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Verotoxins induce apoptosis in human renal tubular epithelium derived cells

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Verotoxins induce apoptosis in human renal tubular epithelium derived cells. Apoptosis mediated by verotoxins (VTs) has been identified in a renal carcinoma cell line, ACHN cells, which are an *in vitro* model of renal tubular epithelial cells. ACHN cells express the renal tubular marker CD24 as well as globotriaosyl ceramide/CD77, the receptor for VTs. VT binding to the ACHN cell surface was confirmed by positive staining with antibodies to the VTs. Treatment of ACHN cells with VTs induced prompt growth inhibition and cell death, and fragmentation of the genomic DNA in cells, typical of apoptosis, was observed. The expression of apoptotic antigen 7A6 detected by APO2.7 antibody in ACHN cells further supports the occurrence of apoptosis as a result of VT treatment. Cycloheximide enhanced VT-mediated apoptosis of ACHN cells, suggesting a strong correlation between the inhibition of protein synthesis and VT-mediated apoptosis. Moreover, tumor necrosis factor-α had a synergistic effect on VT-mediated apoptosis in ACHN cells. Considering the above evidence together with the clinical evidence showing the presence of apoptosis in the renal epithelium of a HUS patient, our results suggest a VT-induced apoptotic mechanism in normal renal tubular epithelium that may contribute to the pathogenesis of hemolytic uremic syndrome.

The hemolytic uremic syndrome (HUS) is characterized by the triad of hemolytic anemia, acute renal failure, and thrombocytopenia [1]. The epidemic form of the HUS, which is preceded by a diarrheal prodrome, has been shown to be strongly associated with infection by verotoxin (VT) producing strains of *Escherichia coli* (VTEC) [2]. Although the details of the pathogenesis of HUS are still unclear, the damage of endothelial cells of glomerular capillaries and renal arterioles mediated by VTs has been postulated to play an important role [3, 4].

Several lines of evidence, however, imply that renal tubular impairment also contributes to the development of HUS [5–10]. For an example, we previously demonstrated that VTs are cytotoxic for renal carcinoma line ACHN, an *in vitro* model of renal tubular epithelial cells, which is 10^6- to 10^7-fold more sensitive to them than human umbilical cord vascular endothelial cells (HUVECs) [10].

In this study, we further characterized VT-mediated ACHN cell damage and found that apoptotic cell death was part of the process. The finding described in this paper could point out a new mechanism of cell damage of renal tubular epithelium involved in the pathogenesis of HUS caused by VTEC.

Key words: ACHN cells, cell death, hemolytic uremic syndrome, anemia, VTEC.

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METHODS

Materials

VT-1 and VT-2 were prepared as described previously [11, 12]. The ACHN cell line was obtained from the American Type Culture Collection (ATCC CRL 1611). The mouse monoclonal antibodies used in this study were CD10 (IF-6 [13]), CD24 (L30 [14]), CD34 (QBEnd10; Coulter/Immunotec, Inc., Westbrook, MA, USA), APO2.7 (Coulter), and CD77 (38.13; Coulter). Antiserum for VT-1 and VT-2 were prepared as described previously [11, 12]. Fluorescein-conjugated secondary antibodies were purchased from Jackson Laboratory, Inc. (West Grove, PA, USA). The human recombinant cytokines including tumor necrosis factor (TNF)-α, interleukin-1 (IL-1)β, and interferon (INF)-γ, were purchased from Pepro Tech EC Ltd. (London, UK).

Immunofluorescence study

ACHN cell suspensions were produced by treating the cells with PBS containing 0.1% NaN_3 for five minutes, followed by pipetting. The cells were then stained with monoclonal antibodies and analyzed by flow cytometry (EPICS Profile and EPICS-XL; Coulter) as described previously [13]. To detect VT binding, one million of ACHN cells in suspension were incubated with and without 0.1 μg/ml of VT-1 or VT-2 for one hour at 4°C. The cells were then washed intensively with PBS and stained with either control normal rabbit serum or polyclonal anti-VT-1 (VT-2) antiserum. To detect 7A6 antigen, which is located in the cytoplasm, ACHN cells were harvested by trypsinization and fixed followed by permeabilization prior to staining with PE-Cy5-conjugated APO2.7 antibody, as described previously [15].

MTT assay

To assess growth and viability, ACHN cells were plated on 96-well plates (Corning, Inc., Corning, NY, USA) at a concentration of 1×10^4 cells in 100 μl of complete medium per well, with

and without VT-1 (VT-2) or cytokines. MTT assays were performed as described previously [16].

DNA fragmentation assay

ACHN cells were assayed for DNA ladder formation by gel electrophoresis. After treatment with and without VTs, DNAs were extracted from the cells, separated in 1% agarose gel by electrophoresis and examined under UV light as described previously [17].

