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Cross-Talk between the Pathways Leading to the Induction of Apoptosis and the Secretion of Tumor Necrosis Factor-α in Ricin-Treated RAW 264.7 Cells

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Ricin induced apoptotic nuclear morphological changes in mouse macrophage cell line RAW264.7 cells at concentrations sufficient to cause severe protein synthesis inhibition. Ricin also induced the release of tumor necrosis factor-α (TNF-α) from this cell line in a dose-dependent manner but the profile was bell-shaped. However, the isolated galactose-specific ricin B-chain had no such effects. These results suggest that the receptor-binding of ricin through the B-chain is not enough, and subsequent attack on the intracellular target, *i.e.*, the 28S ribosomal RNA (rRNA), by the A-chain of internalized ricin is required for the effects of ricin. Z-D-CH_2-DCB, a caspase family inhibitor, showed potent inhibition of the release of TNF-α from RAW264.7 cells as well as blockage of the induction of apoptosis by ricin. Furthermore, SB202190, a specific P38 mitogen-activated protein (MAP) kinase inhibitor that strongly inhibits the release of TNF-α, also showed significant inhibition of ricin-induced apoptosis. These results suggest that there may be cross-talk between the pathways leading to the release of TNF-α and apoptosis. Time course analysis revealed that the activation of p38 MAP kinase started prior to the induction of TNF-α release and apoptosis. Since the activation of p38 MAP kinase in ricin-treated RAW264.7 cells was not prevented by Z-D-CH_2-DCB, the activation of p38 MAP kinase may occur upstream of the caspase cascade. Among the other protein synthesis inhibitors examined, modeccin and anisomycin, which can trigger a ribotoxic stress response similar to ricin, induced the release of TNF-α, but emetine and cycloheximide did not. These results suggest that the specific attack on the 28S ribosomal RNA and the resulting ribotoxic stress response may trigger the multiple signal transduction pathways through the activation of p38 MAP kinase, which in turn leads to TNF-α release and apoptosis.

Key words: apoptosis, caspase-cascade, macrophage, MAP kinase, ricin, TNF-α.

Abbreviations: TNF-α, tumor necrosis factor-α; rRNA, ribosomal RNA; MAP kinase, mitogen-activated protein kinase; PBS, phosphate-buffered saline; Z-D-CH_2-DCB, carbobenzoxy-Asp-1-yl-[(2,6-dichlorobenzoyl)oxy]methane; FBS, fetal bovine serum; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid.

Ricin is a natural protein toxin present in castor beans (*Ricinus communis*), and consists of two structurally and functionally different chains (A and B) that are linked together through a single disulfide bond. The A-chain (32 kDa) is *N*-glycosidase, which specifically cleaves the *N*-glycosidic bond of a single adenine residue in the 28S ribosomal RNA (rRNA), and eventually inhibits cellular protein synthesis. The B-chain is a galactose or *N*-acetylgalactosamine specific lectin (*1–3*). The potent cytotoxicity of ricin is due to the collaboration of the two chains. Namely, the intoxication pathway of ricin consists of (i) binding to cell-surface receptors *via* the B-chain, (ii) receptor-mediated endocytosis and intracellular transport through the vesicular system, (iii) translocation of the enzymatically active A-chain across the vesicle membrane to the cytosol, and (iv) enzymatic inactivation of the 28S rRNA (*4–6*).

Recent studies have demonstrated that ricin induces apoptosis in several different cell lines (*7–10*). Although the detailed mechanism remains unclear, one can speculate that the cytotoxic stress caused during ricin-intoxication may trigger the intracellular signal transduction leading to apoptosis.

Apart from its cytotoxic effects, ricin also induces the release of TNF-α and interleukin-1β from human peripheral-blood mononuclear cells (*11*). Furthermore, Iordanov *et al*. recently reported that ricin activates the stress-activated protein kinase (SAPK/JNK1) (*12*). Thus, it seems likely that ricin not only inhibits cellular protein synthesis but also stimulates multiple intracellular signaling pathways that may be responsible for the induction of apoptosis or secretion of cytokines. However, the relationship between the protein synthesis inhibition by ricin and ricin-mediated induction of signal transduction is totally unknown, and which pathway is responsible for the transmission of the signals produced by ricin for apoptosis or secretion of cytokines has not been clarified. To gain an insight into the relationship between the pathways leading to the induction of apoptosis and the release of TNF-α, and the involvement of MAP kinase in these pathways, we investigated the effect of ricin on mouse macrophage cell line RAW264.7 using caspase

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family and specific MAP kinase inhibitors. Our results suggest that there is cross-talk between the apoptosis pathway and the pathway leading to the secretion of TNF-α.

