International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 14 (3), pp. 001-006, March, 2020. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Vibrio parahaemolyticus' thermostable direct hemolysin disrupts actin and activates caspase-3 in Rat-1 cells

Rochman Naim[1]*, Fachriyan Hasmi Pasaribu[2], Tetsuya Iida[3] and Takeshi Honda[3]

[1]Faculty of Medicine and Health Sciences, Universiti Sultan Zainal Abidin, Jalan Sultan Mahmud, 20400 Kuala Terengganu, Terengganu, Malaysia.

[2]Department of Animal Diseases and Veterinary Public Health, Faculty of Veterinary Medicine, Institut Pertanian Bogor, Bogor, Indonesia.

[3]Department of Bacterial Infections, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan.

Accepted 17 January, 2020

In our previous studies, Rat-1 cells treated with *Vibrio parahaemolyticus* thermostable direct hemolysin (TDH) developed morphological changes including shrinkage and rounding of the cells. Such alterations suggest that TDH stimulates reorganization of the cellular cytoskeleton. In the current study, the effects of TDH on actin cytoskeleton, chromatin, and caspase-3 were evaluated. Rat-1 cells treated with TDH 5 µg/ml showed redistribution of actin with loss of stress fibers, a floccular staining pattern, cellular membrane blebbing, and cell rounding as assessed by fluorescent actin-staining. This actin redistribution was time dependent. Actin was rapidly disappeared within 10 min after TDH exposure, and the maximal effect was detected by 30 min. TDH-treated cells also showed chromatin condensation which is time dependent. The changes were also detected by as early as 10 min, with the maximal effect by 20 min. Moreover, caspase-3 was activated in TDH-treated Rat-1 cells by time-dependent manner. Pretreatment with a caspase inhibitor (ZVAD-FMK) partially reduced the activation of caspase-3 in the TDH-treated cells. All of the changes found in this study appear to be linked, and the findings, especially activation of caspase-3 in TDH-treated Rat-1 cells, provide a new insight into TDH-mediated cytotoxicity.

Key words: Thermostable direct hemolysin (TDH), *Vibrio parahaemolyticus*, Rat-1 cells, actin disruption, caspase-3 activation, chromatin condensation.

INTRODUCTION

Thermostable direct hemolysin (TDH) is considered as an important virulence factor in *V. parahaemolyticus* gastroenteritis (Honda and Iida, 1993) and is a dimmer composed of two identical subunit molecules of approximately 21 000 Da (Takeda et al., 1978). Genes encoding TDH have been sequenced and cloned (Iida and Yamamoto, 1990). A number of biological properties have been attributed to TDH including hemolytic activity, enterotoxicity, cytotoxicity and cardiotoxicity (Honda and Iida, 1993).

It is well established that TDH has potent cytotoxic activity which have been observed with various cultured cells. In cultured mouse myocardial and mouse melanoma cells, TDH induced morphological damages, including cell shrinking and condensation of the nuclei of both cells (Goshima et al., 1978). In the various eukaryotic cultured cells, TDH induces its cytotoxic effects, and Rat-1 cells were highly sensitive to TDH among 15 cell lines examined (Tang et al., 1997). In the previous study, we reported that Rat-1 cells exposed to TDH showed morphological changes including detachment of cells from their neighbors, apparent loss of cell cytoplasm with shrinkage of most of the cells and

*Corresponding author. E-mail: rnaim@unisza.edu.my. Tel: +60(9)6275665. Fax: +60(9)6275639.

reduction in the size of nuclei (Naim et al., 2001a, b). TDH also induced apoptosis in Rat-1 cells (Naim et al., 2001b).

Several studies on human cultured cells dealing with TDH cytotoxicity have been reported. In human embryonic derived cells (Tang et al., 1997) and in human Caco-2 intestinal cells (Raimondi et al., 2000), TDH was cytotoxic. Recently, it was reported that TDH is also cytotoxic in cultured human epithelial cells, Intestine 407 and HeLa cells (Naim and Pasaribu, 2009).

Although several studies of TDH were conducted on cultured cells, the cytotoxic mechanism of this toxin still remains unclear. In the present study, we demonstrated the effects of TDH on actin cytoskeleton of cultured cells as well as on the chromatin and the caspase-3.

MATERIALS AND METHODS

TDH purification

TDH was purified by a previously described method (Tang et al., 1994).

