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ORIGINAL ARTICLE

Burkholderia pseudomallei infection induces the expression of apoptosis-related genes and proteins in mouse macrophages

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Apoptosis-based polymerase chain reaction array;
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Caspases

Background/Purpose: In this study, we addressed whether the production of apoptosis-related genes and proteins is induced in mouse macrophages infected with *Burkholderia pseudomallei* cells.
Methods: Mouse macrophages were infected with *B. pseudomallei* cells at 0.5 hours, 1 hour, 2 hours, 4 hours, and 6 hours, respectively, followed by real-time polymerase chain reaction (PCR) array analysis. The amount of apoptosis-related proteins (caspase-3, caspase -8, caspase -9, Bax, and Bcl-2) was confirmed by Western blot.
Results: After infection, an increase of these proteins was observed. The expression levels of other apoptosis-related genes were also determined by PCR array. Experimental results revealed that the messenger RNA levels of tumor necrosis factor ligand (e.g., *tnfsf10* and *tnfrs10b*) and *fas* were increased, whereas the expression levels of some antiapoptosis genes such as *Birc5*, *Hells*, and *Bnip3* were decreased.
Conclusion: Our study results demonstrate that the apoptosis-related genes and proteins in mouse macrophages were modulated by *B. pseudomallei*.

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Introduction

Burkholderia pseudomallei is the causative agent of a broad spectrum of diseases collectively known as melioidosis. This disease occurs in tropical areas, especially in southeast Asia and northern Australia.[1] As a facultative and intracellular bacterium, *B. pseudomallei* can multiply in both phagocytic and nonphagocytic cells.[2,3] The bacterium can spread from cell to cell and can also induce apoptotic death in infected host cells.[4] Apoptosis is mediated by many enzymes and signal transduction pathways.[5] The first group of enzymes involved in apoptosis, including apoptosis induced by bacterial pathogens, is caspases.[6] Caspases are proteases that are shown to cleave many cellular proteins that result in membrane changes, including the alteration of membrane asymmetry, mitochondrial changes, and DNA fragmentation.[5]

Although *B. pseudomallei* has been shown to induce apoptotic death in host cells,[4,7,8] the involvement of other apoptosis-related genes/proteins has not yet been determined. Therefore, in the present study, a mouse macrophage cell line was selected as an *in vitro* model to investigate whether other unpublished apoptosis-related genes and/or proteins are involved in *B. pseudomallei*-induced death.

Materials and methods

Cell line and cell culture

Mouse macrophage cell line (RAW264.7) was cultured and maintained in Dulbecco's modified Eagle medium (GIBCO BRL, Grand Island, New York, USA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA) and 2 mM L-glutamine (Sigma Chemical Co., St. Louis, MO, USA). Cells were cultured at 37°C in a humidified incubator under an atmosphere of 5% CO_2.

Bacterial growth conditions

The *B. pseudomallei* strain VGH19 was isolated from the blood cells of melioidosis patients with acute septicemia in Kaohsiung Veterans General Hospital, Taiwan. The bacterial cells were cultured in tryptic soy broth medium and incubated with shaking at 37°C overnight in a tightly capped tube (50 mL). Bacterial cells were harvested by centrifuging at 6000*g* for 2 minutes. The pellets were washed with 1 mL of phosphate-buffered saline, and centrifugation was repeated as stated previously. The bacterial concentration was determined based on its optical density (OD) at 650 nm.

Infection of macrophages

On the day prior to infection, macrophages were cultured in medium at an initial seeding of 1×10^6 cells/mL in a 6-well plate. The culture medium was removed, and 1 mL of fresh medium was added prior to infecting the cells with bacteria at a multiplicity of infection (MOI) of 10:1. Subsequently, the macrophages were infected for the indicated time as stated in the "Results" section.

