Ceramide and glutathione define two independently regulated pathways of cell death initiated by p53 in Molt-4 leukaemia cells

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The tumour suppressor p53 induces cell death by launching several pathways that are either dependent on or independent of gene transcription. Accumulation of the sphingolipid ceramide and reactive oxygen species are among these pathways. Cross-regulation of these two pathways is possible owing to the demonstrated inhibition of neutral sphingomyelinase by glutathione, the predominant cellular antioxidant, and has been observed in some cytokine-dependent cell-death models. In a model of irradiation-induced cell death of Molt-4 leukaemia cells, it was found that ceramide accumulation and glutathione depletion were dependent on p53 up-regulation. The loss of p53 owing to expression of the papilloma virus E6 protein inhibited both pathways after irradiation. However, in this model, these two pathways appeared to be independently regulated on the basis of the following observations: (1) glutathione supplementation or depletion did not alter irradiation-induced ceramide

accumulation, (2) exogenous ceramide treatment did not induce glutathione depletion, (3) glutathione depletion was dependent on new protein synthesis, whereas ceramide accumulation was independent of it and (4) caspase activation was required for ceramide accumulation but not for glutathione depletion. Furthermore, caspase 9 activation, which is dependent on the release of mitochondrial cytochrome *c*, was not required for ceramide accumulation. This suggested that a caspase, other than caspase 9, was necessary for ceramide accumulation. Interestingly, Bcl-2 expression inhibited these pathways, indicating a possible role for mitochondria in regulating both pathways. These findings indicate that these two pathways exhibit cross-regulation in cytokine-dependent, but not in p53-dependent, cell-death models.

Key words: apoptosis, ceramide, DNA damage, glutathione, p53, signal transduction.

INTRODUCTION

Genotoxic damage in cells triggers the accumulation of the tumour suppressor p53 [1]. The functions of p53 include participation in DNA damage repair, induction of cell-cycle arrest and promotion of cell death. The ability of p53 to induce cell death was demonstrated to be either dependent on or independent of transcription and to involve apoptosis as well as necrosis [2–4]. Several genes that are specifically induced by p53 were discovered to play a potential role in mediating its effects on cell death. These include members of the Bcl-2 family of proteins Bax, Noxa and PUMA, the death receptors Fas, DR5 and Pidd, and several other proteins including PERP, p53AIP1 and p53/pw1 [5]. Each of these genes was demonstrated to be required, under select conditions, for the full effect of p53-induced cell death. Additionally, a number of p53-induced genes were discovered to share the ability to either generate ROS (reactive oxygen species) potentially or respond to oxidative stress [6].

Several inducers of cell death, including γ -irradiation, chemotherapeutic agents and TNF- α (tumour necrosis factor- α), also induce ROS accumulation in many cell types [7–9]. ROS were found to be downstream mediators of p53-dependent cell death [10,11]. The major cellular defence against the deleterious effects of ROS is glutathione, present at high concentrations (mM range) in most cell types [12]. Glutathione depletion was shown to increase the sensitivity of various cell types to chemotherapeutic

agents or γ -irradiation [13]. Some of the agents that induce ROS generation also promote accumulation of the sphingolipid ceramide [14]. The role of ceramide as a regulator of stress response and cell death has been shown in various models, including yeast [15,16]. Possible cross-regulation of the oxidative response and the ceramide pathways emerged with the discovery that glutathione inhibits the activation of N-Smase (neutral magnesium-dependent sphingomyelinase), an important enzyme in regulated generation of ceramide from membrane sphingomyelin [17]. Indeed, several studies demonstrated that, in response to specific stimuli, some cells respond by ROS generation, leading to glutathione depletion and subsequent sphingomyelinase-dependent generation of ceramide [11,18–20].

In a previous study, we had shown that ceramide accumulates in response to actinomycin D or γ -irradiation in a p53-dependent manner [21]. We decided to investigate whether p53 also regulates the oxidative pathway in the same model and whether the two pathways were involved in cross-regulation in response to γ -irradiation.

