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ORIGINAL ARTICLE

Caspase-3-dependent Cell Death in B lymphocyte Caused by *Pseudomonas aeruginosa* Pyocyanin

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ABSTRACT

Pseudomonas aeruginosa is a Gram negative bacterium that can cause fatal infection in immunocompromised patient. This is an opportunist pathogen which is associated with some dental infections. *Pseudomonas aeruginosa* produces pyocyanin that functions as an important virulent factor in bacterial invasion. It can be identified in the lesion tissue and capable to induce cellular damage in endothelial cell, respiratory, neutrophil, and lymphocytes. B lymphocyte plays a significant role in the immune response of periapical infection; however, its cellular and molecular response to pyocyanin is unclear. **Objective:** To investigate cellular responses of B lymphocyte to the exposure of pyocyanin and the role of caspase-3 in its molecular mechanism. **Methods:** B lymphocytes (Raji cells) were cultured, and in five replications were exposed to various concentrations of pyocyanin for 24 h. MTT assay was performed to analyze the cytotoxicity effect of pyocyanin. Cell morphological analysis using phase contrast microscope were done in separate experiments. Immunocytochemical analysis was carried out for the identification of active caspase-3 protein expression, to study the mechanism involved in pyocyanin-induced cellular damage, **Results:** It showed that cell viability was decreased in pcyocyanin-treated groups. Pyocyanin induced cell death on B lymphocyte in a dose-dependent manner. Statistical analysis using ANOVA demonstrated significant difference between groups with p=0.000. Nuclear fragmentation was observed in pyocyanin-induced cell death; furthermore, caspase-3 was expressed clearly in cell cytoplasm after 24 h incubation. **Conclusion:** Pyocyanin is capable of inducing cell death on B lymphocyte. Caspase-3 may play an important role in the molecular mechanism of pyocyanin-induced cell death.

Key words: apoptosis; caspase-3 activation; nuclear fragmentation; *Pseudomonas aeruginosa*

INTRODUCTION

Pseudomonas aeruginosa is a Gram negative bacterium wich widely habitates in the environment. This species can be found in human oral cavity.[1] A study by Barben and Schmid (2004) demonstrated that *P. aeruginosa* also can be identified in dental clinics, in watering system of contaminated dental unit.[2] Despite the classification of *P. aeruginosa* as a commensal bacterium, this species is related with nosocomial infections in hospitalized patients, patients with ventilator, immunocompromised patients, and the elderly.[1,3,4]

As an opportunist pathogen, *P. aeruginosa* is atributed with some virulent components. *Pseudomonas aeruginosa* virulence factors play an important role in bacterial colonization, cell survival, and invasion to host cell.[5] One of bacterial invasion factors is pyocyanin. This cytotoxin is responsible for the bacterial invasion, induction of inflammatory reaction, and cellular damage on respiratory epithelium cells, apoptotic cell death on neutrophil, and persistent infection caused by *P. aeruginosa*.[6-8] In adition, pyocyanin also cause apoptotic cell death on neutrophil. This ability facilitate the bacterium to excuse from the host protection mechanism and lead to persistent infection.[7,8]

Apoptosis also known as programmed cell death. This occurs as cellular response to various stimulants including inflammation caused by bacterial infection.[9,10]

Death mechanism in apoptosis is regulated by activation of endoproteases named caspases that classified as initiator caspases and executioner caspases. The initiator caspase (caspases-2, -8, -9, and -10) induces activation of executioner caspases-3, -6, and -7 resulting irreversible morphological change such as DNA fragmentation.[10]

Periapical infection represents as a frequent case in dental and periodontium infection. Unsuccessful endodontic treatment for periapical lesion is caused by surviving bacteria that reside in the pulp tissue or periapical area. *P, aeruginosa* was identified from the colonies isolated from periapical lesion,[11,12] however, cellular response of host tissue needs more investigations.

Inflammatory reaction in periapical tissue ocurrs as a response to persistent bacterial infection or exposure of bacterial toxins in periapical tissue.[13] T cell as well as B cell are responsible for the immune response mechanism to antigen. Presentation of antigen-antibody complex on B cell surface stimulates T cell activation that finally initiate of B cell activation.[14] These cells play important role in immune response of periapical tissue against bacterial invasion.[15] In case of increased caspase-3 activity, B cell pr oliferation was decreased. It was demonstrated by the results of in vivo study on caspase-3-deficient mice that caspase-3 regulated homeostasis in B cell.[16]

Despite the presence of *P. aeruginosa* in periapical lesion, its mechanism to induce cellular response or tissue damage remains still unclear. This study focussed to investigate cellular and molecular mechanism how *P. aeruginosa* pyocyanin induce cell damage on B cell in order to find out part of pathogenesis of infection caused by *P. aeruginosa*. The cell viability, morphological change, and activation of caspase-3 protein were assessed in this study.

