Phosphatidylcholine-specific phospholipase C and phospholipase D are respectively implicated in mitogen-activated protein kinase and nuclear factor κ B activation in tumour-necrosis-factor- α -treated immature acute-myeloid-leukaemia cells

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Tumour necrosis factor- α (TNF α) has been reported to induce potent growth inhibition of committed myeloid progenitor cells, whereas it is a potential growth stimulator of human CD34⁺ CD38[−] multipotent haematopoietic cells. The present study was aimed at evaluating the respective role of two phospholipases, phosphatidylcholine-specific phospholipase C (PC-PLC) and phospholipase D (PLD) in the response of the CD34⁺ CD38[−] KG1a cells to TNF α . In these cells TNF α triggered phosphoinositide 3-kinase (PI3K)-dependent PC hydrolysis within 4–8 min with concomitant production of both diacylglycerol (DAG) and phosphocholine (P-chol). DAG and P-chol production was accompanied by extracellular-signal-related protein kinase-1 ('ERK-1') activation and DNA-synthesis stimulation. PC-PLC stimulation was followed by PI3K-independent PLD activation

with concomitant phosphatidic acid (PA) production followed by PA-derived DAG accumulation and sustained nuclear factor κ B (NF- κ B) activation. PLD/NF- κ B signalling activation played no role in the TNF α proliferative effect and conferred no consistent protection of KG1a cells towards antileukaemic agents. Altogether these results suggest that, in KG1a cells, TNF α can stimulate in parallel PC-PLC and PLD, whose lipid products activate in turn mitogen-activated protein kinase (MAP kinase) and NF- κ B signalling respectively. Finally, our study suggests that PC-PLC, but not PLD, plays a role in the TNF α proliferative effect in immature myeloid cells.

Key words: diacylglycerol, ERK, phosphocholine, PI3K, PKC.

INTRODUCTION

Tumour necrosis factor- α (TNF α) overproduction occurs during infections, inflammatory diseases or malignancies. TNF α exhibits a wide range of biological activities, including adverse effects on blood-cell production. However, the effect of TNF α on haematopoiesis is not unique. Indeed, previous studies have documented that, whereas TNF α exerts a direct cytotoxic effect on committed progenitors, it is a potential growth stimulator of human immature myeloid cells and decreases their ability to differentiate [1,2]. Thus, in a long-term bone-marrow culture system, it was shown that TNF α increased the number of multipotent progenitor cells and supported colony formation from acute-myeloid-leukaemia (AML) blasts plated at high cell concentration without further addition of exogenous growth factors [1,3]. Further studies indicated that the 55 kDa TNF receptor (TNF-R55) exclusively mediates the stimulatory effects on normal primitive progenitors [2], whereas both TNF-R55 and TNF-R75 participate in TNF α -mediated stimulation of AML blast cells [4]. Moreover, TNF α induced resistance to transforming growth factor- β (TGF β) [5] and decreased all-*trans*-retinoic acid-induced colony-formation inhibition in CD34⁺ KG1

cells [6]. However, owing to the difficulty in obtaining sufficient numbers of immature myeloid cells, the signalling pathways involved in TNF α -induced proliferation and survival enhancement are not well understood. For this reason, established AML cell lines derived from early stage of myelopoiesis may represent alternative cellular models for investigating TNF α -activated signalling pathways involved in proliferation and survival. In fact, TNF α induced proliferation of the CD34⁺ Mo7e and TF-1 cells [7,8]. However, these cell lines exhibit an intermediate CD34⁺ CD38⁺ phenotype and are derived from megakaryocytic and erythroid compartment respectively. For this reason, KG1a cells, which express a more immature CD34⁺ CD38[−] phenotype, appear to be more representative of the stem-cell compartment. TNF α has been reported to stimulate their growth [9]. However, the mechanism by which TNF α exerts this effect remains unclear.

The present study was undertaken to evaluate the role of phosphatidylcholine (PC) hydrolysis in TNF α -induced KG1a cell proliferation. This hypothesis was based on previous studies which have shown that, in various cellular models, TNF α triggers PC hydrolysis by stimulating PC-specific phospholipase C (PC-PLC) [10,11] or phospholipase D (PLD) activities [12,13]. More-

Abbreviations used: AML, acute myeloid leukaemia; DAG, diacylglycerol; DNR, daunorubicin; EMSA, electrophoretic-mobility-shift assay; FCS, foetal-calf serum; IMDM, Iscove's modified Dulbecco's medium; MAP kinase, mitogen-activated protein kinase; NF- κ B, nuclear factor κ B; PA, phosphatidic acid; PC, phosphatidylcholine; P-chol, phosphocholine; PC-PLC, PC-specific phospholipase C; PEt, phosphatidylethanol; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLD, phospholipase D; SM, sphingomyelin; TNF-R55, p55 TNF receptor; TNF α , tumour necrosis factor- α ; ERK, extracellular-signal-related protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DTT, dithiothreitol; DiC₈, 1,2-dioctanoyl-sn-glycerol.

