J. Biochem. 129, 521–527 (2001)

Apoptosis of CTLL-2 Cells Induced by an Immunosuppressant, ISP-I, Is Caspase-3–Like Protease–Independent[1]

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Received December 4, 2000; accepted January 12, 2001

In our previous study, the sphingosine-like immunosuppressant ISP-1 was shown to induce apoptosis in the mouse cytotoxic T cell line CTLL-2. In this study, we characterized the ISP-1–induced apoptotic pathway. Although caspase-3–like protease activity increases concomitantly with ISP-1–induced apoptosis in CTLL-2 cells, the apoptosis is not inhibited by caspase-3–like protease inhibitors, *i.e.* DEVD-cho and z-DEVD-fmk. In contrast, sphingosine-induced apoptosis in CTLL-2 cells is caspase-3–like protease–dependent. A caspase inhibitor with broad specificity, z-VAD-fmk, protects cells from apoptosis induced by ISP-1, indicating that ISP-1–induced apoptosis is dependent on caspase(s) other than caspase-3. Overexpression of Bcl-2 or Bcl-xL suppresses the apoptosis induced by ISP-1, although sphingosine-induced apoptosis is not efficiently inhibited by Bcl-2. Finally, ISP-1–induced mitochondrial depolarization, which is thought to be a checkpoint dividing the apoptotic pathway into upstream and downstream stages, is not inhibited by DEVD-cho, but is inhibited by z-VAD-fmk. These data suggest that a pathway dependent on caspase(s) other than caspase-3 is involved upstream of mitochondrial depolarization in ISP-1–induced apoptosis.

Key words: Bcl-2, caspase-3, CTLL-2, ISP-1, sphingosine.

Recent reports have shown that sphingolipids are involved in several cellular biological functions, including adhesion, differentiation, growth, and apoptosis (*1–6*). Ceramide, sphingosine and sphingosine-1-phosphate, which are metabolic products of sphingolipids, are thought to be upregulated by several stimuli and to serve as second messengers in signal transduction pathways. Increasing the cellular level of ceramide through exogenous addition or stimulation of the sphingomyelin cycle by a Fas ligand, TNF-α, UV or γ-irradiation causes apoptosis in Jurkat T cells and several other cell lines (*7–14*). Sphingosine upregulation also induces growth inhibition and apoptosis in several cell lines (*15–17*). On the other hand, sphingosine-1-phosphate is involved in the proliferation of 3T3 fibroblast cells induced by platelet-derived growth factor, and in the survival of PC12 pheochromocytoma cells induced by neuronal growth factor (*18, 19*). These findings indicate that the regulation of intracellular ceramide, sphingosine and sphingosine-1-phosphate is important for cell survival and cell death.

In addition to an increase in sphingolipids, a decrease in sphingolipids is also a signal for apoptosis. A sphingosine-like immunosuppressant, ISP-1, has been shown to be a potent inhibitor of serine palmitoyl transferase (SPT), which catalyzes the first step of sphingolipid biosynthesis. Treatment of cells with ISP-1 has been shown to be effective for inhibiting the overall *de novo* biosynthesis of sphingolipids, and, as a consequence, treated cells have a decreased level of intracellular sphingolipids (*20*). ISP-1 treatment induces apoptosis in a mouse cytotoxic T cell line, CTLL-2 (*21*) and rat Purkinje cells (*22*), and G2/M arrest in the mouse pro B cell line, LyD9 (*23*). Apoptosis in a temperature-sensitive SPT mutant of CHO cells is also induced when the cells are cultured at a non-permissive temperature (*24*). Inhibition of proliferation by ISP-1 has been observed in *Saccharomyces cerevisiae*, and the cloning of a serine/threonine kinase was found to overcome this inhibition (*25*).

Recently, many proteins have been shown to act on the apoptotic pathway. Among them, the cascade of members of

[1] This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (B-12140202), a Grant-in-Aid for Scientific Research on Priority Areas (A-12033206) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a Grant-in-Aid for Scientific Research (B-11557182) from the Japan Society for the Promotion of Sciences and a Grant-in-Aid for Scientific Research from the Welfide Medical Research Foundation.

