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Involvement of c-*Jun* NH_2-Terminal Kinase Activation and c-*Jun* in the Induction of Apoptosis by the Ether Phospholipid 1-*O*-Octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine

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ABSTRACT

The ether phospholipid 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3; edelfosine) is a potent inducer of apoptosis in human tumor cells. We show that ET-18-OCH_3-induced apoptosis is associated with activation of the c-Jun NH_2-terminal kinase (JNK) signaling. The addition of ET-18-OCH_3 to distinct human leukemic cells (HL-60, U937, and Jurkat), which undergo rapid apoptosis on treatment with ET-18-OCH_3, induced a dramatic and sustained increase in the of c-*jun* mRNA level that was associated with activation of activator protein-1 transcription factor. We found that ET-18-OCH_3 induced a persistent activation of JNK in HL-60 cells that was detected before the onset of apoptosis, the latter being assessed by DNA fragmentation and by the appearance of phosphatidylserine on the external leaflet of the plasma membrane. The inductions of JNK after HL-60 monocyte/macrophage differentiation and ET-18-OCH_3-mediated apoptosis were distinguished by the different activation patterns, transient versus persistent, respectively. ET-18-OCH_3 analogues unable to induce apoptosis failed to activate JNK. ET-18-OCH_3-dependent JNK activation was not detected in K562 cells, which did not undergo apoptosis on treatment with ET-18-OCH_3. Phorbol myristate acetate inhibited both ET-18-OCH_3-induced apoptosis and sustained JNK activation; thus, persistent JNK activation by ET-18-OCH_3 is associated with the capacity of this ether phospholipid to induce apoptosis. Furthermore, antisense oligonucleotides directed against c-*jun* blocked ET-18-OCH_3-induced apoptosis, indicating a role for c-Jun in this apoptotic response. These data indicate that JNK activation and c-Jun are involved in the induction of apoptosis by ET-18-OCH_3.

Synthetic ether phospholipids, characterized by the presence of an ether bond in position *sn*-1 of the glycerol backbone, are showing promise as a new class of clinical cancer chemotherapeutic drugs (Munder and Westphal, 1990; Houlihan *et al*., 1995). Some of these lipid molecules are being used as purging agents in autologous bone marrow transplantation (Koenigsmann *et al*., 1996) due to their antineoplastic activities and high selectivity for tumor cells. Several of these compounds are scheduled for, or currently undergoing, phase I/II clinical evaluation (Houlihan *et al*., 1995). The ether phospholipid ET-18-OCH_3 (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; edelfosine) is a synthetic analogue of 2-lysophosphatidylcholine and shows a selective cytotoxic action against transformed cells (Munder and Westphal, 1990; Houlihan *et al*., 1995; Mollinedo *et al*., 1997). An important finding in the elucidation of the processes involved in the antineoplastic effect of ET-18-OCH_3 was its action as a potent inducer of apoptosis in tumor cells (Diomede *et al*., 1993, 1994; Mollinedo *et al*., 1993a, 1997), and this apoptotic action seems to account for the previously reported cytotoxic effects exerted by this ether phospholipid (Mollinedo *et al*., 1997). Although the direct apoptotic action of ET-18-OCH_3 on the cancer cell has been established (Diomede *et al*., 1993, 1994; Mollinedo *et al*., 1993b, 1997), the molecular mechanisms that initiate the active programmed cell death in ET-18-OCH_3-treated tumor cells remain largely unknown. One of the first putative targets for

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ABBREVIATIONS: JNK, c-Jun NH_2-terminal kinase; AP-1, activator protein-1; CAT, chloramphenicol acetyltransferase; ERK, extracellular signal-regulated kinase; FITC, fluorescein isothiocyanate; GSH, glutathione; GST, glutathione S-transferase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TK, thymidine kinase; ITS, insulin/transferrin/sodium selenite; MAPK, mitogen-activated protein kinase; SEK, mitogen-activated protein kinase kinase/c-Jun NH_2-terminal kinase kinase; PMA, phorbol-12-myristate-13-acetate; SDS, sodium dodecyl sulfate; TNF-α, tumor necrosis factor-α; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

the action of ET-18-OCH_3 was considered to be the lipid metabolism, because this ether phospholipid affects lipid biosynthesis and reduces the levels of phosphatidylcholine (Modolell *et al*., 1979; Boggs *et al*., 1995a). In this regard, it has been recently reported that ET-18-OCH_3 behaves as a negative regulator of *de novo* phosphatidylcholine synthesis, acting at the CTP:phosphocholine cytidyltransferase step (Boggs *et al*., 1995b), and it also is a potent inhibitor of coenzyme A-independent transacylase (Winkler *et al*., 1996). Nevertheless, some studies show a lack of correlation between decreases in phospholipid metabolism and the sensitivity of different cell lines to ET-18-OCH_3, suggesting that the lipid perturbation by ET-18-OCH_3 is unlikely to be the underlying cause for its antineoplastic effect (Lu and Arthur, 1992a, 1992b).

The JNKs, also known as stress-activated protein kinases, are members of the MAPK-related family (Karin, 1995; Gupta *et al*., 1996) and are activated in response to a variety of cellular stresses (Verheij *et al*., 1996; Zanke *et al*., 1996). The JNK protein kinases phosphorylate the NH_2-terminal transactivation domain of c-Jun at Ser63 and Ser73, causing increased c-Jun transcriptional activity (Hibi *et al*., 1993). Thus, JNK can mediate the effect of extracellular stimuli on c-Jun and thereby acts as a physiologically relevant regulator of AP-1 transcriptional activity. JNK activation in turn requires its phosphorylation by mitogen-activated protein kinase kinase 4/c-Jun NH_2-terminal kinase kinase, also referred to as SEK1 (Lin *et al*., 1995). Mitogen-activated protein kinase kinase 4/c-Jun NH_2-terminal kinase kinase itself is phosphorylated and activated by the upstream kinase MAPK/ERK kinase kinase 1 (Yan *et al*., 1994). Thus, the stress-activated protein kinase/JNK pathway involves sequential activation of the proteins MAPK/ERK kinase kinase 1, SEK1, JNK, and c-Jun. A number of agents able to induce DNA degradation, including tumor necrosis factor, ceramide, *cis*-platinum, γ-radiation, or UV radiation, recently have been reported to activate JNK (Sluss *et al*., 1994; Chen *et al*., 1996 Verheij *et al*., 1996; Zanke *et al*., 1996). Furthermore, transient and stable expression of a dominant-negative kinase-inactive SEK1 construct impairs both activation of JNK and the apoptotic response triggered by various cell stressors (Verheij *et al*., 1996; Zanke *et al*., 1996). These observations are consistent with the involvement of JNK in the initiation of apoptosis.

We reported previously that ET-18-OCH_3 is able to induce the expression of *fos* and *jun* proto-oncogenes and to activate the transcription factor AP-1 in human leukemic cells (Mollinedo *et al*., 1994a). In this previous study, we observed that the steady state mRNA level of c-*jun* was dramatically increased on ET-18-OCH_3 treatment (Mollinedo *et al*., 1994a). On these grounds, and because JNK activation is involved in the induction of c-*jun* (Karin, 1995), we examined whether JNK stimulation and c-Jun could be implicated in the apoptotic response induced by ET-18-OCH_3.

In the current study, we found that ET-18-OCH_3 increases dramatically the steady state levels of c-*jun* mRNA and induces a persistent JNK activation. The results reported here establish an association between ET-18-OCH_3-induced apoptosis and JNK activation. Furthermore, we found that treatment of cells with antisense oligonucleotides directed against c-*jun* proto-oncogene protects cells from cell death induced by ET-18-OCH_3, indicating a role for this gene on the induction of apoptosis by ET-18-OCH_3.

Materials and Methods

Chemicals and reagents. ET-18-OCH_3 was from R. Berthold (Biochemisches Labor, Bern, Switzerland) and from Laboratorios INKEYSA (Barcelona, Spain). ET-18-OCH_3 analogues were kindly provided by Dr. P. G. Munder (Max-Planck-Institut für Immunbiologie, Freiburg, Germany). ET-18-OCH_3 and its analogues were dissolved at 500 μg/ml as a stock solution in RPMI-1640 culture medium containing 10% (v/v) heat-inactivated fetal calf serum by heating at 50° for 30 min. The clear solutions were sterilized by filtration through a sterile filter (pore size, 0.22 μm) and stored at 4°. Human recombinant TNF-α was kindly provided by Dr. G. Adolf (Boehringer Research Institute, Vienna, Austria). RPMI-1640 culture medium, fetal calf serum, and L-glutamine were purchased from GIBCO BRL (Gaithersburg, MD). Antibiotics were from Laboratorios Llorente (Madrid, Spain). Annexin-V-FLUOS to examine phosphatidylserine exposure was from Boehringer-Mannheim Biochemica (Mannheim, Germany). The Fluorescein Apoptosis Detection System kit for TUNEL assays was purchased from Promega (Madison, WI). [α-^{32}P]dCTP (3000 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), and D-*threo*-[*dichloroacetyl*-1-^{14}C]chloramphenicol (54.6 mCi/mmol) were purchased from Amersham (Buckinghamshire, UK). PMA, GSH-agarose, and pyrithione were from Sigma Chemical (St. Louis, MO). Guanidine thiocyanate was from Fluka (Buchs, Switzerland). Formaldehyde was from J. T. Baker Chemicals B.V. (Deventer, Holland). Acrylamide, bisacrylamide, ammonium persulfate, and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine were from BioRad (Richmond, CA). All other chemicals were from Sigma or Merck (Darmstadt, Germany).

