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Lack of ceramide generation in TF-1 human myeloid leukemic cells resistant to ionizing radiation

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Abstract

The mechanism(s) by which ionizing radiation (IR) induces cell death is of fundamental importance in understanding cell sensitivity and resistance. Here we evaluated the response to IR of two subclones of the autonomous human erythromyeloblastic cell line TF-1: TF-1-34 (which expresses CD34) and TF-1-33 (which lacks CD34). In clonogenic assays, TF-1-34 cells were found to be relatively less sensitive to IR compared to TF-1-33 cells based on the D_0 determination (3.01 *vs* 1.56 Gy). Furthermore, after IR at 12 Gy, TF-1-33 cell viability decreased by ~50% within 24 h, whereas TF-1-34 cell growth was unaffected during this time. Gradual loss of TF-1-34 cell viability was observed only after 48 h. Morphological and molecular analysis revealed that TF-1-33 cells died of apoptosis, and TF-1-34 cells of delayed reproductive cell death. While IR produced comparable amounts of DNA double strand breaks (DSB) in both cell lines, TF-1-34 retained DSB much longer than TF-1-33 suggesting that radioresistance and the defective apoptotic response of TF-1-34 cells was not related to a higher DNA repair capacity. However, the lack of an apoptotic response in TF-1-34 was correlated to the absence of a sphingomyelin (SM)-ceramide (CER) signaling pathway. Indeed, IR triggered in TF-1-33 cells but not in TF-1-34, SM hydrolysis (25% at 12 Gy) and CER generation (>50%) through the activation of neutral but not acid sphingomyelinase. Synthetic cell permeate CER induced apoptosis in both TF-1-33 and TF-1-34 cells. This study indicates that alterations of the SM-CER signaling pathway can significantly influence the cell death process as well as the survival of acute myeloid leukemia cells after IR exposure.

Keywords: Apoptosis; resistance; myeloid leukemia; irradiation; ceramide

Abbreviations: PBS, phosphate buffered saline; DAPI, 4′,6′-diamidino 2-phenylindole; EDTA, ethylene diaminetetraacetic acid; SM, sphingomyelin; CER, ceramide; SMase, sphingomyelinase; TNF, tumor necrosis factor; IR, ionizing radiation; DSB, double-strand breaks; AML, acute myeloid leukemia

Introduction

In acute myeloid leukemia (AML), IR is commonly used as part of the conditioning regimen, before autologous or allogeneic bone marrow transplantation. While little is known about the response of immature AML cells to IR at both the cellular and molecular levels, it is believed that IR contributes to the eradication of the most immature residual leukemic cell population. However, studies in mice and subhuman primates have demonstrated that normal immature hematopoietic cells are relatively resistant to IR compared to more mature myeloid cells (Ploemacher *et al*, 1992). Therefore, it is conceivable that immature AML cells also display natural resistance to IR compared to more mature compartments of leukemic myelopoiesis.

DNA double-strand breaks are believed to be the principal (or most important) lesions underlying IR-induced cell death. They are caused by either direct interaction of IR with DNA or indirect action via the production of free radicals following the radiolysis of water (Radford, 1986). Above a certain threshold dose, delineated by the shoulder of the survival curve, irradiated cells fail to correctly repair DNA. The residual DNA breaks lead to chromosome aberrations, rendering cells incapable of sustained cell division (Radford, 1986). However, at lower IR doses, where cell survival is detectable, the onset of cell death generally occurs after one or more cell divisions (Hofer, 1970) which lead to the terms mitotic or delayed reproductive cell death (Chang and Little, 1991).

IR-induced cell death has also been associated with the induction of apoptosis. In fact, the inductibility of apoptosis by IR is generally correlated with radiosensitivity (Meyn *et al*, 1993; Ling *et al*, 1994). In contrast to mitotic cell death, apoptosis is a relatively rapid and irreversible process which occurs within a few hours following IR exposure. Although documented in proliferating cell lines as well as tumor cells (Bump *et al*, 1994), IR-induced apoptosis was originally described in nonmitotic cells such as lymphocytes, which lead to the term of interphase cell death (Yamada and Ohyama, 1988; Soldatenkov *et al*, 1989).

The recent description of ceramide (CER) as a potential mediator of IR-induced apoptosis has attracted a great deal

of attention (Haimovitz-Friedman *et al*, 1994; Santana *et al*, 1996). This signaling cascade consists in SM hydrolysis through the activation of a sphingomyelinase (SMase) with concomitant generation of CER which can mediate the apoptotic process (Santana *et al*, 1996; Hannun, 1996). In fact, the SM-CER pathway has been implicated in the apoptotic process of tumor cells treated with an increasing number of cytotoxic agents including TNFα (Obeid *et al*, 1993), antibodies directed against the Fas/APO-1 protein (Cifone *et al*, 1994), stress-inducing agents such as UV and H_2O_2 (Verheij *et al*, 1996) as well as antileukemic agents (Jaffrézou *et al*, 1996; Zhang *et al*, 1996). However, as far as IR is concerned, it remains unclear whether or not the activation of the SM-CER cycle occurs in human tumor cells. If this were the case, it is conceivable that any alteration of this pathway, upstream and/or downstream of CER generation, may inhibit IR-induced apoptosis, and subsequently favor mitotic cell death; a delayed and less drastic response which would allow for DNA repair and perhaps increase the fraction of surviving tumor cells.

In order to address this important question, we evaluated the cellular response to IR of TF-1-34 cells, an autonomous subclone of the erythro-myeloblastic TF-1 AML cell line that expresses CD34 (an early differentiation antigen), compared to TF-1-33, another TF-1 subclone which lacks CD34 expression. TF-1-34 cells were found to be relatively less sensitive to IR than TF-1-33 cells based on the 90% lethal dose. We show herein that irradiated TF-1-34 cells exhibited mitotic cell death while TF-1-33 cells displayed an apoptotic interphase cell death. Moreover, the lack of TF-1-34 apoptotic response was correlated to the failure of the induction of the SM-CER pathway. Our study indicates that the alteration of the SM-CER signaling pathway can significantly influence the cell death process as well as the survival of AML cells after IR exposure.

Results

Cell survival after irradiation

TF-1-34 and TF-1-33 cells were exposed to increasing IR doses and assayed for their capacity to form colonies in methyl cellulose. Clonogenic assays were performed to obtain a quantitative assessment of the ability of irradiated cells to survive (Figure 1A). Radiosensitivity can be expressed in terms of D_0 for the two cell lines. The D_0 were 3.01 Gy (α=0.23 Gy^{-1}, β=3.02 $\times 10^{-2}$ Gy^{-1}) for TF-1-34 and 1.56 Gy (α=0.59 Gy^{-1}, β=3.11 $\times 10^{-2}$ Gy^{-1}) for TF-1-33 ($P<0.01$). The D_0 determinations suggest that the sub-clone TF-1-34 is more resistant to IR than TF-1-33.