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over, PC breakdown products, including PC-derived diacylglycerol (DAG), phosphocholine (P-chol) and phosphatidic acid (PA), have been involved in cell proliferation [14,15]. DAG and P-chol act through a variety of kinases including mitogen-activated protein kinase (MAP kinase) or transcription factors such as nuclear factor κ B (NF- κ B) [11,16]. Previous studies have extensively documented the role of MAP kinase in cellular proliferation, whereas more recent studies have suggested a role for NF- κ B in cell survival and tumour progression [17–20]. In the present study we investigated the role of PC breakdown products in MAP kinase and NF- κ B activation as well as their possible implication in mitogenesis and survival in TNF α -treated KG1a cells.

EXPERIMENTAL

Materials

Iscove's modified Dulbecco's medium (IMDM), penicillin, streptomycin and fetal-calf serum (FCS) were from Gibco BRL (Cergy-Pontoise, France). Human recombinant TNF α was supplied from PeproTech-Tebu (Le Perray-en-Yvelines, France). Daunorubicin (DNR) was from Roger Bellon (Neuilly/Seine, France). [*methyl*- 3 H]Thymidine (79 Ci/mmol), [9,10 (n)- 3 H]-palmitic acid (53 Ci/mmol) and [*methyl*- 3 H]choline chloride (83 Ci/mmol) were purchased from Amersham (Les Ulis, France). Silica-gel 60 TLC plates were from Merck (Darmstadt, Germany). D609 (xanthogenate tricyclodecan-9-yl) and LY-294002 were purchased from Calbiochem (San Diego, CA, U.S.A.). Wortmannin, PLC (from *Bacillus cereus*), cabbage (*Brassica oleracea*) PLD, PMA, myelin basic protein, calphostin C and chelerythrin were from Sigma (St Quentin-Fallavier, France). Cell-permeant DAG [1,2-dioctanoyl-*sn*-glycerol (DiC $_8$)] was provided by Alexis (Cogen, France). Anti-p85 phosphoinositide 3-kinase (PI3K) (06-195) polyclonal antibody was from UBI (Lake Placid, NY, U.S.A.). Anti-(extracellular-signal-related protein kinase-1) (anti-ERK-1) polyclonal antibody (and to a lesser extent ERK-2) (C-16) and anti-phosphotyrosine (PY-20) were from Santa-Cruz/Tebu (Le Perray en Yvelines, France). [γ - 32 P]ATP was purchased from ICN (Orsay, France). En 3 Hance was from Dupont De Nemours (Les Ulis, France). NF- κ B (5'-AGTTGAGGGGCTTTCCAGGC-3') consensus oligonucleotides were supplied by Promega (Charbonnières, France).

Cell culture

The human myeloblastic cell line KG1a, purchased from the American Type Culture Collection (Rockville, MD, U.S.A.), was cultured in IMDM supplemented with 20% (v/v) heat-inactivated FCS, 2 mM glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin. Cell stocks were routinely screened for mycoplasma (mycoplasma PCR kit; Stratagene, La Jolla, CA, U.S.A.). Phenotypic analysis showed that KG1a cells were CD34 $^+$ CD38 $^-$ CD33 $^-$ CD41 $^-$ HLA-DR $^-$ P-glycoprotein $^+$ and Rhodamine123 dull [21]. Moreover, these cells expressed both TNF-R55 and TNF-R75 [9].

Proliferation assay

KG1a cells (2×10^5 cells/200 μ l per well) were resuspended in IMDM supplemented or not with 1% FCS and incubated with the various effectors in a fully humidified incubator containing 5% CO $_2$ at 37 °C for 24 h. [3 H]Thymidine (0.5 μ Ci/ml) was then added 6 h before harvesting. The incorporation of radioactivity

was determined by liquid-scintillation counting. Assays were carried out in triplicate.

Drug cytotoxicity studies

Cells were pretreated with TNF α for 12 h, then co-treated with TNF α and DNR for 4 h. After washing, cells (30 000 cells/100 μ l) were seeded in 96-well plates and incubated for 48 h at 37 °C. A 100 μ l of portion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (2 mg/ml) was added for 2 h, then supernatant was removed and DMSO (100 μ l) was added. Finally the absorbance was read at 540/690 nm.

Analysis of cellular phospholipids

Quantification of phospholipids, ceramide, DAG and sphingomyelin (SM) was performed by labelling cells to isotopic equilibrium with [9,10(n)- 3 H]palmitic acid (0.5 μ Ci/ml). Similarly, PC, SM and P-chol were quantified after metabolic labelling with [*methyl*- 3 H]choline (0.5 μ Ci/ml). After 48 h incubation, cells were washed and resuspended in serum-free medium for kinetic experiments, and continuously exposed to TNF α or the other effectors for the indicated times. Cellular lipids were extracted from aliquots (5×10^6 cells per point) by the method of Bligh and Dyer [22] and separated by TLC using chloroform/methanol/acetic acid/formic acid/water (65:30:10:4:2, by vol.), followed by a second step using chloroform/methanol/acetic acid (95:5:5, by vol.) as developing solvent system. Radioactive spots were scraped off and the amount of radioactivity was determined by liquid-scintillation counting. The amount of P-chol was determined in the aqueous phase of [3 H]choline-labelled cell extracts by TLC using the solvent system methanol/0.5% NaCl/ammonia (28%) (50:50:5, by vol.) [23]. After autoradiography with En 3 Hance, the radioactive spots were scraped off and counted for radioactivity. Statistical analyses were performed by Student's *t*-test.

