Pyocyanin-induced neutrophil apoptosis modulated by the presence of a bacterial sonicate and LPS in vitro

Dr. Chateen I. Ali Pambuk

College of Dentestry , University of Tikrit

Abstract

The paradigm of pathogen-driven neutrophil apoptosis is exemplified by the *Pseudomonas aeruginosa* toxic metabolite, pyocyanin that induce a dramatic acceleration of neutrophil apoptosis in Cystic fibrosis lung disease. Whether the course of pyocyanin-induced neutrophil apoptosis can be modulated or not by bacterial cell wall product present significantly in inflammatory foci is unknown.

Materials and methods : Purification of neutrophils and preparation of exotoxin pyocyanin, bacterial sonicate and LPS-HE were performed according to many standard methods. Apoptotic neutrophils were identified microscopically based on morphological changes characteristic of apoptosis.

Results: Pyocyanin-induced neutrophil apoptosis was significantly delayed at earlier time points of 6 hr. in the presence of bacterial sonicate recording of a neutrophil apoptosis rate of 24.8% when compared to 42.3% of apoptosis in the absence of bacterial product. Combinatorial preincubation of LPS-HE with sonicate cell wall product more decreased synergistically the apoptotic rate to reach 17.1% at the same time point of treatment.

Conclusions: Our findings suggest that bacterial sonicate most probably cell wall components are an antiapoptotic stimulus for delaying pyocyanin-induced neutrophil apoptosis in vitro, thereby may contribute to attenuate the neutrophilic inflammation in lungs of Cystic fibrosis patients.

Keywords : Pyocyanin, Pseudomonas aeruginosa, Neutrophil apoptosis, LPS .

Introduction

Neutrophils are the most abundant cell type among circulating white cells and constitute the first line of host defense against invading microorganisms. Neutrophils have a very short life span in circulation (6–8 h) and in tissue (1–4 days). In inflammatory site Pathogens are removed by reactive oxygen species and proteases in neutrophil phagosome. When neutrophils are overactivated, these toxic products can also be released extracellularly and cause substantial bystander tissue damage. [1,2].However resolution of acute inflammation involves the programmed cell death (apoptosis) of infiltrating these inflammatory cells, predominantly neutrophils. Perturbation of neutrophil apoptosis during bacterial infection has focused largely on the delay of this process in response to bacterial products, notably LPS [3].During inflammation, neutrophil apoptosis can be markedly delayed by granulocyte/macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), interferon-γ, or toll-like receptor agonists, such as lipopolysaccharide (LPS). [4-6]

The concept of pathogen-driven neutrophil apoptosis was identified as a mechanism of immune evasion. In this respect it is demonstrated that a *Pseudomonas* exotoxin, pyocyanin, induced dramatic acceleration of neutrophil apoptosis [7]. *P. aeruginosa* also generates highly diffusible, toxic, secondary metabolites, known as phenazines, and is the only organism to produce a specific phenazine, named pyocyanin [8]. However, pyocyanin-induced apoptosis is associated with production of reactive oxygen intermediates (ROI), which may have a specific role in induction of neutrophil apoptosis [9], and a central role for oxidative stress in pyocyanin-induced apoptosis was identified in the nematode, *Caenorhabditis elegans* [10].The Induction of neutrophil apoptosis by *Pseudomonas aeruginosa* exotoxin pyocyanin that observed by Usher et al.[7] also confirmed by our previous study [11] . Furthermore in pneumonia mice model, causing extensive tissue damage. [12] The induction of inappropriate or premature apoptosis of the host innate immune cells, particularly neutrophils by pyocyanin culminates in the depletion of cell numbers and function and thus impairing host defense and favoring bacterial persistence [12]

The main objective of the present study was to further elucidate in vitro effect of bacterial cell wall components i.e. bacterial sonicate and LPS-HE (Lipopolysaccharide heat extracted) upon pyocyanin-induced neutrophil apoptosis. In the sense of a providing evidences that, firstly: an antiapoptotic factor LPS [13] recently failed to inhibit neutrophil apoptosis induced by proapoptotic factor pyocyanin [7], and secondly : to address an antiapoptotic effect of bacterial cell wall products in

vitro, which are present in inflammatory site in infected lung with *P. aeruginosa* in chronic pulmonary inflammations .