EXPERIMENTAL

Cell culture and cell-death assays

Molt-4 human leukaemia cell lines were obtained from A. T. C. C. (Manassas, VA, U.S.A.) and grown in RPMI 1640, supplemented

Abbreviations used: BSO, L-buthionine S,R-sulphoximine; C₆, ceramide, N-hexanoylsphingosine; CHX, cycloheximide; ER, endoplasmic reticulum; FBS, foetal bovine serum; LEHD-FMK, Leu-Glu-His-Asp-fluoromethylketone; N-Smase, neutral magnesium-dependent sphingomyelinase; PARP, poly(ADP-ribose) polymerase; ROS, reactive oxygen species; TNF- α , tumour necrosis factor- α ; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone.

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with 10 % (v/v) FBS (foetal bovine serum). Molt-4-LXSN, -E6 and -Bcl-2 cells were developed as described previously and maintained in selection media [21,22]. Cell death was assayed by uptake of Trypan Blue. Apoptosis was verified by assaying for cleavage of PARP [poly(ADP-ribose) polymerase] after Western-blot analysis using a rabbit polyclonal antiserum (Boehringer Mannheim, Mannheim, Germany) as described below. *D-erythro-C₆* ceramide, referred to as *C₆* ceramide (*N*-hexanoylsphingosine), was purchased from Matreya (State College, PA, U.S.A.). Treatment with ceramide was performed in the presence of 2 % FBS to minimize binding by serum proteins. The caspase inhibitors Z-VAD-FMK (benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone) and LEHD-FMK (Leu-Glu-His-Asp-fluoromethylketone) were purchased from Bachem (King of Prussia, PA, U.S.A.) and Enzyme Systems (Livermore, CA, U.S.A.) respectively.

Western-blot analysis for p53 and PARP

Western-blot analysis was performed as described in [21]. Briefly, cell lysates were prepared from 2×10^6 cells using the following buffer: 1 % SDS, 5 % (v/v) glycerol, 1.5 % (v/v) 2-mercaptoethanol and 20 mM Tris/HCl (pH 7.4). The lysates were boiled for 10 min or to solubilization, and aliquots were removed for determination of protein concentration (Bio-Rad assay). Equal amounts of total protein (50 μ g) were run on an SDS/10 %-(w/v)-polyacrylamide gel for PARP and caspase 9 gels and 12 % for p53 gels and then transferred to nitrocellulose paper. Blots were stained with Ponceau S to verify equal loading. Molt-4 lysates were probed with anti-human wild-type p53 (Upstate Biotechnology), PARP or caspase 9 antibodies (Santa Cruz Biotechnology).

Measurement of apoptosis

Apoptosis was measured in cells using a commercially available ELISA kit (Roche, Mannheim, Germany) according to the manufacturer's instructions, which detects the cytoplasmic level of histone-associated DNA fragments (mono- and oligonucleosomes) that are generated after endonuclease activation during apoptosis.

Glutathione measurement

Cells were seeded at 0.5×10^6 cells/ml in a 25 cm² flask, kept overnight and then treated accordingly. At the desired time points, cells were harvested, washed twice with ice-cold PBS and then lysed in 500 μ l of metaphosphoric acid using a 27.5 gauge needle. Proteins were separated from the supernatant by centrifugation at 2500 g for 10 min at 4 °C. Glutathione levels were measured using a commercially available kit (Oxis, Portland, OR, U.S.A.).

Ceramide measurement

Lipids were collected by the method of Bligh and Dyer [23]. Ceramide was measured with a modified diacylglycerol kinase assay using external ceramide standards as described previously [24]. Briefly, 80 % of the lipid sample was dried under N₂. The dried lipid was solubilized in 20 μ l of octyl- β -D-glucoside (7.5 %, w/v)/dioleoyl phosphatidylglycerol (25 mM) micellar solution by several cycles of sonication in a bath sonicator, followed by resting at room temperature (25 °C) for 15–20 min. The reaction buffer was prepared as a 2 $\times$ solution containing 100 mM imidazole/HCl (pH 6.6), 100 mM LiCl, 25 mM MgCl₂ and 2 mM EGTA. The following were added to the lipid micelles:

