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Recombinant YopJ induces apoptotic cell death in macrophages through TLR2

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ABSTRACT

Bacterial species evolved evasive maneuvers to bypass their recognition by the receptors primarily TLRs of the innate immune cells. We have reported that 3 μg/ml of recombinant YopJ when provided extracellularly induced apoptosis in murine peritoneal macrophages in vitro. The present investigations demonstrate the role of TLR2 in apoptotic signals induced by rYopJ protein in murine peritoneal macrophages. The role of TLR2 in rYopJ induced macrophage apoptosis was shown by neutralization experiments and its co-immunoprecipitation with downstream molecule MyD88. The observed functional consequence of TLR2 neutralization were the inhibition of caspase-8 and caspase-3 activation, change in mitochondrial membrane potential ($\Delta\psi m$) and DNA fragmentation induced by rYopJ in macrophages. Further, rYopJ induced enhanced expression of IRAK-4, FADD, phosphorylation of IκB and p38 MAP kinase in macrophages. Pharmacological inhibitor of p38 MAP kinase and neutralization of TLR2 with neutralizing antibodies significantly inhibited the rYopJ induced caspases activation and DNA fragmentation, suggesting the possible involvement of TLR2 and p38 MAP kinase in rYopJ induced macrophages apoptosis.

1. Introduction

Bacterial infections are a major cause of mortality and morbidity worldwide (Hinman, 1998). Evocation of an adequate immune reaction against bacterial pathogens critically depends on appropriate sensing and recognition of the invading organism by host immune cells. Pattern recognition receptors (PRRs) recognize the conserved molecules among many different types of pathogens; these molecules are referred to as pathogen associated molecular pattern (PAMPs) (Areschoug and Gordan, 2009). The toll like receptors (TLRs) are subfamily of PRRs. To date 10 TLRs have been described in humans and 12 in mice (Georgel et al., 2009). All of them have structural homology but are able to recognize the unrelated diverse molecules (Akira et al., 2001; Takeda et al., 2003; Werling et al., 2009). Activation of TLR provokes rapid onset of immune reactions by triggering the signaling cascades using several intracellular molecules, including myeloid differentiation factor-88 (MyD88), IL-1 receptor-associated kinases (IRAKs), TNF receptor-associated factor (TRAF) 6 and mitogen activated protein kinases (MAPKs), leading to activation and further production of various cytokines, adhesion molecules, and antiapoptotic factors (Chu et al., 1997; Jiang et al., 2005; Karin and Lin., 2002; Liu et al., 1996; Wang et al., 1996, 1998). Among TLRs, TLR2 has a broader role as a pattern recognition receptor and is implicated in inflammatory/apoptotic responses to lipoprotein/lipopeptides from different bacterial species (Derrick and Morris, 2007; Lopez et al., 2003; Takeda et al., 2003; Takeuchi et al., 1999). Heterodimerization of TLR2 with other TLRs like TLR1 and 6 increases diversity in pathogen recognition by TLR2 (Ozinsky et al., 2000; Takeuchi et al., 2001, 2002). The bacteria *Yersinia pestis* is known for causing "Black death" in human history (Perry and Fetherston, 1997). During infection macrophages are the primary target of *Y. pestis* (Monack et al., 1997). It harbors a 70 kb plasmid which encodes for the type three secretion apparatus (T3SS) and effector proteins. Among six effectors, one effector protein, YopJ is known to induce apoptosis in macrophages by preventing activation of NFkB pathway (Cornelis and Wolf-Watz, 1997; Zhang et al., 2005). Recently, it is reported that YopJ acts as an acetyltransferase thus preventing the activation of signaling molecules by phosphorylation (Mukherjee et al., 2006). The implication of TLRs in the apoptotic response to YopJ has been not well studied. Previous studies have suggested the involvement of TLR4 in the apoptotic response of macrophages to yersinia infection but it was either with plasmid encoded YopJ or with bacteria producing YopJ in cultures (Haase et al., 2003; Zhang and Bliska, 2003). In both cases the protein YopJ does not get exposed to the surface receptors i.e. TLRs. It directly translocated in to the host cytosol by bacteria and once inside the host cytosol the YopJ blocks the survival pathway by preventing the activation of NF-κB and MAP kinase signaling molecules. In the absence of survival pathway the lipopolysaccharide of the invad-

