Recombinant YopJ induces apoptosis in murine peritoneal macrophages \textit{in vitro}: involvement of mitochondrial death pathway

Ashok Kumar Pandey and Ajit Sodhi

School of Biotechnology, Faculty of Science, Banaras Hindu University, Varanasi-221005, India

Keywords: cell death, rYopJ, \textit{Yersinia}

Abstract

\textit{Yersinia} species during infection adhere to host immune cells primarily to macrophages and employ its secretory proteins known as \textit{Yersinia} outer proteins to trigger death in infected cells. In the present study, it is shown that recombinant \textit{Yersinia} outer protein J (rYopJ) could induce apoptosis in murine peritoneal macrophages \textit{in vitro} as assessed by morphological features, internucleosomal DNA fragmentation, change in mitochondrial membrane potential (MMP) (\(\Delta \psi m\)), activation of caspases and Annexin V binding. rYopJ-induced cell death was dose and time dependent. Pre-treatment with broad-spectrum caspase inhibitor Z-VAD-FMK, caspase-3 inhibitor Ac-DEVD-CHO and caspase-8 inhibitor Z-IETD-FMK prevented the change in MMP and DNA fragmentation, suggesting caspase-dependent apoptosis of rYopJ-treated macrophages. Blocking the endocytosis by pre-treatment of cells with cytochalasin B did not prevent the rYopJ-induced macrophages apoptosis. The data further suggest that rYopJ-induced apoptosis is mediated by molecules upstream of caspase-8 and relay through mitochondrial pathway involving Bax, Bcl-2, activation of caspase-8 and caspase-3, Bid and polyadenosine diphosphate-ribose polymerase cleavage, cytochrome c release and DNA fragmentation.

