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Escherichia coli verotoxin 1 mediates apoptosis in human HCT116 colon cancer cells by inducing overexpression of the GADD family of genes and S phase arrest

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Abstract The *Escherichia coli* verotoxin 1 (VT1) inhibits protein synthesis, cell proliferation, and damages endothelial cell in the hemolytic uremic syndrome. VT1 can specifically bind and act on endothelial cells as well as on many tumor cells because these cells express its high affinity receptor, globotriaosylceramide. This indicates that VT1 may have both antiangiogenic and antineoplastic activities. We investigated this potential of VT1 by incubating several colon cancer cell lines with VT1 for different time periods and found that HCT116 cells were especially sensitive to VT1. A combination of morphological studies, flow cytometry, DNA laddering and annexin V staining confirmed that VT1 irreversibly arrests these cells in S phase within 24 h and prolonged incubation triggers DNA fragmentation. Concomitant to the activation of the S phase checkpoint, increased levels of mRNA and proteins of growth arrest and DNA damage-inducible gene family that include GADD34, GADD45α, and GADD45β was observed. Interestingly, no significant changes in expression of key cell cycle related proteins such as cdk2, cdk4, p21, p27, and p53 was found during the S phase arrest and apoptosis. We therefore suggest that GADD proteins might play an important role in VT1 induced S phase arrest and programmed cell death in HCT116 cells.

Keywords: Verotoxin; Cell cycle; Apoptosis; GADD; Microarray analysis

1. Introduction

Verotoxin 1 (also known as VT1 or Shiga toxin-1 [Stx-1]), a major cytotoxin produced by VT-producing *Escherichia coli* (VTEC) and enterohemorrhagic *E. coli* plays an important role in the pathogenesis of hemolytic uremic syndrome (HUS) following an enteric infection [1]. VT1 is known to be cytotoxic and damages the endothelial cell lining of the glomeruli, and epithelial cells of the tubule of the kidney, as well as other tissues and organs that express the specific receptor, globotriaosylceramide (Gb3), of the toxin [2]. VT1 action is thought to involve the induction of a hemorrhagic diarrhea, neurological damage and apoptosis in some *E. coli* diseases, including HUS. Although the exact mechanism of apoptotic cell death is not yet known, a variety of cell types including Burkitt's lymphoma, HeLa cells and renal tubular epithelial cells undergo apoptosis upon exposure to VT1 [3–5].

In the past 20 years, several bacterial toxins have emerged as powerful therapeutic agents with possible applications in cancer, neurological and non-neurological diseases. Currently, botulinum toxins are used for the treatment of dystonia, spasticity, and muscle overactivity disorders [6]. Anthrax toxin was shown to inhibit mitogen-activated protein kinase in both normal melanocyte and melanoma cells, but can only triggers apoptosis in the latter [7]. Most importantly, the potential use of VT1 as a clinical agent for malignant meningiomas is now under investigation [8].

In the work described here, we observed that the human colon cancer cell line HCT116 was especially sensitive to VT1. These cells provided a means to investigate whether VT1 induced apoptosis in human colon cancer cells in vitro. On the basis of DNA degradation pattern, flow cytometry, and morphological studies, we have shown for the first time to our knowledge that incubation with VT1 induced a dramatic accumulation of HCT116 cells in S phase followed by programmed cell death, in a dose and time dependent manner. Moreover, S-phase arrest was accompanied by increased mRNA and protein levels of the growth arrest and DNA damage (GADD) family of genes, including GADD34, GADD45α, and GADD45β. Taken together, these observations suggest that *E. coli* Verotoxin 1 induced a p53/p21-independent cell cycle arrest and apoptosis in HCT116 colon cancer cells and that GADD proteins might play an essential role in these events.

2. Materials and methods

2.1. Cell lines and cell synchronization

HCT116 cells were grown as monolayer in McCoy medium (Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin and 10 U/mL penicillin G. For

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S-phase synchronization by double thymidine block, 5×10^5 cells were treated with 2.5 mM thymidine (Wako Pure Chemical, Osaka, Japan) for 20 h. Cells were then washed once with PBS and twice with medium and grown for 6 h. Thymidine (2.5 mM) was added again for an additional 20 h. Release of the second thymidine block was performed by washing twice with PBS and three times with medium. Cells were collected after 18 h for flow cytometry analysis.

