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DIFFERENTIAL EFFECTS ON CASPASE MEDIATED APOPTOSIS OF HELA CELLS INDUCED BY DIFFERENT *PSEUDOMONAS AERUGINOSA* CULTURE FRACTIONS

Carmen Iordache[1], Coralia Bleotu[2], Alina Holban[2], Mariana Lixandru[1], Ani Cotar[1], Veronica Lazar[2], Felicia Antohe[3], Mariana Carmen Chifiriuc[1,2]

[1]National Institute for Research in Microbiology and Immunology, Cantacuzino, Spl. Independentei 103, cod 060631, Bucharest Romania
[2]University of Bucharest, Faculty of Biology, Microbiology Immunology Department, Alea. Portocalelor 1-3, Bucharest Romania
[3]Institute of Cellular Biology and Pathology "N. Simionescu", 050568, Bucharest, Romania

ABSTRACT : The aim of this work was to investigate intracellular survival of *Pseudomonas aeruginosa* clinical strains in non-phagocytic, epithelial cells and the capacity to induce apoptosis of the host cell using qualitative (evaluation of virulence factors expression, adherence to HeLa cells) and quantitative assays (invasion assay, qRT-PCR). Taken together the results demonstrated that all tested strains adhered to HeLa cells, exhibiting a diffuse, aggregative or mixed diffuse-aggregative pattern with 20-70% adherence rates. The assays confirmed that *P. aeruginosa* had the ability to enter and invade the epithelial, non-phagoytic cells, probably due to the pores forming enzymes (hemolysins, lecithinase, lipase) released by the analyzed strains. Two strains proved the ability to induce apoptosis of HeLa cells, inducing an increased expression of caspase 3 and Bax genes, correlated with a decreased level of the anti-apoptotic factor Bcl-2. The apoptotic gene expression levels were also analyzed for HeLa cell treated with cell free culture supernatants, responsible for decreased expression levels of caspase 3 and caspase 9 genes. The results corroborate well with other reported data, proving the ability of these bacteria to penetrate non-phagocytic cells and to induce changes at molecular level, including apoptosis. The cell free culture supernatants did not demonstrate the ability to induce apoptosis by caspase – mediated pathways, leading us to the hypothesis that the host cells apoptosis is requiring cell to cell contact, probably implicating the activation of a type III secretion system responsible for the intracellular release of pro-apoptotic factors.
Key words: apoptosis, *Pseudomonas aeruginosa*, caspases, soluble fraction

INTRODUCTION

Besides classical effects described during eukaryotic cells infection with bacteria (internalization or phagocytosis of bacteria, defensine secretion, release of cytokines or reactive oxygen species), there are also mechanisms in which some bacterial strains could induce apoptosis in the host cells (Weinrauch et al., 1999). Apoptotic cells, systematically inactivate, disassemble and degrade their structural and functional components in order to silently eliminate them. Although in many infectious, apoptosis of the mammalian cells could help the host to defense against bacteria, reported data showed that bacterial pathogens induce apoptosis of the host cell in order to facilitate their access to different tissues. Bacteria – induced apoptosis is a consequence of some complex interactions between host cell proteins and bacterial proteins. It was demonstrated that bacteria are able to activate some pro-apoptotic proteins, as caspases, to inactivate anti-apoptotic proteins, as NFkB or MAP kinases, or to stimulate the formation of ligand - receptor sinaling complexes at the surface of the infected cells.