50 μ l of 2 $\times$ reaction buffer, 0.2 μ l of 1 M dithiothreitol, 5 μ g of diglycerol kinase membranes and dilution buffer (10 mM imidazole, pH 6.6/1 mM diethylenetriaminepenta-acetic acid, pH 7) to a final volume of 90 μ l. The reaction was started by adding 10 μ l of 2.5 mM [γ -³²P]ATP solution (specific activity, 75 000–200 000 c.p.m./nmol). The reaction was allowed to proceed at 25 °C for 30 min. Lipid extraction was performed by the method of Bligh and Dyer [23] and a 1.5 ml of aliquot of the organic phase was dried under N₂. Lipids were then resuspended in 50 μ l of methanol/chloroform (1:9, v/v), and 25 μ l was spotted on a 20 cm silica-gel TLC plate. Plates were developed with chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1), air-dried and subjected to autoradiography. The radioactive spots corresponding to phosphatidic acid and ceramide phosphate, the phosphorylated products of diacylglycerol and ceramide respectively, were identified by comparison with known standards. Spots were scraped into a scintillation vial containing 4 ml of scintillation fluid and counted on a scintillation counter. Linear curves of phosphorylation were produced over the concentration range 0–960 pM of external standards (dioleoyl glycerol and *C₃* ceramide; Sigma). Ceramide levels were always normalized to lipid phosphate, which was measured by the method of Rouser et al. [25]. It is important to note that, under these conditions, there was total conversion of ceramide and diacylglycerol into their phosphorylated products, and there was no change in the specific activity of the diacylglycerol kinase enzyme.

Unless indicated otherwise, results shown are representative of at least three independent experiments with nearly identical results.

RESULTS

In a previous study, we showed that, in Molt-4 cells, ceramide accumulates in response to γ -irradiation or actinomycin D treatment in a p53-dependent manner [21]. Using the same model of Molt-4 cells that expresses either the retroviral vector LXSN or the papilloma virus E6 protein that induces ubiquitination-dependent down-regulation of p53, we extended these earlier findings by examining a wider dosage range of γ -irradiation. It was found that up to a dose of 10 Gy, there was a dose-dependent increase in p53 accumulation in Molt-4-LXSN cells, but not in Molt-4-E6 cells (Figure 1A). This was accompanied by dose-dependent cell death in Molt-4-LXSN cells, but not in Molt-4-E6 cells, confirming that γ -irradiation induced p53-dependent cell death at the doses used (Figure 1B). Next, the time course of p53 and ceramide accumulation in response to γ -irradiation at 5 Gy was determined. It was found that p53 accumulated to almost maximal levels by 2 h in Molt-4-LXSN cells (results not shown). It was also found that ceramide started to accumulate at 6 h after irradiation in Molt-4-LXSN cells and continued to increase with time, reaching 6–7-fold of baseline levels by 24 h (Figure 1C). As shown previously, there was minimal accumulation of ceramide in Molt-4-E6 cells, indicating that this was p53-dependent. It was decided to examine whether glutathione depletion was also induced in a p53-dependent manner. Molt-4-LXSN and -E6 cells were irradiated at 5 Gy and glutathione levels were measured at several time points. As shown in Figure 1(D), cellular glutathione levels were depleted after irradiation in a p53-dependent manner, since no glutathione depletion occurred in Molt-4-E6 cells. Glutathione depletion in Molt-4-LXSN cells started at 8 h and reached 30 % of control levels by 24 h. Therefore, in Molt-4 cells, γ -irradiation induced p53-dependent cell death, ceramide accumulation and glutathione depletion.

Figure 1 Dependence of ceramide accumulation and glutathione depletion after irradiation on p53

(A) p53 up-regulation induced by γ -irradiation. Molt-4-LXSN and -E6 cells were irradiated at different doses as indicated. Proteins were extracted 6 h after irradiation and p53 levels were assayed by Western-blot analysis. (B) Cell death induced by γ -irradiation. Experimental details are as in (A), but cell death was determined by Trypan Blue uptake at 24 h. (C) Ceramide levels post-irradiation. Molt-4-LXSN and -E6 cells were irradiated at 5 Gy and ceramide levels were measured at the indicated time points. (D) Glutathione levels post-irradiation. Experimental details are as in (C), but glutathione levels were measured at the indicated time points.

The ceramide and oxidative pathways have been linked by a number of previous studies. Glutathione was shown in some studies to inhibit membrane N-Smase activation, whereas other studies showed that ceramide treatment induced the generation of ROS. Therefore we decided to investigate possible cross-regulation of glutathione depletion and ceramide accumulation

Table 1 Effect of GSH supplementation on post-irradiation ceramide levels

Molt-4 cells were irradiated at 5 Gy in the presence or absence of 10 mM GSH added 2 h before irradiation. Then, ceramide levels were measured at the indicated time points.

