Enhancement of Diphtheria Toxin-induced Apoptosis in Vero Cells by Combination Treatment with Brefeldin A and Okadaic Acid

Izumi Kusano, Ai Kageyama, Tadashi Tamura, Tatsuya Oda*, and Tsuyoshi Muramatsu

Division of Biochemistry, Faculty of Fisheries, Nagasaki University, Bunkyo-machi, Nagasaki 852-8521, Japan

ABSTRACT. In the present study, we compared the abilities of ricin and diphtheria toxin to induce apoptosis in Vero cells. The cytolsysis and DNA fragmentation by ricin paralleled its protein synthesis inhibitory activity. However, unlike ricin, diphtheria toxin could induce neither cytolsysis nor DNA fragmentation in Vero cells up to very high concentration, in spite of the fact that Vero cells were even more sensitive to protein synthesis inhibition by diphtheria toxin than ricin. Interestingly, coexistence of brefeldin A (BFA) and okadaic acid (OA) significantly enhanced diphtheria toxin-mediated cytolsysis and DNA fragmentation without affecting the activity of protein synthesis inhibition. Ammonium chloride almost completely abolished the ability of diphtheria toxin to induce apoptosis in the presence of BFA and OA as well as the protein synthesis inhibitory activity. The mutant CRM 197, which does not catalyze the ADP ribosylation of elongation factor-2 (EF-2), failed to induce apoptosis in Vero cells even in the presence of BFA and OA. Thus, translocation of diphtheria toxin into the cytosol and subsequent enzymatic inactivation of EF-2 may be necessary steps to induce apoptosis. Taken together our results suggest that protein synthesis inhibition by toxins is not sufficient to induce apoptosis, and underlying mechanisms of apoptosis induction may be distinct between ricin and diphtheria toxin. Since a morphological change in the Golgi complex was observed in Vero cells treated with BFA and OA, modulation of the Golgi complex by these reagents may be partly responsible for enhanced apoptosis induction by diphtheria toxin.

Key words: diphtheria toxin/apoptosis/brefeldin A/okadaic acid/Golgi complex/ricin

Protein toxins of plant (ricin, abrin and modeccin) and bacterial (diphtheria toxin, Pseudomonas toxin and Shiga toxin) origin inhibit protein synthesis following receptor-mediated endocytosis (Goldstein et al., 1985; Middlebrook and Dorland, 1984; Olsnes and Sandvig, 1988; Olsnes et al., 1990; Sandvig et al., 1989). After intracellular trafficking through the vesicular system, the enzymatically active components of these toxins are eventually translocated into the cytosol to reach their intracellular targets, i.e., the 28S RNA of 60S ribosomal subunit for ricin, abrin, modeccin, and Shiga toxin, or elongation factor 2 for Pseudomonas toxin and diphtheria toxin. Yoshida et al. (Yoshida et al., 1990; Yoshida et al., 1991) have shown that brefeldin A (BFA), which affects the structure and function of Golgi apparatus (Doms et al., 1989; Fujiwara et al., 1986; Oda et al., 1987), blocks the intoxication of Vero cells and other cell lines by ricin, modeccin and Pseudomonas toxin but has no effect on the cytotoxicity of diphtheria toxin, suggesting an involvement of the Golgi apparatus in the intoxication process of these toxins except for diphtheria toxin. Diphtheria toxin is a 58 kDa single polypeptide produced by Corynebacterium diphtheriae whereby the carboxyl-terminal B fragment has affinity for a specific cell-surface receptor (Cieplak et al., 1987; Mekada et al., 1988) and acts as a carrier for the amino-terminal A fragment, which possesses the enzymatic activity for protein synthesis inhibition (Reaves and Banting, 1992; Sandvig and Olsnes, 1981; Sandvig and Olsnes, 1982). The diphtheria holotoxin or its A fragment is known to be translocated to the cytosol from endosomes following acidification. Thus, NH4Cl inhibits the cytotoxicity of diphtheria toxin by increasing the pH in

*To whom correspondence should be addressed: Tatsuya Oda, Division of Biochemistry, Faculty of Fisheries, Nagasaki University, Bunkyo-machi, Nagasaki 852-8521, Japan.
Tel: +81-95-847-1111, ext. 3163, Fax: +81-95-844-3516
E-mail: t-oda@net.nagasaki-u.ac.jp

Abbreviations: BFA, brefeldin A; OA, okadaic acid; EF-2, elongation factor-2; MEM, minimal essential medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; LDH, lactate dehydrogenase; NBD-ceramide, N-[7-(4-nitrobenzo-2-oxa-1,3-diazole)-6-aminocaproyl]sphingosine; TNF, tumor necrosis factor; CHA, cycloheximide.
the intracellular vesicles (Sandvig and Olsnes, 1981; Sandvig and Olsnes, 1982).

