1,25-Dihydroxyvitamin D₃ Protects Human Leukemic Cells from Tumor Necrosis Factor-induced Apoptosis via Inactivation of Cytosolic Phospholipase A₂

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ABSTRACT

The mechanism by which tumor necrosis factor (TNF) induces death of cancer cells appears to involve the activation of cytosolic phospholipase A₂ (cPLA₂). U937 human leukemia cells treated with 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) become resistant to TNF, an effect that is independent of cell cycle status and expression of TNF receptors or BCL-2. In this study, TNF produced a dose- and time-dependent enhancement of [³H]arachidonic acid release in U937 cells. The amount of [³H]arachidonic acid released was positively associated with TNF-induced apoptosis. Both immunofluorescence microscopy and Western blotting of cell subcompartments demonstrated translocation of cPLA₂ from the cytosol to the cell membrane in response to TNF. In addition, TNF up-regulated expression of cPLA₂ mRNA. An antisense oligonucleotide to cPLA₂ and the cPLA₂ inhibitor 4-bromophenacyl bromide significantly inhibited TNF-induced cytotoxicity. Prior incubation of cells with 1,25(OH)₂D₃ significantly inhibited (a) TNF-induced [³H]arachidonic acid release and apoptosis, (b) TNF-induced translocation of cPLA₂ to the membrane, and (c) the up-regulation of cPLA₂ mRNA with TNF. Furthermore, the inhibitory effect of 1,25(OH)₂D₃ was not reversed by inhibitors of transcription or translation. The data suggest that activation of cPLA₂ is involved in TNF-induced apoptosis of leukemic cells. 1,25(OH)₂D₃ directly inhibits cPLA₂ translocation and mRNA up-regulation induced by TNF. Disruption of cPLA₂ activation may represent a possible mechanism whereby leukemic cells can become resistant to TNF-mediated killing.

INTRODUCTION

Among multiple biological functions exhibited by TNF² (1, 2), the phenomenon of TNF-induced cell death has attracted great attention. TNF can cause cell death in several types of tumor cells in vitro; however, the vast majority of tumor cells are resistant to TNF-mediated cell killing (3–5). Furthermore, several reports have shown that the resistance of tumor cells to TNF-mediated killing can be overcome by cotreatment with inhibitors of DNA transcription or protein synthesis (6, 7). These studies postulated the existence of cellular proteins, called “TNF-resistant proteins,” which control resistance to cell killing by TNF. When the synthesis of these proteins was disrupted, cells became susceptible to TNF killing (8, 9). Several intracellular pathways have been demonstrated to be involved in TNF-initiated cytotoxic processes, including the reduction of glutathione levels (10), free radical generation (11), and activation of the caspase system (12, 13). TNF-mediated cell killing also seems to be accompanied by activation of cytosolic phospholipase A₂ (cPLA₂), which is thought to be crucial for the cytotoxic response to TNF (6, 14–16). cPLA₂ shows high selectivity for arachidonoyl-containing phospholipids, and its activation leads to the release of arachidonic acid from cell membrane phospholipids at the sn-2 position (17, 18). Reactive oxygen radicals, the metabolic products generated by arachidonic acid acting as intracellular toxins, may eventually contribute to TNF-mediated cytotoxicity (11, 19). Alternatively, cPLA₂ may activate sphingomyelinase to generate ceramide, a known component of the apoptotic pathway (20, 21). Interestingly, it has been shown recently that activation of cPLA₂ is downstream of caspase activity in TNF-induced apoptosis of MCF and WEHI-164 cells (22).

The protective role of the transcription factor NF-κB against apoptotic killing by TNF has been demonstrated recently (23, 24). Several lines of evidence have suggested that resistance to TNF killing is attributable to the constitutive expression of protective proteins in resistant cell lines, such as manganese superoxide dismutase (25), major heat shock protein 70 (26), and zinc finger protein A20 (27). There is some evidence that TNF-resistant proteins prevent the activation of cPLA₂ by TNF (28), although these proteins are as yet unidentified.