DNA fragmentation was also investigated *in situ* by the TUNEL method using a TACS™ 2 TdT(TBL) *in situ* apoptosis detection kit (Trevigen, Inc., Gaithersburg, MD, USA). Experiments were performed according to the manufacturer's protocol.

To evaluate relative DNA contents, cells were stained with propidium iodide (PI; Sigma-Aldrich Fine Chemicals, St. Louis, MO, USA) then analyzed on by flow cytometry as described previously [18].

RESULTS

Cell surface phenotype of ACHN cells

ACHN is a cell line derived from human renal tubular epithelium adenocarcinoma. As reported in several papers including our own, the epithelial components of renal tubules can be classified according to the pattern of expression of hematopoietic cell surface antigens [19–21]. Thus, we first examined ACHN cells by using antibodies against CD leukocyte antigens. As shown in Figure 1 A, ACHN cells were strongly positive for CD24 by flow cytometry, but negative for CD10. ACHN cells were also found to express low levels of Gb3/CD77, the functional receptor for VTs [22–24]. Based on the distribution of various molecules in normal renal tissue [21], ACHN cells in culture may correspond to the epithelium of the distal tubule.

Verotoxin binding to ACHN cells

Since ACHN cells were found to express Gb3/CD77 on their cell surface, we investigated whether the cells bind VTs. As shown in the Figure 1B upper panels, rabbit anti-VT-1 antiserum labeled ACHN cells pretreated with VT-1 as shown by flow cytometry. On the other hand, ACHN cells not incubated with VT-1 failed to react with anti-VT-1 antiserum, demonstrating the specificity of the antiserum. In addition, anti-VT-2 antiserum did not show any binding to VT-1-pretreated ACHN cells (data not shown). Similar results were obtained by using a combination of VT-2 and anti-VT-2 antiserum on ACHN cells (Fig. 1B, lower panels).

Cytotoxic effect of verotoxins on ACHN cells

Next we investigated whether VTs affect the growth and survival of ACHN cells. When incubated with VT-1 or VT-2, the viable number of ACHN cells assessed by MTT assay was markedly decreased in a dose and time dependent manner (Fig. 2). The reduction of cell growth after treatment with VTs was due to cell death as confirmed by trypan blue staining (data not shown), showing a high sensitivity of ACHN cells to the cytotoxic effect of VTs. VT-1 was significantly more cytotoxic to ACHN cells than was VT-2 after three days of incubation ($P < 0.01$). Each experiment was repeated more than three times independently and similar results were obtained. Since VT-1 is more cytotoxic to ACHN cells than VT-2, the following experiments were mainly performed with VT-1.