MATERIALS AND METHODS

Materials—Ricin was isolated from small castor beans as described by Mise *et al*. (*13*). The caspase family protease inhibitor, carbobenzoxy-Asp-1-yl-[(2,6-dichlorobenzoyl)oxy]methane (Z-D-CH_2-DCB), was obtained from Peptide Institute, Osaka. SB202190, a specific p38 MAP kinase inhibitor, was purchased from Calbiochem (La Jolla, CA, USA). Anisomycin, actinomysis, emetine, and cycloheximide were obtained from Sigma Chemical (St. Louis, MO, USA). Modeccin was obtained from Inland Laboratories (Austin, TX, USA). H33258 (Hoechst 33258) was purchased from Wako Chem. (Tokyo). [^{3}H]Leucine (60 Ci/mmol) was obtained from NEN Research Products (Boston, MA, USA).

Cell Culture—RAW264.7 cells (mouse macrophage-like cell line) and L929 cells (mouse fibrosarcoma cell line) were obtained from the American Type Culture Collection (Rockville, MD, USA). These cells were grown as monolayers in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 μg/ml), and streptomycin (100 μg/ml) (growth medium). The cells were subcultured 1 day 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 5×10^4 cells/well in 0.2 ml of medium on 48-well plates. One day later, varying concentrations of ricin or other toxins were added to the cells followed by incubation for 24 h in the growth medium at 37°C. After removal of the medium, the cells were incubated with 1 μCi/ml of [^{3}H]leucine for 45 min at 37°C in leucine-free medium. The incorporation of [^{3}H]leucine into perchloric acid/phosphotungstic acid–insoluble materials was determined as described previously (*14*). The results were expressed as the percentage of the incorporation in control cells incubated without toxin but otherwise treated in the same way.

TNF Assay—The TNF-α level in each sample was determined by means of a cytotoxicity assay for TNF using TNF-α-sensitive L929 mouse fibrosarcoma cells (*15*). Briefly, L929 cells were plated on a 96-well culture plate (3×10^4 cells/well). To the adherent cells, test samples in RPMI 1640 medium supplemented with 10% FBS containing actinomycin D (2 μg/ml) were added. After 24 h incubation at 37°C, cell viability was determined by means of the Alamar blue assay (*16*). Serial dilutions of recombinant mouse TNF-α were also included in each assay. In addition to the bioassay, the TNF-α levels in culture medium were independently determined by means of an enzyme-linked immunosorbent assay (ELISA).

Nuclear Staining—Nuclear morphological changes in cells undergoing apoptosis were detected by staining with the DNA-binding fluorochrome bisbenzimide (Hoechst 33258), as described previously (*17*). In brief, cells (6×10^5 cells/dish) (35 mm) were incubated with varying concentrations of ricin in RPMI 1640 medium containing 10% FBS for 24 h at 37°C. The cells were washed with PBS and subsequently fixed with 1% glutaraldehyde for 30 min at room temperature, and then were stained with Hoechst 33258 (40 μM) for 5 min at room temperature and observed under an Olympus BX-60 fluorescence microscope. Cells with three or more chromatin fragments were considered as apoptotic ones.

DNA Fragmentation Assay—The cell monolayers in dishes (35 mm) (6×10^5 cells/dish) (35 mm) were incubated with ricin in RPMI 1640 medium containing 10% FBS for the indicated periods of time at 37°C. The cells were washed once with PBS and then lysed in 1 ml of ice-cold lysis buffer (0.5% Triton X-100, 10 mM Tris-HCl, pH 8.0, 10 mM EDTA). Samples were subsequently centrifuged for 30 min at 13,000 ×*g* to separate DNA fragments (supernatant) from intact DNA (pellet). The DNA contents of the supernatant and pellet fractions were determined using diphenylamine reagent, as described previously (*19*).