Materials an d methods

Bacterial isolate:

Psudomonas aeruginosa isolate used in the present study was previously isolated from cystic fibrosis patient (provided kindly by the hospital laboratory of Kirkuk General Hospital . Bacterial isolate was kept on nutrient agar slants(Oxoid,U.K) at 4°C. For all following experiments each freshly streaked out bacteria were grown for 24 hrs. at Loria B (LB) agar or Trypticase soy broth (TSB) (HIMEDIA Lab. India), and then suspended in sterile normal Ringers solution to 0.1 U at an optical density of 650nm (A_{650}) giving 10^8 CFU/ml ;150 µl of this suspension containing approximately 1.5×10^7 CFU as measured by dilution plating[11].

Isolation of neutrophils

Peripheral blood was collected in sterile heparinized tubes from healthy adult volunteers. Heparinized whole blood (4ml) was mixed with 5 ml supplemented RPMI-1640 or PBS. The mixture was carefully layered over 4 ml of ficoll-hypaque (lymphocyte separation medium; Flow laboratories : UK) in a 20- ml conical centrifuge tube and centrifuged for 20 min at 2000 rpm (900 g, 4°C), the layer between ficoll and the upper layer (containing RPMI-1640 and serum) containing mononuclear cell fraction was removed[14].

Neutrophil Purification

The red blood cells and polynorphonuclear cells left by ficoll-Hypaque density gradient centrifugation at the bottom were removed and RBCs were removed by hypotonic lysis. After centrifugation at 1500 rpm for 10 min. the supernatant was removed and the deposited cells were suspended carefully with 5 ml of heparin-HBSS solution [15]. Neutrophils were isolated and resuspended in HBSS-H, and stored on ice in Hanks buffered salt solution containing 0.1 %(wt/vol) dextrose. The purity and cell viability of neutrophils were consistently > 98% as assessed by Trypan Blue exclusion assay [16]. Purified neutrophils were suspended at concentration of 2×10^5 / ml in RPMI-1640, with 10% FCS and with penicillin and streptomycin (100 U/L). the age of neutrophils in culture was calculated, designating this stage as time 0. Neutrophils were incubated in 96-well Falcon plates at 37°C to be utilized in further experiments [7].

Pyocyanin preparation.

For experimental purpose , 100 µl of bacterial growth was transferred on to 3.5-cm-diameter Brain Heart infusion (BHI) (HIMEDIA Lab. India) agar plate containing 4 ml BHI agar. The plate was incubated for 24 hrs. at 37°C. the P. aeruginosa lawn-bearing agar from each plate was diced and extracted into chloroform and vacuum-dried to a powdery residue. The residue was dissolved in chloroform

and acidified with an equal volume of 0.1 M HCl. The red acidic form of pyocyanin was converted into the blue form using 0.5 M NaOH. This blue form of pyocyanin, now in the aqueous phase, was extracted into chloroform and this cycle repeated 3 times. The blue form of pyocyanin which was extracted into chloroform the last time was vacuum dried. The pyocyanin thus partially purified was stored in ethanol in dark at –20°C. The concentration of pyocyanin quantitated by utilizing its known absorption spectrum and absorption coefficient values as elucidated earlier by Watson *et al.*. Before use, the ethanol solvent was removed and when completely dry, pyocyanin was reconstituted in RPMI-1640 and used in a final concentration of 55 µM [17,18].

Assessment of neutrophil apoptosis

Neutrophils (2×10^5 cells/ml) were incubated in the presence or absence of exotoxin pyocyanin or LPS-HE (neutrophils preincubated with LPS for 1 hr. before adding pyocyanin), and bacterial sonicate at 37°C for 18 h in RPMI 1640–10% FBS in Falcon plates. After incubation, cells were and stained with Giemsa stain. A minimum of 300 neutrophils/slide was examined by light microscopy on duplicate and apoptotic neutrophils were identified based on morphological changes characteristic of apoptosis, such as chromatin condensation, formation of rounded nuclear profiles, cell shrinking, membrane blebbing, and presence of cytoplasmic vacuolization [19].

