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Apoptosis and Phagocytosis Activity of Macrophages Infected by *Mycobacterium tuberculosis* Resistant and Sensitive Isoniazid Clinical Isolates

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Abstract

Mycobacterium tuberculosis (M.tb) is the main causative pathogen that cause the pulmonary tuberculosis. Intracellular *M.tb* was reported able to induce macrophages apoptosis, which may have crucial role in the regulation of immun response against *M.tb* infection. As an intracellular bacteria, *M.tb* able to live and replicate within macrophages. Phagocytosis is the first step to achieved this condition. The induction of macrophages apoptosis by INH resistant and sensitive *M.tb* clinical isolates, and H37Rv was studied. The macrophages apoptosis level were measured using an Ag-capture ELISA for histone and fragmented DNA (Cell Death Detection ELISA^{plus}, Roche Diagnostic GmBH). Phagocytosis activity also analyzed, after staining using fluorescence dye (AcriFluor™, Scientific Device Lab.). The results showed that there was no significantly different between INH resistant and sensitive *M.tb* clinical isolates in respect their ability to induce apoptosis. The phagocytosis activity among the clinical isolates was shown to be strain dependent, and undistinguishable between the *Mtb* clinical isolates. There was no association between macrophages apoptosis level and the phagocytosis activity. These data suggested that among the virulent *Mtb* clinical isolates, the ability to induce macrophages apoptosis and phagocytosis were consistently in comparable level

Keywords: *Mycobacterium tuberculosis*, apoptosis, phagocytosis, macrophages, isoniazid

Introduction

Mycobacterium tuberculosis (*M.tb*), the main causative pathogen of tuberculosis (TB), is responsible for eight million incidences of TB and killing more than 1.7 million peoples per year worldwide (WHO, 2005). TB as world's health problem becomes more complicated as the multi drug resistant (MDR) TB occurs, especially to isoniazid (INH) and rifampicin (WHO, 2004).

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INH is powerful and most widely used among anti-tuberculosis drugs, which interferes with nearly every metabolic pathway in *M.tb* (Zhang, 2004). There was no agreement among the scientist about the target molecule of INH in killing the *M.tb*. However, accumulated data suggested strongly that mycolic acid synthesis selectively inhibited by INH and correlated with their lethal effect (Slayden and Barry, 2000). There is a correlation between inhibition of mycolic acid synthesis and *M.tb* viability (Takayama *et al.*, 1978).

M.tb is an intracellular microbe which able to live and replicate in the macrophages, which was defined its pathogenocity.

These process is start from the phagocytosis event. A mannose-containing lipoglycan of the *M.tb* cell wall, the terminal mannose-capped lipoarabinomannan (ManLAM), has been implicated in the regulation of several of these processes. The presence of ManLAM on the mycobacterial surface places this molecule in an ideal position to mediate the initial interactions between *M.tb* and macrophages, which in turn facilitates the phagocytosis (Hunter and Brennan, 1990; Kang *et al.*, 2005)

M.tb infection could induce human alveolar macrophages and monocytes-macrophages apoptosis (Keane *et al.*, 1997; Klingler *et al.*, 1997; Placido *et al.*, 1997). There is an inverse correlation between mycobacterial virulence with infected macrophages apoptosis level. Virulent *M.tb* induces little apoptosis as compare to attenuated strains (Keane *et al.*, 2000). Macrophages apoptosis has direct correlation with killing of intracellular bacilli (Molloy *et al.*, 1994; Oddo *et al.*, 1998). Apoptosis of the macrophages is crucial for innate immunity against *M.tb* and eradication of intracellular *M.tb*.

Previous reports showed that the *M.tb* cell wall is very important in its pathogenicities. Furthermore, the accumulated data suggested that INH target molecule reside in the cell wall, apoptosis were induced by component of the cell wall, and the phagocytosis also was facilitated by the component of cell wall. It is also reported that the disturbance of mycolic acid biosynthesis may resulted in the disturbance of *M.tb* cell wall structure. The aim of this work is to study the induction of macrophages apoptosis by *M.tb* resistant and sensitive INH clinical isolates. Furthermore, the association between the apoptosis level and phagocytosis activity of the macrophages was explored.

Materials and Methods

Mycobacterium tuberculosis clinical isolates.

M.tb isolates were obtained from patients attend to the primary health care centers in Yogyakarta. The *M.tb* were cultured on Lõwenstein-Jensen (LJ) medium. Sensitivity of the *M.tb* clinical isolates to INH were tested using the agar proportional method on LJ medium (Freixo *et al.*, 2002). INH sensitivity were tested with concentrations of 0.1 μg/ml and 1 μg/ml. The colonies of the *M.tb* were only observed after 3 weeks incubation on LJ medium. Two INH resistant *M.tb* clinical isolates (R1 and R2) and two INH sensitive *M.tb* clinical isolates (S1 and S2) were chosen for further analysis in this work.