Cytotoxicity assays

Macrophages were seeded onto 96-well plates at a density of 10^4 cells/well and were incubated for 24 hours at 37°C. The medium was then replaced with fresh serum-free medium containing bacterial cells at an MOI of 10:1 and subsequently incubated for 24 hours. A solution of MTT (final concentration: 0.5 mg/mL) was then added, and the plates were incubated for an additional 3 hours. At the end of the incubation with MTT, the medium was removed, and the formazan crystals were dissolved in dimethyl sulfoxide. The OD was measured at 570 nm (reference filter: 690 nm) using a microplate-reading spectrofluorometer. Viability was determined by comparing the ODs of the wells containing the dicrotophos-treated cells with those of the untreated cells. The results are expressed as the mean values of at least three independent experiments.

Western blot analysis

Protein extracts (30 μg) were resolved in 12% sodium dodecyl sulfate-polyacrylamide gel, transferred to polyvinylidene difluoride membrane, and then blocked with 3% bovine serum albumin (BSA) at room temperature for 1 hour. After removal from the BSA, the membranes were incubated overnight and the indicated proteins were then washed three times with 1× Tris-buffered saline—Tween 20 buffer (pH 7.4) solution. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated antimouse antibody (1:5000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 hour at room temperature and were washed three times again as mentioned previously. The density of the bands was measured with ImageQuant TL7.0 software (GE Healthcare, Salt Lake City, UT, USA). Experiments were performed in triplicate to ensure reproducibility.

Total RNA isolation

The isolated RNAs in the infected and uninfected cells were used with the Trizol reagent (Invitrogen, Grand Island, NY), according to the manufacturer's protocol. The quantity and purity of RNA were measured spectrophotometrically (BioPhotometer, Eppendorf, Hamburg, Germany). The RNA was considered suitable for further processing if the A_{260}-to-A_{280} ratio was between 1.8 and 2.0. The RNA integrity was determined by 1.8% agarose gel electrophoresis.

Pathway-specific real-time polymerase chain reaction array

RT2 Profiler Mouse Apoptosis PCR Array (Catalog No. PAMM-012, SABiosciences, Frederick, MD, USA) was used for comparing the expression of apoptotic genes between the infected cells and the untreated cells. The *Ct* values for the 84 genes were normalized to five housekeeping genes (*gusb*, *hprt1*, *hsp90ab1*, *gapdh*, and *actb*). Gene-expression differences in the polymerase chain reaction (PCR) expression array were determined using the $2^{-\triangle\triangle ct}$ method. Changes in gene expression were represented as a fold increase/decrease. These experiments were performed in duplicate.

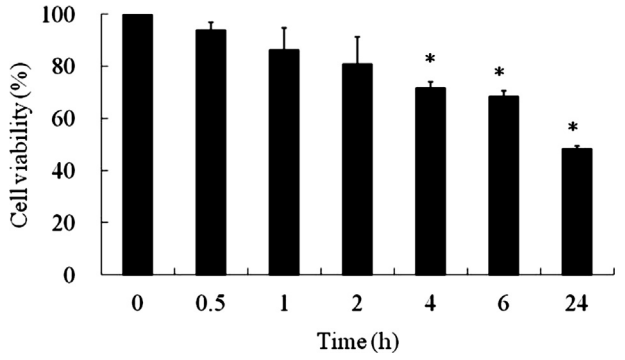

Figure 1. The viability of *Burkholderia pseudomallei*-infected macrophages. Mouse macrophage cell line (RAW264.7) infected with *B. pseudomallei* at a multiplicity of infection of 10:1 for different time intervals. Values represent the mean ± standard deviation from at least three independent analyses. * $p < 0.05$ is a significant difference compared with the control group. h = hours.

Real-time reverse transcription PCR (qRT-PCR)

In brief, the 10 μL reaction mixtures contained 2 μL Master Mix, 2 μL of 0.75 μM forward and reverse primers (a pair of primers for each target gene shown in Table 1), and 6 μL complementary DNA (cDNA) sample. Each sample was run in triplicate. The QRT-PCR program consisted of an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of amplification and quantification at 95°C for 10 seconds, 60°C for 15 seconds, and 72°C for 10 seconds. At the end of the program, a melting curve analysis was performed. At the end of each RT-PCR run, data were subjected to automated analysis, and an amplification plot was generated for each cDNA sample. The results are reported as mean fold changes in gene expression compared with housekeeping genes if the fold change is greater than two.