In vitro PI3K assay

The phosphorylated phosphoinositides were evaluated as previously described [24]. Briefly, exponentially growing cells were incubated in IMDM supplemented with 1% FCS for 48 h. After various times of incubation with TNF α in serum-free medium, PI3K was immunoprecipitated prior to assay. After washing, the pellets were resuspended with 30 μ l of HEN buffer (20 mM Hepes/0.4 mM EGTA/0.4 mM Na $_2$ HPO $_4$), 10 μ l (10 μ g) of phosphatidylinositol (PtdIns) (previously sonicated in 5 mM Hepes) and 10 μ l of reaction buffer {50 mM MgCl $_2$ /250 μ M ATP/[γ - 32 P]ATP (0.3 μ Ci/sample)} and incubated for 15 min at room temperature. The reaction was stopped by the addition of 15 μ l of 4 M HCl. Lipids were extracted with methanol/chloroform (1:1, by vol.) and separated by TLC in chloroform/methanol/NH $_4$ OH (7.3%) (90:70:20, by vol.). After autoradiography, the phosphorylated PI fractions were scraped off and quantified by liquid-scintillation counting.

Analysis of PA and PLD-catalysed transphosphatidylolation reaction

Evaluation of PA and phosphatidylethanol (PEt) was performed as previously described [10]. Briefly, cells were labelled to isotopic equilibrium with [9,10(n)- 3 H]palmitic acid (0.5 μ Ci/ml). After 48 h incubation, cells were washed and resuspended in serum-free medium for kinetic experiments, and continuously exposed to TNF α . In order to evaluate the PLD-catalysed transphosphatidylolation reaction, 1% ethanol was added before TNF α treatment. Cellular lipids were then extracted from aliquots

(5×10^6 cells per point) by the method of Bligh and Dyer [22] and separated by TLC using chloroform/methanol/acetic acid (90:10:10, by vol.). PMA (100 nM), added to the cells for 30 min, was used as a positive control.

ERK-1 activity

After treatment of cells (5×10^6 /ml) with TNF α for 12 h, cells extracts were prepared by lysing cells in buffer containing 20 mM Hepes, pH 7.4, 0.5 M EDTA, 125 mM NaCl, 0.1 % Nonidet P40, 1 mM NaVO₃, 2 μ g/ml leupeptin, 2 μ g/ml aprotinin, 1 mM PMSF, 0.5 μ g/ml benzamidine and 1 mM dithiothreitol (DTT). Cell extracts (150–200 μ g) were immunoprecipitated with 0.3 μ g of anti-ERK-1 for 60 min at 4 °C. Immune complexes were collected by incubation with Protein G–Sepharose beads for 60 min at 4 °C. The beads were extensively washed with lysis buffer and twice with kinase buffer [20 mM Hepes (pH 7.4)/1 mM DTT/25 mM NaCl]. Kinase assays were performed for 15 min at 32 °C using myelin basic protein as a substrate in 20 mM Hepes (pH 7.4)/10 mM MgCl₂/1 mM DTT/[γ -³²P]ATP (10 μ Ci). Reactions were stopped by the addition of 15 μ l of 2 $\times$ SDS sample buffer, boiled for 5 min and the samples were subjected to SDS/10 % (w/v)-PAGE. Phosphorylated myelin basic protein levels were analysed by autoradiography, and the corresponding bands excised and quantified by liquid-scintillation counting.

ERK-1/ERK-2 tyrosine phosphorylation

After treatment of cells (5×10^6 /ml) with TNF α for 3, 6, 12 and 24 h, cells extracts were prepared by lysing cells in buffer containing 50 mM Tris, pH 8, 5 mM EDTA, 150 mM NaCl, 1 % Triton X-100, 1 % sodium deoxycholate, 0.1 % SDS, 10 mM β -glycerophosphate, 50 mM NaF, 1 mM NaVO₃, 2 μ g/ml leupeptin, 2 μ g/ml pepstatin, 0.1 mM PMSF and 1 mM DTT. Cell extracts (150–200 μ g) were immunoprecipitated with 3 μ g of anti-PY-20 overnight at 4 °C. Immune complexes were collected by incubation with Protein G–Sepharose beads for 60 min at 4 °C. The beads were extensively washed with lysis buffer and resuspended in 15 μ l of 2 $\times$ SDS sample buffer, boiled for 5 min and the samples subjected to SDS/12.5 % (w/v)-PAGE. Then proteins were transferred on to a nitrocellulose membrane, probed with anti-ERK-1 (and, to a lesser extent, anti-ERK-2), and analysed for chemiluminescence.

Preparation of nuclear extracts

Cells (5×10^6 /ml) were treated separately with the different effectors and inhibitors (TNF α , D609, LY294002 and ethanol) for various times, and nuclear extracts were prepared as previously described [11]. Briefly, cellular pellets were washed twice in PBS and suspended in 400 μ l of lysis buffer [10 mM Hepes (pH 7.8)/10 mM KCl/0.1 mM EDTA/0.1 mM EGTA/1 mM DTT/1 mM PMSF/2 μ M pepstatin/0.6 μ M leupeptin/aprotinin (1 μ g/ml)/0.6 % Nonidet P40]. After 15 min on ice, the nuclear pellets were recovered after centrifugation at 1200 *g* and resuspended in 50 μ l of 20 mM Hepes (pH 7.9)/0.4 M NaCl/1 mM EDTA/1 mM EGTA and the tubes were incubated on ice for 30 min with intermittent mixing. These nuclear extracts were then centrifuged for 30 min at 20000 *g* and supernatants were either used immediately or stored at –20 °C for later use.