Cell line and cell culture conditions

Rat-1 cells were maintained under standard cell culture conditions, grown as monolayer culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and gentamicin (100 µg/ml). They were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Actin and nuclear stainings of TDH-treated cells

Cells grown on glass coverslips in six-well plates until approximately 85% confluent were treated with TDH in DMEM for 0-30 min in a 5% CO_2 incubator at 37°C. Cells not treated with TDH served as controls. After treatment, both control and toxin-treated cells were fixed with 3% paraformaldehyde. Then the cells were subjected to actin and nuclear stainings. Actin cytoskeleton of both cells was stained by using rhodamine-phalloidin (Molecular Probes). For nuclear staining, the cells were incubated with Hoechst 33258 (1 µg/ml) at room temperature for 1 min. Images were taken with a fluorescence microscope Olympus BX-50 linked to a camera.

Caspase-3 activity measurements in untreated and TDH-treated Rat-1 cells

Rat-1 cells were grown until approximately 90% confluent, and then the cells were made in suspensions by using DMEM. The cell suspensions were treated with TDH 5 µg/ml for 0-30 min in a 5% CO_2 incubator at 37°C, and cell suspensions were not treated with TDH served as control. Caspase activities in both control and TDH-treated cells were then measured by flow cytometry using PhiPhiLux-G_2D_2 kit (OncoImmunin). The procedures for analysis of caspase activities by flow cytometry were performed as recommended by the manufacturer. Briefly, 75 µl of 10 µM substrate solution was added to each of untreated and TDH-treated Rat-1 cells and then the cells were incubated in a 5% CO_2 incubator at 37°C for 60 min before flow cytometric analysis. For experiments with a caspase inhibitor, cells were pre-incubated with a broad-spectrum caspase inhibitor Z-Val-Ala-Asp-fluoromethylketone (ZVAD-FMK) at a concentration of 100 µM for 2 h before treated with and without TDH.

RESULTS

Effects of TDH on actin cytoskeleton of Rat-1 cells

The effect of 5 µg/ml of TDH on F-actin structure in Rat-1 cells was assessed after 0 to 30 min of exposure. Figures 1A and 1C showed rhodamine-phalloidin staining of F-actin of untreated Rat-1 cells. Fluorescence light microscopy showed long actin-containing bundles in Rat-1 cells. The distribution of actin filaments in the cells was not limited to the bundles, they were also observed along the cell surface membrane. These control cells showed uniform and organized, linear-stress F-actin fibers. In contrast, cells treated with TDH revealed distinct separation from each other with membrane blebbing, loss of stress fibers with a floccular pattern of F-actin staining, and an increased cell globular appearance (Figures 1B, 1D and 1E). The small blebs appeared in various regions of the cell border (Figure 1E). Exposure of Rat-1 cells to TDH induced a generalized collapse of the actin cytoskeleton to a rounded perinuclear position (Figure 1B)

Figure 2 shows the time course of the TDH-treated Rat-1 cells. The morphological changes of F-Actin were observed. A rapid and marked disappearance of the actin filaments occurred within 10 min after exposure to TDH (Figure 2B). Within 20 min, the actin filaments were no longer seen in the cytoplasm and the cells were shrunken (Figure 2C). The maximal effect evident was detected by 30 min and the cells were totally shrunken and rounded (Figure 2D). After treating with TDH, condensation of the nuclei in Rat-1 cells was also seen. Strong chromatin condensation corresponding to an enhancement of the fluorescence intensity could be observed in the cells.

These changes were also detected by as early as 10 min, with the maximal effect by 20 min (Figures 2F, 2G and 2H).

Intracellular Caspase-3 activation in TDH-exposed Rat-1 cells

To measure whether TDH caused the activation of caspase-3, quantitation of intracellular caspase-3 in Rat-1 cells treated with and without TDH was determined by flow cytometry. As shown in Figure 3, Rat-1 cells treated with TDH showed higher caspase-3 activity in time-dependent manner compared to the untreated cells. In untreated cells, the mean fluorescence intensity (MFI) of caspase-3 measured by flow cytometry was (1) 32.19 ± 0.42 (Figure 3). After 10 min treatment of the cells with TDH, caspase-3 was slightly activated, (2) 38.41 ± 1.36 (Figure 3). Caspase-3 in the cells was highly activated after 30 min treatment with TDH, (3) 173.36 ± 5.03