Introduction

Although the outbreak of plague has been restricted to a greater extent, the use of \textit{Yersinia} species as a biological weapon still exist. \textit{Yersinia pestis} is the causative agent of bubonic plague; \textit{Yersinia pseudotuberculosis} and \textit{Yersinia enterocolitica} cause adenitis, septicemia and gastrointestinal syndromes (1–3). Pathogenic \textit{Yersinia} species carry a 70-kb virulence plasmid, known as pYV (plasmid of \textit{Yersinia} virulence), that encodes the type III secretion system (T3SS) and effectors known as \textit{Yersinia} outer proteins (Yops). There are five Yops (YopE, YopH, YopM, YpkA/YopO and YopJ/YopP) common in all species, while the sixth, YopT, is only present in \textit{Y. enterocolitica} (4). These effectors are directly translocated into the host cell cytoplasm, by adhered bacteria, through a specialized needle-like structure associated with T3SS (5, 6). Once inside the host cytoplasm, Yops causes disruption of cytoskeleton, block nuclear factor-kB (NF-kB) and mitogen-activated protein kinase activation pathways, inhibit cytokine production and induce cell death through apoptosis (7–11). One specific effector, called YopJ in \textit{Y. pestis} and \textit{Y. pseudotuberculosis} and YopP in \textit{Y. enterocolitica}, is a 32-kDa protein which inhibits production of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-\(\alpha\)) and IL-8 and induces macrophage apoptosis \textit{in vitro} (10,12–14). YopJ/P-induced apoptosis has been shown to play a role in the establishment of a systemic infection in mouse infection model (15). YopJ/P belongs to a cysteine protease class, having proteolytic activity toward ubiquitin-like proteins (16). It acts by removing ubiquitin moieties from critical proteins like TAK-1, IKKb, TRAF-2, TRAF-6 and I\(\kappa\)B\(\alpha\), thereby preventing activation of NF-kB pathway (17–21). In absence of survival pathway, endotoxin (LPS) from infecting bacteria activates the apoptosis pathway (22). Recent reports suggested that instead of acting as proteases, YopJ and its homologs like YopA from \textit{Vibrio parahaemolyticus} act as an acetyltransferase by acetylating serine/threonine residues on IKKs and MKKs activation site, thus preventing phosphorylation and subsequent activation by kinases (23, 24). Apoptosis is characterized by cell shrinkage, chromatin condensation, internucleosomal degradation of the DNA and disassembly into membrane-enclosed vesicles (25). Apoptotic cell death is mediated by activation of caspases (26) leading to cleavage of polyadenosine...
diphosphate-ribose polymerase (PARP) (27). Caspases are activated by two major pathways: extrinsic (death receptor) and intrinsic (mitochondrial) death pathway. The extrinsic pathway is activated by binding of ligand (CD95 ligand, TNF-α) to death receptors on cell surface leading to activation of caspase-8 and subsequently in the cleavage of pro-caspase-3, -6 and -7 (28). The mitochondrial apoptosis pathway is initiated by various stimuli (anti-cancer drugs or prostaglandins, etc.), which results into disruption of mitochondrial membrane and release of various pro-apoptotic factors (29–31). Caspase-8-mediated generation of truncated Bid links the death receptor signaling pathway to the mitochondrial apoptotic pathway (32, 33). It has been reported that purified recombinant *Yersinia* outer protein J (rYopJ) is catalytically active and can cleave ubiquitin–7-amino-4-methylcoumarin (AMC) and block MKK and Pbs2 activation during *in vitro* assay (20, 34). Previously, most of the work done on the YopJ-induced macrophages apoptosis has been carried out with either using different *Yersinia* strains or plasmid construct expressing YopJ. Also, the apoptotic effect of YopJ has been studied on either RAW or J774A.1 macrophage cell lines (12, 35). The present study has been carried out on murine peritoneal macrophages, which are terminally differentiated, non-dividing cells and constitute the major group of leukocytes, crucial to immunosurveillance against malignancies and invading pathogens (36, 37). However, no data exist on direct effect of rYopJ on murine peritoneal macrophages in *in vitro*. Several studies have demonstrated the cytotoxic effect of purified recombinant proteins from *mycobacterium* on macrophages (38, 39). This led us to study, if rYopJ from *Y. pestis* could induce apoptosis in murine peritoneal macrophages *in vitro* or not and if so, through which pathway? In this report, we present evidence that rYopJ can induce apoptosis in murine peritoneal macrophages by activation of caspases and death signal through mitochondrial pathway. rYopJ induced cleavage of pro-caspase-8, Bid, up-regulated pro-apoptotic factor Bax and down-regulated anti-apoptotic factor Bcl-2. It is further observed that rYopJ-induced change in mitochondrial membrane potential (MMP) and DNA fragmentation was inhibited by broad-spectrum caspase inhibitor Z-VAD-FMK and by caspase-8 inhibitor Z-IETD-FMK. Blocking caspase-3 did not prevent the activation of caspase-8, suggesting that rYopJ-induced apoptosis is mediated by molecules upstream of caspase-8, through a pathway involving Bax, Bcl-2, activation of caspase-8 and caspase-3, Bid
and PARP cleavage, cytochrome c release and DNA fragmentation.

**Methods**

**Mice**

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

**Cell cultures and reagents**

Macrophages were cultured in RPMI 1640 medium supplemented with heat-inactivated FCS (10%), penicillin (100 U ml⁻¹), streptomycin (100 U ml⁻¹) and gentamycin (20 µg ml⁻¹) at 37°C in humidified air containing 5% CO₂. Cytochalasin B, proteinase K, polymixin B, medium RPMI 1640, TRI reagent, L-NMMA, L-arginine, LPS, caspase-3 and caspase-8 fluorimetric assay kit, caspase-3 inhibitor Ac DEVD-CHO and most of the other reagents were obtained from Sigma–Aldrich Chemicals, St Louis, MO, USA. FCS was purchased from Biological Industries, Haemek, Israel. Broad-spectrum caspase inhibitor Z-VAD-FMK, caspase-8 inhibitor Z-IETD-FMK and caspase-9 inhibitor Z-LEHD-FMK were purchased from Imgenex Co., San Diego, CA, USA. Nucleosome ELISA Kit and MitoCapture Apoptosis detection kits were from Calbiochem, La Jolla, CA, USA. Annexin V apoptosis detection kit, 4',6-diamidino-2-phenylindole (DAPI) mounting media, polyclonal antibodies against caspase-8, -3, Bax, Bcl-2, PARP, Bid, apoptosis protease activating factor-1 (Apaf-1), actin, FITC-conjugated anti-Bax, TRITC-conjugated anti-Bcl-2, FITC-conjugated anti-rabbit IgG, HRP-conjugated anti-rabbit and anti-goat IgG were obtained from Santa Cruz Biotechnology Inc., CA, USA. One-step reverse transcription (RT)–PCR kit was from QIAGEN, Hilden, Germany. Mouse primers for Bcl-2, Bax and GAPDH were purchased from GENSET Singapore Biotech. Pvt. Ltd, Singapore. Purified, rYopJ (32.5 kDa) of *Y. pestis* was provided by Dr H. V. Batra, Head, Division of Microbiology, DRDE, Gwalior, Madhya Pradesh, 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 (40). All the reagents were endotoxin free as determined by the Limulus amoebocyte lysate assay (sensitivity limit, 0.1 ng ml⁻¹).

