Journal of Cellular Biochemistry 81:128–142 (2001)

Activation of the Caspase Cascade During Stx1-Induced Apoptosis in Burkitt's Lymphoma Cells

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Abstract Shiga toxin 1 (Stx1) produced by *Escherichia coli* has been reported to induce apoptosis in many different cell types, including Burkitt's lymphoma (BL) cells. Since it has been established that the caspases play essential roles as the effector molecules in the apoptotic process in most cases, we examined the kinetics of caspase activation during the process of Stx1-mediated apoptosis of BL cells. Using Ramos BL cells that are highly sensitive to Stx1-mediated cytotoxicity, we observed that multiple caspases, including caspase-3, -7, and -8 were promptly activated following Stx1 treatment, as indicated by both the procaspase cleavages and enhancement of cleavage of the tetra-peptide substrates of the caspases. In addition, the inhibition assay revealed that caspase-8 is located upstream of both caspase-3 and -7, suggesting that Stx1-mediated apoptosis utilizes a similar caspase cascade to that involved in Fas-mediated apoptosis. Neither anti-Fas mAb nor TNF-α, however, affected the Stx1-mediated apoptosis of Ramos cells. Although the precise mechanism of Stx1-mediated activation of caspase-8 is still unclear, we have demonstrated that crosslinkage of CD77, a functional receptor for Stx1, with specific antibody is sufficient to induce activation of caspase-8. Our findings should provide new insight into the understanding of the molecular basis of Stx1-mediated cell injury. J. Cell. Biochem. 81:128–142, 2001.

Key words: Burkitt's lymphoma; apoptosis; CD77; Shiga toxin; caspase

Shiga toxin (Stx) is an enterotoxin produced by Stx producing-*Escherichia coli* (STEC), such as O157:H7. Stx is believed to play a crucial role in the pathogenesis of complications associated with STEC-induced hemorrhagic colitis, including hemolytic uremic syndrome, neurologic disorders, and pulmonary involvement [Monnens et al., 1978; Fong et al., 1982; Karmali et al., 1985; Argyle et al., 1990; Brandt et al., 1994; Siegler et al., 1995]. Recent studies have shown that Stx can directly injure several different cell types, including vascular endothelial cells, Burkitt's lymphoma (BL) cells [Mangeney et al., 1993], renal tubular epithelial cells [Kiyokawa et al., 1998; Taguchi et al., 1998], and pulmonary epithelial cells [Uchida et al., 1999], most of which are present in the target organs of STEC infection.

Grant sponsor: The Organization for Drug ADR Relief, R&D Promotion and Product Review of Japan; Grant number: 96-5; Grant sponsor: The Japan Health Sciences Foundation: Grant number: 11106 & 21107; Grant sponsor: The Ministry of Health and Welfare of Japan; Grant number: 12C-01; Grant sponsor: Science and Technology Promotion Bureau for Organized Research Combination System of Japan; Grant number: 6070.

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Received 5 October 2000; Accepted ? ? ? ?

This article published online in Wiley InterScience, December XX, 2000.

Stx binds specifically to the glycosphingolipid receptor, globotriaosylceramide (Gb3), also known as cluster of differentiation (CD)77 or blood group pk, expressed on the cell surface of definite species of cells and leads to cell death [Lingwood et al., 1987]. The cytotoxic effects of Stx were originally thought to be due to the inhibition of protein synthesis mediated by the A-subunit of the toxin which possesses RNA *N*-glycohydrolase activity [Obrig et al., 1987; Endo et al., 1988; Saxena et al., 1989]. Recently, however, several reports have claimed that apoptosis is involved in the Stx-mediated cell death [Sandvig et al., 1992; Mangeney et al., 1993; Inward et al., 1995; Williams et al., 1997]. Furthermore, the B-subunit, or, the binding subunit, of Stx has been shown to be sufficient to induce apoptosis in Burkitt's lymphoma (BL) lines [Mangeney et al., 1993], suggesting the presence of an alternate mechanism for Stx-mediated cell death that is independent of protein synthesis inhibition in these lines. The observation that intracellular signaling events in BL cells are triggered by the cross-linking of CD77 may further lend support to this notion [Taga et al., 1997], although the precise mechanism underlying the Stx-mediated apoptosis of BL cells still remains unclear.

Apoptosis is a form of cell death that is characterized by specific morphological changes such as cell shrinkage, condensation of nuclei and loss of microvilli [Wyllie et al., 1980a] as well as the biochemical features of chromosomal DNA cleavage [Wyllie, 1980b; Wyllie et al., 1984; Compton, 1992]. Although a number of distinct pathways that mediate apoptosis have been identified, caspases (cysteine proteases) are thought to be essential as effector molecules in the apoptotic process in most cases [Henkart, 1996; Martin et al., 1996; Muzio et al., 1997]. The caspase family is composed of more than ten family members and is classified into several subfamilies according to the substrate specificities [Thornberry et al., 1997]. Caspases exist in the cells as inactive proenzymes and become activated upon cleavage, and heterotetramers of the cleaved subunits eventually compose the active enzyme [Chinnaiyan et al., 1996; Fraser et al., 1996].

A number of studies have shown that caspases themselves form a regulatory cascade which transduces the apoptotic signals. For example, in the case of Fas (CD95)- or TNF-α receptor (TNF-R)-mediated apoptosis, the upstream caspase (caspase-8) is activated by ligand binding to the receptor, which subsequently cleaves other caspases such as caspase-3 located downstream in the cascade [Fraser et al., 1996; Nagata, 1997]. Once activated, the downstream caspases cleave various cellular substrates [Martin et al., 1995a; Nagata et al., 1997] such as actin, poly(ADP-ribose) polymerase (PARP), fodrin, lamin, and an inhibitor of caspase-activated DNase (ICAD), all of which are responsible for the apoptosis of the cells [Nicholson et al., 1995; Tewari et al., 1995; Martin et al., 1995b; Orth et al., 1996; Enari et al., 1998].

In an attempt to clarify the downstream events in the apoptosis of BL cells induced by Stx1, we examined the kinetics of caspase activation in BL cells following Stx1 treatment. In this study, we showed that stimulation of apoptosis by Stx1 induces the activation of multiple caspases, including caspase-3, -7, and -8 in Ramos BL cells. A more detailed study revealed that caspase-8 is located upstream of caspase-3, indicating the involvement of a similar caspase cascade in the Stx1-mediated apoptosis of BL cells to that in Fas- or TNF-R-mediated apoptosis.

EXPERIMENTAL PROCEDURES

Cells and Reagents

The BL-derived cell line Ramos, the monocytic cell line THP-1, the acute T cell leukemia cell line Jurkat and the histiocytic lymphoma cell line U-937 were obtained from either the Japanese Cancer Research Resources Bank (Tokyo, Japan) or Riken Cell Bank (Tsukuba Science city, Ibaragi, Japan). The cells were cultured in RPMI1640 medium supplemented with 10% fetal calf serum at 37°C in a humidified 5% CO_2 atmosphere. *Escherichia coli* Stx1 was prepared as described previously [Noda et al., 1987]. The monoclonal antibodies (mAbs) used in this study were anti-caspase-2 (Transduction Laboratories, TDL, Lexington, KY), anti-caspase-3 (TDL), anti-caspase-7 (TDL), anti-caspase-8 (Medical & Biological Laboratories Co., LTD., MBL, Nagoya, Aichi, Japan), anti-caspase-9 (Pharmingen, San Diego, CA), anti-PARP (Biomol Research Laboratories, Inc., Plymouth Meeting, PA), anti-ICAD (MBL), anti-CD95 (Coulter/Immunotech, Inc., Westbrook, MA), anti-TNF-R1 and -R2 (Genzyme/Techne, Cambridge, MA), anti-CD77

(1A4, a generous gift from Dr. S. Hakomori of University of Washington, Seattle, WA and Otsuka Assay Laboratories, Kawauchi-cho, Tokushima, Japan) and anti-β-actin (Seikagaku Co., Tokyo, Japan) antibodies. Rabbit antisera against activated caspase-3 (Pharmingen) and caspase-6 (Stress Gen Biotechnologies Co., Victoria, BC, Canada) were also used. Secondary Abs, including fluorescein-conjugated and enzyme-conjugated Abs, were purchased from Jackson Laboratory, Inc. (West Grove, PA). The peptide inhibitors of caspase used in this study were z-Tyr-Val-Ala-Asp-fluoromethyl ketone (z-YVAD-fmk, specific for caspase-1, MBL), z-Trp-Glu-His-Asp-fmk (z-WEHD-fmk, specific for caspase-1, -4, -5, MBL), z-Asp-Glu-Val-Asp-fmk (z-DEVD-fmk, for caspase-3, MBL), z-Ile-Glu-Thr-Asp-fmk (z-IETD-fmk, for caspase-8, MBL), z-Leu-Glu-His-Asp-fmk (z-LEHD-fmk, for caspase-9) and z-Val-Ala-Asp-fmk (z-VAD-fmk, for a broad spectrum of caspases, Bachem AG, Bubendori, Switzerland). TNF-α was obtained from Pepro Tech EC Ltd. (London, UK). The cell lysates prepared from either human fibroblasts or Hela cells, as positive controls for immunoblotting, were obtained from TDL. All chemical reagents were obtained from Sigma-Aldrich Fine Chemicals (St. Louis, MO), unless otherwise indicated.