Abbreviations: rYopJ, recombinant yersinia outer protein J; TLR, toll like receptor; MAPK, mitogen-activated protein kinases; PAMP, pathogen associated molecular pattern; PRR, pattern recognition receptors; IRAK, IL-1 receptor-associated kinases; SB 202190, inhibitor of p38 MAP kinase; EDTA, ethylene diamine tetra acetic acid; PMSF, phenyl methyl sulfonyl fluoride.

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ing bacteria triggers the TLR4 mediated apoptosis in the infected cell. YopJ has been shown to potentiate the apoptotic signaling of LPS by inhibiting the activation of NF-κB (Ruckdeschel et al., 2001). The present study uses purified recombinant YopJ protein free from endotoxin and suggested that rYopJ when exposed to the cell surface could induce activation/apoptosis in macrophages. Several studies have demonstrated the cytotoxic effect of purified recombinant proteins from mycobacterium on macrophages and demonstrated the involvement of TLRs in the recognition of bacterial virulence proteins but no study has been done on recombinant YopJ protein (Derrick and Morris, 2007; Lopez et al., 2003). We have recently reported that 3 μg/ml of rYopJ protein when provided extracellularly induces apoptotic cell death of murine peritoneal macrophages and suggested but not demonstrated the possible involvement of TLRs in the recognition of rYopJ protein (Pandey and Sodhi, 2009). In the present study we demonstrate the role of TLR2 and elucidated its downstream signaling molecule in rYopJ induced macrophages apoptosis. The involvement of TLR2 has been shown by using neutralizing antibodies and its co-immunoprecipitation with downstream molecule MyD88 in the rYopJ treated macrophages. The functional consequence of TLR2 neutralization resulted in the inhibition of caspase-8 and caspase-3 activation, change in mitochondrial membrane potential ($\Delta\psi m$) and DNA fragmentation induced by rYopJ in macrophages. Further it is observed that rYopJ induces the phosphorylation of p38 MAP kinase and IκB molecules in macrophages. Pharmacological inhibition of p38 MAP kinase and neutralization of TLR2 significantly reduced the rYopJ induced activation of caspases, demonstrating the involvement of TLR2 and p38 MAP kinase in rYopJ induced macrophages apoptosis. Inhibition of NF-κB did not affect the rYopJ induced apoptosis in macrophages.

2. Materials and methods

2.1. Mice

Inbred strains of Balb/c mice of either sex at 8–10 weeks of age were used for obtaining peritoneal macrophages.

2.2. Cell cultures and reagents

Macrophage were cultured in RPMI 1640 medium supplemented with heat-inactivated fetal calf serum (10%), penicillin (100 U/ml), streptomycin (100 U/ml) and gentamycin (20 μg/ml) at 37 °C in humidified air containing 5% CO_2. Medium RPMI 1640, TRI-reagent, MTT, curcumin and most of the other reagents were obtained from Sigma–Aldrich Chemicals, St Louis, MO, USA. Fetal calf serum was purchased from Biological Industries, Haemek, Israel. PD98059, SB202190, SP600125, Nucleosome Elisa Kit, TUNEL assay kit, Mitocapture apoptosis detection kit, was purchased from Calbiochem, La Jolla, CA, USA. Polyclonal antibodies against Actin, TLR1, TLR6, MyD88, FADD, p-IκB, p-p38, caspase-8, 3, FITC conjugated anti-rabbit IgG, HRP-conjugated anti-rabbit, anti-goat IgG and DAPI mounting medium were obtained from Santa Cruz Biotechnology Inc., CA, USA. Neutralizing antibodies against TLR2 (clone: T2.5) and TLR4 (MTS 510) was obtained from eBiosciences, San Diego, CA, USA. Purified, recombinant YopJ protein (32.5 kDa) of *Y. pestis* was provided by Dr. H.V. Batra, Head, Division of Microbiology, DRDE, Gwalior, MP, India. The protein has been cloned and over expressed in *Escherichia coli* by induction to IPTG. Recombinant protein was purified using nickel-NTA column chromatography and was observed as single band on SDS-PAGE (Khushiramani et al., 2006). All the reagents were endotoxin-free as determined by the limulus amoebocyte lysate assay (sensitivity limit, 0.1 ng/ml).