METHODS

Culture of B cell line

A commercial B lymphocyte cell line (Raji cell) was obtained from cell collection in Laboratorium Penelitian dan Pengujian Terpadu (LPPT) Unit I, Universitas Gadjah Mada, Yogyakarta. The cell was cultured in RPMI 1640 (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% FBS, 100 IU/ml penicillin and 10 µg/ml streptomycin at 37°C with 5% CO_2. Three days cell culture that reach confluent monolayer was harvested after trypsination by using 0.25% trypsin in PBS. These cells were transferred into 96-well microplate for cell viability assay. For the detection of cell morphology and expresion of caspase-3 protein, the cells were seeded in a 24-well microplate. All cultures were incubated overnight prior to the experiments.

Cell viability assay

Pyocyanin (P0046) was purchased from Sigma, St. Louis, MO, USA. That toxin powder was dissolved in DMSO at a concentration of 1 mg/mL. For cell stimulation, pyocyanin was prepared in antibiotic-free culture medium at the final concentration of 1, 5, 10, 50, and 100 µg/mL. Overnight Raji cell culture (2×10^3 cells/well) in five replicates were exposed to pyocyanin in culture medium and incubated for 24 h at 37°C with 5% CO_2. As for the negative control group, the cells were incubated with culture medium without pyocyanin. MTT assay was performed to measure pyocyanin's cytotoxicity to B cell. The absorbance value of culture supernatant of each group was measured by using microplate reader (Bio-Rad) at 570 nm with the reference wave length of 630 nm. The percentage of viable cells was determined according to the formula that was used by Coskun, et al as follows: % viability is an optical density value of treated cells devided by that value of un-treated cells, multiply by 100%.[17]

Cell morphological analysis

Raji cell was cultured on a 24-well microplate at cell density of 2×10^4 cells/well in triplicate. The culture then was incubated with culture medium containing 5 µg/mL pyocyanin for 24 h. The pyocyanin treated culture and negative control group were removed from the culture plate and then transfered to fresh microcentrifuge tubes. The cells were resuspended with fresh culture medium and placed on a glass slide for cell morphology observation under a phase contrast microscope. Nuclear shape was observed as a representative parameter for cell morphological change.

Detection of active caspase-3 protein expression

In order to investigate the involvement of caspase in cell death mechanism induced by pyocyanin, we performed immunostaining for active caspase-3 protein. Raji cell was cultured in a 24-well microplate and incubated with complete culture medium overnight. Cell stimulation with 5 µg/mL pyocyanin was performed in the following day for another 24 h at 37°C. Other cultures were cultured with culture medium only as negative control, whereas the others were treated with 0.5625 µg/ml Doxorubicin as positive control for caspase-3 expression. Following the treatment, the cells were removed from culture plate and transferred to fresh tubes. Immunocytochemistry was applied to detect active caspase-3 using rabbit monoclonal antibody anti-caspase-3 (Abcam, Cambridge, UK). The staining was done according to the previous published method with slight modifications for non-adherence cell.[18] The expression of active caspase-3 proteins was observed by using a light microscope. The protein marker was identified as brown particle in cytoplasm of activated cells.

Statistical analysis

Data of cell viability test was presented as percentage of viable cells in pyocyanin-treated and control groups. Normality and homogeneity tests were performed prior to analysis for mean difference using one way

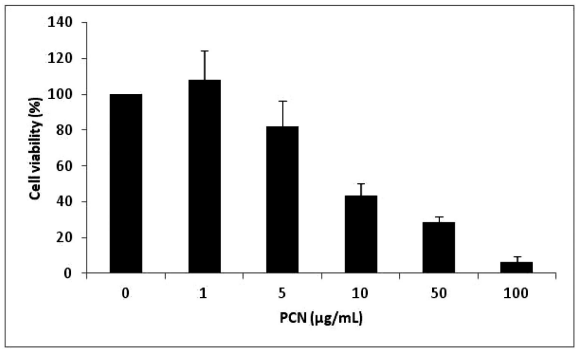

Figure 1. Cell viability of pyocyanin-treated and negative control groups after 24h incubation. Percentage of viable cells decreased at increasing concentrations of pyocyanin. The results are representative from three independent experiments.

