Research Article

Open Access   CrossMark

Induction of apoptosis in cultured intestinal epithelial cells by adhesin of salmonella enterica serovar typhimurium

Abstract

A mannose sensitive adhesin (~31kDa) of *Salmonella enterica* serovar Typhimurium (Fim+ve strain) was purified by sequential chromatography on Mono Q HR 5/5 anion exchange column and Superose 12 HR 10/30-gel filtration column in the FPLC system. This adhesin could induce morphological changes in human intestinal epithelial cells (INT-407), which might be due to induction of apoptosis as assessed by cell death detection ELISA. This finding was further substantiated by the appearance of DNA ladder in the adhesin treated INT-407 cells. Moreover, up regulated expression of phosphorylated JNK, p53, Bax as well as activated caspases (-9 & -3) was also observed in lysate of the cells cultured with adhesin. Bcl-2, an anti-apoptotic regulator was down regulated in the adhesin triggered cells, although it was present in the cells cultured in absence of the adhesin. Thus, the mannose sensitive adhesin may be a major contributor to *Salmonella* induced death-signaling pathway in human intestinal epithelial cells.

Keywords: *s.enteric* serovar typhimurium, adhesion, apoptosis, *escherichia coli*

Sujata Ghosh, Lipi Singh

Department of Experimental Medicine and Biotechnology, Postgraduate Institute of Medical Education and Research, India

Correspondence: Sujata Ghosh, Department of Experimental Medicine and Biotechnology, Postgraduate Institute of Medical Education and Research, Chandigarh 160 012, India,, Tel 91-0172-2755236, Fax 91-0172-2747403, Email suassi1@gmail.com

Received: April 28, 2016 | **Published:** June 05, 2016

Introduction

Bacterial virulence mechanisms stem from the selective pressure of host environment and *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) is an ideal example of this.[1] *S. typhimurium* causes self-limiting gastroenteritis and poses serious health problem in the regions of unhygienic conditions. Adhesin mediated attachment of enteric pathogens to host intestinal mucosa is an important early event, leading to colonization and infection.[2,3] *S. typhimurium* possesses mannose sensitive type-1 fimbriae, which can bind to specific cell surface carbohydrate structures and thus are responsible for the adherence of the pathogen to intestinal epithelial cells. Adhesin mediated colonization of the epithelial cells by *Salmonella* initiates the activation of diverse signal transduction pathways, leading to invasion of the epithelial barrier.[4,5] Subsequently, macrophages are infected by this organism and ultimately cell death occurs by apoptosis.[6] Apoptosis is a programmed cell death, mediated by different signals originating from intracellular as well as extracellular milieu.[7] A wide range of bacterial virulence determinants including pore forming toxins, other toxins with enzymatic activity and effector proteins released by type-III secretion system, are known to induce apoptosis.[8,9]

It has been reported that type I fimbriae of uropathogenic *Escherichia coli* along with lipopolysaccharide could also trigger oxygen-dependent apoptosis in human neutrophils.[10] Mulvey et al.[11] have shown that uropathogenic *E.coli* expressing type I fimbriae containing the mannose sensitive Fim H adhesin could also bring about exfoliation of the host bladder cells through an apoptosis like mechanism.[11] The importance of the adhesin in the colonization of the enteropathogenic bacteria is a well established fact but its role in transducing signals and ultimately leading to the apoptosis of intestinal epithelial cells has not been reported. Hence, this study has been focused on the role of the mannose sensitive adhesin of *S. typhimurium* host cell death signaling pathways.

Materials and methods

Bacterial strain, cell line and culture conditions

The strain of *S. typhimurium* (fim+ve) was obtained from Dr.

Philip T. Loverde (State University of New York, Buffalo). Bacteria were grown on Luria agar for 48h at 37 °C. INT-407 (human embryo small intestinal epithelial cell line) was obtained from National Centre for Cell Science (Pune), grown in RPMI medium containing fetal bovine serum (10%), L-glutamine (5mM), streptomycin (100µg/ml) and penicillin (100µg/ml) at 37 °C in a CO_2 incubator.

