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Regular Paper

Effects of Ceramide Inhibition on Radiation-induced Apoptosis in Human Leukemia MOLT-4 Cells

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Ceramide/Fumonisin B1/MOLT-4/Sphingomyelinase/X irradiation.

In the present study, using inhibitors of ceramide synthase (fumonisin B_1), ketosphinganine synthetase (L-cycloserine), acid sphingomyelinase (D609 and desipramine) and neutral sphingomyelinase (GW4869), the role of ceramide in X-ray-induced apoptosis was investigated in MOLT-4 cells. The diacylglycerol kinase (DGK) assay showed that the intracellular concentration of ceramide increased time-dependently after X irradiation of cells, and this radiation-induced accumulation of ceramide did not occur prior to the appearance of apoptotic cells. Treatment with D609 significantly inhibited radiation-induced apoptosis, but did not inhibit the increase of intracellular ceramide. Treatment with desipramine or GW4869 prevented neither radiation-induced apoptosis nor the induced increase of ceramide. On the other hand, fumonisin B_1 and L-cycloserine had no effect on the radiation-induced induction of apoptosis, in spite of significant inhibition of the radiation-induced ceramide. From these results, it was suggested that the increase of the intracellular concentration of ceramide was not essential for radiation-induced apoptosis in MOLT-4 cells.

INTRODUCTION

It is important to understand the pathways responsible for cell death by ionizing radiation in the light of its use as an anticancer agent or a diagnostic tool. Several cellular pathways are known to exist in radiation-induced apoptosis depending on the cell type.[1–3] It has been shown that a large number of genotoxic stimuli and cellular stress elevate the intracellular level of ceramide.[4–7] The mechanisms for the elevation of ceramide has been described to involve two major metabolic routes; *de novo* synthesis from palmitoyl-CoA and hydrolysis of sphingomyelin via acid and neutral sphingomyelinases[6] (Fig. 1). Fumonisin B_1 and L-cycloserine are known to be inhibitors of ceramide synthase and ketosphinganine synthetase, respectively. D609 and desipramine are known to be acid sphingomyelinase inhibitors and GW4869 is a neutral sphingomyelinase inhibitor. The ceramide induced by cytotoxic stimuli has been generally considered to be an intermediator for apoptosis, since lymphoblastic cells from patients with Niemann-Pick disease, an inherited deficiency in acid sphingomyelinase, and cells from acid sphingomyelinase-deficient mice exhibit a defect in radiation-induced apoptosis.[8] Loss of the ceramide generation from neutral sphingomyelinase also confers resistance to radiation-induced apoptosis.[9] Furthermore, *de novo* ceramide generation contributes to etoposide-induced apoptosis in MOLT-4 cells.[10] In contrast, the contribution of ceramide to the apoptotic signaling triggered by TNF and Fas ligand is still uncertain. De Maria *et al*. have shown that acid sphigomyelinase is required for Fas-mediated apoptosis in lymphoblastic cells from patients with Niemann-Pick disease,[11] but Cock *et al*. have found that acid sphigomyelinase is not required.[12] In the data obtained by using inhibitors of sphingomyelinases and *de novo* ceramide synthesis, there is no relationship between cellular ceramide generation and the extent of Fas- or TNF-induced apoptosis in U937 and Jurkat cells.[13]

Our previous paper reported that the radiation-induced apoptosis in MOLT-4 cells depended on the expression of Fas, followed by the subsequent formation of DISC (death-inducing signaling complex) and the activation of caspase-3.[14] We also showed that the induction of apoptosis by treatment of MOLT-4 cells with cell-permeable ceramide analogs 5 μM C2 ceramide and 50 μM C_6 ceramide, was quite low when compared with that by 7.5 Gy of X-rays and that the inhibition of *de novo* ceramide synthesis by fumonisin B_1