Assay for Apoptosis

To assess their growth and viability, the cells were plated on 96-well plates (Corning, Inc., Corning, NY) at a concentration of 1×10^5 cells in 100 μl of complete medium per well with or without Stx1. After incubation for the indicated periods, MTT assays were performed as described previously [Hansen et al., 1989].

To quantitate the apoptotic cells, cells treated and not treated with Stx1 were stained with propidium iodide (PI) and analyzed by flow cytometry (EPICS XL, Coulter) as described previously [Gong et al., 1994]. Apoptotic cells were also identified by staining with FITC-labeled annexin V using MEBCYTO®-Apoptosis Kit (MBL) according to the manufacturer's protocol. Nuclear DNAs were extracted and DNA ladder formation was examined as described previously [Mangeney et al., 1993]. The activity of the caspases was measured using Colorimetric Protease Assay kits with specific substrates for each caspase (MBL) according to the manufacturer's protocol.

Immunofluorescence Study and Immunoblotting

Immunofluorescence study was performed as described previously [Fujimoto et al., 1988]. Immunoblotting was also performed as described previously [Kiyokawa et al., 1997]. Briefly, cell lysates were prepared by solubilizing the cells in lysis buffer (20 mM Na_2PO_4, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% aprotinin, 5 mM PMSF, 100 mM NaF, and 2 mM Na_3VO_4). After centrifugation, supernatants were obtained, and the protein concentration in each cell lysate was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Fifty μg samples of each cell lysate were applied for electrophoretic separation by SDS-polyacrylamide gel and the fractions were transferred onto a nitrocellulose membrane using a semi-dry transblot system (Bio-Rad). After blocking with 5% skim milk in PBS, the membranes were incubated with an appropriate combination of primary and secondary Abs as indicated, washed thoroughly, and then examined with an enhanced chemiluminescence reagent system (ECL, Amersham Life Science, Little Chalfont, Buckinghamshire, UK). The results obtained from a 1-min exposure of the ECL-treated membrane to film are presented, unless otherwise indicated.

RESULTS

Stx1 Promptly Induces Apoptosis of BL-Derived Ramos Cells

We first confirmed whether Stx1 treatment indeed induces the apoptosis of BL-derived cells. As shown in Figure 1A, the viable Ramos cell count, as assessed by MTT assay, was markedly decreased following Stx1 treatment in a dose- and time-dependent manner. Stx1 at 50 pg/ml was sufficient to induce more than 95% cell reduction after one day, indicating the high sensitivity of Ramos cells to this toxin. Since two other BL-derived lines, Daudi and Raji, were less sensitive to Stx1 under our culture conditions (data not shown), Ramos cells were used throughout this study.

The following results clearly show that the death of Ramos cells induced by Stx1 was a result of apoptosis. First, most Ramos cells exhibited cleaved nuclei when examined morphologically 9 h after Stx1 treatment (Fig. 1B, right panel). No such figures were observed in

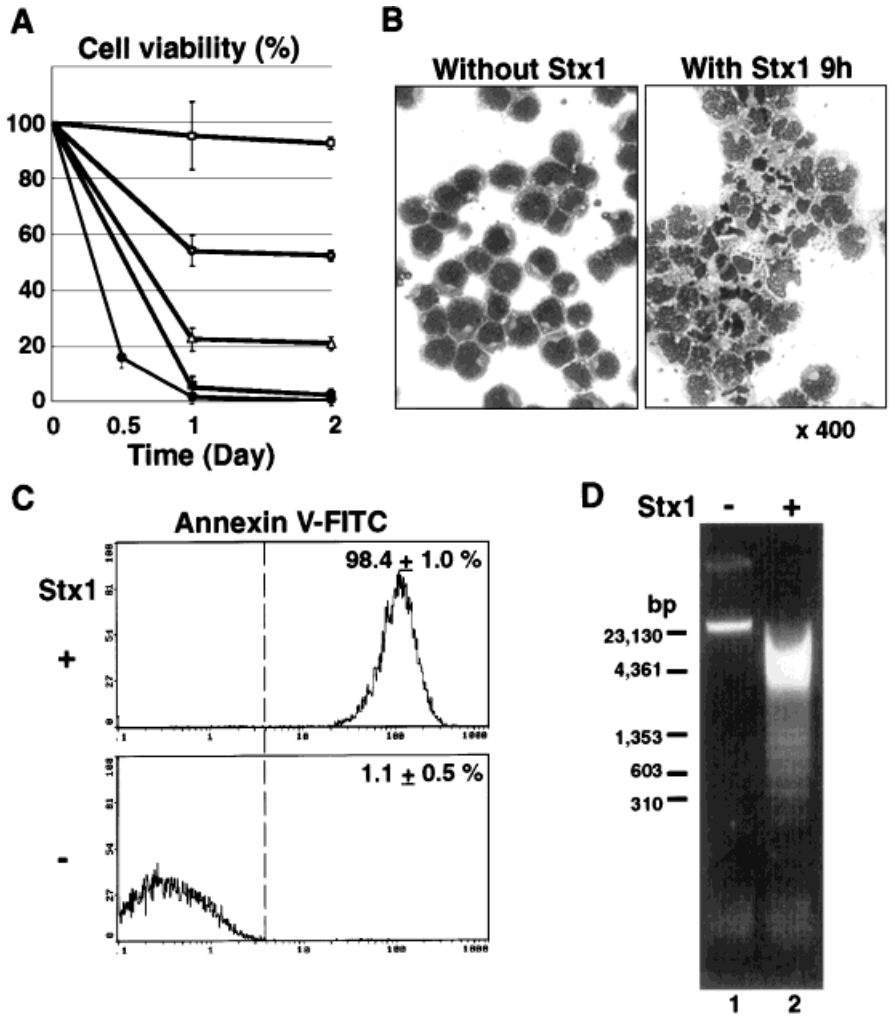

Fig. 1. Stx1 induces apoptosis of Ramos cells. **A**: Ramos cells (1×10^5 cells per 100 µl of medium per well) were incubated with different concentrations of Stx1, as indicated. After incubation for the periods indicated, viable cell counts were estimated by MTT assay and expressed as ratios to that of untreated cells. Experiments were performed in triplicate, and the means ± SD of the values are presented. □, with 0.8 pg/ml of Stx1;○, with 3.1 pg/ml of Stx1; △, with 12.5 pg/ml of Stx1;■, with 50.0 pg/ml of Stx1;●, with 100.0 pg/ml of Stx1. **B**: Ramos cells were cultured in medium alone (left panel) or in the presence of 100 pg/ml of Stx1 (right panel) for 9 h, cytocentrifuged and Giemsa-stained, and their morphological appearance was examined by light microscopy. As compared with untreated cells (left panel), cells treated with Stx1 (right panel) show characteristic chromatin condensation and cleaved nuclei. Magnification × 400. **C**: Ramos cells were either treated (upper panel) or left untreated (lower panel) with 100 pg/ml of Stx1 for 12 h. After staining with annexin V conjugated with FITC, the frequency of apoptotic cells was examined by flow cytometry. The means ± SD of the cells bound annexin V, i.e., those undergoing apoptosis, are presented. **D**: DNA ladder formation after treatment with Stx1 was examined. Ramos cells were either treated (lane 2) or not treated with (lane 1) 100 pg/ml of Stx1 for 24 h. DNAs were extracted and 1.5 µg of DNA from each sample was electrophoresed on 1% agarose gel.

untreated cells (Fig. 1B, left panel). Second, flow-cytometric assay revealed that Stx1 treatment induced a marked increase in annexin-V-binding to Ramos cells (Fig. 1C), indicating the exposure of phosphatidylserine residues in the outer leaflet of the plasma membrane, an early event during apoptosis. Third, we examined whether the cleavage of nuclear DNA occurred following Stx1 treatment. As shown in Figure 1D, DNA prepared from Ramos cells treated with Stx1 showed oligonucleosomal ladder fragmentation on agarose gel electrophoresis. Cleavage of the nuclear DNA in Stx1-treated Ramos cells was also confirmed by the detection of subploid cells with PI staining (Fig. 2A). PI staining revealed that

