Changes of phospholipase D activity in TNF- α and anti-Fas/Apo1 monoclonal antibody induced apoptosis in HL-60 and A20 cells

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Abbreviations: FBS, fetal bovine serum; PC, phosphatidylcholine; PEt, phosphatidylethanol; PLC, phospholipase C; PLD, phospholipase D; PKC, protein kinase C; TNF, tumor necrosis factor; PMA, phorbol 12-myristate-13-acetate

Abstract

The changes of phospholipase D (PLD) activity were investigated during the courses of apoptotic process induced by tumor necrosis factor (TNF)- α or anti-Fas/Apo1 antibody in human premyelocyte HL-60 and murine B cell lymphoma A20 cells. The treatment of recombinant TNF- α to HL-60 cells resulted in the increased PLD activity as determined by the phosphatidylethanol formation in the presence of 1% ethanol. The enhancement of PLD activity was also observed in the anti-Fas/Apo1 monoclonal antibody-treated A20 cells. However, the activity of PLD was maximized when HL-60 and A20 cells were treated with either TNF- α or anti-Fas/Apo1 monoclonal antibody for 6 h. Both TNF- α and anti-Fas/Apo1 monoclonal antibody increased PLD activity in a dose-dependent manner up to 200 U/ml and 200 ng/ml, respectively. When the intracellular activity of protein kinase C (PKC) was interrupted by treatment of calphostin-C, both the PLD activation and the apoptosis induced by TNF- α and anti-Fas/Apo1 monoclonal antibody appeared to be inhibited. Since PKC is reported to activate PLD, the results indicate that the intracellular signaling cascade via PLD may play a role in the induction of apoptosis induced by TNF- α and anti-Fas/Apo1 monoclonal antibody.

Keywords: apoptosis, anti-Fas monoclonal antibody, phospholipase D, protein kinase C, tumor necrosis factor- α

Introduction

Apoptosis or programmed cell death is a naturally occurring event found in healthy organisms (Ellis *et al.*, 1991). Apoptosis is an indispensable mode of physiological cell death to gain the maintenance of proper numbers and types of cells. One of the key features that distinguish apoptosis from accidental necrosis is that apoptosis is intrinsically and extrinsically regulated biological phenomena which occur during metamorphosis, embryogenesis, endocrine-dependent tissue atrophy, tumor regression and normal tissue turnover. Apoptosis is the normal physiological mechanism of cell death characterized by cytoplasmic blebbing, cell shrinkage, nuclear condensation and DNA fragmentation into multiple nucleosomes prior to cell death. Various stimuli including cytokines (Elias *et al.*, 1988; Laster *et al.*, 1988; Bellomo *et al.*, 1992; Wright *et al.*, 1992), glucocorticoids (Wyllie, 1980), calcium ionophores (McConkey *et al.*, 1989), γ -radiation (Sellins *et al.*, 1991) and metabolic inhibitors (Barry *et al.*, 1990; Martin *et al.*, 1990) can induce apoptosis in certain cell types. It has been thought that cells have a mechanism converging the various extracellular signals to a common intracellular signals in apoptotic process, but the detailed mechanism of apoptosis has been not clearly understood yet.

Tumor necrosis factor (TNF)- α , a polypeptide secreted by activated macrophages and monocytes, is originally described on the basis of its cytotoxic or cytostatic effects on tumor cell lines (Trinchieri *et al.*, 1986). Since the polypeptide can induce divergent responses in various cell types including stimulation of proliferation, differentiation and death, it may also activate multiple signaling pathways in different cell types (Wright *et al.*, 1992). Moreover, it has been reported that TNF receptors have the homologous domains found in various transmembrane receptor superfamily, such as nerve growth factor receptor and Fas/Apo1 receptor. The activation of Fas/Apo1 receptor can also elicit apoptosis in mouse thymus (Watanabe-Fukunaga *et al.*, 1992) and these TNF homologous receptors have been thought to transmit a common signal leading to apoptosis.