Bacterial sonicate

Psudomonas aeruginosa sonicate was prepared by suspending the bacteria in PBS and sonicating three times for 45 s on ice with a sonicater (Cole-Parmer Instrument Co: Chicago, US) at 400 W. The sonicates were centrifuged at 20,000 rpm for 30 min at 4°C. Then, the supernatant was filtered through a 0.2-µm filter and stored in aliquots at −20°C.[20] . From the results of the previous experiments the most suitable concentration of bacterial sonicate to be used in the stimulation experiments was chosen: 1×10^7 bacteria/ml of neutrophil suspension at multiplicity of Infection (MOI) of 50.

Lipopolysacharide heat extract (LPS-HE) preparation

One a prepared aliquot of bacteria containing 2×10^7 bacteria per 10 µl (stock solution) was previously grown on TSA .The bacterial suspension in physiological saline was sonicated and heated for 1 hr at 100° C and centrifuged at 20.000 rpm for 20 min at 4° C. The supernatant after dialysis was used as LPS-HE . Aliquots of 100 µl equivalent to 2×10^6 bacteria/ml of neytrophil suspention were used in experiments [21].

Statistical Analysis

All data are presented as the mean _ SE. ANOVA was used to identify

initial statistically significant differences in apoptotic rates. Differences were judged statistically significant when *p* values were ≤ 0.05.

Results

The influence of LPS-HE on pyocyanin induced neutrophil apoptosis:

To gain insight into the predictive interaction between of LPS-HE and pyocyanin induced neutrophil apoptosis, time course experiment at 3,6,9,12 and18 hrs. were performed in the presence of aliquots finaly gave a concentration approximately 55µM of the exotoxin pyocyanin previously shown to have biological effect to induce neutrophil apoptosis [7,11]. The results revealed morphological alterations of neutrophils indicative for apoptosis i.e cell rounding, membrane blebbing, loss of cytoplasm as early as 3 hr. after incubation of pyocyanin with 7.4 % of neutrophil apoptosis .Furthermore, apoptosis significantly increased in the following hrs. to reach 42.3 % at 6 hr. time point (P≤0.05) when compared with control (9.7%). A complete cell death (90%) was observed within 12 hr. after incubation. In the presence of LPS-HE no significant change was observed as an antiapoptotic factor recording 10.7%, 36% and 89.5% of neutrophil apoptosis at 3,6 and 12 hr. exposure, respectively. After 12 h in culture, LPS-HE remarkably retarded constitutive apoptosis (10.1%) as compared with control (14.3%, $p < 0.05$) (Fig. 1). For all early time points studied (6 h and before), both control and pyocyanin-treated cell populations remained trypan blue negative. At later time points (>12 h), there was a significant secondary necrosis of pyocyanin-treated neutrophils, as assessed by trypan blue staining (data not shown).

Evaluation the effect of bacterial sonicate on pyocyanin induced neutrophil apoptosis

We hypothesized that the bacterial products that are present pathologically , upon the death of bacteria and apoptotic and necrotic neutrophils, at inflammatory site in cystic fibrosis patient and chronic pulmonary diseases (----)may significantly affect the course of neutrophil apoptosis namely pyocyanin induced neutrophil apoptosis . To test this the same time course experiment in the previous section was pursued in the presence of bacterial sonicate. Unlike the earlier time points of 6 hr. a significant reduction in neutrophil apoptosis (24.8%) in the presense of a bacterial sonicate were observed when compared with control , and when compared with the presence of the bacterial sonicate alone (6.7%) (Fig.2) . A complete cell death (90%) was observed within 16 hr. after incubation The same secondary necrosis of pyocyanin-treated neutrophils, as

assessed by trypan blue staining was also remarkable in this experiment.