Statistical analysis

The mean parameters from three independent samples were obtained, and the differences of the mean parameters in both the control and test groups from the three independent experiments were analyzed using a one-way analysis of variance. If a significant *F* value was obtained, Dunnett multiple comparison tests were then conducted. A *p* value <0.05 was considered to be significant.

Results

B. pseudomallei induces macrophage (RAW264.7) cell death

To determine the cytotoxic effect of *B. pseudomallei* on macrophages, the cells were infected with bacterial cells

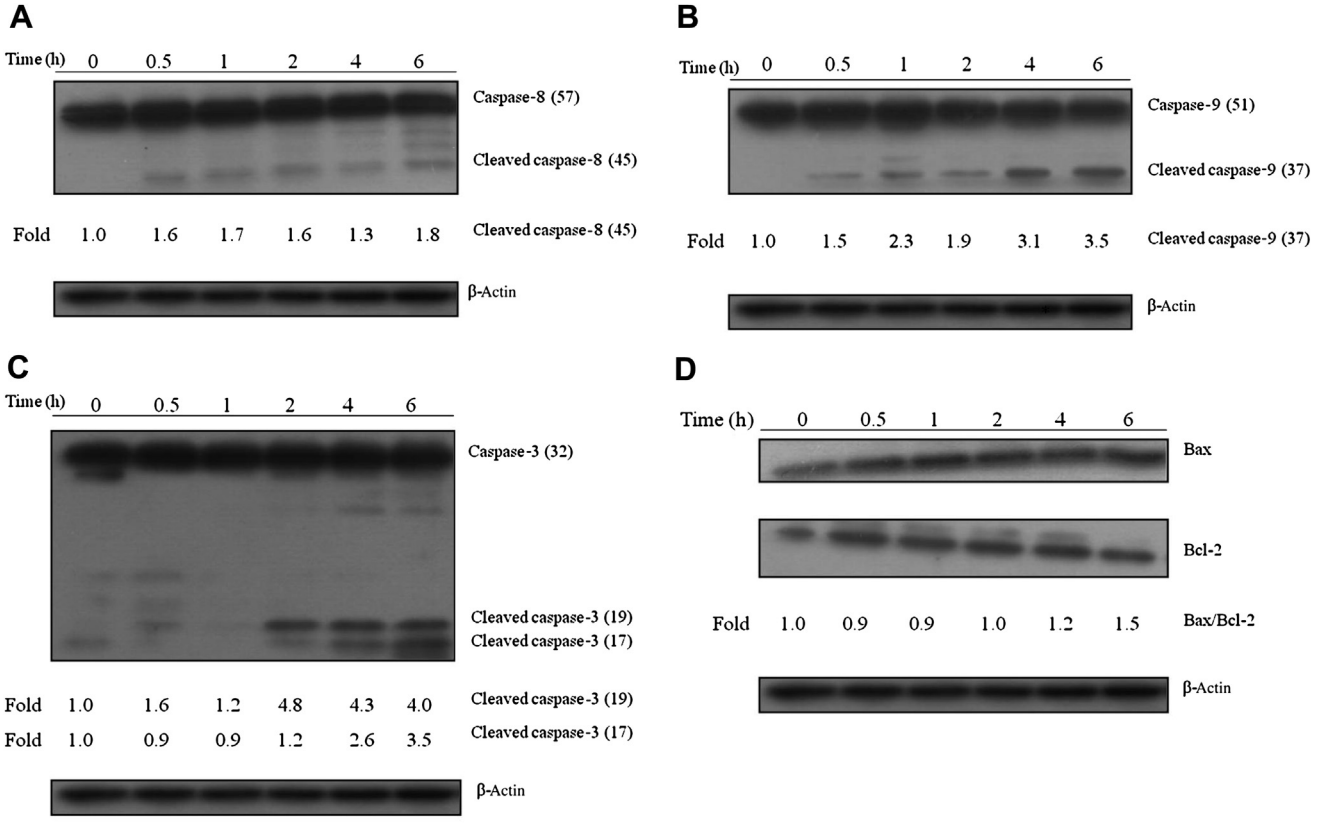

Figure 2. Western blot analysis of (A) caspase-8, (B) caspase-9, (C) caspase-3, and (D) Bax and Bcl-2 protein production level. Mouse macrophages were infected with *Burkholderia pseudomallei* at a multiplicity of infection of 10:1 for different time intervals. The relative production level between Bax and Bcl-2 is shown in panel D. h = hours.

at an MOI of 10:1 for 0.5 hours, 1 hour, 2 hours, 4 hours, 6 hours, or 24 hours, and cell viability was measured by MTT colorimetric assay (Fig. 1). The results showed that the percentage of viable cells decreased as the incubation time increased after the infection of the macrophages with *B. pseudomallei*. No significant cell death occurred during an initial 2-hour infection; however, 22% of macrophages were dead after a 6-hour infection ($p < 0.05$).

Expression of caspase-3, caspase-8, and caspase-9 in *B. pseudomallei*

The amount of production of caspase-8 and caspase-9 was analyzed by Western blot. As shown in Fig. 2A and B, the levels of caspase-8 and caspase-9 expression were significantly increased as early as 0.5 hours and appeared up to 6 hours after exposure to *B. pseudomallei* in comparison with uninfected control cells. In addition, we also determined the activity of caspase-3 in this *in vitro* model of infection with *B. pseudomallei*. Fig. 2C showed that the cleaved form of caspase-3 (32 kDa, 17 kDa, and 19 kDa) persisted through 6 hours of the infection.