2.2. VT1 preparation

VT1 was purified by a modification of a method previously described involving polymyxin B extraction, and immunoaffinity column chromatography using anti-VT1 rabbit IgG [9]. To detect the presence of VT1 and/or VT2, VTEC-reversed passive latex agglutination (VTEC-RPLA) assay (Denka Seiken Co., Tokyo, Japan) was performed according to the manufacturer's instructions. Briefly, purified VT1 samples were diluted with the supplied diluent. Latex particles sensitized with rabbit polyclonal anti-VT1 or anti-VT2 IgG antibody were mixed in appropriate wells. Plates were sealed, incubated at room temperature, and examined for agglutination after 12–18 h. The positive and negative control toxins supplied with the kit were employed in each assay. Heat inactivated (80 °C, 15 min) VT1 was not detected by this kit, and VT2 contamination in the VT1 sample was not observed.

2.3. DNA fragmentation

Both fragmented DNA and high molecular weight intact genomic DNA were extracted from 5×10^5 cells using the suicide-Track™ DNA ladder isolation kit (Oncogene, Madison, WI). 1.5% Agarose gel electrophoresis was followed by ethidium bromide staining.

2.4. Annexin V-FITC apoptosis detection assay

The cells were harvested and incubated with VT1 as described above and washed twice with phosphate-buffered saline (PBS). Staining was carried out using the annexin V-FITC apoptosis detection kit (BioVision, Mountain view, CA). Briefly, 2×10^5 cells were resuspended in 1 × binding buffer and incubated with annexin V-FITC and propidium iodide (PI) for 5 min in darkness at room temperature. Annexin V binding was analyzed by FACScan cytometer (Becton–Dickinson, San Jose, CA) equipped with a FITC signal detector FL1 (Ex = 488 nm, green) and PI staining by the phycoerythrin emission signal detector FL2 (Ex = 585 nm, red). Percentage of apoptotic cells was calculated from the total (10^4 cells) using FlowJo 4.5.2 software.

2.5. Nucleosome enzyme immunoassay

HCT116 cells were treated with VT1 as described in Fig. 1C. The presence of nucleosomes in the cytoplasm was determined by ELISA using mouse monoclonal antibodies directed against DNA and histones according to the manufacturer's protocol (Roche, Penzberg, Germany). Enrichment of mono- and oligonucleosomes were calculated as: enrichment factor = mU of the sample (dying/dead cells)/mU of the corresponding control (viable cells) where mU = absorbance [10^{-3}].

2.6. Cell cycle analysis

For the analysis of DNA content by flow cytometry, cells were trysinized, washed in PBS and stained by PI using the CycleTEST and DNA reagent kits (Becton–Dickinson, San Jose, CA). This method involves dissolving the cell membrane, eliminating nuclear proteins, stabilizing chromatin and staining by PI. Fluorescent light emitted (at 585 nm) from PI-stained nuclei was detected by the FACScan's fluorescence 2 (FL2) detector. Percentage of cells (from a total of 10^4 cells) at different phases of the cell cycle was determined using the FlowJo 4.5.2 software and the Watson analysis.

2.7. Immunoblotting

For immunoblotting, cells treated with or without VT1, were washed with cold PBS and pelleted. The cells were lysed in buffer X + BSA (100 mM Tris–HCl, pH 8.5, 250 mM NaCl, 1% (v/v) NP-40, 1 mM EDTA, 1 μg/ml aprotinin, 2 mg/ml BSA). Whole cell protein lysates were solubilized in loading buffer, subjected to SDS–PAGE, and transferred to nitrocellulose followed by incubation with the antibodies as mentioned in the figure legends.