Viability assays

Unirradiated TF-1-34 and TF-1-33 cells presented a doubling time of approximately 24 h when seeded at a cellular concentration of 4×10^5/ml. Under these conditions TF-1 cells grew exponentially until day 4 after which cells became confluent and rapidly died between day 5 and day 6 (Figure 1B). In preliminary experiments, cells were exposed to varying doses of IR (2, 6, 12, and 20 Gy) and cell viability was measured by trypan blue exclusion. The dose of 12 Gy was determined as the minimal dose required to achieve total loss of cell viability within 5 days in both TF-1-34 and TF-1-33. However, TF-1-34 and TF-1-33 cells exposed to 12 Gy exhibited dramatically different responses. Indeed, irradiated TF-1-34 cell growth was unaffected during the first 24 h, then was reduced until day 3 after which a gradual loss of cell viability was observed; no viable cell was detectable at day 5. In contrast, in TF-1-33 loss of cell viability was observed as soon as 24 h ($\sim$50%) and dropped as a linear function of the time with no viable cell detectable at day 3. These findings suggested that after 12 Gy IR exposure TF-1-34 cells proceeded through a complete cell cycle followed by cell death whereas TF-1-33 death occurred within one doubling time.

Characterization of IR-induced cell death

Twenty-four-hours after exposure to 12 Gy IR, DAPI staining of TF-1-33 cells revealed morphological changes characteristic of apoptosis with reduction of cell volume, chromatin fragmentation and apoptotic bodies whereas TF-1-34 remained essentially unchanged. At 48 h, while the majority of TF-1-33 cells were apoptotic, TF-1-34 morphological changes consisted in cell volume increase, plurinucleated giant cells but no apoptotic features (Figure 1C). At 72 h, while no viable TF-1-33 cells were detected, the TF-1-34 cell population consisted of both necrotic and plurinucleated giant cells.

Spectrofluorometric measurement of DNA fragmentation showed that IR induced a dose and time dependent DNA fragmentation in TF-1-33 cells. This was detectable as soon as 6 h with a maximum of 34% DNA fragmentation at 48 h for 12 Gy IR (Figure 2A). No significant DNA fragmentation was detected in TF-1-34 cells whatever the time of analysis (up to 5 days) and the IR dose (up to 20 Gy). Similar results were found after a 6 Gy irradiation (data not shown).

DNA gel electrophoresis confirmed IR-induced oligonucleosomal DNA fragmentation in TF-1-33 but not in TF-1-34 cells. Indeed, in TF-1-33 conventional agarose gel electrophoresis revealed DNA laddering which was faintly visible at 6 h but clearly detectable at 48 h for 12 Gy IR (Figure 2B). In contrast, no DNA laddering was observed in TF-1-34 cells up to 96 h. Since it has been reported that internucleosomal DNA fragmentation, which follows DNA macrofragmentation of chromatin loops, can be absent in certain forms of apoptosis (Oberhammer *et al*, 1993), we also investigated DNA macrofragmentation using pulsed-field gel electrophoresis. These experiments revealed DNA fragments comprised between 600 Kbp and 50 Kbp which preceded DNA ladder formation in TF-1-33 but not in TF-1-34 cells (Figure 2C). Finally, analysis of phosphatidylserine externalization by flow cytometry, using annexin V-fluorescein isothiocyanate, showed significant annexin-positive TF-1-33 cells ($>$20%) as soon as 3 h post-12 Gy IR, whereas TF-1-34 cells remained negative throughout the experiment (data not shown).

Cell cycle analysis

PI-BrdU double staining showed major cell cycle changes in the two TF-1 subclones after IF exposure; however, significant differences between TF-1-33 and TF-1-34 were found (Figure 3). Irradiated TF-1-33 cells (12 Gy) displayed a decrease in G1 content, an accumulation in S phase cells and a moderate G2-M block as soon as 12 h, followed by a progressive increase of subG1 cell content (ADN $<2n$ which was consistent with the detection of apoptotic cells and DNA fragmentation. Altogether these results suggested that IR-triggered apoptosis mainly occurred in S phase accumulated cells. This was confirmed by PI-dUTP-FITC double staining (data not shown). In TF-1-34 cells exposed to the same dose, we observed no modification of the cell cycle during the first 24 h (a result which is consistent with the doubling of the population during this time period) thereafter the most significant cell cycle change consisted in a G2-M block and