Our previous studies have shown that prior incubation of the U937 human leukemia cell line with 1,25(OH)₂D₃ for 48 h can confer resistance to TNF-mediated killing, an effect that appears to be independent of cell cycle status and expression of TNF receptors or BCL-2 (29). This observation suggests that leukemic cells may readily acquire resistance to TNF-mediated apoptosis, possibly in association with differentiation. Understanding the mechanisms by which these cells became resistant to TNF may indicate the processes by which human leukemia escapes immune surveillance. In this report, we demonstrate that cPLA₂ activity is involved in TNF-mediated killing of U937 cells. 1,25(OH)₂D₃ protects cells from TNF-induced killing by inhibiting the activation of cPLA₂, preventing its translocation from the cytosol to the cell membrane and inhibiting expression of cPLA₂ mRNA. The effect of 1,25(OH)₂D₃ does not appear to involve synthesis of TNF-resistant proteins, thereby making it less likely to be dependent on differentiation.

MATERIALS AND METHODS

Reagents. Recombinant human TNF-α, MTT, BPB, CHI, Act D, RNase, and proteinase K were all purchased from Sigma Chemical Co. (St. Louis, MO). 5,6,8,9,11,12,14,15-[³H]Arachidonic acid (1 mCi/ml) was purchased from Amersham. 1,25(OH)₂D₃ was a kind gift from Dr. K. Colston (Department of Biochemistry, St. George’s Hospital Medical School, London, United Kingdom).

Cell Line and Culture Methods. The human monocytic leukemia cell line U937 was cultured in RPMI 1640 with 10% FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a 5% CO₂ incubator. Cells were seeded at 0.3 × 10⁶ cells/ml in a 24-well plate and primed with 1,25(OH)₂D₃ (10⁻⁸ M) for 48 h. For assays with DNA transcription or protein synthesis inhibitors, U937 cells were preincubated with Act D or CHI for 3 h before 1,25(OH)₂D₃ treatment. Cells were harvested and centrifuged, and the cell pellets were re suspended in fresh medium for different studies.

Detection of DNA Degradation by Gel Electrophoresis. Cells were harvested and washed twice in HBSS and then lysed in lysis buffer consisting of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.5% N-lauroylsarcosine with proteinase K (0.5 mg/ml) for 1 h at 56°C followed by RNase (0.25 mg/ml) incubation for 1 h at 56°C. Thereafter, 1 volume of a 2:1 mixture of

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chloroform and isooamy alcohol was added and mixed thoroughly for 5 min by repeated inversion. After centrifugation at 800 x g for 4 min, the majority of the upper aqueous phase was carefully removed and transferred to clean tubes. One and one-half volumes of Tri-s-EDTA buffer were added, and then the mixture was centrifuged at 13,000 x g for 10 min. The supernatant was collected and resuspended in 2 volumes of cold (4°C) 100% ethanol and mixed by repeated inversion until DNA was precipitated. The DNA was pelleted by centrifugation at 13,000 x g for 10 min and dried before dissolving in Tri-s-EDTA buffer. Horizontal 1% agarose gel electrophoresis was performed at 6 V/cm for 2 h. The DNA in the gel was visualized under UV light after staining with ethidium bromide (1 μg/ml).

Measurement of [3H]Arachidonic Acid Release from U937 Cells. Cells were homogenized with a Dounce (Jencons, UK) tissue grinder. The nuclei and debris, followed by centrifugation at 8000 x g for 30 min to remove the HD. 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 5% of the total. To confirm that the increase in [3H]arachidonic acid in the supernatant of TNF-treated cells was not attributable 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 an additional 6 h before both the supernatant and the pellet were counted by liquid scintillation. The amount of [3H]arachidonic acid in both supernatant and pellet fractions were identical for both untreated and TNF-treated cells, indicating that reuptake of [3H]arachidonic acid from supernatant, once released, is negligible.

