Porins were extracted and purified according to the method described I previous reports [6]. The procedure was potentiated further by applying gel filtration chromatography through Sephacryl-S- 200 gel [7] to increase the purity of the final porins preparations. The final preparation resulted after purification steps contained porins in a concentration of 3.2 mg/ml as estimated by the absolute method [8]. The contaminating lipopolysaccharides were detected by thiobarbituric acid assay [9].
3. Anti proliferation caused by incubation with multiple concentrations of porins was measured through the use of Cell proliferation ELISA, BrdU (colorimetric) kit. It is colorimetric immuno-assay for the quantification of cell proliferation based on the measurement of BrdU incorporation during DNA synthesis. Manufactured by Roche (Germany). Cat no. 11647229001.
4. Application of different concentrations of porins for three different cell lines, namely HL-60, NIH/3T3 and HFFC.Buffers, solutions and media were prepared according to the literature [10]. The cells of the three cell lines were seeded in different tissue culture vessels and treated with kit component according to the instructions of the manufacturing company (Roche).

Results

The results of the current study showed that when HL-60 cells were considered, there were significant differences when the control group is compared to the porins treated groups of 15 and 20 µg/ml concentrations and after 24 hrs of incubation. On the contrary, when HL-60 cells were incubated with the porins of multiple concentrations for 72 hrs, the mean of the absorbance values, clearly indicated that there is a significant difference between the control groups as compared with all the treatment groups. In addition, 25 µg/ml porins concentration yields the most significant effect on cell proliferation as compared to the other treatment groups. Moreover, porins concentration is not a critical factor in inducing apoptosis within this cell line as far as 120 hrs incubation periods is considered since it is proved statistically that there was no significant difference when all

treatment groups compared with each others. Furthermore, all porins treatment groups show nearly the same effect on cell proliferation as compared with the control group. Different incubation times applied throughout the study for each particular porins concentration were compared to find out which incubation period was most effective and suitable, within a fixed porins concentration treatment, to elicit maximal apoptotic or anti proliferative response. The results of the present study revealed that there was a significant difference when mean absorption values after 120 hrs of incubation at 10μ g/ml porins treatment group was compared to those of 24 and 72 hrs. Furthermore, the most significant effect exerted by porins treatment when the cells were incubated for 72 hrs with 25 μg/ml porins concentration compared to the other incubation periods. The rest of the comparisons yield no significant differences when the three incubation periods for the rest of the porins treatment group rather than that of the 10 and 25 μg/ml treatment groups were compared Table (1).

Table (1): Mean of absorption values for HL-60 cells after being incubated with different concentrations of porins for three periods of time

Porins conc. μg/ml	Mean of absorbance values after 24 hrs incubation ± SE	Mean of absorbance values after 72 hrs Incubation ± SE	Mean of absorbance values after 120 hrs Incubation ± SE
0	0.6833± 0.01[a] A	0.6933 ± 0.1[a] A	0.58 ± 0.08 [a] A
5	0.5567 ± 0.17[a] AB	0.3467 ± 0.03 [a] B	0.22 ± 0.07 [a] B
10	0.4667 ± 0.11[a] AB	0.3133 ± 0.03 [a] B	0.1833 ± 0.05 [b] B
15	0.3433 ± 0.04 [a] B	0.2567 ± 0.01 [a] B	0.2633 ± 0.09 [a] B
20	0.2967 ± 0.04 [a] B	0.27 ± 0.04 [a] B	0.2267 ± 0.1 [a] B
25	0.33867 ± 0.08 [a] AB	0.08 ± 0.06 [b] C	0.2033 ± 0.07 [a] B

Different capital letters [rows (concentration)] denote significant differences ($p < 0.05$). Different small letters [columns (time)] denote significant differences ($p < 0.05$).

The description of the results obtained after working with NIH/3T3 cells revealed that there was a significant difference ($p <0.05$) between control group and all porins concentrations treatment groups. In addition, there was no significant difference among treatment groups when compared with each other. Moreover, NIH/3T3 cells react very clearly and showed profound apoptotic response after being incubated with increasing porins concentrations for 72 hrs and there was significant difference of the mean absorption values belonging to control and treatment groups of (10, 15, 20, 25) μg/ml as they were statistically compared. Furthermore, NIH/3T3 cells were also treated with the same porins concentrations used throughout the study and were also incubated for 120 hrs and the results showed that there were no significant differences, except for the 25 μg/ml porins concentration treatment group when its result compared with the control group. In addition, the comparison between mean absorption values of control group and all the

porins concentrations treatment groups results in a less profound effect. NIH/3T3 cells were tested for their ability to manifest the antiproliferative effect characteristic of the porins treatment. It is pointed out also that significant differences resulted when incubation periods of 24 and 120 hrs at (10, 15, 20, 25) µg/ml porins concentrations were compared to the results of the 72 hrs incubation period of the correspondent porins concentrations on the other hand. The results showed clearly that 72 hrs incubation period was the optimum time required to manifest the anti proliferative or apoptotic effect resultant of incubation with various porins concentrations Table (2).