Recent studies have demonstrated that protein toxins such as diphtheria toxin, *Pseudomonas* toxin, and ricin, aside from their known activity of protein synthesis inhibition, can cause target cell lysis and induce DNA fragmentation in a process reminiscent of apoptosis (Chang et al., 1989; Griffiths et al., 1987; Komatsu et al., 1998; Komatsu et al., 2000; Morimoto and Bonavida, 1992; Oda et al., 1999). Although the mechanism of apoptotic events caused by these toxins is still unclear, several compounds or conditions have been reported to modulate protein toxin-induced cell lysis or apoptosis. For instance, cycloheximide (CHA) inhibits ricin-induced cell lysis but did not affect diphtheria toxin-mediated cell lysis (Morimoto and Bonavida, 1992; Sandvig and van Deurs, 1992). In contrast, 3-aminobenzamide, which is reported to inhibit diphtheria toxin-induced lysis of U937 cells (Chang and Wisnieski, 1990), had no protective effect against the ricin-induced lysis in MDCK cells (Sandvig and van Deurs, 1992). Moreover, our recent study has demonstrated that diphtheria toxin and CHA were less effective as compared to ricin in terms of inducing DNA fragmentation and cytolysis in U937 cells, even at the concentration that causes severe inhibition of protein synthesis (Komatsu et al., 2000). These results suggest that apoptosis induction by these protein synthesis inhibitors is not a direct consequence of protein synthesis inhibition. It seems likely that the underlying mechanism for apoptosis induction may be distinct depending on the cell types and apoptotic stimuli.

To gain insight into the mechanism of toxin-induced apoptosis, we compared the cytotoxic effects of ricin and diphtheria toxin on Vero cells so as to view the relationship between their activity of protein synthesis inhibition and their ability to cause cytolysis and DNA fragmentation. Here we report that diphtheria toxin is far less effective than ricin in terms of the induction of apoptosis as judged by the cytolysis and DNA fragmentation, despite the higher sensitivity of Vero cells against diphtheria toxin-mediated protein synthesis inhibition than ricin. We also report that diphtheria toxin-induced cytolysis and DNA fragmentation are highly enhanced by the combination treatment with BFA and okadaic acid (OA).

**Materials and Methods**

**Materials**

Ricin was isolated from small castor beans as described by Mise et al. (Mise et al., 1977). Diphtheria toxin was purchased from Swiss Serum and Vaccine Institute (Berne, Switzerland). Brefeldin A (BFA) was purchased from Pierce (Rockford, IL). Okadaic acid (OA) was purchased from Wako Pure Chem. Co. (Tokyo, Japan). The fluorescent tetrapeptide substrate of caspase-3 (Ac-DEVD-MCA) was obtained from the Peptide Institute, Inc. (Osaka, Japan). [3H]Leucine (60 Ci/mmol) was obtained from NEN Research Products (Boston, MA). NBD-ceramide [N-7-(4-nitrobenzo-2-oxa-1,3-diazole)-6-aminocaproylsphingosine] was purchased from Molecular Probes, Inc. (Eugene, OR).

**Cell culture**

Vero cells were from the American Type Culture Collection (Rockville, MD). Cells were grown as monolayers in α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum, 10 μg each of adenosine, guanosine, cytidine, and thymidine per ml of medium, penicillin (100 μg/ml) and streptomycin (100 μg/ml) as described previously (Oda and Wu, 1993). The cells were subcultured 2 days before use by treatment with 0.1% trypsin - 0.05% EDTA in phosphate-buffered saline (PBS).