Fluorescence Microscopy. Cells were mounted on slides by cytopsin, fixed with 1% paraformaldehyde for 20 min, and then incubated with anti-human cPLA2 mAb (100 μg/ml; Santa Cruz Biotechnology) for 60 min, followed by incubation with FITC-conjugated antibody for 45 min. After washing, a coverslip was mounted with 90% glycerol-PBS with 4,6-diamidino-2-phenyl-indole for counterstaining. The subcompartmental location of cPLA2 was visualized using a Leica DM RXA microscope coupled to a Perceptive Scientific International Ltd. image analysis system and confirmed by confocal microscopy. All images were captured with a 100X objective using a monochrome charge-coupled device camera and automated filter wheel capture.

Preparation of Membrane Proteins and Analysis by Western Blot. Plasma membrane was prepared as described by Wu et al. (17). Briefly, cells were washed three times with cold HBSS and rinsed in 0.5 ml of homogenization buffer (50 mM HEPES (pH 8.0), 1 mM EDTA, 1 mM EGTA, 50 μg/ml leupeptin, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 10 μM phosphoramidon, 10 μg/ml soybean trypsin inhibitor, and 100 μg/ml aprotinin). The cells were homogenized with a Dounce (Junons, UK) tissue grinder. The homogenates were first centrifuged at 1000 x g for 10 min to discard the nuclei and debris, followed by centrifugation at 8000 x g for 30 min to remove mitochondria. The supernatants were finally centrifuged at 100,000 x 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 protein were separated on 8% SDS-polyacrylamide gels. The separated proteins were electrophoretically transferred onto a nitrocellulose membrane. The blots were stained with Ponceau S solution (Sigma Chemical Co., Poole, United Kingdom) to monitor the equal loading proteins for each lane. After destaining, the blots were incubated in blocking solution consisting of 5% nonfat dry milk in phosphate-buffered NaCl solution and 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-cPLA2 mAb. The immunoblots were then incubated with goat anti-mouse IgG conjugated to peroxidase for 60 min and visualized with enhanced chemiluminescence detection reagents (Amersham, Amnham, United Kingdom).

Detection of cPLA2 mRNA by RT-PCR. RT-PCR was used to detect cPLA2 mRNA in a semiquantitative manner in preference to Northern blotting because of the low levels of message under several experimental conditions. Total cellular RNA was extracted by an RNAsol B single-step guanidinium thiocyanate-phenol-chloroform extraction method and reverse transcribed into cDNA at 42°C for 60 min using avian myeloblastosis virus reverse transcriptase and random hexamer primers. The cDNA was amplified via PCR using Thermus aquaticus DNA polymerase. The reaction was carried out for 35 cycles in 25 μl of reaction mixture using a step program (94°C, 45 s; 56°C, 45 s; and 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 stained with ethidium bromide. The cPLA2 primer pair amplified a 554-bp PCR product confirming the identity of the PCR product as cPLA2 cDNA. Primers were composed of the following sequences: 5'-TTG CAA ACT GCC TCA GCA TCA G-3' and 5'-CTC TAG TCC GTT CAA GGA AC-3'. The β-actin primer pair amplified a 400-bp product and was composed of the following sequences: 5'-GAT GAG GTT GAA GGT AGT TT-3' and 5'-TGC TAT CCT TCG GCC TGT CTT AT-3'. The RT-PCR experimental conditions were identical for both cPLA2 and β-actin. Normalization of RNA by β-actin was confirmed by running PCR for 25 cycles.

Inhibition by Antisense Oligonucleotide to cPLA2. The human cPLA2 antisense oligonucleotide (5'-TAC AGT AAA TAT CTA CAA GTA AGT-3') was directed against the initiation site. The nonsense oligonucleotide (5'-CCT ACT GAG GTG AAT TTG ATG-3') was a random sequence of the antisense bases.