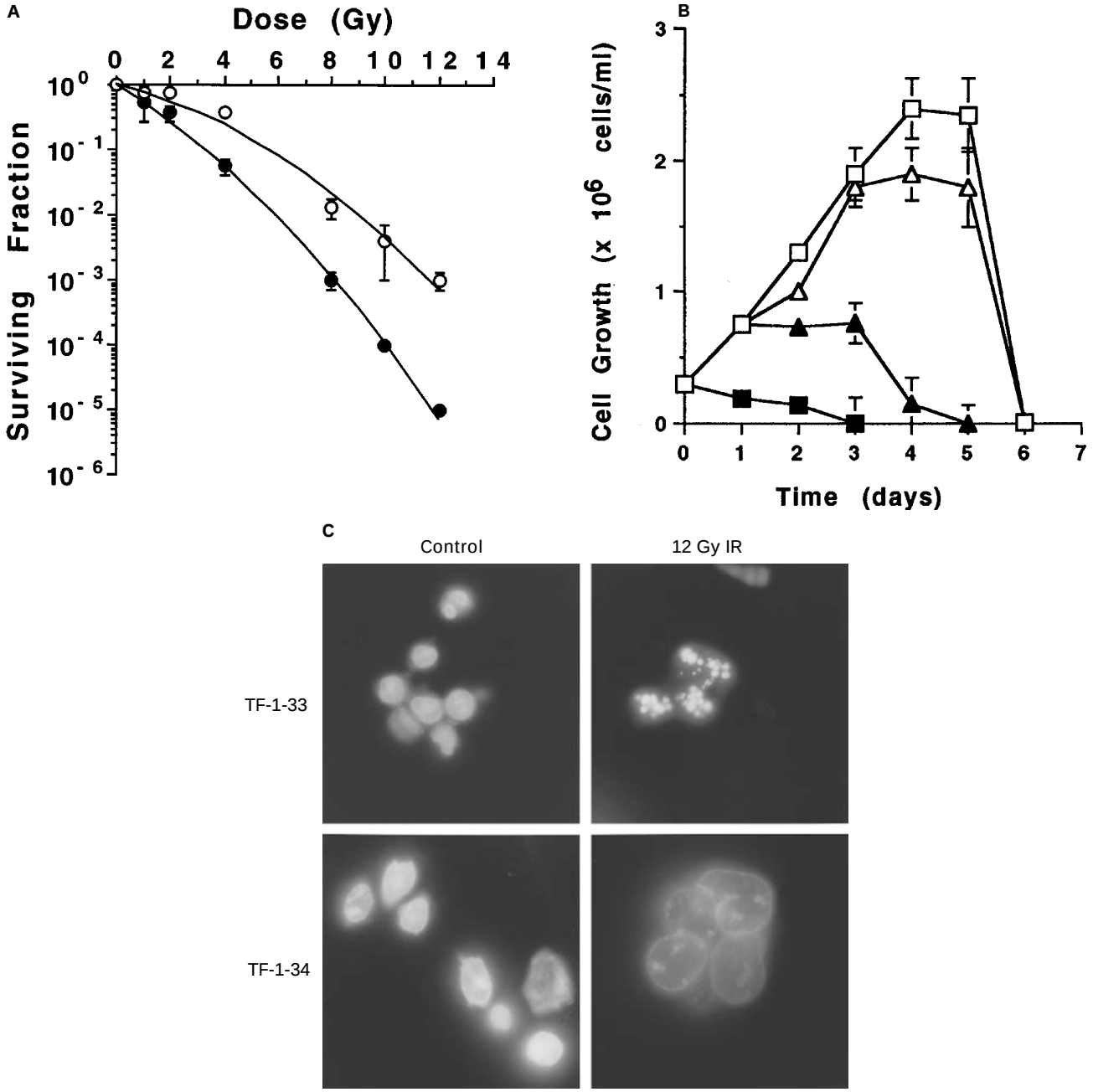

Figure 1 Characterization of IR induced cell death. (**A**) Clonogenic survival of TF-1-33 (●) and TF-1-34 (○) cells treated with graded doses of IR. Irradiated cells were cultured in methylcellulose for 7 days at 37°C. Colonies containing more than 20 cells were enumerated. Points are the mean of triplicate determination ± S.E.M. (**B**) Viability curves for TF-1-33 (■,□) and TF-1-34 (▲,△) cells treated with (closed) or without (open symbol) IR at the dose of 12 Gy. After IR, cells were counted each 24 h, using trypan blue. Points are the mean of triplicate determination ± S.E.M. (**C**) Morphological analysis of IR induced cell death. TF-1-33 and TF-1-34 cells were treated with or without 12 Gy IR, respectively. After 48 h, cells were stained with 0.1 μg/ml DAPI for 30 min and viewed at a magnification of 100 ×

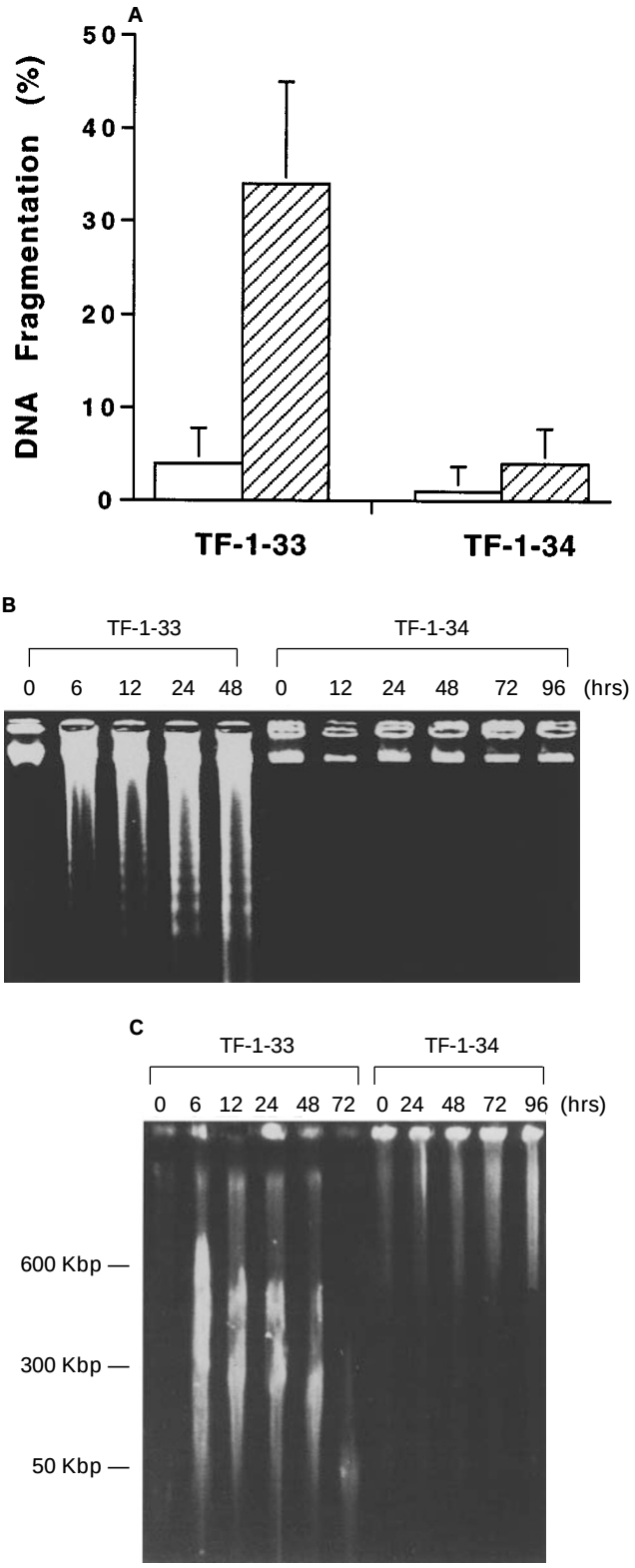

Figure 2 Detection of DNA fragmentation in TF-1-33 and TF-1-34 cells after γ-IR at the dose of 12 Gy. (**A**) Control cells (▭) or irradiated cells (▨) were harvested after 48 h incubation and quantitative DNA fragmentation was determined by the spectrofluorimetric DAPI procedure (see Materials and Methods). Data are the mean $\pm$ S.E.M. of three independent determinations. (**B**) Apoptosis-associated DNA oligonucleosomal fragmentation in TF-1-33 and TF-1-34 cells treated by IR. The formation of oligonucleosomal fragments was determined at various time points by lysing cells, and submitting the DNA to 1.8% agarose gel electrophoresis. Results are representative of five independent experiments. (**C**) DNA macrofragmentation in TF-1-33 and TF-1-34 cells after IR. DNA of treated or untreated cells was extracted at various time points (see Materials and Methods), and submitted to 1% agarose electrophoresis pulsed-field electrophoresis. Results are representative of four independent experiments

the appearance of hyperploid cells with DNA content >4n detectable at 48 h which progressively increased in parallel with the loss of cell viability. Altogether these results suggested that irradiated TF-1-34 cells proceeded through a normal cell cycle, reentered into G1, achieved complete DNA replication before they were transiently blocked at the G2-M transition and died from abortive mitosis.