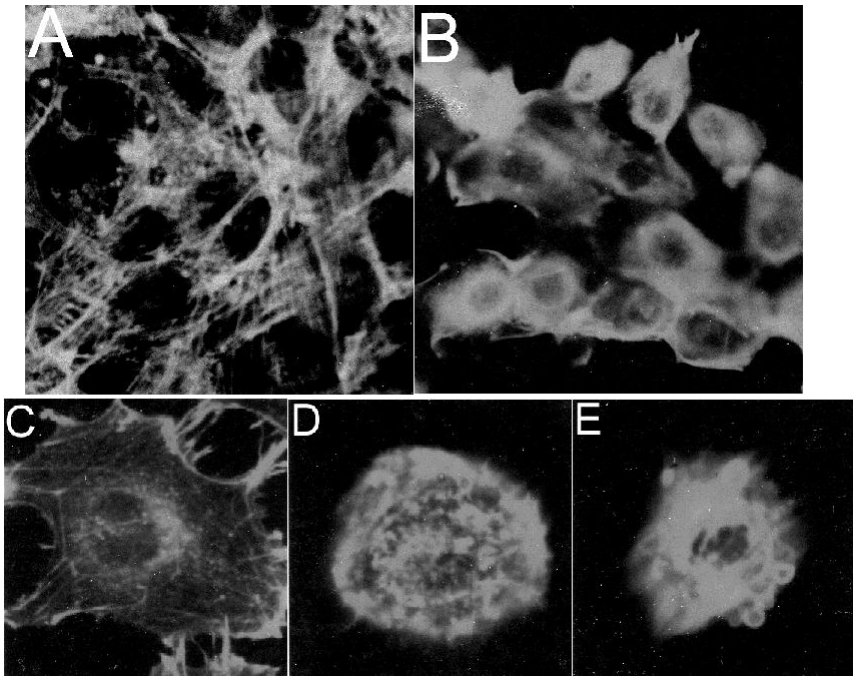

Figure 1. Effect of TDH on the actin cytoskeleton of Rat-1 cells as observed by immunofluorescence microscopy. Cells were stained with rhodamine-phalloidin and photographed at a magnification of 400 for A and B, and 1000 for C, D, and E. A and C are untreated control cells. B, D, and E are cells treated with TDH 5 µg/ml for 30 min. Control cells in smooth-edged clusters with organized stress fibers contrast with TDH-treated cells, which show membrane blebbing, loss of stress fibers, and a floccular pattern of F-actin staining.

(Figure 3). The pre-incubation of cells with a caspase inhibitor ZVAD-FMK could partially reduce the activation of caspase-3 in the cells after treatment 30 min with TDH, (4) 92.70 ± 1.05 (Figure 3).

DISCUSSION

In the previous study, we reported that TDH induces apoptosis in Rat-1 cells (Naim et al., 2001b). The active phase of apoptosis is the execution phase which is characterized by the hallmark morphologic features of apoptosis, including membrane blebbing, chromatin condensation, and DNA fragmentation (Mills et al., 1999). A family of cystein proteases called caspases play a critical role in the the apoptotic program cell death (Pop and Salvesen, 2009). Like many other cellular proteases, caspases are synthesized as inactive proenzymes (Yi and Yuan, 2009) that can be activated upon an apoptotic signal. In this study we found that TDH activates caspase-3 in the cells with the time-dependent manner. Preincubation of the cells with broad-spectrum caspase inhibitor ZVAD-FMK partially reduced activation of caspase-3 by TDH. This study confirms our previous study that ZVAD-FMK was not able to inhibit apoptotic signaling in TDH-exposed Rat-1 cells since it might be not sufficient to totally reduce activation of caspases caused by TDH (Naim et al., 2001b).

There is evidence that caspase-3 contributes to the drastic morphological changes of apoptosis by proteolysing and disabling selected cellular proteins or a number of key substrates. Several substrates involved in regulating actin architecture have been identified. Gelsolin is cleaved by caspase-3 and the cleaved fragment can disrupt actin filaments (Kothakota et al., 1997). Alpha-fodrin is cleaved during apoptosis by caspase-3 and this cleavage could contribute to the remodeling of the cell (Janicke et al., 1998a). Caspase-3-mediated cleavage of p21-activated kinase 2 (PAK2) was implicated in the morphological changes associated with apoptosis (Rudel and Bokoch, 1997).