Electrophoretic-mobility-shift assays (EMSAs)

EMSAs were performed by incubating 4 μ g of nuclear extract with 50000 c.p.m. of ³²P-end-labelled NF- κ B (5'AGTTGAG-

GGGCTTTCCAGGC-3') consensus oligonucleotides in a reaction buffer [2 mM Hepes (pH 7.5)/50 mM NaCl/0.5 mM EDTA/1 mM MgCl₂/4 % glycerol/0.5 mM DTT/poly(dI-dC) (1 μ g/ml)/BSA (2 μ g)]. After 20 min incubation, the DNA–protein complex formed was separated from free oligonucleotides by electrophoresis through a low-ionic-strength native 4 %-(w/v)-polyacrylamide gel. Electrophoresis was carried out in buffer containing 6.7 mM Tris/HCl, pH 7.9, 3.3 mM sodium acetate, 2 mM EDTA at 10 V/cm for 3 h at room temperature with buffer recirculation. The gel was dried and autoradiographed with intensifying screens.

RESULTS

TNF α induces early DAG and P-chol production due to PC-PLC activity stimulation

KG1a cells were cultured in IMDM supplemented with 1 % FCS and prelabelled with [³H]palmitic acid or [³H]choline for 48 h, and then treated with 20 ng/ml TNF α . Intracellular PC, P-chol and DAG contents were determined at various time points (0–60 min). As shown in Figure 1(A), TNF α stimulated a rapid

Figure 1 Effects of TNF α on PC metabolism in KG1a cells

Cells were incubated in 1 % FCS for 48 h with [³H]palmitate or [³H]choline (0.5 μ Ci/ml), washed, and treated with TNF α (20 ng/ml) for the indicated times. Intracellular levels of PC (A), DAG (B) and P-chol (C) were analysed as described in the Experimental section. Results are means $\pm$ S.D. for three independent experiments. The asterisk represents significant differences ($P < 0.05$) compared with control (zero time).

Table 1 Effect of D609 on TNF α -induced DAG and P-chol production in KG1a cells

Cells were incubated in 1% FCS for 48 h with [3 H]palmitate or [3 H]choline (0.5 μ Ci/ml), washed, pretreated with D609 (1 h, 10 μ g/ml) and then treated with TNF α (20 ng/ml). Intracellular levels of DAG and P-chol were measured at peak (4 min) as described in the Experimental section. Results are means $\pm$ S.D. for three independent experiments. The asterisk represents significant differences ($P < 0.05$) compared with controls.

Addition	[3 H]Lipids (% of control)	
	DAG	P-chol
None	100 $\pm$ 4	100 $\pm$ 7
TNF α	225 $\pm$ 16*	119 $\pm$ 4*
D609	100 $\pm$ 6	100 $\pm$ 12
TNF α + D609	85 $\pm$ 15	91 $\pm$ 9

loss of PC ($\approx 10\%$ of unstimulated values within 4–8 min). In parallel, TNF α induced the release of DAG and P-chol which reached a maximum of $\approx 230\%$ and $\approx 120\%$ of unstimulated values respectively (Figures 1B and 1C). Measurement of the radioactivity extracted from the TLC plates after [3 H]choline labelling revealed that the changes in PC and P-chol levels were similar (≈ -26590 c.p.m./ 5×10^6 cells versus $\approx +24850$ c.p.m./ 5×10^6 cells for PC and P-chol respectively), suggesting that P-chol primarily originated from PC. In contrast, during the first 1 h of stimulation, we were unable to detect PA production as well as PLD-catalysed transphosphatidylolation, which could argue in favour of PLD activation (results not shown).

To confirm further TNF α -induced PC-PLC stimulation, we used an inhibitor of this phospholipase, D609 [25,26]. A 1 h preincubation with D609 alone (10 μ g/ml) did not influence DAG, P-chol and PC basal levels (results not shown). As shown in Table 1, D609 completely abrogated TNF α -induced DAG and P-chol production. Preincubation of cells for 1 h with D609 also resulted in the prevention of the TNF α -stimulated PC hydrolysis (results not shown). Altogether, our results suggest that TNF α stimulated within 4 to 8 min DAG and P-chol production through PC-PLC-mediated PC hydrolysis. Since TNF α -induced KG1a cell proliferation was detected only after 24–72 h incubation [9], the link between early DAG production and increase in DNA synthesis remained uncertain. For this reason, we monitored intracellular DAG levels over a prolonged incubation time period.