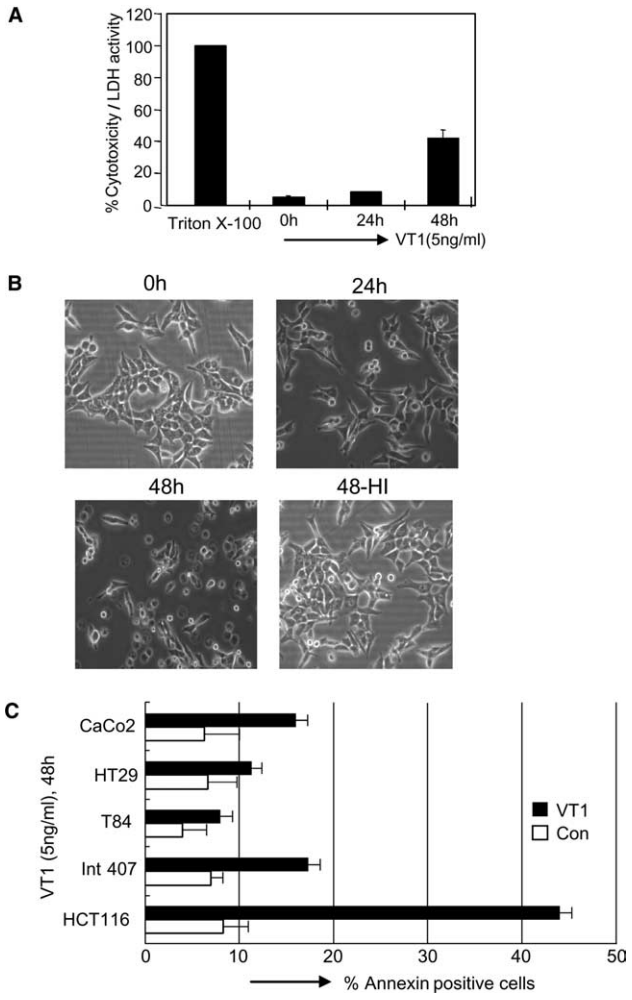

Fig. 1. Relative sensitivity of human colon cancer cells to VT1. (A) The graph indicates the percentage of annexin V positive cells (from a total of 10^4 cells). All cell lines were treated with VT1 at low dose (5 ng/ml) for 48 h. Annexin V-FITC staining and cell counting were done as described in Section 2. Standard deviations are shown with error bars from mean of at least three different experiments. (B) HCT116 cells were incubated with VT1 (5 ng/ml) for 0, 24, and 48 h. Untreated cells were lysed by Triton X-100 to ensure maximum release of LDH as a positive control. LDH release into the culture medium was measured according to the manufacturer's instruction (CytoTox96 kit, Promega). Results are representative of at least three independent experiments. (C) Morphological changes in HCT116 cells treated with 5 ng/ml VT1 for 0, 24, 48 h and with HI-VT1 for 48 h (magnification 400×). Images were captured by an Olympus 1 × 70 inverted microscope fitted with an Olympus Camedia C-4040 digital camera.

2.8. Cytotoxicity assays

HCT116 cells were grown at 37 °C in 5% CO_2 in McCoy's medium supplemented with 10% fetal bovine serum. Before incubation with VT1, cells were washed with phosphate-buffered saline (PBS, pH 7.2) and further incubated with DMEM without phenol red. The release of lactate dehydrogenase (LDH) into the medium was assayed using the CytoTox96 Non-radioactive Cytotoxicity Kit (Promega) according to the manufacturer's instructions. At 0, 24 and 48 h post incubation with VT1, the supernatants were collected, and the release of LDH was quantified. LDH release (% cytotoxicity) was calculated using the following equation: $\{(OD_{490}$ experimental release $- OD_{490}$ spontaneous release$)/(OD_{490}$ maximum release $- OD_{490}$ spontaneous release$)\} \times 100$. The spontaneous release is the amount of LDH released from the cytoplasm of uninfected cells, whereas the maximum release is the amount released by total lysates of uninfected cells by Triton X-100.

2.9. Microarray gene chip analysis

HCT116 cells were treated with VT1 (5 ng/ml) for 0, 24, 36 h. Total RNA was extracted (RNeasy Kit, Qiagen) and double stranded cDNA was synthesized from 5 μg of total RNA using the Superscript system (Invitrogen, CA) primed with a T7-(dT)-24 primer. To prepare biotin labeled cRNA from this cDNA, an in vitro transcription reaction was performed in the presence of T7 RNA polymerase and biotinylated ribonucleotides (Enzo Diagnostics, NY). The cRNA product was purified (RNeasy Kit, Qiagen), fragmented, and hybridized to a human genome focus array chip, as per the manufacturer's instruction (Affymetrix, CA). The chips were washed and scanned with a GeneArray scanner (Affymetrix). The color intensity of gene expression was generated with the Gene Spring6.0 software (Silicon Genetics).

3. Results

3.1. Relative sensitivity of colon cancer cell lines to VT1

Host sensitivity to VT1 varies according to the specific cellular expression of its Gb3 receptor [10]. VT1 was also shown to reduce cell viability in a dose and time-dependent manner. To study the relative sensitivity of colon cancer cells, five cell lines including four colon cancer cell lines were monitored for 48 h after exposure to a low concentration (5 ng/ml) of VT1. Cellular shrinkage and cell rounding, two typical morphologic changes occur in apoptosis, started appearing in HCT116 cells from 24 h of VT1 treatment and a significant increase in these characteristics were observed after 48 h of incubation. Incubating the same cells with heat inactivated (HI) VT1 (80 °C for 15 min) produced no significant morphological changes (Fig. 1A), also excluding the possibility of LPS contamination in the VT1 preparation.

To understand if this cytotoxic effect was, at least in part, due to apoptosis, all cell lines were stained with annexin V-FITC. Fluorescence analysis showed that 40% of HCT116 cells were apoptotic compared to only 5–18% in other colon cancer cell lines (Fig. 1B). Therefore, we used the HCT116 cell line in our further studies aimed to understand the mechanism of VT1 mediated programmed cell death.