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Abbreviations: FCS, fetal calf serum; IL-2, interleukin-2; SPT, serine palmitoyl transferase; FSC, forward light scattering; MCA, 7-amino-4-methylcoumarin; YVAD-MCA, acetyl-Tyr-Val-Ala-Asp-MCA; DEVD-MCA, acetyl-Asp-Glu-Val-Asp-MCA; YVAD-cho, acetyl-Tyr-Val-Ala-Asp-aldehyde; DEVD-cho, acetyl-Asp-Glu-Val-Asp-aldehyde; z-DEVD-fmk, carbobenzoxy-Asp-Glu-Val-Asp-fluoromethylketone; z-VAD-fmk, carbobenzoxy-Val-Ala-Asp-fluoromethylketone; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid; DTT, dithiothreitol; PMSF, α-phenyl-methylsulfonyl fluoride; ICE, interleukin-1β converting enzyme; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

the cysteine protease family, caspases, is thought to be indispensable for apoptosis to occur (*26–29*). Caspase-3, a member of this family, is the most downstream in the protease cascade, and the inhibition of caspase-3 by synthetic peptide inhibitors often prevents apoptosis induced by various stimuli. On the other hand, Bcl-2 and Bcl-xL are known to be apoptosis inhibitory proteins. They function as suppressors of mitochondrial dysfunction related to apoptosis (*30–32*), and inhibit the release of cytochrome *c* (*33, 34*) and AIF (*35*), apoptosis-inducing factor, from mitochondria.

In the case of sphingolipid-related apoptosis, exogenously added ceramide- or sphingosine-induced apoptosis has been reported to be dependent on caspase-3 (*36, 37*). Bcl-2 overexpression does not prevent sphingosine-induced apoptosis but does prevent ceramide-induced apoptosis (*38*). However, the mechanism underlying the apoptosis induced by ISP-1 has not been clarified yet.

In the present study, we examined the effects of caspase inhibitors, Bcl-2 and Bcl-xL, on the apoptotic pathway induced by ISP-1, and show that the ISP-1 induced apoptosis pathway is distinguishable from other sphingolipid-related apoptosis pathways.

MATERIALS AND METHODS

Materials—ISP-1 was obtained as described previously (*20*) and stored as a methanol solution at −20°C. Sphingosine and 7-amino-4-methylcoumarin (MCA) were purchased from Sigma. Recombinant human IL-2 was a generous gift from Yoshitomi Pharmaceutical Industries. YVAD-MCA, DEVD-MCA, YVAD-cho, and DEVD-cho were purchased from Peptide Institute Inc. z-DEVD-fmk was from MBL. z-VAD-fmk was from Enzyme Systems Products. Anti Bcl-2 was from Calbiochem. Anti Bcl-xL was from Transduction Laboratories. Anti Bax was from Upstate Biotechnology. JC-1 was from Molecular Probes.

Cell Culture—The mouse cytotoxic T cell line CTLL-2 (*39*) was obtained from the American Type Culture Collection. The cells were maintained in RPMI 1640 containing 10% FCS and 50 units/ml IL-2. For the induction of apoptosis, cells were cultured at either 2.5×10^4 cells/ml in the presence of 50 nM ISP-1 or 1.0×10^5 cells/ml in the presence of 5 μM sphingosine. Cell viability was determined by the erythrosine exclusion assay, in which more than 200 cells are counted and the viability is estimated (*21*). For flowcytometric analysis of apoptosis, the cells were stained with propidium iodide using a Cycle TEST™ PLUS DNA Reagent Kit (Becton Dickinson) as described previously (*21*), and then examined with a FACScan™ flowcytometer (Becton Dickinson).

Measurement of Caspase Activity—Cells were washed twice with phosphate-buffered saline (PBS) and then once with extraction buffer (50 mM PIPES-NaOH, pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 20 μM cytochalasin B, 1 mM PMSF, 50 μg/ml antipain). A cytosolic extract was prepared by sonication after two rounds of freeze-thawing in extraction buffer. Aliquots of the extract supernatant, each containing 12 μg protein, were diluted with ICE standard buffer (100 mM HEPES-KOH, pH 7.5, 10% sucrose, 0.1 mg/ml ovalbumin, 0.1% CHAPS, 10 mM DTT) and then incubated at 30°C for 30 min with 2 μM fluorogenic substrate. Caspase activity was measured with excitation at 380 nm and emission at 460 nm using an RF-1500 spectrofluorophotometer (Shimadzu).

DNA Transfection—The human Bcl-2- and chicken Bcl-xL-cDNA–containing plasmids were generous gifts from Dr. Y. Tsujimoto (Osaka University). The *Xho*I fragment containing human Bcl-2 cDNA was subcloned into the $pCXN_2$ vector (*40*). The *Eco*RI fragment containing chicken Bcl-xL cDNA was subcloned into the $pCXN_2$ vector. These constructs were transfected into CTLL-2 cells by electroporation. After 24 h, G418 (0.5 mg/ml) was added to the cells, and the clones were then isolated and analyzed for Bcl-2 overexpression with a flowcytometer using a specific antibody (Pharmingen), and for Bcl-xL by RT-PCR using specific primers.