Caspase-3 has also been shown to cleave a number of structural proteins involved in maintaining nuclear architecture and integrity. Caspase-3 mediated DNA fragmentation by cleaving DNA fragmentation factor (DFF-45) (Liu et al., 1997) and activating the caspase-activated deoxyribonuclease (CAD) that translocates into the nucleus where it degrades the DNA by specifically cleaving and inactivating ICAD, the inhibitor of CAD (Sakahira et al., 1998; Enari et al., 1998). In another study, using the MCF-7 breast carcinoma cell line lacks caspase-3 due to the functional deletion of the

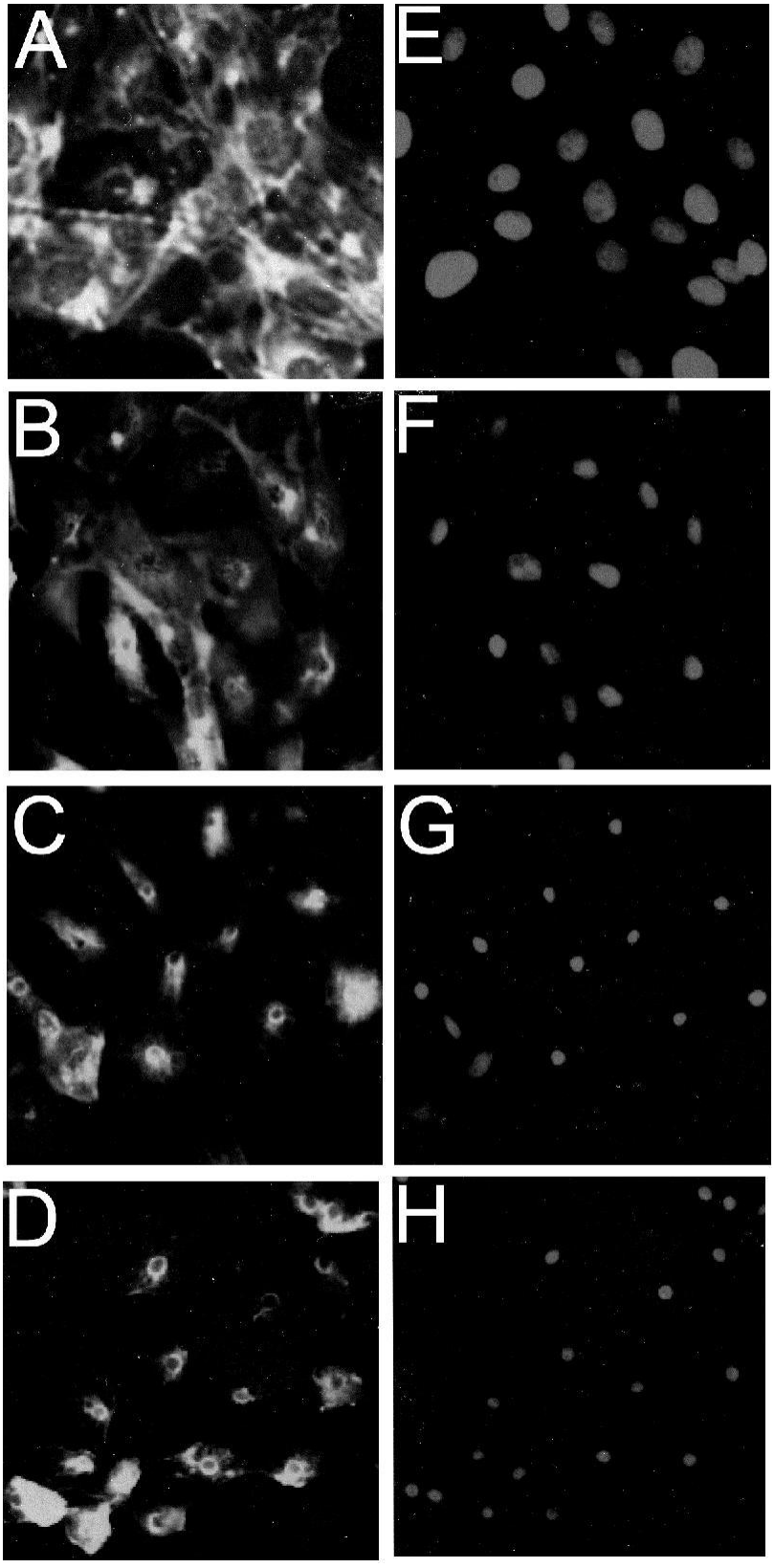

Figure 2. Time course of TDH-treated cells. Subconfluent Rat-1 cells were untreated (A, E) and treated with TDH (5 µg/ml) for 10 min (B, F), 20 min (C, G), and 30 min (D, H). Both untreated and TDH-treated cells were then stained with actin (A-D) and nuclear (E-F) stainings.