Purification of fimbrial adhesin

The method of Moch et al.[12] (with slight modifications) was used to isolate the fimbrial adhesin of *S. typhimurium*.[12] Briefly, the bacteria cultured on Luria agar were collected, washed and suspended in 10mM Tris/HCl (pH 7.8) buffer. This was followed by detachment of the fimbriae from the bacteria by omnimixing (10min) at 8-10 °C and centrifugation at 12000 x g for 30min.The supernatant containing fimbriae was further centrifuged (227,000 x g, 2.5h) and the pellet (fimbriae-adhesin complex) so obtained was suspended in 10mM phosphate buffer (pH 7.4)/ 150mM NaCl (PBS) containing 2.6mM KCl and 5mM EDTA. Subsequently, the fimbriae-adhesin complex was heated at 70 °C for 1h and centrifuged (1,40,000 x g, 2h).

The supernatant was found to contain the mannose specific adhesin as assessed by its mannose sensitive hemagglutinating activity (HA) with guinea pig erythrocytes.[13] The adhesin containing supernatant was dialyzed and concentrated by lyophilisation. Further, the crude adhesin (5.9mg) was purified using Mono Q HR 5/5 anion exchange column in FPLC system (Pharmacia, Sweden). Briefly, the crude adhesin was loaded on the Mono Q column, pre-equilibrated in 20mM Tris /HCl (pH 7.2) buffer (buffer-A) with a flow rate of 0.5ml/min. After washing the column with buffer- A (till absorbance at 280nm of the eluent was less than 0.002), elution of the bound protein was achieved with a linear gradient of 0-750mM NaCl in the same buffer. The fractions were collected, dialyzed, lyophilized and estimated for the protein content.[14] Each fraction was analysed for mannose sensitive hemagglutination activity (HA) with guinea pig erythrocytes and the fraction showing mannose sensitive HA was further purified by Superose 12 HR 10/30 column in FPLC system. The column was pre-equilibrated with 50mM Tris/HCl (pH 7.2) / 150mM NaCl with a flow rate of 0.5ml/min and the elution was done with the same

buffer. The fractions were collected, dialyzed and concentrated. The protein content of each fraction was estimated. All the fractions were analyzed for mannose sensitive HA and the fraction showing mannose sensitivity was subjected to sodium dodecyl sulphate polyacrylamide (10%) gel electrophoresis (SDS-PAGE)[15] for the assessment of purity as well as confirmation of apparent molecular mass (Mr).

Detection of morphological changes

INT-407 cells (0.25 x 10^6 cells/ ml/ well) were grown in monolayer overnight in 6-well cell culture plates (Greiner, Germany) and incubated in serum free medium for 6h. Following this, the cells were cultured in presence of different doses of the adhesin (0.1, 0.25 & 0.5µg) for 20h. Cells cultured without adhesin were taken as control. The morphological changes of the cells were assessed under inverted microscope (40x).

Evaluation of apoptosis

Cell death detection (CDD) ELISA kit (Roche, Germany) was used to assess the extent of adhesin-induced apoptosis in INT-407 cells.[16] For the assay, serum starved INT-407 cells (5 x 10^4 cells/500µl medium/well of 24-well plates) were treated with various doses of the adhesin (0.025-0.2µg) for 20h. Cells cultured in absence of the adhesin were taken as control. After incubation, the cells were detached from the plate using PBS, centrifuged (1200 rpm, 5min) and washed. Subsequently, the cells were suspended in 250µl of incubation buffer (supplied in the kit), incubated for 30min and the lysate was centrifuged (9000 rpm, 20min). The supernatant (100µl) was collected, diluted (1:10 in the incubation buffer) and subjected to ELISA as per instructions of the manufacturers. The apoptotic index (directly related to percentage of cell death) was calculated as the ratio of the optical density of the adhesin triggered cells to that of control.

Detection of apoptotic DNA ladder

Apoptotic DNA ladder kit (Roche, Germany) was used to assess the pattern of DNA in INT-407 cells (10^6 cells/ml medium/well of 6 well-plates) cultured with and without adhesin under the same condition as described earlier. After washing with PBS, the cells were lysed with lysis buffer [10mM Tris/HCl (pH 4.4) / 6M guanidine-HCl / 10mM Urea / 20% TritonX-100] (10min, 15-20 °C). Subsequently, isopropanol was added to the lysate and the mixture was shaken followed by isolation of DNA in separating unit (filter tube with glass fiber fleece) by centrifugation (8000rpm, 1min). After washing with washing buffer, DNA was eluted from the separating unit using pre-warmed (70 °C) elution buffer. Finally, DNA was analysed by agarose gel (1.5%) electrophoresis[17] and visualized under UV light in gel documentation system (Biorad Laboratories, Italy).