Immunoblot Analysis of Activated p38 MAP Kinase—Cell monolayers (2×10^6 cells/dish) (60 mm) in RPMI 1640 medium containing 10% FBS were treated with ricin (6.1 ng/ml) at 37°C. After the indicated periods of time, the cells were washed twice with PBS, scraped off, and pelleted by centrifugation. The pelleted cells were resuspended in 100 μl of extraction buffer (10 mM HEPES, 150 mM NaCl, 1 mM EGTA, 1% CHAPS, 1 mM phenylmethylsulfonyl fluoride, 10 mM aprotinin, 10 mM leupeptin, 10 mM pepstatin, pH 7.4), and then sonicated for 2 min at 4°C. Following centrifugation (21,000 ×*g* for 10 min at 4°C), the extract was mixed with SDS-sample buffer and boiled for 5 min. Samples containing 50 μg of protein were subjected to SDS-PAGE in a 5–20% gradient polyacrylamide gel. The proteins were then transferred to a PVDF membrane. Western blotting with antibodies recognizing phosphorylated p38 MAP kinase (Boehringer Mannheim) and suitable peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech) was performed. The effect of caspase family inhibitor Z-D-CH_2-DCB on the ricin-induced activation of p38 MAP kinase at 6 h was determined in the same way as described above.

RESULTS

Relationship Between Ricin-Induced Protein Synthesis Inhibition and Apoptotic Nuclear Morphological Changes in RAW264.7 Cell—As shown in Fig. 1A, ricin inhibited protein synthesis in RAW264.7 cells in a concentration-dependent manner, 10 ng/ml ricin causing nearly 90% inhibition of protein synthesis in 24 h. From the dose-response curve, the IC_{50} of ricin for this cell line was estimated to be 5.1 ng/ml. In a similar concentration range, ricin also induced apoptotic nuclear morphological changes, and this effect of ricin was maximum at 10 ng/ml (Fig. 1B).

Effect of Ricin on the Release of TNF-α from RAW264.7 Cells—It has been reported that ricin induces the release of tumor necrosis factor-α (TNF-α) by human peripheral blood mononuclear cells in a dose- and time-dependent manners (*11*). As shown in Fig. 2, ricin induced the release of TNF-α from RAW264.7 cells in a concentration-dependent manner on 24 h incubation. The maximum release was attained with 6.1 ng/ml ricin. Increas-

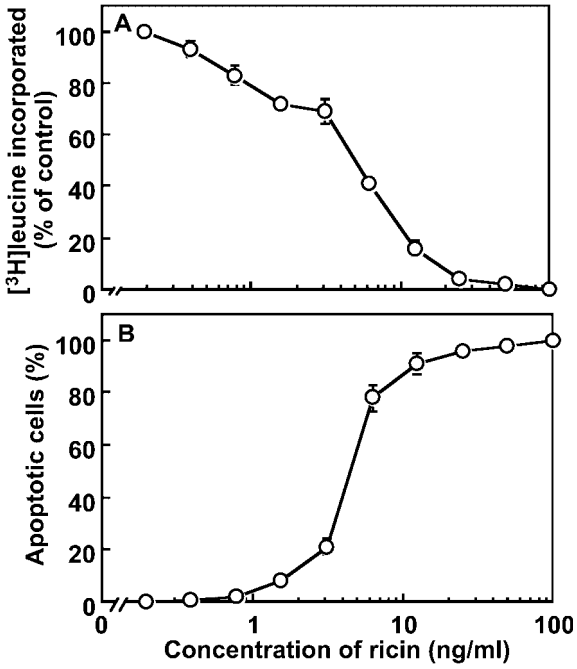

Fig. 1. **Ricin-induced protein synthesis inhibition and apoptotic nuclear morphological changes in RAW264.7 cells.** (A) RAW264.7 cells grown on 48-well plates (5×10^4 cells/well) were incubated with varying concentrations of ricin in RPMI 1640 medium containing 10% FBS for 24 h at 37°C. For measurement of protein synthesis, the cells were labeled with [^{3}H]leucine for 45 min in leucine-free RPMI 1640 medium. Each point represents the average of triplicate measurements. (B) RAW264.7 cells (6×10^5 cells/dish) (35 mm) were incubated with varying concentrations of ricin in RPMI 1640 medium containing 10% FBS at 37°C. After 24 h, the cells were fixed with 1% glutaraldehyde, stained with Hoechst 33258 (40 μM), and viewed under a fluorescence microscope. Cells with three or more chromatin fragments were considered to be apoptotic. Each point represents the average of triplicate measurements.