The combinatorial effect of of LPS-HE plus bacterial sonicate on pyocyanin induced neutrophil apoptosis

Further estimation of antiapoptotic effect of bacterial sonicate this time in combination with LPS-HE were prudent . To test this the same time course experiment in the previous section was pursued in the presence of bacterial sonicate plus LPS-HE. By 6 hrs. after challenging neutrophils, more than 80% of neutrophils were survived with 17.1% of pyocyanin –induced neutrophil apoptosis was recorded ($P \leq 0.05$), while the results of the challenge without pyocyanin (LPS-HE plus bacterial sonicate alone**)** was not comparable with a control (9.5%) (Fig.3). A complete cell death (90%) was observed within 18 hr. after incubation . In addition, constitutive neutrophil apoptosis is delayed remarkably(21.9%) under the exposure to LPS-HE plus bacterial sonicate alone in combination when compared to the control(23.2%) at 24 and 20 hrs. time exposure respectively. .The same secondary necrosis of pyocyanin-treated neutrophils, as assessed by trypan blue staining was also remarkable in this experiment.

Discussion

The main aim of this study was to compare levels of Pyocyanin-induced neutrophil apoptosis in the presence of bacterial LPS and sonicated Pseudomonas aeruginosa lung isolate obtained from cystic fibrosis patient. Our hypothesis being that levels of apoptosis may be modulated by the dominant micro-organism colonizing the lung along with their products and hence influence the inflammatory milieu of the lung. We found significant differences in levels of neutrophil cell death in vitro induced by cytotoxin pyocyanin in the presence of LPS-HE plus bacterial sonicate .

The levels of Antiapoptotic factor at inflammatory site may play a key role in protecting neutrophils from accelerated apoptosis. The presence of several of these factors, most notably bacterial LPS and immune mediators , is often upregulated . This response plays an important role in maintaining cell viability and function under such conditions. Bacterial LPS have been found to have potent and long-lasting antiapoptotic effects upon neutrophils and are produced in significant quantities by *P. aeruginosa* [(7)].the results of the present study revealed of no inhibitory effect of LPS-HE on pyocyanin-induced neutrophil apoptosis. In consistent with the present work, A study performed by Usher et. al, also found the same result [(7)].

Regarding the constitutive neutrophil apoptosis our results clearly showed a remarkable but nonsignificant ($p > 0.05$) retardation after 12 hr. incubation of neutrophils with LPS-HE when compared with control. On the other hand Usher found a significant inhibition of constitutive neutrophil apoptosis after 5 hr. incubation of neutrophils with LPS. This is may be due to the method of LPS preparation and unpurity because we had a shortage in Limulus amebocyte lysate kit to evaluate the accurate concentration of LPS. Previous studies in neutrophils have also supported the suggestion that TNF-α, GM-CSF, and LPS can cause a delay in neutrophil apoptosis [22,23].

Many interesting studies have shown that lipoproteins from diverse bacterial sources activate a variety of host defense cell types, including monocytes, macrophages, neutrophils, etc., and stimulate their proinflammatory cytokine production [24]. LPS is restricted to Gram-negative organisms, whereas BLP is produced by the complete spectrum of bacterial pathogens [25]. In fact, it has been shown that lipoproteins are the most abundant protein in the cell wall of certain Gram-negative bacteria (_700,000 molecules/cell) and may outweigh LPS as a cell wall component by as much as 4-fold[26] .
In this study we described also the ability of *Pseudomonas aeruginosa* sonicate to profoundly reduce pyocyanin proapoptotic effect upon neutrophils. At a time points of 6 hr. a significant reduction in neutrophil apoptosis (24.8%) in the presense of a bacterial sonicate were observed when compared with control. In a recent study results indicated a potent inhibitory effects of the ubiquitous bacterial cell wall component bacterial lipoprotein(BLP) on PMN apoptosis [27] to a similar degree, as has been previously observed with LPS [13].

Our findings also were clear that PMN apoptosis is profoundly modified in a specific fashion by a bacterial wall components but not LPS that are present along with bacterial sonicat because LPS-HE has failed to secure neutrophils to undergo apoptosis by pyocyanin. The particular cell wall component causing the above effect need to be elucidated.