Cytotoxicity Assay. TNF-induced cytotoxicity of U937 cells pretreated with different inhibitors was determined by MTT assay as described previously (30–32). The IC50 was calculated from a logaritmic regression curve of the results for at least four separate experiments.

Statistical Analysis. Results are expressed as mean ± SD obtained from multiple experiments. Statistical analysis was performed using paired Student’s t test for grouped data. In all circumstances, P < 0.05 was considered statistically significant.

RESULTS

TNF Can Functionally Activate cPLA2 in U937 Cells. TNF can induce apoptosis in U937 cells, as shown previously (29). Cells may undergo apoptosis by TNF through PLA2-dependent and -independent lytic responses (33). To investigate whether such effects of TNF in U937 cells are mediated via a PLA2-dependent pathway, the activation of cPLA2 was determined by measuring the release of [3H]arachidonic acid from prelabeled U937 cells after exposure of cells to TNF. Fig. 1A showed that spontaneous release of [3H]arachidonic acid from radioabeled U937 cells remained at a steady level between 8 and 48 h of culture. Treatment of cells with TNF (2 ng/ml) resulted in a significant increase in [3H]arachidonic acid release as a time-dependent manner. Exposure of U937 cells to TNF for 24 h at concentrations ranging from 0.03 to 10 ng/ml led to a dose-dependent release of [3H]arachidonic acid (Fig. 1B). [3H]Arachidonic acid release reached a maximum level at approximately 24 h and corresponded to approximately twice the spontaneous release (control level (P < 0.01), suggesting that TNF induces cPLA2 activation, thereby inducing [3H]arachidonic acid release.

cPLA2 Activation Is Required for TNF-Induced Apoptosis: Protection by the PLA2 Inhibitor BPB and an Antisense Oligonucleotide to cPLA2. As described previously, TNF induced apoptotic cell death in U937 cells (29). BPB (5.0 μM), a PLA2 inhibitor, effectively inhibited TNF-induced cytotoxicity (Fig. 2) as measured by the MTT assay. A phosphorothioate-modified antisense oligonucleotide to cPLA2 (20 μM) directed against the initiation codon also effectively inhibited TNF-induced apoptosis of U937 cells. The antisense oligonucleotide directed against the initiation codon also effectively inhibited TNF-induced apoptosis of U937 cells.
INACTIVATION OF cPLA₂ BY 1,25(OH)₂D₃ IN TNF RESISTANCE

1,25(OH)₂D₃ still inhibits TNF-induced cPLA₂ activation in the presence of inhibitors of transcription and translation. As shown in Fig. 1, U937 cells released significantly more [³H]arachidonic acid when challenged with TNF than resting cells; i.e., TNF increased [³H]arachidonic acid release to 191% of background levels (P < 0.01). 1,25(OH)₂D₃ had no effect on resting [³H]arachidonic acid release (data not shown) but inhibited [³H]arachidonic acid release in response to TNF (Fig. 5). Again, we investigated whether inhibitory proteins are involved in the protection against TNF-induced activation of cPLA₂ evoked by 1,25(OH)₂D₃. CHI at 10 ng/ml did not increase resting or TNF-induced cPLA₂ activation. In addition, the inhibitory effect of 1,25(OH)₂D₃ persisted after preincubation with CHI (data not shown). Both CHI at a higher concentration of 300 ng/ml and Act D at 1 ng/ml were then preincubated for 3 h before 1,25(OH)₂D₃ treatment. In the presence of CHI at this concentration, TNF-induced [³H]arachidonic acid release was enhanced by 236 and 192% in unprimed and 1,25(OH)₂D₃-primed cells, respectively.