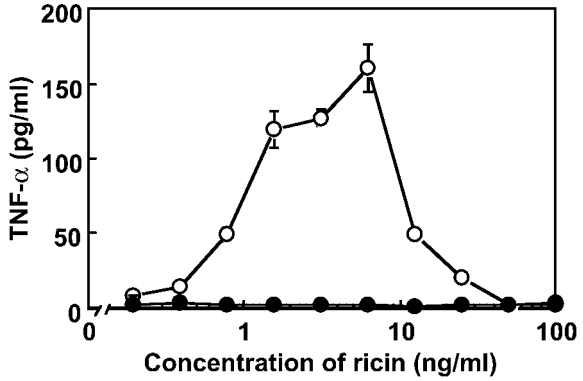

Fig. 2. **Effect of ricin on the release of TNF-α from RAW264.7 cells.** RAW264.7 cells on 96-well plates (3×10^4 cells/well) were incubated with varying concentrations of intact ricin (open circles) or heat-inactivated ricin (solid circles) in RPMI 1640 medium containing 10% FBS at 37°C. After 24 h, the amounts of TNF-α in the cell-free supernatants were measured by ELISA as described under "MATERIALS AND METHODS."

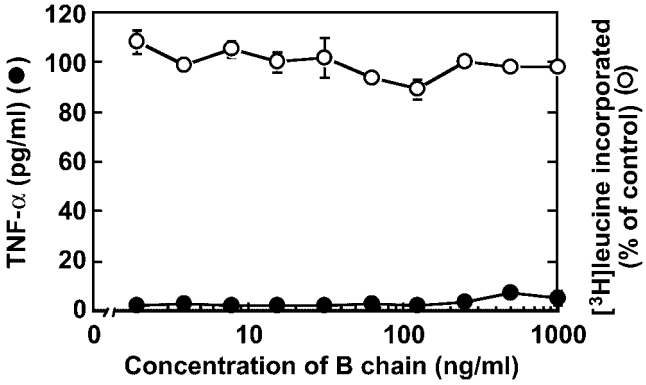

Fig. 3. **Protein synthesis inhibitory activity of the B chain and its effect on the release of TNF-α in RAW264.7 cells.** The protein synthesis inhibitory activity of B chain (open circles) in RAW264.7 cells was measured as described in the legend to Fig. 1, and its effect on the release of TNF-α (solid circles) was examined as described in the legend to Fig. 2. Each point represents the average of triplicate measurements.

ing concentrations beyond this point resulted in a progressive decrease from the maximum effect on the induction of the release of TNF-α. Heat-inactivated ricin did not have a significant effect up to 100 ng/ml (Fig. 2).

Effects of Isolated B-Chain on Protein Synthesis and Secretion of TNF-α in RAW264.7 Cells—The protein synthesis inhibitory activity of the isolated ricin B-chain was measured in a similar to as for ricin as described above (Fig. 1). As shown in Fig. 3, the B-chain had no effect up to 1,000 ng/ml, indicating that there was no contamination by traces of intact ricin in the B-chain preparation. The B-chain was also ineffective in terms of the induction of the release of TNF-α from RAW264.7 cells (Fig. 3).

Relationship between Protein Synthesis Inhibition and Induction of Release of TNF-α by Cycloheximide, Emetine, Anisomycin, and Modeccin in RAW264.7 Cells—To obtain a clue as to the mechanism of ricin-induced release of TNF-α from RAW264.7 cells, we examined the abilities of other protein synthesis inhibitors with different modes of action to induce the release of TNF-α as well as their abilities to inhibit protein synthesis. As shown in Fig. 4, anisomycin and modeccin, which are known to attack ribosomal 28S RNA similar to ricin, induced the release of TNF-α from RAW264.7 cells, the dose response curves being bell-shaped as seen for ricin. However, cycloheximide and emetine completely failed to induce the release of TNF-α from RAW264.7 cells over the wide range of concentrations causing from partial to severe inhibition of protein synthesis.