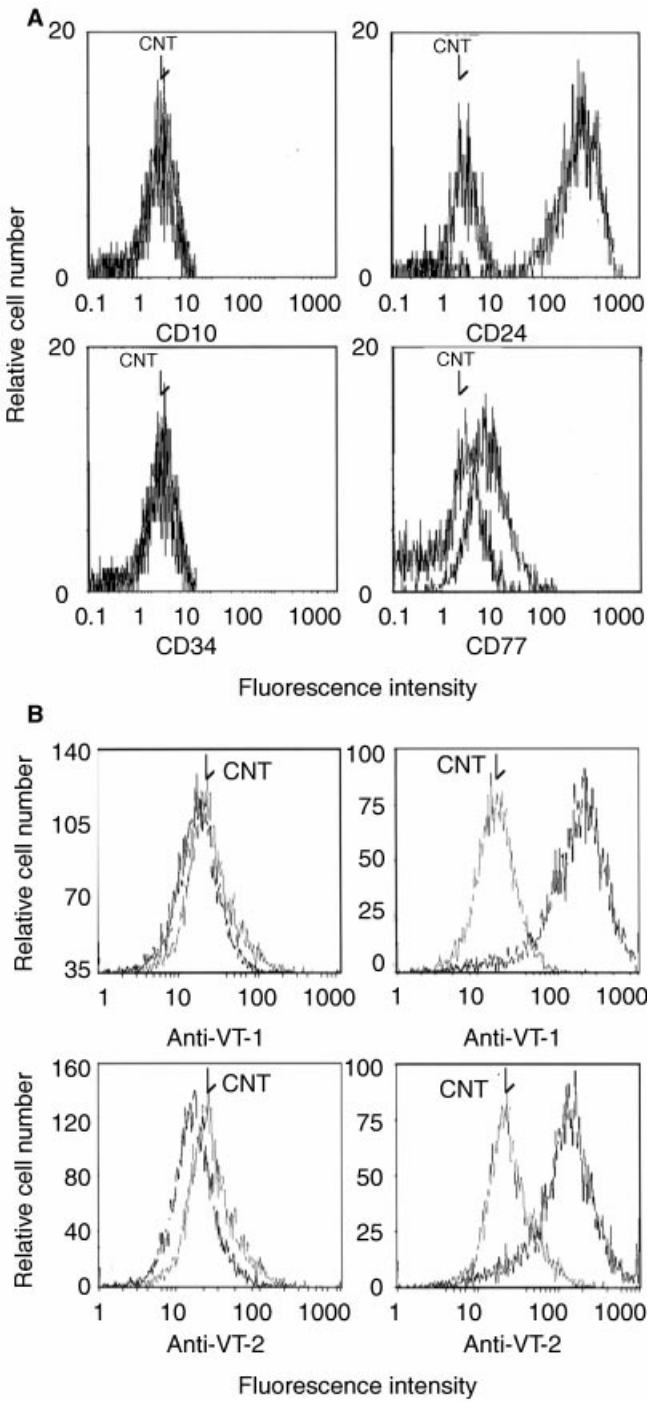


Fig. 1. Analysis of antigen expression on the ACHN cell surface and detection of verotoxin (VT) binding. (*A*) ACHN cells in suspension were stained with specific monoclonal antibodies as indicated in the Figure and analyzed by flow cytometry as described in **Methods** section. The histogram obtained was superimposed on that of the negative control (indicated as CNT, the cells stained with isotype matched control immunoglobulin) and displayed. X-axis, fluorescence intensity; Y-axis, relative cell number. (*B*) To test VT binding, after incubation with (right panels) and without (left panels) 0.1 μg/ml of VT-1 (upper panels) or VT-2 (lower panels) for one hour at 4°C, ACHN cells in suspension were stained with either rabbit anti-VT-1 anti-serum (upper panels) or anti-VT-2 anti-serum (lower panels) and analyzed by flow cytometry. The histograms obtained are displayed essentially as in A.

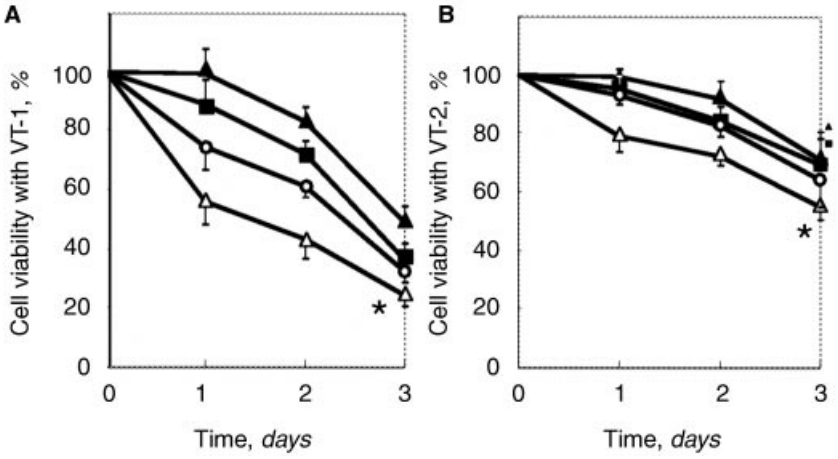


Fig. 2. Cytotoxic effect of verotoxin (VT)-1 on ACHN cells. ACHN cells (1×10^4 cells per 100 μl of medium per well) were incubated with different concentrations of VT-1 or VT-2 as indicated. After incubation for the periods indicated, viable cell numbers were estimated by MTT assay as described in **Methods** and presented as the ratio against untreated cells. Experiments were performed in triplicate, and mean ± SD values are presented. *VT-1 showed significantly greater cytotoxicity than VT-2 after three days treatment at each concentration, as verified by statistical analysis ($P < 0.01$).

Detection of apoptosis in the verotoxin-induced cell death of ACHN cells

We conducted several experiments to investigate the possibility that the cytotoxic effect of VTs involves an apoptotic pathway. First, we examined the morphological appearance of ACHN cells during VT-1 treatment. When ACHN cells were cultured in the presence of VT-1, they showed condensed chromatin (Fig. 3A-b) and cleaved nuclei (Fig. 3A-c), that is, findings typical of apoptosis. Moreover, typical apoptotic bodies were observed in cells that floated after VT-1 treatment (Fig. 3A-d). However, no such observations were made in untreated cells (Fig. 3A-a).

Next, we tested whether cleavage of nuclear DNA, a major feature of apoptosis, occurs during the VT-induced death of ACHN cells. As shown in Figure 3B, DNA prepared from ACHN cells pretreated with VT-1 exhibited an oligonucleosomal ladder fragmentation pattern on agarose gel electrophoresis. Cleavage of the nuclear DNA in ACHN cells treated with VT-1 was also confirmed by *in situ* detection by the TUNEL method, as shown in Figure 3C. Some ACHN cells clearly incorporated biotinylated nucleotides, indicating the occurrence of DNA fragmentation induced by VT-1. Moreover, VT-1-induced cleavage of the nuclear DNA in ACHN cells was further confirmed by detecting subploid cells with PI-staining (Fig. 4B).