Pseudomonas (P.) aeruginosa is an opportunistic human pathogen, resistant to many antibiotics, causing a wide range of clinical symptoms and infections, frequently in immunocompromised patients. Epithelial cell apoptosis induced by *P. aeruginosa* contribute to the host defense. Bacteria - induced apoptosis occurs via a specific mechanism that could be useful for the infected organ, allowing host cells to engulf apoptotic bodies containing bacterial entities. Since apoptosis is not accompanied by local inflammation, this mechanism allows the removal of bacteria from the lungs or other infected organs, without the occurrence of inflammation negative effects. In contrast, *P. aeruginosa* internalization without activation of apoptotic mechanisms, allows bacteria to block phagosome maturation, to survive intracellularly and even to multiply, protecting bacteria against any immune response (Grassm'e H et al., 2000).
The aim of this work was to investigate the intracellular survival of *P. aeruginosa* clinical strains in non-phagocytic epithelial cells and the influence of *P. aeruginosa* bacterial culture and of soluble molecules accumulated in the cell free culture supernatants in order to establish their ability to induce epithelial cells apoptosis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. aeruginosa* pathogenic strains isolated from different clinical sources from patients admitted to the Institute of Heart Disease Prof. C.C. Iliescu were used. Isolates were identified using automatic Vitek II system. Subsequently strains were maintained in the "*Culture Collection of Microbiology Laboratory"* of the Faculty of Biology. For further experiments, the bacteria were grown for 18 hours in nutrient broth.

Cell free cultures

Soluble mediators were collected after 18 hours from *P. aeruginosa* bacterial cultures inoculated in nutrient broth. Bacterial suspension was centrifuged at 5000xg and the resulting bacterial supernatant was sterilized by filtration (Φ = 0.22 mm). Bacterial filtrates were kept at 4 ˚ C until use.

pH adjustment

Since eukaryotic cell metabolism is influenced by pH fluctuations, in order to equalize conditions of the experiment, pH of bacterial culture was adjusted to 7.2, -7.4 using 10N NaOH or 0.1N HCl solutions.
Cell culture. The HeLa cell line was cultivated at 37^0C with 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 50 µg/ml penicillin and 50 µg/ml streptomycin. Cultures at 80% confluence were routinely split 1:5 in 10 cm culture dishes.
Evaluation of virulence factors expression. Bacterial strains grown for 18 hours in nutrient broth were evaluated for the following virulence factors expression: haemolysins, other pore forming toxins (lecithinase, lipase), proteases (caseinase, gelatinase), DNA-se and mucinase. Detection of haemolysin production was performed by spotting the strains in 5% sheep blood agar medium. After incubation at 37^0C for 24h the colorless area around the colony revealed the released haemolysis activity. For the investigation of lipase production the strains were spotted on 1 % Tween 80 agar as a substrate and incubated at 37^0 C until 7 days. An opaque (precipitation) zone around the spot was registered as positive reaction; for lecithinase production, the cultures were spotted into 2.5% yolk agar and incubated at 37^0 C for 7 days. An opaque (precipitation) zone around the spot indicated the lecithinase production; the DN-ase production was studied on DNA supplemented agar. The strains were spotted and after incubation at 37^0 C for 24 h, a drop of HCl 1N solution was added upon the spotted cultures; a clearing zone around the culture was interpreted as positive reaction; the caseinase activity was determined using 15% soluble casein agar as substrate. The strains were spotted and after incubation at 37^0 C for 24 h, a white precipitate surrounding the growth indicating casein proteolysis; the mucinase production was determined using pig stomach mucine (final concentration of 1%) incorporated in brain heart agar with 2% NaCl. The strains were spotted and incubated at 35^0 C for 48 h; the enzyme activity was noticed by the presence of a clear area around the culture spot; the clear area became more evident when some Lugol drops were poured upon. For production of gelatinasse the strains were spotted in the solid medium and incubated with gelatin 24h at 37^0C. The presence of a precipitation area around the culture growth indicated proteolysis of gelatin by an active gelatinase.

Adherence to HeLa cells. For the adherence assay, Cravioto's adapted method was used (Cravioto et al., 1979). Briefly, the HeLa cell monolayers were washed 3 times with PBS and 1 ml of fresh medium without antibiotics was added to each well. Suspension of *P. aeruginosa* from bacterial mid-logarithmic phase cultures grown in nutrient broth was adjusted to 10^7 CFU/ml and 1 ml was used for the inoculation of each well. The inoculated plates were incubated for 2 hours at 37ºC. After incubation, the monolayers were washed 3 times with PBS, briefly fixed in cold ethanol (3 min), stained with Giemsa solution (1:10) for 20 min. The plates were washed, dried at room temperature overnight, and examined microscopically (magnification, ×2500) with immersion oil to evaluate the adherence index and patterns. Three distinct adherence patterns could be identified: localized adherence, in which bacteria attach to and form microcolonies in distinct regions of the cell surface; diffuse adherence, in which bacteria adhere evenly to the whole cell surface, and aggregative adherence, in which aggregated bacteria attach to the cell in a stacked-brick arrangement.