Recently, there are several lines of evidence suggesting that the apoptotic process involves signal transduction mechanism found in embryonic development and cell proliferation (Dowd *et al.*, 1992; Song *et al.*, 1992). The well-known components in signal transduction pathway are reported to mediate various apoptotic signals within cells. For example, increased levels of intracellular signaling molecules like Ca²⁺ and inositol trisphosphate (Agarwal *et al.*, 1993), activation of protein kinase C

(PKC) (Pongacz *et al.*, 1996) and inhibitors of PKC like sphingosine (Sakakura *et al.*, 1996) are associated with apoptotic process.

Accumulating evidences have recognized intracellular phospholipases as the key elements in signal transduction in a variety of cells. It was initially thought that phosphatidylcholine (PC) hydrolysis in response to agonist stimulation was mainly resulted from activation of phospholipase A2 and phospholipase C (PLC) since the main products of PC hydrolysis were arachidonic acid and diacylglycerol. However, more recent studies identified phospholipase D (PLD) as a major enzyme responsible for PC hydrolysis because phosphatidate phosphohydrolase action on phosphatidic acid, a primary product of PLD, could account for the increased intracellular diacylglycerol level following agonist treatment (Exton, 1994). Furthermore, activation of PLD brings to the activation of various intra-cellular signaling cascades, such as increase of cytosolic Ca^{2+} (Salmon and Honeyman, 1980), stimulation of DNA synthesis (Moolenaar *et al.*, 1986), enhanced transcription of *c-fos* and *c-myc* (Moolenaar *et al.*, 1986) and activation of phosphatidylinositol-4,5-bisphosphate specific PLC (Knauss *et al.*, 1990).

Hence, a question has arisen whether PLD might be involved in TNF receptor- and Fas-mediated apoptosis, a yet another important biological phenomenon in eukaryotic cells. In this report, we examined the changes of PLD activity during the courses of the receptor-mediated apoptosis.

Materials and Methods

Materials

TNF- α and primulin were purchased from Sigma (St. Louis, MO). Anti-Fas monoclonal antibody was obtained from Pharmingen (San Diego, CA). Calphostin-C was from RBI (Natick, MA). [^3H]-palmitic acid was purchased from NEN (Boston, MA). Fetal bovine serum (FBS), penicillin/streptomycin solution and RPMI-1640 medium were from Gibco-BRL (Gaithersburg, MD). All other chemical agents were of analytical grade.

Cell culture

HL-60 human premyelocytes and A20 murine B cell lymphoma obtained from American Type Culture Collection were maintained in RPMI-1640 medium containing 10% (v/v) FBS, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. Cells were grown for 2 days at 37°C in a humidified CO_2 controlled (5%) incubator and the cultures were washed with RPMI-1640 medium supplemented with 0.1% (w/v) bovine serum albumin, 2 mM glutamine, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (serum-free medium). The cultures were then incubated in the

serum-free medium at 37°C for 1 day before stimulation with agonists.

Determination of PLD activity

PLD activity was determined by the formation of phosphatidylethanol (PEt) as described elsewhere (Jung *et al.*, 1997). Briefly, cells were cultured on 6-well plates, labeled with 2 $\mu\text{Ci}/\text{well}$ of [^3H]-palmitic acid, and treated with TNF- α or anti-Fas monoclonal antibody in the presence of 1% (v/v) ethanol. For the inhibition of PKC, cells were pretreated with 1 μM of calphostin-C for 30 min before stimulating with the agonists. Then, the cells were quickly washed with ice-cold phosphate buffered saline (PBS) and suspended in ice-cold methanol. Lipids were extracted according to the procedures of Bligh and Dyer (1959), and PEt was separated by a thin layer chromatography using a solvent system of ethyl acetate/iso-octane/acetic acid/water (110:50:20:100, v/v). The PEt band was identified with 0.002% (w/v) primulin in 80% (v/v) acetone, scraped and then counted using a scintillation counter.

DNA fragmentation analysis

Cells on 6 well plates were treated with TNF- α or anti-Fas monoclonal antibody for 6 h to induce apoptosis. After the treatment, 1×10^6 cells were then washed with 10 ml of PBS and collected by centrifugation. The cell pellets were resuspended in 1 ml of PBS, and 0.2 ml of cytosolic DNA extraction buffer (0.5% Triton X-100, 20 mM EDTA and 5 mM Tris-HCl, pH 7.4) was added. Extraction of cytosolic DNA was achieved by vortex mixing the cell pellet for 1 min. After centrifugation for 15 min at 15,000 *g* in micro-centrifuge, the supernatant containing fragmented DNA was subjected to phenol/chloroform extraction. The precipitation of fragmented DNA was obtained by addition of 0.1 volume of 3 M sodium acetate (pH 5.0) and the precipitate was washed with 80% cold ethanol. To evaluate DNA fragmentation, DNA samples were electrophoresed in a 1.5% agarose gel and visualized by ethidium bromide staining.