Recently, a 38 kDa protein, 7A6, detected by APO2.7 antibody was identified as an early stage marker of the apoptotic process in various cells [18]. As shown in Figure 4A, APO2.7 clearly stained ACHN cells treated with VT-1. No staining was observed when APO2.7 was reacted with non-treated ACHN cells. These findings clearly showed that apoptosis had indeed occurred in ACHN cells following treatment with VT-1.

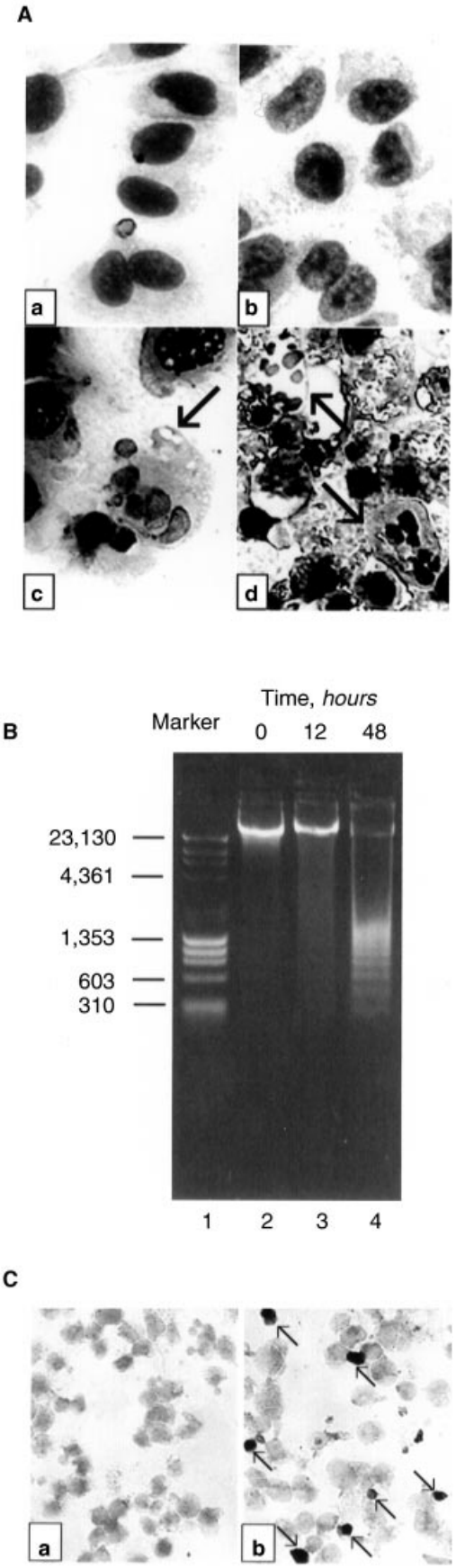
Since we observed a significant difference between VT-1 and VT-2 in cytotoxic effects on ACHN cells, we compared the abilities of VT-1 and VT-2 to induce apoptosis in these cells. When the occurrence of apoptosis was evaluated by the either appearance of 7A6 antigen (Fig. 4A) or the detection of subploid cells (Fig. 4B), again, VT-1 showed significantly higher efficiency of apoptosis induction in ACHN cells than did VT-2. This was verified by statistical analysis ($P < 0.001$).

To better understand the mechanism of VT-mediated apoptosis in ACHN cells, we conducted further experiments. First, we examined the influence of cell concentration on VT-induced apoptosis. ACHN cells were first seeded at different cell concentrations and then treated with VT-1. Subploid cell appearance was markedly reduced as the cell concentration increased (Fig. 5), showing the susceptibility of ACHN cells to VT-induced apoptosis to be affected by initial cell concentration. We next examined the effect of a protein synthesis inhibitor on VT-mediated apoptosis in ACHN cells. Cycloheximide alone induced apoptosis in ACHN cells at doses exceeding 25 μg/ml (data not shown). As shown in Figure 6, a low dose (10 μg/ml) of cycloheximide did not induce apoptosis but did increase the induction of subploid cells by VT-1 when added at the same time ($P < 0.05$). We also tested Actinomycin D, a RNA synthesis inhibitor, and observed similar results (data not shown). Each experiment was repeated more than three times independently and similar results were obtained. These findings strongly suggests that protein synthesis inhibition is closely related to VT-mediated apoptosis in ACHN cells.

Effect of tumor necrosis factor-α on the verotoxin-mediated cytotoxicity toward ACHN cells

As certain cytokines including TNF-α and IL-1β reportedly enhance the cytotoxicity of VTs toward vascular endothelial cells *in vitro* [3, 25–27], we tested whether or not these cytokines synergistically affect the cytotoxicity of VTs to ACHN cells. No significant reduction of cell viability was observed when ACHN cells were treated with TNF-α alone (Fig. 7A, columns 1 and 2). However, TNF-α was found to enhance the cell viability reduction caused by VT-1 (Fig. 7A, columns 3 and 4, $P < 0.05$). Similar results were obtained when IL-1β and INF-γ were used (data not shown).