Table 1. Summary of Bonferroni Multiple Comparison Test to percentage of viable B cells in negative control and pyocyanin-treated group

		Control	Treatment µg/mL			
		1	5	10	50	100
Control						
Treatment (µg/mL)	1	1.000				
	5	0.880	0.040*			
	10	0.000*	0.000*	0.000*		
	50	0.000*	0.000*	0.000*	0.320	
	100	0.000*	0.000*	0.000*	0.000*	0.019*

*Significance at $p<0.05$

ANOVA. Multiple Comparison using Bonferroni Least Significant Different test was done to analyze mean differences between sub-groups. Statistical significance was set at $p<0.05$.

RESULTS

Effect of pyocyanin on viability of B lymphocyte

The results of this study demonstrated that pyocyanin induced cell damage on B lymphocyte. Means and standard deviation of percentage of viable cells are presented in Figure 1.

The result of cell viability assay was then analyzed for homogeinity and normality tests using Levene test and Saphiro-Wilk test, respectively. Significance value was determined at $p<0.05$. The Levene test showed no-significant difference between groups, indicating that the variance of the data was homogeneous. Furthermore, the normality test using Saphiro-Wilk test resulted *p* values of 0.161; 0.068; 0.949; 0.675; 0.306 from the negative control, 1 µg/mL-, 5 µg/mL-, 10 µg/mL-, 50 µg/mL-, and 100 µg/mL-treated group. These results demonstrated that the data was normaly distributed.

Based on the results of homogeneity and normality tests, the data were then analyzed using one way Anova. The obtained result showed that there was significant difference on cell viability caused by the treatment of cells using pyocyanin. The significance value was 0.000 at significance level of 0,005. The following analysis was performed using least significant different test (Bonferroni). The results of this analysis are presented in Table 1.

As summarized in Table 1, it is demonstrated that there was no significant difference at $p<0.05$ between negative control and all pyocyanin-treated groups; also between 10 µg/mL pyocyanin- treated group and 50 µg/mLpyocyanin- treated group. Other 2 groups comparison showed significant difference. These results indicate that the viability of B cell was decreased with increasing concentrations of pyocyanin.

Cell morphological changes induced by pyocyanin

The result of cell morphologycal analysis using phase contrast microscope is shown in Figure 2. Untreatead B lymphocytes showed round cells with intact nuclei, but the stimulated cells showed nuclear fragmentation.

As shown in Figure 2, the cells treated with pyocyanin developed fragmentation in their nuclei. These occurred consistently in some cells in treated groups. Some of the nulei that showed morphological changes became irregular, and others were fragmented. Cellular response of B lymphocyte to the exposure of pyocyanin indicated cellular damage leading to cell death. In order to identify the mechanism of pyocyanin-induced cell death, the involvement of caspase, as molecular marker of apoptotic cell death was investigated in this study. The result showed that acitive caspase-3 was detected in pyocyanin-treated cells (Figure 3).

The result of immunocytochemical analysis demonstrate qualitative data of the expression of active caspase-3 protein on pyocyanin-treated B lymphocytes. As shown in Figure 3, active caspase-3 protein were observed in cell cytoplasm on pyocyanin (B) and Doxorubicin (C) after 24 h stimulation. It corresponds to the previous results that 24h stimulation with pyocyanin could induce cell death on B cell.

DISCUSSION

The results of this study suggest that *P. aeruginosa* cytotoxin, pyocyanin, could cause cell damage on B lymphocyte. The cytotoxicity of pyocyanin were also confirmed by previous studies on bronchial epithelium, pulmonary epithelium, and neutrophil.[6-8] Pyocyanin induced production of inflammatory mediators and caused cellular change on bronchial epithelium. Those

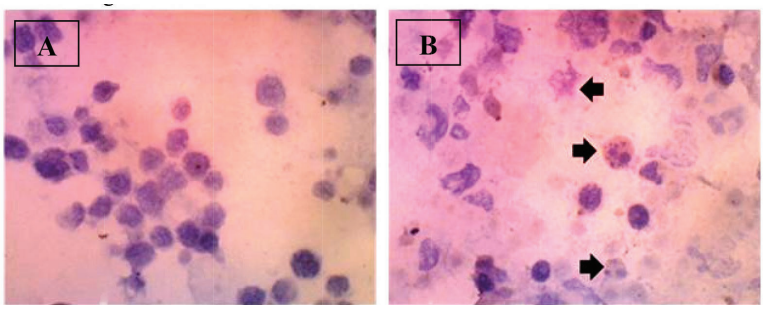

Figure 2. Photomicrograph of B lymphocytes in control and pyocyanin-treated cells. The cells were incubated for 24 h and proceeded for analysis of nuclear morphology. Intact nuclei were observed in control cells (A); and nuclear fragmentation can be identified in pyocyanin-treated cells (B). Arowhead indicates nuclear fragmentation.