Time (h)	Ceramide/phosphate (pmol/nmol)		
	Control	Irradiated – GSH	Irradiated + GSH
0	1.59 $\pm$ 0.25	1.59 $\pm$ 0.25	1.59 $\pm$ 0.25
8	1.28 $\pm$ 0.01	1.53 $\pm$ 0.08	1.36 $\pm$ 0.05
14	1.02 $\pm$ 0.07	2.93 $\pm$ 0.18	3.22 $\pm$ 0.24
24	1.08 $\pm$ 0.03	8.82 $\pm$ 0.23	7.53 $\pm$ 0.07

Table 2 Effect of GSH depletion on post-irradiation ceramide levels

Cells were treated with 100 μ M BSO or vehicle for 2 h before irradiation. Then, ceramide levels were measured at the indicated time points.

Time (h)	Ceramide/phosphate (pmol/nmol)			
	Control	BSO	Irradiated – BSO	Irradiated + BSO
0	0.94 $\pm$ 0.52	0.94 $\pm$ 0.52	0.94 $\pm$ 0.52	0.94 $\pm$ 0.52
8	1.22 $\pm$ 0.18	1.18 $\pm$ 0.06	1.52 $\pm$ 0.04	1.72 $\pm$ 0.12
14	1.44 $\pm$ 0.15	1.43 $\pm$ 0.11	5.52 $\pm$ 1.34	5.12 $\pm$ 0.45
24	1.45 $\pm$ 0.36	1.61 $\pm$ 0.08	12.64 $\pm$ 1.22	12.85 $\pm$ 0.64

in this model. Since ceramide accumulation coincided with glutathione depletion, it was possible that glutathione depletion led to ceramide accumulation via activation of neutral sphingomyelinase activity or ceramide accumulation led to glutathione depletion via generation of ROS or both pathways were independently regulated by p53. We decided to examine whether glutathione replenishment affected ceramide accumulation. Molt-4 cells were treated with glutathione (10 mM) before γ -irradiation. This maintained glutathione levels in irradiated cells at 85% of non-treated cells up to 24 h after irradiation (results not shown). Ceramide levels were measured at several time points and compared with control cells that were irradiated in the absence of exogenous glutathione (Table 1). It was found that ceramide levels at early and late time points were not affected by exogenous glutathione pretreatment. In another approach, the glutathione-depleting agent L-buthionine *S,R*-sulphoximine (BSO) was utilized to decrease chemically intracellular levels of glutathione. Treatment of Molt-4 cells with 100 or 500 μ M BSO resulted in a rapid decrease in cellular glutathione levels down to 10% of control by 24 h (results not shown). Examination of the effects of BSO-induced depletion of glutathione on the levels of ceramide (Table 2) showed that there was no change in cellular ceramide levels up to 24 h after treatment. When cells pretreated with BSO were irradiated, there was no difference in the levels of ceramide that accumulated up to 24 h when compared with irradiated control cells (Table 2). These studies indicated that glutathione depletion was unlikely to trigger ceramide accumulation and that the membrane N-Smase that is regulated by glutathione did not play a role in p53-dependent ceramide accumulation in these cells after irradiation. To verify this, the membrane activity of N-Smase was measured after irradiation. It was found that baseline N-Smase activity was 7.4 ± 0.69 nmol $\cdot$ mg $^{-1}$ $\cdot$ h $^{-1}$, whereas at 24 h after irradiation the activity was 6.15 ± 0.68 nmol $\cdot$ mg $^{-1}$ $\cdot$ h $^{-1}$.

Another possibility that was investigated was whether ceramide accumulation led to the depletion of glutathione. Cell-permeable

Table 3 Effect of C₆ ceramide treatment on GSH levels

Molt-4 cells were treated with the indicated concentrations of C₆ ceramide and GSH levels were measured at the indicated time points.

Time (h)	Glutathione (nmol/mg of protein)		
	Control	Ceramide (10 μ M)	Ceramide (20 μ M)
0	52.15 $\pm$ 6.69	52.15 $\pm$ 6.69	52.15 $\pm$ 6.69
4	61.92 $\pm$ 10.1	65.29 $\pm$ 3.74	61.99 $\pm$ 5.89
8	55.83 $\pm$ 3.28	53.65 $\pm$ 4.53	61.21 $\pm$ 3.88
24	59.98 $\pm$ 11.59	61.05 $\pm$ 5.83	59.21 $\pm$ 1.32

synthetic C₆ ceramide was used to treat Molt-4 cells and cellular glutathione levels were measured at several time points (Table 3). It was found that, at the concentrations used to induce apoptotic cell death in these cells, C₆ ceramide had no effect on the glutathione content of cells. These experiments indicated that ceramide accumulation was unlikely to contribute significantly to glutathione depletion in the first 24 h after irradiation.