TNF α induces delayed DAG production due to PLD activity stimulation

KG1a cells were cultured in IMDM supplemented with 1% FCS and pre-labelled with [3 H]palmitic acid or [3 H]choline for 48 h, and then treated with 20 ng/ml TNF α . Intracellular DAG, PA, and PEt were determined at various times. Results were expressed as percentage of controls (TNF α -untreated cells harvested at the same time of incubation). As shown in Figure 2(A), TNF α induced delayed DAG accumulation which was detected at 12 h, and then increased by $50 \pm 27\%$ at 24 h. These results were confirmed by using the DAG kinase assay (results not shown). During this period of time we were unable to detect any significant changes in P-chol levels, and co-treatment with D609 did not inhibit DAG accumulation (Figure 2A). These results suggest that TNF α -induced delayed DAG accumulation was not related to PC-PLC stimulation. However, we observed an increase in PA which was detected at 3 h and peaked at 6 h

Figure 2 Effect of TNF α on DAG and PA production in TNF α -treated KG1a cells

(A) After incubation of the cells in 1% FCS for 48 h with [3 H]palmitate (0.5 μ Ci/ml), washing, pretreatment with D609 (●) or not (○), and treatment with TNF α (20 ng/ml), intracellular rates of DAG production were determined. (B) PA (□) and PEt (■) were evaluated as described in the Experimental section. Results are means $\pm$ S.D. for three independent experiments. The asterisk represents significant differences ($P < 0.05$) compared with control (zero time).

(Figure 2B), suggesting PLD activation. To provide further evidence that PLD was involved in late DAG production, we next assessed PLD activity more directly by investigating the possible formation of labelled PEt, which results from PLD's transphosphatidylolation activity in the presence of ethanol. In cells treated with 1% ethanol, TNF α increased PEt (Figure 2B). The magnitude of PA or PEt increases was similar to that measured in PMA-treated KG1a cells (results not shown). Altogether, these results suggest that, in KG1a cells, TNF α induced a progressive increase in PLD activity responsible for phospholipid hydrolysis, release of PA, and subsequent DAG accumulation.

Role of PI3K in TNF α -induced DAG production

Since on the one hand TNF α induces PI3K activation in a number of non-haematopoietic-cell models [27,28], and on the other hand PI3K has been found to be involved in PLD activation by growth factors [29], we hypothesized that TNF α -induced DAG production could be regulated by PI3K. In fact, as depicted in

Figure 3 Role of PI3K in TNF α -induced DAG production in KG1a cells

(A) Effect of TNF α on PI3K activity. Cells were incubated in 1% FCS for 48 h, washed in serum-free medium, then pre-treated with (■) or without (□) wortmannin (100 nM, 30 min) and treated with TNF α (20 ng/ml). (B) Effect of wortmannin on TNF α -induced DAG generation. Cells pretreated (■) or not (□) with wortmannin (30 min, 100 nM) and treated with TNF α (20 ng/ml). PI3K activity and intracellular rate of DAG production were measured as described in the Experimental section. Results are means $\pm$ S.D. for three independent experiments. The asterisk represents significant differences ($P < 0.05$) compared with untreated cells.

Figure 3(A), TNF α stimulated PI3K activity within 3–10 min. Moreover TNF α -induced PI3K stimulation was totally inhibited by wortmannin, a potent PI3K inhibitor (Figure 3A). We found that both wortmannin and LY294002, another PI3K inhibitor, had no influence on PA and delayed DAG production (results not shown). However, pretreatment of KG1a cells with PI3K inhibitors resulted in the inhibition of TNF α -induced early DAG (Figure 3B) and P-chol production, whereas they had no influence on basal DAG (results not shown). These results strongly suggest that TNF α -induced PI3K stimulation is a critical event for PC-PLC stimulation, but does not seem to be involved in TNF α -induced PLD activation.

Role of PC breakdown products in TNF α -induced ERK activation

On the basis of the effect of PC breakdown products on a variety of kinases, including MAP kinase [16], we hypothesized that

Figure 4 Role of PC-PLC and PI3K in TNF α -induced ERK activation in KG1a cells

(A and B) Cells were treated with TNF α (20 ng/ml), and time-dependent activation of ERK-1 was evaluated either by myelin basic protein (MBP) kinase activity (A) or by phosphotyrosine immunoprecipitation and probing with ERK-1 (to a lesser extent ERK-2) (B). (C) Cells were pre-treated with LY294002 (1 h, 20 μ M), D609 (1 h, 10 μ g/ml), calphostin C (1 h, 100 nM), chelerythrin (1 h, 10 μ M) or ethanol (EtOH; 0.3%), then treated for 12 h with TNF α (20 ng/ml). After immunoprecipitation with anti-ERK-1, samples were analysed for MBP kinase activity as described in the Experimental section. Results are means $\pm$ S.D. for three independent experiments. The asterisk represents significant differences ($P < 0.05$) compared with control.