Primary culture of macrophages derived from peripheral blood

Peripheral blood mononuclear cells were isolated using standard gradient centrifugation method of Histopaque® (Sigma Diagnostic Inc.) from heparin-treated blood of healthy and non-smoker donors after informed consent were granted. Mononuclear cells were suspended with complete medium which was consist of RPMI 1640 supplemented with L-glutamine, without sodium bicarbonate (GIBCO), 10% fetal bovine serum (Invitrogen Corp.), antifungal, and antibiotics. The cells were plated in polystyrene tissue culture disc (Nunc™) and then incubated for 2 h at 37°C with 5% CO_2. After incubation, non-adherent cells were removed by three times washing with RPMI 1640. Adherent monocytes were collected by vigorous pippeting and subjected for viability test and cell counting after trypan blue staining. Freshly isolated monocytes were suspended in complete medium and re-plated to the 24 wells polystyrene tissue culture disc at a concentration of 5 X 10^5/ml and incubated at 37°C with 5% CO_2. The cells were ready to be infected at the fourth

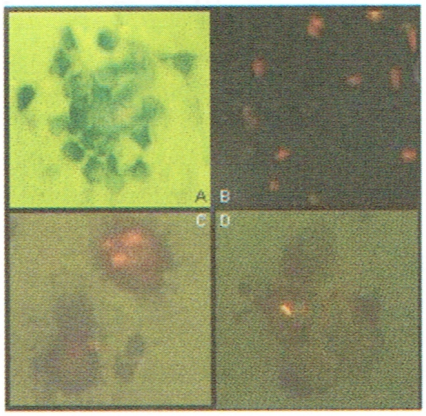

Figure 1. Mycobacterium tubercolosis infected macrophages. A. Microscopic appearance of macrophages cells culture obtained from the peripheral blood. **B**. *M.tb* colonies were dissolved in ringer lactate solution. Solitary cellular solution was needed to assure a reliable multiplicity of infection (MOI) achievement during infection. **C.** A higher MOI was observed in the macrophage cells which was infected with INH resistant *M.tb* (R1) as compare to (**D**.) the macrophage cells which was infected with INH resistant *M.tb* (R2).

uishable. There was no significantly difference between INH-resistant *M.tb* clinical isolates and INH-sensitive *M.tb* clinical isolates in respect of their ability to induce the macrophages apoptosis.

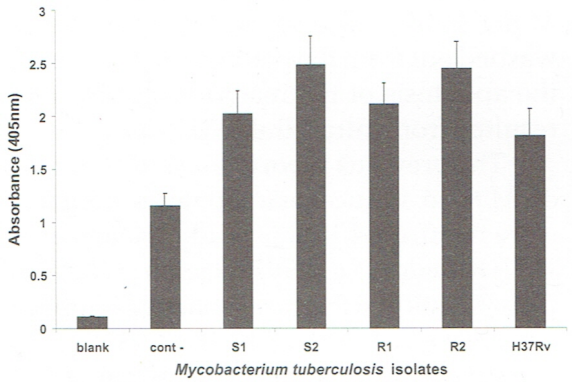

Figure 2. Macrophages cells culture were infected either with *M.tb* clinical isolates sensitive (S1 and S2) or resistant (R1 and R2) to INH, or *M.tb* H37Rv with MOI 1-10. Negative control macrophages were not infected with *M.tb*. Blank denotes absorbance measurement using only buffer without cells lysate. After 4 h infection the extracellular *M.tb* were removed. Macrophages with intracellular *M.tb* were incubated at 37 °C and 5 % CO_2 for 72 h. Macrophages apoptosis level was measured using Cell Death Detection ELISA^{plus} (Roche). Absorbance at 405 nm corresponded to the apoptosis level of macrophages. The data were collected from duplicate measurements of five independent experiments and showed as means with standard error means (SEM).

The *M.tb* isolates that were used in this work were virulent strains of mycobacteria. H37Rv is a standard *M.tb* virulent strain which widely used in many reports. This data suggested that virulent isolates consistently induced the macrophages apoptosis in comparable level (Keane *et al.*, 2000).

In order to clarify the association of phagocytosis with the *M.tb* -induced macrophages apoptosis, the phagocytosis levels of *M.tb* into the macrophages were studied. Intracellular *M.tb* were counted for 200 microscope field and the data were shown as mean of five independent experiments (Figure 3). The data showed a strain dependent pattern of phagocytosis level, though there was no significantly difference between the strains. No significantly difference of phagocytosis level also observed between INH resistant and sensitive *M.tb* clinical isolates and H37Rv (Mann-Whitney test, $p>0.05$).