The regulation of glutathione depletion and ceramide accumulation in response to irradiation was explored further. Depending on the system examined, new protein synthesis has been found either necessary or unnecessary for cell death [26,27]. The requirement for new protein synthesis in response to irradiation-induced Molt-4 cell death was examined. CHX (cycloheximide) treatment of Molt-4 cells by itself induced a slight increase in cell death by 24 h (Figure 2A). When cells were irradiated in the presence of CHX, there was significant attenuation of cell death at 24 h but not at earlier time points. This suggested that new protein synthesis was partially needed for cell death in this system. This confirms a recently published study showing that protein synthesis is needed for irradiation-induced cell death in Molt-4 cells [28]. The requirement of new protein synthesis for ceramide accumulation was subsequently examined. Treatment with CHX by itself induced ceramide accumulation, reaching approx. 5-fold of baseline levels at 24 h. When ceramide levels in cells irradiated in the presence or absence of CHX were compared, it was observed that ceramide accumulated earlier in the presence of CHX, but eventual levels at 24 h were equivalent (Figure 2B). This indicated that ceramide accumulation was not dependent on new protein synthesis.

CHX treatment of Molt-4 cells resulted in a gradual accumulation of glutathione (Figure 2C). When glutathione levels were compared in cells irradiated in the presence or absence of CHX, it was found that, in the presence of CHX, glutathione depletion was completely inhibited and glutathione levels increased instead. This suggested that, unlike ceramide accumulation, glutathione depletion in response to irradiation of Molt-4 cells was dependent on new protein synthesis.

It is now evident that p53 launches more than one pathway of apoptotic as well as necrotic cell death [3,4]. Some of these pathways engage the 'extrinsic' pathway of apoptotic cell death by up-regulation of death receptors such as Fas, and other pathways engage the 'intrinsic' pathway that is regulated at the mitochondrial level by up-regulation of proapoptotic members of the Bcl-2 family of proteins such as Bax. Both of these pathways involve activation of various caspases that contribute to apoptosis as either initiators (e.g. caspase 8, 2 or 9) or effectors (e.g. caspase 3 or 7). They can function at either a premitochondrial (e.g. caspase 8 or 10) or post-mitochondrial (e.g. caspase 9 or 3) level. Bcl-2 functions at the mitochondrial, nuclear and endoplasmic-membrane levels to prevent apoptotic as well as necrotic

Figure 2 Effects of protein synthesis inhibition on irradiation-induced cell death, ceramide accumulation and glutathione depletion

(A) Effect of protein synthesis inhibition on irradiation-induced cell death. Molt-4 cells were irradiated at 5 Gy in the presence or absence of 5 μ g/ml CHX added 2 h before irradiation. Cell death was assessed by Trypan Blue uptake at the indicated time points and presented as percentage of the total number of cells. (B) Effect of protein synthesis inhibition on irradiation-induced ceramide accumulation. Cells were treated as in (A) and ceramide levels were measured at the indicated time points. (C) Effect of protein synthesis inhibition on irradiation-induced glutathione depletion. Cells were treated as in (A) and glutathione levels were measured at the indicated time points.

cell death [29], triggered by many inducers, including those that signal via p53. Therefore it was important to evaluate how Bcl-2 and caspases regulated p53-dependent changes in ceramide and glutathione levels. Molt-4 cells that overexpress Bcl-2 (Molt-4-Bcl-2) were irradiated and compared with control cells. Expression of Bcl-2 almost completely blocked cell death in response to γ -irradiation at a dose that induced p53-dependent

Table 4 Expression of Bcl-2 or inhibition of caspases prevents irradiation-induced cell death, but differentially affects ceramide accumulation and glutathione depletion

Molt-4 cells were treated with 100 μ M Z-VAD-FMK 2 h before irradiation at 5 Gy. Alternatively, Molt-4-Bcl-2 cells stably transfected with Bcl-2 were used. Cell death, as determined by Trypan Blue uptake, and ceramide and glutathione levels were measured at 24 h after irradiation and were compared with irradiated control or vector cells respectively.