Fig. 1. A. time-dependent release of [³H]arachidonic acid by U937 cells in response to TNF. U937 cells were labeled with [³H]arachidonic acid as described in “Materials and Methods.” The labeled cells were exposed to media or TNF (2 ng/ml) for the times indicated, and then the supernatant was collected and the radioactivity released was analyzed. B. dose-dependent release of [³H]arachidonic acid by U937 cells in response to TNF. Prelabeled U937 cells were treated with different doses of TNF for 24 h. The values of each point represent mean cpm of three separate experiments; bars, SD.

oligonucleotides were approximately 20.98 and 28.14% greater than those of TNF-treated cells, respectively (P < 0.01), whereas a nonsense oligonucleotide had no effect (Fig. 2). These data indicate that activation of PLA₂ is causally associated with TNF-induced apoptosis in this cell model.

1,25(OH)₂D₃ Confers Resistance to TNF-induced Killing: Lack of Reversal by Inhibitors of Transcription or Translation. In agreement with our previous observations, 1,25(OH)₂D₃ (10⁻⁸ M) conferred significant resistance to TNF-induced apoptosis in U937 cells, as shown by the MTT assay, with IC₅₀ increasing from 0.58 to 3.26 (data not shown). This was confirmed by DNA fragmentation (Fig. 3). Inhibition of protein synthesis with either CHI (10 ng/ml) or Act D (0.1 ng/ml) rendered U937 cells more sensitive to TNF-induced apoptosis (Figs. 3 and 4), implying that TNF-resistant proteins do contribute to a certain degree to the protection of this cell line against TNF. However, the relative reduction in IC₅₀ for TNF by either CHI or Act D was identical, irrespective of whether the cells had been treated with 1,25(OH)₂D₃; e.g., IC₅₀ for TNF fell by 70% from 0.50 to 0.15 with CHI and by 58% to 0.21 with Act D. After treatment with 1,25(OH)₂D₃, IC₅₀ fell from 3.34 to 1.2 with CHI and to 1.42 with Act D, falls of 64 and 57%, respectively. This suggests that 1,25(OH)₂D₃ can induce the same degree of TNF resistance independently of inhibition of protein or mRNA synthesis and implies that new protein synthesis plays little role in the mechanism of resistance induced by 1,25(OH)₂D₃.

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Fig. 2. Effects of phospholipase A₂ inhibitor BPB and an antisense oligonucleotide to cPLA₂ on TNF-induced cytotoxicity of U937 cells. U937 cells were treated with BPB (5.0 μM) for 24 h or with 20 μM phosphorothioate-modified antisense or nonsense oligonucleotides for 6 h in FCS-free medium, followed by further incubation for 24 h in 5% FCS medium. Then treated cells were exposed to TNF at 2 ng/ml. BPB and antisense oligonucleotide to cPLA₂ alone failed to induce significant growth inhibition (<5%). Results are the means of at least four separate experiments (**, P < 0.01. U937 cells treated with TNF compared with control, BPB or antisense oligonucleotide treated U937 cells compared with TNF-treated cells); bars, SD.

Fig. 3. Effect of 1,25(OH)₂D₃ on TNF-induced cytotoxicity of U937 cells. Shown are results of agarose gel electrophoresis of DNA extracted from untreated U937 cells (Lane 1) and cells treated with 5 ng/ml TNF (Lane 2), 5 ng/ml TNF plus 300 ng/ml CHI (Lane 3), 10⁻⁸ M 1,25(OH)₂D₃ alone (Lane 4), 1,25(OH)₂D₃ plus 5 ng/ml TNF (Lane 5), and 1,25(OH)₂D₃ plus TNF and 300 ng/ml CHI (Lane 6). U937 cells were incubated with 300 ng/ml CHI for 1 h before preincubation with 1,25(OH)₂D₃ and then exposed to 5 ng/ml TNF for another 4 h.
cPLA2 in the plasma membrane fraction appeared to become stronger. cPLA2 was found to be associated with both cytosol and membrane. Using a specific anti-cPLA2 mAb. In unstimulated cells, constitutive cell membrane as a discrete ring (Fig. 6A), suggesting that TNF treatment (5 ng/ml, 12 h), cPLA2 staining appeared surrounding the distributed throughout the cytoplasm (Fig. 6A). By contrast, after TNF treatment of 1,25(OH)2D3, on TNF-induced cPLA2 activation is not mediated by cPLA2 activation. Second, even at a higher concentration of CHI at 10 ng/ml can increase TNF-induced cytotoxicity but has no effect on cPLA2 activity. The data suggest that, first, CHI at the lower concentration of 10 ng/ml can increase TNF-induced cytotoxicity but has no effect on cPLA2, it directly inhibits TNF-mediated changes in intracellular localization.