**Macrophage isolation and cytotoxicity assay**

Macrophage monolayers were prepared as described previously (41) Peritoneal exudates cells 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% CO₂ in a CO₂ 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.

Cell cytotoxic activity of rYopJ was measured by 3-(4,5-dimethylthiazol-2-yl-2, 5-diphenyl tetrazolium bromide) (MTT) assay (42). Briefly, macrophages (10^6 cells per well) were cultured in 24-well culture plates in 1 ml RPMI 1640 medium. After overnight culture, the cells were washed and then treated with various doses of rYopJ for different time intervals in fresh medium. To exclude the possibility of cytotoxicity by rYopJ due to endotoxin contamination, macrophages were either treated with scrambled rYopJ (boiled for an hour) or proteinase K (20 μg ml^-1) for 24 h. After completion of treatment, 50 μl of 5 mg ml^-1 MTT solution was added to the monolayers 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 formazan formed was measured spectrophotometrically (540 nm). The percentage cytotoxicity was calculated by the formula:

\[
\text{percentage cytotoxicity} = \frac{C-T}{C} \times 100
\]

where C, absorbance ‘control’ represents macrophages incubated in medium alone and T, absorbance ‘experimental’ represents macrophages treated with the purified rYopJ.

**Immunofluorescence staining**

Peritoneal macrophage monolayers on cover glass were treated with rYopJ (3 μg ml^-1) for 18 h. To evaluate nuclear morphology, the cells on cover glass were fixed with 4% PFA, stained with DAPI (10 μg ml^-1) in PBS for 20 min and then observed under fluorescence microscope (Olympus BX61, Olympus optical Co. Ltd, Tokyo, Japan).

For staining Bcl-2 and Bax, macrophage monolayers on cover glass were treated with rYopJ (3 μg ml^-1) for stipulated time intervals. After treatment, monolayers were washed with warm incomplete medium. The cells were then fixed in 4% PFA for 10 min at room temperature, washed...
(three times) in PBS and permeabilized with 0.1% Triton X-100 for 5 min at room temperature. Cells were washed twice in PBS and incubated in PBS containing 1% BSA for 30 min, blocking the non-specific-binding sites. Subsequently, the cells were incubated with anti-Bax-FITC or anti-Bcl-2-TRITC antibody overnight at 4°C, washed with chilled PBS containing 0.05% Triton-X-100 (three times), observed and photographed with Olympus fluorescence microscope.

For rYopJ immunostaining, macrophage monolayers on cover glass were incubated with rYopJ (3 μg ml⁻¹) for 2 h at 37°C in a CO₂ incubator. After incubation, the monolayers were washed three times with warm PBS and fixed in 4% PFA for 10 min at room temperature. After fixation, under non-permeabilized conditions, cells were washed in PBS and monolayers blocked with 1% BSA for 30 min. These monolayers were incubated with anti-rYopJ antibody in PBS, 1% BSA for 2 h at room temperature. After washing, cells were incubated with FITC-conjugated anti-rabbit secondary antibody for 1 h at room temperature, washed three times with PBS and the cover glass mounted immediately with DAPI medium. The cells were observed and photographed with Olympus fluorescence microscope.

