Dual Roles of Sphingolipids in Signaling of the Escape from and Onset of Apoptosis in a Mouse Cytotoxic T-cell Line, CTLL-2

(Received for publication, October 30, 1995, and in revised form, November 27, 1995)

Sachiko Nakamura, Yasunori Kozutsumi, Yidi Sun, Yukie Miyake, Tetsuro Fujita, and Toshisuke Kawasaki

From the Department of Biological Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyoku, Kyoto 606, Japan and the Department of Pharmaceutical Manufacturing Chemistry, Faculty of Pharmaceutical Sciences, Setsunan University, Nagato, Hirakata, Osaka 573-01, Japan

In our previous study, the sphingosine-like immunosuppressant, ISP-1, was found to suppress the proliferation of an interleukin-2-dependent cytotoxic T cell line, CTLL-2, through the inhibition of serine palmitoyltransferase, which catalyzes the committed step of sphingolipid biosynthesis. Analysis of the effect of ISP-1 by flow cytometry revealed that the ISP-1-dependent decrease in cell number was not due to inhibition of the cell cycle progression of CTLL-2 cells but to the induction of apoptosis of the cells. The ISP-1-induced apoptosis was inhibited by the addition of sphingosine (2 μM), suggesting that this ISP-1-induced apoptosis is triggered by the decrease in the intracellular levels of sphingolipids caused by the inhibition of serine palmitoyltransferase. However, another interleukin-2-dependent cell line, F7, which was derived from a mouse pro-B cell line, did not show ISP-1-dependent apoptosis, indicating that the effect of ISP-1 may be specific for a certain type of T cell lineage such as CTLL-2. On the other hand, a high dose of sphingosine (5 μM) by itself induced the apoptosis of CTLL-2 cells. This sphingosine-dependent apoptosis was also observed with F7 cells. These results provide evidence that the intracellular levels of sphingolipids play an important role in the signaling of the escape from and onset of apoptosis of CTLL-2 cells.

Several lines of evidence have suggested that sphingosines are involved in apoptosis. Exogenously added cell-permeable ceramides induce the apoptosis of U937 human leukemia cells (1) and several other cell lines (2). Later, a natural ceramide was shown to induce apoptosis when added as a cell-permeable form in a mixture of dodecane and ethanol (3). Furthermore, the level of intracellular ceramide was shown to increase in the cases of tumor necrosis factor-α-induced apoptosis of human neutrophils (11) and phorbol ester-induced apoptosis of HL-60 cells (12). A new potent immunosuppressant, ISP-1, is a fungal product that has a structure very similar to that of sphingosine (13). ISP-1 inhibited the mouse allogenic mixed lymphocyte reaction and allo-reactive cytotoxic T lymphocyte generation in vivo, exhibiting a potency of 10–100-fold greater than that of cyclosporin A, the most widely used immunosuppressant. Unlike cyclosporin A and FK506 (another popular immunosuppressant), ISP-1 did not interfere with IL-2 production in the mixed lymphocyte reaction (13), but instead it suppressed the IL-2-dependent growth of the mouse cytotoxic T cell line, CTLL-2 (14). This suppression is most probably triggered by a reduction of the intracellular levels of sphingolipids due to the inhibition of serine palmitoyltransferase, which catalyzes the first step of sphingolipid biosynthesis, i.e. the condensation of serine and palmitoyl-CoA to ketodihydrosphingosine (14).

In this paper, we report that the overall growth suppression of CTLL-2 cells by ISP-1 is not due to inhibition of cell proliferation but to the apoptosis of the cells caused by inhibition of the de novo synthesis of sphingosine. This is the first evidence that apoptosis is caused by a reduction in the intracellular levels of sphingolipids.

EXPERIMENTAL PROCEDURES

Materials—ISP-1 was obtained as described previously (14) and stored as a methanol solution at −20 °C. Recombinant human IL-2 was kindly provided by Yoshitomi Pharmaceutical Industries, Tokyo. All other biological reagents were purchased from Sigma.