The adherence index was expressed as the ratio between the number of the eukaryotic cells with adhered bacteria and 100 eukaryotic cells counted on the microscopic field.

Invasion assay. Bacterial suspension were inoculated on HeLa cells grown on two T25 well plates and incubated at 37^0C for two hours. After that, in one plate 500µl/well of 1mg/ml gentamycin solution in PBS) was added to kill extracellular bacteria, and further incubated for another hour in the same conditions. The other plate was maintained in standard conditions (in the initial culture medium). After incubation, plates were washed 3 times with PBS and permeabilized with 0.1% Triton X-100 for 5 min, at 37^0C. Serial dilutions of suspended cells harvested from the plate wells (with and without gentamycin) were seeded on solid media in order to establish the adhesion, plus invasion indexes (C.F.U./ml) as describeb by Lazar et al., 2002.

RNA extraction and qRT-PCR reaction. To demonstrate the bacterial changes induced at molecular level in the designed experimental model, approximately $5x10^5$ HeLa cells were seeded in T25 plates. After 24 hours, the cell monolayer was covered with bacterial culture harvested after 18 hours and diluted 1:3 in eukaryotic cells specific medium (experimental model 1) or cell free supernatants (experimental model 2) and incubated at 37^0 C. For the first experimental model, the contact time was 2 hours while for the second experimental model the incubation time was 24 hours.

After the specified incubation times, 6 ml culture medium were collected from each plate (4 ml HeLa cell culture + 2 ml bacterial suspension / bacterial cell free culture supernatants) and transferred into 15 ml centrifuge tubes. Cells adhered to the bottom plate were detached by enzymatic treatment by incubation with 1 ml 0.25% trypsin (preheated to 37˚C), 5 min at 37 ˚C followed by vigourous mechanical pipetting for several times. Cell suspension was added into the same centrifuge tubes. The suspensions were then centrifuged 5 minutes at 300xg. The cell sediment was further used for RNA extraction. Total RNA extraction was performed using *SV Total RNA Isolation System Z3105* (Promega, Madison, USA) and reverse transcription was performed using MultiScribe Reverse Transcriptase Kit (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems, California, USA) following manufacturer's protocol.

50ng/ml cDNA from each sample was used in real time PCR reaction. Real Time PCR was carried out on ABI 7300 Real Time PCR System using Taqman Universal PCR Master Mix (Applied Biosystems) and pre-validated Taqman Gene Expression Assays kit (Applied Biosystems), with human Bcl2 (Assay ID: Hs00153350_m1); Bax (Assay ID: Hs00180269_m1); Mcl1 (Assay ID: Hs03043899_m1); caspase 3 (Assay ID: Hs00234387_m1); caspase 9 (Assay ID: Hs00154260_m1) as target gene and human GAPDH as endogenous control (Assay ID: Hs99999905_m1). Each sample was done in triplicates. Results were analyzed with RQ study software (Applied Biosystems). The $\Delta\Delta C_T$ method recommended by the manufacturer (Applied Biosystems) was used to compare the relative expression levels of pro-/anti-apoptotic mRNA. Expression of interest genes was normalized based on the levels of mRNA for human GAPDH. The normalized gene expression level of HeLa cells was set as 1, to which the expression levels of samples were compared and then presented as fold changes. The gene expression levels were plotted as log10 values, therefore, the expression level of the calibrator samples appear as 0 in the graph.