Apoptosis assays
FITC-Annexin V and propidium iodide (PI) and MitoCapture apoptosis detection kits were used to detect apoptosis in macrophages under fluorescence microscope. For Annexin V-FITC and PI staining, macrophage monolayers on cover glass were incubated for 3 and 18 h with rYopJ (3 μg ml⁻¹). The cells were washed with PBS and incubated in 1× assay buffer, Annexin-V-FITC and PI as per the protocol described in the Annexin V apoptosis detection kit. After 20 min, the monolayers were washed with PBS and observed under the fluorescence microscope. The green fluorescent cells were scored as apoptotic cells. Untreated cells served as control.

For detection of mitochondrial membrane depolarization (Δψm), macrophage monolayers were incubated with 1 μg ml⁻¹ of MitoCapture cationic dye at 37°C for 30 min. Cells were photographed using fluorescence microscope with excitation wavelengths of 500 and 570 nm. Cells exhibiting green fluorescence were counted as apoptotic cells.

Quantification of oligonucleosome unit resulted due to DNA fragmentation
The Nucleosome ELISA Kit was used to quantify free oligonucleosome unit resulted due to rYopJ-induced DNA fragmentation in macrophages as recommended by the manufacturer. Briefly, macrophages treated with rYopJ were washed and lysed. The lysates were used to measure free oligonucleosome units by antigen capture ELISA. The optical density at 405 nm was read in ELISA plate reader (Emax, Molecular Devices, Menlo Park, CA, USA). Triplicate wells were assayed for each condition.

To check the caspase-dependent DNA fragmentation in rYopJ-treated macrophages, cells were pre-treated with broad-spectrum caspase inhibitor Z-VAL-FMK (25 μM), caspase-8 inhibitor Z-IEHD-FMK (50 μM) and caspase-3 inhibitor Ac-DEVD-CHO (100 μM) in incomplete medium for 1 h, after that medium was replaced and macrophages were further incubated in complete media with rYopJ for 18 h. Free oligonucleosome DNA fragment was quantified according to the manufacturer’s instruction manual.

Caspases activity assays
The caspase-3 and caspase-8 activity was determined by using fluorimetric assay kit. Briefly, macrophages were pre-treated with caspase-8 inhibitor Z-IEHD-FMK (50 μM) or caspase-3 inhibitor Ac-DEVD-CHO (100 μM) for an hour in
incomplete medium. The medium was replaced and macrophages were incubated in fresh complete medium with rYopJ (3 μg ml⁻¹) for 12 h. The cells were then lysed as per the instructions provided. Five microlitres of cell lysates were added to the wells of 96-well flat-bottom plate. For blank, 5 μl of assay buffer was used. To some wells, 200 μl of caspase-3 or caspase-8 inhibitor was added. A total of 200 μl of reaction mixture was then added gently to each well. Plate was covered for an hour in dark and read at 360 nm excitation and 460 nm emission at 37°C in a Microplate Fluorescence Reader (FLx 800, Bio-Tek Instruments, Inc., Winooski, VT, USA). The results are expressed as nanomoles of AMC per milligram of protein per 60 min.

Preparation of cell lysates and immunoblotting

The macrophages with or without treatment were washed with ice-cold PBS saline containing 1 mM Na₃VO₄ and then lysed in 50 μl of lysis buffer [20 mM Tris–HCl, pH 8, 137 mM NaCl, 10% glycerol (v/v), 1% Triton X-100 (v/v), 1 mM Na₃VO₄, 2 mM EDTA, 1 mM phenylmethylsulphonylfluoride, 20 μM leupeptin and 0.15 U ml⁻¹ aprotonin] for 20 min at 4°C. The lysates were centrifuged (13 000 × g at 4°C) for 15 min and the supernatant (containing Triton X-100-soluble proteins) was separated on 10% SDS–polyacrylamide gels at 20 mA. The separated proteins were transferred to nitrocellulose membrane (45 min at 200 mA) using Bio-Rad mini transblotter and immunoblotted with primary antibody, incubated with secondary antibody conjugated with HRP and visualized by the chemiluminescence western blotting kit (Santa Cruz Biotechnology Inc.). Cytosolic extract was prepared using a previously reported method and used for western blotting against cytochrome c antibody (43). To monitor equal loading of protein, western blot analysis using antibody directed against actin was done for each experiment as shown in lower panels.
RNA isolation, RT–PCR