Cells and culture conditions. The human leukemic cell lines used in this study were promyelocytic HL-60, promonocytic U937, T lymphoid Jurkat, and chronic myelogenous K562. These cells were grown in RPMI-1640 culture medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 24 μg/ml gentamicin. Cells were incubated at 37° in a humidified atmosphere of 5% CO_2/95% air. Ether phospholipids were added to the cell cultures at 3 or 5 μg/ml for the times indicated in the respective figures.

Analysis of DNA fragmentation in agarose gels. To assess apoptosis, we isolated fragmented DNA as described previously (Mollinedo *et al*., 1993a). In brief, $\approx 2 \times 10^6$ cells were washed with phosphate-buffered saline and then lysed with 200 μl of hypotonic detergent buffer (10 mM Tris·HCl, pH 7.5, 1 mM EDTA, 0.2% Triton X-100) for 30 min at 4°. Nuclei and cell organelles were removed by centrifugation in a microfuge for 20 min, and the supernatant, containing the DNA released into the cytosol due to DNA fragmentation, was incubated with RNase A (75 μg/ml) for 45 min at 37° and then with proteinase K (200 μg/ml) in the presence of 0.5% SDS for additional 45 min at 37°. The DNA was extracted, precipitated, and analyzed by electrophoresis on 1% agarose gels as described previously (Mollinedo *et al*., 1993a).

TUNEL assay. Apoptosis also was analyzed *in situ* by the TUNEL technique using the Fluorescein Apoptosis Detection System Kit (Promega) according to the manufacturer's instructions, labeling the 3′-OH ends generated by DNA fragmentation through incorporation of fluorescein-12-dUTP (Gavrieli *et al*., 1992). Fluorescent cells were visualized and scored with a Zeiss LSM 310 laser scan confocal microscope.

Phosphatidylserine exposure. Phosphatidylserine exposure at the external surface of the cell was measured by the binding of FITC-labeled annexin V according to the protocol outlined by the manufacturers in the Annexin-V-FLUOS reagent (Boehringer-Mannheim). Then, cells were analyzed with Becton Dickinson (Le Pont de Claix, France) FACScan and FACStar-Plus flow cytometers.

[^{3}H]Thymidine incorporation and TUNEL assay. Cell proliferation was monitored according to [^{3}H]thymidine (Amersham) incorporation as described previously (Mollinedo *et al*., 1993a).

Northern blot. Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method. Samples of 20 μg of RNA were electrophoresed on 0.9% (w/v) agarose-formaldehyde gels and then transferred to Hybond-N nylon membranes (Amersham) as described previously (Mollinedo *et al*., 1994a). ^{32}P-labeled cDNA probes were prepared using the random hexanucleotide priming method (oligo-labeling kit; Pharmacia Biotech, Uppsala, Sweden) to a specific radioactivity $\geq 7 \times 10^8$ cpm/mg of cDNA. cDNA probes for c-*fos* and c-*jun* (Mollinedo and Naranjo, 1991) were kindly provided by Dr. P. Sassone-Corsi (Laboratoire de Genetique Moleculaire des Eucaryotes, Center National de la Recherche Scientifique, Strasbourg, France) and Dr. R. Bravo (Squibb Institute, Princeton, NJ). The plasmid pAc 18.1, used as a probe for β-actin, was used as a control probe as described previously (Mollinedo and Naranjo, 1991). Conditions for blot hybridization and washing have been described elsewhere (Mollinedo *et al*., 1994a). Quantitative analysis of the autoradiograms was performed by integration of peak areas after scanning with a PDI computing densitometer (Pharmacia).

Electroporation and CAT assay. Jurkat cells (20×10^6) in the exponential phase of growth were electroporated as described previously (Mollinedo *et al*., 1994b) with 25 μg of the expression vector AP-1-TK-CAT plasmid (Angel *et al*., 1987), kindly provided by Dr. M. Karin (University of California, San Diego, CA). The AP-1-TK-CAT plasmid contains a single copy of the phorbol ester-responsive element (AP-1 site) inserted upstream to position -109 of the herpes simplex virus thymidine kinase promoter and fused to the structural gene coding for the CAT gene, used as reporter gene (Angel *et al*., 1987). Experimental conditions for CAT assay and thin layer chromatography have been described elsewhere (Mollinedo *et al*., 1994b). After autoradiogram development, the percentage of acetylation of each treatment was determined by scraping off both the acetylated and nonacetylated chloramphenicol and counting radioactivity.

Solid-phase JNK assay. Protein kinase assays were carried out using a fusion protein between GST and c-Jun (amino acids 1–223) as a substrate of JNK, as described previously (Hibi *et al*., 1993) with slight modifications. Cells ($3–5 \times 10^6$) were resuspended in 200 μl of extract buffer (25 mM HEPES, pH 7.7, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Cells were incubated for 30 min in continuous rotation at 4° and then microfuged at 12,000 rpm for 10 min. The pellets were discarded, and the supernatants, representing cell extracts, were diluted with 600 μl of dilution buffer (20 mM HEPES, pH 7.7, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.05% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Mixtures were incubated for 10 min on ice and then microfuged at 12,000 rpm for 10 min. The cell extracts were mixed with 20 μl of a suspension in dilution buffer of GSH-agarose beads, to which GST-c-Jun were freshly bound. Mixtures were rotated overnight at 4° in an Eppendorf tube and pelleted by centrifugation at 12,000 rpm for 1 min. After four 1-ml washes in dilution buffer containing 50 mM NaCl, to remove kinases that have weaker affinity to bind c-Jun(1–223) than JNK, the pelleted beads were resuspended in 30 μl of kinase buffer (20 mM HEPES, pH 7.7, 2 mM dithiothreitol, 20 mM β-glycerophosphate, 20 mM $MgCl_2$, 0.1 mM Na_3VO_4, 20 mM ATP) and incubated with 4 μCi of [γ-^{32}P]ATP. After 30 min at 37°, the reaction was terminated by washing with dilution buffer containing 50 mM NaCl and microfugation at 12,000 rpm for 1 min. Then, the beads were boiled with 10 μl of 5× SDS-polyacrylamide gel sample buffer to elute the phosphorylated proteins, which were subsequently resolved in an SDS-10% polyacrylamide gel, followed by autoradiography. These conditions have been shown previously to enable specific binding of JNK to c-Jun NH_2-terminal domain (Hibi *et al*., 1993).

In-gel kinase assay for JNK activation. This assay was performed according to Hibi *et al*. (1993) with slight modifications. Cells were lysed with extract buffer as above and centrifuged at 12,000 rpm for 10 min, and the supernatants, representing the cell extracts, were saved. Protein content was determined in these extracts using a BioRad protein assay kit. Approximately 60 μg of protein was loaded onto an SDS-10% polyacrylamide gel, which was polymerized in the presence of GST-c-Jun ($\approx$100 μg/ml). After electrophoresis, the gel was washed twice for 15 min each with 100 ml of 50 mM HEPES, pH 7.6, and 20% (v/v) 2-propanol to remove SDS and then twice for 15 min each with 50 mM HEPES, pH 7.6, 5 mM β-mercaptoethanol, and 0.05% Tween 20, supplemented first with 3 M urea and then with 1.5 M urea and finally with 0.75 M urea. The proteins were renatured by washing the gel in 50 mM HEPES, pH 7.6, and 0.05% Tween 20 at 4°. The kinase reaction was performed by incubating the gel for 1 hr at 30° in kinase buffer supplemented with 20 μM ATP and 100 μCi [γ-^{32}P]ATP. Finally, the gel was washed with 100 ml of 5% trichloroacetic and 1% sodium pyrophosphate at room temperature, followed by drying and autoradiography.

Western blot. Mininuclear extracts were obtained from 3×10^6 cells as described previously (Mollinedo *et al*., 1993b), and proteins (30 μg) were separated through an SDS-12% polyacrylamide gel under reducing conditions, transferred to nitrocellulose filters, and subjected to immunological detection. Low range prestained protein molecular mass standards (BioRad) also were run in the same gel. After electroblotting and blocking for 1 hr in 2% powdered nonfat dry milk in TBS buffer (50 mM Tris·HCl, pH 8.0, 150 mM NaCl), the nitrocellulose filters were incubated overnight with sheep anti-human/c-Jun antibody (Cambridge Research Biochemicals, Cheshire, UK) at a dilution of 1:250 in TBS containing 0.05% Tween 20. Signal was developed after incubation with a rabbit anti-sheep immunoglobulin coupled to peroxidase and using 1.7 mM 3,3′-diaminobenzidine with 0.03% (v/v) H_2O_2 in 50 mM Tris·HCl, pH 7.4, as a substrate solution. A rabbit antiserum to the Fos M peptide, kindly provided by Dr. J. Jain (Dana-Farber Cancer Institute, Boston, MA), was used for Fos detection.