Assessment of the expression of pro- and anti-apoptotic markers

The adhesin induced expression of marker proteins of apoptotic pathway in INT-407 cells was assessed by Western immunoblotting. Briefly, the cells (10^6) were cultured with and without adhesin as mentioned before. After detachment of the cells from each well, the cells were washed and suspended in 270µl lysis buffer [10mM HEPES buffer (pH 7.5) containing 150mM NaCl, glycerol (10%), $NaVO_4$ (10mM), Triton X-100 (0.6%) & cocktail protease inhibitors (30µl)] and incubated for 30min at 4 °C.[18] The lysate was centrifuged (10,000rpm, 10min) to remove the cell debris and the supernatant was estimated for the protein content.[19] The lysate obtained from cells (adhesin treated as well as untreated) was run separately on SDS-PAGE (10%) under reducing conditions.[15] This was followed

by electrophoretic transfer of the protein bands in the gel onto polyvinylidene difluoride (PVDF) membrane.[20] After blocking in 20mM Tris/HCl (pH 7.2) / 150mM NaCl (TBS) containing 5% skim milk [SM] , the membrane strips were incubated (3h, 37 °C) separately in presence of specific antibodies [Santa Cruz Biotechnology (CA, USA)] against p53 [p53 (E-19)], Bax [Bax (P-19)], Bcl-2 [Bcl-2 (N-19)], Caspase-9 [Caspase 9 p10 (H-83)], Caspase-3 [Caspase-3 p20 (N-19)], each diluted to 1:400 in TBS-SM and phosphorylated JNK (Thr 183 / Tyr 185), diluted to 1:200 in the same buffer. The membrane strips were washed with TBS containing 0.05% Tween-20 (TBST), followed by TBS and incubated at 37 °C for 1h with HRP-conjugated respective secondary antibody (diluted to 1:1000 in TBS-SM). After washing, the strips were developed using 3,3'-Diaminobenzidine-tetrahydrochloride (0.05%)/H_2O_2 (1µl/ ml) in TBS.

Statistical analysis

The data was analyzed by standard statistical methods [mean and standard deviation (SD) wherever applicable]. One-way ANOVA (Tukey HSD test) was applied to analyze the data. The probability value (p) of <0.05 was considered to be significant.

Results

The fimbrial adhesin of *S. typhimurium*(fim+ve) was purified using a simple approach. In this process, heating of the fimbriae-adhesin complex (70 °C, 1h) followed by ultra-centrifugation yielded mannose specific adhesin rich supernatant. The elution profile of the crude adhesin by anion exchange chromatography is shown in Figure 1A. The fraction eluted at 22.8 min (11.4 ml) with 66% of NaCl showed mannose sensitive HA with guinea pig erythrocytes. The yield of the Mono Q eluted fraction containing the mannose specific adhesin was 0.534mg. When this fraction was further chromatographed on Superose 12HR 10/30 column, it revealed four peaks (Figure 1B). The peak 1 eluted at 14.5min showed the mannose sensitive HA and a single band of ~31kDa (Figure 1C). The yield of the purified mannose specific adhesin was 0.135mg.
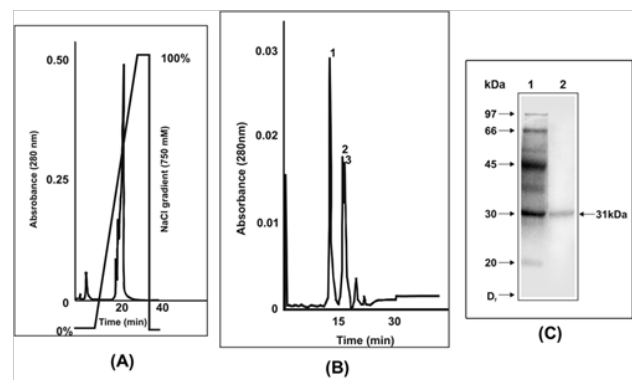

Figure 1A Elution profile of crude adhesin on Mono Q HR 5/5 column. The fraction eluted at 22.8 min contains the mannose sensitive adhesin.

Figure 1B Elution profile of Mono Q purified adhesin on Superose 12 HR 10/30 column. The purified adhesin eluted at 14.5 min (peak 1).

Figure 1C Electrophoretic pattern of the purified adhesin on SDS-PAGE.

Lane 1: Molecular weight marker

Lane 2: Purified adhesin.