Cells and Cell Culture—The mouse cytotoxic T cell line, CTLL-2 (15), was obtained from the American Type Culture Collection. The F7 cell line (16) was kindly provided by Dr. T. Taniguchi, Tokyo University, Tokyo. These cells were maintained in RPMI 1640 containing 10% fetal calf serum and 50 units/ml IL-2. The administration of ISP-1 and sphingosine to the cells was performed as described previously (14).

Cell Proliferation Assay—Cells were cultured at 5 × 10^3 cells in 500 μl of medium in the presence or absence of ISP-1. After incubation for 48 h, the viable cell number was determined by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (17). The absorbance increased in proportion to the viable cell number, within the range examined in this experiment.

Flow Cytometry Analysis—Cells were cultured as indicated, harvested, stained with propidium iodide using a Cyte TEST™ PLUS DNA Reagent Kit (Becton Dickinson), and then subjected to flow cytometry with a FACScan™ flow cytometer (Becton Dickinson) for measurement of the cell cycle progression and apoptosis.

DNA Fragmentation Analysis—DNA fragmentation was analyzed by agarose gel electrophoresis as described by Selens and Cohen (18). Briefly, fragmented genomic DNA was extracted by incubating cells in 10 mM Tris-HCl buffer, pH 7.4, containing 10 mM EDTA and 0.5% Triton X-100 at 4°C for 10 min. Under these conditions, intact genomic DNA was not extracted. The cell suspension was centrifuged, and the resultant supernatant was incubated for 1 h at 37°C in the presence of RNase (400 μg/ml) and then for an additional 1 h in the presence of proteinase K (400 μg/ml). The fragmented DNA was precipitated with isopropl alcohol and then analyzed by agarose gel electrophoresis.

Cell Morphology—For morphological analysis, cells were fixed with 1% glutaraldehyde overnight and then collected by centrifugation and resuspended in phosphate-buffered saline. The cell nuclei were stained
effect of sphingosine, a compound located downstream of ceramide, which inhibits the conversion of sphingosine to ceramide (24), which is the direct trigger of the apoptosis of CTLL-2 cells, the effect of sphingosine on cell cycle progression of CTLL-2 cells significantly but instead dramatically increased the hypodiploid DNA peaks, which are commonly found in apoptosis (19). In order to confirm that the cells were indeed the process of apoptosis, the chromosomal DNA profiles were investigated. As shown in Fig. 2A, lane 2, genomic DNA obtained from ISP-1-treated cells gave a ladder on agarose gel electrophoresis, which is a characteristic of apoptosis specific internucleosomal DNA fragmentation (20), while this fragmentation was not seen in mock-treated cells (lane 1). Further confirmation of apoptosis was the morphological changes of nuclei detected in ISP-1-treated cells. As shown in Fig. 3B, ISP-1-treated CTLL-2 cells showed morphological changes typical of apoptosis including condensed chromatin and fragmented nuclei revealed upon DNA staining with Hoechst 33342 (160 μM) and then examined under a fluorescent microscope (Olympus BX 50).

RESULTS AND DISCUSSION

Although ISP-1 was demonstrated to be a potent inhibitor of serine palmitoyltransferase, which catalyzes the first step of sphingolipid biosynthesis, i.e. the condensation of serine and palmitoyl-CoA into ketohexosamine (14), the mechanism underlying the inhibition of cell growth by ISP-1 was not clear. In an attempt to elucidate the mechanism underlying the suppression of CTLL-2 cell growth by ISP-1, the effect of ISP-1 on cell cycle progression was investigated by the flow cytometric method. As shown in Fig. 1B, incubation of the cells in the presence of 47 nM ISP-1 for 48 h did not affect the cell cycle progression of CTLL-2 cells significantly but instead dramatically increased the hypodiploid DNA peaks, which are commonly found in apoptosis (19). In order to confirm that the cells were indeed the process of apoptosis, the chromosomal DNA profiles were investigated. As shown in Fig. 2A, lane 2, genomic DNA obtained from ISP-1-treated cells gave a ladder on agarose gel electrophoresis, which is a characteristic of apoptosis specific internucleosomal DNA fragmentation (20), while this fragmentation was not seen in mock-treated cells (lane 1). Further confirmation of apoptosis was the morphological changes of nuclei detected in ISP-1-treated cells. As shown in Fig. 3B, ISP-1-treated CTLL-2 cells showed morphological changes typical of apoptosis including condensed chromatin and fragmented nuclei revealed upon DNA staining with Hoechst 33342. All these results indicated that the ISP-1 dependent decrease in the number of CTLL-2 cells was not due to inhibition of the proliferation of CTLL-2 cells but to the acceleration of cell death by apoptosis.

