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**References**

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Failure to Activate Cytosolic Phospholipase A<sub>2</sub> Causes TNF Resistance in Human Leukemic Cells

Yu-Ling Wu, Xu-Rong Jiang, Adrian C. Newland, and Stephen M. Kelsey<sup>1</sup>

Activation of cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) by TNF has been shown to be an important component of the signaling pathway leading to cell death. The role of cPLA<sub>2</sub> in the cytotoxic action of TNF was investigated in a panel of human leukemic cell lines. TNF could activate cPLA<sub>2</sub> only in U937 and HL60 TNF-sensitive leukemic cells, but not in KG1a, CEM, and CEM/VLB<sub>100</sub> cells that are relatively resistant to TNF. Pretreatment with 4-bromophenacyl bromide, a cPLA<sub>2</sub> inhibitor, rendered U937 and HL60 cell lines resistant to the cytotoxic effect of TNF. Immunoblot and reverse-transcriptase PCR demonstrated that cPLA<sub>2</sub> expression was detectable at both transcriptional and translational levels in all leukemic cell lines studied, although CEM and CEM/VLB<sub>100</sub> cells expressed cPLA<sub>2</sub> mRNA and protein at lower levels. The protein synthesis inhibitor, cycloheximide, increased TNF-induced cPLA<sub>2</sub> activity and cytotoxicity in both CEM and CEM/VLB<sub>100</sub> cell lines. Low levels of cPLA<sub>2</sub> activity in the KG1a cell line could be activated by the cPLA<sub>2</sub> activator mellitin, or the calcium ionophore A23187. The data suggest that cPLA<sub>2</sub> activity is involved in TNF-induced cytotoxicity in leukemic cells. Resistance to TNF-induced cytotoxicity may involve either protein inhibitors that act upstream of cPLA<sub>2</sub> in the TNF-signaling pathway or constitutive defects of cPLA<sub>2</sub> itself, possibly involving calcium utilization.


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Materials and Methods

Reagents

Human rTNF-α, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), cycloheximide (CHI), 4-bromophenacyl bromide (BPB), mellitin, and saponin were all purchased from Sigma (Dorset, U.K.). Ionophore A23187 was purchased from Calbiochem (Nottingham, U.K.). [5,6,8,9,11,12,14,15-3H]arachidonic acid (1 mCi/ml) was purchased from Amersham (Amersham, U.K.).

Cell line and culture methods

Cell lines were cultured in RPMI 1640 with 10% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a 5% CO<sub>2</sub> incubator.
Cytotoxicity assay
TNF-induced cytotoxicity was determined by MTT assay, as previously described (23). Resuspended cells were plated in a volume of 90 μl at 1.5 × 10^5/well in the presence or absence of CHI or BPP. After incubation with different concentrations of TNF at 37°C for 48 h, 10 μl MTT (5 mg/ml in PBS, pH 7.2) was added to each well. Following a further 4-h incubation at 37°C, 150 μl of 0.04 N HCl in isopropanol was added to each well. Once the dark blue formazan had been dissolved, the absorbance of each well was measured with a Titertek Multiskan MCC 340 microplate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm. Cytotoxicity by MTT conversion at each concentration of TNF was compared with untreated (control) cells (shown as 100%). The ID_{50} value was taken as the concentration of drug required to produce a 50% inhibition of cell growth. This was calculated from a logarithmic regression curve of the results for at least five separate experiments.

Measurement of [3H]arachidonic acid release
Cells were labeled with 0.3 μCi/ml [5,6,8,9,11,12,14,15-3H]arachidonic acid and incubated at 37°C for 18 h. The unincorporated [3H]arachidonic acid was removed by washing three times with HBSS. Cells were then resuspended in fresh media in 24-well plates in triplicate. For experiments using the protein synthesis inhibitor CHI, the inhibitor was incubated with cells for 3 h before the addition of TNF. For experiments involving melilitin, the agent was added 3 h before the addition of TNF, such that cells were exposed to the agent for 27 h in total by the time of assay. After centrifugation at 4000 × g for 15 min, 0.4 ml of the supernatant was collected, mixed with 3.6 ml of scintillation fluid, and counted by liquid scintillation. To control for the nonenzymatic release of radiolabeled material from lysed cells, cells that had incorporated [3H]arachidonic acid were incubated with media for 18 h, then rapidly frozen at −70°C and thawed at 37°C. After thawing, the media were removed and processed for liquid scintillation counting, as described above. In all experiments, the average release of [3H]arachidonic acid from cells incubated with media only was less than 7% of the total. To confirm that the increase in [3H]arachidonic acid in the supernatant of TNF-treated cells was not due to the failure of dying cells to take up [3H]arachidonic acid, cells were treated with or without TNF for 8 h and then labeled with [3H]arachidonic acid for a further 6 h before both the supernatant and the pellet were collected, mixed with 3.6 ml of scintillation fluid, and counted by liquid scintillation. The amounts of [3H]arachidonic acid in both supernatant and pellet fractions were identical for both untreated and TNF-treated cells, indicating that re-uptake of [3H]arachidonic acid from supernatant, once released, is negligible.