Detection of Mitochondrial Depolarization—2×10^5 cells were suspended in 200 μl of medium with 5 μg/ml of JC-1, incubated at 37°C for 10 min, washed two times with PBS, and resuspended in 1 ml of PBS. Red and green fluorescence emission was analyzed with a FACScan™ flowcytometer.

RESULTS

Caspase-3–Like Protease Inhibitors Have No Effect on ISP-1–Induced Apoptosis—To determine the possible involvement of members of the caspase family in the apop-

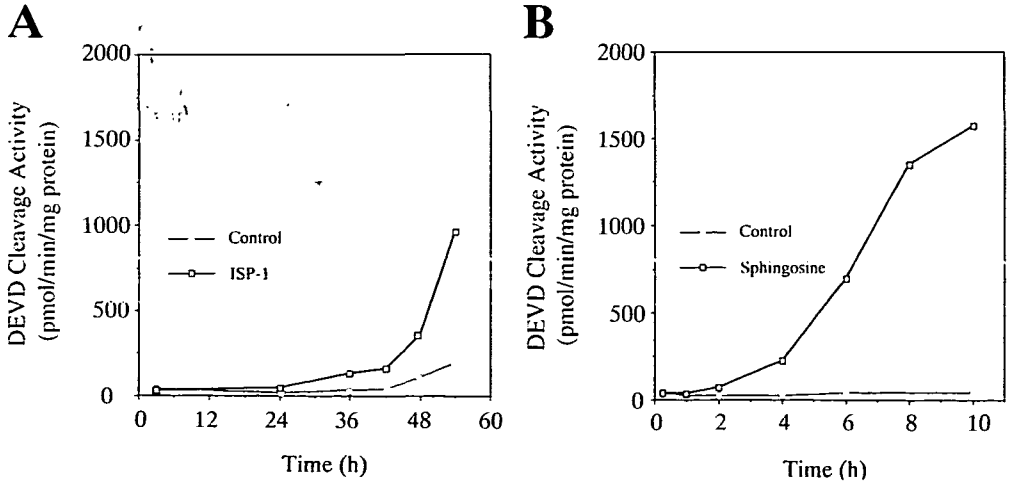

Fig. 1. **Increase of caspase-3–like activity in ISP-1– and sphingosine-treated CTLL-2 cells.** Cells were incubated with ISP-1 (A), or sphingosine (B) in order to induce apoptosis. After incubation, caspase-3–like activity was measured with a fluorescence substrate, DEVD-MCA, as described under "MATERIALS AND METHODS." At least three independent experiments were performed, each giving similar results. A representative example is shown in this figure.

totic pathway triggered by ISP-1, the activity of caspase-3–like protease, which is known to participate in most apoptosis pathways, was measured in lysates of CTLL-2 cells after treatment with ISP-1 using a specific tetrapeptide substrate, DEVD-MCA. The caspase-3–like protease activity started to increase rapidly after 42 h, although the activity in untreated cells also started to increase 54 h after mock treatment (Fig. 1A). The increased activity of the mock-treated cells was most likely induced by an insufficiency of growth factors or nutrients due to cell overgrowth, since cells exhibiting lower confluency did not show any apoptotic features (data not shown). The increase in the caspase-3–like activity caused by ISP-1 correlates with the elevated number of dead CTLL-2 cells (Figs. 1A and 3A). To demonstrate the involvement of caspase-3–like protease directly, the effects of caspase-3–like protease inhibitors on ISP-1–induced apoptosis were examined by flowcytometric analysis of hypodiploid nuclei and by detecting loss of viability. Surprisingly, the ISP-1–induced increases in both hypodiploid nuclei and loss of viability were not inhibited by DEVD-cho, a specific inhibitor of caspase-3–like protease, although z-VAD-fmk, an inhibitor with broad specificity, inhibited both (Figs. 2A and 3A). To exclude the possible degradation of DEVD-cho during incubation, the inhibitor was added repeatedly 0, 24, 36, and 44 h after the addition of ISP-1. Even with this inhibitor treatment, ISP-1–induced apoptosis was not inhibited (Figs. 2B and 3B). Furthermore, z-DEVD-fmk, an irreversible caspase-3–like protease inhibitor, had no effect on the apoptosis induced by ISP-1 (data not shown). In contrast, CTLL-2 cells in which apoptosis was induced by sphingosine, another form of sphingolipid-related apoptosis induction, showed increasing caspase-3–like protease activity (Fig. 1B), and the apoptosis was inhibited by both DEVD-cho and z-VAD-fmk (Figs. 2C and 3C). This is consistent with the case of sphingosine-induced apoptosis in HL60 cells (*37*). These results suggest that a pathway dependent on caspase(s) other than caspase-3–like protease is involved in CTLL-2 apoptosis induced by ISP-1.