In order to determine whether or not inhibition of the condensation of serine and palmitoyl-CoA into ketohexosamine is the direct trigger of the apoptosis of CTLL-2 cells, the effect of sphingosine, a compound located downstream of ketohexosamine biosynthesis, was investigated. Although sphingosine is a potent inhibitor of ceramide synthase (21–23), the inhibition of cell growth by ISP-1 is caused by the ISP-1 action (14), suggesting that ceramide is not the downstream effector that is directly involved in apoptosis. However, Fumonisin B1, which inhibits the conversion of sphingosine to ceramide (24), did not inhibit the effect of exogenously added sphingosine on the ISP-1 action (14), suggesting that ceramide is not the downstream effector and that sphingosine derivatives with a
Regulation of Apoptosis by Sphingolipids

Procedures.

through the IL-2 receptor and the function of ISP-1. However, the possibility that F7 cells have a specific ability to incorporate sphingolipids from the fetal calf serum in the culture medium could not be ruled out. Since the proliferation of stimulated T-lymphocytes was inhibited by ISP-1 (14), we then tried to identify the type of cells that is susceptible to ISP-1. When mouse splenic T cells were stimulated with concanavalin A in the presence or absence of ISP-1, CD4-negative and CD8-positive cells were more sensitive to ISP-1 than CD4-positive and CD8-negative cells (data not shown). Taken together, ISP-1-induced apoptosis is not specific for IL-2-dependent cells but may be a characteristic of a CD8-positive and CD4-negative cytotoxic T cell lineage. In sharp contrast to the cell type-specific expression of ISP-1-induced apoptosis, sphingosine-induced apoptosis is observed in many cell types, including Chinese hamster ovary cells (27), human neutrophils (11), and HL-60 cells (12), as well as the CTL-2 and F7 cells examined in this study. The effect of sphingosine may be associated with the protein kinase C inhibitory activity of sphingosine (27), because dimethylsphingosine, which is also a protein kinase C inhibitor, induced the apoptosis of CTL-2 cells (data not shown), as reported in human neutrophils (11) and HL-60 cells (12). However, the detailed mechanism of the involvement of protein kinase C in the sphingosine-induced apoptosis remains to be elucidated.

Acknowledgments—We thank Yoshitomi Pharmaceutical Industries, Ltd. for providing the recombinant human IL-2 and Dr. T. Taniguchi of Tokyo University for the F7 cell line.