	Cell death (% of total)	Ceramide/phosphate (pmol/nmol)	GSH/protein (nmol/mg)
Vector-Control	3 $\pm$ 1.41	1.59 $\pm$ 0.17	27.73 $\pm$ 1.90
Vector-Irradiated	43.5 $\pm$ 6.71	9.62 $\pm$ 0.60	8.03 $\pm$ 4.39
Bcl-2-Control	2 $\pm$ 1.33	1.68 $\pm$ 0.09	36.04 $\pm$ 3.07
Bcl-2-Irradiated	5.6 $\pm$ 3.43	2.34 $\pm$ 0.21	38.96 $\pm$ 2.14
Control	4.88 $\pm$ 1.93	1.80 $\pm$ 0.48	34.17 $\pm$ 0.76
Irradiated	42.0 $\pm$ 8.98	8.135 $\pm$ 1.40	12.23 $\pm$ 2.48
Irradiated + Z-VAD	9.25 $\pm$ 2.63	3.435 $\pm$ 0.68	14.34 $\pm$ 1.84

cell death (Table 4). Similarly, inhibition of caspase activity using the pan-caspase inhibitor Z-VAD-FMK almost totally abrogated cell death. This confirms previous studies showing that p53-dependent cell death is caspase-dependent and can be inhibited by Bcl-2 [30,31]. Moreover, neither Bcl-2 overexpression nor caspase inhibition by Z-VAD interfered with p53 up-regulation after irradiation, indicating that they were not functioning 'upstream' of p53 (results not shown). When ceramide levels were measured at 24 h post-irradiation, it was found that Bcl-2 inhibited ceramide accumulation almost completely (Table 4). Similarly, examination of glutathione levels showed that Bcl-2 blocked glutathione depletion in response to p53 up-regulation after irradiation (Table 4). This indicated that Bcl-2 functioned at a point 'upstream' of both ceramide accumulation and glutathione depletion.

When the involvement of caspases in these pathways was examined, it was found that inhibition by Z-VAD significantly blocked ceramide accumulation at 24 h post-irradiation (Table 4). However, caspase inhibition by Z-VAD had no effect on glutathione depletion (Table 4). Although Bcl-2 inhibited both ceramide accumulation and glutathione depletion, only ceramide accumulation was dependent on caspase activation.

Recently, it was shown that ceramide accumulation, induced by targeted bacterial sphingomyelinase expression to several cell compartments other than mitochondria, did not induce apoptotic cell death [32]. Cytochrome *c* release and apoptotic cell death were seen only when ceramide accumulated in the mitochondria. This was inhibited by Bcl-2 expression. Several studies have also shown that caspase 9, whose activation is dependent on the release of cytochrome *c* from mitochondria, is an essential caspase in p53-dependent apoptotic cell death [30]. Therefore it became important to examine the relationship of caspase 9 activation to ceramide accumulation. Caspase 9 was activated after irradiation as demonstrated by cleavage of the procaspase to the active fragments (Figure 3A). Significant inhibition of this activation was achieved by exogenous treatment with the specific caspase 9 inhibitor LEHD-FMK (Figure 3A). PARP, which is cleaved by effector caspases, was also cleaved progressively after irradiation (Figure 3A). This was similarly inhibited by LEHD. When cell death, as measured by Trypan Blue uptake, was assessed in irradiated cells in the presence or absence of LEHD, there was significant but not complete inhibition of cell death by LEHD (Figure 3B). This indicated that, in this system, caspase 9 is an important, but not the only, transducer of p53-

Figure 3 Ceramide accumulation without caspase 9

(A) Inhibition of caspase 9 and PARP cleavage by LEHD-FMK. Molt-4 cells were treated with 40 μ M LEHD-FMK for 2 h before irradiation at 5 Gy and compared with untreated irradiated cells at the indicated time points. Caspase 9 and PARP cleavage was determined by Western-blot analysis. The native fragments (procaspase 9 and 116 kDa PARP) and the respective cleaved fragments (p32 and 89 kDa PARP) are indicated. (B) Partial prevention of irradiation-induced cell death by LEHD-FMK. Cells were treated as in (A), and cell death was measured by Trypan Blue uptake at the indicated time points. (C) Lack of inhibition of irradiation-induced ceramide accumulation by LEHD-FMK. Cells were treated as in (A) and ceramide levels were measured at the indicated time points.