Effects of SB202190 and Z-D-CH_2-DCB on Ricin-Induced Release of TNF-α and Apoptosis—Ricin has been reported to activate MAP kinase family cascades through a ribotoxic stress response (*12*). We used the specific p38 MAP kinase inhibitor SB202190 (*18*) to determine whether or not the p38 MAP kinase pathway is involved in the ricin-induced secretion of TNF-α and apoptosis. We also examined the effect of caspase family inhibitor Z-D-CH_2-DCB, which is a potent inhibitor of ricin-induced apoptosis (*17*), on both the secretion of TNF-α and induction of apoptosis. Figure 5 is representative of several repeated experiments, and shows the effects of SB202190 and Z-D-CH_2-DCB on the secretion of TNF-α and induction of apoptosis in ricin-treated RAW264.7 cells. In the

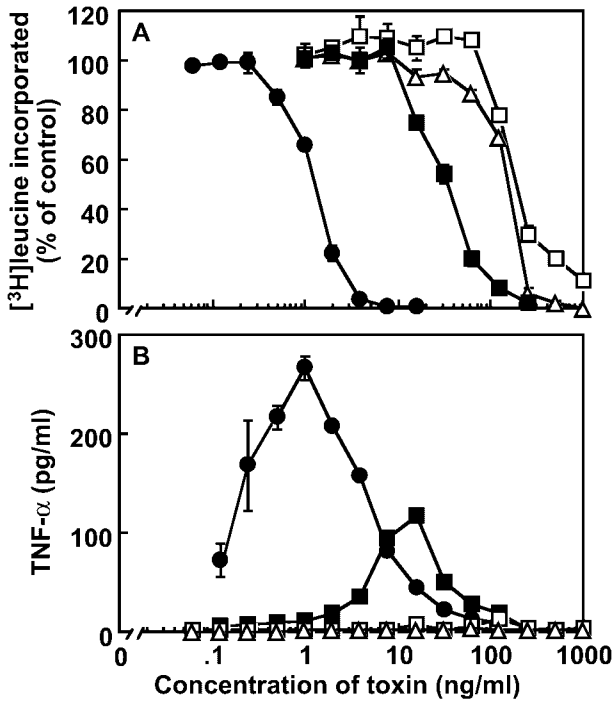

Fig. 4. **Protein synthesis inhibitory activities of cycloheximide, emetine, anisomycin, and modeccin and their effects on the release of TNF-α in RAW264.7 cells.** (A) The protein synthesis inhibitory activities of cycloheximide (open squares), emetine (open triangles), anisomycin (solid squares), and modeccin (solid circles) in RAW264.7 cells were measured as described in the legend to Fig. 1. (B) The effects of cycloheximide (open squares), emetine (open triangles), anisomycin (solid squares), and modeccin (solid circles) on the release of TNF-α were examined as described in the legend to Fig. 2. Each point represents the average of triplicate measurements.

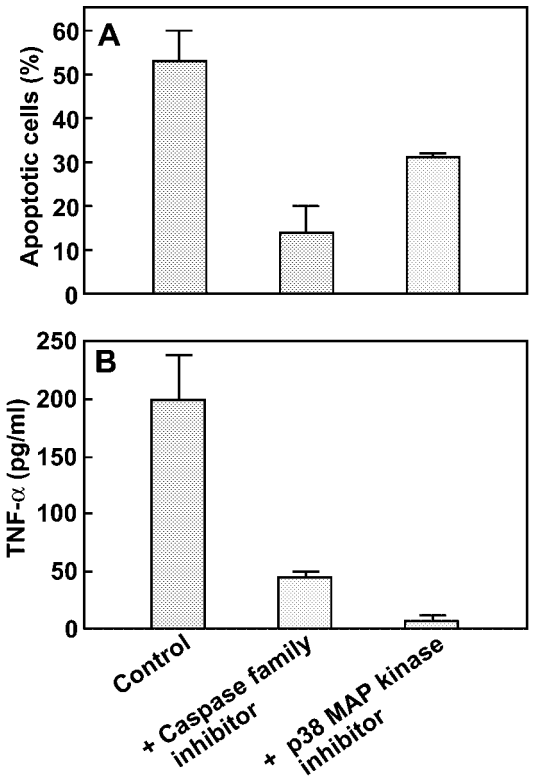

Fig. 5. **Effects of Z-D-CH_2-DCB and SB202190 on the ricin-induced release of TNF-α and apoptotic nuclear morphological changes in RAW264.7 cells.** (A) Cells in 35 mm-dishes (6×10^5 cells/dish) were incubated with or without ricin (6.1 ng/ml) in the presence or absence of 125 μM Z-D-CH_2-DCB or 15 μM SB202190 in RPMI 1640 medium containing 10% FBS at 37°C. After 24 h, apoptotic cells were counted as described in the legend to Fig. 1. (B) Cells on 96-well plates (3×10^4 cells/well) were incubated with or without ricin (6.1 ng/ml) in the presence or absence of 125 μM Z-D-CH_2-DCB or 15 μM SB202190 in RPMI 1640 medium containing 10% FBS at 37°C. After 24 h, the amounts of TNF-α in the cell-free supernatants were measured by ELISA as described under "MATERIALS AND METHODS." Each column represents the average of triplicate measurements.