Oligonucleotides. c-*fos* sense (5′-TTCTCGGGCTTCAACGCA-3′), c-*fos* antisense (5′-TGCGTTGAAGCCCGAGAA-3′), c-*jun* sense (5′-ACTGCAAAGATGGAAACG-3′), c-*jun* antisense (5′-CGTTTC-CATCTTTGCAGT-3′), and a random oligonucleotide (5′-ACCGT-TCGCTGTTATCTT-3′) were synthesized by using phosphorothioate linkages. The antisense nucleotide sequences were complementary to the first 18 bases after the AUG sequences of human mRNAs for c-*fos* (accession no. K00650) and c-*jun* (accession no. J04111) obtained from the GenBank EMBL database. The corresponding sense oligonucleotides and the random oligonucleotide were used as a control. Oligonucleotides penetrated into the cells without any treatment, as described by Loke *et al*. (1989). HL-60 cells at an initial concentration of 1.2×10^5 cells/ml were either untreated or treated with specific oligonucleotides in serum-free ITS medium composed of 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite in RPMI-1640, containing 0.4% (w/v) bovine serum albumin, 2 mM glutamine, 100 units/ml penicillin, and 24 μg/ml gentamicin. Oligonucleotides were added at a final concentration of 50 μg/ml and incubated for 48 hr in serum-free ITS medium. Then, cells were incubated in the absence or presence of 5 μg/ml ET-18-OCH_3 for 4 hr in serum-free ITS medium and assayed for DNA fragmentation and Western blot analysis. In addition, cells pretreated for 48 hr with random, sense, and antisense c-*jun* oligonucleotides also were assayed for ET-18-OCH_3-induced JNK activation and [^{3}H]thymidine incorporation as indicated above.

Results

c-Jun expression induced by ET-18-OCH_3. We reported previously that ET-18-OCH_3 induces *jun* and *fos* proto-oncogenes and activates AP-1 transcription factor in

HL-60, U937, and Jurkat human leukemic cells (Mollinedo *et al*., 1994a), which are highly sensitive to undergoing rapid apoptosis by this ether phospholipid (Mollinedo *et al*., 1993a, 1997).Because c-*fos* and c-*jun* have been implicated in the induction of apoptosis in various systems (Colotta *et al*., 1992; Ham *et al*., 1995), we analyzed and quantified the respective increases in the steady state mRNA levels of both c-*jun* and c-*fos* in three ET-18-OCH_3-sensitive human leukemic cell lines (U937, HL-60, and Jurkat) (Mollinedo *et al*., 1993a, 1994a, 1997) on treatment with ET-18-OCH_3. We corroborated our previous findings (Mollinedo *et al*., 1994a), showing that ET-18-OCH_3 induces a dramatic and sustained increase in the c-*jun* mRNA level, whereas the c-*fos* mRNA level was weakly or hardly increased (Mollinedo *et al*., 1994a). Because U937, HL-60, and Jurkat cells undergo apoptosis after only 6 hr of treatment with 3 μg/ml ET-18-OCH_3 (Mollinedo *et al*., 1993a, 1994a, 1997), we compared the relative increases in the steady state levels of c-*jun* and c-*fos* mRNA on treatment with ET-18-OCH_3 before the onset of apoptosis (Fig. 1A). After 4 hr of treatment with ET-18-OCH_3 (3 μg/ml), we observed a maximal induction in c-*jun* and c-*fos* expression occurring before the onset of apoptosis (Mollinedo *et al*., 1994a; data not shown). At this incubation time, we found relative inductions of 27-fold (U937), 38-fold (HL-60), and 17-fold (Jurkat) in the c-*jun* mRNA steady state levels on treatment with ET-18-OCH_3, whereas the relative increase in the c-*fos* mRNA steady state level was <5-fold in the three distinct human leukemic cell lines (Fig. 1A). Thus, we found that ET-18-OCH_3 is an extremely potent inducer of c-*jun* expression. We also found, corroborating our previous findings (Mollinedo *et al*., 1994a), that ET-18-OCH_3 activated AP-1 transcriptional activity when used at a concentration able to induce apoptosis, as assessed by CAT assays (Fig. 1B). This activation of AP-1 enhancer activity by ET-18-OCH_3 resulted in lower than that observed when cells were incubated with PMA (Fig. 1B), a strong activator of AP-1 transcription factor. PMA, unlike ET-18-OCH_3, induces a strong increase in the c-*fos* mRNA steady state level in these cell lines (Mollinedo *et al*., 1993b; data not shown). The relative weaker activation of AP-1 transcriptional activity in relation to the extremely high induction of c-*jun* proto-oncogene observed on ET-18-OCH_3 treatment (Fig. 1) could be explained by the very weak induction of c-*fos* in ET-18-OCH_3-treated cells (Fig. 1), because the AP-1 transcription factor containing c-Jun/c-Fos shows a higher DNA binding capacity and transcriptional activity than the c-Jun homodimers (Karin, 1995).

ET-18-OCH_3-induced apoptosis is associated with JNK activation. The c-*jun* proto-oncogene is positively autoregulated by its own gene product, once it is properly phosphorylated (Karin, 1995). Because JNK activates c-Jun transcriptional activity (Hibi *et al*., 1993), candidate inducers of the JNK pathway include agents that cause sustained induction of c-Jun, such as the ether phospholipid ET-18-OCH_3 (Mollinedo *et al*., 1994a). On the other hand, several studies suggest that JNK activation may be related to apoptosis (Sluss *et al*., 1994; Chen *et al*., 1996; Verheij *et al*., 1996; Zanke *et al*., 1996). Fig. 2A shows that 5 μg/ml ET-18-OCH_3 induced a very rapid DNA internucleosomal fragmentation in HL-60 cells after only a 3-hr treatment. On these grounds, we examined JNK activation in HL-60 cells treated with ET-18-OCH_3. To this aim, we used a GST fusion protein containing amino acids 1–223 of c-Jun, GST-c-Jun-(1–223). This fusion protein was bound through its GST moiety to GSH-agarose beads to generate an affinity matrix to precipitate JNK activities from HL-60 cell lysates. The precipitated complexes were washed and subjected to solid-phase kinase assay. As shown in Fig. 2B, GST-c-Jun phosphorylation was observed after 30–60 min of ET-18-OCH_3 incubation, and the response was further increased with the incubation time and was found to be persistent (Fig. 2B). A strong JNK activation was obtained after 2 hr of treatment with ET-18-OCH_3 (Fig.

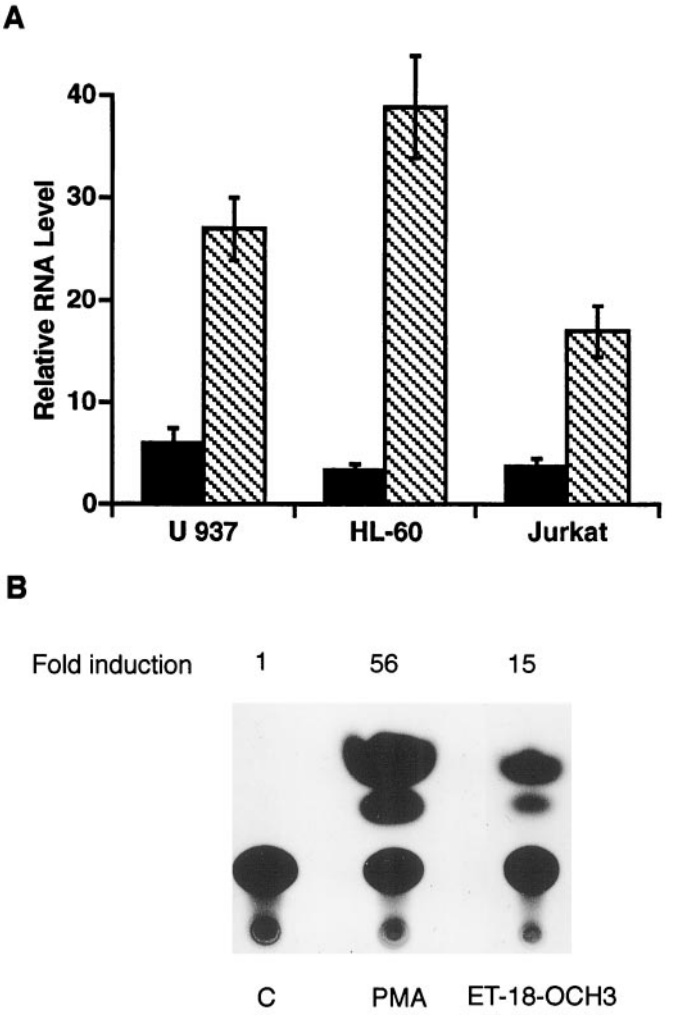

Fig. 1. Expression of c-*jun* and c-*fos* proto-oncogenes and induction of AP-1 enhancer activity on ET-18-OCH_3 treatment. A, Relative induction of c-*fos* and c-*jun* genes in ET-18-OCH_3-treated human leukemic cells. c-*fos* (■) and c-*jun* (▨) steady state mRNA contents were quantified by densitometric scanning of three independent autoradiograms of Northern blots performed after treatment of the indicated cell types with 3 μg/ml ET-18-OCH_3 for 4 hr. Untreated control cells were run in parallel. Each value was corrected for the β-actin mRNA content, and the fold induction was obtained by comparison with the corresponding value in untreated control cells. Data are shown as mean ± standard error of three independent experiments. B, Induction of AP-1 enhancer activity by ET-18-OCH_3 in Jurkat cells as measured by CAT assay. Jurkat cells were electroporated with AP-1-TK-CAT plasmid and treated with PMA (10 ng/ml) or ET-18-OCH_3 (3 μg/ml). Untreated control cells (*C*) were run in parallel. After 24 hr, cells were disrupted, and CAT activity was determined as described in Materials and Methods. Values are representative of three experiments and represent fold induction in AP-1 enhancer activity based on the ratio between the percentage of acetylation in each treatment and the corresponding percentage of acetylation in the control unstimulated cells (*C*).