Results

Effects of TNF- α and anti-Fas monoclonal antibody on PLD activity

When HL-60 cells prelabeled with [^3H]-palmitic acid were challenged with TNF- α for PLD activation in the presence of 1% ethanol, [^3H]-PEt was produced as a result of PLD-catalyzed transphosphatidyl reaction. As shown in Figure 1A, TNF- α -induced activation of PLD was first detected after 1 h of TNF- α treatment to HL-60 human myelocytes. Upon prolonged incubation with TNF- α up to 6 h, TNF- α -induced [^3H]-PEt formation was increased to approximately 2.5-fold of control. Phorbol 12-myristate-

Figure 1. Time course of TNF- α - and anti-Fas monoclonal antibody-induced PLD activity in HL-60 and A20 cells. HL-60 cells (A) and A20 cells (B) grown on 6 well plates were labeled with 1 μ Ci/ml of [3 H]-palmitic acid in serum free RPMI 1640 medium for 24 h. Then, the cells were pre-treated with 1% (v/v) ethanol and stimulated with 200 U/ml of TNF- α (A) or 200 ng/ml anti-Fas monoclonal antibody (B) for each indicated times. As a positive control for PLD activation, 200 nM PMA was treated for 30 min. The agonist treated cells were quickly washed with ice-cold PBS and lipids were extracted as described in 'Materials and Methods'. The [3 H]-PEt bands were separated by a thin layer chromatography, scraped and counted in a liquid scintillation counter.

13-acetate (PMA) was also used for a positive control, where it induced about 6-fold rise in PLD activity as compared to control. Figure 1B shows the effect of anti-Fas monoclonal antibody treatment on PLD activity in A20 murine B cell lymphoma culture. Anti-Fas antibody also induced fairly late activation of PLD in contrast to PMA, which increased the PLD activity after 30 minute of treatment. The relative differences in the folds of PMA-induced PLD activation between HL-60 and A20

Figure 2. Dose-dependent effects of TNF- α and anti-Fas monoclonal antibody on PLD activity. Various concentrations of TNF- α or anti-Fas monoclonal antibody were added to [3 H]-palmitic acid labeled HL-60 human premyelocytes (A) and A20 cells (B) for 6 h. After stimulations, the cells were harvested in ice-cold PBS and lipids were extracted. The radioactive spots corresponding to [3 H]-PEt were scraped and counted in a liquid scintillation counter.

cells might be resulted from cell type-specific responsiveness to PMA.

The dose response pattern of TNF- α - and anti-Fas monoclonal antibody-induced PLD activity was also examined (Figure 2A and 2B). TNF- α increased PLD activity in a dose-dependent manner and almost maximum rise in PLD activity was obtained at 200 U/ml in HL-60 cells. In A20 cells, treatment of anti-Fas monoclonal antibody also increased PLD activity in a dose-dependent

manner, starting at 50 ng/ml and then reaching nearly saturated value at the dose of 200 ng/ml.

Calphostin-C inhibition of TNF- α - and anti-Fas-monoclonal antibody induced PLD activation

Calphostin-C, a well-known inhibitor of PKC (Westermann *et al.*, 1996), was used in this study to examine its effect on TNF- α - and anti-Fas monoclonal antibody-induced PLD activation. As shown in Figure 3, pretreatment of calphostin-C for 30 min resulted in the perturbation of TNF- α - and anti-Fas monoclonal antibody-induced PLD activation. This result might suggest that the agonist-induced increases in PLD activity are correlated with

Figure 3. Effect of calphostin-C on TNF- α - and anti-Fas monoclonal antibody-induced PLD activities. HL-60 (A) and A20 (B) cells were labeled with 1 μ Ci/ml of [3 H]-palmitic acid and stimulated for 6 h with 200 U/ml TNF- α or 200 ng/ml anti-Fas monoclonal antibody, respectively. To determine the effect of calphostin-C, a protein kinase inhibitor, on the enhanced PLD activities by the agonists, 1 μ M of calphostin-C was pretreated for 30 min before the addition of TNF- α or anti-Fas monoclonal antibody.