Bcl-2 and Bcl-xL Prevent ISP-1–Induced Apoptosis—Bcl-

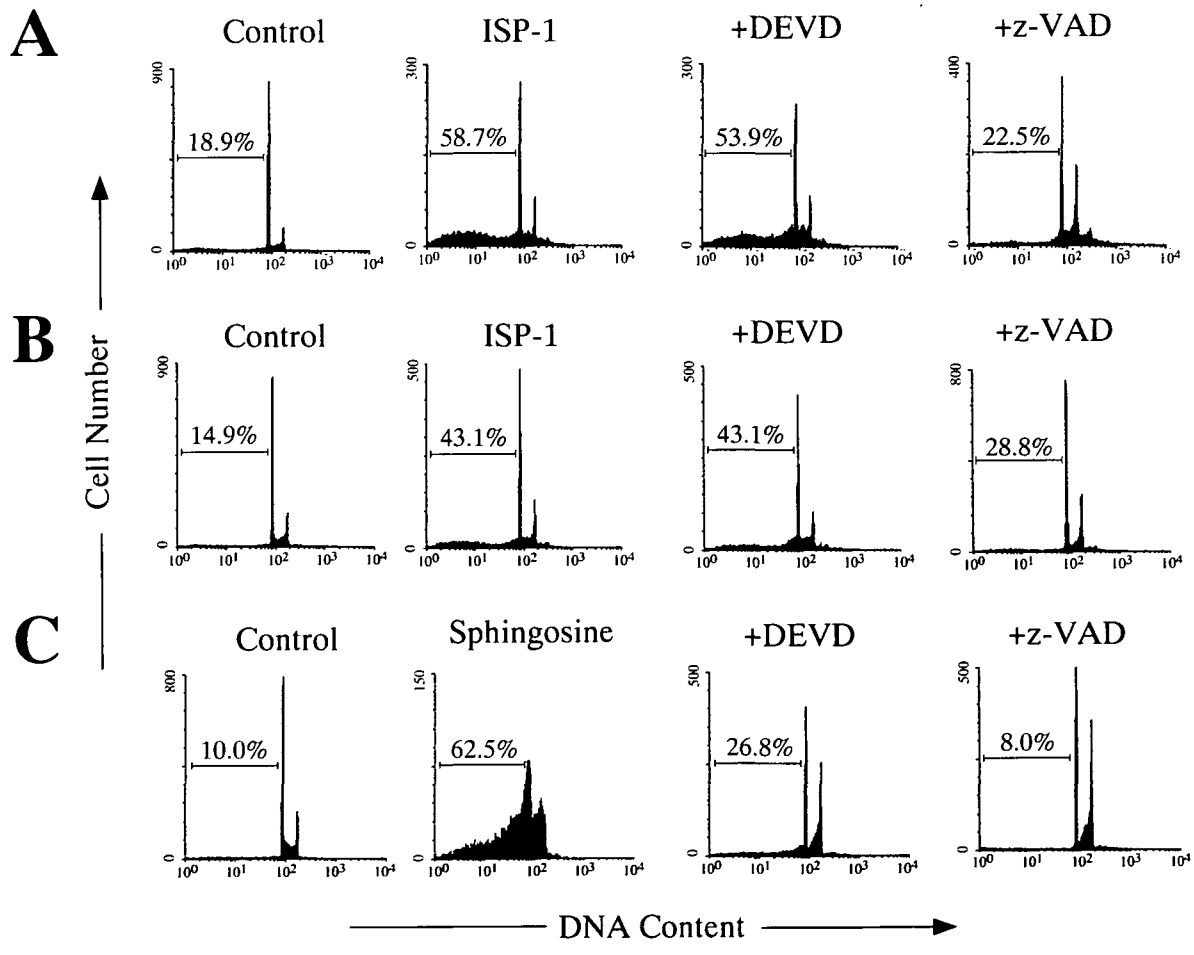

Fig. 2. **Effects of caspase inhibitors on ISP-1– and sphingosine-induced hypodiploid DNA peaks in CTLL-2 cells.** (A) Cells were incubated with ISP-1 for 52 h, and a caspase inhibitor (200 μM) was added to the cells 38 h after the addition of ISP-1. Similar results were obtained when the caspase inhibitor was added 0 h or 24 h after the addition of ISP-1. (B) Cells were incubated with ISP-1 for 52 h. For treatment with YVAD-cho or DEVD-cho, the inhibitor (100 μM) was added successively at 0, 24, and 36 h after the addition of ISP-1, with an extra addition (200 μM) after 44 h to give a final inhibitor concentration at 500 μM. In the case of z-VAD-fmk, the inhibitor (40 μM) was added at 0, 24, and 36 h after the addition of ISP-1, with an extra addition (80 μM) after 44 h to give a final concentration at 200 μM. (C) Cells were preincubated with a caspase inhibitor (200 μM) for 1 h and then treated with sphingosine for 9 h. After incubation, the cells were stained with propidium iodide and analyzed as described under "MATERIALS AND METHODS."

