Alkyl-Lysophospholipids Activate the SAPK/JNK Pathway and Enhance Radiation-induced Apoptosis

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ABSTRACT

Alkyl-lysophospholipids (ALPs) represent a new class of antitumor drugs that induce apoptotic cell death in a variety of tumor cell lines. Although their precise mechanism of action is unknown, ALPs primarily act on the cell membrane, where they inhibit signaling through the mitogen-activated protein kinase (MAPK) pathway. Because stimulation of the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) pathway is essential for radiation-induced apoptosis in certain cell types, we tested the effect of ALPs in combination with ionizing radiation on MAPK/SAPK signaling and apoptosis induction. Here, we present data showing that three ALPs, 1-O-octadecyl-2-O-methyl-ω-glycerol-3-phosphocholine, hexadecylphosphocholine, and the novel compound octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate (D-21266) induce time- and dose-dependent apoptosis in the human leukemia cell lines U937 and Jurkat T but not in normal vascular endothelial cells. Moreover, in combination with radiation, ALPs strongly enhance the induction of apoptosis in both leukemic cell lines. All tested ALPs not only prevented MAPK activation, but, like radiation, stimulated the SAPK/JNK cascade within minutes. A dominant-negative mutant of c-Jun inhibited radiation- and ALP-induced apoptosis, indicating a requirement for the SAPK/JNK pathway. Our data support the view that ALPs and ionizing radiation cause an enhanced apoptotic effect by modulating the balance between the mitogenic, antiapoptotic MAPK, and the apoptotic SAPK/JNK pathways. This type of modulation of specific signal transduction pathways in tumor cells may lead to the development of new therapeutic strategies.

INTRODUCTION

Synthetic membrane-permeable ALPs, also referred to as ether lipids, have been studied as antitumor agents for more than a decade. Examples of these compounds include Et-18-OCH₃ and HePC. The latter has been successfully applied as a topical drug for the treatment of cutaneous metastases (1–3). The precise mechanism of the anti-proliferative effect of ALPs has not yet been fully established. Unlike the classical chemotherapeutic drugs that target the nuclear DNA, ALPs primarily act at the cell membrane. Due to their inherent resistance to phospholipase activities, ALPs accumulate persistently in the plasma membrane and other subcellular membranes (4), where they interfere with mitogenic signaling at different levels of the MAPK signal transduction pathway (5, 6). These effects include: disturbance of the continuous, rapid turnover and biosynthesis of natural phospholipids (7–9); reduction of phospholipase C-mediated inositol 1,4,5-triphosphate formation; and calcium release and inhibition of PKC (10–14).

ALPs have recently become the target of renewed interest because of their capacity to induce apoptosis in various cell types (9, 15–18). For Et-18-OCH₃, it has been suggested that the apoptotic effect is highly selective for malignant cells (9, 16, 17) and that this selectivity is causally related to the cellular uptake of the compound (9). Moreover, ALPs appear to enhance the cytotoxic effect of classical cytostatic regimes (11, 19). For example, in two human epithelial carcinoma cell lines, HePC and Et-18-OCH₃ were found to enhance radiation-induced inhibition of colony formation to supra-additive levels (19).

Ionizing radiation has been shown to induce apoptosis in a large variety of cell types (20–24). One of the signaling pathways that have been implicated in mediating radiation-induced apoptosis is the SAPK/JNK cascade. We and others have shown that this pathway is rapidly activated upon exposure to various stress stimuli and precedes the appearance of nuclear apoptotic features (20, 24–27). Furthermore, activation of this pathway appeared to be essential in transducing death signals because disruption of the pathway by dominant-negative mutants abrogated radiation- and stress-induced apoptosis (24, 28–32). Whereas stimulation of the SAPK/JNK cascade has been associated with apoptosis induction, activation of MAPK is essential for cell growth and differentiation and may counteract apoptotic signaling. In fact, the balance between the pro-apoptotic SAPK/JNK pathway and the antiapoptotic MAPK cascade may be critical in a cell’s decision to activate the death or survival pathway (33, 34). In this respect, it has been shown that Et-18-OCH₃ not only inhibits MAPK signaling but also increases c-Jun gene expression in Jurkat T cells, thereby enhancing the transcriptional activity of the AP-1 complex (35). Because c-Jun is a downstream target of SAPK/JNK, this effect of ALP may contribute to the apoptotic response.