This adhesin at a dose of 2µg was found to induce distinct morphological changes in INT-407 cells (10^6 cells/ml) at 20h (Figure 2B) as compared to untreated cells (Figure 2A). It was observed that the cells were detached from the substratum and became round

in appearance.In CDD- ELISA, the intestinal epithelial cells (5×10^4 cells/500µl) cultured with different doses of the adhesin (0.25-0.2µg) showed variation in absorbance at 492nm due to difference in the number of mono- and oligonucleosomes. The apoptotic index of the cells cultured in presence of adhesin at the dose of 0.025µg, 0.05µg, 0.1µg and 0.2µg was found to be higher as compared to the cells cultured in absence of the adhesin (Figure 3). The result clearly indicated that 0.1µg of the adhesin could induce maximum apoptosis (1.56 folds) in INT-407 cells (5×10^4cells/ 500µl) at 20h. Thus, 2µg mannose sensitive adhesin/ 10^6 INT-407 cells/ ml were used in study of all other parameters. DNA fragmentation is known as the hallmark of apoptosis. Thus, the pattern of DNA isolated from the human intestinal epithelial cells cultured with and without adhesin was analysed on 1.5% agarose gel electrophoresis (Figure 4). DNA isolated from the adhesin triggered cells revealed a ladder like appearance (lane 2), whereas no such ladder was observed in case of untreated INT-407 cells (lane 1).

Further, the level of expression of different regulatory and effector molecules of apoptotic pathway was assessed in the adhesin treated INT-407 cells. Maximum expression of phosphorylated JNK was observed in the lysate of the cells cultured in presence of the adhesin, while it was absent in the lysate of untreated cells (Figure 5a). The other proapoptotic molecules such as p53 and Bax were found to be expressed only in the adhesin triggered cells (Figure 5b & 5c) but not in control cells. However, Bcl-2 (antiapoptotic molecule) expression was noticed only in the lysate prepared from the cells cultured in absence of the adhesin (Figure 5d). The proteolytic degradation of effector molecules namely procaspase-9 and procaspase-3 was prominent in the lysate of the adhesin treated INT-407 cells, while the lysate of the untreated cells showed no such cleaved products of these molecules (Figure 5e & 5f).
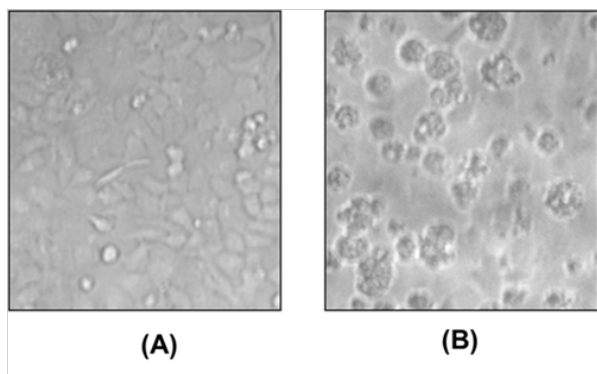

Figure 2 Photomicrograph showing the morphological effects induced by the adhesin on INT-407 cells. (A) Control cells (B) INT-407 cells treated with the purified adhesin.

Discussion and Conclusion

Bacterial adhesins have become the focus of much interest of researchers; since these are known to play a crucial role in adherence, an early event in the disease process.[21] *S. typhimurium* possesses mannose sensitive adhesin at the tip/ lateral position of its type-1 fimbriae and these strains have been reported to adhere in significantly higher number as compared to non-fimbriated strains.[22]

The mechanism of salmonellosis is highly complex which involves the interaction of *Salmonella* with many different cell types like M cells, enterocytes and macrophages. The *Salmonella* strains capable of causing gastroenteritis invade and destroy enterocytes after passage through M cells. Though, M cell-*Salmonella* association may play a