Total RNA was isolated from the murine peritoneal macrophages by TRI reagent in accordance with the supplier’s instructions. The RNA was reverse transcribed using one-step RT–PCR kit (QIAGEN, Germany) and amplified by PCR using the specific murine primers. The thermocycler conditions were 28 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, after which an additional extension step at 72°C for 5 min was included. Electrophoresis of amplified DNAs was carried out on a 2% agarose gel and stained with ethidium bromide. The sequences of primers specific for murine genes are as follows: Mouse Bax—forward primer, 5'-TGCAGAGGATGATTGCTGAC-3' and reverse primer, 5'-GGAGGAAGTCCAGTGTCCAG-3'; Mouse Bcl-2—forward primer, 5'-AATGTCCAGGTGGGTCAGAG-3' and reverse primer, 5'-TCCTGCTGGATCTGCCTAGT-3'; and Mouse GAPDH—forward primer, 5'-GGTGAAGGTCGGTGATTT-3' and reverse primer, 5'-GATGCCAAGTTGTCATGGATGTCC-3'.

Statistical analysis

The statistical significance of difference between the test groups was analyzed by Student’s t-test (two tailed). P-value of <0.05 is considered significant.

Results

rYopJ-mediated cytotoxicity in murine peritoneal macrophages in vitro

rYopJ produced a single band on SDS–PAGE (10%) with Coomassie Brilliant Blue R-250 staining (Fig. 1A). Dot immuno blotting using an antibody specific for rYopJ (Provided by Dr H. V Batra) was done to check the specific reactivity (Fig. 1B). The percentage cytotoxicity of the rYopJ on macrophages was quantitatively determined by MTT assay (42). rYopJ induced significant cytotoxicity toward macrophages in a dose- and time-dependent manner (Fig. 1C and D). rYopJ boiled for an hour or proteolytic degraded by digestion with proteinase K (20 l/gm l⁻¹) for 30 min did not induce death in macrophages. Similarly, pre-treatment of rYopJ with polymixin B did not prevent the rYopJ-induced cell death of macrophages. Higher dose of LPS (30 l/gm l⁻¹) was cytotoxic to macrophages. Boiled LPS did not inhibit the cell death of macrophages; however, pre-treatment of macrophages with polymixin B significantly inhibited LPS-induced cell death. Recombinant YopM protein used at the same concentration as rYopJ (3 l/gm l⁻¹) did not show cytotoxic activity on macrophages (Fig. 1E). These findings indicate that recombinant protein was not contaminated with endotoxin.

rYopJ induces apoptosis in murine peritoneal macrophages in vitro

To confirm that macrophage cell death triggered by rYopJ is due to apoptosis, typical feature of apoptosis like exposure of phosphatidylserine on the outer surface of cell membrane as an early marker of apoptosis, nuclear morphology by DAPI staining and DNA fragmentation was investigated. Macrophages were treated with various doses of rYopJ for different time intervals and free oligonucleosome unit due to DNA fragmentation was quantified by Nucleosome ELISA as described in Methods. It is observed that rYopJ-induced DNA fragmentation in macrophages was dose and time-dependent. Fig. 6. (A) Effect of cytochalasin B and caspase inhibitors on rYopJ (3 µg ml⁻¹) induced DNA fragmentation in murine peritoneal macrophages. Macrophages were pre-treated with either cytochalasin B (10 µM) or broad-spectrum caspase inhibitor Z-VD-FMK (25 µM) or caspase-3 inhibitor Ac-DEVD-CHO (100 µM) or caspase-8 inhibitor Z-IETD-FMK or caspase-9 inhibitor Z-LEHD-FMK (20 µM) for an hour washed and then further treated with rYopJ for 18 h. The number of free oligonucleosome unit was quantified by Nucleosome ELISA. Untreated cells were taken as control. Each bar represents the standard error of three independent experiments. (B) Photomicrograph of immunofluorescence staining of rYopJ in murine peritoneal macrophages. Macrophage monolayers were treated with rYopJ (3 µg ml⁻¹) for 2 h, fixed under non-permeabilized condition, incubated with anti-rYopJ and then stained with FITC-anti-rabbit secondary antibody as described in experimental procedures. Untreated macrophages were taken as control. The figures are representative of three independent experiments with similar result. DIC, differential interference contrast; FL, fluorescence.
dependent and dose 3 µg ml⁻¹ (Fig. 2A) was found to induce maximum fragmentation at 18 h (Fig. 2B). A dose of 4 µg ml⁻¹ and higher doses of rYopJ induced a rapid cell death of macrophages within 6–12 h of treatment. rYopJ-induced apoptosis was further confirmed by the externalization of phosphatidylserine on surface of cell membrane by fluorescence microscope using FITC-conjugated Annexin V and staining with PI. It was observed that at early hours (3 h of rYopJ treatment), macrophages were brightly stained for Annexin V only. Both Annexin V and PI staining exhibited late stages of apoptosis in macrophages incubated with rYopJ for 16 h (Fig. 2C). Chromatin condensation and nuclear fragmentation was also observed by DAPI staining of macrophages treated with 3 µg ml⁻¹ of rYopJ for 18 h (Fig. 2D). Macrophages stained for Annexin V and PI as observed under fluorescence microscope were counted from four different areas and their mean was expressed as percentage apoptotic cells (Fig. 2E).