RESULTS AND DISCUSSION

P. aeruginosa, regarded until recently as an exclusively extracellular pathogen, express virulence factors that confer it the ability to adhere and invade eukaryotic cells. *In vitro* studies on different types of cell lines showed invasive capacity of these bacteria (Chifiriuc et al., 2008). Our previous studies (Chifiriuc et al., 2008) showed that some *P. aeruginosa* strains proved the ability to invade the epithelial, non-phagocytic cells. The examination of fluorescence labeled HeLa cells infected with *P. aeruginosa* confirmed the results of the quantitative assay of viable, internalized bacteria. *P. aeruginosa* invasion was consistent with active cellular events such as the cytoplasm wrinkling and the induction of thin, lamellar membrane pseudopodes formation. The present results confirm the reported data on HeLa cells. The qualitative assay of the bacterial adherence to the cellular substrate demonstrated that all tested strains adhered to HeLa cells, exhibiting a diffuse, aggregative or mixed diffuse-aggregative pattern and 20-70% adherence rates (Table no. 1). The quantitative viable cell counts assay sustained the previous results and confirmed that *P. aeruginosa* strains had the ability to invade and multiply in the epithelial, non-phagoytic cells (Fig. 1). These data are also supported by the demonstration of membrane pores forming enzymes' presence (hemolysins, lecithinases, lipases) providing the ability of these bacteria to enter the host cells (Table no. 1).

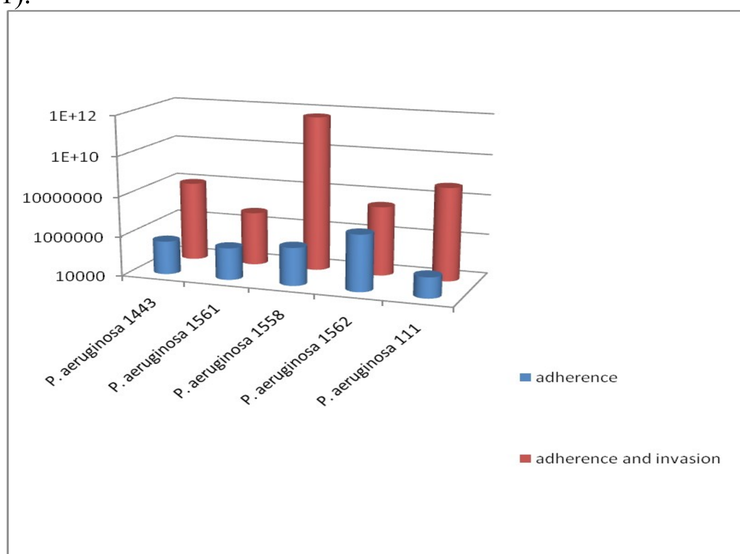

Figure 1. The comparative level of the adherence ability and adherence and invasion ability of the *P. aeruginosa* strains to the cellular substrate represented by HeLa cells

The demonstration of adhesion ability, invasion and survival of bacteria in infected HeLa cells led us to the investigation of the possible changes occurred at molecular level in infected eukaryotic cells. The gene expression of Bcl-2 family namely the anti-apoptotic proteins Bcl-2 and Mcl-1, and the pro-apoptotic protein Bax was evaluated. In addition, expression of two genes that encoded for caspases 3 and 9 were also quantified.

The qPCR results, regarding the gene expression were different for the tested strains for the present study. In case of whole viable bacterial culture, only 2 out of 6 analyzed strains proved the ability to induce apoptosis of HeLa cells, showing an increased expression of caspase 3 and Bax genes that correlates with the decreased level of the anti-apoptotic factor Bcl-2 (Fig. 2)

Table no. 1. Expression of virulence soluble factors in *P. aeruginosa* strains

Strain no.	Adherence Pattern and index	DN-ase	Caseinase	Amilase	Lecithinase	Lipase	Mucinase	Haemolysins	Gelatinase
P. aeruginosa 1443	diffuse-aggregative 70%	++	+++	+++	+	-	-	++	-
P. aeruginosa 1561	diffuse-aggregative 20%	++	+++	+++	+	+	±	++	-
P. aeruginosa 1558	Aggregative 30%	-	-	+	-	-	-	+	+
P. aeruginosa 1562	Diffuse 40%	-	++	+++	-	+	-	+	-
P. aeruginosa 111	Diffuse 30%	-	+	++	-	+	-	++	-

-” absence of enzyme, “±” low level enzyme expression, “+” enzyme expression, “++”, “+++” high level enzyme expression