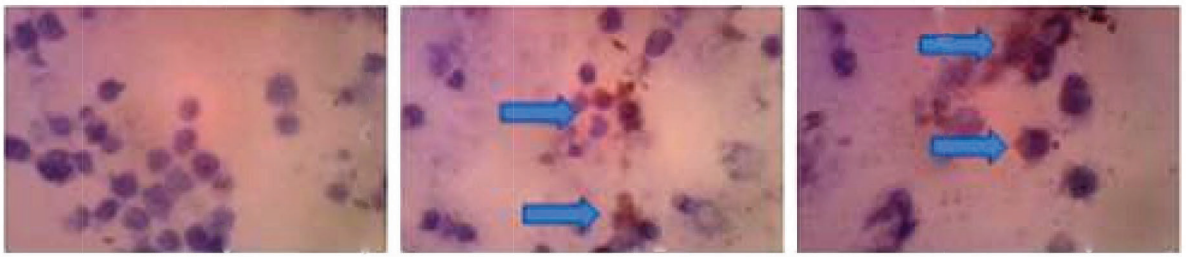

Figure 3. B lymphocyte from negative control group (A), pyocyanin-treated group (B), and Doxorubicin-treated group (C). After 24 h cell stimulation, active caspase-3 protein (brown particles) were observed in B and C, indicating apoptotic cell death. The result was representative from 3 independent experiments. Arrowhead indicates caspase-3 proteins.

mechanisms involved disturbance of intracellular antioxidant function that result in the inhibition of catalase activity.[6] Implication of death mechanism on B cell induced by pyocyanin indicates disturbance in the immune responses played by this cell. B lymphocyte plays an important role in humoral immune system. According to Murphy et al., decreased cell viability on lymphocyte may disturb immune response.[19]

Cellular and molecular change may specifically be observed within cell death mechanism. This study revealed that nuclear morphology of B cell was changed under pyocyanin stimulation. The change was observed as nuclear fragmentation, a typical nuclear change in apoptotic cell death. It indicated that pyocyanin may cause apoptotic cell death on B lymphocyte. This result is supported by study done by Bewley et al.[20] Those study demonstrated that other cytotosin, pneumolysin, produced by *Streptococcus pneumoniae* induced apoptotic cell death on macrophage. They demonstrated that pneumolysin caused apoptotic cell death on macrophage marked by nuclear fragmentation. Macrophage cell death was observed 20 h after post challenge with pneumolysin. That was caspase-3 dependent cell death mechanism.[20] In our study, nuclear fragmentation was observed 24 h after pyocyanin treatment.

In this study, active caspase-3 protein was detected in pyocyanin-treated B cells. This evidence is in line with the result of study by Usher et al. (2002). Usher et al. demonstrated that pyocyanin induced apoptotic cell death 5 h after stimulation of neutrophil using 50 µM toxin.[7] Caspase-3 activation may be induced by the increase of reactive oxygen species and decreased intracellular cAMP.[21] This protein functions as a key factor in apoptosis execution. The active caspase-3 may enter nucleus from the pore generated by caspase-9 and cleave lamin A and Fodrin. This mechanism may cause release of substrate for the degradation of DNA. Cleavage of Lamin A by caspase-3 can cause chromatin condensation, whereas the breakdown of fodrin may lead to formation of apoptotic bodies.[22]

It is confirmed by this study that pyocyanin can induce apoptotic cell death on B lymphocyte. At least the cell death mechanism induced by pyocyanin is regulated by activation of caspase-3. Its activation may act as one of the sequences of the whole molecular mechanism of nuclear morphological change. The results of this study may contribute to explain the pathological mechanism of *P. aeruginosa* in causing persistent infection in periapical lesion. However, the complete molecular mechanism in persistent periapical infection is still unclear which needs further clarification.

CONCLUSION

It is concluded from this study that pyocyanin is capable of inducing cell death in B lymphocyte. Caspase-3 may play an important role in the molecular mechanism of pyocyanin-induced cell death.

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