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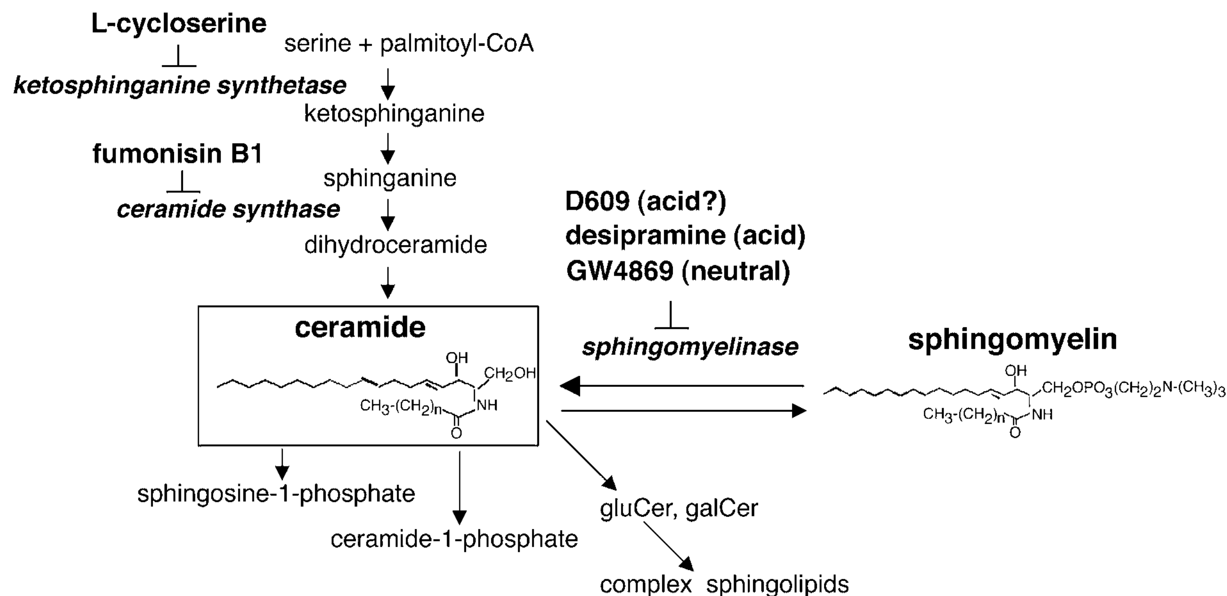

Fig. 1. Two pathways of ceramide generation. *De novo* ceramide synthesis and hydrolysis of sphingomyelin via acid and neutral sphingomyelinases.

had no effect on the induction of apoptosis by X irradiation.[15] These results prompted us to examine whether ceramide-related signal transduction pathways are involved in the death receptor-mediated apoptosis in MOLT-4 cells exposed to X rays.

In the present study, we undertook to elucidate the role of ceramide in the induction of apoptosis in irradiated MOLT-4 cells by comparing changes in both apoptosis and intracellular ceramide concentration after application of the metabolic ceramide inhibitors such as fumonisin B_1 (an inhibitor of ceramide synthase), L-cycloserine (an inhibitor of ketosphinganine synthetase), D609 and desipramine (inhibitors of acid sphingomyelinase) and GW4869 (an inhibitor of neutral sphingomyelinase). In addition, the changes in the ceramide concentrations before and after X irradiation were measured.

MATERIALS AND METHODS

Cell culture and regents

A human leukemia cell line, MOLT-4 (RIKEN Cell Bank, Tsukuba, Japan) was cultured in RPMI 1640 medium with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2-air. Fumonisin B_1, L-cycloserine, D609 and desipramine were purchased from Sigma (St. Louis, MO) and GW4869 from Calbiochem (San Diego, CA). Reagents for the assay of diacylglycerol kinase (DGK) were purchased as follows: Easytide™ solution of [γ-^{32}P]ATP (3000 Ci/mmol) from New England Nuclear (Boston, MA), *E. coli* diacylglycerol kinase (DGK) and *n*-octyl–β-D-glucopyranoside (β-OG) from Calbiochem, L–α-dioleoylphosphatidylglycerol (DOPG) from Avanti Polar Lipids (Alabaster, AL) and silica gel 60 TLC plates from Merck (Darmstadt, Germany).

Fluorescence microscopic observation of apoptotic cells

X irradiation was performed with a Shimazu HF-320 X-ray generator at 200 kVp and 20 mA with a 2.0 mm Al filter at a dose rate of 3.0 Gy/min, which was determined using Fricke's chemical dosimeter. Cells were treated with 50 µM fumonisin B_1, 300 µM L-cycloserine, 50 µg/ml D609, 10 µM desipramine or 1.43 µM GW4869 30 min before X irradiation at 37°C. GW4869 was used by solubilizing its suspension in dimethyl sulfoxide with 5% methane sulfonic acid.[16] After the cells were incubated with medium containing each drug for the indicated period of time, they were collected and washed in $Ca^{2+}Mg^{2+}$-free PBS [PBS(–)], and fixed with 1% glutaraldehyde/PBS and stained with 40 µg/ml propidium iodide (PI) for 15 min in the dark. Apoptotic cells with morphological changes such as fragmentation and condensation of chromatin were counted in at least 300 cells, using an Olympus BX50 microscope (Tokyo, Japan) with reflected-light fluorescence.