2B), before DNA fragmentation, occurring after 3 hr of treatment (Fig. 2A). Identical results were obtained using a GST-c-Jun-(1–79) fusion protein (data not shown). Both the apoptotic response and the JNK activation induced by ET-18-OCH_3 were found to be dose dependent and were well correlated (Fig. 3). Thus, HL-60 underwent apoptosis on treatment with 3 μg/ml ET-18-OCH_3, and this response was dramatically increased at 5 μg/ml ET-18-OCH_3 (Fig. 3A). ET-18-OCH_3-induced JNK activation was observed after cell incubation with a concentration of 1 μg/ml of the ether phospholipid, and it was particularly strong after treatment with 3 and 5 μg/ml ET-18-OCH_3 (Fig. 3B). In this regard, it is interesting to note that 1 μg/ml ET-18-OCH_3 has been reported as the threshold concentration in the inhibitory effect on DNA synthesis shown by this ether phospholipid (Mollinedo *et al.*, 1993a). As shown in Figs. 2 and 3, ET-18-OCH_3-induced apoptosis as well as JNK activation were time and dose dependent, and JNK activation always preceded the induction of internucleosomal DNA fragmentation.

Apoptosis also is accompanied by a loss of membrane phospholipid asymmetry, resulting in the exposure of phosphatidylserine on the outer leaflet of the plasma membrane. This phosphatidylserine externalization is considered to be an early process in the apoptotic response, and it seems to play an important role in the recognition and removal of the apoptotic cells by macrophages (Koopman *et al.*, 1994). Expression of phosphatidylserine at the surface of the cell can be monitored by flow cytometry using the binding of FITC-labeled annexin V to phosphatidylserine (Koopman *et al.*, 1994). We found that the appearance of internucleosomal DNA degradation (Fig. 2A) preceded the exposure of phosphatidylserine to the cell outer leaflet (Fig. 4) in ET-18-OCH_3-treated HL-60 cells. In these cells, we observed an intense DNA degradation, a hallmark in the apoptotic response, after 3 hr of ET-18-OCH_3 treatment (Fig. 2A), whereas at this incubation time, only 1.5% of the cells were positive for phosphatidylserine exposure, as assessed by FITC-annexin V binding (Fig. 4). A time course of phosphatidylserine translocation in ET-18-OCH_3-treated HL-60 cells indicated that ≈19% and ≈42% of cells expressed phosphatidylserine after 6 and 9 hr of treatment, respectively (Fig. 4). Interestingly, ≈19% and ≈57% of HL-60 cells underwent DNA fragmentation after 3 and 6 hr of treatment with ET-18-OCH_3, respectively, as assessed by TUNEL analysis.

The above results clearly indicate that JNK activation is an early process in the apoptotic response and precedes DNA degradation and phosphatidylserine exposure (compare Fig. 2B with Figs. 2A and 4), two well known features of the apoptotic response.

We used an in-gel kinase assay to determine the size of the JNK protein kinase or kinases that resulted activated by ET-18-OCH_3. Crude extracts of unstimulated and ET-18-

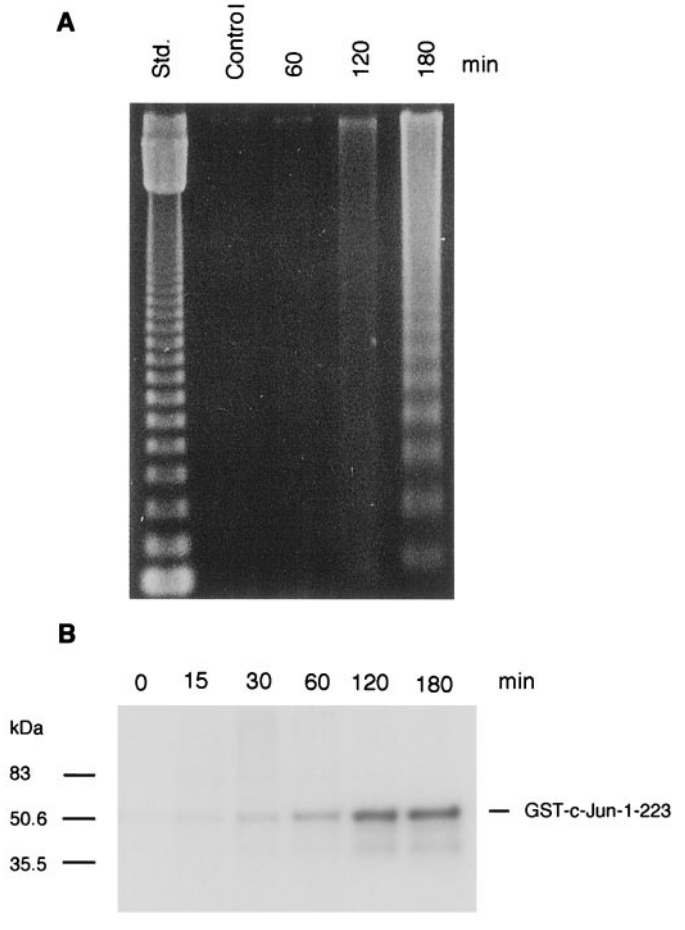

Fig. 2. ET-18-OCH_3 induces rapid apoptosis and persistent JNK activation in HL-60 cells. HL-60 cells were treated with 5 μg/ml ET-18-OCH_3 for the indicated times and assayed for DNA fragmentation (A) or for JNK activation (B) as described in Materials and Methods. Control untreated cells were run in parallel in the same gels. Fragmented DNA from 6×10^5 cells was loaded in each lane of the agarose gel. A, 123-base pair DNA ladder was used as standard (*Std*). B, Position of phosphorylated GST-c-Jun-1–223 is indicated. *Left*, molecular masses (in kDa) of protein markers. Values are representative of three experiments.

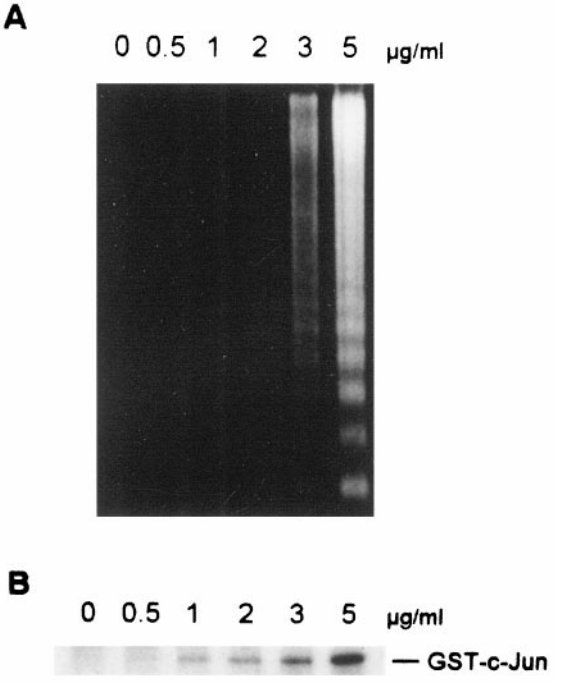

Fig. 3. Dose response of ET-18-OCH_3-induced DNA fragmentation and JNK activation. HL-60 cells were incubated for 4 hr with increasing concentrations of ET-18-OCH_3 and assayed for DNA fragmentation (A) or JNK activation (B) as described in Materials and Methods. Control untreated cells were run in parallel in the same gels. Fragmented DNA from 6×10^5 cells was loaded in each lane of the agarose gel. A, 123-base pair DNA ladder was used as standard. B, Position of phosphorylated GST-c-Jun-1–223 (*GST-c-Jun*) is indicated. Values are representative of three experiments.