dependent cell death. Since cell death in response to irradiation can be a combination of necrosis and apoptosis and Trypan Blue uptake is an indicator of cell-membrane leakiness seen during both necrosis and late apoptosis in cell culture experiments (as opposed to *in vivo*, where phagocytosis of apoptotic cells occurs before membrane leakiness), the effect of LEHD on the prevention of irradiation-induced apoptotic cell death was specifically measured. Using an assay that measures the level of released mono- and oligonucleosomes in the cytoplasm after apoptotic cell death, it was found that irradiation increased the level of nucleosomes from 1 in control to 26.6 and 60.5 arbitrary units at 14 and 24 h respectively. Co-treatment of irradiated cells with LEHD or Z-VAD resulted in nucleosome

levels of 0.4 and 0.9 (LEHD) or 0.3 and 0.4 (Z-VAD) at 14 and 24 h respectively. This suggested that LEHD and Z-VAD had equivalent effects on the inhibition of endonucleases, whose activation is a hallmark of apoptotic cell death. Ceramide levels were then measured in the presence of LEHD. In response to irradiation, ceramide accumulated to similar levels in the presence or absence of LEHD (Figure 3C). Moreover, treatment of Molt-4 cells with cell-permeable C_6 ceramide at 20 μ M resulted in the release of cytochrome *c* from the mitochondrial fraction (results not shown). These studies indicated that caspase 9 activation was not necessary for ceramide accumulation and a different caspase that is inhibited by Z-VAD was responsible for triggering ceramide accumulation.

DISCUSSION

Most tumours involve one or more levels of dysregulation of the p53 pathway, particularly after exposure to chemotherapeutic agents during therapy [33]. Understanding the signalling mechanisms of p53 is of central importance in developing new therapeutic approaches that engage the downstream mediators and bypass the p53 defect. It is now clear that not all the p53-induced events (dependent on or independent of transcription) operate in response to all stimuli and in all cell types. Ample evidence is available that there are stimulus- and tissue-specific responses to p53 up-regulation [34–36].

Several lines of evidence suggest that cellular levels of ceramide play an important role in the regulation of cell death in response to γ -irradiation: (1) irradiation induces p53-dependent ceramide accumulation in a dose- and time-dependent manner, (2) exogenous ceramide induces apoptotic cell death in the same cell types that are sensitive to irradiation at physiologically achievable concentrations, (3) increasing the cellular ceramide levels pharmacologically sensitizes cancer cells to the effects of irradiation and (4) defects in ceramide generation correlate with resistance to irradiation [21,37–39]. Similarly, there is increasing evidence of an important role for ROS generation and cellular glutathione levels in response to γ -irradiation [13,40,41].

In the present study, we show that γ -irradiation induced ceramide accumulation and glutathione depletion within several hours in a p53-dependent manner. In previous studies, coupling of glutathione depletion with ceramide accumulation in response to TNF- α was demonstrated in astrocytes, MCF7 and L929 cells [18,20,42]. It was shown that N-Smase was inhibited by glutathione, which, when depleted, resulted in the activation of N-Smase and generation of ceramide. Exogenous replenishment of glutathione resulted in the inhibition of ceramide accumulation, whereas depletion of glutathione led to sphingomyelin hydrolysis and generation of ceramide. In the present study, it was found that glutathione replenishment of Molt-4 cells before irradiation did not affect ceramide accumulation, whereas glutathione depletion by BSO did not induce ceramide accumulation (Tables 1 and 2). This indicates that glutathione depletion is not upstream of ceramide in response to γ -irradiation of Molt-4 cells. Although N-Smase was involved in ceramide generation in response to cytokines in several cell types and, specifically in Molt-4 cells, in response to serum deprivation [43], it did not contribute significantly to irradiation-induced ceramide generation as shown by direct measurement of this activity in response to irradiation. This suggests that this enzyme has signal-specific and possibly cell-type-specific roles and that alternative pathways of ceramide accumulation are engaged in response to irradiation.

In previous studies, ceramide treatment was shown to induce the generation of ROS in isolated mitochondria or in intact cells at late

time points [44–46]. However, in the current model, ceramide did not contribute to sufficient ROS generation to deplete glutathione levels in the first 24 h after treatment (Table 3). This indicates that ceramide is not upstream of glutathione depletion in the first 24 h after irradiation. However, a later role for ceramide in modulating the redox pathway in this model cannot be excluded. Thus, in this model, the early signalling pathways characterized by ceramide accumulation and glutathione depletion appear to be distinct.