REFERENCES

1. Obeid, L. M., Linardic, C. M., Karolak, L. A., and Hannun, Y. A. (1993) Science 259, 1769–1771
2. Jarvis, W. D., Kolesnick, R. N., Fornari, F. A., Traynor, R. S., Gewirtz, D. A., and Grant, S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 73–77
3. Ji, L., Zhang, G., Umematsu, S., Akahori, Y., and Hirokawa, N. (1995) FEBS Lett. 358, 211–214
4. Schütze, S., Hämmerle, M., Machleidt, T., Berkovic, D., Wiegmann, K., and Kronke, M. (1992) Cell 71, 765–776
5. Wiegmann, K., Schütze, S., Kampen, E., Hämmerle, M., Machleidt, T., and Kronke, M. (1992) J. Biol. Chem. 267, 17997–18001
6. Yang, Z., Costanzo, M., Golde, D. W., and Kolesnick, R. N. (1993) J. Biol. Chem. 268, 20520–20523
7. Hrenda-Friedman, A., Kan, C. C., Ehleiter, D., Persaud, R. S., McLoughlin, M., Fuku, Z., and Kolesnick, R. N. (1994) J. Exp. Med. 160, 525–535
8. Cifone, M. G., DeMaria, R., Roncaolì, P., Rippo, M. R., Azuma, M., Lanier, L. L., Santoni, A., and Testi, R. (1993) J. Exp. Med. 177, 1547–1552
9. Gill, B., Nishikata, S., Chanc, G., Detwolch, T. L., and Ochi, A. (1994) Immunol. Rev. 142, 113–126
10. Uematsu, S., Akahori, Y., and Hirabayashi, Y. (1995) Cancer Res. 55, 691–697
11. Fujita, T., Inoue, K., Yamamoto, S., Ikumoto, T., Sasaki, S., Toyama, R., Yoneda, M., Hoshino, Y., and Okumoto, T. (1994) J. Antibiot. (Tokyo) 47, 208–213
12. Miyake, Y., Zou, C. Y., Nakanuma, S., Fujita, T., and Kawasaki, T. (1994) Biochem. Biophys. Res. Commun. 212, 396–403
13. Gillis, S., and Smith, R. S. (1987) Cell 50, 154–164
14. Shibuya, H., Yonezawa, M., Nishimiyab-Tsui, J., Matsumoto, K., and Taniguchi, T. (1992) Cell 70, 57–67
15. Selle, K., Ishihara, N., Oishi, M., and Kase, H. (1993) J. Immunol. Methods 164, 255–261
16. Selis, K. S., and Cohen, J. J. (1987) J. Immunol. 139, 3199–3206
17. Migliorati, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. (1991) J. Immunol. Methods 139, 271–279
18. Gunji, H., Hase, R., and Kufe, D. (1992) Clin. Invest. 99, 591–596
19. Merrill, A. H., Nimkar, S., Nalunak, D., Hannun, Y. A., Loomis, C. B., Bell, R. M., Tyagi, S. R., Lambicki, D., Stevens, V. L., Hunter, R., and Liotta, D. C. (1989) Biochemistry 28, 3318–3345
20. Kahan, W. A., Dobkowski, R., Tonn, Y., and Hannum, Y. A. (1990) Biochem. Biophys. Res. Commun. 172, 683–693
21. Igarashi, Y., Kitamura, K., Toyokuni, T., Dean, B., Fenderson, B., Ogawa, T., and Hakomori, S. (1990) J. Biol. Chem. 265, 5385–5396
22. Sinha, V. L., Owens, N. E., Winton, E. F., Kinkade, J. M., and Merrill, A. H., Jr. (1994) J. Biol. Chem. 269, 3475–3491
23. Wiegmann, K., Schütze, S., Kampen, E., Hämmerle, M., Machleidt, T., and Kronke, M. (1992) J. Biol. Chem. 267, 17997–18001
24. Schroeder, J. J., Crane, H. M., Xia, J., Liotta, D. C., and Merrill, A. H., Jr. (1991) J. Immunol. Methods 139, 271–279
25. Sinha, V. L., Owens, N. E., Winton, E. F., Kinkade, J. M., and Merrill, A. H., Jr. (1990) Biochem. Biophys. Acta 1051, 37–45

Fig. 4. Flow cytometry analysis of propidium iodide-stained F7 cells. Cells were cultured with the methand vehicle (A), 47 nM ISP-1 (B), or 5 μM sphingosine (C) for 48 h (A and B) or 8 h (C). After treatment, the cells were analyzed as described under “Experimental Procedures.” Bar 1, apoptotic cells; bar 2, G0/G1 phase cells; bar 3, S phase cells; bar 4, G2/M phase cells.