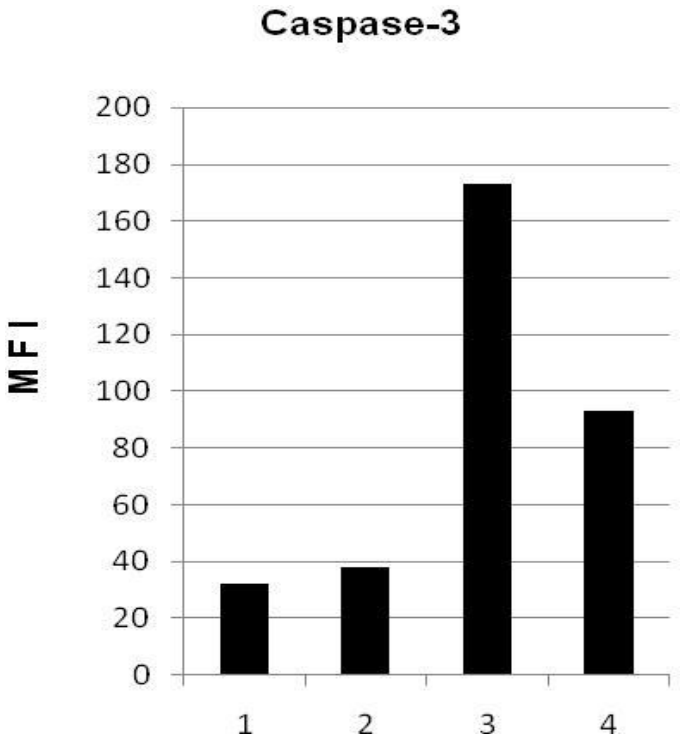

Figure 3. Caspase-3 activation in TDH-treated Rat-1 cells with or without pretreatment with ZVAD-FMK. (1) Cells were not treated with TDH. (2) Cells were treated with TDH 5 µg/ml for 10 min. (3) Cells were treated with TDH 5 µg/ml for 30 min. (4) Cells pretreated with ZVAD-FMK 100 µM were treated with TDH 5 µg/ml for 30 min. MFI, mean fluorescence intensity (arbitrary units) as measured by flow cytometry.

CASP-3 gene, it was demonstrated that caspase-3 is required for DNA fragmentation and membrane blebbing (Janicke et al., 1998b).

The characteristics of cytotoxicity in response to TDH in this study are the disassembly of filamentous actin, chromatin condensation, and caspase-3 activation. Caspase-3 was slightly activated within 10 min in the cells after exposure to TDH, and at the same time the morphological changes of filamentous actin and chromatin condensation were also detected. The maximal effect evident was detected by 30 min when caspase-3 was highly activated by TDH. At that time, the cells were totally shrunken and rounded, and the chromatin was strongly condensed. We speculate that all of these changes are linked. Activation of caspase-3 may not from direct effects of TDH but from the activation of a signal transduction pathway in target cells which activates caspase-3. Activated caspase-3 will then cleave selected cellular proteins involved or required for morphological changes (actin disruption and blebbing) and chromatin condensation.

In mammalian cells, the pathways by which caspase-3 is activated have been pinned down to two major branches; one is initiated by cell surface receptor and another by mitochondria (Green, 1998; Kluck et al., 1997; Porter and Janicke, 1999). TDH may trigger one of these two signal transduction pathways in target cells. The findings in this study, particularly activation of caspase-3 by TDH, provide a new insight into the mechanism of TDH-mediated cytotoxicity, but the present study can not exclude the real pathway which TDH activates caspase-3 and it is needed to be further investigated.

ACKNOWLEDGEMENTS

Rochman Naim would like to thank Yuke, Zakiyyah, Faqih, Aminah, Salmah, and Farhan for continuous encouragement. The work was partially supported by Faculty of Veterinary Medicine, Institute Pertanian Bogor during the study.

REFERENCES

Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S (1998). A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature, 391: 43-50.

Fabbri A, Falzano L, Frank C, Donelli G, Matarrese P, Raimondi F, Fasano A, Fiorentini C (1999). *V. parahaemolyticus* thermostable direct hemolysin modulates cytoskeletal organization and calcium homeostasis in intestinal cultured cells. Infect. Immun., 67: 1139-1148.