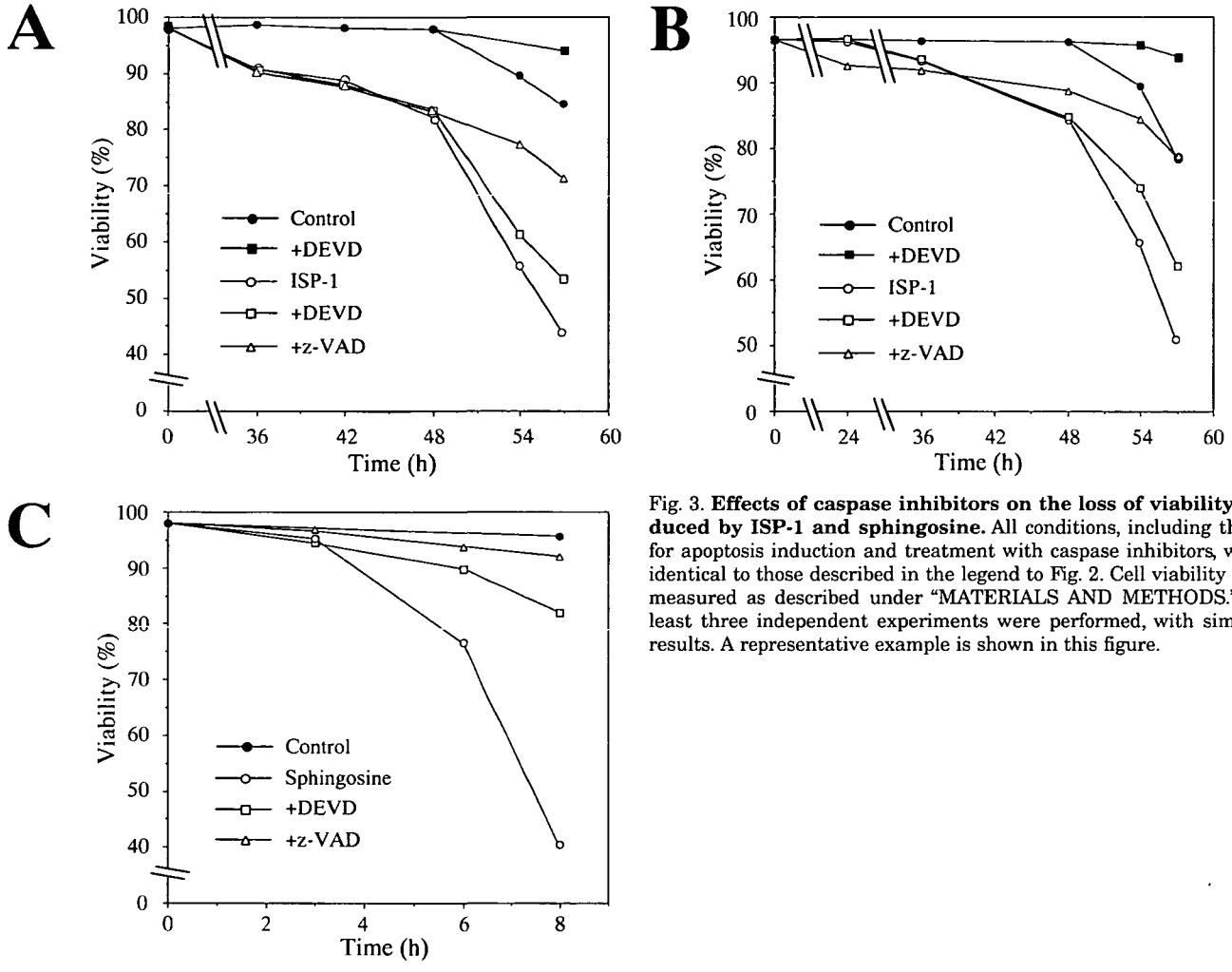

Fig. 3. **Effects of caspase inhibitors on the loss of viability induced by ISP-1 and sphingosine.** All conditions, including those for apoptosis induction and treatment with caspase inhibitors, were identical to those described in the legend to Fig. 2. Cell viability was measured as described under "MATERIALS AND METHODS." At least three independent experiments were performed, with similar results. A representative example is shown in this figure.