LDH, a stable cytosolic enzyme, was shown to be released upon cell lysis during necrosis and at later stages of apoptosis [11]. Consistent with the annexin V staining result, LDH activity in HCT116 cells was increased up to 45% only after 48 h of VT1 incubation (Fig. 1C).

3.2. DNA fragmentation in HCT116 cells exposed to VT1

Based on LDH activity and phase contrast microscopy further studies were extended by DNA fragmentation and annexin V staining. Total DNA (genomic and fragmented) was prepared from VT1-exposed HCT116 cells after 0, 24, 48 h, and from cells treated with HI VT1 for 48 h. Electrophoretic patterns indicating DNA fragmentation were seen in cells treated with VT1 for 48 h, but not in 0 h cells. The DNA also remained intact in cells after 24 h of incubation with VT1 and 48 h of incubation with HI-VT1 (Fig. 2A). T84, another colon cancer cell line, was resistant to VT1-induced apoptosis, since no apoptotic ladders were observed using the condition described above (Fig. 2B). The fragmented DNA formed a ladder of starting with 190 bp and up, suggesting that cleavage occurred at the internucleosomal regions, characteristic to cells commit programmed cell death.

To support and extend the DNA fragmentation experiments, we performed double staining using annexin V-FITC and PI followed by flow cytometry. Fig. 2C shows that in cells treated with VT1 for 0, 24, and 48 h, 18.7% and 33% cells were positive (right-bottom quadrant) for phosphatidyl-serine, representing early stages of apoptosis. No significant changes were observed either in control and or in HI-VT1-treated cells indicating that the percentage of apoptotic cells in the total cell population was the same when different methods to detect apoptosis were applied.

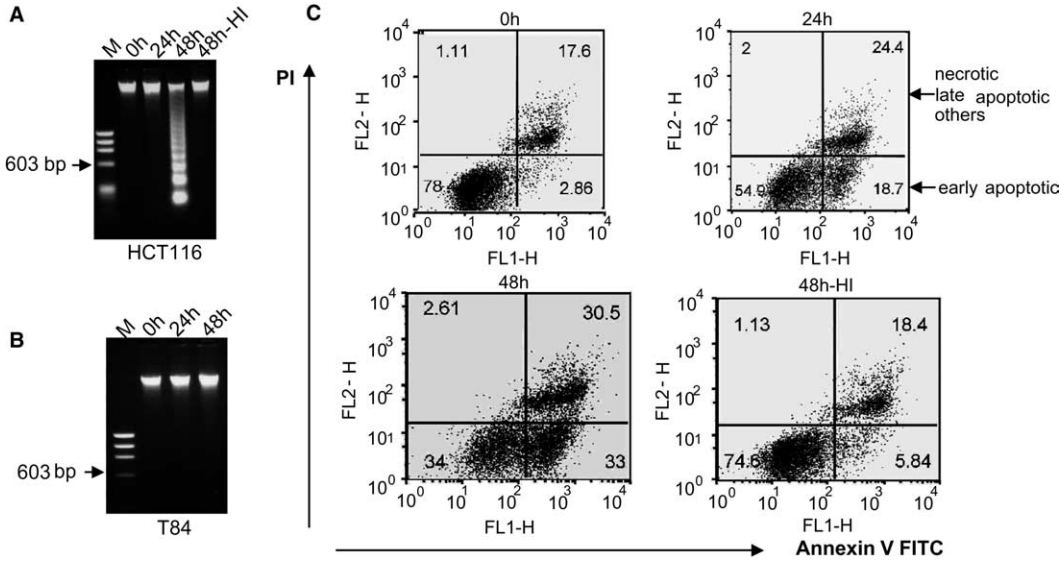

Fig. 2. DNA fragmentation and annexin V binding analysis showing induction of apoptosis. (A) Agarose gel (1.5%) electrophoresis of DNA isolated from HCT116 and T84 cells either untreated or treated with VT1 (5 ng/ml) and HI-VT1 (5 ng/ml) for 24, and 48 h followed by ethidium bromide staining. (B) HCT116 cells were incubated with VT1 as described above. Percent apoptotic cells was detected by analyzing annexin V-FITC and PI binding with by flow cytometry and using the FlowJo 4.5.2 software. Viable cells do not display annexin V or PI staining (lower left quadrant), early apoptotic cells show annexin V staining, but exclude PI (lower right quadrant), and necrotic, or late apoptotic cells are both annexin V and PI positive (upper right quadrant).