DNA repair

DSB are believed to be responsible for IR-induced lethality. To investigate the possible relation between DSB and TF-1 cells response to IR, the induction of DSB was measured by pulsed-field agarose gel electrophoresis in each cell line. The induction of DSB from 1 to 10 Mbp increased linearly with the dose, and was similar, if not identical, in TF-1-33 and TF-1-34 cells (Figure 4). The DNA repair capacities of the two cell lines were also measured 3 h after 12 Gy, as DSB rejoining is generally virtually complete in IR sensitive cells within 2–3 h at 37°C (Dhermain *et al*, 1995; Foray *et al*, 1996). In these experiments we found that TF-1-33 cells exhibited much higher DNA repair capacity than TF-1-34 cells. Indeed, DSB were totally removed within 3 h in TF-1-33 cells whereas the percentage of residual DSB in TF-1-34 was as high as 50%.

SM hydrolysis and CER generation

To ascertain whether IR could activate the SM cycle in TF-1 cells, cells were prelabeled with ^{3}H-choline to equilibrium for 48 h. As shown in Figure 5, 12 Gy exposure triggered a SM cycle (hydrolysis and resynthesis) in TF-1-33 but not in TF-1-34. In TF-1-33 cells about 25% SM hydrolysis was detected 2 min after irradiation and SM content returned to baseline level at 8 min post-irradiation. SM hydrolysis was detected for doses as low as 2 Gy and its magnitude reached about 30% for 20 Gy (Figure 5A). In order to identify the product of SM hydrolysis triggered by IR in TF-1-33 cells, intracellular CER was quantitated by the diacylglycerol kinase method. Basal CER levels was similar for both TF-1-33 and TF-1-34 cells with 308.25 pmol/mg, $\pm$ 80.29. As shown in Figure 9, we found a significant burst in intracellular CER concentrations (over 50% at 12 Gy) which was observed concurrently with SM hydrolysis, i.e. at 2–4 min after irradiation. However, no CER production was detected in TF-1-34 cells (Figure 5B). Furthermore, treatment with 25 μM exogenous C6-CER induced 25$\pm$3% and 28$\pm$4% DNA fragmentation at 24 h in the two TF-1 subclones, indicating that the apoptotic signaling pathway downstream of CER was intact in TF-1-34 cells.

SMase stimulation

Since the product of IR-induced SM hydrolysis is CER, the enzyme involved in this reaction is a SMase. Both neutral

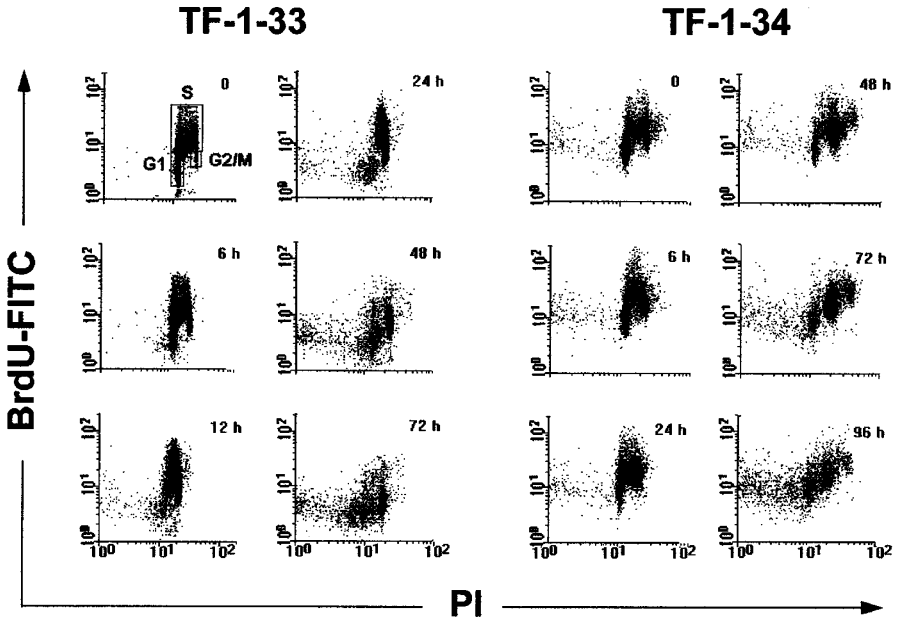

Figure 3 Cell-cycle changes in TF-1-33 and TF-1-34 cells after a 12 Gy IR exposure. Irradiated or unirradiated cells were stained at the indicated time with the BrdU, harvested, fixed and double stained with an FITC-conjugated anti-BrdU antibody and propidium iodide. Cell-cycle analysis was performed on a fluorescence-activated cell sorter. BrdU-FITC fluorescence is on the Y-axis, and DNA content, measured by propidium iodide fluorescence, is on the X-axis

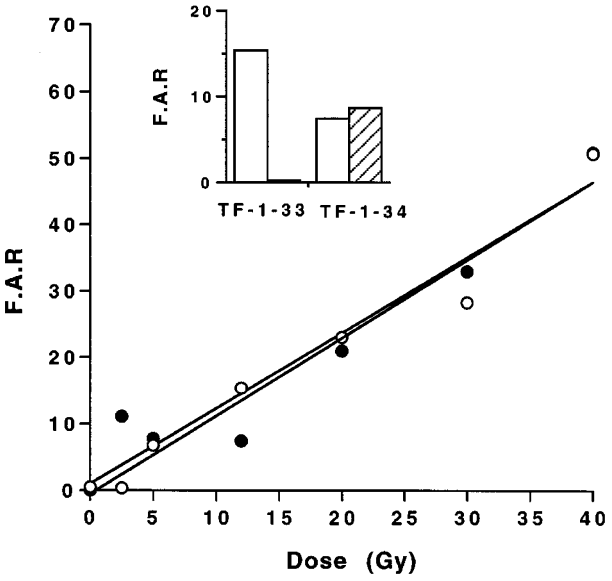

Figure 4 Presence of DSB measured in TF-1-33 and TF-1-34 cells after IR. Quantification of methyl-^{14}C-thymidine-labeled DNA fragments released from the wells after pulsed-field gel electrophoresis was performed for TF-1-33 (○) and TF-1-34 (●). Percentage of the fraction of activity released (FAR) as a function of IR dose. *Insert:* Repair of DSB measured in TF-1-33 and TF-1-34 cells after IR. Radio-labeled DNA fragments released from the wells after pulsed-field gel electrophoresis were fixed and FAR analysis were performed directly (▭) after IR at 12 Gy or after 3 h at 37°C (▨)

(Haimovitz-Friedman *et al*, 1994) and acidic (Santana *et al*, 1996) SMases have been reported to be activated in IR-triggered SM hydrolysis, depending on the cell type. To determine the identity of the SMase(s) implicated in the SM cycle in TF-1-33 cells, measured at 2 min (SM hydrolysis peak) both acidic SMase and neutral, magnesium-dependent SMase activities as previously described (Wiegmann *et al*, 1994; Jaffrézou *et al*, 1996). As shown in Figure 6, IR exposure (12 Gy) resulted in a 25% stimulation of neutral SMase activity in TF-1-33 cells but not in TF-1-34, whereas acid SMase activity remained unchanged.