**Measurement of protein synthesis inhibition**

Cells were inoculated at a density of 5×10^5 cells/well in 0.2 ml of medium using 48-well plates. One day later, the monolayer culture was pretreated with or without BFA and/or OA in serum-free α-MEM containing 35 μM BSA for 1 h at 37°C, followed by the addition of varying concentrations of ricin or diphtheria toxin. After another 3 h incubation at 37°C, the medium of each well was replaced by leucine-free medium containing 1 μCi/ml [3H]leucine, and then the cells were incubated for 45 min at 37°C. The incorporation of [3H]leucine into perchloric acid/phosphotungstic acid-insoluble materials was determined as described previously (Oda and Wu, 1995). The results were expressed as percentage of incorporation in control cells incubated without toxin but otherwise treated in the same way.

**DNA fragmentation assay**

The cell monolayers in dishes (35 mm) (6×10^4 cells/dish) were pretreated with or without BFA and/or OA in α-MEM containing 35 μM BSA for 1 h at 37°C, followed by the addition of ricin or diphtheria toxin. After 15 h incubation at 37°C, the cells were washed once with PBS and lysed in 1 ml of ice-cold lysis buffer (0.5% Triton X-100, 10 mM Tris-HCl, pH 8.0, 20 mM EDTA). Samples were subsequently centrifuged for 30 min at 13,000 x g to separate DNA fragments (supernatant) from intact DNA (pellet). DNA contents of the supernatant and pellet fractions were determined using diphenylamine reagent as described previously (Komatsu et al., 2000).

**Cytolytic assay**

The cytolytic activity of toxins was measured by lactate dehydrogenase (LDH) release assay in which LDH released from lysed cells was determined by the measurement of 2-((p-iodophenyl)-3-((p-nitrophenyl))-5-phenylethrazolium chloride reduction as described previously (Komatsu et al., 2000). In brief, 2×10^4 cells/well in a 96-well plate in α-MEM containing 35 μM BSA were pretreated with or without BFA and/or OA in α-MEM containing 35 μM BSA for 1 h at 37°C, followed by the addition of varying concentrations of ricin or diphtheria toxin. After another
20 h incubation at 37°C, the plates were centrifuged (1,500×g, 10 min), and then the supernatant (50 μl) of each well was subjected to LDH assay.

**Nuclear staining**

Vero cells grown on glass coverslips were pretreated with or without BFA and/or OA in α-MEM containing 35 μM BSA for 1 h at 37°C, followed by the addition of ricin or diphtheria toxin. After another 15 h incubation at 37°C, the cells were washed with PBS, and subsequently fixed with 1% glutaraldehyde for 30 min at room temperature. After washing with PBS, the cells were stained with Hoechst 33258 (40 μM) for 5 min at room temperature, and observed by Olympus BX-60 fluorescence microscope.

**Peptide cleavage assay**

The cell monolayers in dishes (35 mm) (6×10^5 cells/dish) were pretreated with or without BFA and/or OA in α-MEM containing 35 μM BSA for 1 h at 37°C, followed by the addition of ricin or diphtheria toxin. After 15 h incubation at 37°C, the cells were washed once with PBS and suspended in ice-cold 200 μl of extraction buffer (10 mM HEPES/KOH buffer, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF) as described (Nicholson et al., 1995). After repeated freezing and thawing, cell debris were removed by centrifugation at 13,000×g at 4°C for 20 min. The supernatants were incubated with 10 μM fluorescent substrate at 37°C for 10 min, and then cleavage of peptide was analyzed with excitation at 380 nm and emission at 460 nm.

**NBD-ceramide labeling of cells**

NBD-ceramide-BSA conjugates were prepared in a serum free medium according to Lipsky and Pagano (Lipsky and Pagano, 1985). Vero cells plated on glass coverslips were washed once with serum-free medium, incubated for 30 min at 37°C with NBD-ceramide-BSA (16.6 μM), and washed three times with the growth medium. Labeled cells were immediately observed under fluorescence microscope.

**Results**

**Comparison between toxin-mediated protein synthesis inhibition and their abilities to induce apoptosis as measured by DNA fragmentation, cytolysis, and morphological changes**

It has been shown that ricin and diphtheria toxin cause programmed cell death or apoptosis in addition to their ability of inhibition of protein synthesis (Chang and Wisnieski, 1990; Griffiths et al., 1987; Komatsu et al., 1998; Komatsu et al., 2000; Morimoto and Bonavida, 1992; Oda et al., 1999; Sandvig and van Deurs, 1992). To gain the insight into the relationship between protein synthesis inhibition and induction of apoptosis by toxins, we first compared toxin-mediated protein synthesis inhibition with toxin-