presence of SB202190, potent inhibition of the ricin-induced secretion of TNF-α and a partial but significant decrease in the number of the cells with apoptotic nuclear morphological changes were observed. Interestingly, Z-D-CH_2-DCB also inhibited the secretion of TNF-α and markedly prevented the ricin-induced apoptosis.

Time Course Analysis of Ricin-Induced Activation of p38 MAP Kinase, TNF-α Release, and DNA-Fragmentation in RAW264.7 Cells—To examine the temporal relationship between ricin-induced biochemical changes, we performed time course studies on p38 MAP kinase activation, TNF-α release, and DNA fragmentation in ricin-treated RAW264.7 cells. As shown in Fig. 6, a significant level of activated p38 MAP kinase was detected after 3 h incubation with ricin, the maximum level being reached at 6 h, followed by a gradual decrease. The induction of TNF-α release started just after the maximum level of p38 MAP kinase activation was attained, whereas DNA-fragmentation was observed as a relatively later event.

Effect of Z-D-CH_2-DCB on Ricin-Induced Activation of p38 MAP Kinase—We next examined the effect of Z-D-CH_2-DCB on the ricin-induced activation of p38 MAP kinase at 6 h. As shown in Fig. 7, the activation levels of p38 MAP kinase in ricin-treated RAW264.7 cells in the presence and absence of Z-D-CH_2-DCB were not significantly different.

DISCUSSION

Early research on ricin was mainly focused on the cytotoxic mechanism underlying the cellular protein synthesis inhibition. Recent studies, however, demonstrated that ricin induces apoptotic cell death in various cell lines. Various biochemical apoptotic features such as DNA fragmentation, typical nuclear morphological changes, increased caspase-3-like activity, cytochrome *c* release from mitochondria, and PARP cleavage have been observed in ricin-treated cells (*7–10*, *17*, *19*). We recently found that a brefeldin A–resistant mutant cell line derived from Vero cells (BER-40) is highly resistant to ricin-induced apoptosis as compared to parental Vero cells, even though both cell lines show similar sensitivities to ricin-mediated inhibition of protein synthesis (*20*). In BER-40 cells, the extents of apoptotic events caused by ricin such as cytolysis, nuclear morphological changes, and DNA fragmentation were less than in Vero cells, and no significant apoptotic signaling events such as increases in caspase-3 and -9-like activities, the release