role during the infectious process, the bulk of prokaryotic-eukaryotic interactions are likely to occur over the surface of the epithelial cells, the major cell type throughout the ileum.[23] The best characterized *Salmonella* induced host response is the dramatic rearrangement of cytoskeletal and plasma membrane at the point of bacteria host cell contact,[24] where the major role of adhesin is of prime consideration. It has been reported that *Salmonella* could induce apoptosis in murine macrophages in a caspase-1 dependent fashion and Sip B, a protein of type III secretion system was found to be responsible for such cell death.[25,26] Further, studies have shown that Pathogenicity Island 2 as well as virulence plasmid of *Salmonella* is essential for induction of apoptosis in epithelial cells.[27] However, no report exists in the literature regarding apoptosis in the intestinal epithelial cells induced by the mannose sensitive adhesin, a potentially important virulence factor of *Salmonella*. Thus, the study on the role of the mannose specific adhesin of *S. typhimurium* on apoptosis in human intestinal epithelial cell line is essential.
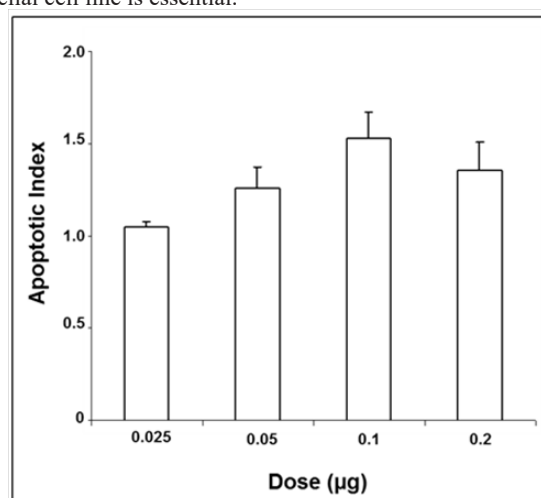

Figure 3 Apoptosis induced by different doses of the Salmonella adhesin in INT-407 cells as evaluated by cell death detection ELISA.
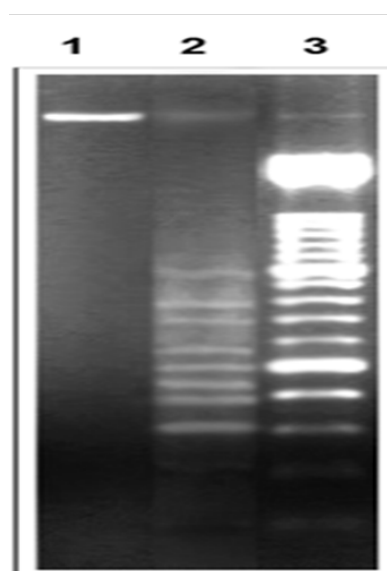

Figure 4 Agarose gel electrophoresis for the analysis of DNA.

Lane 1: Cells only

Lane 2: Cells cultured in presence of the adhesin

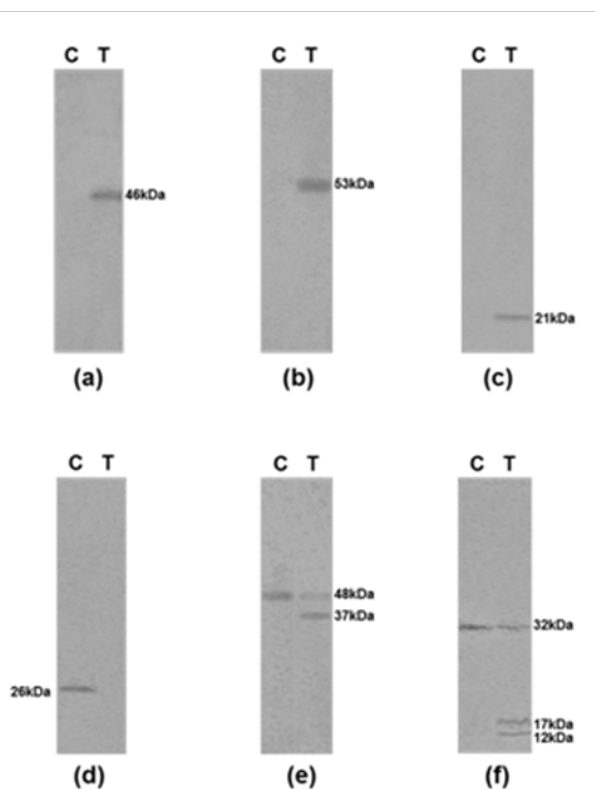Lane 3: Molecular weight marker XIV (100bp ladder).