![Fig. 1. Comparison of the abilities of ricin (□) and diphtheria toxin (●) to induce protein synthesis inhibition (A), DNA fragmentation (B), and cytolysis (C) in Vero cells. (A) Cells grown in 48-well plates (5×10^4 cells/well) were incubated with varying concentrations of ricin or diphtheria toxin in α-MEM containing 35 μM BSA for 3 h at 37°C, and labeled with [3H]leucine (1 μCi/ml) for 45 min in leucine-free medium for the measurement of protein synthesis. (B) Cells grown in dishes (35 mm) (6×10^5 cells/dish) were incubated with varying concentrations of ricin or diphtheria toxin in α-MEM containing 35 μM BSA for 15 h at 37°C. The extent of DNA fragmentation of each treated cells was determined by diphenylamine assay as described under Materials and Methods. (C) Cells grown in 96-well plates (2×10^5 cells/well) were incubated with varying concentrations of ricin or diphtheria toxin in α-MEM containing 35 μM BSA for 20 h at 37°C, and then the extent of cytolysis of each treated cells was assessed by LDH release assay as described under Materials and Methods. Each point represents an average of duplicate determination.**
mediated cytolysis and DNA fragmentation in Vero cells. As shown in Fig. 1, the extent of protein synthesis inhibition by ricin paralleled its cytolytic activity monitored by LDH release. Nearly maximum level of cytolysis was observed at 10 ng/ml ricin, a concentration that caused significant inhibition of protein synthesis. Furthermore, DNA fragmentation was also induced by ricin at a similar concentration range in inhibition of protein synthesis (Fig. 1B). After 20 h exposure to ricin, membrane blebbing and formation of apoptotic bodies, characteristic features of apoptosis were also seen in Vero cells (Fig. 2). However, neither DNA fragmentation nor cytolytic activity were induced by diphtheria toxin up to very high concentration, in spite of the fact that Vero cells were more sensitive to diphtheria toxin than ricin in terms of inhibition of protein synthesis (Fig. 1A). Consistent with these results, no apoptotic morphological changes were observed in Vero cells after 20 h diphtheria toxin treatment (Fig. 2). These results suggest that protein synthesis inhibition by toxins is not sufficient to induce apoptotic changes and that the strong ability of ricin to induce apoptosis in Vero cells may be due to certain steps present in its own intoxication mechanism which may lack in diphtheria toxin.

**Effect of BFA and OA on diphtheria toxin-induced cytolysis and DNA fragmentation in Vero cells**

In the course of the studies to find certain reagents or conditions which affect the toxin-induced apoptosis, we found that diphtheria toxin-induced apoptosis was markedly enhanced in the presence of BFA and OA. Our preliminary experiments revealed that the effective concentrations of BFA and OA are 36 nM and 25 nM, respectively. This concentration of each reagent was used throughout the experiment. As shown in Fig. 3, in the presence of both 36 nM BFA and 25 nM OA, diphtheria toxin caused cytolysis of Vero cells with similar dose-dependent profile of ricin (Fig. 1C), while no significant effects of either of these reagents alone were seen (data not shown). Furthermore, the combination of BFA and OA also enhanced diphtheria toxin-induced DNA fragmentation, while each reagent alone had no significant effect (Fig. 4).

**Effect of BFA and OA on diphtheria toxin-induced nuclear morphological changes in Vero cells**

During exposure to diphtheria toxin in the presence of BFA and OA, gradual rounding of adherent Vero cells was observed, and those rounded cells showed a typical apoptotic nuclear morphological change as examined by Hoechst 33258 staining, whereas diphtheria toxin alone or BFA and OA themselves did not cause significant nuclear morphological changes in Vero cells (Fig. 5).

![Fig. 2. Effect of ricin and diphtheria toxin on cellular morphology in Vero cells. Adherent cells were incubated with 10 ng/ml ricin or 10 ng/ml diphtheria toxin in α-MEM containing 35 μM BSA for 20 h at 37°C. Phase contrast micrographs of control (A), diphtheria toxin-treated cells (B), and ricin-treated cells (C). The bar indicates 20 μm.](image)
Effect of BFA and OA on protein synthesis inhibitory activity of diphtheria toxin in Vero cells

In contrast to the enhancement of diphtheria toxin-induced apoptotic changes by BFA and OA, no significant effects of these reagents on diphtheria toxin-mediated inhibition of protein synthesis were observed (Fig. 6). These results suggest that BFA and OA may modulate the steps leading to apoptosis rather than the processes leading to the inhibition of protein synthesis. Such modulation may result in sensitizing Vero cells against diphtheria toxin-induced apoptosis.