Table (2): Mean of absorption values for NIH/3T3 cells after being incubated with different concentrations of porins for three periods of time

Porins conc. µg/ml	Mean of absorbance values after 24 hrs Incubation ± SE	Mean of absorbance values after 72 hrs Incubation ± SE	Mean of absorbance values after 120 hrs incubation± SE
0	0.9223 ± 0.04 [a] A	0.43 ± 0.17 [a] A	0.61 ± 0.09 [a] A
5	0.6033 ± 0.05 [a] B	0.3933 ± 0.16 [a] A	0.4233 ± 0.09 [a] A
10	0.56 ± 0.07 [a] B	0.07 ± 0.08 [b] B	0.4367 ± 0.04 [ab] A
15	0.53 ± 0.11[a] B	0.1 ± 0.09 [b] B	0.3667 ± 0.06 [ab] A
20	0.4233 ± 0.08 [a] B	0.08 ± 0.12 [b] B	0.3533 ± 0.09 [ab] A
25	0.45 ± 0.02 [a] B	0.09 ± 0.1 [b] B	0.31 ± 0.11 [ab] B

Different capital letters [rows (concentration)] denote significant differences ($p < 0.05$). Different small letters [columns (time)] denote significant differences ($p < 0.05$).

The results obtained by the aid of Cell Proliferation ELISA, BrdU (colorimetric) kit after experimentation with the normal human foreskin fibroblast cell line for 24 hrs reveal the development of an apoptotic response but with significance manifested only when cells were treated with 20 and 25 µg/ml and the comparison among treatment group themselves showed no significant difference. Human foreskin fibroblast cells were also incubated with the porins for 72 hrs and treated with the same kit components after the end of the incubation period which results in somewhat weird cellular reaction since all porins concentrations except 20 µg/ml showed an apoptotic effect with little significance whereas the 20 µg/ml porins concentration caused the cells to manifest a much profound apoptotic effect compared to the control group after which, and at 25 µg/ml porins concentration the cells seem to be rescued and reveal reaction similar to the other porins concentrations. The results after 120 hrs of incubation revealed the presence of significant differences when comparing the control group with the porins concentrations groups of 15, 20, 25 µg/ml separately, despite non significant, the results show also that 25 µg/ml porins concentration is the most effective in eliciting an apoptotic phenotype. For the idea

about the reactive characteristics of the human foreskin fibroblast cells to be completed, comparison was further expanded to involve the applied time of incubation in order to find out the optimum time required to get the maximal apoptotic or anti proliferative effect. The results revealed that there is no significant difference for the porins concentrations up to 15 µg/ml when the three incubation periods were compared. Expectedly, the cells reaction is manifested by profound apoptosis when 20 and 25 µg/ml porins concentrations were considered with 120 hrs of incubation at 20 µg/ml porins concentration showing the maximal apoptotic response Table (3).

Table (3): Mean of absorption values for human foreskin fibroblasts cells after being incubated with different concentrations of porins for three periods of time

Porins conc. µg/ml	Mean of Abs. values after 24 hrs Incubation ± SE	Mean of Abs. values after 72 hrs Incubation ± SE	Mean of Abs. values after 120 hrs incubation± SE
0	0.1333 ± 0.03[a] A	0.26 ± 0.03[a] A	0.23 ± 0.03[a] A
5	0.1166 ± 0.03[a] A	0.2133 ± 0.03[a] AB	0.16 ± 0.03[a] AB
10	0.0933 ± 0.03[a] A	0.1866 ± 0.02[a] AB	0.1566 ± 0.006[a] AB
15	0.0833 ± 0.04[a] A	0.1633 ± 0.02[a] AB	0.1233 ± 0.003[a] B
20	0.02 ± 0.01[a] B	0.09 ± 0.02[b] B	0.1333 ± 0.01[c] B
25	0.01 ± 0.01[a] B	0.18 ± 0.02[b] AB	0.09 ± 0.01[b] B

Different capital letters [rows (concentration)] denote significant differences ($p < 0.05$). Different small letters [columns (time)] denote significant differences ($p < 0.05$).