3.3. VT1 induces irreversible DNA and chromatin disruption

VT1 binds to the cellular receptor Gb3 and is internalized into the cytoplasm. The entire internalization process requires more than an hour of incubation with the cells [12]. To test whether the findings described above resulted from specific functions exerted by VT1, HCT116 cells were incubated with VT1 for 0, 0.5 and 2 h. Cells were then washed extensively to remove unbound toxin and incubated for another 48, 47.5 and 46 h, respectively, in fresh medium without VT1. DNA fragmentation was occurred in cells (Fig. 3A) those were exposed with VT1 for 2 h, indicating that the removal of extracellular VT1 were unable to halt the signaling events initiated in these cells in the first 2 h. A progressive increase in the percentage of rounded/shrunken cells was observed and eventually approximately 70–80% of the cell population detached from the culture dish 72 h after washing VT1 (data not shown). Therefore, once VT1 could enter the host cell cytoplasm, its effects are irreversible.

The cytoplasm of the apoptotic cells is enriched in mono and oligonucleosomes owing to the DNA degradation that occurs prior to the breakdown of the plasma membrane. We observed such response in VT1-treated cells by a quantitative sandwich-enzyme-immunoassay that can specifically determinate the mono and oligonucleosomes in the cytoplasmic fraction of cell lysates, by using mouse monoclonal antibodies directed against DNA and histones. The enrichment factor (see the calculation in Section 2) of nucleosomes in the cytoplasm of HCT116 cells treated with VT1 for 48 h increased 4-fold compared to control cells and HI-VT1 treated cells (Fig. 3B).

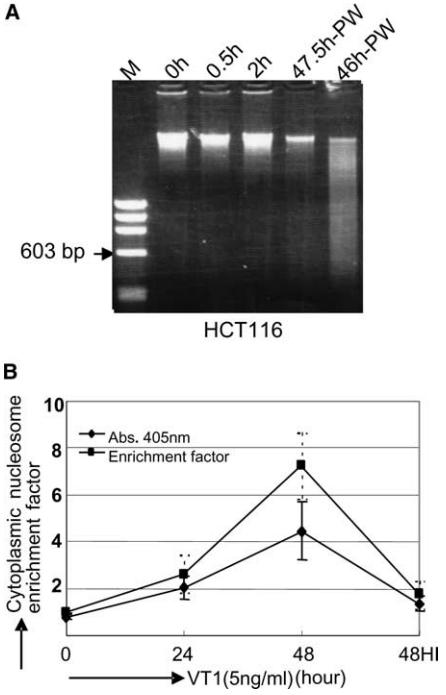

Fig. 3. Enrichment of nucleosomes in the cytoplasm of cells treated with VT1. (A) DNA fragmentation analysis was done as described in Fig. 2A. 46-h PW (46 h after washing) indicates that HCT116 cells were incubated with VT1 for 2 h and then washed with PBS and fresh mediaum to remove unbound toxins, followed by 46 h incubation in fresh medium. (B) HCT116 cells were treated as described in Fig. 2A. After cell lysis and centrifugation, the cytoplasmic fractions were diluted 1:10 with incubation buffer (ELISA kit; Roche, Penzberg, Germany) and tested for nucleosome presence using mouse monoclonal antibodies specific for DNA and histones.

3.4. VT1 activates the S phase checkpoint

It has been suggested that activation of cell cycle checkpoints is often involved in the initiation of apoptosis [13]. To examine the effect of VT1 on cell cycle progression, we analyzed the DNA content of HCT116 cells treated with VT1 and HI-VT1 by flow cytometry. After 18 and 24 h of treatment with VT1, there was a gradual increase in the S phase population (Fig. 4A), from 15% at 0 h to 28% at 18 h and 40% at 24 h, accompanied by a sharp decline in the number of cells in G1 phase (from 73% at 0 h to 34% at 24 h). Importantly, in cells treated with VT1 for 48 h a sub-G1 (>G1) peak appeared indicating apoptosis (48 h in Fig. 4B). Cells treated with HI-VT1 exhibited normal cell cycle distribution (Fig. 4B). This suggests that a small number of cells escaped from the S phase arrest and undergo apoptosis. When comparing the characteristics of S phase arrest caused by thymidine and VT1, flow cytometry analysis showed that 18 h after the release from the thymidine block, cells previously accumulating in S phase progressed normally through the cell cycle, while a high proportion of VT1 treated cells still remained arrested in S phase (Fig. 4C). Based on the combination of cell cycle experiments and DNA fragmentation analysis, we conclude that VT1 exposure exerts leave a permanent, irreversible effect on these cells.