Caspase 9 is part of the initiation caspases pathway, and its activation will induce activation of specific effector caspases, like caspase 3. The low expression of initiator caspase 9 in the HeLa infected cells (Fig. 2) as compared with the controls, may lead to at least two hypothesis: i) the apoptosis was prompted by initiating caspases independent pathway in which the effector caspases (including caspase 3) were activated by other pro-apoptotic factors or ii) apoptosis induced after 2 hours from bacterial infection spreads rapidly once initiated, and reached the final stage, when the caspase 9 activity was no longer needed. Chai et al. (2008) investigated the influence of *P. aeruginosa* infection on monocytes’ apoptosis, by quantifying the expression of certain genes, such as those for Bcl-2, Bax, caspases 3 and 9 using RT-PCR and Western blotting methods. The results proved that *P. aeruginosa* could induce apoptosis in eukaryotic cells in a time and dose dependent manner, by Bax gene over expression and Bcl-2 gene inhibition, which induced high levels of cytochrome c release, and increased levels of caspases 3 and 9 gene expression in human cell line U937.

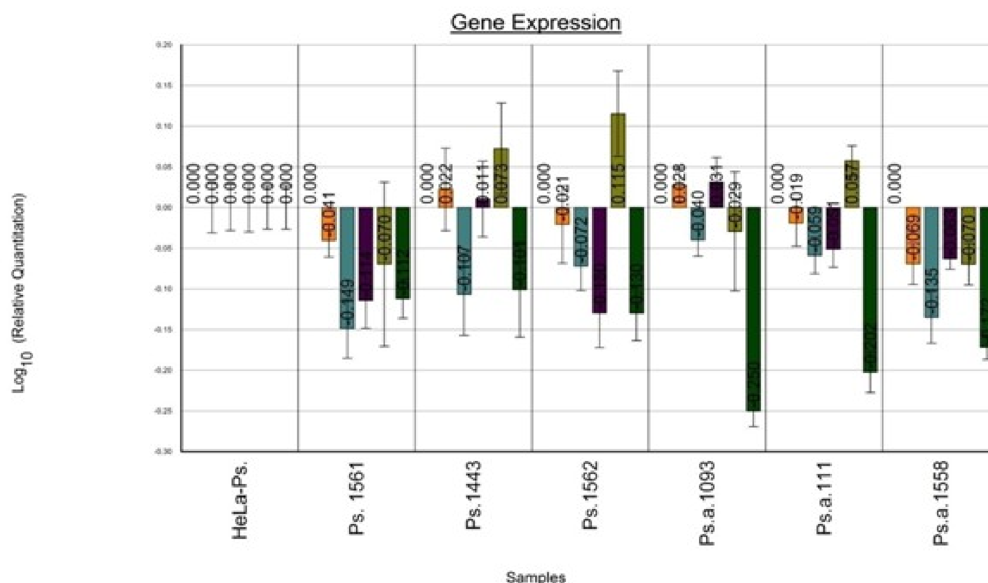

Figure 2. Gene expression (caspase 3-orange, caspase 9-lavander green, Bax-violet, MCL 1- light green and Bcl-2 –dark green genes) in HeLa cell infected with *P. aeruginosa* whole culture

Numerous other results showed that apoptosis of eukaryotic cells could be induced not only by the presence of whole bacterial culture, but also by some soluble bacterial compounds. For example pyocyanin, an exopigment produced by some *P. aeruginosa* strains dramatically accelerated neutrophil apoptosis both *in vivo* and *in vitro* as well, adversely affecting the level of host defense and favoring bacterial persistence (Bianchi and Prince, 2008). Subsequent studies performed on neutrophils demonstrated that pyocyanin could induce lysosomal dysfunction, mitochondrial membrane permeability, activation of caspases, and destabilization of MCL-1 protein, resulting in immediate onset of apoptosis (Prince et al., 2008).

Autoinducers like acyl homoserin lactone type are signaling molecules synthesized by Gram-negative bacteria which were used to coordinate gene expression through a process called "*quorum sensing*" . Recent observations have shown that autoinducers are involved not only in controlling gene expression but also in altering gene expression in mammalian cells. These alterations include modulation of proinflammatory cytokines expression, phagocytosis and apoptosis (Tateda, 2003).