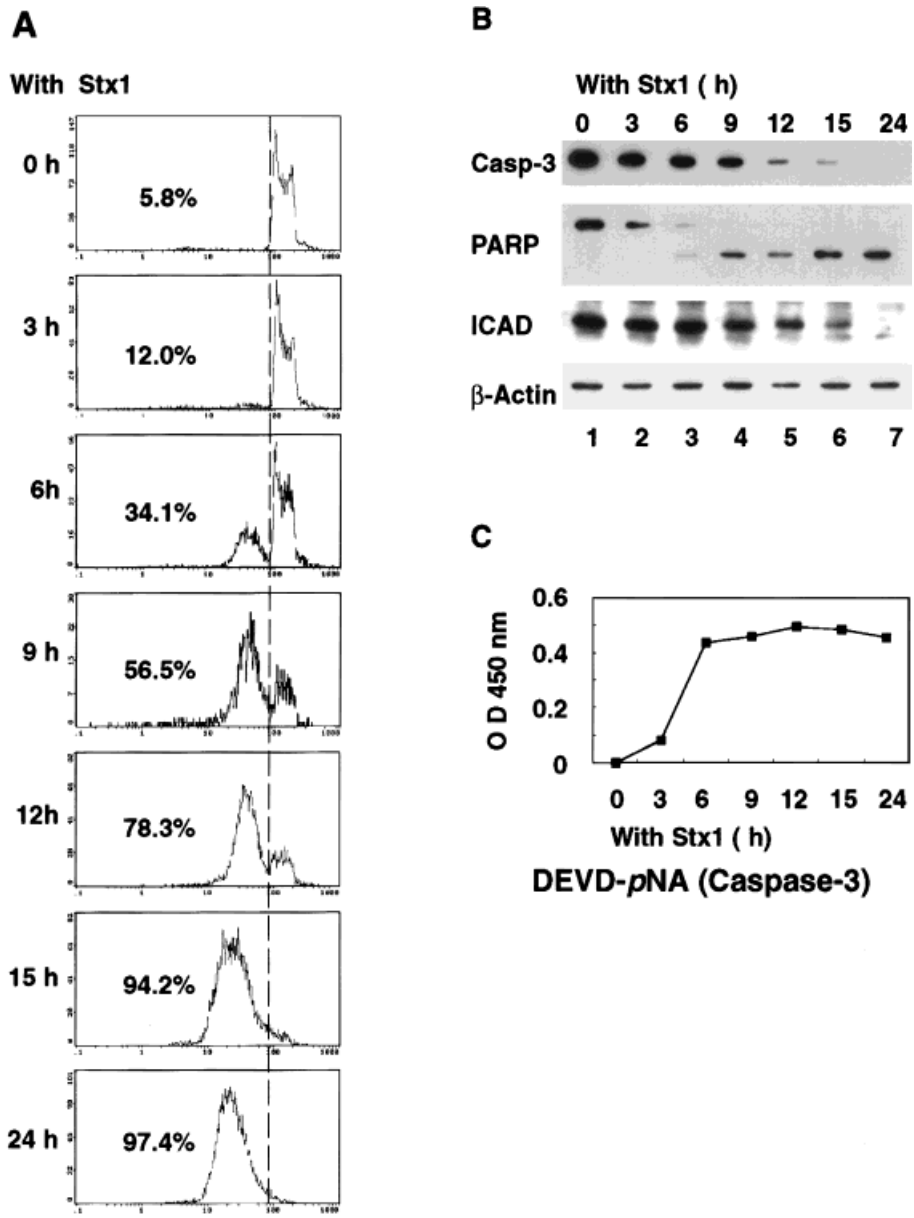

Fig. 2. Activation of caspase-3 during the process of Stx1-mediated apoptosis in Ramos cells. **A**: After culture in medium alone (0 h) or in the presence of 100 pg/ml of Stx1 for the periods indicated, Ramos cells were fixed and stained with PI, and analyzed by flow cytometry. The percentage of subploid cells, i.e., those undergoing apoptosis, are indicated. **B**: In parallel, cell lysates were prepared and their protein contents were determined. Fifty micrograms of protein per lane were analyzed by SDS-PAGE and subsequently immunoblotted with mAbs against caspase-3, PARP or ICAD. The cell lysates were also examined with anti-β-actin mAb as the internal control. **C**: Simultaneously, the cleavage activity of DEVD-*p*NA (specific for caspase-3-like protease) was monitored by colorimetric assay.

subploid cells promptly increased in number after treatment with Stx1, the majority of the cells becoming subdiploid after 15 h incubation (Fig. 2A). All these data indicate that Stx1 very effectively induces apoptosis of Ramos cells.

Induction of Caspase Activity During the Process of Stx1-Induced Apoptosis in BL Cells

Recently, a number of studies have shown that caspases act as common effectors of the apoptotic process induced by various distinct

apoptotic stimuli [Henkart, 1996]. In particular, caspase-3 (CPP32) was found to be a central apoptosis-related protease in different types of apoptosis in various cell systems [Los et al., 1995; Nicholson et al., 1995; Enari et al., 1996; Henkart, 1996]. Thus, we investigated whether these caspases were also activated during the apoptotic process in BL cells induced by Stx1.

We examined the activation of caspase-3 during the process of Stx1-mediated apoptosis in Ramos cells by two distinct methods. First, we tested the cell lysates by immunoblotting with a specific mAb against caspase-3. As shown in Figure 2B, the protein level of the caspase-3 proenzyme rapidly decreased in parallel with the appearance of subploid cells (Fig. 2A), indicating cleavage of the caspase-3 proenzyme during the process of Stx1-mediated apoptosis in Ramos cells. Consistent with this, prompt cleavage of PARP and ICAD, both of which are known to be substrates of caspase-3, was also observed (Fig. 2B). No significant change was observed in the protein level of β-actin, indicating that the amounts of protein loaded onto each lane were comparable (Fig. 2B). Second, we examined the DEVD-*p*NA (a tetrapeptide substrate of caspase-3-like protease) cleavage activity by colorimetric assay. As shown in Figure 2C, DEVD-*p*NA cleavage activity was also detected in the cell lysate of Stx1-treated Ramos cells in parallel with the appearance of subploid cells. The elevation of this activity was well consistent with the cleavage of the caspase-3 proenzyme detected by immunoblotting as mentioned above (Fig. 2B). On the basis of these results, we concluded that treatment with Stx1 induces activation of the caspase-3 protease in Ramos cells.

Next, we examined whether other caspases are also activated following Stx1 treatment in Ramos cells. When the same samples as described above were examined by immunoblotting with specific mAbs against caspase-7 and -8, rapid cleavage of these caspase proenzymes was observed (Fig. 3A), indicating that Stx1 treatment also activates these caspases in Ramos cells. On the other hand, the protein level of the caspase-2 proenzyme showed no significant change during the process of Stx1-mediated apoptosis in Ramos cells (Fig. 3A). Inconsistent with the results of immunoblotting, however, we observed a significant elevation of the cleavage activities of VDVAD-*p*NA, which are highly specific substrates of caspase-2, by colorimetric assay (Fig. 3C). Since VDVAD is also reported to be cleaved by caspases-3 and -7 [Thornberry et al., 1997], we considered that the elevation of the VDVAD-*p*NA cleavage activity is mainly attributable to the activation of caspase-3 and/or -7 but not of caspase-2.

In contrast, caspase-6 proenzyme was not detectable in Ramos cells by immunoblotting, while the anti-caspase-6 Ab used in this study detected a significant expression of caspase-6 proenzyme in human fibroblasts as a positive control (Fig. 3B). Although an elevation of the VEID-*p*NA (a tetrapeptide substrate highly specific for caspase-6) cleavage activity was observed by colorimetric assay (Fig. 3C), it was suggested to be mainly due to the activation of caspase-8, rather than that of caspase-6, which can also cleave this substrate [Thornberry et al., 1997].

In comparison with an abundant expression in Hela cells as a positive control, only a faint caspase-9 proenzyme was detected in Ramos cells by immunoblotting (Fig. 3B). Consistently, a colorimetric assay with LEHD-*p*NA (a tetrapeptide substrate highly specific for caspase-9) indicated no significant elevation of the caspase-9 cleavage activity in Ramos cells after treatment with Stx1 (Fig. 3C,D). Since a significant elevation of the cleavage activity of this substrate was observed in camptothecin-treated Jurkat cells as a positive control (Fig. 3D), we concluded that caspase-9 was not involved in the Stx1-mediated apoptosis.

We also examined the possibility of concerns of caspase-1, -4, and -5 during Stx1-induced apoptosis. However, the colorimetric assay using YVAD-*p*NA (tetrapeptide substrates highly specific for caspase-1) and WEID-*p*NA (tetrapeptide substrates specific for caspase-1, -4, and -5) revealed no significant activation of these caspases in Ramos cells after treatment with Stx1 (Fig. 3D). Since a significant elevation of the cleavage activity of these substrates was observed in DTT-treated THP-1 cells as a positive control (Fig. 3D), we concluded that caspase-1, -4, and -5 are not involved during the process of Stx-mediated apoptosis in Ramos cells.

Effect of Peptide Inhibitors of Caspases on Stx1-Mediated Apoptosis of Ramos Cells

To confirm the participation of the caspases, next we examined the effects of peptide