3.5. VT1 treatment up-regulates GADD family proteins and mRNA

To further investigate the possible mechanism(s) of this S phase arrest and apoptosis in cells treated with VT1, we employed cDNA microarray analysis to analyze global changes in gene expression. Due to the large number of genes modulated during 24 and 36 h after VT1 exposure, the data were filtered and 4-fold changes (compare to uninduced) and greater at both 24 and 36 h of VT1 incubation are shown. Among such changes, the induced genes contain those that were previously shown to be associated with the cell cycle, DNA damage and apoptosis (Fig. 5A). To determine whether the changes in RNA levels reflect a change in the protein produced, we analyzed the levels of various cell cycle/apoptosis regulating proteins by western blotting (Fig. 5B). We found that the level of three GADD family genes GADD45α, GADD45β, and GADD34, the activating transcription factor (ATF3) and one cell cycle related phosphatase, CdkN3, gradually increased in cells treated with VT1 for 24 and 36 h (Fig. 5A). Interestingly, the protein levels of key cell cycle regulators such as cdk2, cdk4, p21, p27, and p53 did not changed significantly as the result of prolonged VT1 treatment. Consistent with mRNA induction, western blot analysis also revealed a VT1-mediated upregulation of GADD45α, GADD45β, and GADD34. The expression level of housekeeping genes (GAPDH and β-actin) and protein did not change in microarray and western blot during this period of HCT116 cell incubation with VT1 (Fig. 5A and B).

4. Discussion

E. coli verotoxin 1 (VT1) is a potent cytotoxin involved in the pathogenesis of hemorrhagic colitis and is known to cause microvasculopathies of the colon. VT1 causes damage

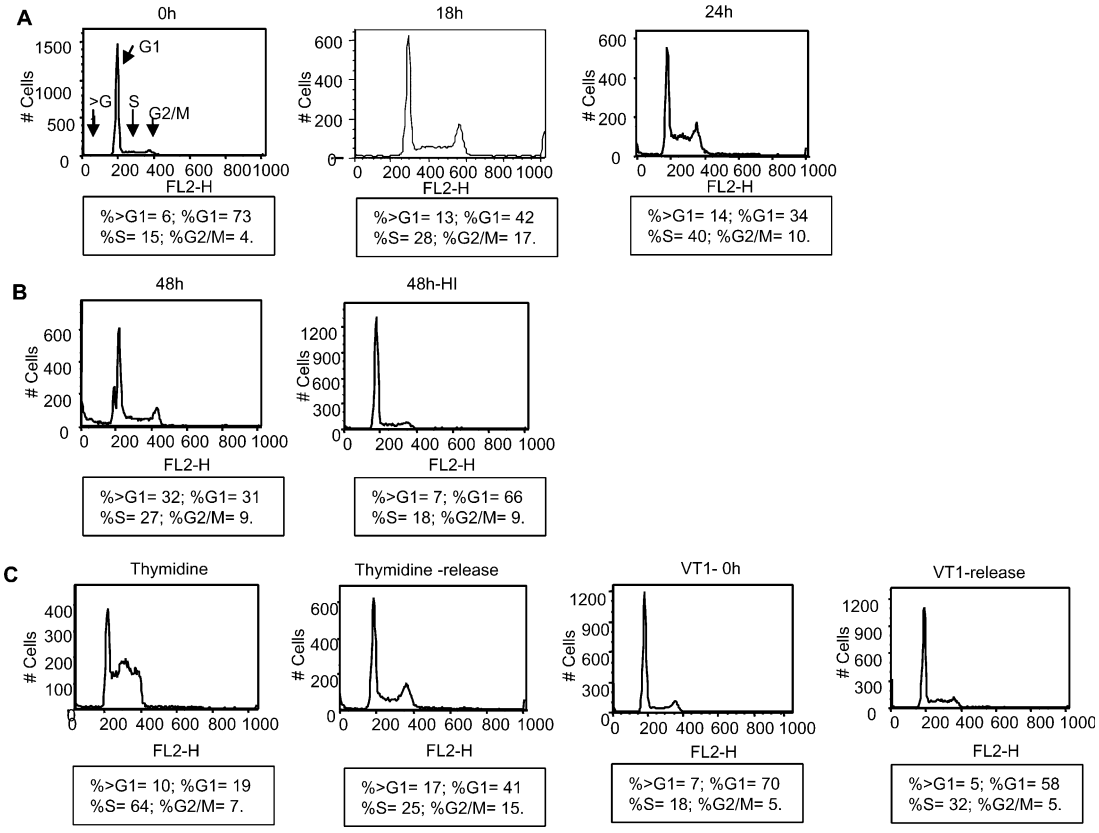

Fig. 4. Cell cycle progression of HCT116 cells after treatment with VT1. (A,B) Cells were treated as described in Fig. 2C, then fixed, stained with PI, and examined for DNA content by flow cytometer. The peaks represent the cells in G0–G1, S, and G2-M. Subdiploid apoptotic DNA is marked >G1. A total of 10^4 nuclei, per sample, analyzed. (C) Cells were grown in the presence of thymidine (see Section 2) and VT1. To release from thymidine and VT1 toxin, cells were washed extensively with PBS and medium and incubated for an additional 18 h and cell cycle analysis was performed as described above.