Detection of apoptotic cells by annexin V-FITC/propidium iodide (PI) flow cytometry

After cells were treated with drugs and X-irradiated as described above, they were collected by centrifugation at 1,000 rpm for 5 min at 4°C. The pellet was washed with PBS(–) and stained with binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM $CaCl_2$) containing a fluorescein isothiocyanate (FITC) conjugate of annexin V for 10 min in the dark. Then the cells were washed and resuspended in 1 ml of binding buffer containing PI solution

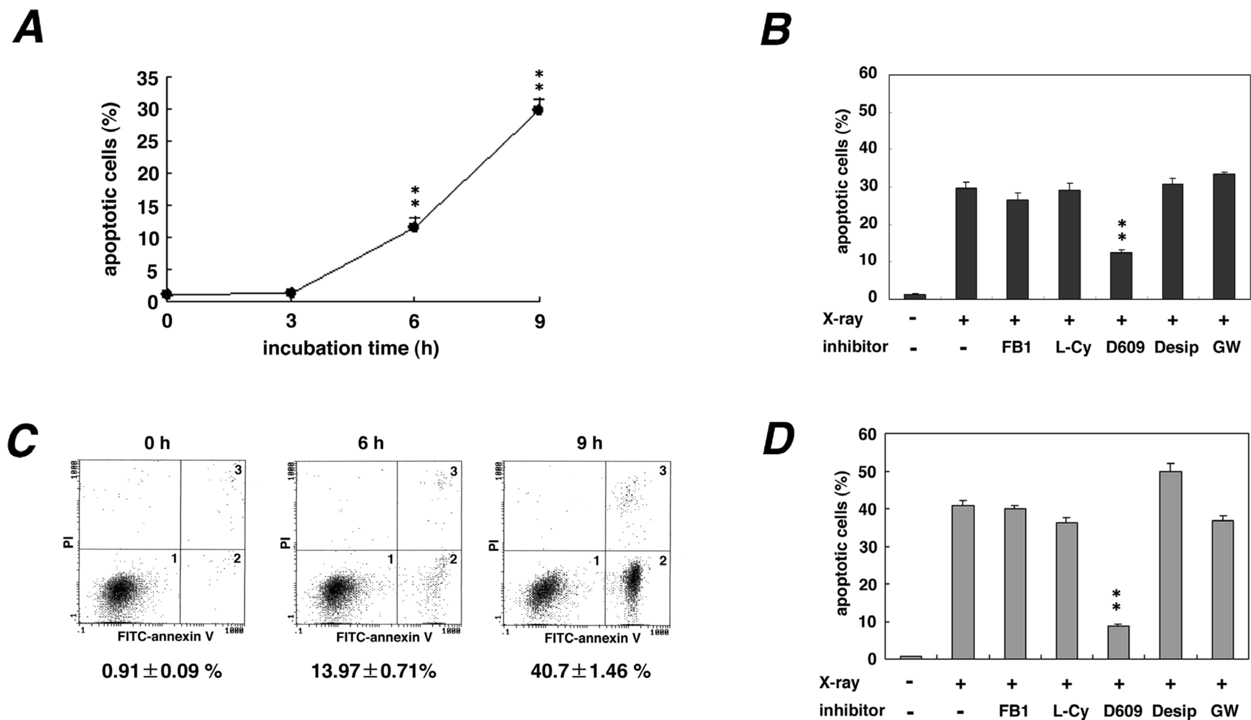

Fig. 2. The time course of radiation-induced apoptosis and the effects of ceramide metabolic inhibitors on the apoptosis. (A) The time-dependent increase in apoptotic cells after X irradiation with 7.5 Gy. Cells with morphological changes (chromatin fragmentation and condensation) among 300 PI-stained cells were counted under a fluorescent microscope. Data represent mean ± SEM from three independent experiments (n=3) **, P < 0.01 (B) Apoptosis in MOLT-4 cells was observed 9 h after X irradiation with 7.5 Gy without or with 50 mM fumonisin B_1 (FB1), 300 μM L-cycloserine (L-cy), 50 μg/ml D609, 10 μM desipramine (Desip) or 1.43 μM GW4869 (GW). Apoptotic cells were detected by PI staining. Data represent mean ± SEM (n = 3). **, P < 0.01. (C) Annexin V-FITC/PI detection of apoptosis induced by 7.5 Gy of X rays at the indicated times. See Methods for the explanation of each quadrant. Percentage of apoptotic cells (mean ± SEM, n=3) in quadrant 2 (high FITC/low PI: see Methods) was indicated below each panel. (D) Apoptosis in MOLT-4 cells was observed 9 h after X irradiation with 7.5 Gy without or with 50 μM fumonisin B_1 (FB1), 300 μM L-cycloserine (L-cy), 50 μg/ml D609, 10 μM desipramine (Desip) or 1.43 μM GW4869 (GW). Apoptotic cells were detected by annexin V-FITC/PI flow cytometry. Data represent mean ± SEM (n=3). **, P < 0.01