Figure 5 Western Blot analysis for expression of pro- and anti- apoptotic markers. (a) JNK, (b) p53, (c) Bax, (d) Bcl-2, (e) Caspase-9 and (f) Caspase-3 in INT-407 cells in absence (C) and in presence (T) of the adhesin.
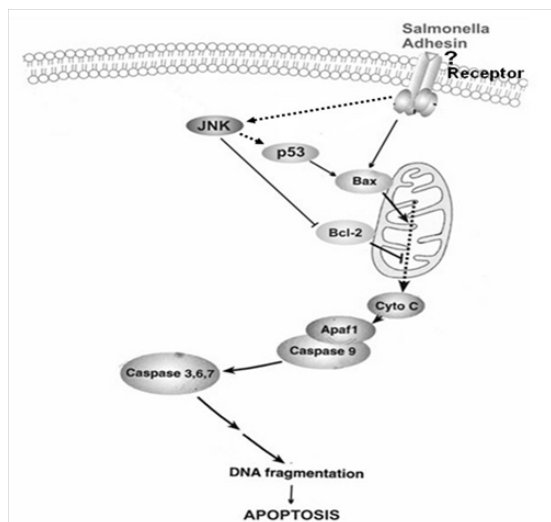

Figure 6 Proposed model for the mode of action of the Salmonella adhesin. The mannose sensitive adhesin of *S. typhimurium* could activate JNK which in turn phosphorylated p53 leading to the upregulation of Bax. The phosphorylated JNK could deactivate Bcl-2 resulting in the release of cytochrome C from the mitochondria and activation of various caspases such as caspase-9 and caspase-3 leading to DNA fragmentation and ultimately apoptosis.

In the present study, the mannose sensitive adhesin was purified using a combination of differential centrifugation followed by sequential chromatography in the FPLC system. The adhesin induced morphological alterations in INT-407 cells tempted us to look for whether this change could be due to the induction of apoptosis in the cells by the adhesin or not. This was confirmed by CDD-ELISA, which resulted in a dose dependent increase in apoptotic index of INT-407 cells treated with the adhesin. Further, the adhesin induced formation of DNA ladder in the cells supported this finding. In the present study, the adhesin was found to induce apoptosis in INT-407 cells at about 20h. Paesold et al.[27] have also reported that *Salmonella dublin* could induce apoptosis at 24-33h in human colon epithelial cells. However, apoptosis in murine macrophages by *Salmonella* was found to be a very rapid process.[26]

Our next attempt was to investigate the intracellular pathways through which the apoptosis could be induced in the adhesin triggered INT-407 cells. Thus, the level of the expression of different regulators as well as effectors of apoptosis was assessed. The c-Jun N-terminal kinase (JNK) cell signaling pathway is known to be activated in response to a variety of insults including radiation, heat shock, osmotic stress and inflammatory cytokine.[28] In our study, the phosphorylated JNKs i.e. the activated JNKs were expressed in the cells cultured in presence of the adhesin. Activated JNK is known to phosphorylate the substrates such as c-Jun and p53 and induce apoptosis through multiple mechanisms including modification of transcriptional regulation of proteins in Bcl-2 family.[29]

Available data suggests that p53 suppresses the growth by two mechanisms namely cell cycle arrest and apoptosis.[30] In one of the studies, using the inducible p53 expressing cell line, it was found that low level of p53 could inhibit proliferation whereas high level could stimulate apoptosis.[31] In the present study, the level of p53 was found to be upregulated in the adhesin treated human intestinal epithelial cells. Thus, our observations are well corroborated with the previous report. The p53 could also induce apoptosis by increasing the expression of Bax.[32] Bax is a proapoptotic member of Bcl-2 family that can antagonize the protective role of Bcl-2 (an anti-apoptotic member) with the formation of Bax/ Bcl-2 heterodimers. Bax can also bind to itself, forming Bax/ Bax homodimers and the Bax/Bcl-2 ratio determines the fate of a cell.[33] Pastorino et al. have reported that over expression of Bax in jurkat cells could induce the mitochondrial permeability transition.[34] This event was accompanied by upregulation of cytosolic cytochrome-C, activation of pro-caspases, fragmentation of DNA and apoptosis. In the present study, Bax was found to be expressed in the adhesin triggered INT-407 cells, while Bcl-2 was down regulated.

Activation of Bax is known to release cytochrome C from mitochondria into cytosol, which then binds and activates apoptotic protease activating factor-1. This is followed by activation of procaspase-9 (48kDa), an important member of cysteine aspartic acid protease (Caspase) family. Upon activation, procaspase-9 is cleaved into a large active subunit (37 kDa), which in turn cleaves cytoskeletal as well as nuclear proteins and induces apoptosis.[35,36]

In the present investigation, the activated form of procaspase-9 was found to be expressed as the large subunit (37kDa) in Western immunoblot of the lysate, obtained from the adhesin treated INT-407 cells. Caspase-3, an important executioner of apoptosis, is known to be responsible for proteolytic cleavage of many key proteins.[37] In this study, the presence of the 17kDa and 12kDa bands of caspase-3 in the Western immunoblot of the lysate obtained from cells cultured in presence of the adhesin clearly revealed the adhesin induced-activation of procaspase-3 in INT-407 cells.