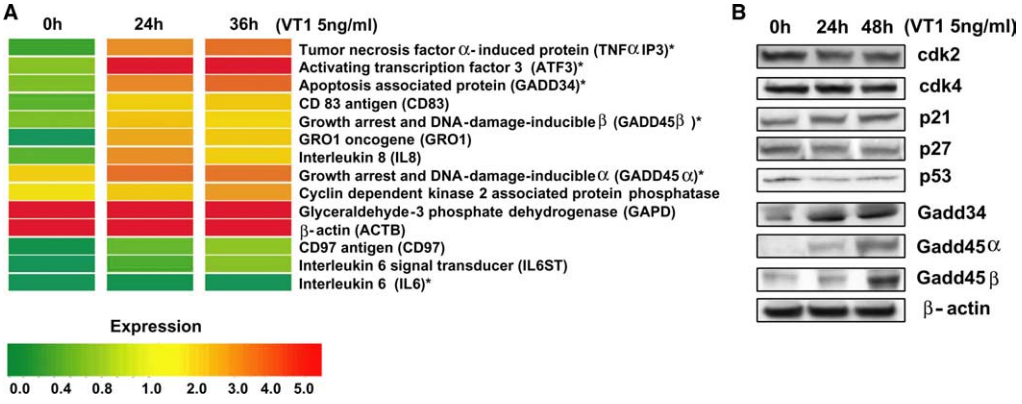

Fig. 5. Effect of VT1 treatment on gene transcription and protein expression in HCT116 cells. (A) Cells were untreated, or treated with VT1 (5 ng/ml) for 24 and 36 h. Biotin labeled cRNA prepared from cells was hybridized to spotted human genome microarray chips. High quality spots (signal strength of three times over background) were organized into gene expression clusters. Genes those changed more than 2-fold were given a color image based on intensity of gene expression. (B) Cells were either untreated, or treated with VT1 (5 ng/ml) for 24 and 48 h. Whole cell protein extracts were solubilized in loading buffer, boiled for 3 min at 100 °C, subjected to 10% SDS–PAGE, and transferred to nitrocellulose membrane before immunoblotting with antibodies as indicated in the figure.

to glomerular endothelial cell lining as well as apoptosis in variety of cell types that express its specific Gb3 receptor [14]. The exact mechanism(s) by which VT1 trigger the apoptotic signal are not well defined [4,5]. Interestingly, expression of the VT1 receptor Gb3 on human cancer cell lines including renal carcinoma, melanoma, prostate cancer, and testicular tumors suggest that further studies on the mechanism of VT1-mediated signaling pathway(s) might be beneficial if VT1 has an antineoplastic potential. Recent studies have shown that in mice harboring tumor xenografts originating from Gb3-positive human renal, prostate cancer and malignant meningiomas, VT1 induces apoptosis and rapid elimination of these

xenografts [8,15]. Although VT1 exerts its cytotoxic effect upon recognition of the Gb3 glycolipid receptor of tumor cells and tumor neovasculature, the rationale for using VT1 in antitumor clinical application will only be possible if its functional mechanism is unraveled. Aiming to this, we studied the mechanism of VT1 action in a VT1-sensitive human colon cancer cell line HCT116. DNA fragmentation, annexin V staining and nucleosomal (DNA plus histones) assays showed that VT1 kills these cells through an apoptotic pathway. Flow cytometric analysis showed an increase in the subG0/G1 cell population, a hallmark of apoptosis, when these cells were incubated with VT1 for 48 h. Unlike the reversible S phase blocker thymidine, releasing cells from the VT1 block after 6 h of incubation by extensive washing made no difference in the ongoing signaling events that ultimately led to S phase arrest and eventually to apoptosis (Figs. 3 and 4). Within this time period, the processes of Gb3 recognition by VT1 and the subsequent internalization of VT1 by endocytosis were completed and the signaling events initiated in these cells by VT1 are irresistible.

Apoptosis is regarded as one of the important defense mechanisms in the event of cell and DNA damage, because it triggers a series of controlled signaling mechanisms including cell cycle arrest and DNA repair to protect the genome from defective DNA synthesis [16]. One of the principal causes of S phase arrest is DNA damage [17]. The S phase arrest induced by the VT1 toxin is most likely the consequence of irreversible DNA damage. It is possible that part of such cells damaged beyond the capacity to repair their DNA, progress to programmed cell death, since after 48 h a sub-G1 population became evident.