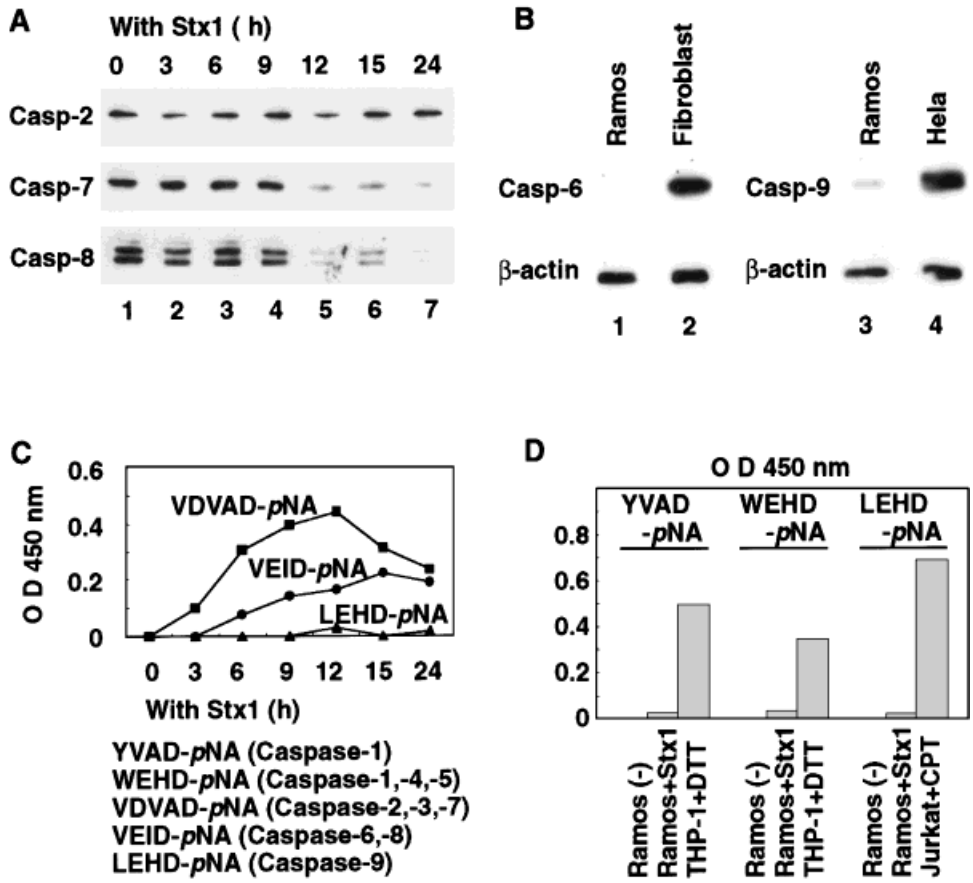

Fig. 3. Activation of other caspases during the process of Stx1-mediated apoptosis in Ramos cells. **A**: The same sample as that used for obtaining the data of Figure 2B was examined by immunoblotting with specific mAbs against caspase-2, -7, and -8. **B**: Cell lysates were prepared from untreated Ramos cells and immunoblotted with mAbs against caspase-6 (lane 1) or caspase-9 (lane 3) as in Figure 2B. Cell lysates prepared from human fibroblasts (Fibroblast, lane 2) and Hela cells (lane 4) were also examined as positive controls for the expression of caspase-6 and caspase-9, respectively. **C**: In parallel with A, the cleavages of VDVAD-*p*NA, VEID-*p*NA, and LEHD-*p*NA were monitored by colorimetric assay similarly as described in the legend for Figure 2C. **D**: The cell lysates were prepared from Ramos cells treated and untreated with 100 pg/ml of Stx1 for 12 h and examined by colorimetric assay to measure the cleavage of YVAD-*p*NA, WEHD-*p*NA and LEHD-*p*NA as in the legend for Figure 2C. As a positive control for the cleavage activity of YVAD-*p*NA and WEHD-*p*NA, the cell lysate prepared from THP-1 cells treated with 20 mM DTT at 37°C for 1 h was examined. The cell lysate prepared from Jurkat cells treated with 2 μM camptothecin was also examined as a positive control for the cleavage activity of LEHD-*p*NA. Ramos (−), untreated Ramos cells; Ramos+Stx1, Ramos cells treated with Stx1, THP-1+DTT, THP-1 cells treated with DTT; Jurkat+CPT, Jurkat cells treated with camptothecin.

inhibitors of the caspases on the induction of apoptosis by Stx1. When the appearance of apoptotic cells was monitored by staining with FITC-labeled annexin V, apoptosis of more than 95% of the Ramos cells was observed after 12-h incubation with Stx1 (Fig. 4). When Ramos cells pretreated with z-DEVD fmk (a tetrapeptide inhibitor of caspase-3-like protease) were incubated with Stx1, the number of annexin-V-positive cells was significantly reduced as compared with that in the cells treated with Stx1 alone (Fig. 4).

In contrast, pretreatment with z-YVAD fmk (tetrapeptide inhibitor of caspase-1-like protease), z-WEID fmk (tetrapeptide inhibitor for caspase-1, -4, and -5) or z-LEHD fmk (tetrapeptide inhibitor for caspase-9) did not cause any reduction in the number of apoptotic cells as evaluated by annexin-V-binding (Fig. 4), indicating that z-DEVD fmk specifically inhibited the Stx1-mediated apoptosis of Ramos cells. The data also indicate that caspase-1, -4, -5, and -9 were not involved in this apoptotic process. When we tested the effect of z-VAD

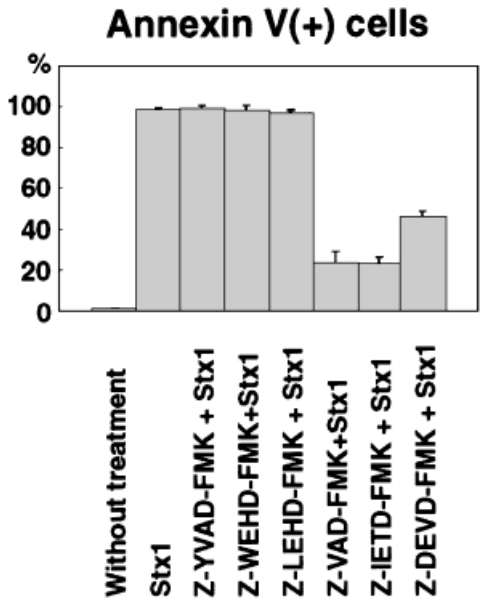

Fig. 4. Effect of peptide inhibitors on Stx1-mediated apoptosis of Ramos cells. After 12-h incubation with different combinations of 100 pg/ml of Stx1 and 75 μM of each tetrapeptide inhibitor as indicated, the incidence of apoptotic cells was determined as the percentage of FITC-labeled annexin-V-binding cells as mentioned in the legend for Figure 1D and indicated.

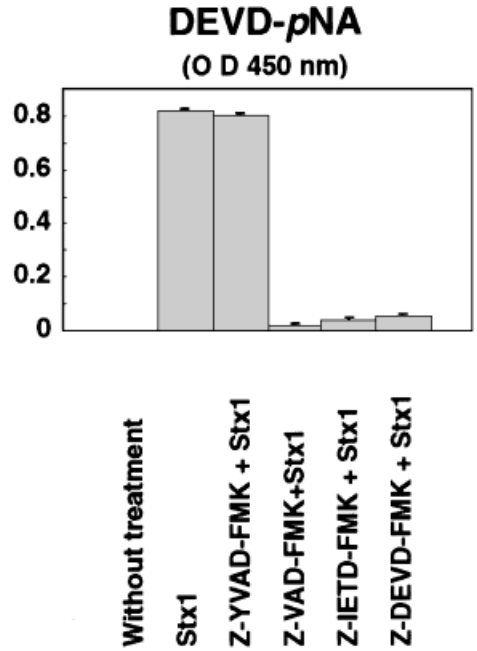

Fig. 5. The effects of peptide inhibitors on the cleavage of DEVD-*p*NA induced by Stx1 treatment in Ramos cells. The cell lysates were prepared from the same sample as that used to obtain the data of Figure 4 and examined by colorimetric assay to measure the cleavage of DEVD-*p*NA as described in the legend for Figure 2C.

fmk (a tripeptide inhibitor of a broad range of caspases) and z-IETD fmk (a tetrapeptide inhibitor of caspase-8), however, greater inhibition of apoptosis than that by z-DEVD fmk was observed (Fig. 4). These data indicate that while caspase-3 is one of the caspases that is involved in the Stx1-mediated apoptosis of Ramos cells, the inhibition of caspase-3 alone is not sufficient to completely prevent the apoptosis.

Caspase-8 is Located Upstream of Caspase-3 and -7 in the Apoptotic Cascade Activated by Stx1 in Ramos Cells

To clarify the cascade of the caspases that is activated during the Stx1-mediated apoptosis in Ramos cells, we examined the effect of peptide inhibitors on the Stx1-mediated activation of the caspases. First, we examined the effect of the peptide inhibitors on the activation of caspase-3 induced by Stx1. When evaluated by the DEVD-*p*NA cleavage activity using a colorimetric assay, Stx1 treatment after pretreatment with z-DEVD-fmk significantly inhibited the activation of caspase-3 (Fig. 5). In contrast, z-YVAD-FMK did not affect the activation of caspase-3 induced by Stx1-treatment (Fig. 5), indicating that caspase-3 activation is specifically inhibited by z-DEVD-fmk. Furthermore, both z-VAD-fmk and z-IETD-fmk also inhibited the Stx1-mediated activation of caspase-3 (Fig. 5). We also confirmed by immunonblotting that both z-VAD-fmk (Fig. 6A) and z-IETD-fmk (Fig. 6B,C) also inhibited the cleavage of the caspase-3 proenzyme after Stx1-treatment. In contrast, z-DEVD-fmk did not inhibit the cleavage of the caspase-8 proenzyme (data not shown), indicating that caspase-8 is located upstream of caspase-3 in the caspase cascade activated by Stx1-treatment. Next, we tested the effect of the peptide inhibitors on the activation of caspase-7 induced by Stx1. When evaluated by immunoblotting, both z-VAD-fmk and z-IETD-fmk, but not z-DEVD-fmk, inhibited the Stx1-mediated cleavage of caspase-7 in Ramos cells (Fig. 6D,E). These data indicate that both caspase-3 and caspase-7 are located downstream of caspase-8, and that the cleavage of caspase-7 is independent of caspase-3.