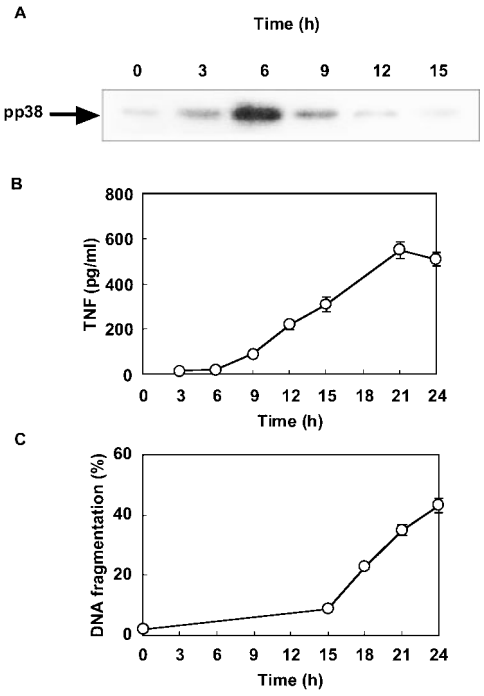

Fig. 6. **Time course analysis of the activation of p38 MAP kinase (A), release of TNF-α (B), and DNA-fragmentation (C) in ricin-treated RAW264.7 cells.** (A) Cells in 60 mm dishes (2×10^6 cells/dish) in RPMI 1640 medium containing 10% FBS were treated with ricin (6.1 ng/ml) for the indicated periods of time, and then cell lysates were prepared and analyzed by immunoblotting for changes in the level of activated p38 MAP kinase as described under "MATERIALS AND METHODS." (B) Cells on 96-well plates (3×10^4 cells/well) were incubated with ricin (6.1 ng/ml) for the indicated periods of time, and then the amounts of TNF-α in each cell-free supernatant was measured as described in the legend to Fig. 2. (C) Cells grown in dishes (35 mm) (6×10^5 cells/dish) were incubated with ricin (6.1 ng/ml) in RPMI 1640 medium containing 10% FBS for the indicated periods of time at 37°C. The extent of DNA fragmentation in each lot of treated cells was determined by means of the diphenylamine assay as described under "MATERIALS AND METHODS."

Fig. 7. **Effect of Z-D-CH_2-DCB on the ricin-induced activation of p38 MAP kinase in RAW264.7 cells.** Cells in 60 mm dishes (2×10^6 cells/dish) in RPMI 1640 medium containing 10% FBS were treated with ricin (6.1 ng/ml) in the absence (b) or presence (C) of 125 μM Z-D-CH_2-DCB at 37°C. After 6 h, cell lysates were prepared for immunoblotting analysis with antibodies recognizing phosphorylated p38 MAP kinase (pp38) as described under "MATERIALS AND METHODS." (a) Control untreated cells.

of cytochrome *c* from mitochondria, and the cleavage of PARP were observed under the conditions under which these changes were evidently induced in parental Vero cells. These results suggest that the entire apoptotic signal transduction mechanism in BER-40 cells becomes resistant. These findings also prompt us to speculate that a certain intracellular signal transduction pathway that may be triggered during ricin-intoxication is involved in ricin-induced apoptosis besides the pathway leading to protein synthesis inhibition. Furthermore, we have found that ricin and anisomycin potently induced apoptosis in U937 cells, but diphtheria toxin and cycloheximide were less effective even at the concentrations at which these toxins cause almost complete inhibition of protein synthesis (*21*). Thus, it seems likely that protein synthesis inhibition does not necessarily result in apoptosis, and induction of apoptosis by ricin is not a simple or straightforward consequence of protein synthesis inhibition.

Apart from its cytotoxic effects, ricin induces the release of TNF-α and interleukin-1β by human peripheral-blood mononuclear cells in dose- and time-dependent manners (*11*). Ricin-induced release of TNF-α has also been observed in macrophage cell line J774A.1 cells (*22*). Similar to ricin, it has been shown that several bacterial toxins induce the secretion of cytokines and it is considered that the toxin-mediated release of cytokines may play a role in the pathogenesis of such bacterial infections (*23*). For instance, verotoxin, which has the same enzymatic activity and intracellular vesicle trafficking pathway as ricin, induces the release of interleukin-1β, TNF-α, interleukin-6, and interleukin-8 by both human monocytes and human macrophages (*24*, *25*), and the release of interleukin-8 by the Caco-2 cell line (*26*), although the underlying mechanism remains to be clarified.

The results presented in this study show that ricin induces intensive apoptosis in macrophage cell line RAW264.7 cells, as reflected by nuclear morphological criteria as well as severe protein synthesis inhibition (Fig. 1). Treatment of RAW264.7 cells with ricin also resulted in the induction of the release of TNF-α. This effect was dose-dependent but exhibited a bell-shaped profile, and the maximum level of TNF-α secretion was attained at the concentration at which only partial inhibition of protein synthesis and apoptotic nuclear morphological change were induced (Figs. 1 and 2). Probably, a partial toxic dose of ricin is required to stimulate the secretion of TNF-α, and increasing the dose beyond a certain critical point decreases this activity due to the severe toxic effect. It was confirmed that this activity is not due to lipopolysaccharide contaminating the ricin preparation, since heat-inactivated ricin had no effect. Furthermore, the isolated ricin B-chain completely failed to stimulate TNF-α secretion (Figs. 2 and 3). Thus, it appears that the enzymatically active ricin A-chain is deeply involved in the pathway leading to the secretion of TNF-α, whereas the B chain is required for the binding to the cell membrane and the internalization of the A-chain. In contrast to our findings, it has been reported that the isolated carbohydrate binding B-chain of cytotoxic mistletoe lectin with β-galactoside specificity induced increased secretion of TNF-α by human mononuclear cells, similar to the complete lectin (*27*). Although the reason for this discrepancy is unclear at present, it has been shown that the B subunit of verotoxin failed to induce the secretion of cytokines from monocytes (*25*), and the *N*-glycosidase activity of the A subunit was essential for IL-8 induction (*26*). Thus, we prefer the idea that intracellular expression of the specific enzymatic activity of the A-chain is important for induction of the secretion of cytokines in the case of the protein toxins

which consist of an enzymatically active A-chain and a saccharide-binding B-chain.