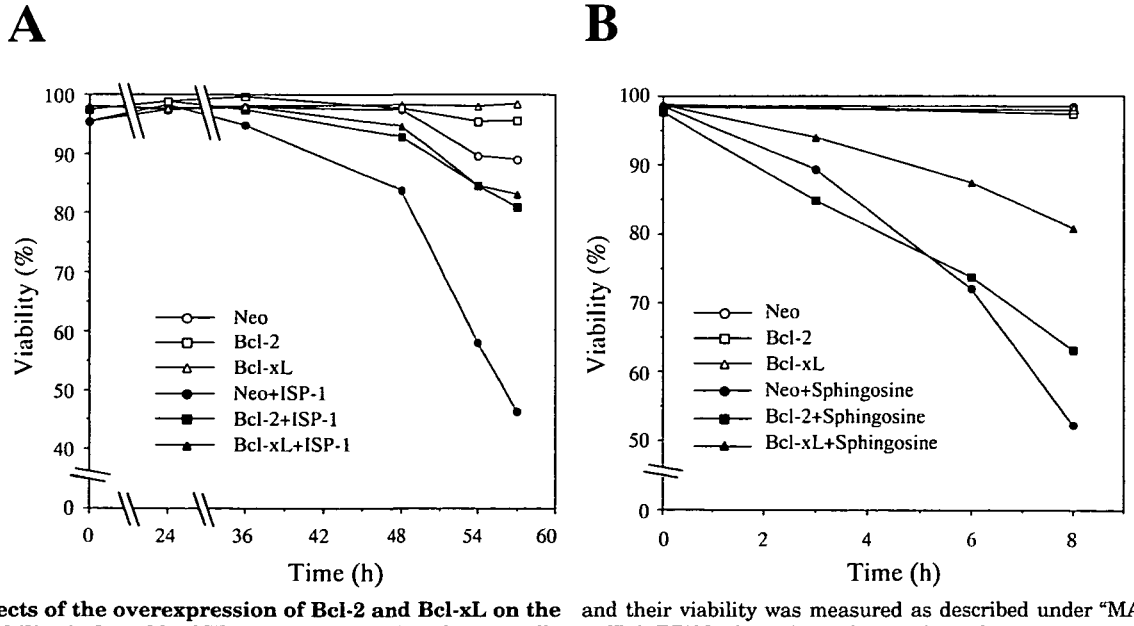

Fig. 4. **Effects of the overexpression of Bcl-2 and Bcl-xL on the loss of viability induced by ISP-1 and sphingosine.** CTLL-2 cells overexpressing Bcl-2 or Bcl-xL and vector transfectants (Neo) were incubated with ISP-1 (A) or sphingosine (B) for the indicated times, and their viability was measured as described under "MATERIALS AND METHODS." At least three independent experiments were performed, with similar results. A representative example is shown in this figure.