Discussion

The present study shows that 12 Gy of IR induces interphase apoptotic cell death in the erythro-myeloblastic TF-1-33 but not in TF-1-34 cells. IR-induced cell death has been associated with the induction of apoptosis which is generally correlated with radiosensitivity (Meyn *et al*, 1993; Ling *et al*, 1994). However, as our study shows, a defective apoptotic response to IR may not have significant consequences in terms of IR-induced cytotoxicity above a certain threshold dose. Indeed, at high doses (12–20 Gy), IR was very efficient in eliminating 'resistant' TF-1-34 cells as evidenced by both viability and clonogenic assays. Within this dose-range, we observed that after one round of cell division, TF-1-34 cells died following a prolonged arrest in G2-M. Morphological studies identified plurinucleated cells with hyperploid DNA cellular content, suggesting that some cells proceeded to abnormal mitosis before dying. Such a delayed reproductive cell death (Radford, 1986), also termed mitotic death (Chang and Little, 1991), which was first described in IR treated cells, has also been observed in cells treated with low doses of some anticancer compounds such as bleomycin (Tounetki *et al*, 1993). These findings suggested that TF-1-34 cell death

resulted from a failure to induce a G2-M block-DNA repair mechanism during the first post-IR cell cycle, which subsequently lead to mitotic cell death (mitotic catastrophe). The reason for which the G2 checkpoint did not occur is unclear. Indeed, G2 arrest is commonly observed within the first cell cycle post-IR exposure. This early response to IR has been associated with the rapid and transient phosphorylation-mediated inactivation of $p34^{cdc-2}$ (Kharbanda *et al*, 1994). Therefore, it could be speculated that TF-1-34 cells display defective IR-mediated $p34^{cdc-2}$ regulation.

The prevailing paradigm of the lethal effects of IR identifies DNA DSB as the critical lesions leading to cell death (Radford, 1986). Accordingly, it is generally believed that the inherent resistance of cells to IR is due primarily to their ability to repair DNA damage. In fact, in some mutant cell lines, decreased DNA repair resulted in enhanced sensitivity to IR-induced cytotoxicity (Warters *et al*, 1995). Since TF-1-34 cells were found to be more radioresistant than TF-1-33 cells, we compared IR-induced DNA damages in the two clones after 12 Gy IR exposure. We observed that IR produced comparable amounts of DSB of 1 to 10 Mbp.

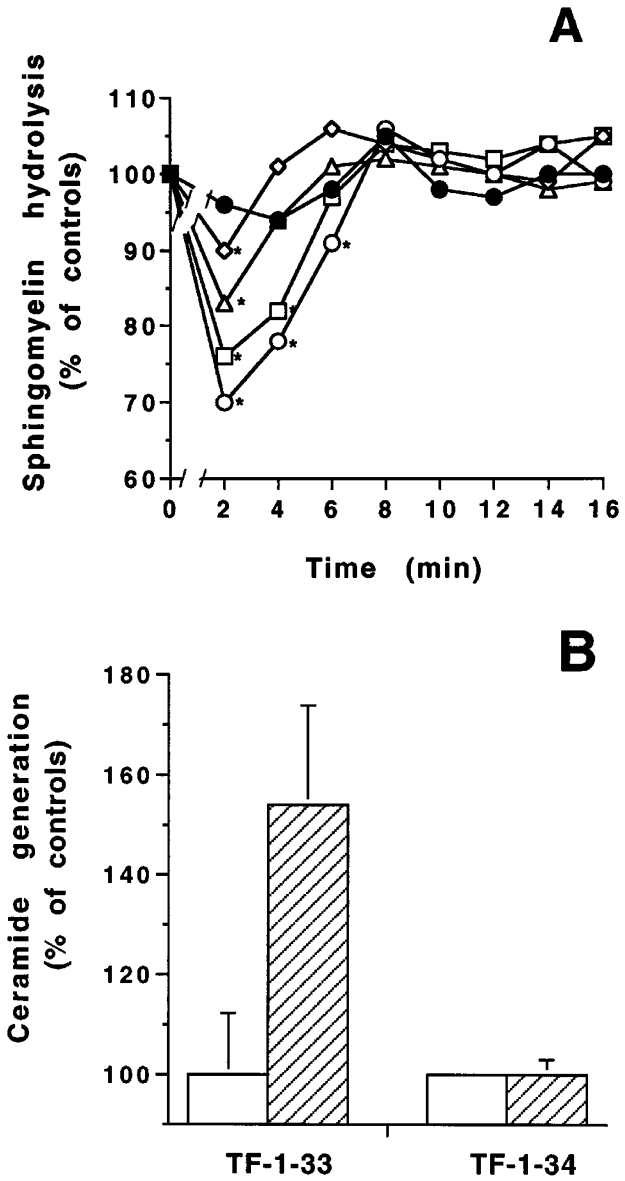

Figure 5 Induction of SM hydrolysis and CER generation in TF-1-33 and TF-1-34 cells treated with IR. (**A**) SM levels were estimated in TF-1-33 (open symbols) and TF-1-34 (closed symbols) cells prelabeled with methyl-^{3}H-choline for 48 h. Cells were washed, resuspended in serum-free medium and irradiated at 2 Gy (◇), 6 Gy (△), 12 Gy (□) and 20 Gy (●,○). Following incubation, aliquots were collected and lipids were extracted. Labeled SM was quantitated as described in Materials and Methods). Results are representative of three to six independent experiments and expressed as a percentage of control. S.E. $<$10% (asterisks mean significant hydrolysis; $P<0.01$). (**B**) CER levels in control (▭) or irradiated cells (▨) were quantitated by the diacylglycerol kinase assay (see Materials and Methods). Results represent peak CER generation observed at peak SM hydrolysis. Values are the mean of triplicate determinations $\pm$ S.E.M. and are representative of three independent experiments