Detection of TNFR expression by flow cytometry
Cell surface TNFR expression was assessed by indirect immunofluorescence using the mouse anti-human TNFR-P55 mAb (Genzyme, Cambridge, MA). Briefly, cells were collected, washed twice with PBS containing 1% BSA (1% BSA-PBS), then incubated with 10 μg/ml mouse anti-human TNFR-P55 mAb (Genzyme, Cambridge, MA) at 4°C for 45 min. Cells were then fixed with 1% paraformaldehyde at 4°C for 20 min to prevent receptor internalization. Nonspecific mouse IgG1 was used as a negative control. After washing twice with 1% BSA-PBS, cells were incubated with mouse phycoerythrin-conjugated rabbit anti-mouse F(ab')2 fragments (Dako, Glostrup, Denmark) at 4°C for 30 min. Cells were analyzed by flow cytometry (FACSscan; Becton Dickinson, San Jose, CA) and the results are expressed as mean fluorescence intensity and percentage of positive cells.

Preparation of membrane proteins and analysis by Western blot
Cells were washed three times with cold HBSS and rinsed in 0.5 ml homogenization buffer (50 mM HEPES, pH 8, 1 mM EDTA, 1 mM EGTA, 50 mM/leupeptin, 1 mM DTT, 0.5 mM PMSF, 10 mM phosphoramidon, 10 μg/ml soybean trypsin inhibitor, and 100 μg/ml aprotinin). Cells were then homogenized with a dounce tissue grinder (Jencons, Leighton Buzzard, UK). The homogenates were centrifuged at 1,000 × g for 10 min to discard the nuclei and debris, followed by centrifugation at 8,000 × g for 30 min to remove mitochondria. The supernatants were finally centrifuged at 100,000 × g for 60 min to produce a soluble fraction (crude cytosol) and a particulate fraction (pellet). The membrane pellets were resuspended in homogenization buffer and stored at −70°C. Forty micrograms of crude cytosol and membrane proteins were separated on 8% SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. The blots were stained with porcine-S solution (Sigma) to monitor the equal loading of proteins for each lane. After destaining, the blots were incubated in blocking solution consisting of 5% fat-free dry milk in PBS containing 0.1% Tween-20 at room temperature for 1 h, followed by incubation at room temperature for 2 h with a 1:100 mouse anti-human cPLA_{2} mAb (100 μg/ml) (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoblots were then incubated with goat anti-mouse IgG1 conjugated to peroxidase for 60 min and visualized with enhanced chemoluminescence detection reagents (Amersham).

Detection of cPLA_{2} mRNA by RT-PCR
Total cellular RNA was extracted by RNAzol B single step guanidinium thiocyanate-phenol-chloroform extraction method and reverse transcribed into cDNA at 42°C for 60 min using AMV reverse transcriptase and random hexamer primers. The cDNA was amplified via PCR using Taq DNA polymerase. The PCR reaction for cPLA_{2} was conducted for 35 cycles in 25 μl of reaction mixture using a step program (94°C, 45 s; 56°C, 45 s; 72°C, 1.5 min), followed by a 10-min final extension at 72°C. The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The cPLA_{2} primer pair amplified a 554-bp PCR product and was composed of the following sequences: 5'-TGG CAA ACT GCC TGC TCA GCA TCA G-3'; 5'-CTC TAG TCC TCC GTTCAA GGA AC-3'. The β-actin primer pair amplified a 400-bp product and was composed of the following sequences: 5'-GAT GGA GTT GAA GGT AGT TT-3'; 5'-TGG TAT CCA GGC TGT GCT AT-3'. The reverse transcription and PCR experimental conditions were identical for cPLA_{2} and β-actin.

Statistical analysis
Results are expressed as mean ± SD obtained from multiple experiments. Statistical analysis was performed using two-sided paired Student’s t test for grouped data. In all circumstances, a p value less than 0.05 was considered statistically significant.