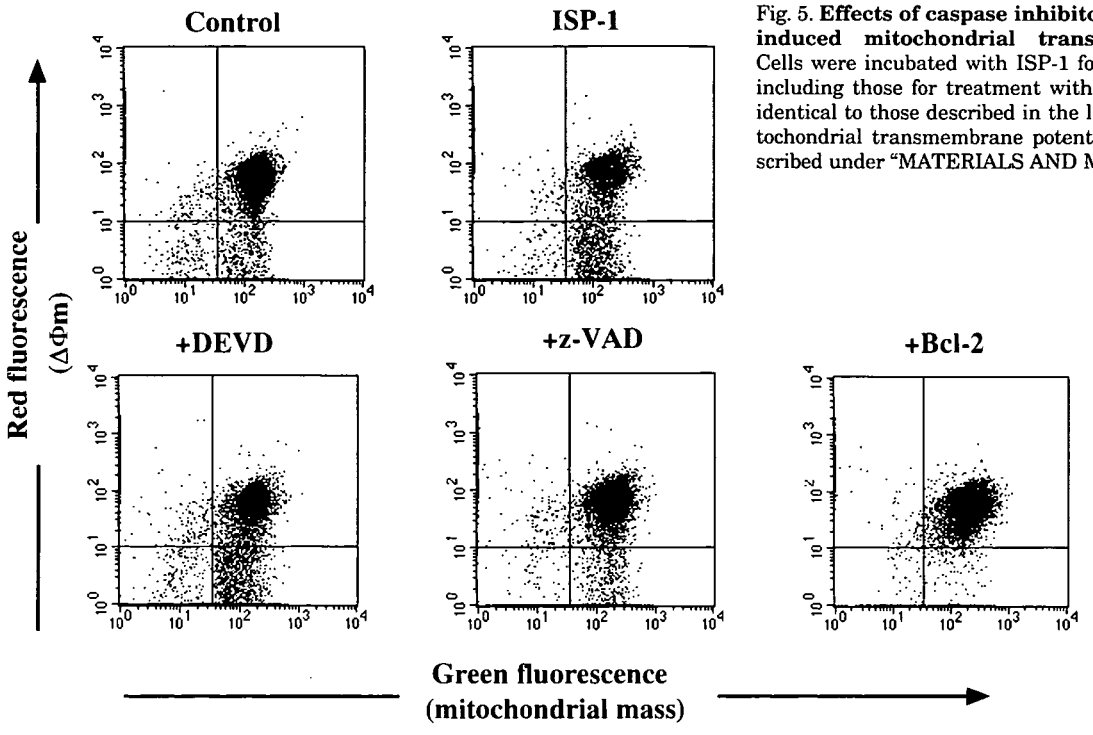

Fig. 5. **Effects of caspase inhibitors and Bcl-2 on ISP-1–induced mitochondrial transmembrane potential.** Cells were incubated with ISP-1 for 51 h. Other conditions, including those for treatment with caspase inhibitors, were identical to those described in the legend to Fig. 2B. The mitochondrial transmembrane potential was measured as described under "MATERIALS AND METHODS."

2 and Bcl-xL are members of the Bcl-2 family, which is known to inhibit many types of apoptosis. To determine the effects of Bcl-2 and Bcl-xL, stable transfectants of CTLL-2 cells overexpressing either Bcl-2 or Bcl-xL were developed and treated with ISP-1 or sphingosine. As shown in Fig. 4A, Bcl-2 and Bcl-xL overexpressing transfectants exhibited marked resistance to ISP-1. Although Bcl-xL expression significantly prevented the apoptosis induced by sphingosine, Bcl-2 expression did not effectively inhibit sphingosine-induced apoptosis until at least 8 h (Fig. 4B), which is consistent with the case of sphingosine-induced apoptosis of the leukemic cell line, TF1 (*38*).

ISP-1–Induced Mitochondrial Depolarization—The mitochondrial depolarization that occurs during apoptosis induced by ISP-1 was measured using the cationic carbocyanine dye JC-1. JC-1 normally exists as a monomer emitting green fluorescence at low concentration, but its potential-driven mitochondrial accumulation results in a dimeric configuration emitting red fluorescence. Therefore, the red fluorescence emission of JC-1 can be used to determine mitochondrial membrane potential (*41*). As shown in Fig. 5, a decrease in the red fluorescence emitted by JC-1 was observed in ISP-1–treated CTLL-2 cells, suggesting that ISP-1–induces mitochondrial depolarization. Mitochondrial depolarization is known to be a checkpoint in many types of apoptosis, and the apoptosis pathway can be divided into those parts downstream and upstream of depolarization. The mitochondrial depolarization induced by ISP-1 is resistant to DEVD-cho but sensitive to z-VA-fmk (Fig. 5). Bcl-2 overexpression also prevents mitochondrial depolarization (Fig. 5). These results suggest that the upstream stage is dependent on caspase(s) other than caspase-3–like protease, although the mechanism of the downstream stage has not been determined.