Anti-Fas mAb and TNF-α did not Affect the Stx1-Mediated Apoptosis of Ramos Cells

Since both Fas-mediated and TNF-R-mediated apoptotic stimuli activated caspase-8, we

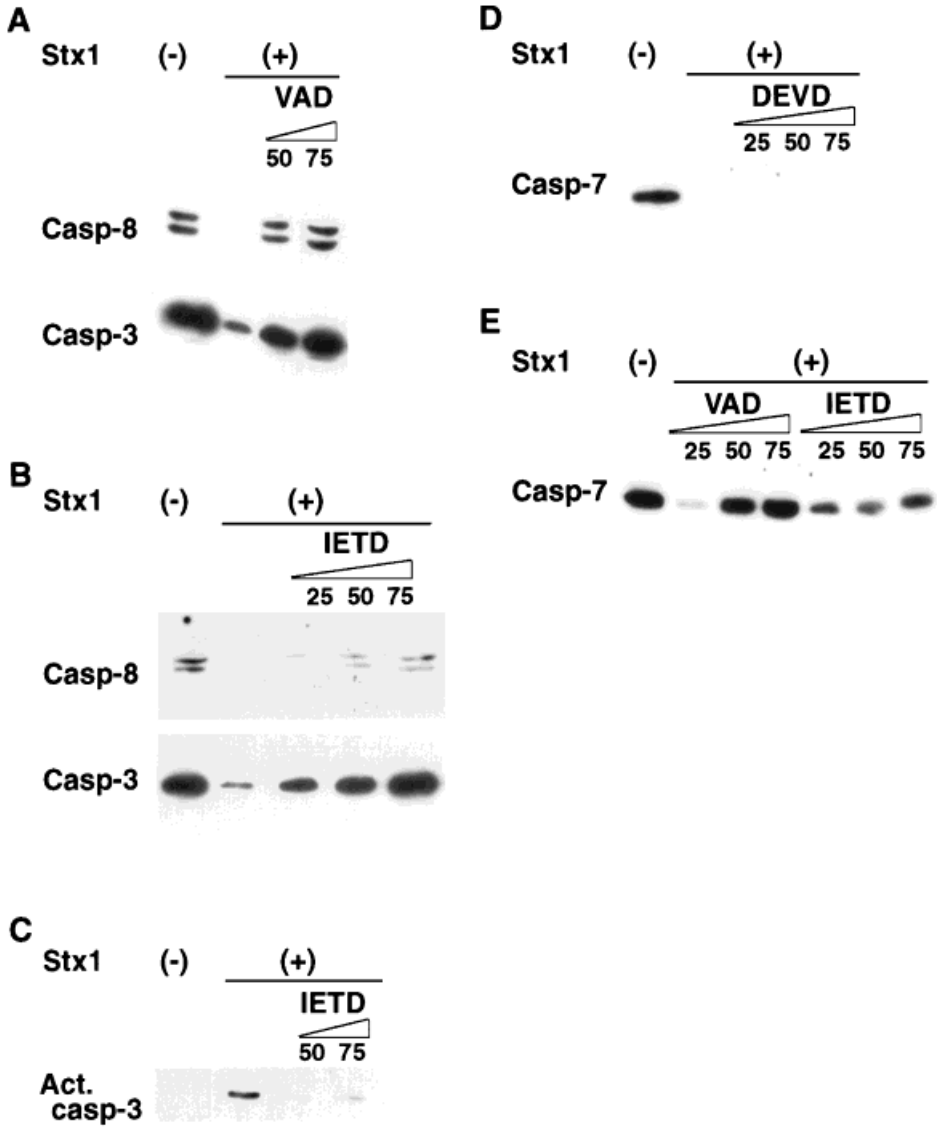

Fig. 6. The effects of peptide inhibitors on the cleavage of procaspases induced by Stx1 treatment in Ramos cells. **A**: Cell lysates were prepared from Ramos cells treated or not treated with 100 pg/ml of Stx1 in the presence and absence of the indicated amounts (μM) of VAD-fmk and examined by immunoblotting with either anti-caspase-8 (Casp-8, upper panel) or anti-caspase-3 (Casp-3, lower panel) mAb similarly as described in the legend for Figure 2B. **B**: Cell lysates prepared from Ramos cells treated with Stx1 and IETD-fmk were also examined similarly. **C**: Same sample preparation was examined by immunoblotting with anti-activated caspase-3. **D**: The cell lysates prepared from Ramos cells treated with Stx1 and DEVD-fmk were examined by immunoblotting with anti-caspase-7. **E**: The same sample preparation as that used to obtain the data of Figure 6A–C was examined by immunoblotting with mAb against caspase-7.

next examined whether these stimuli affect the Stx1-mediated apoptosis of Ramos cells. As shown in Figure 7A, flow-cytometric analysis revealed that Ramos cells express a certain amount of Fas on their cell surface. Although the anti-Fas mAb used in this study induced apoptosis of Fas-expressing Jurkat T cells (Fig. 7C), this mAb alone did not induce apoptosis of Ramos cells (Fig. 7C). Furthermore, treatment with anti-Fas mAb did not affect the apoptosis of Ramos cells mediated by Stx1 (Fig. 7C).

We also examined the effect of TNF-α. Flow-cytometric analysis revealed that an apparent expression of TNF-R1 or -R2 was not detectable on the surface of Ramos cells (Fig. 7B). In agreement with the expression level of the receptors, TNF-α did not induce apoptosis in

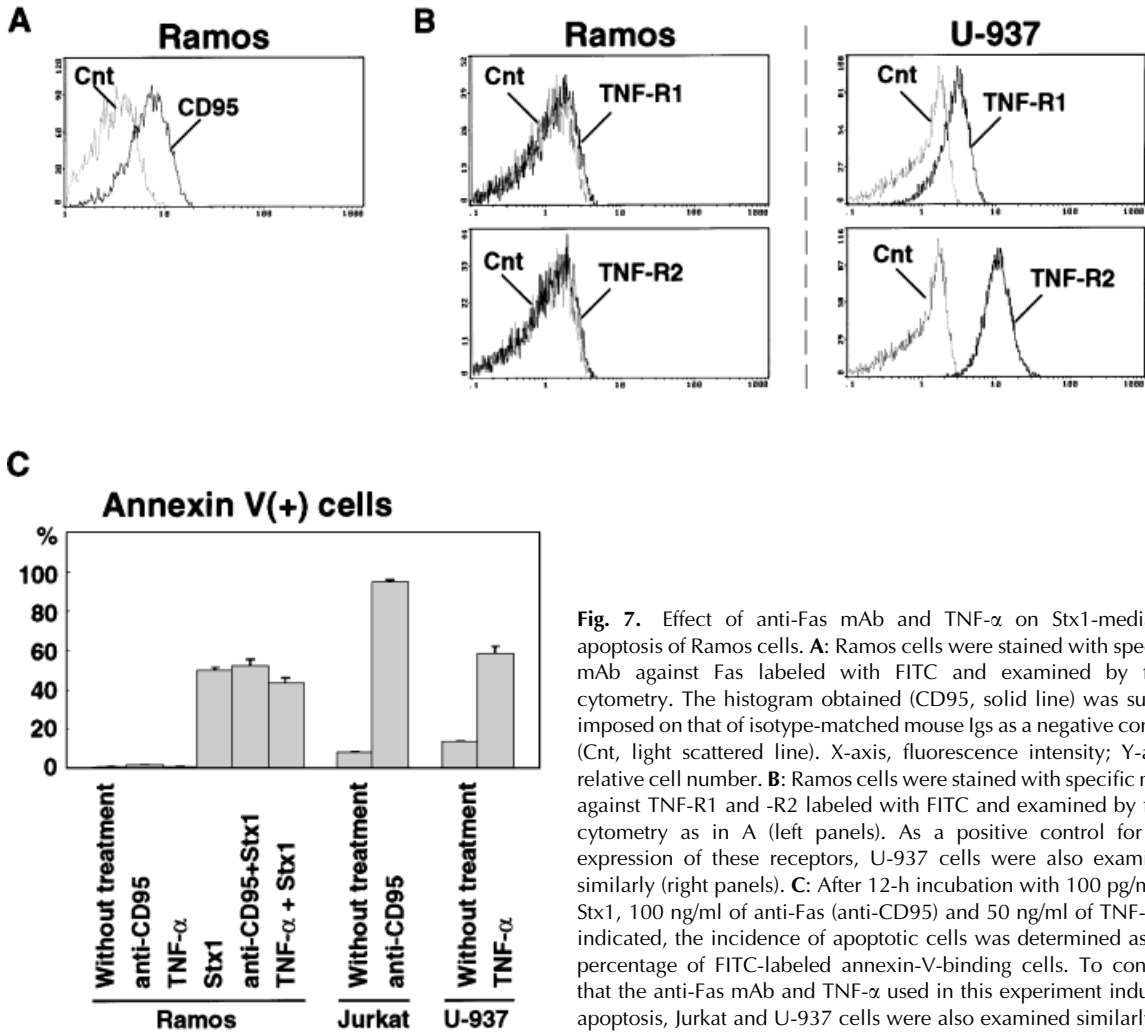

Fig. 7. Effect of anti-Fas mAb and TNF-α on Stx1-mediated apoptosis of Ramos cells. **A**: Ramos cells were stained with specific mAb against Fas labeled with FITC and examined by flow cytometry. The histogram obtained (CD95, solid line) was superimposed on that of isotype-matched mouse Igs as a negative control (Cnt, light scattered line). X-axis, fluorescence intensity; Y-axis, relative cell number. **B**: Ramos cells were stained with specific mAb against TNF-R1 and -R2 labeled with FITC and examined by flow cytometry as in A (left panels). As a positive control for the expression of these receptors, U-937 cells were also examined similarly (right panels). **C**: After 12-h incubation with 100 pg/ml of Stx1, 100 ng/ml of anti-Fas (anti-CD95) and 50 ng/ml of TNF-α as indicated, the incidence of apoptotic cells was determined as the percentage of FITC-labeled annexin-V-binding cells. To confirm that the anti-Fas mAb and TNF-α used in this experiment induced apoptosis, Jurkat and U-937 cells were also examined similarly.