Goshima K, Owaribe K, Yamanaka H, Yoshino S (1978). Requirements of calcium ions for cell degeneration with a toxin (vibriolysin) from *V. parahaemolyticus*. Infect. Immun., 22: 821-832.

Green DR (1998). Apoptotic pathways: the roads to ruin. Cell, 94: 695-698.

Honda T, Iida T (1993). The pathogenicity of *V. parahaemolyticus* and the role of themostable direct hemolysin and related hemolysins. Rev. Med. Microbiol., 4: 106-113.

Iida T, Yamamoto K (1990). Cloning and expression of two genes encoding highly homologous hemolysins from a Kanagawa-phenomenon-positive *V. parahaemolyticus* T4750 strain. Gene, 93: 9-15.

Janicke RU, Ng P, Sprengart ML, Porter AG (1998a). Caspase-3 is required for alpha-fodrin cleavage but dispensable for cleavage of other death substrates in apoptosis. J. Biol. Chem., 273: 15540-15545.

Janicke RU, Sprengart ML, Wati MR, Porter AG (1998b). Caspase-3 is required for DNA fagmentation and morphological changes associated with apoptosis J. Biol. Chem., 273: 9357–9360.

Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science, 275: 1132-1136.

Kothakota S, Azuma T, Reinhard C, Klippel A, Tang J, Chu K, McGarry TJ, Kirschner MW, Koths K, Kwiatkowski DJ, Williams LT (1997). Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. Science, 278: 294-298.

Liu X, Zou H, Slaughter C, Wang X (1997). DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. Cell, 89: 175-184.

Mills JC, Stone NL, Pittman RN (1999). Extranuclear apoptosis: The role of the cytoplasm in the execution phase. J. Cell Biol., 146: 703-707.

Naim R, Iida T, Takahashi A, Honda T (2001a). Monodansylcadaverine inhibits cytotoxicity of *V. parahaemolyticus* thermostable direct hemolysin on cultured rat embryonic fibroblast cells. FEMS Microbiol., Lett., 196: 99-105.

Naim R, Pasaribu FH (2009). Thermostable direct hemolysin of *V. parahaemolyticus* induces morphological changes and disrupts actin in cultured human epithelial cells. Afr. J. Microbiol. Res., 3: 727-730.

Naim R, Yanagihara I, Iida T, Honda T (2001b). *V. parahaemolyticus* thermostable direct hemolysin can induce an apoptotic cell death in Rat-1 cells from inside and outside of the cells. FEMS Microbiol. Lett., 195: 237-244.

Pop C, Salvesen G (2009). Human caspases: activation, specificity, and regulation. J. Biol. Chem., 284: 21777–21781.

Porter AG, Janicke RU (1999). Emerging roles of caspase-3 in apoptosis. Cell Death Differ., 6: 99-104.

Raimondi F, Kao JP, Florentini C, Fabbri A, Donelli G, Gasparini N, Rubino A, Fasano A (2000). Enterotoxicity and cytotoxicity of *V. parahaemolyticus* thermostable direct hemolysin in *in vitro* systems. Infect. Immun., 68: 3180-3185.

Rudel T, Bokoch GM (1997). Membrane and morphological changes in apoptotic cells regulated by caspase-mediated activation of PAK2. Science, 276: 1571-1574.

Sakahira H, Enari M, Nagata S (1998). Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. Nature, 391: 96-99.

Salvesen GS, Riedl SJ (2008). Caspase mechanisms. Adv. Exp. Med. Biol., 615: 13–23. doi:10.1007/978-1-4020-6554-5_2.

Takeda Y, Taga S, Miwatani T (1978). Evidence that thermostable direct hemolysin of *V. parahaemolyticus* is composed of two subunits. FEMS Microbiol. Lett., 4: 271-274.

Tang G, Iida T, Inoue H, Yutsudo M, Yamamoto K, Honda T (1997). A mutant cell line resistant to *V. parahaemolyticus* thermostable direct hemolysin (TDH): its potential in identification of putative receptor for TDH. Biochim. Biophys. Acta., 1360: 277-282.

Tang G, Iida T, Yamamoto K, Honda T (1994). A mutant toxin of *V. parahaemolyticus* thermostable direct hemolysin which has lost hemolytic activity but retains ability to bind to erythrocytes. Infect. Immun., 62: 3299-3304.

Yi CH, Yuan J (2009). The Jekyll and Hyde Functions of Caspases. Dev. Cell., 16: 21–34. doi:10.1016/j.devcel.2008.12.012.