To our knowledge, this is the first demonstration that glutathione depletion can be p53-dependent. The fact that this requires new protein synthesis suggests that it is dependent on genes that are transcriptionally up-regulated by p53, such as the p53-induced genes described previously [6]. In contrast, ceramide accumulation does not require new protein synthesis and, hence, may be independent of genes that are transcriptionally up-regulated in response to irradiation.

Bcl-2 was shown previously to block apoptosis induced by exogenous ceramide in Molt-4 cells, but not to inhibit ceramide accumulation in response to treatment with vincristine [22]. This contrasts with our finding that Bcl-2 completely blocks ceramide accumulation in this cell line in response to irradiation. The most probable explanation is that the pathways of ceramide generation in response to different stimuli may be different and that Bcl-2 selectively inhibits them. Importantly, Bcl-2 overexpression inhibits ceramide accumulation and glutathione depletion, but does not interfere with p53 accumulation in response to irradiation. This indicates that the effects of Bcl-2 are not due to the regulation of p53 itself, but are probably due to the regulation of p53 targets. Also, Bcl-2 is reported to regulate mainly the mitochondrial checkpoint of apoptosis [29]. However, there is recent evidence that it plays important regulatory roles at the levels of ER (endoplasmic reticulum) and the nuclear membrane and that it inhibits necrosis [47]. In this model, where Bcl-2 appears to function both upstream (blocking ceramide accumulation) and downstream (blocking ceramide-induced apoptosis) of ceramide in response to irradiation, we speculate that it regulates another checkpoint (ER or nuclear membrane) in addition to mitochondria, the presumed target of ceramide action.

Caspases were shown previously to function either upstream or downstream of ceramide accumulation and several studies have placed ceramide downstream of caspase 8 and upstream of caspase 3 [48,49]. Inhibition of caspase 8 by the cowpox virus protein crmA resulted in the inhibition of ceramide accumulation in response to TNF- α , but not in response to actinomycin D [48; W. El-Assaad and G.S. Dbaibo, unpublished work). In Molt-4 and other cells, exogenous ceramide was found to activate caspase 3 [50]. In the present study, we found that using Z-VAD-FMK for the inhibition of multiple caspases resulted in the inhibition of ceramide accumulation, whereas the specific caspase 9 inhibitor LEHD-FMK had no effect on ceramide accumulation, although it completely blocked apoptotic cell death and caspase 9 and PARP cleavage. This is important because caspase 9 was described as an essential 'initiator' caspase in response to p53 up-regulation [30]. Thus, our findings indicate that another caspase is activated by p53, leading to ceramide accumulation before activation of caspase 9. In preliminary studies, we found that inhibition of caspase 2 or caspase 8 by specific inhibitors partially inhibited ceramide accumulation in response to irradiation. These ongoing studies will help delineate the caspase-dependent pathway of ceramide accumulation triggered by p53.

A role for ceramide in regulating necrosis in addition to apoptosis has been proposed [51]. In this regard, it is important to note that, whereas LEHD completely inhibited apoptotic cell death as measured by PARP cleavage and DNA fragmentation, it only

partially inhibited total cell death, which includes necrosis, as measured by Trypan Blue uptake (Figure 3). Thus the persistent increase in ceramide levels in the presence of LEHD is associated with significant non-apoptotic or necrotic cell death in response to irradiation. Further studies are needed to delineate the role of ceramide in this non-apoptotic cell death.

In conclusion, the present study demonstrates that, in response to γ -irradiation, in this model, there is p53-dependent cell death that is preceded by p53-dependent ceramide accumulation and glutathione depletion. These two pathways are regulated independently as evidenced by the different responses to protein synthesis inhibition and caspase inhibition and the lack of cross-regulation. Although these two pathways have been shown to be cross-regulated in response to TNF- α through glutathione-regulated N-Smase, this does not seem to be the case in p53-dependent cell death, probably owing to the absence of a role for this enzyme in p53-regulated pathways and the compartmentalization of ceramide production and glutathione depletion. Future studies are aimed at delineating the mechanisms of p53-dependent ceramide accumulation, as this may provide a key to novel therapeutic approaches for resistant cancers.

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