DISCUSSION

In this study, the apoptosis of CTLL-2 cells induced by an immunosuppressant, ISP-1, was characterized. ISP-1–induced apoptosis is not inhibited by caspase-3–like protease specific inhibitors, although Bcl-2 protects the cells from apoptosis, in sharp contrast to the case of apoptosis induced by sphingosine, which is caspase-3–like protease–dependent and is not effectively inhibited by Bcl-2. These results indicate that ISP-1 and sphingosine induce apoptosis in different ways, although their structures are similar (*20*). In our previous report (*21*), we postulated that ISP-1 and sphingosine induce apoptosis through downregulation and upregulation of sphingolipids, respectively, a hypothesis consistent with the results of this study. The sensitivity to caspase-3–like protease inhibitor also distinguishes ISP-1–induced apoptosis from ceramide-induced apoptosis, another sphingolipid-related form of apoptosis (*36*).

ISP-1–induced apoptosis is independent of caspase-3–like protease but dependent on some other caspase(s) because a caspase inhibitor with broad specificity inhibits apoptosis. Furthermore, the pathway dependent on caspase(s) other than caspase-3–like protease is located upstream of mitochondrial depolarization. However, the pathway upstream of depolarization is usually caspase-independent, except in Fas- and TNFR-mediated apoptosis. Fas-mediated mitochondrial depolarization is known to be dependent on caspase-8, which promotes the cleavage of the proapoptotic protein Bid, leading to a mitochondrial loss of function (*42–44*). Caspase-8–like protease(s) might, therefore, be involved upstream of the depolarization induced by ISP-1.

The pathway downstream of the ISP-1–induced mitochondrial depolarization was not characterized in this

study. However, it can be assumed that the downstream pathway is either dependent on caspase(s) other than caspase-3–like protease or caspase-independent, because both the whole pathway and the upstream stage are dependent on caspase(s) other than caspase-3–like protease. Apoptosis-inducing factor (AIF) is a 50 kDa protein associated with the mitochondrial inner space that is released during mitochondrial depolarization (*35*). AIF is sufficient to induce phenomena typical of nuclear apoptosis in a cell-free and cytosol-free system in a caspase-independent manner. AIF might, therefore, be involved downstream of depolarization in ISP-1–induced apoptosis, if the pathway downstream of the ISP-1–induced mitochondrial depolarization is caspase-independent. Mitochondrial depolarization leads to the release of cytochrome *c* as well as AIF (*33*, *34*). Cytochrome *c* has the ability to activate caspase-3 (*45*). The increase in the activity of caspase-3–like protease, which occurs concomitant with apoptosis induced by ISP-1, may be due to cytochrome *c* released from mitochondria. Another candidate for the apoptosis mediator of the downstream pathway is Bax, which is a member of the Bcl-2 family and acts as a proapoptotic protein. Bax inhibits the anti-apoptotic action of Bcl-2 (*46*), and Bax itself causes apoptosis in a caspase-independent manner (*47*). However, no significant difference in the intracellular levels of Bax was found between control and ISP-1–treated cells (data not shown).

Although most apoptosis pathways are known to be caspase-3–dependent, a few have been shown to be independent. Apoptosis induced by IL-3 deprivation in murine leukemic cell line 32D is inhibited by z-VAD-fmk, but not by z-DEVD-fmk (*48*). DEVD-cho has no effect on apoptosis induced by buprenorphine hydrochloride in NG108-15 nerve cells (*49*). Furthermore, apoptosis induced by exogenous NO donors in RES cells, which are RAW 264.7 macrophages resistant to apoptosis induced by inducible NO synthase, is not inhibited by DEVD-cho despite increasing caspase-3–like activity during apoptosis (*50*), in close correspondence with the case of ISP-1–induced apoptosis. Analyses of caspase-3–deficient mice have demonstrated that their thymocytes are still sensitive to several apoptotic stimuli, although the apoptosis of neuronal cells is prevented (*51*). Caspase-3 substrates, including PARP and DFF45, are cleaved even in MCF-7 cells, which have a functional deletion of the caspase-3 gene, when the cells are stimulated with TNF-α and staurosporin (*52*). Under these conditions, other DEVD-sensitive caspases, such as caspases-2 and -7, are not activated, implying that protease(s) other than caspase-3 exhibit are able to cleave these substrates (*52*).

Thus, ISP-1–induced apoptosis of CTLL-2 cells is distinct from sphingosine- or ceramide-induced apoptosis. However, it remains to be determined which sphingolipids are directly involved in the apoptosis induced by ISP-1.

We would like to thank Dr. T. Fujita (Setsunan University) for providing us with the ISP-1, Dr. J. Miyazaki (Osaka University) for the pcXN2 vector, and Yoshitomi Pharmaceutical Industries Ltd. for the recombinant human IL-2.

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