In this report, we describe the apoptotic effect of ionizing radiation and ALPs in U937 and Jurkat T cells. We tested the hypothesis that a combined treatment of both modalities would lead to higher levels of apoptosis than after single agent treatment due to a shift in the SAPK/MAPK signaling balance. Three ALPs were tested: Et-18-OCH₃, HePC, and the recently developed HePC analogue octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate (ASTA compound D-21266; Fig. 1). The latter compound is currently undergoing clinical Phase I evaluation as an oral drug. We show here that all three ALPs that were tested inhibit MAPK signaling, activate the SAPK/JNK pathway, and enhance radiation-induced apoptosis. Whereas human leukemic cells were highly sensitive to the apoptotic effect of ALPs, normal human and bovine endothelial cells remained unaffected, providing a basis for selective and efficient tumor cell kill.

MATERIALS AND METHODS

Reagents. Antibodies against SAPK/JNK1 (C17) were from Santa Cruz Biotechnology (Santa Cruz, CA). [γ-32P]ATP (3 mCi/mmol) was from Amersham (Buckinghamshire, England). Antibodies against p44 and p42 MAPks (ERK1 and ERK2) were from New England Biolabs (Beverly, MA). HePC was purchased from Sigma Chemical Co. (Zwijndrecht, the Netherlands). Et-18-OCH₃ was from Biomol (Plymouth Meeting, PA). D-21266 was kindly provided by ASTA Medica AG (Frankfurt, Germany). ALPs were diluted in serum-free medium.

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3 The abbreviations used are: ALP, alkyl-lysophospholipid; Et-18-OCH₃, 1-O-octadecyl-2-O-methyl-ω-glycerol-3-phosphocholine; HePC, hexadecylphosphocholine; PKC, protein kinase C; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; HUVEC, human umbilical vein endothelial cell; BAEC, bovine aortic endothelial cell; GST, glutathione S-transferase.
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Fig. 1. Chemical structures of ALPs. A, HePC. B, Et-18-OCH3. C, D-21266 [octadecyl-(1,1-dimethyl-piperidino-4-yl)-phosphate].

Cell Culture and Irradiation Procedure. Human monoblastic leukemia (U937) cells and the human T-lymphoid leukemic Jurkat cell line (J16, kindly provided by Dr. J. Borst, NCI, Amsterdam, the Netherlands) were grown at a density between 0.1 × 10⁶ and 1 × 10⁶ cells/ml in Iscove’s modified Dulbecco’s medium (Life Technologies, Inc., Paisley, Scotland), supplemented with glucose (4.5 g/liter), 10% heat-inactivated FCS, penicillin (50 units/ml), and streptomycin (50 µg/ml). U937 cells stably transfected with TAM-67 (U937/TAM-67 cells; a kind gift from Dr. M. J. Birrer, National Cancer Institute, Rockville, MD; Ref. 24) were cultured in the presence of neomycin sulfate (G418; 400 µg/ml). Cells were washed, resuspended in serum-free medium, and irradiated with γ-rays from a 137Cs radiation source (Von Gahlen B.V., Didam, the Netherlands) at an absorbed dose rate of ~1 Gy/min. Control cells were sham-irradiated. In some experiments, endothelial cells from human umbilical vein (HUVECs) or bovine aortic (BAECs) origin were used. HUVECs (kindly provided by Dr. J. A. van Mourik, CLB, Amsterdam, the Netherlands) were cultured in plastic six-well plates precoated with human fibronectin (2 mg/ml). The medium consisted of an equal mixture of RPMI 1640 and Medium 199 (Life Technologies, Inc.), 20% (v/v) heat-inactivated pooled human serum, 2 mM glutamine (Merck, Darmstadt, Germany), and subcultured. Subcultured cells from passages 1 and 2 were used. The medium was replaced every 3 days. BAECs (kindly provided by Dr. Haimo-vitz-Friedman, Memorial Sloan-Kettering Cancer Center, New York, NY) were grown to confluency in DMEM (Life Technologies, Inc.), supplemented with glucose (1 g/liter), 10% heat-inactivated bovine calf serum, penicillin (50 units/ml), and streptomycin (50 µg/ml). Human recombinant basic fibroblast growth factor (R&D Systems) was added every other day during the phase of exponential growth. Confluent monolayers were used for the experiments.

