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

Eriko TAKAHASHI, Osamu INANAMI, Taketoshi ASANUMA and Mikinori KUWABARA*

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

In the present study, using inhibitors of ceramide synthase (fumonisin B1), 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 B1 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 sphingomyelinases6) (Fig. 1). Fumonisin B1 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 sphingomyelinase is required for Fas-mediated apoptosis in lymphoblastic cells from patients with Niemann-Pick disease,11) but Cock et al. have found that acid sphingomyelinase is not required.12) In the data obtained by using inhibitors of sphingomyelinases and de novo ceramide synthytesis, 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 C6 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 B1...
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 B1 (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% CO₂-air. Fumonisin B1, 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-32P]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 B1, 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 Ca²⁺Mg²⁺-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/pro-pidium 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 CaCl₂) 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. 1. Two pathways of ceramide generation. De novo ceramide synthesis and hydrolysis of sphingomyelin via acid and neutral sphingomyelinases.
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 \([\gamma^{32}\text{P}]\)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\(_2\)O (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 B₁ (FB₁), L-cycloserine (L-Cy), D609, desipramine and GW4869. 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 [³²P]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/10⁶ 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 B₁ 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-³²P]ATP. Thin layer chromatography (TLC) was performed to separate the phosphorylated ceramide. (B) From the autoradiogram of [³²P]ceramide-1-phosphate on the TLC plate, total amounts of ceramide (pmol/10⁶ 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 B₁ (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 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 and the P-glycoprotein inhibitor PSC 833 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 and that treatment with D609 reduced or significantly inhibited it, indicating that redox signaling plays as an antioxidant such as Trolox or N-acetylcystein (NAC) significantly inhibited it, indicating that redox signaling plays as an antioxidant such as Trolox or N-acetylcystein (NAC) significantly inhibited it, indicating that redox signaling plays 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 B1, 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 B1 and L-cycloserine but not by D609, desipramine and GW4869. However, fumonisin B1 and L-cycloserine did not affect radiation-induced apoptosis and only D609 was found to inhibit it as reported elsewhere. 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. However, it has recently been reported that D609 prevents radiation-induced apoptosis by acting as a novel GSH mimic antioxidant. 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. From these results, it seems that the inhibitory effect on radiation-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). There are several reports that apoptosis via Fas occurs independently of ceramide generation. In addition, the substantial reduction in the cell ceramide and sphingomyelinase contents induced by chronic treatment with fumonisin B1 did not affect TNF- or anti-Fas cytotoxicity. Recently, it has been demonstrated that the induction of apoptosis in cis-platin-treated Colon-205 and SKBR3 carcinoma cells is mediated by the activation of caspase-3 without an increase in the amount of ceramide and that the induction of apoptosis in camptothecin-treated prostate cancer LNCaP cells and UV-treated murine melanoma cells is independent of ceramide accumulation 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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