(final concentration of 1 μg/ml) and analyzed using an EPICS ALTRA flow cytometer (Beckman Coulter, Inc., Fullerton, CA). In the FITC/PI diparametric plot, quadrants 1 (low FITC/low PI), 2 (high FITC/low PI), 3 (high FITC/high PI) and 4 (low FITC/high PI) represent the fractions of viable, apoptotic, secondary-necrotic, and primary-necrotic cells, respectively. We took the quadrants 2 cells as apoptotic cells (see Fig. 2C).

Measurement of total endogenous ceramide

For measurement of total endogenous ceramide, the diacyl glycerol kinase (DGK) assay method was employed.[17,18] Briefly, the treated cells were harvested and lipids were extracted from them by the method of Bligh and Dyer.[19] The lipids were incubated at room temperature for 30 min in the presence of β-OG/DOPG micelles, 2 mM dithiothreitol, 5 μg of E. coli DGK, and 2 mM ATP containing 4 μCi of [γ-^{32}P]ATP. After the lipids were again extracted, reaction products were separated using a silica gel 60 TLC plate in chloroform/acetone/methanol/acetic acid/H_2O (10:4:3:2:1). The radioactivity of ceramide 1-phosphate was measured with a Bioimaging Analyzer BAS-2500 (Fuji, Tokyo, Japan). Using a standard curve for non-hydroxy fatty acid (NHFA) ceramide from bovine brain (Sigma), total amounts of ceramide (pmol/10^6 cells) were calculated.

Data analysis

Data were presented as means ± SEM. Comparisons were made by the paired Student's *t*-test. P < 0.05 was considered to be significant.

RESULTS

Effects of ceramide metabolic inhibitors on radiation-induced apoptosis

As shown in Fig. 2A, post-irradiation incubation of MOLT-4 cells exposed to 7.5 Gy of X rays gradually increased the number of apoptotic cells with characteristic

morphological changes such as fragmentation and condensation of chromatin from 3 h to 9 h. To evaluate the contribution to apoptosis of ceramide, MOLT-4 cells were irradiated in the presence of metabolic ceramide inhibitor; fumonisin B_1 (FB1),[20] L-cycloserine (L-Cy),[20] D609,[21,22] desipramine[23] and GW4869.[16,24] Figure 2B shows the effects of these inhibitors on radiation-induced apoptosis in MOLT-4 cells at 9 h after X irradiation. D609 significantly inhibited radiation-induced apoptosis, but the other inhibitors had no effects.

To confirm these effects of metabolic ceramide inhibitors on the radiation-induced apoptosis, we further used a different method of annexin V-FITC/PI flow cytometry to detect the quadrant 2 apoptotic and quadrant 3 secondary-necrotic cells (see Methods). As shown in Fig. 2C, X irradiation induced time-dependent increase in quadrant 2 apoptosis, but not in quadrant 3 secondary-necrosis. Fig. 2D indicates the annexin V-FITC/PI analysis result of effects of the inhibitors on radiation-induced apoptosis. The results also demonstrated that only D609 significantly inhibited radiation-induced apoptosis, and confirmed the morphological apoptosis data in Fig. 2B. Furthermore, these drug treatments combined with X rays did not induce any increase of the cell number in the necrotic fraction (data not shown).

Effects of inhibitors on accumulation of ceramide in MOLT-4 cells exposed to irradiation

Figure 3A shows a typical autoradiographic TLC pattern for [^{32}P]ceramide-1-phosphate originating from endogenous ceramide in X-irradiated MOLT-4 cells. This DGK assay revealed that the spot density derived from ceramide-1-phosphate was obviously increased at 9 h after X irradiation, indicating a radiation-induced increase of the intracellular concentration of ceramide. For quantitative analysis, the ceramide concentration was calculated by the ceramide standard curve and data were expressed as pmol/10^6 cells. As shown in Fig. 3B, the total amount of endogenous ceramide was not altered at 3 h after X irradiation but it increased progressively during a period of 6 h and 9 h after X irradiation, and such elevated levels at both of the times were significantly higher than that at time 0 h. This time course of the endogenous ceramide concentration after irradiation was similar to that of apoptosis as shown in Fig. 2A.