The data of the current study was further analyzed, but this time through comparison of the resulted mean of absorption values shown by the three cell lines enrolled in the study at fixed porins concentration treatment and incubation time regimen. The results of the statistical comparison among the three cell lines enrolled in this study (HL-60, NIH/3T3 and Human foreskin fibroblast) reveal that only human foreskin fibroblast cells had experienced significant apoptotic or anti proliferative effect when compared with the other two cell lines after 24 hrs of incubation. In addition, when 72 hrs incubation period was considered, NIH/3T3 cells and human foreskin fibroblast cells at porins concentration 10 and 20 µg/ml had shown significant difference when compared to HL-60 cells. Moreover, 5 and 10 µg/ml porins concentrations reveal a significant difference when NIH/3T3 cells was compared to both HL-60 and human foreskin fibroblast cells Finally, it is shown that human foreskin fibroblast cells manifested the significant difference when compared with the other two cell lines and when porins concentration was 25µg/ml Table (4).

Table (4): Comparisons of the mean absorption values of the three cell lines at fixed time regimen and constant porins concentrations

Porins concentration (µg /ml)	Time of incubation (hrs)	Mean of absorption values of HL-60 cells ± SE	Mean of absorption values of NIH/3T3 cells ± SE	Mean of absorption values of HFFC ± SE
5	24	0.5567± 0.17 A	0.6033 ± 0.05 A	0.1167± 0.03 B
10		0.4667± 0.11 A	0.56 ± 0.07 A	0.0933 ± 0.03 B
15		0.3433 ± 0.04 A	0.53 ± 0.11 AB	0.083 ± 0.04 B
20		0.2966 ± 0.04 A	0.4233 ± 0.08 A	0.02 ± 0.01 B
25		0.336 ± 0.08 A	0.45 ± 0.02 A	0.01± 0.01 B
5	72	0.3467± 0.03 A	0.3933 ± 0.16 A	0.21± 0.03 A
10		0.3133± 0.03 A	0.07 ± 0.08 B	0.18 ± 0.02 B
15		0.2567 ± 0.01 A	0.1 ± 0.09 A	0.16 ± 0.02 A
20		0.27 ± 0.04 A	0.08 ± 0.12 B	0.09 ± 0.03 B
25		0.08 ± 0.06 A	0.09 ± 0.1 A	0.18± 0.02 A
5	120	0.22± 0.07 A	0.42 ± 0.09 B	0.16 ± 0.03 A
10		0.1833± 0.05 A	0.4366 ± 0.04 B	0.1566 ± 0.06 A
15		0.2633 ± 0.09 A	0.3667 ± 0.06 A	0.123 ± 0.003 A
20		0.2267 ± 0.1 A	0.3533 ± 0.09 A	0.1333 ± 0.01 A
25		0.203 ± 0.07 A	0.31 ± 0.11 A	0.09 ± 0.01 B

Different capital letters (columns) denote significant differences ($p < 0.05$) Discussion

Numerous studies had compared the cytostatic and apoptotic effects of several chemicals, plant's extracts and bacterial cell components on the growth of multiple normal and tumor cell lines. It has been demonstrated that porins are able to induce a marked increase in the level of intracellular Ca^{2+} and in the activity of PLA_2 in various human cells (PMNs and endothelial and mesangial cells) [11]. In conclusion, by allowing a substantial intracellular influx of Ca^{2+} associated with an increase of PLA_2 activity, pathological embedding of porins in the plasma membranes of eukaryotic target cells may act as an effective mechanism of signal transduction responsible for the activation of the genetic program of apoptotic death.

The difference in the response to various chemical and biological substances shown by normal and cancer cell lines and even among cancer cell lines themselves could be explained by the fact that each cell type has its own characteristic metabolic behavior and that each cell line expresses its special and distinct receptors on cell surface which governs the rules and selectivity for binding [12]. In addition, DNA of tumor cells was found in a relaxant shape and the DNA molecule was found in unstable figure because of the longer distance between the H-bonds which connect the two strands of the DNA molecule compared to normal cells and this will subsequently make the DNA molecule of tumor cells more susceptible to the action of DNA interacting compounds [13].
In conclusion, incubation with multiple concentrations of porins and for different periods of time had resulted in an antiproliferative or apoptotic effect indicated by lower proliferation index for treated cells compared to the control ones. It is also shown that when the three cell lines were compared, the HFFC showed more regular antiproliferative effect compared to the other cell lines but after incubation with porins for 24 hrs consistent with the role of this subcellular component being inflammatory and damaging to host tissue cells.
It is worthy to suggest that further studies are required for understanding the exact role of porins as proinflammatory and apoptotic inducing agent who might open new avenues for better understanding of prokaryote – eukaryote interaction and anti cancer therapy.

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