In order to rule out the possibility of this observation being due to direct growth inhibition of ACHN cells by TNF-α, we examined the effect of TNF-α on VT-mediated apoptosis in ACHN cells by detecting apoptotic antigen 7A6. As shown in Figure 7B, TNF-α by itself did not induce the appearance of 7A6 antigens on ACHN, but did markedly enhanced the 7A6 antigen expression induced by VT-1 treatment. These results indicate that TNF-α has a synergistic effect on VT-mediated apoptosis. Each experiment



was repeated more than three times independently and similar results were obtained.

Detection of apoptotic cells in a renal tissue from hemolytic uremic syndrome patient

In vitro observations described above suggest that apoptosis in renal tubular epithelium involved in the pathogenesis of HUS. Since we had the opportunity to examine the frozen renal tissue of a child who died of HUS, we tested the occurrence of apoptosis in tubular epithelium. As shown in Figure 8, cleavage of the nuclear DNA in tubular epithelial cells as well as stromal cells was confirmed by the TUNEL method, clearly indicating the occurrence of apoptosis in renal tissue of a HUS patient.

DISCUSSION

Cytotoxic effects of VT are thought to be mediated by the A-subunit which inhibits protein synthesis [28–30]. Recently, however, several reports have claimed that VTs induce programmed cell death, apoptosis, in several cell lines including Vero, MDCK [31–33] and Burkitt's lymphoma [17]. We demonstrated herein further evidence that VTs induce apoptosis in ACHN human renal adenocarcinoma cells which exhibit the phenotype of distal tubular epithelia. Microscopic nuclear fragmentation, DNA ladder formation, expression of 7A6 antigen, and incorporation of nucleotides by the TUNEL method, all of which occurred following VT-treatment of ACHN cells, constitute features with apoptosis. It is therefore clear that VT induces apoptosis in a variety of cell types, although the mechanism underlying this phenomenon is controversial and remains to be elucidated.

In the case of Vero cells, a complete form of VT is required to induce apoptosis [33], suggesting that inhibition of protein synthesis mediated by the A-subunit is linked to apoptosis. In fact, it was reported that inhibition of protein synthesis caused apoptosis or operated as an apoptosis co-factor in some instances [34–36]. The enhancement of VT-mediated apoptosis of ACHN cells by cycloheximide, an inhibitor of protein synthesis, as described herein, supports this idea and raises the possibility of an unknown pathway by which cells undergo programmed cell death. In

Fig. 3. Detection of apoptosis in ACHN cells after treatment with verotoxin (VT)-1. (*A*) Morphological appearance of Giemsa stained ACHN cells cultured in the presence of VT-1. Cells were cultured on Lab-Tek Tissue Culture Chamber/Slide (Division Miles Laboratories, Inc., Naperville, IL, USA) in medium alone (a, adherent cells) or in the presence of 200 pg/ml of VT-1 (b, for one day, adherent cells; c, for three days, adherent cells; d, for three days, cytocentrifuged floating cells), then stained and examined by light microscopy. Compared with untreated cells (a), cells treated with VT-1 show characteristic condensed chromatin (b), cleaved nuclei (c), and apoptotic bodies (d). Magnification ×600. (*B*) DNA ladder formation after treatment with VT-1. ACHN cells at 50% confluence were treated with (lanes 3 and 4) and without (indicated as 0 hr, lane 2) 200 pg/ml of VT-1. After 12 hours (lane 3) and 48 hours (lane 4) culture, DNAs were extracted as described in the **Methods**, and 1.5 μg of the DNA from each sample was electrophoresed in 1% agarose gel. A mixture of *Hin*dIII-digested λ DNA and *Hae*III-digested ϕ DNA was loaded on the same gel (lane 1), as a molecular weight control. (*C*) The *in situ* detection of nuclear DNA cleavage in ACHN cells after treatment with VT-1. To test the cleavage of nuclear DNA, ACHN cells treated with (b) and without (a) 200 pg/ml of VT-1 for 24 hours were tested by the TUNEL method. Typical positive cells are indicated by the arrow.