Next, the effects of metabolic ceramide inhibitors on the level of the intercellular ceramide were examined at 9 h after X irradiation. As shown in Fig. 3C, treatment of cells with D609, desipramine and GW4869, sphingomyelinase inhibitors, did not inhibit the radiation-induced increase of ceramide. However, fumonisin B_1 and L-cycloserine, *de novo* ceramide synthesis inhibitors, significantly suppressed the increase of ceramide. These results suggested that the radiation-induced increase of ceramide in MOLT-4 cells was, at least partly, due to *de novo* ceramide synthesis.

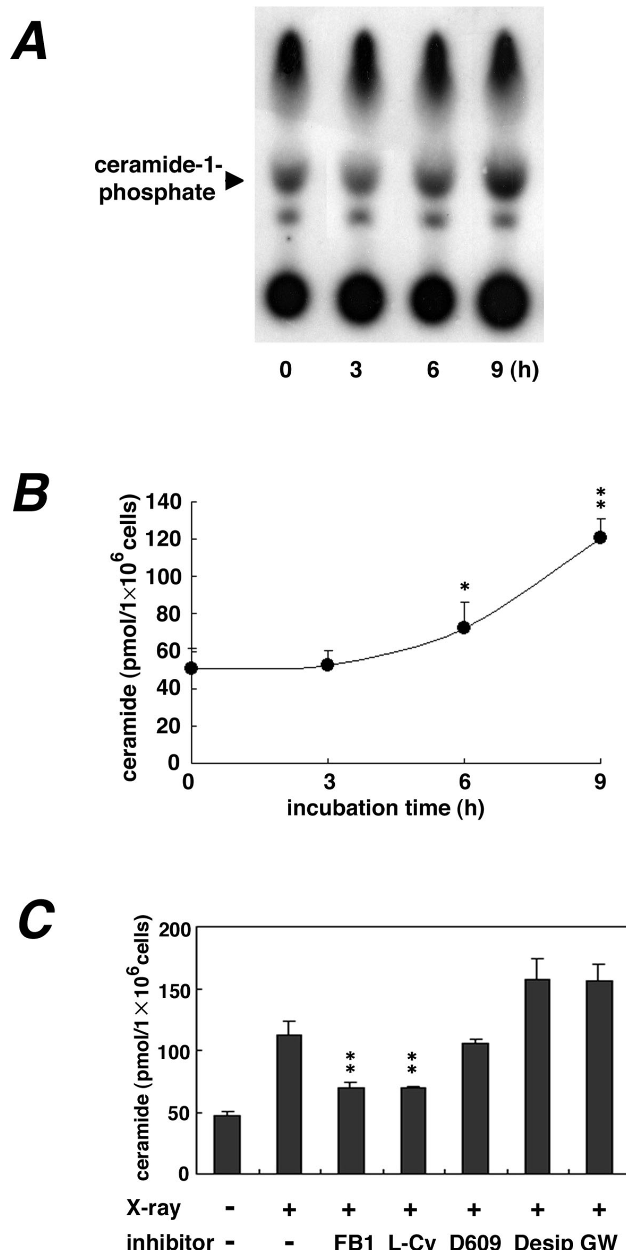

Fig. 3. The time course of radiation-induced ceramide generation and the effects of ceramide metabolic inhibitors on it. (A) Autoradiography of lipids from MOLT-4 cells at 0 h, 3 h, 6 h and 9 h after X irradiation with 7.5 Gy. Lipids were extracted from irradiated cells and incubated with DG kinase and 4 μCi of [γ-^{32}P]ATP. Thin layer chromatography (TLC) was performed to separate the phosphorylated ceramide. (B) From the autoradiogram of [^{32}P]ceramide-1-phosphate on the TLC plate, total amounts of ceramide (pmol/10^6 cells) were calculated by a standard curve for NHFA ceramide. Data represent mean ± SEM (n=3). **, $P < 0.01$; *, $P < 0.05$ (C) The intracellular levels of ceramide in MOLT-4 cells 9 h after X irradiation without or with 50 μM fumonisin B_1 (FB1), 300 μM L-cycloserine (L-Cy), 50 μg/ml D609, 10 μM desipramine (Desip) and 1.43 μM GW4869 (GW). Each column represent mean ± SEM (n=3). **, $P < 0.01$ versus X rays alone.