Previous study reported that *Salmonella dublin* could induce apoptosis of HT-29 (human colon epithelial cells) by caspase-3 activation which is in good agreement with our observations.[27]

Further, studies have shown that Sip B protein of *Salmonella* could induce apoptosis in murine macrophages by activation of caspase-1,[26] while caspase-3 and the regulatory proteins of the Bcl -2 family, the key elements of mitochondrial pathway were not found to be required in this cell death pathway. Hence, it is possible that cross talk among various pathways may be involved in *Salmonella* induced apoptosis in intestinal epithelial cells.

Thus, it can be suggested that probably *S. typhimurium* through its mannose sensitive adhesin can activate the p53 and JNK mediated cell signaling pathways in INT-407 cells resulting in increased expression of Bax, down regulation of Bcl-2 and finally activation of procaspases (-9 & -3) leading to fragmentation of DNA and ultimately apoptosis (Figure 6). In conclusion, it can be speculated that the mannose sensitive adhesin may be a major contributor to *Salmonella* induced apoptosis in intestinal epithelial cells and thus may have a key role in *Salmonella* pathogenesis.

Acknowledgments

None.

Conflicts of Interset

None.

References

1. Jarvelainen HA, Galmiche A, Zychlinsky A. Caspase-1 activationby Salmonella. *Trends Cell Biol.* 2003;13(4):204–209.

2. Pizarro-Cerda J, Cossart P. Bacterial adhesion andentryintohostcells. *Cell.* 2003;124(4):715–727.

3. Grover V, Ghosh S, Sharma N, et al. Characterization of a Galactose Specific Adhesin of Entero aggregative *Escherichia coli*. *Arch Biochem Biophys*. 2001;390(1):109–118.

4. McCormick BA, Miller SI, Carnes D, et al. Transepithelial signaling to neutrophils by *Salmonella*: anovelvirulence mechanism for gastroenteritis. *Infect Immun*. 1995;63(6):2302–2309.

5. Brumell JH, Steele Mortimer O, Finlay BB. Bacterial invasion: force-feeding by *Salmonella*. *CurrBiol*. 1999;9(8):R277–R280.

6. Chen LM, Kaniga K, Galan JE. *Salmonella* spp. Are cytotoxic for cultured macrophages. *Mol Microbiol*. 1996;21(5):1101–1115.

7. Ashida H, Mimuro H, Ogawa M, et al. Cell death and infection: adouble-edged sword for host and pathogen survival. *J Cell Biol.* 2003;195(6):931–942.

8. Hossain Z, Fakruddin Md. Mechanism of host cell death in response to bacterial infections. *J Clin Cell Immunol*. 2008;3(4):128.

9. Cotter PA, DiRita VJ. Bacterial virulence generegulation: Anevolution aryperspective. *Annu Rev Microbiol*. 2000;54: 519–565.

10. Blomgran R, Zheng L, Stendahl O. Uropathogenic *Escherichia coli* trigger oxygen-dependent apoptosis in human-neutrophils through the cooperative effect of type-1 fimbriae and lipopolysaccharide. *Infect Immun*. 2004;72(8):4570–4578.

11. Mulvey MA, Lopez-Boado YS, Wilson CL, et al. Induction and evasion of host defenses by type1-piliateduro pathogenic *Escherichia coli. Science*. 1998;282(5393):1494–1497.

12. Moch T, Hoschutzky H, Hacker J, et al. Isolation and characterization of the alpha-sialyl-beta-2,3-galactosyl-specific adhesin from fimbriated *Escherichia coli*. *Proc Natl Acad Sci U S A*. 1987;84(10):3462–3466.

13. Basu S, Sarkar M, Mandal C. A single step purification of asialic acid binding lectin (AchatininH) from Achatina fulica snail. *Mol Cell Bio chem*. 1987;71(2):149–157.

14. Lowry OH, Rosebrough NJ, Farr AL, et al. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;193(1):265–275.

15. Laemmli UK. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature*. 1970;227(5259):680–685.

16. Singh L, Arora SK, Bakshi DK, et al. Potential role of CXCL10 in the induction of cell injury and mitochondrial dysfunction. *Int J Exp Pathol*. 2010;91(3):210–223.