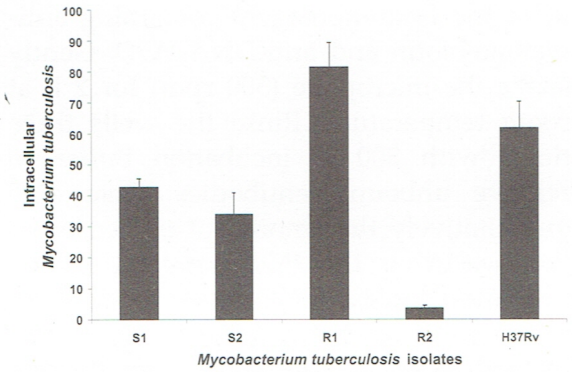

Figure 3. Macrophages cell culture were infected either with *M.tb* clinical isolates sensitive (S1 and S2) or resistant (R1 and R2) to INH, or *M.tb* H37Rv strain. Phagocytosis level were measured by manualy counting the intracellular *M.tb* after stained with fluorescence dye (AcriFluor™, Scientific Device Lab.)

day of culture. Prior to infection the complete medium were replaced with incomplete medium (without antibiotics).

Mycobacterium tuberculosis infection to the macrophages

M.tb colonies were suspended with ringer lactate solution and dissolved by vigorously vortex and repeatedly homogenized with 27G syringe (Stokes *et al.*, 2004). INH- resistant and sensitive *M.tb*, and H37Rv refference isolates were infected to the macrophages primary cell culture for 4 h at approximately multiplicity of infection (MOI) 1 to 10. Extra cellular *M.tb* were removed by three times washing with RPMI 1640 medium.

Macrophages apoptosis measurement

Macrophages with intracellular H37Rv, INH-resistant (R1 and R2) and sensitive (S1 and S2) *M.tb* were incubated for 3 days at 37°C and 5% CO_2. After 3 days, apoptosis were measured using an Ag-capture ELISA for histone and fragmented DNA (Cell Death Detection ELISA^{plus}, Roche Diagnostic GmbH) on cell lysate according to the manufacturer's protocol (Keane *et al.*, 2000). Briefly, transfer 20 µl cell lysate into streptavidin-coated microplate and add 80 µl of the immunoreagent containing anti-histone-biotin and anti-DNA-POD. Gently shake the microplate (300 rpm) for 2 h at room temperature. Rinse the wells three times with 300 µl incubation buffer to remove unbound antibodies. Determine quantitatively the amount of nucleosomes by measuring the POD retained in the immunocomplex. POD was determined photo metrically with ABTS solution after incubation on a plate shaker for 20 min. Measure the absorbance at 405 nm. Duplicate measurements were performed in five independent experiments.

Macrophages phagocytosis activity measurement

The macrophages phagocytosis activity were measured by counting the MOI that was achieved during infection of macrophages with *M.tb*. The intracellular *M.tb* were stained with fluorescence dye (AcriFluor™, Scientific Device Lab.) and were counted under fluorescence microscope. Using this fluorescence dye the intracellular *M.tb* were succeeded to be visualized without disturb the macrophages integrity. Intracellular *M.tb* counting was performed at 200 microscope fields (400 X magnification) in every experiment. Duplicate measurements were performed in five independent experiments.

Results and Discussion

Macrophages cells derived from peripheral blood primary culture were established by obtaining the adderence cells and removing the floating cells in the culture medium. Fluorescence dye was employed to visualized intracellular *M.tb* without disturb the macrophages integrity. Figure 1 shows the intracellular *M.tb* were clearly observed, facilitated a manual counting of intracellular *M.tb*. Extra cellular *M.tb* were clearly washed out from the plates to make sure that the apoptosis of the macrophages in solely resulted from intracellular *M.tb* induction.

The previous reports showed the ability of *M.tb* to induce macrophages apoptosis were confirmed in this study (Keane *et al.*, 1997; Klingler *et al.*, 1997; Placido *et al.*, 1997). Figure 2 shows the macrophages apoptosis level that were induced by *M.tb* H37Rv, *M.tb* sensitive to INH, and *M.tb* resistant to INH clinical isolates. The macrophages apoptosis level that were induced by these *M.tb* isolates were remarkably higher as compare to the negative control that was not infected by *M.tb*. It was noticed that the ability of those *M.tb* isolates to induce apoptosis of macrophages were undisting-

Intracellular *M.tb* counting was performed at 200 microscope fields (400 time magnificence). The data were collected from five independent experiments and showed as means with standard error means (SEM).

Previously, significantly different phagocytosis level were reported by Schlesinger (1993) between virulent and avirulent mycobacteria. Our data showed that phagocytosis level were undistinguishable between virulent *M.tb* strains.

The association between macrophages apoptosis level and the phagocytosis activity were measured using Spearman's test. It was observed a negative association between the two variables. The *M.tb* clinical isolates that phagocytosed easier (more intracellular *M.tb*) seemed induce less apoptosis to the macrophages, though this phenomenon was not statistically significant ($P>0.05$).

Conclusion

M.tb clinical isolates were able to induce the macrophages apoptosis *in vitro*. The ability of *M.tb* virulent strains to induce macrophages apoptosis was not attributed with its sensitivity to isoniazid. There was also no significantly difference between INH-resistant and sensitive *M.tb* in respect with its phagocytosis into the macrophages. Negative association between level of apoptosis and level of phagocytosis activity was not significantly observed in this work.

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