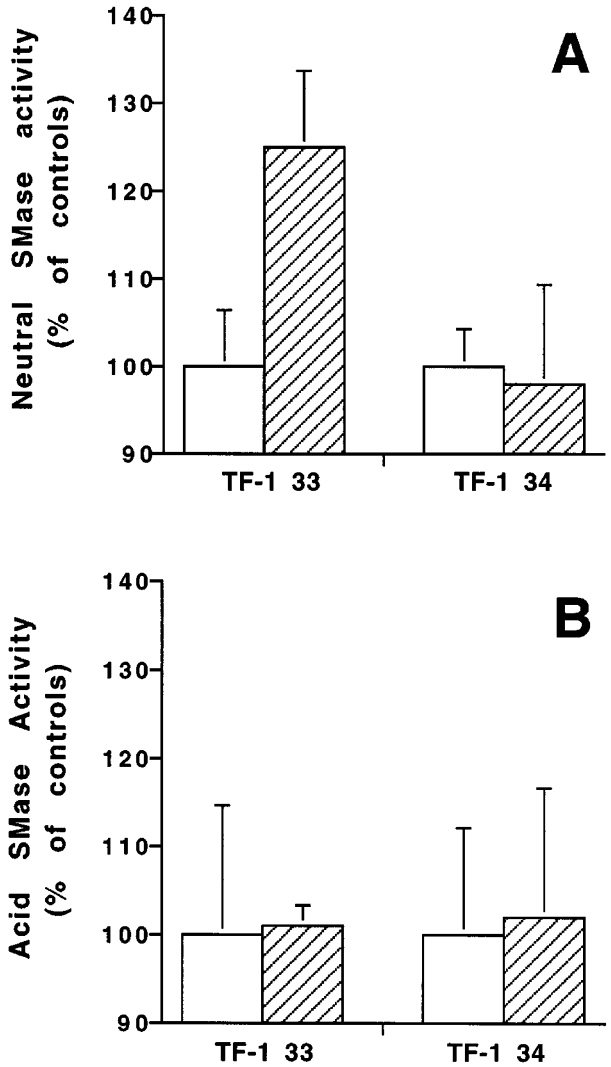

Figure 6 Effect of IR on SMases activities in TF-1-33 and TF-1-34 cells. Control cells (▭) or irradiated cells (▨) at 12 Gy were harvested at various time points and enzyme assays were performed as described in Materials and Methods. Results represent peak SMases activities observed at peak SM hydrolysis. Values are the mean of triplicate determinations and are representative of four independent experiments

However, TF-1-34 retained these DSB much longer than TF-1-33. The lack of DNA repair in TF-1-34 cells may have other important implications. Human hematopoietic progenitor cells have previously been shown to possess, in certain cases, low DNA repair enzymes such as O^6-alkylguanine-DNA transferase (Gerson *et al*, 1996). In our study, after 6 and 12 Gy exposure, an important fraction of TF-1-34 cells entered into S phase and achieved DNA replication before DSB were removed whereas TF-1-33 cells were arrested in S phase before dying by apoptosis. One could speculate that the absence of apoptosis in TF-1-34 cells may have severe mutagenic consequences when these cells are treated with lower doses (for example 6 Gy) thereby allowing for recovery of cell growth of a non negligible fraction (10%) of the cell population. Furthermore, these observations also demonstrate that while TF-1-33 were able to repair large >1 Mbp DSB initially directly induced by IR, this cell line was not able to prevent DNA chromatin loop fragmentation of 50–600 Kbp which appeared during the apoptotic process. Indeed, DNA macrofragmentation of 50–600 Kbp is a direct consequence of an apoptotic pathway (Oberhammer *et al*., 1993), and is not influenced by DNA repair capacity.

The lack of an apoptotic response of TF-1-34 could also be due to abnormal expression of proteins involved in the regulation of IR-induced apoptosis such as Bcl-2 (Sentman *et al*, 1991) or p53 (Fan *et al*, 1994). However, Bcl-2 expression was found similar in both TF-1-33 and TF-1-34, as assessed by Western blot and flow cytometry analysis (data not shown). However, we cannot rule out that other bcl-2 family genes play a role in the defective apoptotic response of TF-1-34 cells. For example, overexpression of Bcl-x_L by cytotoxic drug exposure confers resistance to IR-induced internucleosomal DNA fragmentation (Datta *et al*, 1995). Whether or not TF-1-34 and TF-1-33 cells display differences in bcl-2 related gene expression remains to be investigated. Nevertheless, it seems unlikely that such differences (if any) may account for the lack of CER generation in TF-1-34 cells. Indeed, as far as Bcl-2 is concerned, several recent reports demonstrated that Bcl-2 blocked CER-induced apoptosis but did not interfere with CER generation induced by cytotoxic agents (Martin *et al*, 1995; Zhang *et al*, 1996; Smith *et al*, 1996; Allouche *et al*, 1997). As far as p53 is concerned, it should be noted that a mutated p53 gene has been documented in the parental TF-1 cell line (Sugimoto *et al*, 1992), and we have confirmed altered p53 mRNA in both TF-1-34 and TF-1-33 by reverse transcriptase-polymerase chain reaction single-strand conformational polymorphism analysis (Jaffrézou J.P., Bruno A.P. and Laurent G, unpublished observations). Thus, it can be reasonably speculated that IR-induced apoptosis in TF-1-33 cells can occur via a p53-independent mechanisms as it has been demonstrated for other cellular models (Strasser *et al*, 1994). Moreover, mutated p53 may explain the lack of G1 arrest in irradiated TF-1-33 and TF-1-34 cells since the p53 product is required for IR-triggering G1 checkpoint (Kuerbitz *et al*, 1992) through $p21^{WAF1/CIP1}$ activation (Hunter, 1993). Indeed, we did not observe p21 induction in either cell line after 12 Gy irradiation (data not shown).

Since sensitivity to IR did not appear to correlate with DNA repair, and in light of the rising interest in apoptotic signaling pathways, we elected to evaluate the potential implication of CER in our two cellular models. CER has been implicated as a second messenger for a variety of apoptotic stimuli, including TNFα (Obeid *et al*, 1993), Fas ligand (Cifone *et al*, 1994) chemotherapeutic agents (Bose *et al*, 1995; Jaffrézou *et al*, 1996; Zhang *et al*, 1996), heat shock and oxidative stress (Verheij *et al*, 1996), and IR (Haimovitz-Friedman *et al*, 1994; Santana *et al*, 1996). In these studies, CER generation correlated closely with apoptosis. Moreover, treatment of cells with bacterial SMase or with cell-permeate CER analogs mimicked the apoptotic response. Using human myeloid leukemia cell lines, our study supports the hypothesis that CER acts as a second messenger in the signaling pathway of IR-induced apoptosis not only in normal, but also in tumor cells. Indeed, we found that in TF-1-33 cells, IR-triggered apoptosis and CER generation were dose-related while neither CER production nor apoptosis was detected in TF-1-34 cells. Moreover, the observation that both cell lines were sensitive to cell permeate C6-CER induced apoptosis further supports the role of endogenous CER in IR-induced apoptosis.