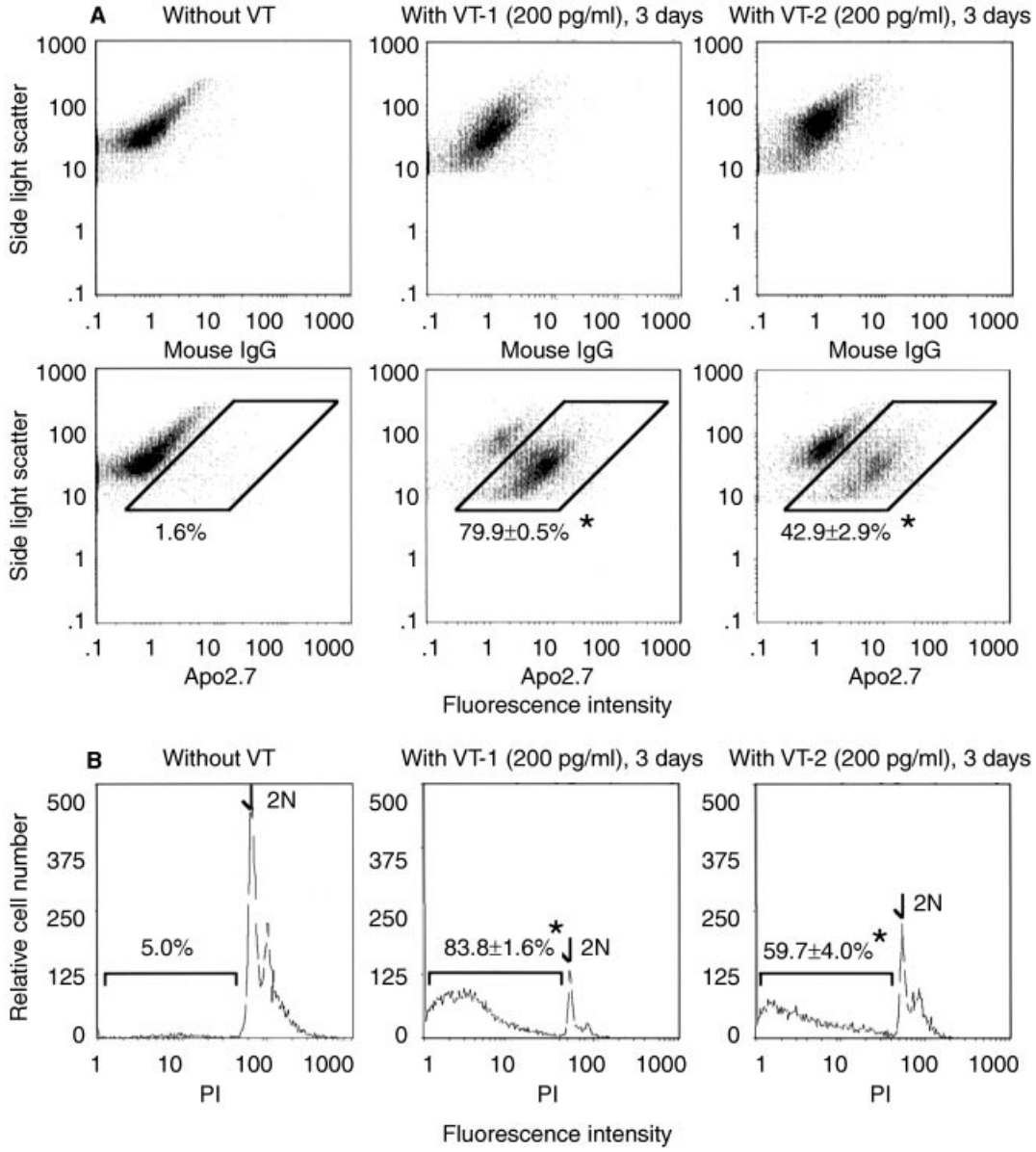


Fig. 4. Comparison of the efficiency of inducing apoptosis between verotoxin (VT)-1 and VT-2. (*A*) Induction of apoptotic antigen 7A6. After three days of culture in medium alone (left panels) or in the presence of 200 pg/ml of VT-1 (center panels) or 200 pg/ml of VT-2 (right panels), ACHN cells were fixed and stained with either APO2.7 monoclonal antibody (lower panels) or isotype matched control immunoglobulin (upper panels), then analyzed by flow cytometry as described in the **Methods** section. To identify positive cells more clearly, the fluorescence intensity was displayed as a two dimensional histogram against side light scatter. The ratios of positive cells, enclosed in the parallelogram, are indicated. Experiments with VT-treated cells were performed in triplicate, and the means ± SD are presented. X-axis, fluorescence intensity; Y-axis, side light scatter. *VT-1 induced significantly greater apoptosis than VT-2, as verified by statistical analysis ($P < 0.001$). (*B*) Detection of subploid cells. ACHN cells, prepared under experimental conditions identical to those of panel A, were fixed and stained with PI, then analyzed by flow cytometry. The ratios of subploid cells, that is, those undergoing apoptosis, are indicated. Experiments with VT-treated cells were performed in triplicate, and the means ± SD are presented. VT-1 induced significantly greater apoptosis than VT-2, as verified by statistical analysis (*$P < 0.001$).

contrast, the B-subunit of VT is sufficient to induce apoptosis in Burkitt's lymphoma lines [17], suggesting the presence of an alternate mechanism for VT-mediated apoptosis, which is independent of protein synthesis inhibition in these lines. A recent report describing intracellular signaling events triggered by cross-linking of CD77 may support this idea [37].