We further compared ricin to other protein synthesis inhibitors such as anisomycin, emetine, cycloheximide, and modeccin in terms of the induction of TNF-α. Our results showed that anisomycin and modeccin could induce the release of TNF-α in a dose-dependent manner similar to ricin, whereas emetine and cycloheximide could not at all over the wide range of the concentrations examined (Fig. 4). It seems likely that these results may be due to the different modes of action of these protein synthesis inhibitors, and the way by which a toxin inhibits protein synthesis may be more important rather than the extent of the inhibition of protein synthesis. Regarding the relationship between protein synthesis inhibition and induction of the stress-activated signaling pathway, it has been demonstrated that anisomycin and ricin, which commonly act on 28S rRNA, potently activates the stress-activated protein kinase (SAPK/JNK1) (*12*), but not cycloheximide or emetine. Furthermore, it has been proposed that a novel stress signaling pathway called the ribotoxic stress response starts in mammalian cells on damage to the 28S rRNA, and 28S rRNA itself functions as a sensor for the stress induced by a subset of agents such as ricin and anisomycin. SAPK/JNK1 is a member of the mitogen-activated protein (MAP) kinase family of proline-directed serine/threonine protein kinases including p38 MAP kinase (*28*). Modeccin is also a toxic lectin belonging to the type 2 ribosome-inactivating protein family and exhibits *N*-glycosidase activity toward 28S rRNA like ricin. Therefore, it appears that the abilities of ricin, modeccin, and anisomycin to induce the release of TNF-α by RAW264.7 cells may be attributable to their abilities to trigger a ribotoxic stress response *via* a specific attack on 28S rRNA. Interestingly, it was recently reported that trichothecene mycotoxins that inhibit protein synthesis by binding to the ribosomal peptidyltransferase site can trigger a ribotoxic stress response that activates c-Jun N-terminal kinase and p38 MAP kinase, and induces apoptosis in a Jurkat human T-lymphoid cell line (*29*). In agreement with these findings, ricin-treatment resulted in the significant activation of p38 MAP kinase (Fig. 6), and the specific p38 MAP kinase inhibitor SB202190 blocked both ricin-induced apoptosis and the secretion of TNF-α (Fig. 5). Consequently, one can speculate that damage to the 28S rRNA triggers the ribotoxic stress response with subsequent initiation of signal transduction *via* MAP kinase family members, and p38 MAP kinase may be deeply involved in both ricin-mediated apoptosis and the secretion of TNF-α. This notion may be partly supported by the finding that Shiga toxin-mediated secretion of IL-8 from intestinal epithelial cells is strongly inhibited by SB202190 (*30*). More interesting is the observation that caspase family inhibitor Z-D-CH_2-DCB not only blocked ricin-induced apoptosis, but also the secretion of TNF-α from RAW264.7 cells. These results suggest that there is cross-talk between the caspase-mediated protease-cascade and p38 MAP kinase-mediated signaling pathway in ricin-treated RAW264.7 cells. Furthermore, the results of kinetics analysis showed that the activation of p38 MAP kinase started after 3 h ricin treatment, and subsequently the release of TNF-α occurred after a very short lag time, whereas DNA-fragmentation, a typical apoptotic change, was a relatively later event (Fig. 6). Since caspase family inhibitor Z-D-CH_2-DCB had no effect on the ricin-induced activation of p38 MAP kinase, it seems likely that the activation of p38 MAP kinase occurs upstream of the caspase-activation cascade. Although the precise relationship between the caspase-activation pathway and the signaling pathway mediated by the MAP kinase family remains unclear, this is the first finding suggesting a connection between caspase- and MAP kinase- signaling systems. Further studies on ricin-induced apoptosis and the secretion of TNF-α from RAW264.7 cells may reveal unique aspects of apoptosis in connection with the signal transduction pathway leading to the secretion of cytokines.

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