An interestining observation was the combination blockade of neutrophil apoptosis provided the greatest attenuation of the proapoptotic effect of exotoxin pyocyanin. At 6 and 9 hrs. post incubation of neutrophils with a combination of LPS-HE plus bacterial sonicate , there was a profound demonstrable delay in neutrophil apoptosis, although LPS alone clearly showed no effect upon pyocyanin-induced neutrophil apoptosis.
This is may be attributed to may be to increased concentration of LPS that affected in combination the neutrophil apoptosis constitutively and induced by pyocyanin proapoptotic effect.

The observations in the current study represent an important first step to a better understanding of the influence of bacterial sonicate and cell wall component on pyocyanin-induced neutrophil apoptosis in vitro by defining the clinical relevance and the evaluation the degrees of neutrophil apoptosis in vivo in cystic fibrosis and

chronic pulmonary inflammation. However, the observational design of the current study did not allow us to explore the particular bacterial cell wall product, and the possible mechanism(s) such as the role of specific receptors and intracellular signalling pathways in modulation of neutrophil apoptosis during chronic pulmonary inflammation caused by P. Aeruginosa pyocyanin producer . Further studies will be important to address these issues and will provide important information on the signal transduction pathways modulating neutrophil apoptosis during cystic fibrosis and chronic pulmonary inflammation .

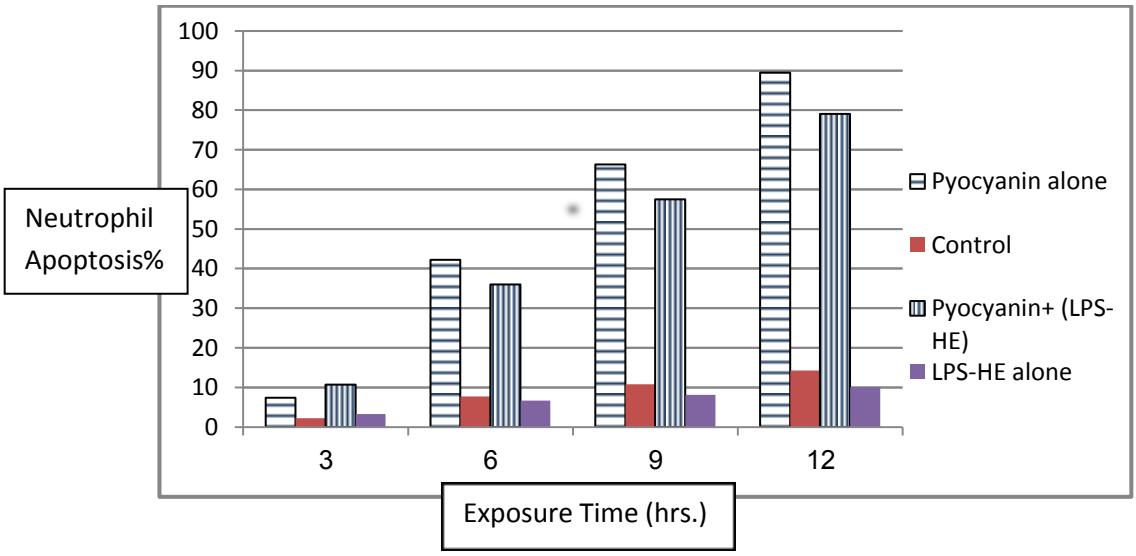

Fig. 1 : Pyocyanin-induced neutrophil apoptosis in the presence of LPS-HE. (p>0.05).