1,25(OH)2D3 Inhibited the Induction of cPLA2 mRNA by TNF.

To determine whether the activation of cPLA2 by TNF was related to the regulation of cPLA2 mRNA, the expression of cPLA2 mRNA in U937 cells was analyzed by RT-PCR. The PCR product of cDNA obtained using primers for cPLA2 ran at 554 bp, confirming its identity. TNF (5 ng/ml) up-regulated cPLA2 mRNA expression in a time-dependent manner in U937 cells (Fig. 8A). 1,25(OH)2D3 significantly inhibited the expression of cPLA2 mRNA induced by TNF (Fig. 8B). The percentages of cPLA2 mRNA to the internal control gene β-actin, as determined by densitometer, were 3.57, 15.63, 3.23, and 3.45%, respectively, in untreated, treated alone, 1,25(OH)2D3-treated alone, and TNF- and 1,25(OH)2D3-treated cells. These results suggest that 1,25(OH)2D3 cannot only modulate the function of the intact protein but can inhibit cPLA2 synthesis at the transcriptional level as well.

DISCUSSION

We have established that TNF activates cPLA2 in the U937 human leukemic cell line. TNF also induces translocation of cPLA2 from the cytosol to the cell membrane and up-regulates production of cPLA2 mRNA. U937 cells were susceptible to TNF-mediated killing, which could be markedly inhibited by both BPB (a PLA2 inhibitor) and an antisense oligonucleotide to cPLA2. BPB inactivates PLA2 by competitive binding to the active site of this enzyme (34, 35). These
results are in agreement with the results of others using dexamethasone and quinacrine as inhibitors of cPLA2 and demonstrate that cPLA2 activity is causally involved in TNF-induced apoptosis of U937 cells. Our data are also consistent with transfection experiments (6, 15) and antisense technology (6), which show that cPLA2 is essential for TNF-induced apoptosis. It has been demonstrated that TNF stimulates arachidonate release in murine C3HA, L929, and WEHI-164 cells (6, 11, 16). It has also been demonstrated that TNF-induced cPLA2 activation in HL60 leukemic cells leads to activation of a neutral sphingomyelinase and generation of ceramide (36). Ceramide is a contender for transduction of the apoptotic signal from the TNF receptor in several cell types (20, 21). The induction of ceramide generation in response to TNF is biphasic, with some occurring in minutes and some after several hours (37). Clearly, only the second phase of ceramide generation is likely to be in any way mediated via cPLA2 activation. Neither BPB nor the antisense oligonucleotide completely inhibited the cytotoxic effect of TNF. This may be attributable to limitations in the accessibility of the reagents used to the cell cytoplasm or may suggest the existence of an alternative pathway, which may bypass cPLA2 in the TNF signal transduction pathway.

TNF may activate cPLA2 through two distinct pathways. Vietor et al. (38) demonstrated that TNF induces activation and tyrosine phosphorylation of mitogen-activated protein kinase in human fibroblasts. cPLA2 is phosphorylated by protein kinase C and the p42 mitogen-activated protein kinase. The phosphorylation of cPLA2 seems to be a rapid response to TNF, which causes only a modest enhancement in cPLA2 activity (39). As treatment of the cells with TNF continues, the increase in cPLA2 activity possibly reflects the generation of newly synthesized cPLA2 protein (40). In our study, we identified that TNF could increase cPLA2 mRNA expression within 2 h. In addition, the observations made by immunofluorescence microscopy and immunoblotting provide evidence that stimulation of cells by TNF was also associated with translocation of cPLA2 from the cytosol to the cell membrane, where lipid hydrolysis takes place.