Apoptosis Assays. Apoptosis was determined by either staining with the DNA-binding fluorochrome bis-benzimide (Hoechst 33258; Sigma; Ref. 36) to detect morphological nuclear changes or by propidium iodide staining and FACScan analysis (37) to determine the percentage of subdiploid apoptotic nuclei.

For the bis-benzimide staining, cells were seeded at 2 × 10⁵ cells/ml, 200 µl/well in round-bottomed, 96-well microtiter plates in serum-free RPMI 1640. Cells were lysed in 200 µl of Nicoletti buffer (0.1% sodium citrate, 0.1% Triton X-100, and 50 µg/ml propidium iodide), and the percentage of apoptotic nuclei, recognized by their subdiploid DNA content, was determined on a FACScan (Becton Dickinson, San Jose, CA) using Lysys II software.

Protein Kinase Assays. SAPK/JNK activation was determined by an immune complex kinase assay, using GST-c-Jun (1–135) as substrate (38). Cells were treated with increasing concentrations of ALP, washed, and lysed in lysis buffer [20 mM HEPES (pH 7.4), 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM DTT, 1 mM Na3VO4, 1% Triton X-100, 10% glycerol, 2 µM leupeptin, 10 mg/ml soybean trypsin inhibitor, and 400 µM phenylmethylsulfonyl fluoride] on ice for 15 min. Lysates were clarified by centrifuging for 10 min at 3000 rpm, normalized for protein content, and subjected to immunoprecipitation with anti-SAPK/JNK1 (C17) conjugated to protein A-Sepharose beads (Pharmacia, Uppsala, Sweden). To 41.5 µl of SAPK/JNK-bound beads in assay buffer were added 4 µl of 1 mg/ml GST-c-Jun (1–135), 4.5 µl of Mg-ATP mix (20 mM MgCl2-25 µM ATP), and 1 µl of [γ-32P]ATP. The reaction was terminated after 20 min at 30°C by addition of 15 µl of Laemmli sample buffer and boiling, and the products were resolved on 12.5% SDS-PAGE. The relative SAPK/JNK activity was determined by quantification of the levels of phosphorylated GST-c-Jun (1–135) after stimulation relative to the control situation, using a Fujix BAS 2000 TR PhosphorImager. The effect of ALP on the time course of radiation (25 Gy)-induced apoptosis was scored at 16 h after the indicated single doses of radiation. Data points, means from three independent experiments; bars, SD.

Fig. 2. Radiation-induced apoptosis in U937 cells. Cells were stained by propidium iodide and apoptosis was determined by FACScan analysis. A, time course of radiation (25 Gy)-induced apoptosis (●). Control cells were sham-irradiated (■). B, dose-effect curve. Apoptosis was scored at 16 h after the indicated single doses of radiation. Data points, means from three independent experiments; bars, SD.

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RESULTS

Radiation- and ALP-induced Apoptosis. In both U937 and Jurkat T cells, ionizing radiation induced a time- and dose-dependent increase in apoptotic cell death, as measured by both bis-benzimide staining and FACScan analysis. The first morphological nuclear changes characteristic for apoptosis were detected after 6 h. Fig. 2 shows the time (Fig. 2A) and dose dependency (Fig. 2B) of radiation-induced apoptosis in U937 cells.