OCH_3-stimulated HL-60 cells were fractionated on the same gel, and after electrophoresis, the proteins were renatured in the gel and incubated with [γ-^{32}P]ATP. As shown in Fig. 5, in-gel kinase assays revealed activation of the 46- and 55-kDa forms of JNK in extracts of HL-60 cells treated with ET-18-OCH_3. This result further demonstrates the JNK activation by ET-18-OCH_3 and indicates that two JNK protein kinases are stimulated by ET-18-OCH_3. In good correlation with the above solid-phase kinase data, we observed a persistent activation of JNK (compare Figs. 2B and 5), which was maintained for ≥6 hr (data not shown).

Because JNK activation also has been found during cell treatment with nonapoptotic stimuli, including mitogenic ones, we analyzed the JNK activation pattern during HL-60 differentiation by TNF-α and compared it with that derived from ET-18-OCH_3 treatment. TNF-α, at a concentration of 100 units/ml, induces HL-60 cells to differentiate toward the monocyte/macrophage lineage (Collins, 1987; data not shown). JNK activation by incubation with 100 units/ml TNF-α displayed a rapid and transient induction pattern (Fig. 6). The addition of TNF-α to HL-60 cells rendered an intense band of phosphorylated GST-c-Jun after 15-min treatment; then, this GST-c-Jun phosphorylation decreased gradually until reaching almost basal levels after 120 min of TNF-α treatment (Fig. 6). Thus, this TNF-α-induced JNK activation time course pattern (transient JNK induction) differed from that obtained when HL-60 cells were incubated with ET-18-OCH_3 (persistent JNK induction) (compare Figs. 2B and 6). These data indicate that duration of JNK induction is regulated differentially in HL-60-monocytic differentiation and apoptosis.

We demonstrated recently that the induction of apoptosis by ET-18-OCH_3 was critically dependent on the molecular structure of the ether phospholipid (Mollinedo *et al*., 1997). In this regard, subtle changes in positions *sn*-2 and *sn*-3 of the glycerol backbone in the ET-18-OCH_3 molecule resulted in a complete loss of its capacity to induce apoptosis (Mollinedo *et al*., 1997). Thus, ET-18-OCH_3 analogues, in which the methoxy group in the *sn*-2 position was replaced by an OH (1-*O*-octadecyl-*rac*-glycero-3-phosphocholine) or H (1-*O*-octadecyl-propanediol-3-phosphocholine) (Fig. 7), were unable to induce an apoptotic response in HL-60 cells (Fig. 8A) (Mollinedo *et al*., 1997). To determine whether the JNK activation by ET-18-OCH_3 was due to its apoptotic effect, we analyzed the effects on JNK activation of the two inactive analogues 1-*O*-octadecyl-*rac*-glycero-3-phosphocholine and 1-*O*-octadecyl-propanediol-3-phosphocholine (Fig. 7). Interestingly, these inactive analogues also were unable to induce JNK activation (Fig. 8B).

Although several human leukemic cell lines have been reported to be sensitive to the action of ET-18-OCH_3 (Diomede *et al*., 1993, 1994; Mollinedo *et al*., 1993a, 1997), we found that ET-18-OCH_3 was unable to elicit an apoptotic response in the human chronic myelogenous K562 leukemic cell line, even after a prolonged incubation time of up to 48 hr (data not shown). This result is in agreement with previous data showing lack of a cytotoxic effect of this ether phospholipid in these cells (Tidwell *et al*., 1981; Diomede *et al*., 1993). We also found that ET-18-OCH_3 failed to activate JNK in K562 cells (Fig. 9), whereas parallel studies indicated that this ether phospholipid promoted a remarkable JNK activation in the drug-sensitive HL-60 cells (Fig. 9).

Phorbol esters have been reported to be inhibitors of apoptosis induced by different agents (McConkey *et al*., 1989; Pérez-Sala *et al*., 1995). We found that PMA prevented ET-18-OCH_3-induced DNA fragmentation in HL-60 cells (Fig. 10A). In good correlation with this protective effect, we found that PMA also abrogated JNK activation induced by ET-18-OCH_3 (Fig. 10B).

PMA, another differentiating agent of HL-60 cells toward the monocyte/macrophage lineage (Collins, 1987), induced by itself a rather small, rapid, and transient increase in JNK activation in HL-60 cells (Fig. 11), but it inhibited the persistent JNK activation promoted by ET-18-OCH_3 (Fig. 11). Thus, when HL-60 cells were incubated in the presence of both PMA and ET-18-OCH_3, the induction of JNK activity was inhibited drastically compared with the JNK activation exerted by ET-18-OCH_3 alone (Figs. 10B and 11). This inhib-

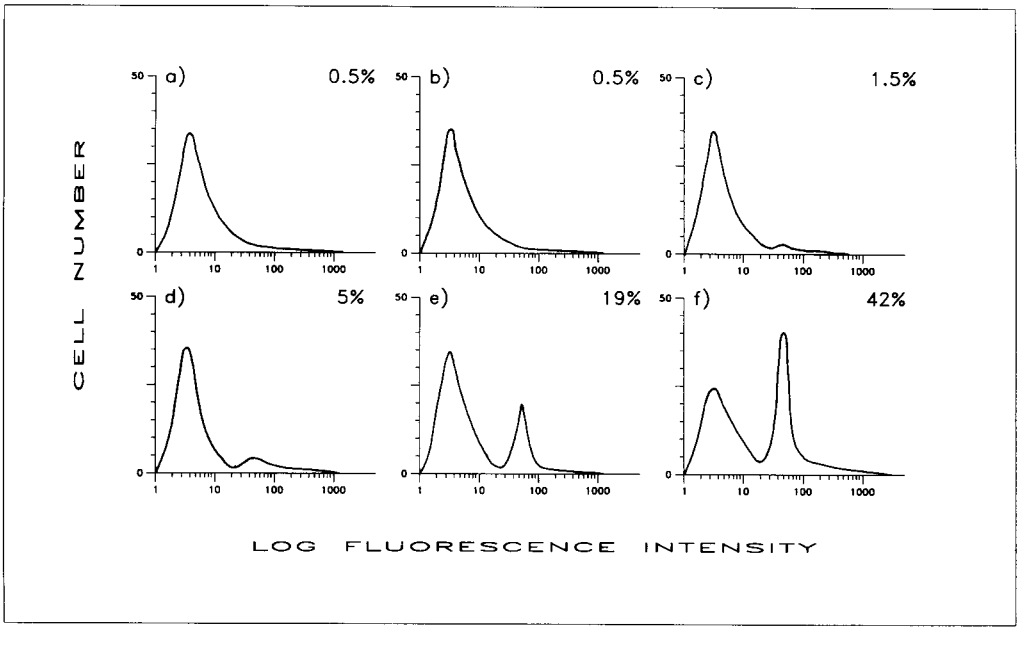

Fig. 4. Flow cytometric analysis of the binding of FITC-labeled annexin V to HL-60 cells treated with ET-18-OCH_3. Untreated (a) and treated HL-60 cells with 5 μg/ml ET-18-OCH_3 for 1 (b), 3 (c), 4 (d), 6 (e), or 9 (f) hr were stained with FITC-labeled annexin V and analyzed by fluorescence flow cytometry as described in Materials and Methods. *Numbers at top right*, percentage of cells that are positive for FITC-annexin V staining. Values are representative of three experiments.

itory effect of PMA, observed when PMA and ET-18-OCH_3 were added simultaneously to the cell culture (Fig. 11), was potentiated further if the cells were pretreated with PMA 15 min before the addition of ET-18-OCH_3 (Fig. 10B).

We also found that Zn^{2+}, acting as an endonuclease inhibitor (Giannakis *et al.*, 1991), blocked ET-18-OCH_3-induced apoptosis (Fig. 10A), indicating that this DNA degradation induced by ET-18-OCH_3 was due to the activation of an endogenous endonuclease. Thus, no internucleosomal DNA degradation was observed when HL-60 cells were treated with ET-18-OCH_3 in the presence of micromolecular concentrations of Zn^{2+} plus a Zn^{2+} ionophore, pyrithione, to facilitate cellular uptake of Zn^{2+} (Fig. 10A).

Requirement of c-Jun in ET-18-OCH_3-induced apoptosis. To examine the role of c-*jun* in the induction of apoptosis by ET-18-OCH_3, we used antisense c-*jun* oligonucleotides. In addition, because c-*fos* has been implicated previously in the induction of apoptosis (Colotta *et al.*, 1992), we used antisense c-*fos* oligonucleotides. Incubation of HL-60 cells in serum-free ITS medium for 48 hr with antisense oligonucleotides directed against c-*jun* or c-*fos* drastically reduced the respective c-Jun and c-Fos protein levels, whereas sense oligonucleotide-treated cells expressed the same protein levels found in untreated cells (Fig. 12D; data not shown). Thus, when HL-60 cells were treated with antisense c-*jun* oligonucleotides for 48 hr before the addition of ET-18-OCH_3, no c-Jun protein was detected (Fig. 12D). The treatment of HL-60 cells with ET-18-OCH_3 slightly increased the protein level of c-Jun, which was not affected by the

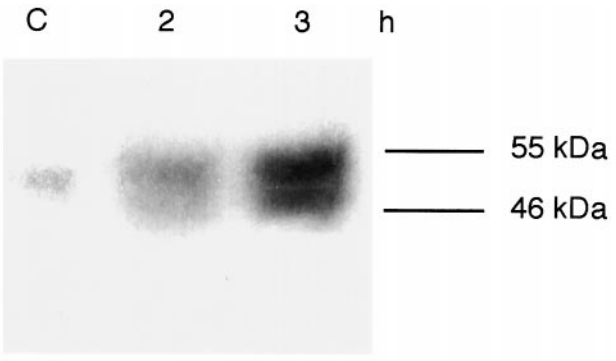

Fig. 5. Identification of the JNK polypeptides activated by ET-18-OCH_3. HL-60 cells were incubated in the absence (*C*) or in the presence of 5 μg/ml ET-18-OCH_3 for the indicated times. Then, cell extracts were prepared, and the JNK polypeptides were visualized after an in-gel kinase assay as described in Materials and Methods. Values are representative of three experiments.