2.3. Isolation and activation of peritoneal macrophage

Macrophage monolayers were prepared as described previously (Biswas et al., 2001). Peritoneal exudates cells (PECs) were harvested from the healthy, inbred strain of BALB/c mice (8–10 weeks old, 20–22 g) using chilled serum-free RPMI 1640 medium and plated in the wells of 24 well plate (Nunc, Roskilde, Denmark). After 2 h incubation at 37 °C in an atmosphere of 5% CO_2 in a CO_2 incubator, the non-adherent cells were removed by washing with warm serum-free medium and the adherent cells were further incubated in complete medium overnight to form macrophage monolayer. More than 95% of the adherent cell population was macrophages as determined by morphology and non-specific esterase staining.

The medium was removed from the wells and macrophage monolayers (10^6 cells/well) were exposed to rYopJ (3 μg/ml). Untreated macrophage monolayers were used as controls. In another set, the macrophage cultures were incubated with various inhibitors for 1 h before rYopJ (3 μg/ml) treatment as indicated in Section 3. Thereafter, macrophage monolayers were used for various experimental procedures. For IκB and MAP kinase experiments, macrophages were serum starved for 6 h.

2.4. Cytotoxicity assay

Cell cytotoxic activity of rYopJ was measured by MTT (4,5-dimethylthiazol-2-yl-2, 5-diphenyl tetrazolium bromide) assay (Mosmann, 1983). Briefly macrophages (10^6 cells/well) were cultured in 24 well culture plates in 1 ml RPMI 1640 medium. After overnight culture, the cells were washed and pretreated with TLRs antibody in incomplete medium for 2 h. Thereafter, medium was removed; macrophages monolayer was washed with warm medium and further incubated in fresh medium with 3 μg/ml rYopJ for 22 h as indicated in Section 3. After completion of treatment, 50 μl of 5 mg/ml MTT solution was added to the monolayer and incubated for 4 h at 37 °C. The MTT reaction was terminated by the addition of 0.04 N HCL in isopropanol. The MTT formazon formed was measured spectrophotometrically (540 nm). The % cytotoxicity was calculated by formula:

$$\text{cytotoxicity}\,(\%) = \frac{C - T}{C} \times 100$$

where C is the absorbance 'control' represent macrophages incubated in medium alone and T is the absorbance 'experimental' represent macrophages treated with the purified rYopJ or rYopJ plus TLRs antibody or antibody alone.

2.5. Terminal deoxynucleotidyl transferase (TdT) mediated nick end labeling (TUNEL) assay

The apoptotic death in macrophages induced by rYopJ were identified by terminal deoxynucleotidyl transferase (TdT)-mediated nick-end labeling (TUNEL) using the TdT-Frag EL DNA fragmentation detection kit (Calbiochem, La Jolla, CA, USA) as per the instructions from manufacturer. Briefly, the macrophages monolayers were treated with rYopJ (3 μg/ml) for 18 h. After then, the cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with 20 μg/ml proteinase K for 5 min, and blocked in 1% BSA for 30 min at room temperature. Wash the cells thrice with 1× TBS buffer and incubate in 60 μl of TdT labeling reaction mixture for 90 min in a humified chamber. After then, incubate the cells in 1× TBS for a minute, mounted with Fluorescein Frag EL mounting media and observed under fluorescence microscope (Olympus BX61, Olympus Optical Co., Ltd., Tokyo, Japan) using DAPI (330–380 nm) and fluorescein filter (465–495 nm).