DISCUSSION

A large number of genotoxic stimuli and cellular stress have been reported to elevate the intracellular level of ceramide[4–7] and the accumulation of ceramide induced by cytotoxic stimuli is considered to act as a second messenger for apoptosis. The ceramide generation is due to *de novo* synthesis by ceramide synthase and/or hydrolysis of sphingomyelin by acid or neutral sphingomyelinases. In MOLT-4 cells, the retinoid N-(4-hydroxyphenyl)retinamide[20] and the P-glycoprotein inhibitor PSC 833[25] were reported to cause apoptotic cell death, which depended on *de novo* ceramide synthesis. In the case of MOLT-4 cells exposed to X rays, it was reported that the accumulation of ceramide was induced by X irradiation[26] and that treatment with D609 reduced or delayed the X-ray-induced decrease of viability and production of the 85 kDa cleaved product of poly-ADP-ribose polymerase (PARP).[21] In the present study, we also measured the total amount of intracellular ceramide by DGK assay and confirmed the radiation-induced accumulation of ceramide in MOLT-4 cells. However, the time course of the radiation-induced increase of the endogenous ceramide concentration paralleled that of radiation-induced apoptosis. If the radiation-induced increase of intracellular ceramide acts as a trigger for induction of apoptosis, the increased response of ceramide should start prior to the appearance of apoptotic cells in X-irradiated MOLT-4 cells. A question is raised whether the radiation-induced increase of intracellular ceramide was responsible for apoptosis in MOLT-4 cells. To address this question, we examined the effects of metabolic ceramide inhibitors fumonisin B_1, L-cycloserine, D609, desipramine and GW4869, on radiation-induced apoptosis and accumulation of ceramide. As shown in Figs. 2B, 2D and 3C, it was demonstrated that the radiation-induced increase of ceramide was inhibited by fumonisin B_1 and L-cycloserine but not by D609, desipramine and GW4869. However, fumonisin B_1 and L-cycloserine did not affect radiation-induced apoptosis and only D609 was found to inhibit it as reported elsewhere.[21] These results indicated the lack of correlation between radiation-induced intracellular ceramide generation and apoptosis. D609 is widely believed to inhibit PC-PLC, resulting in the suppression of ceramide production by acid sphingomyelinase.[27,28] However, it has recently been reported that D609 prevents radiation-induced apoptosis by acting as a novel GSH mimic antioxidant.[29] Furthermore, we demonstrated that a redox signaling mechanism was involved in the radiation-induced apoptosis in MOLT-4 cells and that post-irradiation treatment with an antioxidant such as Trolox or N-acetylcystein (NAC) significantly inhibited it, indicating that redox signaling plays as a key role in radiation-induced apoptosis.[30,31] From these results, it seems that the inhibitory effect on irradiation-induced apoptosis of D609 is due to its antioxidant activity.

We have previously reported that radiation-induced apoptosis of MOLT-4 cells is dependent on the activation of caspase-3 and -8 regulated by *de novo* Fas synthesis and the formation of death-induced signaling complex (DISC).[14] There are several reports that apoptosis via Fas occurs independently of ceramide generation.[13,32–34] In addition, the substantial reduction in the cell ceramide and sphingomyelinase contents induced by chronic treatment with fumonisin B_1 did not affect TNF- or anti-Fas cytotoxity.[13] Recently, it has been demonstrated that the induction of apoptosis in cisplatin-treated Colon-205 and SKBR3 carcinoma cells is mediated by the activation of caspase-3 without an increase in the amount of ceramide[35] and that the induction of apoptosis in camptothecin-treated prostate cancer LNCaP cells and UV-treated murine melanoma cells is independent of ceramide accumulation,[36,37] suggesting that these apoptotic pathways are independent of the ceramide generation in some cancer cells exposed to genotoxic stimuli. These facts and our present results suggest that the increase in intracellular ceramide concentration is not always associated with the induction of apoptosis in tumor cells exposed to genotoxic stimuli.

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REFERENCES

1. Midgley, C. A., Owens, B., Briscoe, C. V., Thomas, D. B., Lane, D. P. and Hall, P. A. (1995) Coupling between gamma irradiation, p53 induction and the apoptotic response depends upon cell type *in vivo*. J. Cell Sci. **108**: 1843–1848.
2. Tamura, T., Ishihara, M., Lamphier, M. S., Tanaka, N., Oishi, I., Aizawa, S., Matsuyama, T., Mak, T. W., Taki, S. and Taniguchi, T. (1995) An IRF-1-dependent pathway of DNA damage-induced apoptosis in mitogen-activated T lymphocytes. Nature **376**: 596–599.
3. Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C., Hooper, M. L. and Wyllie, A. H. (1993) Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature **362**: 849–852.
4. Hannun, Y. and Luberto, C. (2000) Ceramide in the eukaryotic stress response. Trends Cell Biol. **10**: 73–80.
5. Jarvis, W. D. and Grant, S. (1998) The role of ceramide in the cellular response to cytotoxic agents. Curr. Opin. Oncol. **10**: 552–559.
6. Mathias, S., Pena, L. A. and Kolesnick, R. N. (1998) Signal transduction of stress via ceramide. Biochem. J. **335**: 465–480.
7. Bose, R., Verheij, M., Haimovitz-Friedman, A., Scotto, K., Fuks, Z. and Kolesnick, R. (1995) Ceramide synthase medi-