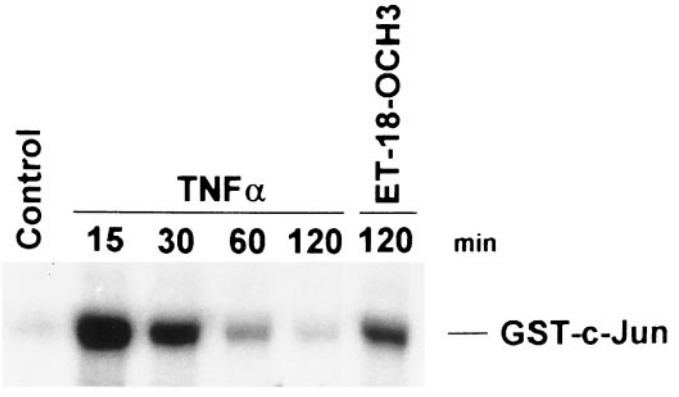

Fig. 6. Rapid and transient JNK activation induced by TNF-α in HL-60 cells. HL-60 cells were treated with 100 units/ml TNF-α or 5 μg/ml ET-18-OCH_3 for the indicated times and assayed for JNK activation as described in Materials and Methods. Control untreated cells were run in parallel in the same gel. The position of phosphorylated GST-c-Jun-1–223 (*GST-c-Jun*) is indicated. Values are representative of three experiments.

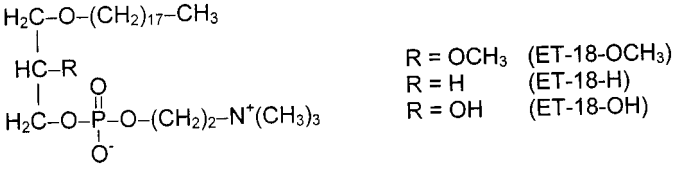

Fig. 7. Chemical structures of the ether phospholipids used in this study. The indicated *sn*-2 substitutions of the glycerol backbone give rise to the ether phospholipids ET-18-OCH_3, ET-18-H, and ET-18-OH.

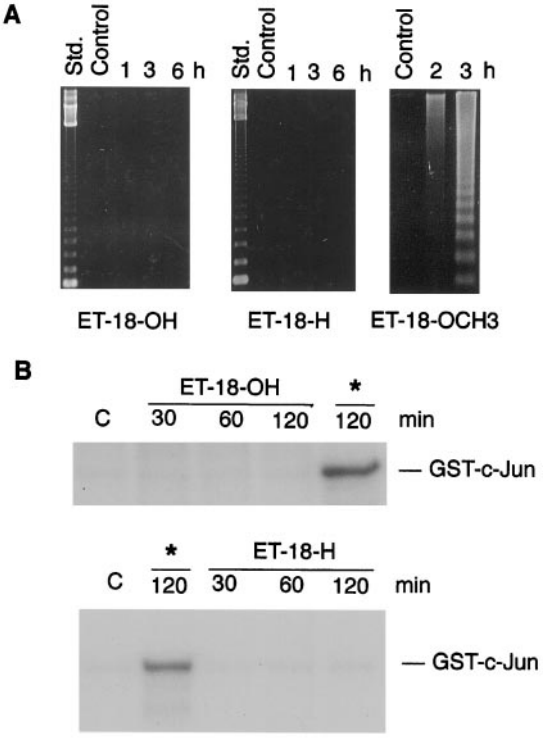

Fig. 8. Molecular structure specificity in the ET-18-OCH_3-induced apoptosis and JNK activation. HL-60 cells were incubated with 5 μg/ml concentration of ET-18-OCH_3 or two closely related ET-18-OCH_3 analogues, ET-18-H and ET-18-OH, for the indicated times and assayed for DNA fragmentation (A) or for JNK activation (B) as described in Materials and Methods. Control untreated cells (*Control* in A; *C* in B) were run in parallel in the same gels. Fragmented DNA from 6×10^5 cells was loaded in each lane of the agarose gels. A, 123-base pair DNA ladder was used as standard (*Std*). B, Position of phosphorylated GST-c-Jun-1–223 (*GST-c-Jun*) is indicated.*, ET-18-OCH_3 and represents the JNK activation induced by treatment of HL-60 cells with 5 μg/ml ET-18-OCH_3 for 2 hr. Values are representative of three experiments.

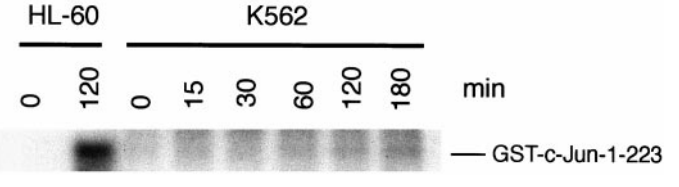

Fig. 9. Lack of JNK activation by ET-18-OCH_3 in K562 cells. HL-60 and K562 cells were treated with 5 μg/ml ET-18-OCH_3 for the indicated times and assayed for JNK activation as described in Materials and Methods. Control untreated cells were run in parallel in the same gels. The position of phosphorylated GST-c-Jun-1–223 is indicated. Values are representative of three experiments.

presence of sense c-*jun* oligonucleotides (Fig. 12D). However, no c-Jun protein was detected when antisense c-*jun* oligonucleotide-treated HL-60 cells were incubated with ET-18-OCH_3 (Fig. 12D). Under these experimental conditions, we found that antisense c-*jun* oligonucleotides blocked the apoptotic response induced by ET-18-OCH_3 (Fig. 12A), whereas ET-18-OCH_3-induced apoptosis was not affected by the presence of antisense c-*fos* oligonucleotides (Fig. 12C). An additional scrambled and random oligonucleotide, with the same base composition as that used for antisense-c-*jun* oligonucleotide (see Materials and Methods), had no effect on ET-18-OCH_3-induced apoptosis or on c-Jun protein level (Fig. 12B; data not shown). These results indicate a role for c-Jun in ET-18-OCH_3-induced apoptosis.

Incubation of HL-60 cells in serum-free ITS medium for 48 hr with antisense c-*jun* oligonucleotides hardly reduced their proliferative capacity (≈12% inhibition), as determined by [^{3}H]thymidine incorporation. Treatment of HL-60 cells with 5 μg/ml ET-18-OCH_3 for 16 hr inhibited cell proliferation by ≈77%. This ET-18-OCH_3 inhibitory effect on cell proliferation was not affected by the presence of sense c-*jun* or random oligonucleotides (≈76% inhibition), and it was slightly diminished (≈57% inhibition) by the presence of antisense c-*jun* oligonucleotides. These results suggest that ET-18-OCH_3 is able to induce two signaling routes leading to apoptosis and inhibition of cell proliferation and that c-Jun would be mainly involved in the apoptotic response. In agreement with this, Boggs *et al*. (1995a) reported that restoration of phosphatidylcholine synthesis through supplementation with lysophosphatidylcholine overrode the cytotoxic but not the cytostatic activity of ET-18-OCH_3.