Expression ratio of Bax/Bcl-2

There is evidence that the Bax-to-Bcl-2 ratio appears to control the relative sensitivity or resistance of many cell types to apoptotic stimuli.[9] Therefore, we determined the production level of Bax and Bcl-2 in infected cells using Western blot analysis. The results revealed that the ratio of Bax to Bcl-2 synthesis increased with incubation time (Fig. 2D).

Differential expression of apoptosis-related genes

We measured the expression level of a set of apoptosis-relevant genes with or without infection in macrophage cells using RT2 Profiler PCR Arrays. Of the 84 apoptotic genes tested, 14 genes showed significant changes in infected cells compared with noninfected cells. Expression of many genes related to cell receptors or ligands was upregulated or downregulated (Table 1). We further adopted a quantitative real-time PCR array to detect the expression of some selected genes (*cd40*, *casp1*, *fas*, and *tnfrsf10b)* after infection of cells with *B. pseudomallei* for 2 hours and 4 hours (Fig. 3). The primers of these genes used in this study were listed in Table 2.

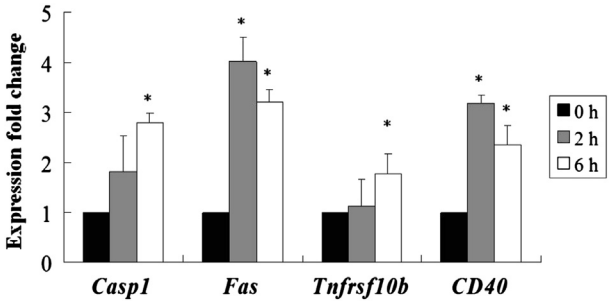

Figure 3. Comparison of the fold change of selected genes in mouse macrophages infected with *Burkholderia pseudomallei* at a multiplicity of infection of 10:1 compared with that of the control group at different time intervals and the analysis of the quantitative real-time polymerase chain reaction. Values represent the mean ± standard deviation from at least three independent analyses. * $p < 0.05$ is a significant difference compared with the control group. h = hours.