We further confirmed that the GADD family of genes was upregulated both at mRNA and protein levels by VT1 and was coupled with the induction of S phase arrest. Growth-arrest and DNA damage-inducible genes were found to be stress response genes often increased when cells are subjected to a stressful environment such as chemical carcinogens, UV radiation, hypoxia, or apoptosis inducing agents such as staurosporine and anti-Fas antibodies [18–20]. The GADD34 protein specifically binds to a wide range of cellular proteins involved in growth arrest, apoptosis and DNA repair [21,22]. ATF3, another stress response gene, plays a critical role in enhancing caspase activation during etoposide-induced apoptosis without affecting the expression of Bcl-2, or Bax [23]. In addition, ATF3 is required for increased expression of GADD34 and the apoptosis inducing protein CHOP (also known as GADD153) [24,25]. It was shown that NF-κB/IκB signaling is a critical regulator of cell survival. Recent work demonstrated that induction of apoptosis by NF-κB inhibitor resulted from induction of GADD45α and γ expression in prostate cancer cells, and a c-Myc dependent downregulation of GADD45α/γ protein expression was associated with NF-κB-mediated escape from programmed cell death in cancer cells [26]. Although the exact biological significance of GADD upregulaion in VT1 treated cells is not known, the effect of VT1 on GADD expression might contribute to the capacity of VT1 to induce S phase arrest and apoptosis. p53 was also analyzed to determine if it is a regulator of the VT1 effect on GADD expression, since this transcription factor is activated under oxidative stress, and DNA damage [27]. Both the RNA and protein expression studies of p53, the p53-related cell cycle inhibitors p21, p27 and kinase Cdk4 imply that GADD expression occurred independently of these factors. Thus in HCT116 cells, VT1 and the bile salt deoxycholate (DOC) induce a similar response by initiating common signaling pathways. When both HCT116 (containing wild type p53) and HCT-15 (containing mutant p53) colon cancer cells were exposed to DOC, DNA damage and apoptosis were observed with increased GADD expression, regardless of their p53 status [28]. Taken together, our data suggest that p53, p21, p27 and Cdk4 might not be a necessary prerequisite for VT1 induced cell cycle arrest and apoptosis in this cell line. On the basis of these observations, we proposed a hypothetical model (Fig. 6) indicating the signaling pathways involving cell cycle arrest, DNA damage, ATF3, GADDs and apoptosis.

Bacterial toxins are considered as potential therapeutic agents for various diseases. When athymic nude mice carrying human melanoma xenograft were treated with the anthrax lethal factor (LeTx), complete tumor regression without apparent side effects were observed [7]. VT1 has also proved highly effective in a xenograft cancer model [12]. The precise mechanism by which VT1 blocks S phase progression of the colon cancer cell line HCT116 remains incompletely defined. However, for the first time to our knowledge, this finding would add the VT1-mediated upregulation of the GADD family of genes to the growing list of apoptotic pathways initiated by this *E. coli* toxin in various cell lines and tumor xenografts. Further studies along these lines are warranted before the use of VT1 as a potential therapeutic agent in experimental medicine.

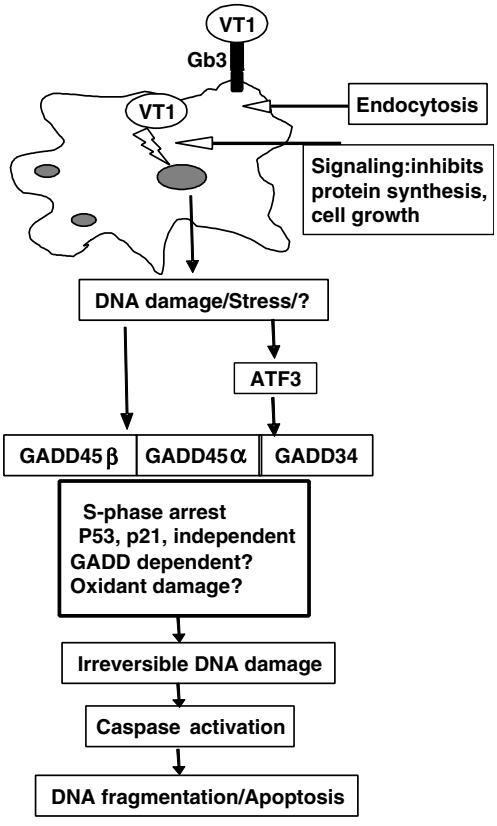

Fig. 6. A proposed pathway for VT1 action in HCT116 cells. A hypothetical model describes the involvement of ATF3 and GADD family members in mediating apoptosis in colon cancer HCT116 cells by VT1.

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