Ramos cell, while this cytokine induced apoptosis of TNF-Rs-expressing U-937 cells (Fig. 7B, C). We further observed no significant effect of TNF-α on the Stx1-mediated apoptosis of Ramos cells (Fig. 7C).

Crosslinkage of CD77 With Specific Ab Induces Activation of Caspase-8

Recent studies have shown that the B-subunit of Stx1 or immobilized anti-CD77 mAb on culture dish can induce apoptosis in BL cells [Mangeney et al., 1994; Taga et al., 1996; Mori et al., 2000]. Thus we tested whether crosslinkage of CD77 mediated by specific mAb induces activation of caspase-8 in Ramos cells. As shown in Figure 8A, 48-h treatment with anti-CD77 mAb 1A4 immobilized on culture dish induced apoptosis in a portion of Ramos cells as assessed by annexin V binding. In parallel with the appearance of annexin-V-bound cells, the protein level of the caspase-8 proenzyme in Ramos cells was marked decreased after treatment with anti-CD77 mAb as assessed by immunoblotting (Fig. 8B). In addition, minor bands with lower molecular weight which correspond to the partially cleaved caspase-8 were observed (Fig. 8B). In contrast, no significant change was observed in the protein level of caspase-8 proenzyme in Ramos cells treated with immobilized isotype-matched mouse Igs (Fig. 8B). The data indicate that caspase-8 is cleaved and activated after crosslinkage of CD77 with specific mAb.

DISCUSSION

In this paper, we have clearly shown that multiple caspases, including caspase-3, -7, and -8, are activated during the process of Stx1-mediated apoptosis in Ramos BL cells. The

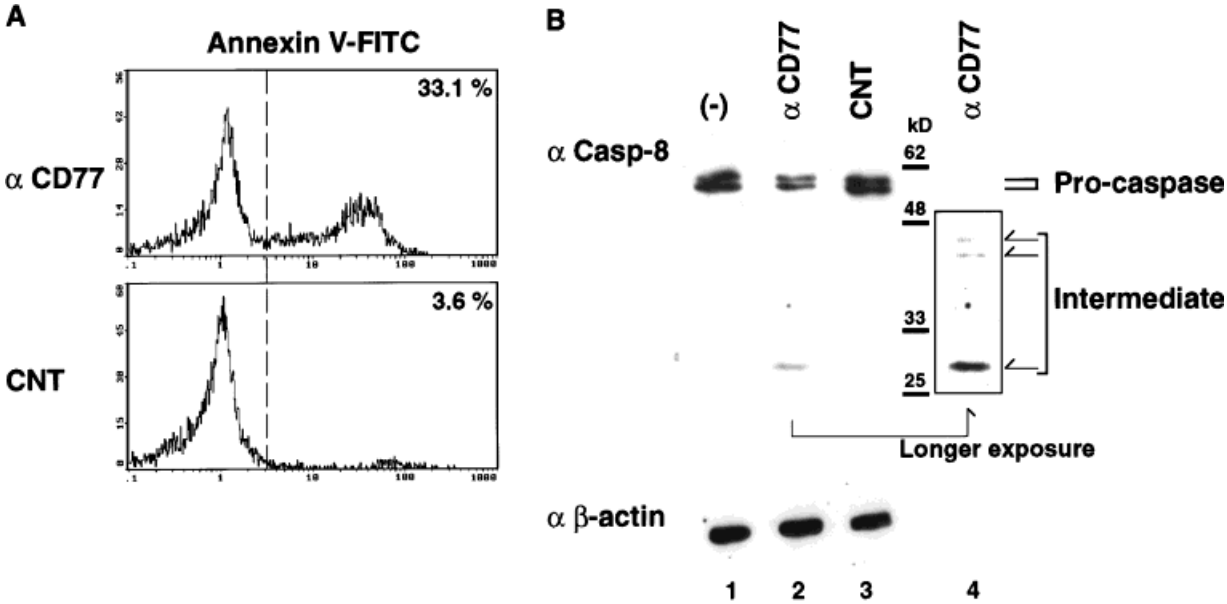

Fig. 8. **A**: Ramos cells were cultured in Petridishes that have been previously coated with rabbit anti-mouse Igs and mouse anti-CD77 mAb 1A4 (α CD77, 5 μg/ml of each, upper panel). After 48-h incubation, the incidence of apoptotic cells was determined by FITC-labeled annexin-V-binding as in Figure 1D. As a negative control, the effect of isotype-matched mouse Igs was also tested similarly (CNT, lower panel). The ratios of the cells bound annexin V are presented. **B**: In parallel, cell lysates were prepared and were examined by immunoblotting with specific mAb against caspase-8 as in Figure 2B. In lane 4, the result obtained by longer (30 min) exposure of the lower part of lane 2 is presented. The bands corresponding to the partially cleaved caspase-8 are pointed by arrows (Intermediate). (−), untreated Ramos cells.

results are summarized in Figure 9. Since a peptide inhibitor of caspase-8 inhibited the cleavage of the proenzymes of caspase-3 and -7 while an inhibitor of caspase-3 did not inhibit the cleavage of the proenzyme of caspase-8, activation of caspase-3 is believed to occur after caspase-8 activation. On the other hand, a specific peptide inhibitor of caspase-3 did not affect the cleavage of the caspase-7 proenzyme, suggesting that the activation of caspase-7 is independent of that of caspase-3. Since an inhibitor of caspase-3 did not fully inhibit the apoptosis of Ramos cells mediated by Stx1, it is most likely that caspase-7 is another caspase in the cascade activated by caspase-8.

The caspases, evolutionally conserved cysteine proteases, are known to be effector molecules of the apoptotic pathway that exists in the cells as inactive proenzymes and become activated upon cleavage [Chinnaiyan et al., 1996; Fraser et al., 1996]. Employing a positional scanning substrate combinatorial library, Thornberry et al. have classified the caspases into three distinct groups based on their individual substrate specificities [Thornberry et al., 1997]. Members of Group I (caspase-1, -4, and -5) that participate in the activation of cytokines, including IL-1 β and IL-18, prefer the tetrapeptide sequence, WEHD. In contrast, the optimal peptide recognition motif of Group II caspases (-2, -3, and -7) is DEXD. Since the DEXD motif is similar or identical to the cleavage sites in several cell maintenance and/or repair proteins that are proteolytically cleaved during apoptosis, all the members of this group of caspases are thought to be effector proteases and destroy essential homeostatic pathways during the apoptotic process. The optimal recognition motif, (L/V)EXD, of Group III caspases (-6, -8, and -9) resembles that at the activation sites of several effector caspase proenzymes implying that these enzymes are present upstream in a proteolytic cascade that serves to amplify the apoptotic signal. This is further supported by the results of several independent researches [Muzio et al., 1996; Boldin et al., 1996a].

Despite extensive homology and similar substrate specificities among caspase subfamily members, distinct biochemical and biological property of each caspase has been demonstrated. For example, mice deficient in caspases-2 or -3, which share very similar tetrapeptide cleavage specificities, exhibit very different phe-

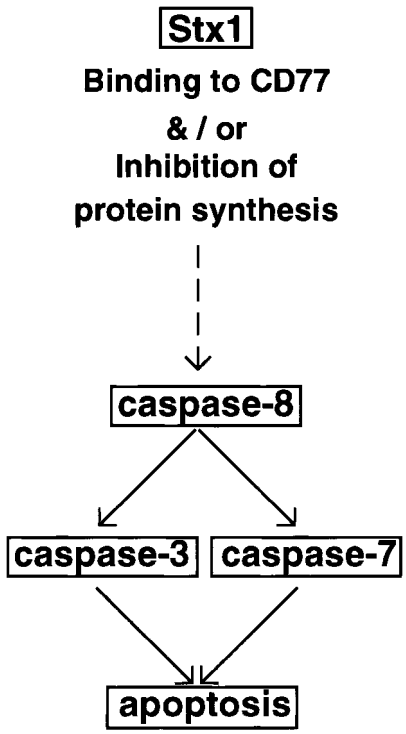

Fig. 9. Activation of the caspase cascade during the process of Stx1-mediated apoptosis of Ramos cells. After the binding of Stx1 to CD77 expressed on Ramos cells, intracellular signal(s) mediated by CD77 that bound to the Stx1 B-subunit and/or protein synthesis inhibition mediated by the Stx1 A-subunit became linked to the aggregation and autoprocessing of procaspase-8. The activated caspase-8 further cleaves and activates the downstream caspases, including caspase-3 and -7, that function as effector caspases in the apoptotic process.

notypes [Kuida et al., 1996; Bergeron et al., 1998]. Consistently, our data indicate that caspase-3 and -7, but not -2, is activated by Stx1-treatment while all of these caspases are expressed in Ramos cells. Similarly, caspase-8, but not caspase-6, is expressed and participates in Stx1-mediated apoptosis. All of the above evidence suggest that the unique features of individual caspases may account for distinct regulation and specialized functions in a tissue and stimulus-specific manner.