17. Sam brook J, Fritsch EF, Maniatis T. *Molecular cloning: A laboratory manual*. (2nd edn), Cold Spring Harbor Laboratory Press, NewYork, USA. 1989.

18. Bui KC, Buckley S, Wu F, et al. Induction of A- and D type cyclins and cdc2 kinase activity during recovery from short-term hyperoxiclung injury. *Am J Physiol*. 1995;268(4 Pt 1):L625–L635.

19. Smith PK, Krohn RI, Hermanson GT, et al. Measurement of protein using bicinchoninic acid. *Anal Biochem.* 1985;150(1):76–85.

20. Towbin H, Staehelin T, Gordon J. Electrophoresis transfer of protein from polyacrylamide gelsto nitrocelluloses heets procedure and same applications. *Proc Natl Acad Sci USA*. 1989;76(9):4350–4354.

21. Goya lA, Bhattacharyya S, Majumdar S, et al. Cellular response induced by a galactose-specific adhesin of enteroaggregative *Escherichia coli* in INT-407cells. *FEMS Immunol Med Micro biol.* 2009;55(3):378–387.

22. Althouse C, Patterson S, Fedorka-Cray P, et al. Type1 Fimbriae of *Salmonella enterica* Serovar Typhimurium Bind to Enterocytes and Contribute to Colonization of Swine *in Vivo*. *Infect Immun*. 2003;71(11):6446–6452.

23. Meyerholz DK, Stabel TJ, Ackermann MR, et al. Early Epithelial Invasion by *Salmonella enterica* Serovar Typhimurium DT104 in the SwineIleum. *Vet Pathol*. 2002;39(6):712–720.

24. Nickerson CA, Goodwin TJ, Terlonge J, et al. Three-Dimensional Tissue Assemblies: Novel Models for the Study of *Salmonella enteric* Serovar Typhimurium Pathogenesis. *Infect Immun*. 2001;69(11):7106–7120.

25. Monack DM, Raupach B, Hromockyj AE, et al. *Salmonella* typhimurium invasion induces apoptosis in infected macrophages. *Proc Natl Acad Sci USA.* 1996;93(18):9833–9838.

26. Hersh D, Monack DM, Smith MR, et al. The *Salmonella* invasion SipB induces macrophage apoptosis by binding to caspase-1. *Proc Natl Acad Sci U S A*. 1999;96(5):2396–2401.

27. Paesold G, Guiney DG, Eckmann L, et al. Genes in the Salmonella pathogenicity island 2 and the Salmonella virulence plasmid are essential for Salmonella-induced apoptosis in intestinal epithelial cells. *Cell Microbiol*. 2002;4(11):771–781.

28. Zarubin T, Han J. Activation and signaling of p38 MAP kinase pathway. *Cell Res*. 2005;15(1):11–18.

29. Bauschmann P, Potopova O, Bar-Shira A, et al. NH2-terminal kinase phosphorylation of p53 and Thr-81 is important of p53 stabilization and transcriptional activities in response to stress. *Mol Cell Biol*. 2001;21(8):2743–2754.

30. Bates S, Vousden KH. Mechanism of p53 mediated apoptosis. *Cell Mol Life Sci*. 1999;55(1):28–37.

31. Nakano K, Wousdan KH. PUMA, a novel proapoptotic gene is induced by p53. *Mol Cell*. 2001;7(3):683–694.

32. O'Reilly MA, Staversky RJ, Stripp BR, et al. Exposure to hyperoxia induces p53 expression in mouse lung epithelium. *Am J Res Cell Mol Biol*. 1998;18(1):43–50.

33. Yang E, Korsmeyer S. Molecular thanatopsis: a discourse on the Bcl-2 family and cell death. *Blood*. 1996;88(2):386–401.

34. Pastorino JG, Tafani M, Rothman RJ, et al. Functional consequences of the sustained or transient activation by Bax of the mitochondrial permeability transition pore. *J Biol Chem*. 1999;274(44):31734–31739.

35. Budihardjo I, Oliver H, Lutter M, et al. Biochemical pathways of caspase activation during apoptosis. *Annu Rev Cell Dev Biol*. 1999;15:269–290.

36. Salvesen GS. Caspases and apoptosis. *Essays Biochem*. 1999;38:9–19.

37. Hengartner MO. The biochemistry of apoptosis. *Nature*. 2000;407(6805):770–776.