Results
Susceptibility of leukemic cells to TNF killing
TNF-mediated cytotoxicity was determined in a panel of human leukemic cell lines, the myeloid cell lines U937, HL60, and KG1a, and the T lymphoblastic cell line CCRF-CEM and its vinblastine-resistant subline CEM/VLB_{100} (Fig. 1). U937 and HL60 cells were relatively resistant. The ID_{50} values for U937 and HL60 cells were 1 to 5 ng/ml of TNF, whereas the TNF ID_{50} values were greater than 100 ng/ml for KG1a, CEM, and CEM/VLB_{100} cells.

TNF acts by binding to either of two cell surface receptors, identified as TNFR-P55 and TNFR-P75. Apoptosis is thought to be mediated by ligand binding to the TNFR-P55. To investigate whether the relative sensitivity of cell lines to TNF was associated...
with difference in expression of TNFR-P55, flow-cytometric analysis of TNFR-P55 expression was performed. TNFR-P55 expression was found in all leukemic cell lines studied (Table I), although to a varying degree. No association between TNFR-P55 expression and sensitivity to TNF was observed.

Activation of cPLA2 by TNF

We tested whether cPLA2 activation was associated with TNF-mediated cytotoxicity by measuring cPLA2-mediated arachidonic acid release from cell membrane. TNF at 2 ng/ml significantly enhanced [3H]arachidonic acid release in TNF-sensitive cells after 24 h (Fig. 2A), whereas no significant increase in [3H]arachidonic acid release was observed in KG1a, CEM, and CEM/VLB100 cell lines. A strong positive correlation was observed between cell death and cPLA2 activity after exposure to TNF (r = 0.95, p < 0.01). Some [3H]arachidonic acid release was observed when cells were exposed to high concentrations of TNF. TNF at 100 ng/ml increased [3H]arachidonic acid release by 12, 25, and 35%, respectively, in the KG1a, CEM, and CEM/VLB100 cell lines.

U937 and HL60 cells pretreated with a cPLA2 inhibitor, BPB, were less susceptible to TNF-mediated cytotoxicity (Fig. 2B). The ID50 values for U937 and HL60 cells treated with BPB increased by approximately 3.5- and 2.1-fold (p < 0.01), suggesting that cPLA2 activity is, at least in part, required for TNF-mediated cytotoxicity in these cells. This has been confirmed using an antisense oligonucleotide to cPLA2 in U937 cells (24).

Expression of cPLA2 protein and mRNA by leukemic cell lines

We next set out to establish whether activation of cPLA2 by TNF was related to constitutive levels of this protein. cPLA2 protein expression in both the cytosol and membrane fractions was assessed by Western blot. cPLA2 protein was detectable in the cytosol of U937, HL60, and KG1a cells (Fig. 3, A and B). CEM and CEM/VLB100 cells, however, expressed small but nevertheless detectable amounts of cPLA2 protein in their cytosol. No cPLA2 was detected in the membrane of CEM or CEM/VLB100 cells by Western blot. However, small amounts were detected by flow cytometry in permeabilized (for cytosol cPLA2) or nonpermeabilized cells.

Table I. TNFR-P55 expression in human leukemic cell lines

<table>
<thead>
<tr>
<th>Cell Types</th>
<th>MFI</th>
<th>% of Positive Cells</th>
</tr>
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<tbody>
<tr>
<td>U937</td>
<td>91.8 ± 53.8</td>
<td>61.4 ± 32.2</td>
</tr>
<tr>
<td>HL60</td>
<td>40.9 ± 28.1</td>
<td>4.3 ± 2.5</td>
</tr>
<tr>
<td>KG1a</td>
<td>152.3 ± 65.7</td>
<td>54.2 ± 27.1</td>
</tr>
<tr>
<td>CEM</td>
<td>97.6 ± 63.5</td>
<td>19.6 ± 12.2</td>
</tr>
<tr>
<td>CEM/VLB100</td>
<td>88.7 ± 42.4</td>
<td>10.9 ± 5.4</td>
</tr>
</tbody>
</table>

* Data represent the MFI (mean fluorescence intensity) ± SD, and mean % ± SD of positive cells from four separate experiments.