Recently, the regulatory cascade of molecules involved in many types of apoptosis has been well characterized. The Fas-mediated apoptotic cascade is one example. Ligand- or Ab-mediated ligation of Fas results in binding of the adaptor molecule, Fas-associated death domain (FADD), to the intracellular tail of Fas [Chinnaiyan et al., 1995; Kischkel et al., 1995; Boldin et al., 1996a]. FADD, in turn, recruits procaspase-8, the first component of the Fas-associated caspases cascade, into death-including signaling complex (DISC) by a homophilic interaction [Muzio et al., 1996; Srinivasula et al., 1996; Boldin et al., 1996b; Muzio et al., 1998]. Procaspase-8 possesses an intrinsic low level of enzymatic activity that can induce clustering-mediated autoprocessing [Muzio et al., 1998]. Thus, immediately after recruitment into the DISC, the single polypeptide procaspase-8 is proteolytically autoprocessed to the active dimeric species composed of large and small catalytic subunits, and then directly cleaves the downstream caspases such as caspase-3 [Srinivasula et al., 1996; Medema et al., 1997; Yang et al., 1998].

Alternatively, caspase-8 initiates caspase-9-dependent pathway leading to the activation of down stream caspases. Activated caspase-8 cleaves BID, a death agonist member of the Bcl-2/Bcl-x_L family, which in turn induces cytochrome c release from mitochondria [Li et al., 1998; Luo et al., 1998]. In response to cytochrome c, caspase-9 binds to Apaf-1, human homologue of *C. elegans* CED-4, and becomes activated [Cecconi et al., 1997; Li et al., 1997], then cleaves and activates pro-caspase-3. The contributions of these two pathways to Fas-induced cell death vary between different cell types, presumably due to different levels of activated caspase-8 and its downstream substrates in a particular cell type [Scaffidi et al., 1998; Yin et al., 1999].

Besides the Fas-Fas ligand system, several examples of apoptotic machinery in which caspase-8 is activated have been reported. For example, TNF-R utilizes similar downstream molecular cascades, including caspase-8 to mediate apoptotic stimuli [Hsu et al., 1995, 1996; Yeh et al., 1998]. Aragane et al. reported that UV light directly stimulates Fas and thereby activates the Fas pathway to induce apoptosis independently of the natural ligand of Fas [Aragane et al., 1998]. In addition to the receptor-mediated activation of caspase-8, polyglutamine expression was also found to activate caspase-8; however the precise mechanism of this process is still unclear [Miyashita et al., 1999; Sanchez et al., 1999].

Considering the aforementioned lines of evidence, our data indicate that Stx1-mediated apoptosis of Ramos cells utilizes a similar cascade of caspases to that involved in Fas-mediated apoptosis. Since the activation of caspase-9 was not detected in the course of

Stx1-mediated apoptosis, it is suggested that Stx1-activated caspase-8 directly cleaves downstream caspases in this case. However, the mechanism of procaspase-8 activation triggered by Stx1 is currently not identified. Although a certain amount of Fas molecules is expressed on the cell surface of Ramos cells, anti-Fas mAb neither induced apoptosis nor affected the Stx1-mediated apoptosis of Ramos cells. TNF-α also did not affect the Stx1-mediated apoptosis of Ramos cells. Taken collectively, the Stx1-mediated activation of caspase-8 is independent of Fas- or TNF-R-linked signalings.

In the molecular mechanism underlying the Stx1-mediated activation of caspase-8 in BL cells, two major factors are possibly involved. First, the A-subunit of Stx1 is required to induce apoptosis in Vero cells [Williams et al., 1997], suggesting that the inhibition of protein synthesis induced by the A-subunit is linked to apoptosis. In fact, it was reported that inhibition of protein synthesis caused apoptosis or operated as an apoptotic co-factor in some instances [Martin et al., 1990; Bazar et al., 1992; Perandones et al., 1993]. Thus, the possibility that the protein synthesis inhibition induced by the Stx1 A-subunit is involved in the mechanism underlying the Stx1-mediated activation of caspase-8 is undeniable.

Secondly, however, recent studies have shown that the B-subunit alone is sufficient to induce apoptosis of BL cells [Mangeney et al., 1994]. In addition, Taga et al. have reported an elevation of the intracellular Ca^{2+} level in BL cells triggered by the crosslinkage of CD77 [Taga et al., 1996]. Together with our recent observations that Stx1 treatment induces activation of the Src family protein tyrosine kinases, including Yes in renal carcinoma ACHN cells [Katagiri et al., 1999] and Lyn in Ramos cells [Mori et al., 2000], it is most likely that CD77 mediates intracellular signals upon binding with the Stx1 B-subunit. We also presented in this study that crosslinkage of CD77 with specific mAb induces cleavage of caspase-8. Thus, it is plausible that Stx1-binding to CD77 is linked to the receptor-mediated aggregation of procaspase-8 via a similar, if not identical, mechanism to that in Fas-mediated apoptosis. Further clarification of the molecular mechanism of caspase-8 activation following Stx1-binding to the cells should be a subject of study in the future.

In conclusion, we report that Stx1-mediated apoptosis involves the sequential activation of a caspase cascade similar to that in Fas-mediated apoptosis. Although the precise mechanism by which caspase-8, the first component in this cascade, is activated still remains unclear, our findings should provide a new insight into the understanding of the molecular basis of Stx1-mediated cell injury.

ACKNOWLEDGMENTS

We thank M. Sone and S. Yamauchi for their excellent secretarial work. We also thank Dr. S. Hakomori (Washington University) and Otsuka Assay Laboratories for gifting CD77 MoAb 1A4.

REFERENCES

Aragane Y, Kulms D, Metze D, Wilkes G, Pöppelmann B, Luger TA, Schwarz T. 1998. Ultraviolet light induces apoptosis via direct activation of CD95 (Fas/APO-1) independently of its ligand CD95L. J Cell Biol 140:171–182.

Argyle JC, Hogg RJ, Pysher TJ, Silva FG, Siegler RL. 1990. A clinicopathological study of 24 children with hemolytic uremic syndrome. Pediatr Nephrol 4:52–58.

Bazar LS, Deeg HJ. 1992. Ultraviolet B-induced DNA fragmentation (apoptosis) in activated T-lymphocytes and Jurkat cells is augmented by inhibition of RNA and protein synthesis. Exp Hematol 20:80–86.

Bergeron L, Perez GI, Macdonald G, Shi L, Sun Y, Jurisicova A, Varmuza S, Latham KE, Flaws JA, Salter JC, Hara H, Moskowitz MA, Li E, Greenberg A, Tilly JL, Yuan J. 1998. Defects in regulation of apoptosis in caspase-2-deficient mice. Genes Dev 12:1304–1314.

Boldin MP, Goncharov TM, Goltsev YV, Wallach D. 1996a. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell 85:803–815.

Boldin MP, Varfolomeev EE, Pancer Z, Mett IL, Camonis JH, Wallach D. 1996b. A novel protein that interacts with death domain of Fas/APO-1 contains a sequence motif related to the death domain. J Biol Chem 270:7789–7795.

Brandt JR, Fouser LS, Watkins SL, et al. 1994. *Eschrichia coli* O157:H7-associateed hemolytic-uremic syndrome after ingestion of contaminated hamburgers. J Pediatr 125:519–526.

Cecconi F, Alvarez-Bolado G, Meyer BI, Roth KA, Gruss P. 1998. Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. Cell 94:727–737.

Chinnaiyan AM, Dixit VM. 1996. The cell-death machine. Curr Biol 6:555–562.

Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM. 1995. FADD, a novel death domain-containing protein, interacts with death domain of Fas and initiates apoptosis. Cell 81:505–512.

Compton MM. 1992. A biochemical hallmark of apoptosis: internucleosomal degradation of the genome. Cancer Metastasis Rev 11:105–119.

Enari M, Talanian RV, Wong WW, Nagata S. 1996. Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. Nature 380:723–726.

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

Endo Y, Tsurugi K, Yutsudo T, Takeda Y, Ogasawara T, Igarashi K. 1988. Site of action of a Vero toxin (VT2) from *Escherichia coli* O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. Eur J Biochem 171:45–50.

Fong JS, De Chadarevian JP, Kaplan BS. 1982. Hemolytic uremic syndrome. Current concepts and management. Pediatr Clin North Am 29:835–856.

Fraser A, Evan G. 1996. A license to kill. Cell 85:781–784.

Fujimoto J, Ishimoto K, Kiyokawa N, Tanaka S, Ishii E, Hata J. 1988. Immunocytological and immunochemical analysis on the common acute lymphoblastic leukemia antigen (CALLA): evidence that CALLA on ALL cells and granulocytes are structurally related. Hybridoma 7:227–236.

Gong J, Traganos F, Darzynkiewicz Z. 1994. A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. Anal Biochem 218:314–319.

Hansen MB, Nielsen SE, Berg K. 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods 119:203–210.

Henkart PA. 1996. ICE family proteases: mediators of all apoptotic cell death? Immunity 4:195–201.

Hsu H, Xiong J, Goeddel DV. 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell 81:495–504.

Hsu H, Shu HB, Pan MG, Goeddel DV. 1996. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell 84:299–308.

Inward CD, Williams J, Chant I, Crocker J, Milford DV, Rose PE, Taylor CM. 1995. Verocytotoxin-1 induces apoptosis in vero cells. J Infect 30:213–218.

Karmali MA, Petric M, Lim C, Fleming DC, Arbus GS, Lior H. 1985. The association between idiopathic hemolytic uremic syndrome and infection by verotoxin producing *Escherichia coli*. J Infect Dis 151:775–782.