TNF α could stimulate ERK. Exponentially growing KG1a cells were treated for various times (0, 3, 6 and 12 h) with TNF α in the presence or absence of D609 or LY294002. TNF α induced a time-dependent stimulation of ERK-1 kinase activity and tyrosine phosphorylation of both ERK-1 and ERK-2, peaking at 12 h (Figures 4A and 4B). Moreover, D609 and LY294002 inhibited TNF α -induced ERK-1 kinase activation, whereas incubation with 0.3% ethanol, which resulted in the inhibition of PA formation due to PLD's transphosphatidylase activity (results not shown), had no effect (Figure 4). This result suggests that PC-derived DAG, but not PA or PA-derived DAG, mediates TNF α -induced ERK-1 stimulation. On the basis of the effect of PC-derived DAG on various protein kinase C (PKC) isoforms, including atypical PKC isoenzymes [30], we also investigated the

Figure 5 Role of PLD on TNF α -induced NF- κ B DNA binding in KG1a cells

(A) Cells were pretreated with ethanol (EtOH; 0.3%), then treated for various times with TNF α (20 ng/ml) or treated with 10 munits/ml exogenous PLD from cabbage for 6 h. (B) Cells were pre-treated with LY294002 (1 h, 20 μ M), D609 (1 h, 10 μ g/ml), then treated for 12 h with TNF α (20 ng/ml). Nuclear extracts were isolated, and EMSAs were performed as described in the Experimental section.

role of PKC in TNF α -induced ERK-1 stimulation in KG1a cells. As shown in Figure 4, we found that calphostin C (100 nM for 1 h), an inhibitor of Ca²⁺-dependent (classical) and Ca²⁺-independent (novel) PKC isoenzymes, as well as chelerythrin (10 μ M for 1 h), which inhibits all PKC isoforms, blocked ERK-1 enzymic activity in TNF α -treated KG1a cells, thus recapitulating the effect of D609 or LY294002. This result suggested that PI3K lipid products and/or PC-derived DAG mediates TNF α -induced MAP kinase activation through a classical or novel PKC isoform.

Role of PC breakdown products in TNF α -induced NF- κ B activation

In a previous study we documented that TNF α stimulates NF- κ B DNA-binding activity in KG1a cells [9]. On the basis of the present findings we hypothesized that either DAG or PA plays a role in TNF α -induced NF- κ B activation. Exponentially growing KG1a cells were treated for various times (0, 1, 3, 6 and 12 h) with TNF α in the presence or absence of D609, LY294002 or ethanol. TNF α induced time-dependent NF- κ B activation, which was maximum after a 12 h incubation. Pretreatment with 0.3% ethanol strongly inhibited TNF α -induced NF- κ B activation at 6 and 12 h (Figure 5A). This result suggested that TNF α -induced PLD stimulation was involved in increased NF- κ B DNA binding. This hypothesis was supported by the fact that treatment with commercial exogenous PLD from cabbage (*Brassica oleracea*) resulted in NF- κ B activation, which peaked at 6 h (Figure 5A). However, as shown in Figure 5(B), both D609 and LY294002 had no effect, suggesting that neither PC-PLC or PI3K stimulation was involved in TNF α -induced NF- κ B activation.

Table 2 Effects of TNF α and lipids on [³H]thymidine incorporation in KG1a cells

Cells were incubated in 1% FCS, then treated with TNF α , DiC₈, P-chol, PC-PLC and PLD with or without D609 (1 h) or wortmannin (30 min), and analysed 24 h later for [³H]thymidine incorporation as described in the Experimental section. Results are means $\pm$ S.D. for three independent experiments. The asterisk represents significant differences ($P < 0.05$) compared with control.

Pretreatment	Addition	[³ H]Thymidine incorporation (% of control)
None	None	100 $\pm$ 7
	TNF α (20 ng/ml)	120 $\pm$ 4*
	TNF α (100 ng/ml)	128 $\pm$ 8*
D609 (10 μ g/ml)	None	88 $\pm$ 3
	TNF α (20 ng/ml)	70 $\pm$ 7*
Wortmannin (100 nM)	None	100 $\pm$ 7
	TNF α (20 ng/ml)	89 $\pm$ 2*
None	DiC ₈ (50 μ M)	128 $\pm$ 7*
	P-chol (25 μ M)	125 $\pm$ 3*
	PC-PLC (10 munits/ml)	120 $\pm$ 2*
	PLD from cabbage (10 munits/ml)	102 $\pm$ 8

Effect of TNF α on KG1a cell proliferation

As described elsewhere [9], TNF α caused a 20–30% increase in thymidine incorporation (Table 2). Moreover, TNF α induced a 27.0 $\pm$ 7.4% increase in KG1a cell number at 24 h as assessed by Trypan Blue assay (results not shown). However, in D609-pretreated cells TNF α induced no more increase in thymidine incorporation, but rather a moderate, but significant, decrease in DNA synthesis, compared with cells treated with D609 alone. In order to confirm the role of endogenous DAG and P-chol in the TNF α mitogenic effect, we evaluated the effect of a cell-permeant DAG (DiC₈) and exogenous P-chol on DNA synthesis. In fact, both DiC₈ (50 μ M) and P-chol (25 μ M) increased [³H]thymidine incorporation, with a maximal stimulation of about 30% (Table 2). Furthermore, natural DAG and/or P-chol generated by treatment with bacterial PC-PLC stimulated DNA synthesis to a level similar to that induced by TNF α (Table 2). Since PI3K inhibitors abrogated TNF α -induced PC-PLC stimulation, these drugs were expected to also influence the KG1a cell proliferative response to TNF α . Indeed, not only wortmannin abrogated TNF α -induced [³H]thymidine incorporation increase but also it induced, in combination with TNF α , a moderate but significant decrease in DNA synthesis, compared with cells treated with PI3K inhibitors alone (Table 2). These observations suggest that TNF α -induced KG1a cell proliferation results from PI3K-mediated PC-PLC stimulation, PC hydrolysis, and subsequent early DAG and/or P-chol generation. Moreover, commercial exogenous PLD from cabbage was unable to stimulate thymidine incorporation (Table 2), and inhibition of PA production by 0.3% ethanol did not decrease the TNF α proliferative effect (results not shown). These results suggest that PLD stimulation is not involved in TNF α -induced KG1a cell proliferation.