PKC-mediated signaling pathway.

Effect of calphostin-C on TNF- α - and anti-Fas monoclonal antibody-induced DNA fragmentation

As a prominent biochemical feature of apoptosis, DNA fragmentation has been described as a hallmark of apoptosis in many studies. Figure 4 reveals the effects of calphostin-C on TNF- α - and anti-Fas monoclonal antibody-induced DNA fragmentation in HL-60 and A20 cells. When HL-60 cells were treated with TNF- α , obvious internucleosomal DNA fragmentation was recognized by 6 h after exposure (lane 3). Anti-Fas monoclonal antibody also efficiently triggered DNA fragmentation by 6 h of treatment (lane 8). But when 1 μ M calphostin-C was pretreated for 30 min and then washed with fresh medium before the addition of each agonists, both TNF- α and anti-Fas monoclonal antibody failed to induce DNA fragmentation (lanes 5 and 10). The calphostin-C alone also failed to induce DNA fragmentation in both HL-60 and A20 cells.

Discussion

In order to find out a possible involvement of PLD activity in the apoptotic process, changes of PLD activity were investigated in HL-60 and A20 cells treated with TNF- α or anti-Fas monoclonal antibody. It was initially thought

Figure 4. Effect of calphostin-C on TNF- α - and anti-Fas monoclonal antibody-induced DNA fragmentation in HL-60 and A20 cells. Cells on 6 well plates were treated with TNF- α or anti-Fas antibody for 6 h. For the PKC inhibition, 1 μ M calphostin-C was pretreated for 30 min before the addition of agonists. Then, the cells were harvested and cytosolic DNA was extracted as described in 'Materials and Methods'. The fragmented DNA was analyzed on 1.5% agarose gel electrophoresis. Lane 1 and 6, 100 bp DNA size marker; lane 2, control HL-60 cells; lane 3, TNF- α -treated HL-60 cells; lane 4, calphostin-C-treated HL-60 cells; lane 5, calphostin-C- and TNF- α -treated HL-60 cells; lane 7, control A20 cells; lane 8, anti-Fas antibody-treated A20 cells; lane 9, calphostin-C-treated A20 cells; lane 10, calphostin-C- and anti-Fas antibody-treated A20 cells.

that both TNF receptor and Fas antigen may deliver a similar death signal, because they share structural homologies in their extracellular and cytoplasmic domains (Tartaglia *et al.*, 1993). However, Schulze-Osthoff *et al.* (1994) reported that cell death signaling pathways mediated by each receptor molecule may occur through distinct signal transduction pathways. They showed that the reactive oxygen intermediates, which is thought to be responsible for cell death induced by TNF (Zimmerman *et al.*, 1989; Schulze-Osthoff *et al.*, 1992; Hennes *et al.*, 1993), does not play a role in Fas-mediated apoptosis. The authors also reported the different susceptibilities to ADP-ribose polymerase inhibitor and Ca^{2+} ionophores A23187 between TNF receptor-mediated and Fas-mediated apoptosis progressions. Meanwhile, TNF signaling is mediated by the second-messenger like molecule ceramide, which is generated by sphingomyelin breakdown by sphingomyelinase (Schütze *et al.*, 1992), just as in the case of Fas-induced apoptosis (Nagata and Golstein, 1995).

In an attempt to find out whether both TNF- and Fas-mediated apoptosis share a common signal pathway via PLD activation, the changes in PLD activity after treatment of TNF- α and agonistic anti-Fas monoclonal antibody were investigated in HL-60 and A20 cells. PLD, first discovered in plant cells, is now recognized as an important signaling enzyme present in many mammalian cells. The activation of PLD can be readily checked by transphosphatidyl reaction, by which the phosphatidyl group of phospholipids is transferred to a primary alcohol such as ethanol (Exton, 1994). As shown in Figure 1, both TNF- α and anti-Fas monoclonal antibody efficiently increased the amount of [^3H]-PEt in [^3H]-palmitic acid labeled HL-60 and A20 cells, respectively. The fairly late activation of PLD by the agonists, is in accordance with the results obtained by De Valck *et al.* (1993), where the TNF-induced PLD activation in L929 cells becomes evident after 6 h of treatment. PC hydrolysis by PLC in U937 cells were also reported by Schütze *et al.* (1991), where the authors observed no changes in phosphatidic acid and choline upon TNF treatment for the short time periods (4 min) and therefore they concluded that PLD activity is not involved in TNF-mediated signaling pathways. This discrepancy may have resulted from the differences in the incubation time of TNF. In the present study, there was efficient enhancement in PLD activity only after several hours of TNF- α and anti-Fas monoclonal antibody treatment.