Ability of CRM 197 to induce DNA fragmentation and cytolysis in Vero cells in the presence of BFA and OA

To gain insight into the relationship between intoxication pathway leading to protein synthesis inhibition and apoptosis induction by diphtheria toxin, we examined the effects of CRM 197, a mutant diphtheria toxin, which does not catalyze the ADP-ribosylation of EF-2 and thus does not inhibit protein synthesis (Uchida et al., 1973). As shown in Table I, CRM 197 could not induce either DNA fragmentation or cytolysis in Vero cells even in the presence of BFA and OA.

Effect of NH₄Cl on diphtheria toxin-mediated cytotoxicities in the presence of BFA and OA in Vero cells

Diphtheria toxin is known to be translocated into the cytosol from endosomes following acidification. Thus, NH₄Cl, a potent inhibitor of endosomal acidification, prevents protein synthesis inhibitory activity of diphtheria toxin (Sandvig and Olsnes, 1981; Sandvig et al., 1984). As shown in Table II, the preventive effect of NH₄Cl against diphtheria toxin-mediated protein synthesis inhibition was confirmed in Vero cells. In addition, in the presence of 20 mM NH₄Cl, Vero cells were almost completely protected from the apoptosis caused by combination of diphtheria toxin, BFA, and OA as judged by cytolysis and DNA fragmentation (Table II). The increase in caspase-3-like activity was also significantly prevented by NH₄Cl in Vero cell treated with diphtheria toxin, BFA, and OA. These results suggest that the enhanced apoptosis by diphtheria toxin in the presence of BFA and OA, much like its protein synthesis inhibitory activity, is dependent on internalization of toxin molecules and that cell-surface-bound toxin itself is not sufficient to trigger apoptotic signals.

Effect of BFA and OA on the localization of NBD-ceramide in Vero cells

Both BFA and OA are known to affect the structure and function of Golgi complex (Dinter and Berger, 1998). To ascertain whether the enhancement of diphtheria toxin-induced apoptosis is due to a modulation of Golgi complex by these reagents, we examined the effect of BFA and OA on the distribution of NBD-ceramide, a Golgi complex-specific fluorescent probe. As shown in Fig. 7, combination treatment with BFA and OA of Vero cells resulted in a change of the distribution of NBD-ceramide from a perinuclear staining to a more diffuse appearance in the cytoplasm.
Discussion

Our recent studies have demonstrated that ricin caused a marked increase in caspase-3-like activity and induced PARP cleavage in human myeloid leukemia U937 cell line (Komatsu et al., 2000). Furthermore, it has been reported that ricin induced DNA fragmentation, another hallmark of apoptosis, and caused eventual cell lysis in several different cell lines (Griffiths et al., 1987; Komatsu et al., 1998; Komatsu et al., 2000; Morimoto and Bonavida, 1993; Oda et al., 1999; Sandvig and van Deurs, 1992). In addition to ricin, it has been shown that bacterial protein toxins diphtheria toxin and Pseudomonas toxin can also induce apoptosis in certain cell lines (Chang et al., 1989; Morimoto and Bonavida, 1992). Thus, it appears that exposure of cells to these potent protein toxins is one way of inducing apoptosis, and analysis of such toxin-induced apoptosis may provide specific and unique aspect of apoptosis.

In the present study we examined the cytotoxic effects of ricin and diphtheria toxin on Vero cells in view of induction of apoptosis in addition to their protein synthesis inhibitory activity. In agreement with previous findings, it was con-
Diphtheria Toxin-induced Apoptosis