Three different ALPs (shown in Fig. 1) were tested for their apoptotic potential. Et-18-OCH₃ (Fig. 1B) is considered as the prototype of these compounds, having a glycerol backbone with an ether-linked long alkyl chain at position sn-1 and an ether-linked methyl group at position sn-2. The other two components, HePC (Fig. 1A) and octadeetyl-(1,1-dimethyl-piperidino-4-yl)-phosphate (ASTA compound D-21266; Fig. 1C) lack a glycerol backbone and have a more linear shape. All three ALPs, like ionizing radiation, induced typical morphological features of apoptosis in a time- and dose-dependent fashion (Figs. 3 and 4). ALP-induced apoptosis increased rapidly after 8 h for HePC and Et-18-OCH₃ and after 16 h for D-21266, reaching values of ~35–40% after 21 h of incubation (Fig. 4A). A steep dose-effect relation between 4 and 12 μM was observed for all three ALPs in both cell types with ED₅₀ between 9 and 10 μM for U937 cells (Fig. 4B) and between 4 and 8 μM for Jurkat T cells (Table 1). HePC and Et-18-OCH₃ appeared to be the most potent compounds, based on ED₅₀, and achieved maximal levels of apoptosis at higher concentrations and at the onset of apoptotic changes. Both radiation- and ALP-induced apoptosis occurred in a caspase-dependent manner because it was associated with PARP cleavage and completely blocked by the broad-spectrum caspase inhibitor zVAD (data not shown).

From the clinical point of view, it was relevant to test the sensitivity of normal vascular endothelium toward ALPs at concentrations that induced apoptosis in the leukemic cells. Table 1 shows that primary endothelial cells (HUVECs and BAECs) were resistant to Et-18-OCH₃-induced apoptosis in the leukemic cells. Table 1 shows that primary endothelial cells (HUVECs and BAECs) were resistant to Et-18-OCH₃ at concentrations as high as 20 μM, whereas in leukemic U937 and Jurkat T cells, high levels of apoptosis were already reached at 12 μM. Similar results were obtained with HePC and D-21266 (data not shown).

Radiation-induced Apoptosis Is Enhanced by ALPs. To test the effect of ALPs on radiation-induced apoptosis, we incubated U937 and Jurkat T cells with different concentrations of the three compounds (4–10 μM) and irradiated with increasing doses of γ-radiation (10, 15, and 20 Gy). At various time points, apoptosis was determined by propidium iodide staining and FACScan analysis. Treatment with a combination of radiation and ALP induced more apoptosis than radiation alone and exceeded the sum of the effects caused by the single agent treatments (Fig. 5). Comparable results were observed in U937 and Jurkat T cells. Statistical analysis of these data revealed an additive effect for the combinations radiation and HePC and radiation and D-21266. For the combination of radiation and Et-18-OCH₃, the effect was synergistic.

ALPs Inhibit MAPK and Activate SAPK/JNK. To assess the antiproliferative action of ALPs in our cell systems, we measured the effect of these compounds on serum-induced MAPK activation. As shown in Fig. 6, serum elicits a very rapid increase in MAPK activity in U937 cells (up to 7.2-fold relative to basal p44/p42 MAPK activity within 6 min). In the presence of Et-18-OCH₃, however, this response was completely blocked. Similar results were obtained with HePC and D-21266 in both cell types (data not shown). As we have shown

Fig. 3. Morphological changes in U937 cells at 16 h after treatment with 20 Gy of ionizing radiation (B), 8 μM of Et-18-OCH₃ (C), or a combination of both (D). Control cells (A) were untreated. Apoptosis was visualized after Hoechst staining by fluorescence microscopy (magnification, ×400).