Having convinced the possible intercalation of PLD activity in TNF- α - and anti-Fas agonistic antibody-induced apoptotic processes, the effects of PKC inhibitor on the TNF- α - and anti-Fas monoclonal antibody-induced PLD activation were investigated. In Figure 3, the effect of calphostin-C, a potent inhibitor of PKC, on the agonists-induced PLD activity was shown. The overt attenuation of PLD activation by calphostin-C supports the idea that

TNF- α and anti-Fas monoclonal antibody induce the activation of PLD in a PKC-dependent manner. The PKC isozymes were reported to control PLD and PLC in various systems, for example, the inhibitors of PKC such as H-7, staurosporine, Ro-31-8220 or sphingosine reduce the effects of agonists and phorbol esters (Liscovitch and Amsterdam, 1989; Exton, 1990; Billah *et al.*, 1990; Van der Meulen and Haslam, 1990; Oslon *et al.*, 1991; Kanaho *et al.*, 1992; Kester *et al.*, 1992; McNulty *et al.*, 1992). However, there is a conflicting evidence showing that staurosporine can inhibit PLD activity induced by PMA, whereas it effectively enhances fMetLeuPhe-induced PLD activity in neutrophils (Reinhold *et al.*, 1990).

Data in Figure 4 clearly indicate that TNF- α - and anti-Fas monoclonal antibody-induced apoptosis is effectively perturbed upon pretreatment of cells with calphostin-C, a highly specific inhibitor of PKC (Jarvis, *et al.*, 1994). It was well known that inhibitors of PKC could block the effects of agonists or PMA on PC-specific PLD or PC-specific PLC because PLD activity is dependent on PKC (Exton, 1990; Billah and Anthes, 1990; Kester *et al.*, 1992). Since some reports have identified calphostin-C as an efficient inducer of apoptosis (Jarvis, *et al.*, 1994; Chmura *et al.*, 1996; Freeman *et al.*, 1996), the effect of calphostin-C on internucleosomal DNA fragmentation was assessed in HL-60 and A20 cells (Figure 4 lane 4 and 9, respectively). In the present study, calphostin-C treatment for 30 min failed to elicit induction of DNA fragmentation at the dose of 1 μM . The relatively short treatment of calphostin-C might be a reason of the different effect of calphostin-C on the induction of apoptosis. However, since there is conflicting evidence showing both positive and negative roles of PKC on apoptotic process (Pongacz *et al.*, 1996; Sakakura *et al.*, 1996), the precise regulation of apoptosis by PKC hence requires further experimental investigations.

Taken together, the data in Figure 3 and Figure 4 indicate that TNF- α - and anti-Fas monoclonal antibody might exert their effects on apoptosis via PLD-dependent mechanisms. Concomitant perturbation of TNF- α - and anti-Fas monoclonal antibody-induced PLD activation and DNA internucleosomal fragmentation by calphostin-C pretreatment led us to hypothesize the possible involvement of PLD activity in the agonist-mediated apoptotic processes. Then what is the possible mode of PLD activation by the agonists? The enhanced level of diacylglycerol by TNF was observed in U937 cells (Schütze *et al.*, 1992) and this increased amount of diacylglycerol by TNF-activated PLC might activate PKC and lead to the subsequent activation of PKC-dependent PLD. Also, there is another possibility that ceramide, a primary product of sphingomyelinase, might activate phosphatidate phosphohydrolase (Gómez-Munoz *et al.*, 1995) and the enhanced activity of phosphatidate phosphohydrolase may in turn increase intracellular diacylglycerol level.

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