FIGURE 2. A, TNF-induced [3H]arachidonic acid release from human leukemic cell lines. Cells were labeled for 18 h, followed by incubation with media (control) or TNF at 2, 10, and 100 ng/ml for 24 h. Data from at least three independent experiments are shown as percentage increase above background levels of [3H]arachidonic acid release. B, Effects of the PLA2 inhibitor BPB on TNF-induced cytotoxicity of U937 and HL60 cell lines. U937 and HL60 cells were pretreated with BPB (5 μM) for 24 h before TNF treatment (**p < 0.01, *p < 0.05 BPB-treated U937 and HL60 cells compared with TNF alone-treated cells). BPB alone (5 μM) produced a growth inhibition less than 5%.
cPLA₂ activation and sensitization to TNF by protein synthesis inhibitor, CHI

Clearly, cPLA₂ was detectable in the KG1a cell line, but this was not activated by TNF. By contrast, CEM and CEM/VLB₁₀₀ cells have low levels of cPLA₂ expression, possibly resulting in the relative resistance of these cell lines to TNF-mediated apoptosis. To further explore the mechanisms by which TNF-associated cPLA₂ activity in these cell lines is regulated, cells were exposed to TNF after a 3-h preincubation with the protein synthesis inhibitor CHI. Our results showed that TNF alone (2 ng/ml) did not produce a significant increase in [³H]arachidonic acid release in KG1a, CEM, and CEM/VLB₁₀₀ cell lines, as shown in Figure 2A. When these cells were treated with both TNF and CHI, however, a significant increase in the release of [³H]arachidonic acid was observed in both CEM and CEM/VLB₁₀₀ cell lines (Fig. 5A). By contrast, only a marginal increase was observed in the KG1a cell line after CHI exposure. These data suggest the presence of a protein that inhibits TNF-induced cPLA₂ activation in the CEM and CEM/VLB₁₀₀ cell lines, but not in KG1a cells.

We also studied whether the relative resistance of KG1a, CEM, and CEM/VLB₁₀₀ cells to cytolysis by TNF was dependent on the maintenance of protein synthesis. CHI (150 ng/ml) rendered both CEM and CEM/VLB₁₀₀ cell lines significantly more susceptible to TNF (Fig. 5B). By contrast, the viability of the KG1a cell line was not altered significantly even when CHI was used at concentrations as high as 300 ng/ml. Our results demonstrate that inhibition of protein synthesis by CHI significantly potentiates TNF-mediated cPLA₂ activation and cytotoxicity in both CEM and CEM/VLB₁₀₀ cell lines, but not in KG1a cells. The data indicate the existence of cellular proteins that may protect both CEM and CEM/VLB₁₀₀ cells against the cytotoxic effects of TNF in these cell lines, possibly by directly inhibiting the activation of cPLA₂.

Mellitin and the calcium ionophore A23187 can directly activate cPLA₂ in KG1a cells

To study whether the inactivation of cPLA₂ in KG1a, CEM, and CEM/VLB₁₀₀ cells is specific to TNF or whether it is a general phenomenon, we studied the actions of two other agents known to induce activation of cPLA₂, the calmodulin antagonist, mellitin, and the calcium ionophore A23187. Mellitin alone induced some activation of cPLA₂ in all three cell lines and had an additive effect with TNF in the CEM and CEM/VLB₁₀₀ cell lines (Fig. 6). The combined effect with TNF in KG1a cells, although not dramatic, was synergistic.

cPLA₂ is localized in the cytoplasm of unstimulated cells, and translocates to the membrane in response to physiologic elevations of intracellular free calcium. A23187 transports calcium across biologic membranes, down its concentration gradient (25, 26). Exposure to A23187 thereby results in an elevation of intracellular free calcium, independent of plasma membrane receptor-mediated events. As shown in Figure 7, A23187 alone could stimulate [³H]arachidonic acid release from the KG1a cell line, and an additive effect of A23187 and TNF (100 ng/ml) was observed. However, A23187 had no effect in the CEM and CEM/VLB₁₀₀ cell lines. These data suggest that at least some of the cPLA₂ protein detectable in KG1a cells is able to be activated by agents other than TNF, although the level of activity is low and not sufficient in itself to result in apoptosis (data not shown). The levels of activation were lower than those that would have been expected given the relatively high level of cPLA₂ protein. This suggests that there...
is a constitutive failure of cPLA₂ activation in these cells. The data also suggest that mellitin, and possibly the calcium ionophore A23187, permit some activation of cPLA₂ by TNF in the KG1a cell line, but only to a minor degree. Neither mellitin or A23187 had any significant effect on TNF-induced cPLA2 activation in CEM and CEM/VLB₁₀₀ cells. The inhibitory factor preventing TNF-induced activation of cPLA₂ in these cell lines appears to be independent of calcium mobilization.