Interestingly, we observed a significant difference between VT-1 and VT-2 in terms of efficiency for inducing apoptosis in ACHN cells. In our experiment, both VTs induced apoptosis in Burkitt's line Ramos with equal efficacy (data not shown), excluding the possibility of loss of activity due to purification procedures. As the amounts of VT-1 and VT-2 capable of binding to ACHN cells are nearly equal, as showed by flow cytometry (Fig. 1B), the different cytotoxicities of VTs may result from either the difference in thresholds of the stimuli or differences in signaling pathways after binding of VTs to the cell surface. In this regard,

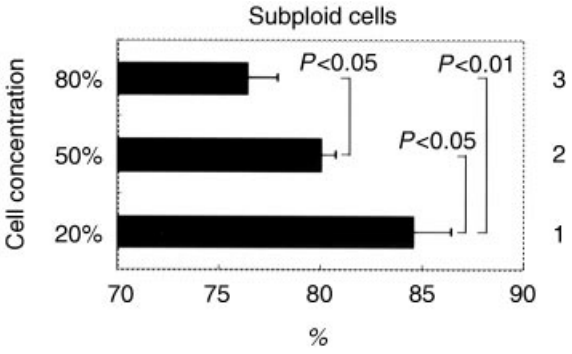


Fig. 5. Effect of initial cell concentration on verotoxin (VT)-mediated apoptosis in ACHN cells. ACHN cells were plated at different cell concentrations (column 1, 20%; column 2, 50%; column 3, 80%) then treated with 200 pg/ml of VT-1 for three days. The subsequent ratios of cells undergoing apoptosis were evaluated by detection of subploid cells as described in Figure 4B. Experiments were performed in triplicate, and the means ± SD are presented. Significant differences in the appearance of subploid cells among concentrations were demonstrated by statistical analysis ($P < 0.05$).

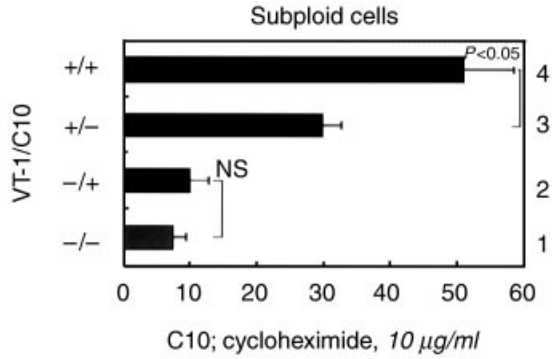


Fig. 6. Effect of low dose of cycloheximide on verotoxin (VT)-mediated apoptosis in ACHN cells. ACHN cells preincubated with (columns 2 and 4) and without (columns 1 and 3) 10 μg/ml of cycloheximide for 12 hours were treated with (columns 3 and 4) and without (columns 1 and 2) 200 pg/ml of VT-1 for an additional 36 hours. The subsequent ratios of cells undergoing apoptosis after treatment were evaluated by detection of subploid cells as described in Figure 4B. Experiments were performed in triplicate, and the mean ± SD are presented. Pretreatment with a low dose of cycloheximide significantly increased the appearance of VT-1 induced subploid cells, as verified by statistical analysis ($P < 0.05$), without affecting cells not treated with VT-1.

it is important to examine whether inhibition of protein synthesis by the A-subunit is solely responsible for apoptosis or the B-subunit is also involved in apoptosis, as in the case of ACHN cells.

In Vero cells, susceptibility to VT was reported to be cell cycle dependent [38]. Similarly, if not identically, the sensitivity of ACHN cells to VT changed according to cell density. Cells at high density were more resistant to VT whereas cells at low density were more susceptible to VT, indicating the operation of a similar regulatory mechanism.

It is generally accepted, based on histopathologic findings [39], that capillary microangiopathy and endothelial damage in the kidney are the major causes of HUS [3, 4]. The findings that endothelial cells express Gb3/CD77 and are susceptible to VTs support this hypothesis [3, 25–27]. However, laboratory data, such as marked elevation of urinary N-acetyl glucosaminidase and β2-microglobulin, both of which are specific markers of tubular function, have provided evidence of impaired renal tubular function in the acute stage of HUS [10]. The histopathologic change in a mouse model of VTEC infection was found to be damage to the renal tubular epithelium, while glomeruli remained unaffected [5–7]. Several reports have described renal tubular epithelia as expressing Gb3/CD77 and binding VTs [8, 9]. Based on these findings, renal tubular epithelium is probably another target of VTs *in vivo*, and it should be viewed as a major site of the renal dysfunction that occurs in the early phase of HUS. The data presented herein, that is, VT-induced cell death in the human renal tubular epithelial line ACHN, raises the possibility of such a mechanism. Since normal renal tubular epithelial cells in primary culture also exhibited sensitivities to VTs (Kiyokawa et al, manuscript submitted), the data obtained in ACHN cells are considered to reflect the features of normal cells.