Discussion

The macrophage—pathogen interactions play an important role in the pathogenesis of bacterial infections. The

Table 1 Quantitative levels of differentially expressed genes related to apoptosis of *Burkholderia pseudomallei*-infected macrophages 6 hours after the infection. Results shown are the relative ratio of gene-expression level in contrast to control cells

Gene	Functional group	Reference sequence	Fold upregulation or downregulation
CD40	TNF receptor family	NM_011611	6.04 ± 2.19
Fas	Death domain family TNF receptor family	NM_007987	5.09 ± 1.14
Casp1	Caspase family CARD family	NM_009807	2.91 ± 0.74
Sphk2	Antiapoptosis	NM_020011	2.80 ± 0.01
Tnfsf10	TNF ligand family	NM_009425	2.78 ± 0.62
Tnfrsf10b	Death domain family TNF receptor family	NM_020275	2.71 ± 0.21
Nol3	CARD family	NM_030152	27.59 ± 11.47
Birc5	Antiapoptosis	NM_009689	−3.17 ± 0.13
Cradd	CARD family Death domain family	NM_009950	−3.09 ± 0.81
Bok	Bcl-2 family	NM_016778	−2.92 ± 0.04
Pycard	CARD family	NM_023258	−2.91 ± 0.45
Hells	Antiapoptosis	NM_008234	−2.65 ± 0.66
Bnip3	Antiapoptosis Bcl-2 family	NM_009760	−2.52 ± 0.29
CD70	TNF ligand family	NM_011617	−2.05 ± 0.57

CARD = caspase-associated recruitment domains; TNF = tumor necrosis factor.

Table 2 DNA sequences of primer pairs used for the quantification of messenger RNA levels of apoptosis-related genes by quantitative real-time PCR array

Gene	Primer sequences
Casp1	Forward 5′-AGAACAAAGAAGATGGCACA-3′
	Reverse 5′-AGATAATGAGGGCAAGACG-3′
Fas	Forward 5′-CTTGCTGGCTCACAGTTAAG-3′
	Reverse 5′-AGGTTGGCATGGTTGACA-3′
Tnfrsf10b	Forward 5′-TTGACTACACCAGCCATTCCAA-3′
	Reverse 5′-CGTCAGTGCAGTTAGAGC-3′
CD40	Forward 5′-TGTACCTGTAAGGAAGGACAAC-3′
	Reverse 5′-CTGATTGGAGAAGAAGCCG-3′
GAPDH	Forward 5′-TCCACTCACGGCAAATTCA-3′
	Reverse 5′-ACCAGTAGACTCCACGAC-3′

PCR = polymerase chain reaction.

proapoptotic mechanisms by these bacteria include the activation of several proapoptotic proteins (e.g., caspases), the inactivation of antiapoptotic proteins, and the upregulation of endogenous receptor/ligand systems.[5] The change of the ratio of Bax to Bcl-2 can stimulate the release of cytochrome c, which activates caspase-9 and caspase-3, and it may govern sensitivity to apoptotic stimuli.[9] In addition to the expression of Bax and Bcl-2, we detected the expression of caspase-3, caspase-8, and caspase-9 in the infected cells (Fig. 2), indicating that these proteins could be involved in apoptosis.

Real-time PCR array results showed that expression level of some of these genes in the infected cells was either upregulated or downregulated. For example, the messenger RNA levels of tumor necrosis factor ligand (e.g., *tnfsf10* and *tnfrs10b*) and *fas* were increased, whereas the expression levels of antiapoptosis genes such as *Birc5*, *Hells*, and *Bnip3* were decreased. Although the exact molecular target and mechanism of apoptosis induction by bacterial infection still need further investigation, our results provided basis for mechanistic studies of the infection of macrophages by *B. pseudomallei*. We further used quantitative real-time PCR to measure the levels of expression of some selected genes (*CD40*, *casp1*, *fas*, and *tnfrsf10b*) in a time-dependent manner. The results of quantitative real-time PCR correspond with those obtained by apoptosis-based pathway real-time PCR array (Fig. 3). Taken together, our results first proved that *B. pseudomallei* induce apoptosis-related genes and proteins in macrophages.

Acknowledgments

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