Treatment of HL-60 cells with antisense c-*jun* oligonucleotides did not block JNK activation (Fig. 13), suggesting that inhibition of the apoptotic response to ET-18-OCH_3 by antisense c-*jun* oligonucleotides occurs at a level downstream of

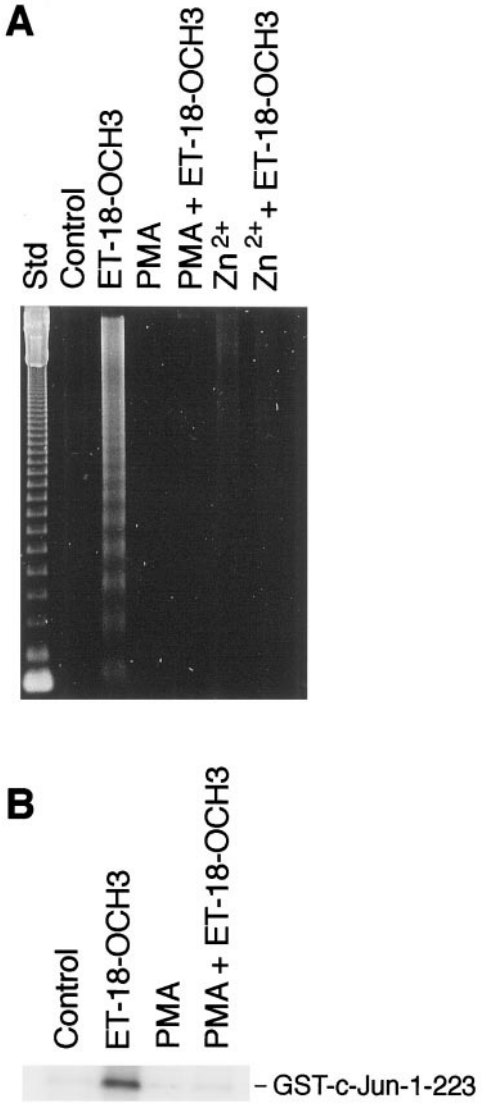

Fig. 10. Suppression of ET-18-OCH_3-induced DNA degradation by PMA and Zn^{2+} and inhibition of ET-18-OCH_3-induced JNK activation by PMA. A, HL-60 cells were incubated for 4 hr in the absence (*Control*) or presence of the indicated agents: 5 μg/ml ET-18-OCH_3, 100 ng/ml PMA, 100 ng/ml PMA plus 5 μg/ml ET-18-OCH_3, 20 μM Zn^{2+} plus 100 μM pyrithione, and 20 μM Zn^{2+} plus 100 μM pyrithione plus 5 μg/ml ET-18-OCH_3. The Zn^{2+} ionophore pyrithione was added to facilitate cellular uptake of Zn^{2+}. PMA, pyrithione, and Zn^{2+} were added 15 min before ET-18-OCH_3. Fragmented DNA was obtained as described in Materials and Methods. Fragmented DNA from 6×10^5 cells was loaded in each lane. A 123-base pair DNA ladder was used as standard (*Std*). B, HL-60 cells were incubated for 2 hr in the absence (*Control*) or presence of the indicated agents (5 μg/ml ET-18-OCH_3, 100 ng/ml PMA, or 100 ng/ml PMA plus 5 μg/ml ET-18-OCH_3) and then assayed for JNK activation as described in Materials and Methods. PMA was added 15 min before ET-18-OCH_3. The position of phosphorylated GST-c-Jun-1–223 is indicated. Values are representative of three experiments.

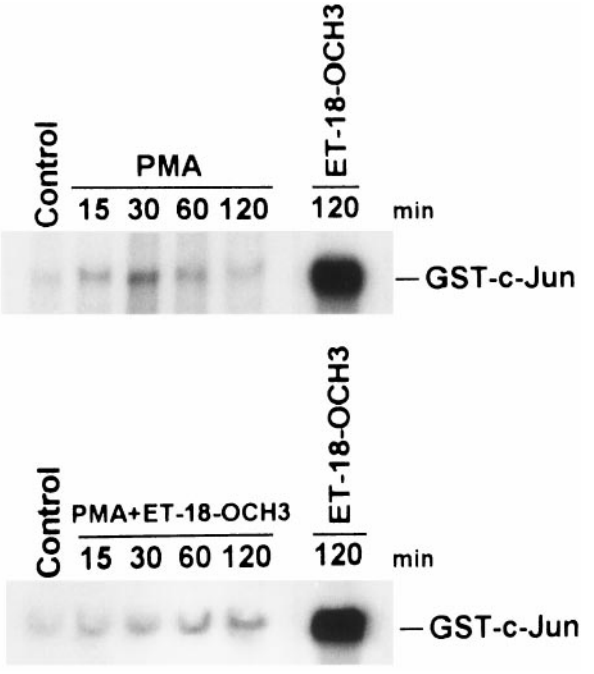

Fig. 11. PMA induces a rapid and transient JNK activation but inhibits the sustained JNK activation induced by ET-18-OCH_3 in HL-60 cells. *Top*, HL-60 cells were treated with 100 ng/ml PMA or 5 μg/ml ET-18-OCH_3 for the indicated times and assayed for JNK activation as described in Materials and Methods. Control untreated cells were run in parallel in the same gel. The position of phosphorylated GST-c-Jun-1–223 (*GST-c-Jun*) is indicated. Experiment shown is representative of three performed. *Bottom*, HL-60 cells were treated with 100 ng/ml PMA and 5 μg/ml ET-18-OCH_3 for the indicated times and assayed for JNK activation as described in Materials and Methods. HL-60 cells treated with 5 μg/ml ET-18-OCH_3 alone for 2 hr were analyzed for JNK activation and run in parallel in the same gel to compare the inhibitory effect of PMA on ET-18-OCH_3-induced JNK activation. Control untreated cells were run in parallel in the same gel. The position of phosphorylated GST-c-Jun-1–223 (*GST-c-Jun*) is indicated. Values are representative of three experiments.

JNK. These results are in agreement with data recently reported by Verheij *et al.* (1996), which showed that stress-induced apoptosis, but not JNK activation, was inhibited in cells expressing a dominant-negative c-Jun mutant.

Discussion

Because (1) ET-18-OCH_3 strongly induces c-*jun* expression (Mollinedo *et al.*, 1994a; current report); (2) JNK activates the transcriptional activity of c-Jun, stimulating c-*jun* transcription (Karin, 1995); and (3) JNK signaling has been reported to be involved in the induction of cell death by different agents in distinct systems (Sluss *et al.*, 1994; Chen *et al.*, 1996; Verheij *et al.*, 1996; Zanke *et al.*, 1996), we suspected that the JNK signaling route might be involved in the induction of apoptosis by ET-18-OCH_3. The results described here indicate for the first time the association of persistent JNK activation with ET-18-OCH_3-induced apoptosis, as well as the requirement of c-Jun for this apoptotic response. Thus, these data unveil a signaling route involved in the induction of apoptosis by ET-18-OCH_3. We found that ET-18-OCH_3 induces a persistent JNK activation in HL-60 cells, which undergo rapid apoptosis on treatment with this ether phospholipid (Fig. 2) (Mollinedo *et al.*, 1993a). This JNK activation may mediate the increased AP-1 activity observed in ET-18-OCH_3-treated cells (Fig. 1) (Mollinedo *et al.*, 1994a). However, ET-18-OCH_3-induced JNK activation is not detected in ET-18-OCH_3-resistant K562 cells. Subtle modifications in the molecular structure of ET-18-OCH_3 that abolish its apoptotic properties also abrogate its capacity to induce JNK activation. In this regard, ET-18-OCH_3 analogues, in which the methoxy group is substituted for an OH or H group in the *sn*-2 position of the glycerol backbone, lack the capacities to activate JNK and induce apoptosis in HL-60 cells. Thus, the results reported here indicate that ET-18-OCH_3-induced JNK activation is associated with the capacity of this ether phospholipid to induce apoptosis.

We also found that this JNK activation seems to precede the onset of apoptosis induced by ET-18-OCH_3. JNK activation was detected before DNA fragmentation and phosphatidylserine externalization, two early and well known processes involved in the apoptotic response. Furthermore, both dose-response and time course analyses indicated that JNK activation preceded DNA fragmentation in ET-18-OCH_3-treated HL-60 cells. On the other hand, our data suggest that in ET-18-OCH_3-treated HL-60 cells, DNA fragmentation, assessed through visualization of the typical internucleosomal DNA fragments in agarose gels and through TUNEL analysis, precedes phosphatidylserine exposure at the outer leaflet of the plasma membrane, as assessed through FITC-labeled annexin V binding.

PMA was found to block both ET-18-OCH_3-induced apoptosis and JNK activation. Although the molecular basis for these effects remains to be determined, the protective effect of PMA against apoptosis has been related to its ability to induce cellular alkalinization (Pérez-Sala *et al.*, 1995). In this regard, an association has been reported between intracellular acidification and DNA degradation (Barry *et al.*, 1993;