Katagiri YU, Mori T, Nakajima H, Katagiri C, Taguchi T, Takeda T, Kiyokawa N, Fujimoto J. 1999. Activation of Src family kinase Yes induced by Shiga toxin biding to globotriaosyl ceramide (Gb3/CD77) in low density, detergent-insoluble microdomains. J Biol Chem 274:35278–35282.

Kischkel FC, Hellbardlt S, Behrmann I, Gerrner M, Pawlita M, Krammer PH, Peter ME. 1995. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins (CAP) form a death–inducing signalling complex (DISC) with the receptor. EMBO J 14:5579–5588.

Kiyokawa N, Lee EK, Karunagaran D, Lin S-Y, Hung M-C. 1997. Mitosis-specific negative regulation of epidermal growth factor receptor, triggered by a decrease in ligand binding and dimerization, can be overcome by overexpression of receptor. J Biol Chem 272:18656–18665.

Kiyokawa N, Taguchi T, Mori T, Uchida H, Sato N, Takeda T, Fujimoto J. 1998. Induction of apoptosis in normal human renal tubular epithelial cells by *Escherichia coli* Shiga toxins 1 and 2. J Infect Dis 178:178–184.

Kuida K, Zheng TS, Na S, Kuan C, Yang D, Karasuyama H, Rakic P, Flavell RA. 1996. Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature 384:368–372.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. 1997. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91:479–489.

Li H, Zhu H, Xu CJ, Yuan J. 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94:491–501.

Lingwood CA, Law H, Richardson S, Petric M, Brunton JL, De Grandis S, Karmali M. 1987. Glycolipid binding of purified and recombinant *Escherichia coli* produced verotoxin in vitro. J Biol Chem 262:8834–8839.

Los MM, Van de Craen M, Penning LC, Schenk H, Westendrop M, Baeuerle PA, Dröge W, Krammer PH, Flers W, Schultze-Osthoff K. Requirement of an ICE/CED-3 protease for Fas/APO-1-mediated apoptosis. Nature 375:81–83.

Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. 1998. Bid, a Bc12 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell 94:481–490.

Mangeney M, Lingwood CA, Taga S, Caillou B, Tursz T, Wiels J. 1993. Apoptosis induced in Burkitt's lymphoma cells via Gb3/CD77, a glycolipid antigen. Cancer Res 53:5314–5319.

Martin SJ, Lennon SV, Bonham AM, Cotter TG. 1990. Induction of apoptosis (programmed cell death) in human leukemic HL-60 cells by inhibition of RNA or protein synthesis. J Immunol 145:1859–1867.

Martin SJ, Green DR. 1995a. Protease activation during apoptosis: death by a thousand cuts? Cell 82:349–352.

Martin SJ, O'Brien GA, Nishioka WK, McGahon AJ, Mahboubi A, Saido TC, Green DR. 1995b. Proteolysis of fodrin (non-erythroid spectrin) during apoptosis. J Biol Chem 270:6425–6428.

Martin SJ, Finucane DM, Amarante-Mendes GP, O'Brien GA, Green DR. 1996. Phosphatidylserine externalization during CD95-induced apoptosis of cells and cytoplasts requires ICE/CED-3 protease activity. J Biol Chem 271:28753–28756.

Medema JP, Scaffidi C, Kischkel FC, Shevchenko A, Mann M, Krammer PH, Peter ME. 1997. FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). EMBO J 16:2794–2804.

Miyashita T, Matsui J, Ohtsuka Y, Mami U, Fujishima S, Okamura-Oho Y, Inoue T, Yamada M. 1999. Expression of extended polyglutamine sequentially activates initiator and effector caspases. Biochem Biophys Res Commun 257:724–730.

Monnens L, van Collenburg J, De Jong M, Zoethout H, van Wieringen P. 1978. Treatment of the hemolytic uremic syndrome. Comparison of results of heparin treatment with the results of streptkinase treatment. Helv pediat Acta 33:321–328.

Mori T, Kiyokawa N, Katagiri YU, Taguchi T, Suzuki T, Sekino T, Sato N, Ohmi K, Nakajima H, Takeda T, Fujimoto J. 2000. Globotriaosyl ceramide (CD77/Gb3) in

the glycolipid-enriched membrane domain participates in the B cell receptor-mediated apoptosis by regulating Lyn kinase activity in human B cells. Exp Hematol (in press).

Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM. 1996. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death–inducing signaling complex. Cell 85:817–827.

Muzio M, Salvesen GS, Dixit VM. 1997. FLICE induced apoptosis in a cell-free system. Cleavage of caspase zymogens. J Biol Chem 272:2952–2956.

Muzio M, Stockwell BR, Stennicke HR, Salvesen GS, Dixit VM. 1998. An induced proximity model for caspase-8 activation. J Biol Chem 273:2926–2930.

Nagata S. 1997. Apoptosis by death factor. Cell 88:355–365.

Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA, Munday NA, Raju SM, Smulson ME, Yamin TT, Yu VL, Miller DK. 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376:37–43.

Noda M, Yutsudo T, Nakabayashi N, Hirayama T, Takeda Y. 1987. Purification and some properties of Shiga-like toxin from *Escherichia coli* 0157 :H7 that is immunologically identical to Shiga toxin. Microb Pathog 2:339–349.

Obrig TG, Moran TP, Brown JE. 1987. The mode of action of Shiga toxin on peptide elongation of eukaryotic protein synthesis. Biochem J 244:287–294.

Orth K, Chinnaiyan AM, Garg M, Froelich CJ, Dixit VM. 1996. The CED-3/ICE-like protease Mch2 is activated during apoptosis and cleaves the death substrate Lamin A. J Biol Chem 271:16443–16446.

Perandones CE, Illera VA, Peckham D, Stunz LL, Ashman RF. 1993. Regulation of apoptosis in vitro in mature murine spleen T cells. J Immunol 151:3521–3529.

Sanchez I, Xu CJ, Juo P, Kakizaka A, Blenis J, Yuan J. 1999. Caspase-8 is required for cell death induced by expanded polyglutamine repeats. Neuron 22:623–633.

Sandvig K, van Deurs B. 1992. Toxin-induced cell lysis: protection by 3-methyladenine and cycloheximide. Exp Cell Res 200:253–262.

Saxena SK, O'Brien AD, Ackerman EJ. 1989. Shiga toxin, Shiga-like toxin II variant, and ricin are all single-site RNA N-glycosidases of 28 S RNA when microinjected into Xenopus oocytes. J Biol Chem 264:596–601.

Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME. 1998. Two CD95 (APO-1/Fas) signaling pathways. EMBO J 17: 1675–1687.

Siegler RL, Loghman-Adham M, Timmons OD. 1995. Acute respiratory failure in the hemolytic uremic syndrome. Clin Pediatr 34:660–662.

Srinivasula SM, Ahmad M, Fernandes-Alnemri T, Litwack G, Alnemri E. 1996. Molecular ordering of the Fas-apoptotic pathway: the Fas/APO-1 protease Mch5 is a CrmA-inhibitable protease that activates multiple Ced-3/ICE-like cysteine proteases. Proc Natl Acad Sci USA 93:14486–14491.

Taga S, Carlier K, Mishal Z, Capoulade C, Mangeney M, Lecluse Y, Coulaud D, Tetaud C, Pritchard LL, Tursz T, Wiels J. 1997. Intracellular signaling events in CD77-mediated apoptosis of Burkitt's lymphoma cells. Blood 90:2757–2767.

Taguchi T, Uchida H, Kiyokawa N, Mori T, Sato N, Horie H, Takeda T, Fujimoto J. 1998. Verotoxins induce apoptosis in human renal tubular epithelium derived cells. Kidney Int 53:1681–1688.

Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS, Dixit VM. 1995. Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell 81:801–809.

Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia-Calvo M, Houtzager VM, Nordstrom PA, Roy S, Vaillancourt JP, Chapman KT, Nicholson DW. 1997. A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J Biol Chem 272:17907–17911.

Uchida H, Kiyokawa N, Taguchi T, Horie H, Fujimoto J, Takeda T. 1999. Shiga toxins induce apoptosis in pulmonary epithelium-derived cells. J Infect Dis 180:1902–1911.

Williams JM, Lea N, Lord JM, Roberts LM, Milford DV, Taylor CM. 1997. Comparison of ribosome-inactivating proteins in the induction of apoptosis. Toxicol Lett 91: 121–127.

Wyllie AH, Kerr JF, Currie AR. 1980a. Cell death: the significance of apoptosis. Int Rev Cytol 68:251–306.

Wyllie AH. 1980b. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284:555–556.

Wyllie AH, Morris RG, Smith AL, Dunlop D. 1984. Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. J Pathol 142:67–77.

Yang X, Chang HY, Baltimore D. 1998. Autoproteolytic activation of pro-caspases by oligomerization. Mol Cell 1:319–325.

Yeh WC, Pompa JL, McCurrach ME, Shu HB, Elia AJ, Shahinian A, Ng M, Wakeham A, Khoo W, Mitchell K, El-Deiry WS, Lowe SW, Goeddel DV, Mak TW. 1998. FADD: essential for embryo development and signaling from some, but not all, inducers of apoptosis. Science 279:1954–1958.

Yin XM, Wang K, Gross A, Zhao Y, Zinkel S, Klocke B, Roth KA, Korsmeyer SJ. 1999. Bid-deficient mice are resistant to Fas-induced hepatocellular apoptosis. Nature 400: 886–891.