Recently, elevated plasma and urinary concentrations of TNF-α, IL-6, and IL-8 have been demonstrated in HUS patients [40–42]. Since TNF-α and IL-1 were reported to up-regulate the expression of Gb3/CD77 on human endothelial cells in culture and to increase their susceptibility to VTs [3, 25–27, 43], a strong correlation between these cytokines and the pathogenesis of HUS has been suggested. The finding that human and murine macrophages bind VTs and subsequently produce these cytokines [44, 45] strongly supports this idea. It is noteworthy that neither TNF-α nor IL-1β alone is directly cytotoxic to endothelial cells, but both become cytotoxic in the presence of co-factors that inhibit protein and RNA synthesis [46], suggesting another synergistic mechanism between cytokines and VTs. The enhancement of VT-mediated apoptosis of ACHN cells by TNF-α, as described herein, further emphasizes the important role of cytokines in the pathogenesis of HUS.

Considering all of the above evidence together, endothelial cell damage does not appear to be the sole cause of HUS, and that to the renal epithelium may also play an important role. Although a direct effect of VTs may be the primary event in damage to these cells, modulation by cytokines should be taken into consideration. Additional basic studies are clearly necessary, but the possibility of apoptosis as a VT-mediated mechanism of cell damage should provide a new approach to understanding the pathogenesis of HUS and to establishing therapeutic strategies. Our finding on the clinical specimen that showed the presence of apoptosis in renal tubules of HUS patient may support this idea.

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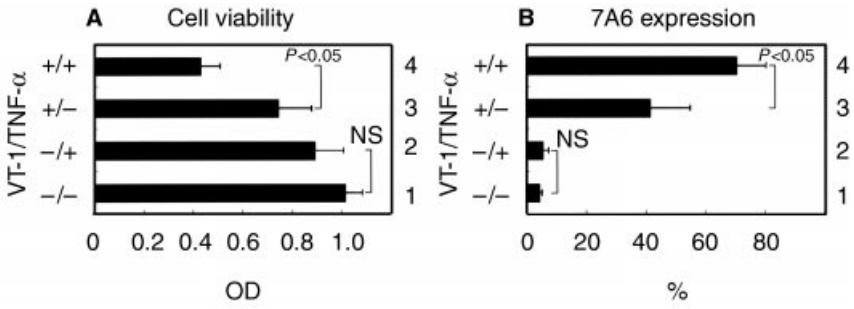

Fig. 7. Effect of tumor necrosis factor (TNF)-α on verotoxin (VT)-mediated apoptosis in ACHN cells. (*A*) The effect of TNF-α on VT-mediated cytotoxicity in ACHN cells. ACHN cells (1×10^4 cells per 100 μl of medium per well) were preincubated with (columns 2 and 4) and without (columns 1 and 3) 20 ng/ml of TNF-α for 12 hours and then treated with (columns 3 and 4) and without (columns 1 and 2) 12.5 pg/ml of VT-1 for an additional 60 hours. Subsequent viable cell numbers were estimated by MTT assay as described in Figure 2 and the averages of OD values are presented. Experiments were performed in triplicate, and the means ± SD are presented. Pretreatment with TNF-α significantly enhanced the cytotoxicity of VT-1 ($P < 0.05$). Although alone TNF-α inhibited the growth of ACHN, the difference was not statistically significant. (*B*) The effect of TNF-α on VT-mediated apoptosis in ACHN cells. ACHN cells, preincubated with (columns 2 and 4) and without (columns 1 and 3) 20 ng/ml of TNF-α for 12 hours, were treated with (columns 3 and 4) and without (columns 1 and 2) 12.5 pg/ml of VT-1 for an additional 60 hours. The cells undergoing apoptosis after treatment were evaluated by the detection of 7A6 antigen expression as described in Figure 4. The ratios of positive cells are indicated on the graph. Experiments were performed in triplicate, and the means ± SD are presented.

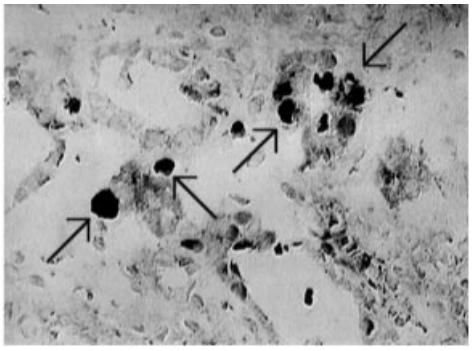

Fig. 8. Detection of apoptotic cells in renal section from hemolytic uremic syndrome (HUS) patient. A renal tissue was obtained from an autopsied case of patient (1 year-9 month-old female) who died of HUS after infection with VTEC O157: H7 (VT-1^+, VT-2^+). To detect the presence of apoptotic cells, a fresh frozen section was examined by TUNEL method as described in Figure 3C. Typical positive cells, which are dark colored, are indicated by the arrow.

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