Statistical Analyses. To characterize the interaction between ionizing radiation and ALPs, we performed calculations using the definition of additivity by Laska et al. (39). This definition is based on the analysis of equi-effect doses (39, 40). If the effect of a combination treatment is equal to the effect expected based on those of its components, it is additive, and if it is superior, it is considered synergistic. Equi-effect doses were calculated from single agent and combined treatment dose effect curves in the range between 0 and 20 μM ALP and between 0 and 50 Gy.

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Fig. 4. ALP-induced apoptosis in U937 cells. Apoptosis was determined by FACScan analysis after propidium iodide staining. A, time course. Cells were incubated with 8 μM HePC ( ), Et-18-OCH₃ (▲), or D-21266 (●). Control cells ( ) were untreated. B, dose-effect curve. Cells were incubated for 16 h with the indicated concentrations of the three ALPs. Data points means from three independent experiments; bars, SD.
previously, MAPK was not significantly activated by radiation in these cells (24).

Because the SAPK/JNK pathway plays an important role in radiation- and stress-induced apoptosis (24, 28, 32, 41), we tested whether ALPs also activate this signaling pathway. As shown in Fig. 7, all three tested ALPs activate the SAPK/JNK pathway in a dose-dependent manner. Fig. 8 shows the time-dependent SAPK/JNK activation in U937 cells by ALP in comparison to radiation. ALP-induced SAPK/JNK activation showed somewhat slower kinetics but was clearly detected in 15 min. Radiation-induced SAPK/JNK activation appeared to be biphasic, as we have recently observed in BAECs as well (41). When both treatments are combined, SAPK/JNK activity is further enhanced by the apparent summation of both stimuli. Fig. 8B also illustrates that both radiation- and ALP-induced SAPK/JNK activation contribute to the combined effect and complement each other over time.

To assess the role of the SAPK/JNK pathway in ALP-induced apoptosis, we used U937 cells stably transfected with the dominant-negative c-Jun deletion mutant TAM-67 (42). Fig. 9 shows that both ionizing radiation- and ALP-induced apoptosis was strongly inhibited in U937/TAM-67 cells, indicating a requirement for SAPK/JNK signaling in apoptosis induction by both stimuli. In contrast, in (normal) HUVECs and BAECs, no significant activation of SAPK/JNK by ALPs (up to 14 μM) was observed (data not shown).

**DISCUSSION**

In these studies, we investigated the effect of membrane-permeable ALPs on radiation-induced apoptosis in human U937 and Jurkat T leukemic cells. Three ALPs that have already shown clinical relevance, were used in our analyses: HePC, Et-18-OCH₃, and D-21266. The first compound, also known as Miltefosine, has been successfully used, at our institute as well as others, as a topical drug for metastatic skin lesions in breast cancer patients (1, 2) and for cutaneous lymphomas (3). The second compound, Et-18-OCH₃ or Edelfosine, has been applied as a purging agent in bone marrow transplantation (43). Finally, compound D-21266, which is structurally related to HePC,
The data presented here are representative of two experiments; a combination of both (○), ALP (●), or a combination of both (▲). The data presented here are representative of two experiments.

For all three compounds we observed an enhancement of radiation-induced apoptosis. In the case of Et-18-OCH₃, the interaction was found to be synergistic. The nature of this enhancing effect is unknown but is clearly dependent on the mode of action of both inducers. Our data show that MAPK activation by serum addition is suppressed by ALPs. This is in agreement with other reports that have shown to be synergistic. The nature of this enhancing effect is unknown but is clearly dependent on the mode of action of both compounds, D-21266 can be administered p.o. with acceptable side effects.

was found to be more active and better tolerated than HePC in preclinical models (10). It has recently entered clinical Phase I studies, including one at our institute. Unlike the other two compounds, D-21266 can be administered p.o. with acceptable side effects.