**rYopJ induces cleavage of caspase-3, PARP, caspase-8 and up-regulates Apaf-1 expression**

rYopJ (3 µg ml⁻¹) treatment of macrophages resulted in activation of caspase-3 and caspase-8 in a time-dependent manner as observed by the expression of cleaved activated form of both caspases by western blotting (Fig. 3A and C). Caspase-3 substrate PARP cleavage and enhanced expression of Apaf-1 were also observed in macrophages treated with rYopJ (Fig. 3B). Caspase-3 and caspase-8 activities were significantly enhanced in macrophages on treatment with rYopJ, as measured by fluorimetric caspase-3 and caspase-8 activity assay kits. rYopJ did not induce caspase-3 activation in macrophages pre-treated with caspase-3 inhibitor Ac-DEVD-CHO (100 µM) and caspase-8 inhibitor Z-IETD-FMK (50 µM) (Fig. 3D). However, rYopJ-induced caspase-8 activity was inhibited in macrophages pre-treated with caspase-8 inhibitor Z-IETD-FMK but not with the caspase-3 inhibitor Ac-DEVD-CHO (Fig. 3E). It suggests that caspase-3 may not be involved in activation of caspase-8 in rYopJ-treated macrophages.

**rYopJ induces Bid cleavage, changes MMP and releases cytochrome c from mitochondria**

The mitochondrial death pathway is mediated through cleavage of Bid by caspase-8. Caspase-8-mediated Bid cleavage was investigated by western blotting. rYopJ (3 µg ml⁻¹)-treated macrophages showed time-dependent decrease in the expression of full-length Bid (Fig. 4A). Full-length Bid was not detected by western blotting in macrophages treated with rYopJ for 12 h. Concomitant with this, increased cytosolic cytochrome c was detected (Fig. 4A). Changes in MMP (Δψm) were also detected in macrophages treated with rYopJ. Macrophages showing green fluorescence suggest cells with changed MMP, whereas cells with normal mitochondria show red fluorescence. The number of macrophages with altered MMP significantly increased with time on treatment with rYopJ. The green fluorescent cells observed under fluorescence microscope were counted from four different area and their mean is expressed as percentage apoptotic cells (Fig. 4B). Pre-treatment of macrophages with caspase-8 inhibitor Z-IETD-FMK inhibited the alteration in MMP (Δψm) induced by rYopJ (data not shown). It suggests that the rYopJ-induced Bid cleavage is required for activation of mitochondrial death pathway in macrophage.

**rYopJ induces up-regulation of Bax and down-regulation of Bcl-2 in murine peritoneal macrophages**

Levels of Bax and Bcl-2 proteins play important role in determining the fate of cells undergoing apoptosis. The expression of both Bax and Bcl-2 in rYopJ (3 µg ml⁻¹)-treated macrophages was checked by western blotting. It is observed that the expression of Bax is significantly enhanced between 3 and 6 h in rYopJ-treated macrophages (Fig. 5A). On the contrary, the expression of anti-apoptotic protein Bcl-2 was observed to be significantly decreased with time. The data obtained from western blotting were further supported by the gene expression of Bax and Bcl-2 as investigated by RT-PCR and immunofluorescence staining (Fig. 5B and C).