As reported previously, 1,25(OH)2D3 rendered U937 cells resistant to cell killing by TNF (29). Modulation of endogenous TNF expression may play a role in this protection, although the precise mecha-
Fig. 7. Effect of TNF on the amount of cPLA2 protein in U937 cell subfracionks primed with 1,25(OH)2D3. Forty micrograms of protein from each sample were run on an 8% SDS-polyacrylamide gel, followed by immunoblotting with anti-cPLA2 antibody as described in "Materials and Methods." The Western blot filters were stained with Ponceau S solution to ensure the equal loading for each lane (not shown). The cPLA2 proteins (110 kDa) in the membrane fraction (A) and in the cytosol fraction (B) are shown as untreated cells (Lane 1), cells treated with TNF (5 ng/ml) for 4 h (Lane 2) and 12 h (Lane 3), cells treated with 1,25(OH)2D3 for 24 h (Lane 4) and 48 h (Lane 5), and cells treated with 1,25(OH)2D3 for 48 h and TNF for 12 h (Lane 6).

Fig. 8. A, time course of cPLA2 mRNA expression in U937 cells treated with TNF. U937 cells were treated with TNF (5 ng/ml) for 0 h (Lane 1), 1 h (Lane 2), 2 h (Lane 3), 4 h (Lane 4), 12 h (Lane 5), 24 h (Lane 6), and 36 h (Lane 7). B, cPLA2 mRNA levels in U937 cells primed with 1,25(OH)2D3 in response to TNF. Lane 1, unstimulated U937 cells; Lane 2, cells treated with TNF (5 ng/ml) for 4 h; Lane 3, cells primed with 1,25(OH)2D3 for 48 h; Lane 4, cells primed with 1,25(OH)2D3 plus TNF for 4 h. The corresponding amplified products of β-actin (400 bp) are shown at the bottom of each lane. Control lanes for non-RT-RNA and for no DNA contained no detectable bands (data not shown), confirming neither genomic contamination nor overall contamination. C, laser densitometric evaluation of the results shown in B. Expression of cPLA2 and β-actin in each lane was quantitated by densitometry and expressed as integrated densitometric units.
activation of cPLA₂ (300 and 1 ng/ml for CHI and Act D, respectively) to the cytotoxic effect of TNF. Thus, protein synthesis inhibitors such as CHI and Act D preferentially sensitize cells to TNF-induced cPLA₂ activation and cytotoxicity, although one effect of TNF is to up-regulate synthesis of cPLA₂ protein. These results confirm that alternative proteins that inhibit TNF-induced cell death do exist, but that these are independent of the cPLA₂ pathway. As a result, it is unlikely that inhibition of cPLA₂ is a result of other processes associated with differentiation and is more likely to be a direct result of 1,25(OH)₂D₃ activity. In addition, these results confirm that cPLA₂ activation is an event that precedes TNF-induced cell death.

Clearly, other mechanisms are involved in the process of TNF-induced apoptosis in U937 cells, because inactivation of cPLA₂ only induces a relative resistance to TNF, which can be overcome by increasing the concentration of TNF. It is known that TNF activates the NFκB family of transcription factors, which in turn regulates synthesis of a number of proteins, including TNF itself. More recently, the activation of NFκB by TNF was found to be protective against cell killing, because inhibition of NFκB nuclear translocation enhanced apoptotic killing by TNF (23, 24). 1,25(OH)₂D₃ has been shown to down-regulate the levels of NFκB proteins and thereby to decrease their transcriptional activity in activated human lymphocytes (43). The role of NFκB proteins in resistance of U937 cells by 1,25(OH)₂D₃ to TNF killing is unknown and warrants further investigation.