Effect of TNF α on KG1a cell protection towards antileukaemic agents

On the basis of the presumed role of NF- κ B in resistance to DNR, a widely used anti-leukaemic agent [17], we hypothesized that TNF α -induced PLD-mediated NF- κ B activation could influence the cytotoxicity of this drug. In these experiments,

Figure 6 Role of TNF α in DNR-induced cytotoxicity in KG1a cells

Cells were pretreated with (●) or without (○) TNF α for 12 h at 20 ng/ml, then treated with various concentrations of DNR. Results are means $\pm$ S.D. for three experiments.

KG1a cells were pretreated with 20 ng/ml TNF α for 12 h, and then co-treated with TNF α and various doses of DNR for 4 h. DNR-induced cytotoxicity was measured after 48 h by MTT assay. As shown in Figure 6, TNF α did not significantly influence DNR cytotoxicity. Similar results were obtained with aracytine, another common antileukaemic agent (results not shown). This result suggests that TNF α -induced PC breakdown plays no role in the chemosensitivity profile of KG1a cells. This hypothesis was further supported by the fact that exogenous PC-PLC or PLD exposure was unable to protect KG1a cells against antileukaemic agents (results not shown).

DISCUSSION

In the present study we show that, in KG1a cells, TNF α can stimulate early PC breakdown, which resulted in DAG and P-chol production. Hydrolysis of PC can be catalysed by either PC-PLC or PLD, yielding DAG and P-chol, or PA and choline, respectively. PA can subsequently be converted into DAG by phosphatidate phosphohydrolase, and choline can be phosphorylated to P-chol by choline kinase [15]. Therefore TNF α -induced DAG and P-chol production could theoretically be related to either PC-PLC or PLD stimulation. The present study provides evidence that TNF α induced a rapid stimulation of PC-PLC activity, but not of PLD. The implication of PC-PLC in early DAG production is supported by several lines of evidence. First, the fact that PC hydrolysis and DAG production occurred concomitantly supports the role of PC as principal DAG source, although we cannot rule out a modest contribution of PI breakdown. Secondly, several considerations strongly argue against the role of PLD in early DAG production: (i) we were unable to detect PA production; (ii) there was no evidence for an early PLD-catalysed transphosphatidyl transfer reaction; (iii) P-chol release paralleled DAG production. Thirdly, D609, a competitive inhibitor of PC-PLC, totally blocked both DAG and P-chol production. The significance of this result depends on the specificity of D609. On the basis of its high *in vitro* specificity, this drug has been considered for years as a reliable probe for assessing PC-PLC-mediated signalling pathways [11]. However, the specificity of D609 has recently been questioned. For example, some studies have shown that high doses of D609 may affect

PLD activity [16,31]. More recently, it has been reported that, under some circumstances, D609 may inhibit SM synthase, which transfers the P-chol group from PC to ceramide, generating SM and DAG [32]. This mechanism may account for the blocking effect of D609 on DAG production. However, this hypothesis is unlikely to explain our results, since: (i) D609-induced SM synthase inhibition has been documented for much higher doses (IC_{50} 100 μ g/ml); (ii) there was no change in ceramide and SM levels in D609-treated KG1a cells (results not shown), suggesting that D609 has no major influence on SM synthase activity in these cells, at least under the conditions used; (iii) this mechanism could not explain the blocking effect of D609 on P-chol production. Altogether our results provide strong evidence that early DAG was derived from PC hydrolysis due to PC-PLC stimulation.

Our study also shows that, in KG1a cells, TNF α stimulates PI3K activity. The mechanism by which TNF α stimulates PI3K in KG1a cells is unclear. It has previously been reported that human TNF α promotes tyrosine phosphorylation of the p85 regulatory subunit of PI3K in a number of non-haematopoietic cellular models through TNF-R55, and that TNF α -induced PI3K activation is mediated by phosphorylation of IRS-1 [27,28]. However, we were unable to detect a modification in the tyrosine phosphorylation status of IRS-1 in TNF α -treated KG1a cells (results not shown), suggesting that other adaptor molecules are involved in the propagation of the signal. Moreover, we show that wortmannin, a potent PI3K inhibitor, inhibits DAG production in TNF α -treated KG1a cells, indicating that PI3K plays a critical role in TNF α -induced PC-PLC activation. To the best of our knowledge, the role of PI3K in PC-PLC stimulation has not been previously documented. These results suggest that, in downstream PI3K activation by cytokines or growth factors, PC hydrolysis may represent a critical step for the propagation of the proliferative signal in haematopoietic cells. The mechanism by which PI3K is involved in PC-PLC stimulation remains undetermined. However, it has been extensively documented that PKC activation results in the stimulation of PC hydrolysis [15]. Furthermore, the PI3K lipid products [PtdIns(3,4) P_2 and PtdIns(3,4,5) P_3] cause a potent stimulation of calcium-insensitive PKC isotypes δ , ϵ and η [33], as well as the calcium-independent phorbol ester/DAG-insensitive PKC family [34]. Therefore one can speculate that lipid products generated upon PI3K stimulation may be responsible for PC hydrolysis through a PKC-dependent mechanism.