For example, recent data showed that N-(3-oxododecanoyl)-L-homoserine lactones (OdDHL), a signaling molecule produced by *Pseudomonas* specifically induce apoptosis of different cell types, including macrophages and neutrophils (Tateda, 2003), in a manner dependent on the mediator concentration (12-50 micro M) and incubation time (1-24 h). OdDHL cytotoxicity was also observed in neutrophils and monocytes cellular lines U-937 and P388D respectively, but not in epithelial types cell lines CCL-185 and HEp-2, which demonstrated the tissue specificity action. HeLa cells treated with OdDHL showed morphological changes specific to apoptotic cells, accompanied by other changes which confirm cells entry in apoptosis (activation of caspases 3 and 8 genes, DNA fragment associated with histones). These data demonstrate that OdDHL is a bioactive molecule in eukaryotic systems and may form the basis for a new paradigm for the new class of compounds with antiproliferative action (Li et al., 2004).

Taking into accoun the reported data concerning the influence of soluble molecules accumulated in the *P. aeruginosa* supernatants on eukariotic cells apoptosis, in our study the gene expression levels were also analyzed for HeLa cells treated with cell free *P. aeruginosa* culture supernatants in order to establish if they are also able to induce apoptosis. Gene expression level for caspase 3 and 9 were in this case lower as compared with control (Fig. 3). Only Bax gene was over expressed, while anti-apoptotic genes (MCL-1 and Bcl-2) were expressed at lower levels than controls. The decreased level of caspases expression could favour the hypothesis that different bacterial molecules from cell free culture supernatants either did not induce apoptosis, or if any apoptosis occurs in HeLa cells, it may be induced by a caspase-independent pathway. Further studies are in progress to confirm these data.

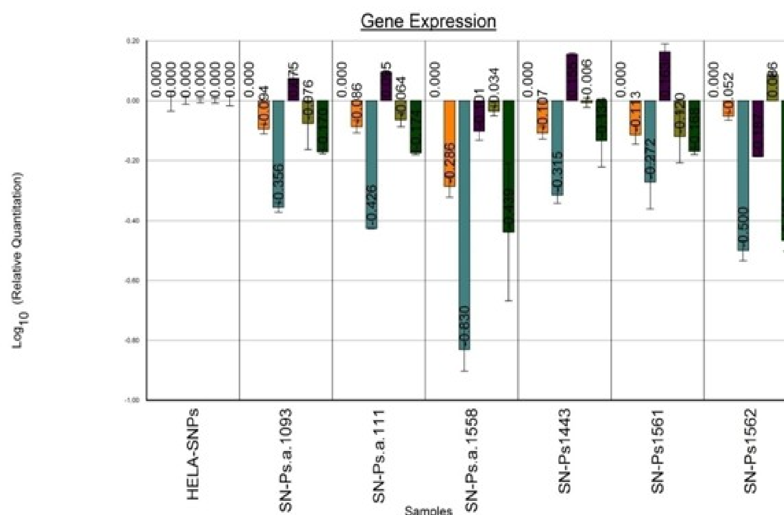

Figure 3. Gene expression (caspase 3-orange, caspase 9-lavander green, Bax-violet, MCL 1- light green and Bcl-2 –dark green genes) in HeLa cell treated with *P . aeruginosa* cell free culture supernatant

Conclusions

The results corroborate very well with recent published literature, showing that *P. aeruginosa* is not exclusively an extracellular pathogen, having also the ability to penetrate non-phagocytic HeLa cells, and to induce changes at molecular level that may initiate apoptosis. The present study did not prove the ability of soluble molecules accumulated in the culture supernatants to induce apoptosis by caspase – mediated pathways, demonstrating that the initiation of host cells apoptosis by this pathway requires a direct cell to cell contact, probably implicating the activation of a type III secretion system responsible for the intracellular release of pro-apoptotic factors. However further studies are needed in order to elucidate the caspase-independent activation pathway of host cells apoptosis.

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