1,25(OH)₂D₃ has functional similarities to glucocorticoids, which are known to have antiinflammatory properties. It has been shown that glucocorticoids suppress cPLA₂ activity by inducing the formation of lipocortins, which bind with high affinity to calcium and phospholipids, thereby decreasing the liberation of arachidonic acid from lipids (44). The mechanism of action of lipocortin is controversial (45, 46). 1,25(OH)₂D₃ may inhibit cPLA₂ activity through the activation of lipocortins, independently of protein synthesis, or possibly through inhibition of the phosphorylation mechanism. The precise mechanism remains to be elucidated. Interestingly, it has been shown recently that IFN-γ mediates monocyctic differentiation in HL60 cells via activation of cPLA₂ and subsequent hydrolysis of sphingomyelin (47). A similar mechanism has been proposed for 1,25(OH)₂D₃. 1,25(OH)₂D₃ is a powerful inducer of monocyctic differentiation in U937 cells and acts synergistically with IFN-γ (41). Our data suggest that the differentiation-inducing properties of 1,25(OH)₂D₃ in U937 cells are not mediated via activation of cPLA₂.

Our data demonstrate that the sensitivity of human leukemic cells to TNF may be exogenously regulated independently of protein synthesis, and further studies are required to answer whether similar changes occur in human leukemia. Inactivation of cPLA₂ may provide a novel mechanism whereby leukemic cells evade immune-mediated killing.

REFERENCES


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Clearly, other mechanisms are involved in the process of TNF-induced apoptosis in U937 cells, because inactivation of cPLA₂ only induces a relative resistance to TNF, which can be overcome by increasing the concentration of TNF. It is known that TNF activates the NFκB family of transcription factors, which in turn regulates synthesis of a number of proteins, including TNF itself. More recently, the activation of NFκB by TNF was found to be protective against cell killing, because inhibition of NFκB nuclear translocation enhanced apoptotic killing by TNF (23, 24). 1,25(OH)₂D₃ has been shown to down-regulate the levels of NFκB proteins and thereby to decrease their transcriptional activity in activated human lymphocytes (43). The role of NFκB proteins in resistance of U937 cells by 1,25(OH)₂D₃ to TNF killing is unknown and warrants further investigation.

1.25(OH)₂D₃ has functional similarities to glucocorticoids, which are known to have antiinflammatory properties. It has been shown that glucocorticoids suppress cPLA₂ activity by inducing the formation of lipocortins, which bind with high affinity to calcium and phospholipids, thereby decreasing the liberation of arachidonic acid from lipids (44). The mechanism of action of lipocortin is controversial (45, 46). 1,25(OH)₂D₃ may inhibit cPLA₂ activity through the activation of lipocortins, independently of protein synthesis, or possibly through inhibition of the phosphorylation mechanism. The precise mechanism remains to be elucidated. Interestingly, it has been shown recently that IFN-γ mediates monocyctic differentiation in HL60 cells via activation of cPLA₂ and subsequent hydrolysis of sphingomyelin (47). A similar mechanism has been proposed for 1,25(OH)₂D₃. 1,25(OH)₂D₃ is a powerful inducer of monocyctic differentiation in U937 cells and acts synergistically with IFN-γ (41). Our data suggest that the differentiation-inducing properties of 1,25(OH)₂D₃ in U937 cells are not mediated via activation of cPLA₂.

Our data demonstrate that the sensitivity of human leukemic cells to TNF may be exogenously regulated independently of protein synthesis, and further studies are required to answer whether similar changes occur in human leukemia. Inactivation of cPLA₂ may provide a novel mechanism whereby leukemic cells evade immune-mediated killing.

REFERENCES


1,25-Dihydroxyvitamin D₃ Protects Human Leukemic Cells from Tumor Necrosis Factor-induced Apoptosis via Inactivation of Cytosolic Phospholipase A₂

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