The identification of the SMase(s) involved in CER generation has proven to be equivocal. Three forms of SMases are candidates for SM hydrolysis. Acid SMase (pH optimum 4.5–5.0) has been associated in apoptosis signaling triggered by TNFα (Wiegmann *et al*, 1994), anti-Fas (Cifone *et al*, 1994), and anti-CD28 (Boucher *et al*, 1995); activation of a membrane bound neutral SMase (pH optimum 7.4) has also been described with TNFα (Wiegmann *et al*, 1994), anti-Fas antibody (Tepper *et al*, 1995), IL-1β (Mathias *et al*, 1993) and daunorubicin (Jaffrézou *et al*, 1996); finally, a cytosolic neutral SMase has been implicated in vitamin D3 induced differentiation (Okazaki *et al*, 1994). A recent study by Kolesnick's group clearly demonstrated that acid SMase-deficient human lymphoblasts as well as lung endothelium, thymus and spleen of acid SMase knockout mice are defective in IR-induced apoptosis (Santana *et al*, 1996). These authors showed that retroviral transfer of acid SMase gene into these cells restored IR-induced CER generation and apoptosis. Surprisingly, these results conflicted with their previous report which suggested that neutral SMase may be involved in CER generation in irradiated bovine endothelial cells (Haimovitz-Friedman *et al*, 1994). In fact, our study identified neutral but not acid SMase as responsible for SM hydrolysis in irradiated TF-1 cells. Therefore, it appears probable that both acid and neutral forms of SMase may mediate IR-induced apoptosis depending on the cellular origin.

The reason for which IR did not induce SMase stimulation in TF-1-34 cells is unclear. However, it has been recently shown that calphostin C and chelerythrine, two potent and relatively specific protein kinase C (PKC) inhibitors, induce CER production and apoptosis through activation of a neutral, but not acid, SMase (Chmura *et al*, 1996). Furthermore, PKC agonists such as phorbol esters, abrogated neutral SMase stimulation, CER generation and

apoptosis in irradiated bovine endothelial cells apoptosis (Haimovitz-Friedman *et al*, 1994). These findings suggest an antagonistic relationship between PKC and neutral SMase activities. Therefore, we hypothesized that TF-1-34 cells may present higher basal PKC activity than TF-1-33 cells. However, the two TF-1 subclones exhibited similar PKC activity (data not shown). Alternatively, it is possible that, in TF-1-34 cells but not in TF-1-33 cells, IR could induce rapid PKC activation due to stimulation of phosphatidylinositol turnover (Uckun *et al*, 1993) or in relation with arachidonic acid production through phospholipase A_2 activation (Hallahan *et al*, 1994); sustained PKC stimulation could be, in turn, responsible for SMase inhibition. These hypotheses are currently being tested in our laboratory.

Based on CD34 expression, it can be assumed that TF-1-34 resistant cells display a less mature phenotype than TF-1-33 cells. We recently reported that other cytotoxic agents such as daunorubicin (Quillet-Mary *et al*, 1996) mitoxantrone (Bailly *et al*, 1997) and TNFα (Bettaïb *et al*, 1996) were also unable to induce apoptosis in CD34 expressing AML cells such as TF-1-34. Since in our study TF-1-34 cells were deficient in DNA repair, cell cycle arrest, and CER generation one could speculate on causality. Perhaps the lack of CER simply reflects the absence (or refusal) of cell damage recognition. Indeed, it is possible that the repression of the apoptotic program is a common feature of immature myeloid leukemia cells which offers a potential survival advantage to these cells compared to the more mature leukemia hemopoietic compartment. This hypothesis needs to be further investigated.

In conclusion, our study shows that IR induces different cellular response in two subclones of the TF-1 myeloid leukemic cell line. At doses comprised between 6 and 12 Gy, TF-1-33 cells engaged an interphase apoptotic cell death most likely by triggering neutral SMase, SM hydrolysis and subsequent CER generation. In TF-1-34 cells IR were unable to induce SMase stimulation and CER generation. The blockage of this signaling pathway may account for the lack of apoptosis and orients the cellular response to a reproductive cell death. While this manuscript was being completed, another group came to similar conclusions as ours, using IR sensitive and resistant murine lymphoid cells (Chmura *et al*, 1997). In concert, our studies reinforce the recent notion that neutral SMase is a critical upstream regulator of IR-induced cytotoxicity. The characterization of the molecular mechanism which acts as a negative regulator of neutral SMase in the resistant cells may lead to the identification of new radiosensitizers which could restore the apoptotic cell death pathway and subsequently increase the cytotoxicity of IR toward naturally resistant AML cells.

Materials and Methods

Cells and cell culture

The human erythro-myeloblastic cell line TF-1 (Kitumara *et al*, 1989) was generously provided by Dr. W. Vainchenker, INSERM U362, Villejuif, France. TF-1 subclones were obtained by GM-CSF withdrawal and limited dilution. Two clones were chosen based on autonomous growth and phenotypic characteristics: TF-1-34 (CD34+, CD33−, CD38+, CD41+, glycophorin A−); TF-1-33 (CD34−, CD33+, CD38+, CD41+, glycophorin A−). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (all from Eurobio, Les Ulis, France). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Cell stocks were screened routinely for *Mycoplasma* (Stratagene Mycoplasma PCR kit, La Jolla, CA).

Drugs and reagents

Aquasafe 300 scintillation cocktail was purchased from Berthold (Elancourt, France). Silica gel 60 thin-layer chromatography plates were from Merck (Darmstadt, Germany). All other drugs and reagents were purchased from Sigma Chemical Co. (St Louis, MO), Carlo Erba (Rueil-Malmaison, France), or Prolabo (Paris, France).

Irradiation

Irradiations were performed using a ^{60}Co source (1.25 MeV, Alcyon, General Electric) at a dose rate of approximately 1 Gy/min. For DNA repair assays cells were irradiated using a ^{137}Cs source (Cis-bio International).

Clonogenic assays

Clonogenic assays were performed as previously described (Laredo *et al*, 1993). Control and irradiated cells were seeded at preestablished optimal densities (1×10^4 and 2×10^4) in 35 mm Petri dishes containing RPMI, 30% fetal calf serum, 0.5 mM β-mercaptoethanol, and 0.9% methyl cellulose. After 7 days, colonies were scored under an inverted microscope. The surviving fraction was calculated at each dose level as the ratio of the colony numbers observed in the irradiated *versus* nonirradiated control. The best fit survival curve was then calculated by computer using the linear quadratic model (Fertil and Malaise, 1981, 1985). Statistical inference was performed using the Wilcoxon *t*-test.