**rYopJ acts extracellularly and induces caspase-dependent apoptosis in murine peritoneal macrophages**

To check whether the caspase activation is necessary for rYopJ (3 µg ml⁻¹)-induced apoptosis, macrophages were pre-treated with broad-spectrum caspase inhibitor Z-VAD-FMK (25 µM) or caspase-8 inhibitor Z-IETD-FMK (50 µM) or caspase-9 inhibitor Z-LEHD-FMK (20 µM) or caspase-3 inhibitor Ac-DEVD-CHO (100 µM) for an hour, incubated with rYopJ for 18 h and subjected to free oligonucleosome unit detection. rYopJ-induced DNA fragmentation was completely prevented in cells pre-treated with broad-spectrum caspase inhibitor Z-VAD-FMK, whereas level of free oligonucleosome was significantly reduced in macrophages pre-treated with caspase-3 and caspase-8 inhibitors (Fig. 6A).

To determine whether rYopJ acts through surface or gets translocated inside macrophages, we localized rYopJ by immunofluorescence staining under non-permeabilized conditions. As shown in Fig. 6(B), fluorescence was only observed at the surface of rYopJ-treated macrophages. Blocking the possible internalization of rYopJ through endocytosis by pre-treatment of macrophages with cytochalasin B (10 µM) did not prevent the rYopJ-induced DNA fragmentation (Fig. 6A).