confirmed that ricin is a potent apoptosis inducer against Vero cells as judged by its ability to induce DNA fragmentation, apoptotic cellular morphological change, and eventual cell lysis (Fig. 1 and 2). These ricin-mediated apoptotic events were caused at the concentration range at which protein synthesis was also severely inhibited. Although the relationship between the intoxication mechanism of ricin leading to the inhibition of protein synthesis and the apoptosis induction is still unclear, our previous study has demonstrated that ricin-mediated cell lysis was inhibited by BFA in BFA-sensitive Vero cells, but not in BFA resistant MDCK cells (Oda et al., 1997). Regarding the effect of BFA on ricin cytotoxicity, it has been shown that, following receptor-mediated endocytosis, ricin molecules are found in the Golgi elements, and that protein synthesis inhibition by ricin is abolished by BFA, which affects the structure and functions of the Golgi apparatus without preventing the binding and subsequent internalization of ricin (Pelham, 1991; Yoshida et al., 1990; Yoshida et al., 1991). Based on these findings, it has been generally considered that vesicle trafficking of ricin molecules through the Golgi region may be involved in the process leading to the inhibition of protein synthesis, especially in the step of translocation of ricin molecule to the cytosol. These findings, together with the results obtained in this study, suggest that the initial pathway of ricin-mediated protein synthesis inhibition is essential for ricin-mediated apoptosis.

In contrast to the potent ability of ricin to induce apoptosis as well as protein synthesis inhibition, diphtheria toxin failed to induce DNA fragmentation and cytolysis in Vero cells (Figs. 1, 2), even though it has been reported that this toxin can induce apoptosis in some other cell lines (Chang et al., 1989; Morimoto and Bonavida, 1992). Thus it appears that the ability of diphtheria toxin to induce apoptosis may depend on cell type. Similar to our results, it has been reported that human K562 cell line is fully resistant to the lytic action of diphtheria toxin even at extremely high concentrations of toxin, where protein synthesis was completely inhibited (Chang et al., 1989). Morimoto et al. have also reported that human ovarian carcinoma SKOV-3 cell line is sensitive to diphtheria toxin-mediated protein synthesis inhibition but resistant to lysis (Morimoto and Bonavida, 1992). Since CRM 197 failed to induce apoptosis (Table I), and NH₄Cl inhibited the diphtheria toxin-induced DNA fragmentation and cytolysis by CRM 197 in the presence or absence of BFA and OA in Vero cells.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NH₄Cl (20 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td></td>
</tr>
<tr>
<td>[³H]leucine incorporated (% of control)</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>100.0 ± 0.0</td>
</tr>
<tr>
<td>+ Diphtheria toxin, BFA, and OA</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>Cytolysis</td>
<td></td>
</tr>
<tr>
<td>LDH released (% of total content)</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>+ Diphtheria toxin, BFA, and OA</td>
<td>55.7 ± 2.4</td>
</tr>
<tr>
<td>DNA fragmentation (%)</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>2.0 ± 0.4</td>
</tr>
<tr>
<td>+ Diphtheria toxin, BFA, and OA</td>
<td>45.7 ± 2.4</td>
</tr>
<tr>
<td>Caspase-3-like activity</td>
<td></td>
</tr>
<tr>
<td>(U/mg cell protein)</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>5.0 ± 2.0</td>
</tr>
<tr>
<td>+ Diphtheria toxin, BFA, and OA</td>
<td>34.7 ± 4.4</td>
</tr>
</tbody>
</table>

Cells were preincubated in the presence or absence of 36 nM BFA and 25 nM OA in α-MEM containing 35 μM BSA for 1 h at 37°C, followed by the addition of 10 ng/ml CRM 197, and then DNA fragmentation and cytolysis were measured as described in the legend to Figure 1.
ria toxin-mediated apoptosis even in the presence of BFA and OA (Table II), diphtheria toxin-mediated protein synthesis inhibition may be a necessary step in the pathway leading to apoptosis. However, the findings described above suggest that protein synthesis inhibition is not sufficient to trigger the apoptosis, and that additional stimuli or certain conditions may be required for diphtheria toxin-mediated apoptosis. This notion may be supported by our finding that coexistence of BFA and OA rendered Vero cells sensitive to diphtheria toxin-induced apoptosis without affecting the protein synthesis inhibitory activity (Fig. 6). Since BFA and OA are known as multifunctional reagents, which action mechanisms of these reagents are mainly responsible for the enhancement of diphtheria toxin apoptosis is still unknown, and the reason for the requirement of two reagents at the same time is also unclear. However, both BFA and OA are potent Golgi-disturbing agents, and the effects of these reagents on Golgi region are well defined (Dinter and Berger, 1998). Recent studies have demonstrated that OA induces a reversible fragmentation of trans-Golgi network (TGN) which behaves differently from the cisternae of the Golgi stack in response to BFA (Horn and Banting, 1994; Lippincott-Schwartz et al., 1991; Reaves and Banting, 1992). Therefore, it is possible that coexistence of BFA and OA may result in profound modulation of total Golgi region including TGN. In fact, fluorescent microscopic observation revealed that morphological change of Golgi apparatus in Vero cells was observed in the presence of BFA and OA as examined with Golgi-specific fluorescent probe NBD-ceramide (Fig. 7). Interestingly, Mancini et al. have recently reported that caspase-2 is localized at the Golgi