The fact that D609 and LY294002 not only blocked TNF α -induced DAG and P-chol production, but also inhibited KG1a cell proliferation, suggests that PC-PLC stimulation plays a critical role in the mitogenic response to TNF α . This hypothesis is supported by three lines of evidence: (i) exposure to exogenous bacterial PC-PLC increased DNA synthesis; (ii) exogenous permeant DAG and P-chol also stimulated mitogenesis; (iii) treatment with TNF α , exogenous PC-PLC, or DAG or P-chol induced similar enhancement of DNA synthesis. In this regard, it should be noted that the KG1a-cell proliferative response, albeit lower than that measured in TNF α -treated M-O7e or TF-1 cells [7], was similar to that observed in a large number of cellular models, including other AML cell lines, untransformed fibroblasts or epithelial tumour cells, after TNF α exposure [7,35–38]. The notion that PC-PLC may mediate cell proliferation has been extensively documented. Indeed, PC-PLC has been shown to be involved in the mitogenic response to various stimuli such as *ras* and *src* oncogenic products, as well as growth factors [38–45]. However, to the best of our knowledge, the implication of PC-PLC in the TNF α mitogenic effect in tumour cells has never been reported.

The exposure of KG1a cells to permeant DAG, P-chol, bacterial PC-PLC, but not PLD, increased DNA synthesis. Taken together, these observations suggest that DAG and/or P-chol mediate the TNF α mitogenic effect. Whereas the second-messenger function of PC-derived DAG has been well established [15], the role of P-chol is more questionable, especially if one considers that P-chol is a major cellular metabolite with a millimolar intracellular concentration [14]. Moreover, the fact that exogenous P-chol may have some biological effects was unexpected, because it does not penetrate through the cell membrane [46]. These results suggest that P-chol may not act directly as an intracellular messenger. In fact, it has been recently shown that exogenous P-chol enhances cell proliferation by regulating an extracellular site for mitogenic mediators, which, in turn, stimulates the PI3K and MAP kinase pathway [46,47]. Our study shows that TNF α stimulated the MAP kinase cascade through a PKC-dependent mechanism. Since it has been documented that DAG, but not P-chol, stimulates PKC, we propose that ERK stimulation and mitogenesis are due to DAG released from PC hydrolysis. This result conflicts with two recent reports from the same group which describe, not only in M-O7e, but also in KG1a cells, the lack of effect of TNF α on the phosphorylation of MAP kinase kinase/ERK kinase ('MEK'), an upstream kinase regulator of ERK [8,48]. The reason for this discrepancy is still unclear.

The present work also shows that TNF α induced delayed PLD stimulation in KG1a cells. Previous studies have documented that TNF α -induced PLD stimulation is a late event which occurs after several hours [12]. The fact that D609 did not inhibit PLD stimulation suggests that PC-PLC and PLD are activated independently. Our study suggests that PLD plays no role in TNF α -induced ERK-1 activation and cell proliferation. Indeed, D609 totally blocked TNF α -induced proliferation, whereas it had no effect on PLD activity. Furthermore, treatment with exogenous PLD failed to increase DNA synthesis. However, we provide evidence that PLD stimulation was involved in TNF α -induced NF- κ B activation. The role of PLD in the regulation of NF- κ B DNA-binding activity has not been previously documented. However, PLD products and downstream kinases, which are involved in NF- κ B-inhibitor ('I κ B') phosphorylation, remain to be determined. In this perspective, it can be speculated that PA (or lyso-PA) appears to play a more important role than PA-derived DAG, since NF- κ B activation preceded DAG production.

The role of TNF α -induced PLD-dependent NF- κ B activation in KG1a cells is unclear. Previous studies provided convincing evidence that NF- κ B activation plays an important role in cell protection against TNF α and cytotoxic agents [17]. However, we found that PLD inhibition did not restore TNF α -induced cytotoxicity, and that TNF α -induced PLD stimulation did not influence the KG1a cell chemosensitivity profile. These results suggest that PLD-dependent NF- κ B activation plays no role in cell survival.

The fact that TNF α may stimulate PC-PLC in leukaemic myeloid cells has been previously reported [10,11,49,50]. However, our study shows for the first time that TNF α -induced PC-PLC stimulation may result in leukaemic-myeloid-cell proliferation. Most previous studies have used the TNF α -sensitive U937 and HL-60 cells, which are both derived from mature stages of haemopoiesis. In these cells, but not in the immature KG1a cells, TNF α triggers SM hydrolysis, ceramide production and apoptosis [9,51]. If these cell lines are representative of haemopoiesis, one can speculate that TNF α induces apoptosis in committed myeloid cells through the SM cycle, whereas it stimulates proliferation in immature myeloid cells

through a PI3K/PC-PLC activation pathway. This hypothesis may have important implications in our understanding of haematological disorders, such as primitive myelodysplastic syndrome, which are characterized by TNF α overproduction by stroma cells, massive apoptosis of myeloid precursors, but enhanced proliferation and expansion of immature myeloid cells [52–54].

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