**Discussion**

The induction of apoptosis or programmed cell death in host cells is a common strategy by which pathogenic bacteria interfere with the host immune response (44). Apoptosis involves the proteolytic action of caspases, which cleaves various substrates inside the cell and causes cell death. The *Yersinia* species signals macrophages and dendritic cells to undergo apoptosis and YopJ is necessary for this cell death (12, 13, 17, 45). However, the signaling pathways leading to *Yersinia*-induced macrophage cell death are poorly understood. In the present study, we have provided evidence that rYopJ can induce apoptosis in murine peritoneal macrophages in vitro. We demonstrate that rYopJ activated caspase-8, caspase-3, induces PARP and Bid cleavage, changes in MMP, cytochrome c release,
up-regulated Apaf-1 expression and DNA fragmentation in murine peritoneal macrophages. rYopJ-induced cytotoxicity was dose and time dependent (Fig. 1A and B). rYopJ-induced apoptosis in macrophages was further validated with Annexin V and PI staining. rYopJ-treated macrophages stained brightly for Annexin V at 3 h. At late stages of treatment (16 h), macrophages stained feebly for Annexin V but brightly for PI, confirming apoptotic death of the macrophages (Fig. 2C). DAPI staining of rYopJ-treated macrophages also exhibited nuclear condensation and fragmentation. Further, DNA fragmentation was quantitatively measured in rYopJ-treated murine macrophages (Fig. 2D). The significantly enhanced DNA fragmentation was dose and time dependent (Fig. 2A and B). Caspases play major role in apoptosis, caspase-3 being the chief executioner molecule (46). Our data indicate that rYopJ induces activation of caspase-3 in macrophages, which was inhibited by caspase-8 inhibitor. Western blot analysis has shown that the expression of full-length Bid decreased in rYopJ-treated macrophages with increased expression of cytosolic cytochrome c. The cleavage of Bid protein is caspase-8 dependent (47). It is observed that rYopJ treatment of macrophages activates caspase-8, resulting in production of truncated Bid, causing the release of cytochrome c from the mitochondria. We suggest that rYopJ interacts with a cell surface receptor, probably TLR-2 to induce apoptosis. There are reports suggesting YopJ may be a TLR-2 ligand and it is already known that bacterial lipoproteins can signal through TLR-2 (43, 48). Zhang and Bliska (49) have also demonstrated the possible role of TLR signaling in Yersinia-induced macrophages apoptosis. Denecker et al. (48) have reported that YopP induces Bid cleavage in macrophage cell line J774.1 and suggested the possible role of caspase-8. Therefore, present observation on the rYopJ-induced activation of caspase-8 in macrophages followed by cleavage of Bid is in agreement with previous reports. Cleaved Bid translocates from the cytoplasm to the mitochondria and induces cytochrome c release (50). It is further observed that rYopJ-induced caspase-8 activity was inhibited in macrophages pre-treated with caspase-8 inhibitor Z-IETD-FMK. However, it was unaffected in macrophages pre-incubated with caspase-3 inhibitor Ac-DEVD-CHO. It shows that caspase-3 may not be involved in activation of caspase-8 in rYopJ-treated macrophages. Increased expression of pro-apoptotic molecule Bax and down-regulation of anti-apoptotic molecule Bcl-2 was also detected by western blotting, immunofluorescence microscopy and RT-PCR. This demonstrates the role of Bax in rYopJ-induced macrophages apoptosis and that enhanced expression of Bax and down-regulation of Bcl-2 observed in rYopJ-treated macrophages may be due to truncated Bid-induced conformational changes of Bax (47). The above observations are further supported by the rYopJ-induced change in MMP (Δψm). rYopJ-induced change in MMP (Δψm) was blocked in macrophages pre-treated with caspase-8 inhibitor Z-IETD-FMK (data not shown). It is, therefore, argued that rYopJ-induced macrophage apoptosis is caspase-8 dependent and is mediated through mitochondrial death pathway. To further establish the involvement of mitochondrial pathway of induced apoptosis of macrophages with rYopJ, the macrophages were pre-treated with caspase-9 inhibitor Z-LEHD-FMK (20 μM) (51). It was further observed that the rYopJ-induced apoptosis of macrophages was significantly suppressed in macrophages pre-treated with caspase-9 inhibitor. Since both truncated Bid and Bax can translocate into mitochondria, causing alteration in MMP and cytochrome c release, it is hypothesized that the up-regulation of Bax accelerate the rYopJ-induced apoptosis in macrophages. The fact that inhibition of caspase activation by their inhibitors prevented the rYopJ-induced apoptosis of macrophages, clearly demonstrates the role of caspases in rYopJ-mediated macrophage apoptosis. Endocytosis can be inhibited in macrophages on treatment with cytochalasin B (52). This procedure was incorporated to inhibit the internalization of rYopJ by macrophages. It was observed that pre-treatment of macrophages by cytochalasin B (10 μM) did not prevent the rYopJ-induced cell death (Fig. 6A). This supports our hypothesis that the rYopJ induces apoptosis in macrophages through a cell surface-mediated mechanism. It is further observed that macrophages treated with rYopJ when immunostained under non-permeable conditions with anti-rYopJ antibody and captured by FITC-anti-rabbit IgG, the bright staining was observed at surface of macrophages. It demonstrates that rYopJ present over cell surface and do not permeate into the cytoplasm (Fig. 6B). The possible interaction or binding of the rYopJ with the receptors (i.e. death receptors or TLRs) present over macrophage surface needed further investigation. The present study demonstrates that murine peritoneal macrophages incubated with purified recombinant YopJ protein from Y. pestis undergo apoptotic cell death. Apoptosis triggered was shown to be caspase dependent and was mediated through mitochondrial death pathway.

Funding

Defence Research and Development Organisation, New Delhi to A.S.

Acknowledgements

We thank Dr H. V. Batra of the DRDE, Gwallor for providing recombinant YopJ protein. A.K.P. is a recipient of University Grants Commission Research Fellowship.

Abbreviations

AMC 7-amino-4-methylcoumarin
Apaf-1 apoptosis protease activating factor-1
DAP 4’,6-diamidino-2-phenylindole
MMP mitochondrial membrane potential
MTT 3-(4,5-dimethylthiazol-2-yl-2, 5-diphenyl tetrazolium bromide)
NF-κB nuclear factor-κ B
PARP polyadenosine diphosphate-ribose polymerase
PI propidium iodide
RT reverse transcription
rYopJ recombinant Yersinia outer protein J
T3SS type III secretion system
TNF-α tumor necrosis factor alpha
YopS Yersinia outer proteins

References

rYopJ induces apoptosis in murine peritoneal macrophages in vitro

7 Black, D. S. and Bliska, J. B. 1997. Identification of p130Cas as a substrate of Yersinia Yoph (Yop51), a bacterial protein tyrosine phosphatase that translocates into mammalian cells and targets focal adhesions. EMBO J. 16:2730.