![Fig. 6. Effect of BFA and OA on diphtheria toxin-mediated protein synthesis inhibition in Vero cells. Cells grown in 48-well plates (5×10^5 cells/well) were preincubated in the absence (●) or presence of 36 nM BFA and 25 nM OA (□) in α-MEM containing 35 µM BSA for 1 h at 37°C followed by the addition of varying concentrations of diphtheria toxin. After further incubation for 3 h at 37°C, cells were labeled with [3H]leucine (1 µCi/ml) for 45 min in leucine-free medium for the measurement of protein synthesis. Each point represents an average of duplicate determination.](image)

![Fig. 7. Effect of BFA and OA on the morphology of the Golgi apparatus in Vero cells. Adherent cells were preincubated with or without diphtheria toxin (10 ng/ml) and/or BFA (36 nM) and OA (25 nM) in α-MEM containing 35 µM BSA for 1 h at 37°C, followed by the addition of 16.6 µM of NBD-ceramide. After 30 min incubation at 37°C, the distribution of NBD-ceramide in live cells was examined by fluorescence microscopy. (A) Control cells (untreated cells), (B) Cells treated with diphtheria toxin alone, (C) Cells treated with diphtheria toxin, BFA, and OA. The bar indicates 20 µm.](image)
macromolecule substrate for caspase-2, during apoptosis (Mancini et al., 2000). Based on these findings, they propose that the Golgi complex, like mitochondria, is an important organelle which senses and transduces apoptotic signals through caspase-2. As described above, ricin molecules are accumulated in Golgi apparatus during its intoxication process, while diphtheria toxin is translocated into the cytosol from the endosomes before going to the Golgi region. Therefore, one can speculate that vesicle trafficking of ricin molecules through the Golgi apparatus may lead to a potent apoptotic signal transduction which may lack in diphtheria toxin intoxication. The different apoptotic mechanisms between ricin and diphtheria toxin may be also supported by the finding that the combination of TNF-α and diphtheria toxin resulted in enhanced cytotoxicity and synergy, whereas ricin did not synergize with TNF-α (Morimoto et al., 1991).

Although the biological significance of protein synthesis in the cells undergoing apoptosis is still controversial (Chow et al., 1995; Khan and Waring, 1993), another possible cause for differences in the abilities of ricin and diphtheria toxin to induce apoptosis may be derived from their different means by which protein synthesis is inhibited. Namely, ricin inactivates ribosomes by acting on 28S ribosomal RNA, while diphtheria toxin inactivates elongation factor EF-2. Regarding the relationship between protein synthesis inhibition and stress-signaling pathway, Iordanov et al. have recently reported that a novel stress signaling pathway (ribotoxic stress response) is initiated in mammalian cells by damage to the 28S ribosomal RNA, and that it is this pathway that leads to activation of stress-activated kinase (SAPK/JNK1) (Iordanov et al., 1997). They also proposed that the 28S ribosomal RNA functions as a sensor for stress induced by a subset of agents that inhibit protein synthesis. Furthermore, it has been reported that ricin showed a strong ability to activate SAPK/JNK1, but diphtheria toxin caused only marginal activation of the kinase under the conditions at which these toxins strongly inhibited cellular protein synthesis (Iordanov et al., 1997). Therefore, it is possible to speculate that the potent ability of ricin to induce apoptosis may be explained partly by its enzymatic action on the 28S ribosomal RNA, and that stress-related signals leading to apoptosis may thereby be triggered. Further studies are required to clarify whether or not MAP kinase activation is involved in ricin-mediated apoptotic induction.

Acknowledgments. We would like to thank Dr. Eisuke Mekada, Osaka University, Research Institute for Microbial Diseases, for generously providing us with a mutant diphtheria toxin, CRM 197.

References


(Received for publication, July 23, 2001 and in revised form, September 13, 2001)