**Discussion**

A substantial body of evidence has suggested that cPLA₂ activation is involved in TNF-mediated cytotoxicity (4, 11). We have shown previously that TNF induces cPLA₂ activation and apoptosis in the U937 cell line, and that the cPLA₂ inhibitor, BPB, or an antisense oligonucleotide to cPLA₂ can protect these cells from TNF-induced death (24). In this study, we have confirmed these observations in the HL60 human myeloblastic cell line. Why the activation of cPLA₂ is necessary for the lytic response is not clear, although a number of hypotheses have been put forward, including arachidonic acid or eicosanoids acting as second messengers, lysosphospholipids acting as agents that disrupt membranes, or active oxygen radicals, generated as by-products of eicosanoid production, acting as intracellular toxins (27). However, different cell lines display differential responses to TNF-mediated cytotoxicity, and the basis for this difference is still unknown. In this study, we have demonstrated that the differential responses to TNF-mediated killing among leukemic cell lines may involve differential activation of cPLA₂.

Whereas cPLA₂ activation and apoptosis were associated in U937 and HL60 cells, the KG1a, CEM, and CEM/VLB₁₀₀ cell lines were relatively resistant to TNF. We therefore set out to establish the mechanisms that regulate cPLA₂ activity in these cell lines. Variations in the amount of [³H]arachidonic acid release from these cell lines could be dependent on the constitutive expression or activity of cPLA₂ itself. Overexpression of cPLA₂ has been shown to increase the sensitivity of a TNF-resistant L929...
served in KG1a cells. These data suggest that cPLA2 in KG1a cells was found in the CEM and CEM/VLB 100 cell lines. These observations may explain, in part, the low level of inducible cPLA2 activity in these cell lines. By contrast, the KG1a cell line, which was more resistant to TNF, expressed more cPLA2 than both CEM and CEM/VLB100 cell lines. This suggests that a mechanism exists whereby cPLA2 in the KG1a cell line is protected from activation by TNF.

Interestingly, prior exposure to the protein synthesis inhibitor, CHI, sensitized the CEM and CEM/VLB100 cell lines to TNF killing, and resulted in greater [3H]arachidonic acid release in response to TNF. These observations suggest that cellular inhibitory proteins exist at or upstream of cPLA2 activation (4). In the presence of CHI, TNF produced a significant increase in [3H]arachidonic acid release in the CEM and CEM/VLB100 cell lines, cPLA2 is efficiently and actively involved in the TNF-signalng pathway. By contrast, inhibiting protein synthesis had no effect on KG1a cells, suggesting either a constitutive defect of cPLA2 itself or in the signaling cascade linking cPLA2 to the TNFR.

Increases in intracellular calcium can translocate cPLA2 to the membrane (25, 26) and enhance its activity. Using the model described above, we would predict that cPLA2 in the CEM and CEM/VLB100 cell lines would be activated by direct stimuli, such as mellitin or the calcium ionophore A23187, whereas cPLA2 in the KG1a cell line would be unaffected. In contrast, both mellitin and A23187 induced low levels of cPLA2 activity in the KG1a cell line, although to nothing like the same degree as could be optimally induced in CEM and CEM/VLB100 cells with TNF and CHI. In fact, mellitin, which acts as a calmodulin inhibitor (28), and A23187 had no more than an additive effect with TNF in CEM and CEM/VLB100 cells, whereas a small synergistic effect was observed in KG1a cells. These data suggest that cPLA2 in KG1a cells is not completely dysfunctional, and some activation may be induced by TNF when calcium mobilization is altered. As anticipated, the ability of TNF to induce cPLA2 activity in CEM and CEM/VLB100 cells is unchanged by disturbing calcium flux.

Some studies have shown that resistance to TNF killing is due to the constitutive expression of cellular proteins that inhibit the TNF-associated lytic pathway in resistant cells. Such proteins include manganese superoxide dismutase (17), major heat-shock protein 70 (18), and the zinc finger protein A20 (19). It is known that TNF activates the nuclear factor-kB family of transcription factors that in turn regulates synthesis of a number of proteins, including TNF itself (15, 16). More recent results suggest a novel caspase-dependent activation pathway for cPLA2 during apoptosis and identify cPLA2 as a mediator of TNF-induced cell death acting downstream of caspases (29).

We propose that cPLA2 activity is involved in TNF-induced cytotoxicity in the human leukemic cell lines used. Resistance to TNF-induced cytotoxicity may involve either protein inhibitors that act upstream of cPLA2 in the TNF-signaling pathway or a constitutive defect of cPLA2 itself, possibly involving calcium utilization.

References