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It has been proposed that apoptosis induction is under tight control of both apoptosis-promoting and apoptosis-inhibiting signals (33, 34). Several lines of evidence from the literature support this proposition. (a) It has been demonstrated that in the presence of nerve growth factor the MAPK pathway is activated and apoptosis is inhibited, whereas the proapoptotic SAPK/JNK pathway is suppressed (32). Conversely, nerve growth factor withdrawal resulted in SAPK/JNK-mediated apoptosis and a concomitant deactivation of the MAPK signaling pathway. (b) It has been demonstrated that the balance between intracellular levels of distinct lipid second messengers and their respective effects on the MAPK and SAPK/JNK pathways control cell survival and cell death (53, 54). (c) Oxidative stress-induced apoptosis was recently shown to increase when MAPK signaling was inhibited, whereas apoptosis decreased when SAPK/JNK activation was blocked (55). (d) PKC-mediated activation of the MAPK pathway by basic fibroblast growth factor or phorbol ester was found to protect against radiation-induced apoptosis both in vitro and in vivo (22, 56). Conversely, pharmacological inhibition of either PKC or MAPK has been shown to enhance radiation-induced apoptosis (22, 57, 58). Collectively, these studies are in line with the concept of balanced signaling and indicate that the MAPK and SAPK/JNK pathways are inversely related, for instance, via cross-talk at the level of lipid second messengers (53, 54). Although it is difficult to assess the relative contribution of these signaling systems to the apoptotic

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response, these data suggest that the enhancement of radiation-induced apoptosis by ALPs is the result of a potent and prevailing apoptotic SAPK/JNK signal elicited by both stimuli. The ability of ALPs to prevent MAPK activation by mitogenic stimuli may further facilitate SAPK/JNK signaling.

It remains to be established how ALPs activate the SAPK/JNK pathway. Multiple upstream activators of SAPK/JNK have been described, including ceramide (reviewed in Ref. 41). This putative second messenger, generated by hydrolysis of sphingomyelin, has been suggested to mediate apoptosis induction in a number of cell systems (23, 59–62), including radiation-induced apoptosis (24, 41, 63). It was recently found in human keratinocytes that HePC interfered with sphingomyelin biosynthesis, resulting in enhanced cellular levels of ceramide (18). Furthermore, HePC-induced apoptosis in these cells was additively increased by exogenous cell-permeable ceramide and could be blocked by Fumonisins B1, a selective inhibitor of ceramide synthase (18). These data suggest that ceramide might mediate HePC-induced apoptosis and raise the possibility that ceramide generation constitutes a common mechanism of ALP- and radiation-induced apoptosis. In this context, it is interesting to note that inhibition of PKC activity, which is one of the documented effects of ALPs (11–14), potentiates the cytotoxic effect of ceramide in human leukemia and squamous cell carcinoma (57, 64).

A number of biological properties of ALPs make this class of drugs attractive for clinical application. (a) We note the differential cytotoxic effect of ALPs on malignant versus normal cells. Whereas tumor cells were found most sensitive to the lethal action of ALPs, nonmalignant cells display no or minimal toxicity (9, 16, 17). These studies show that endothelial cells, which line the inner blood vessel wall, also remain unaffected by high doses of ALPs. This is a relevant finding, because the vascular endothelium will be exposed for prolonged periods of time to high concentrations of these drugs in vivo, when administered systemically. (b) Another attractive biological effect of ALPs relates to the interaction of these drugs with other conventional cytosstatic regimens (11, 19). In combination with ionizing radiation, ALPs cause an enhanced cytotoxic effect both in terms of postmitotic (19) and, as shown in these studies, apoptotic cell death.

In summary, we have shown that three clinically relevant ALPs enhance radiation-induced apoptosis. Evidence is presented that this combination treatment causes a shift in the MAPK/SAPK balance, resulting in a strong prevailing apoptotic signal. Because the cytotoxicity of ALPs shows a selectivity toward malignant cells, the additive effect on radiation-induced apoptosis as described in the present studies may be exploited to increase the therapeutic ratio. With the development of better tolerated compounds like D-21266, it will be possible to design and test more effective anticancer treatment protocols.

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