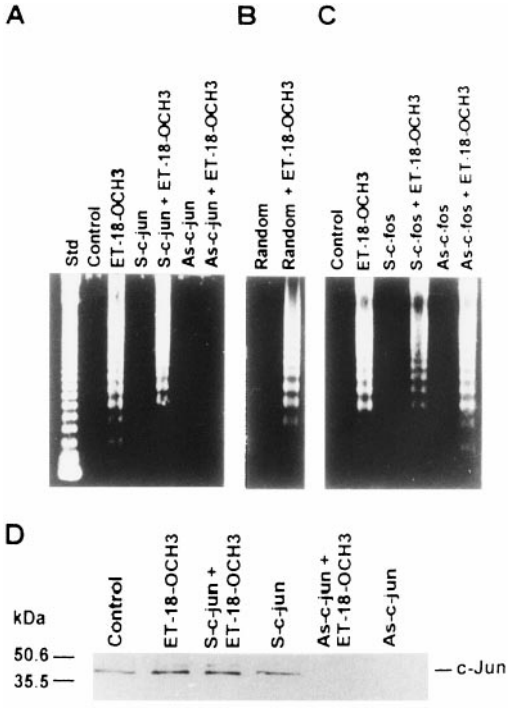

Fig. 12. ET-18-OCH_3-induced apoptosis is prevented by antisense c-*jun* oligonucleotides. A–C, HL-60 cells were grown for 48 hr in serum-free ITS medium in the absence (*Control*) or presence of the indicated specific oligonucleotides as described in Materials and Methods. Then, cells were incubated for additional 4 hr in the absence or presence of 5 μg/ml ET-18-OCH_3, and fragmented DNA was obtained and analyzed as described in Materials and Methods. Sense c-*jun* (*S-c-jun*), 50 μg/ml; antisense c-*jun* (*As-c-jun*), 50 μg/ml; sense c-*fos* (*S-c-fos*), 50 μg/ml; antisense c-*fos* (*As-c-fos*), 50 μg/ml; random oligonucleotide (*Random*), 50 μg/ml. Fragmented DNA from 6×10^5 cells was loaded in each lane. A 123-base pair DNA ladder was used as standard (*Std*). D, Immunoblot analysis of c-Jun protein. HL-60 cells were grown as in A for 48 hr in serum-free ITS medium in the absence (*Control*) or presence of specific oligonucleotides; then, cells were incubated for additional 4 hr in the absence or presence of 5 μg/ml ET-18-OCH_3. Nuclear extracts were prepared as described in Materials and Methods. Equal amounts of nuclear proteins (30 μg) were run on an SDS-12% polyacrylamide gel and analyzed by immunoblotting using a specific anti-c-Jun antibody as described in Materials and Methods. The position of c-Jun protein is indicated. *Left*, molecular masses (in kDa) of protein markers. Sense c-*jun* (*S-c-jun*), 50 μg/ml; antisense c-*jun* (*As-c-jun*), 50 μg/ml.

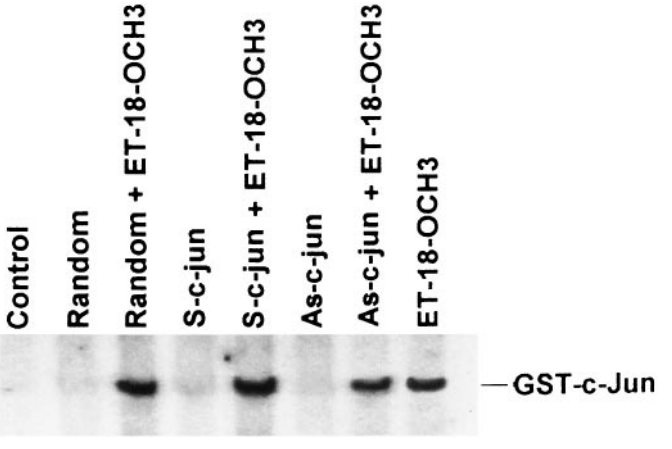

Fig. 13. JNK activation induced by ET-18-OCH_3 is not inhibited by antisense c-*jun* oligonucleotides. HL-60 cells were grown for 48 hr in serum-free ITS medium in the absence (*Control*) or presence of the indicated specific oligonucleotides as described in Materials and Methods. Then, cells were incubated for additional 2 hr in the absence or presence of 5 μg/ml ET-18-OCH_3 and assayed for JNK activation as described in Materials and Methods. Sense c-*jun* (*S-c-jun*), 50 μg/ml; antisense c-*jun* (*As-c-jun*), 50 μg/ml; random oligonucleotide (*Random*), 50 μg/ml. The position of phosphorylated GST-c-Jun-1–223 (*GST-c-Jun*) is indicated. Values are representative of three experiments.

Pérez-Sala *et al*., 1995). Thus, it will be of interest to study a putative relationship between intracellular pH and JNK activation.

The requirement of c-*jun* in ET-18-OCH_3-induced apoptosis was determined by the blockade of apoptosis by antisense c-*jun* oligonucleotides. These results are in agreement with previous reports showing a crucial role for c-Jun in the induction of apoptosis in different systems, as demonstrated by the use of c-Jun dominant-negative mutants, neutralizing antibodies, or antisense oligonucleotides (Colotta *et al*., 1992; Ham *et al*., 1995; Verheij *et al*., 1996). However, antisense c-*fos* oligonucleotides did not prevent ET-18-OCH_3-induced apoptosis, indicating that c-*fos* is not needed for the onset of ET-18-OCH_3-induced apoptosis. Although some previous reports have implicated c-*fos* in programmed cell death (Colotta *et al*., 1992), the results herein described are in agreement with recent evidence from c-Fos-deficient mice demonstrating that c-*fos* is not essential for the induction of apoptosis (Gajate *et al*., 1996).

We found that antisense c-*jun* oligonucleotides were unable to prevent ET-18-OCH_3-mediated JNK activation. In this regard, Verheij *et al*. (1996) reported that expression in U937 and BAE cells of a dominant-negative c-Jun mutant lacking the amino terminus, the portion that is phosphorylated by JNK, blocked stress-induced apoptosis but did not inhibit JNK activation. Taken together, these data indicate that inhibition of the apoptotic response to ET-18-OCH_3 or to stress, by antisense c-*jun* oligonucleotides or by a dominant-negative c-Jun mutant, occurs at a level downstream of JNK. These data also suggest a critical role for c-Jun in these apoptotic processes. The mechanism by which c-Jun could mediate apoptosis is unknown. It is tempting to speculate that activated c-Jun regulates apoptosis through modulation of certain proteins required in the apoptotic response or through sequestration of inhibitors of apoptosis. A putative hypothesis for the c-Jun action on apoptosis might stem from the capacity of c-Jun to complex with different proteins.

Immediate and transient kinetics of JNK activation in response to various stimuli that promote cell activation and proliferation have been reported (Karin *et al*., 1995; Chen *et al*., 1996). We also found that induction of monocyte/macrophage differentiation of HL-60 cells by TNF-α or PMA was accompanied by a rapid and transient JNK activation, reaching a maximum JNK activation after 15 and 30 min of treatment, respectively. However, the JNK activation reported here on ET-18-OCH_3 treatment was delayed and persistent. JNK activation was detected after 30–60 min of ET-18-OCH_3 treatment; increased strongly with the incubation time, being very potent by 2 hr of treatment; and was sustained for a long period of time. Chen *et al*. (1996) recently reported that activation of Jurkat cells with different stimuli rendered a rapid and transient induction of JNK activity, whereas JNK remained persistently activated during γ-radiation- and UV-C-induced apoptosis. Thus, JNK activation occurs in different physiological processes, including cell activation, cell differentiation, and apoptosis. This indicates that JNK can function as a common kinase shared by different physiological processes. The different timing or duration of JNK induction, or both, may lead to opposite outcomes, namely cell activation/proliferation/differentiation (rapid and transient JNK activation) versus apoptosis (sustained JNK activation), as postulated previously (Chen *et al*., 1996). The participation of JNK activation in different physiological outcomes suggests that additional coactivators or JNK-interacting proteins could play a role in promoting a final cell response. The prolonged JNK activation in ET-18-OCH_3 may cause the persistent activation of some cellular factors, such as c-Jun, and results in detrimental effects on the cells.

The JNK protein kinases comprise a family of $\geq$10 isoforms, which correspond to alternatively spliced isoforms derived from the *JNK1*, *JNK2*, and *JNK3* genes (Gupta *et al*., 1996). These JNK isoforms are detected as proteins of 46 kDa (JNK1), 55 kDa (JNK2), and 48 and 57 kDa (JNK3) (Gupta *et al*., 1996). With in-gel assays, we found that ET-18-OCH_3 induces activation of two JNK protein kinase forms with apparent molecular masses of 46 and 55 kDa in HL-60 cells. JNK protein kinases are members of the wide family of the MAPK-related family (Karin *et al*., 1995; Gupta *et al*., 1996), which includes two great subfamilies: MAPK/ERK, mainly involved in mitogenesis and differentiation; and JNK, supposedly involved in growth arrest and cell death. Mitogens are potent ERK activators but activate JNK poorly, and stress stimuli are powerful inducers of JNK but induce ERK relatively weakly (Karin *et al*., 1995; Gupta *et al*., 1996). Our current results demonstrate that ET-18-OCH_3 strongly activates the JNK route in a sustained way. Zhou *et al*. (1996) recently reported that this ether phospholipid inhibited the MAPK/ERK cascade. Thus, ET-18-OCH_3 arrests mitogenic signals (MAPK/ERK) and strongly induces apoptotic signaling pathways (JNK). These actions could explain the rapid and effective induction of apoptosis exerted by this ether phospholipid (Diomede *et al*., 1993, 1994; Mollinedo *et al*., 1993a, 1997).

In conclusion, the current results indicate an association between persistent JNK activation and induction of apoptosis in response to ET-18-OCH_3, suggesting that JNK activation is involved in the initiation of programmed cell death by ET-18-OCH_3. Furthermore, the present data indicate that c-Jun is required for the rapid induction of apoptosis by ET-18-OCH_3, suggesting that activation of c-Jun plays a critical role in this apoptotic response.

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