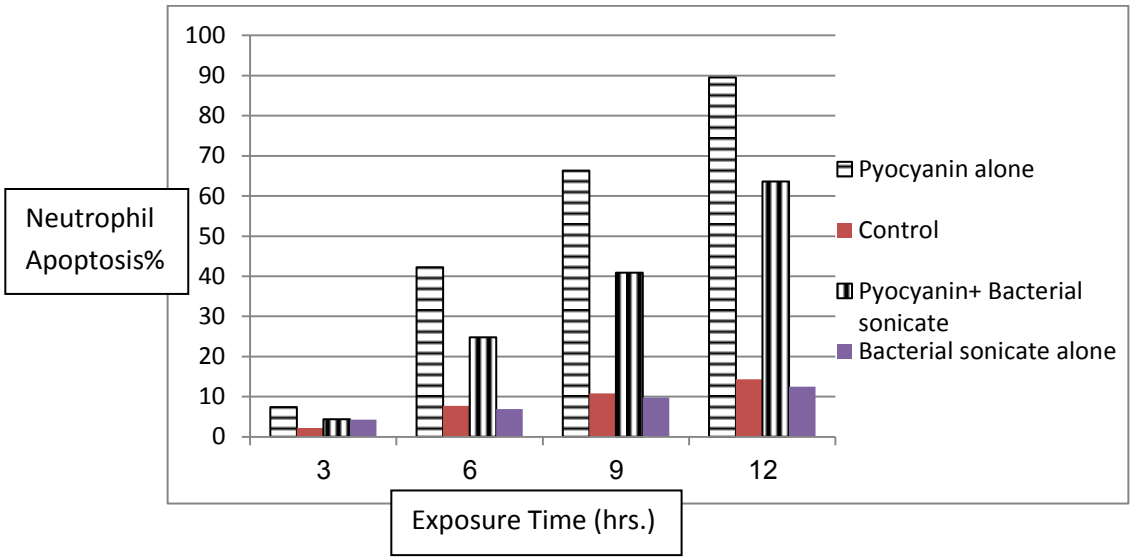

Fig. 2 : Percentile proportion of Pyocyanin-induced neutrophil apoptosis in the presence of bacterial sonicate. ($p \leq 0.05$).

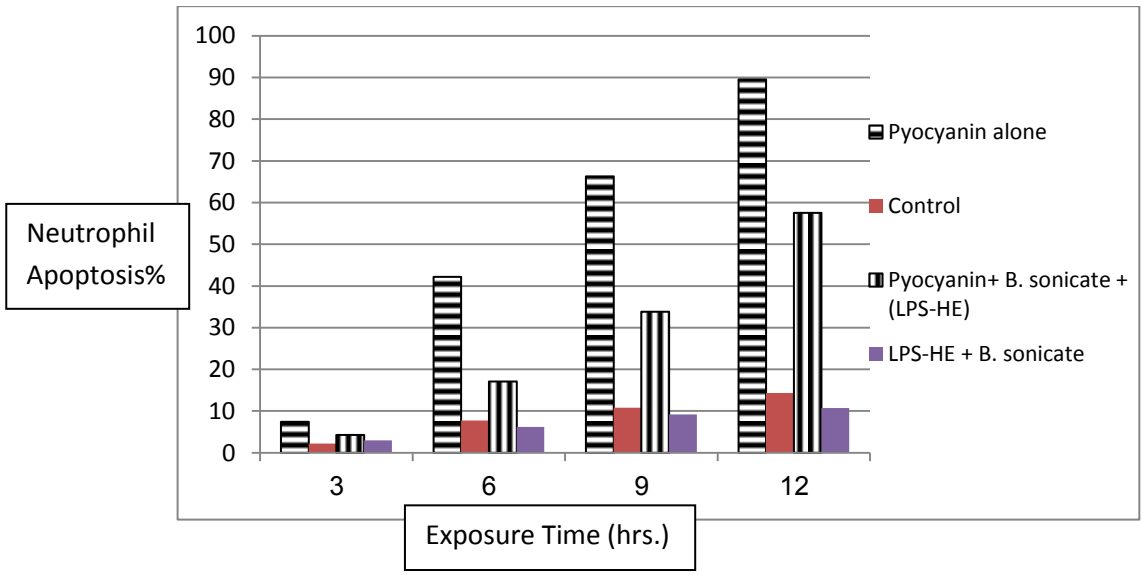

Fig. 3 : neutrophil apoptosis induced by proapoptotic stimuli exotoxin Pyocyanin in the presence of bacterial sonicate and LPS-HE in combination. ($p \leq 0.05$).

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