ates daunorubicin-induced apoptosis: an alternative mechanism for generating death signals. Cell **82**: 405–414.
8. Santana, P., Pena, L. A., Haimovitz-Friedman, A., Martin, S., Green, D., McLoughlin, M., Cordon-Cardo, C., Schuchman, E. H., Fuks, Z. and Kolesnick, R. (1996) Acid sphingomyelinase-deficient human lymphoblasts and mice are defective in radiation-induced apoptosis. Cell **86**: 189–199.
9. Chmura, S. J., Nodzenski, E., Beckett, M. A., Kufe, D. W., Quintans, J. and Weichselbaum, R. R. (1997) Loss of ceramide production confers resistance to radiation-induced apoptosis. Cancer Res. **57**: 1270–1275.
10. Perry, D. K., Carton, J., Shah, A. K., Meredith, F., Uhlinger, D. J. and Hannun, Y. A. (2000) Serine palmitoyltransferase regulates de novo ceramide generation during etoposide-induced apoptosis. J. Biol. Chem. **275**: 9078–9084.
11. De Maria, R., Rippo, M. R., Schuchman, E. H. and Testi, R. (1998) Acidic sphingomyelinase (ASM) is necessary for fas-induced GD3 ganglioside accumulation and efficient apoptosis of lymphoid cells. J. Exp. Med. **187**: 897–902.
12. Cock, J. G., Tepper, A. D., de Vries, E., van Blitterswijk, W. J. and Borst, J. (1998) CD95 (Fas/APO-1) induces ceramide formation and apoptosis in the absence of a functional acid sphingomyelinase. J. Biol. Chem. **273**: 7560–7565.
13. Gamen, S., Anel, A., Pineiro, A. and Naval, J. (1998) Caspases are the main executioners of Fas-mediated apoptosis, irrespective of the ceramide signalling pathway. Cell Death Differ. **5**: 241–249.
14. Takahashi, K., Inanami, O., Hayashi, M. and Kuwabara, M. (2000) Protein synthesis-dependent apoptotic signalling pathway in X-irradiated MOLT-4 human leukaemia cell line. Int. J. Radiat. Biol. **78**: 115–124.
15. Kuwabara, M., Takahashi, K. and Inanami, O. (2003) Induction of apoptosis through the activation of SAPK/JNK followed by the expression of death receptor Fas in X-irradiated cells. J. Radiat. Res. **44**: 203–209.
16. Luberto, C., Hassler, D. F., Signorelli, P., Okamoto, Y., Sawai, H., Boros, E., Hazen-Martin, D. J., Obeid, L. M., Hannun, Y. A. and Smith, G. K. (2002) Inhibition of tumor necrosis factor-induced cell death in MCF7 by a novel inhibitor of neutral sphingomyelinase. J. Biol. Chem. **277**: 41128–41139.
17. Bielawska, A., Perry, D. K. and Hannun, Y. A. (2001) Determination of ceramides and diglycerides by the diglyceride kinase assay. Anal. Biochem. **298**: 141–150.
18. Dobrowsky, R. T. and Kolesnick, R. N. (2001) Analysis of sphingomyelin and ceramide levels and the enzymes regulating their metabolism in response to cell stress. Methods Cell Biol. **66**: 135–165.
19. Bligh, E. G. and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**: 911–917.
20. O'Donnell, P. H., Guo, W. X., Reynolds, C. P. and Maurer, B. J. (2002) N-(4-hydroxyphenyl)retinamide increases ceramide and is cytotoxic to acute lymphoblastic leukemia cell lines, but not to non-malignant lymphocytes. Leukemia **16**: 902–910.
21. Enomoto, A., Suzuki, N., Hirano, K., Matsumoto, Y., Morita, A., Sakai, K. and Koyama, H. (2000) Involvement of SAPK/JNK pathway in X-ray-induced rapid cell death of human T-cell leukemia cell line MOLT-4. Cancer Lett. **155**: 137–144.
22. Wiegmann, K., Schutze, S., Machleidt, T., Witte, D. and Kronke, M. (1994) Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. Cell **78**: 1005–1015.
23. Hundal, R. S., Gomez-Munoz, A., Kong, J. Y., Salh, B. S., Marotta, A., Duronio, V. and Steinbrecher, U. P. (2003) Oxidized low density lipoprotein inhibits macrophage apoptosis by blocking ceramide generation, thereby maintaining protein kinase B activation and Bcl-XL levels. J. Biol. Chem. **278**: 24399–24408.
24. Marchesini, N., Luberto, C. and Hannun, Y. A. (2003) Biochemical properties of mammalian neutral sphingomyelinase 2 and its role in sphingolipid metabolism. J. Biol. Chem. **278**: 13775–13783.
25. Azare, J., Pankova-Kholmyansky, I., Salnikow, K., Cohen, D. and Flescher, E. (2000) Selective susceptibility of transformed T lymphocytes to induction of apoptosis by PSC 833, an inhibitor of P-glycoprotein. Oncol. Res. **12**: 315–323.
26. El-Assaad, W., Kozhaya, L., Araysi, S., Panjarian, S., Bitar, F. F., Baz, E., El-Sabban, M. E. and Dbaibo, G. S. (2003) Ceramide and glutathione define two independently regulated pathways of cell death initiated by p53 in Molt-4 leukaemia cells. Biochem. J. **376**: 725–732.
27. Schutze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K. and Kronke, M. (1992) TNF activates NF-kappa B by phosphatidylcholine-specific phospholipase C-induced "acidic" sphingomyelin breakdown. Cell **71**: 765–776.
28. Cifone, M. G., Roncaioli, P., De Maria, R., Camarda, G., Santoni, A., Ruberti, G. and Testi, R. (1995) Multiple pathways originate at the Fas/APO-1 (CD95) receptor: sequential involvement of phosphatidylcholine-specific phospholipase C and acidic sphingomyelinase in the propagation of the apoptotic signal. EMBO J. **14**: 5859–5868.
29. Lauderback, C. M., Drake, J., Zhou, D., Hackett, J. M., Castegna, A., Kanski, J., Tsoras, M., Varadarajan, S. and Butterfield, D. A. (2003) Derivatives of xanthic acid are novel antioxidants: application to synaptosomes. Free Radic. Res. **37**: 355–365.
30. Inanami, O., Takahashi, K. and Kuwabara, M. (1999) Attenuation of caspase-3-dependent apoptosis by Trolox post-treatment of X-irradiated MOLT-4 cells. Int. J. Radiat. Biol. **75**: 155–163.
31. Hamasu, T., Inanami, O., Tsujitani, M., Yokoyama, K., Takahashi, E., Kashiwakura, I. and Kuwabara, M. (2005) Post-irradiation hypoxic incubation of X-irradiated MOLT-4 cells reduces apoptotic cell death by changing the intracellular redox state and modulating SAPK/JNK pathways. Apoptosis **10**: 557–567.
32. Watts, J. D., Gu, M., Polverino, A. J., Patterson, S. D. and Aebersold, R. (1997) Fas-induced apoptosis of T cells occurs independently of ceramide generation. Proc. Natl. Acad. Sci. USA. **94**: 7292–7296.
33. Hsu, S. C., Wu, C. C., Luh, T. Y., Chou, C. K., Han, S. H. and Lai, M. Z. (1998) Apoptotic signal of Fas is not mediated by ceramide. Blood **91**: 2658–2663.