Morphological analysis

Changes in cellular nuclear chromatin were evaluated by fluorescence microscopy by DAPI staining (Escargueil-Blanc *et al*, 1994).

DNA analysis

DNA was resolved on a 1.8% agarose gel and visualized with ethidium bromide as previously reported (Gong *et al*, 1994). Quantitative DNA fragmentation was determined by the spectrofluorometric DAPI procedure as previously described (McConkey *et al*, 1989; Kapuscinski and Skooczylaski, 1977).

Pulsed-field gel electrophoresis

Agarose plugs were prepared according to a previously published method (Filipski *et al*, 1990). 5×10^6 control and irradiated cells were embedded in 0.7% low-melting agarose plugs and incubated overnight at 50°C in 0.2 M EDTA containing 10 mg/ml proteinase K. The agarose plugs were washed three times for 1 h in 0.2 M EDTA and stored at 4°C prior to analysis. The samples, including a 50–1000 Kbp

pairs lambda DNA standard (Promega Corporation, Madison, USA), were analyzed on a 1% agarose gel (SeaKem FastLane) using a SwitchBack™ Pulse Controller pulsed-field electrophoresis system (Hoefer Scientific Instruments, San Francisco, CA) at 100 V with pulse ramping times from 1 to 50 s (F/R ratio: 2.5 : 1) for 18 h in a 0.5 × TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8). The buffer was recirculated continuously at room temperature. The gels were stained with ethidium bromide (0.5 mg/ml) and photographed under UV illumination.

Cell cycle analysis

Control and irradiated cells were pulsed for 30 min with 25 μM of bromodeoxyuridine (Boehringer Mannheim, Mannheim, Germany), washed in PBS and fixed in 70% ethanol (Minn *et al*, 1995). Fixed cells were then pelleted, washed and further incubated in PBS containing 2.5 M HCl and 0.1% Triton X-100 for 25 min at room temperature. Cells were then washed in neutralization/wash solution (PBS, 0.5% Tween-20) and resuspended in 100 μl antibody-staining solution (PBS, 50% FCS, 0.01% sodium azide). Six microliters of fluorescein isothiocyanate (FITC)-conjugated anti-bromodeoxyuridine antibody (Boehringer Mannheim) was added and cells incubated at room temperature in the dark for 30 min. Cells were washed twice with PBS containing 0.1% sodium azide and resuspended in 500 μl PBS. Ten micrograms of propidium iodide was added and the samples were incubated for 60 min at room temperature in the dark. Samples were analyzed on a fluorescence-activated cell sorter (Facscan, Becton-Dickinson).

Analysis of DNA DSB and DNA repair

Analysis of DSB induction and DNA repair was performed essentially as previously described (Dhermain *et al*, 1995). Cells were radiolabeled by treating for 48 h with 0.3 μCi/ml of methyl^{14}C-thymidine (2.12 MBq/mmole, Amersham, UK). After which, cells were embedded in 0.8% low melting point agarose (BRL, Bethesda, MD, USA). Tubes containing 2 ml of serum-free RPMI medium, and 4–5 agar plugs (approximately 0.8–1.2 μg of DNA) were exposed to γ-radiation (1 Gy/min) from a ^{137}Cs source (IBL 137, Cis-bio international) at a dose range of 0, 2.5, 5, 12, 20, 30 and 40 Gy. After irradiation, cell plugs were treated with 0.5 M EDTA, 2% Sarkosyl, 1 mg/ml proteinase K at 50°C for 24 h, washed twice with Tris-EDTA buffer (1 mM EDTA and 10 mM Tris-HCl, pH 8). Proteinase K activity was stopped by adding 20 μl of 12% PEFA-Block solution for 1 h (Interchim, France). Plugs of control and irradiated samples together with plugs of molecular weight marker, *Schizosaccharomyces pombe* and *Schizosaccharomyces cerevisiae* (BioRad) were inserted into the wells of a 0.8% agarose gel. The gel was placed into the CHEF apparatus (CHEF Mapper™, BioRad) containing permanently circulating 1 × TAE (40 mM Tris acetate and 2 mM EDTA) maintained at 12°C by CHEF Chiller (BioRad). The condition for CHEF electrophoresis were: 74 h total migration time at 2 V/cm with an angle of reorientation +/−53° (106°) and a pulse time of 35 min. After the electrophoretic run the gel was stained in ethidium bromide, destained and visualized by UV transillumination. DNA was quantified by image analysis (Biocom, France) using LECPHOR software. In order to evaluate the radioactivity after electrophoresis when the cells were radioactively labeled, the lanes including the wells (visualized by ethidium bromide staining) were separated and each lane cut into 5 mm segments (22 per lane), each segment was dissolved in 400 μl 1 M HCl at 95°C, neutralized with 1 M NaOH, mixed with 4 ml of scintillation liquid and counted. The fraction of DNA (activity) released from the well (FAR) (Blöcher, 1989) in the unirradiated sample (and its average) was calculated according to the following formula: d.p.m. in the lane of × Gy/d.p.m. in the lane of × Gy+d.p.m. in the well. The fraction of extracted DNA found in the control sample (0 Gy) was systematically substracted from all the fractions of extracted DNA found in the various irradiated samples. Finally, we plotted in the graphs the corrected fractions of extracted DNA (FAR) lane × Gy minus (FAR) lane 0 Gy as a function of irradiation doses.

Metabolic cell labeling and sphingolipid quantitation

SM quantitation was performed by labeling cells to isotopic equilibrium with 0.4 μCi/ml of [methyl-^{3}H]choline (specific activity 81.0 Ci/mmol, DuPont-NEN, Les Ulis, France) for 48 h in complete medium as previously described (Jaffrézou *et al*, 1996; Andrieu *et al*, 1994). Cells were then washed and resuspended in serum-free medium for kinetic experiments. Aliquots were taken for protein determination (Smith *et al*, 1985). Radioactive SM was extracted (Folch *et al*, 1957; Stoffel and Metzner, 1980) and quantitated by scintillation counting. Similar results for SM quantitation were obtained after thin layer chromatography, where SM spots were identified based on Rf and co-migration with authentic standards (data not shown).

Total cellular CER quantitation was quantitated using *E. coli* diaclyglycerol kinase (Amersham, kit no. RPN200) and [^{33}P]γ-ATP (3300 Ci/mmol, Isotopchim, Ganagobie, France) according to previously published procedures (Van Veldoven *et al*, 1992). Statistical analyses were performed by the Student's *t*-test.

Quantitation of acid and neutral SMase activities

SMase assays were performed as previously described (Wiegmann *et al*, 1994) using [choline-methyl-^{14}C]SM (120 000 d.p.m./assay) as substrate (Jaffrézou *et al*, 1996).

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