34. Gewies, A., Rokhlin, O. W. and Cohen, M. B. (2000) Ceramide induces cell death in the human prostatic carcinoma cell lines PC3 and DU145 but does not seem to be involved in Fas-mediated apoptosis. Lab. Invest. **80**: 671–676.
35. Basu, S., Ma, R., Boyle, P. J., Mikulla, B., Bradley, M., Smith, B., Basu, M. and Banerjee, S. (2004) Apoptosis of human carcinoma cells in the presence of potential anti-cancer drugs: III. Treatment of Colo-205 and SKBR3 cells with: cis-platin, tamoxifen, melphalan, betulinic acid, L-PDMP, L-PPMP, and GD3 ganglioside. Glycoconj. J. **20**: 563–577.
36. Akao, Y., Kusakabe, S., Banno, Y., Kito, M., Nakagawa, Y., Tamiya-Koizumi, K., Hattori, M., Sawada, M., Hirabayasi, Y., Ohishi, N. and Nozawa, Y. (2002) Ceramide accumulation is independent of camptothecin-induced apoptosis in prostate cancer LNCaP cells. Biochem. Biophys. Res. Commun. **294**: 363–370.
37. Deng, J., Zhang, H., Kloosterboer, F., Liao, Y., Klostergaard, J., Levitt, M. L. and Hung, M. C. (2002) Ceramide does not act as a general second messenger for ultraviolet-induced apoptosis. Oncogene **21**: 44–52.

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