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Membrane modifications in human erythroleukemia K562 cells during induction of programmed cell death by transforming growth factor β1 or cisplatin

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Transforming growth factor β1 (TGFβ1) and cisplatin induce apoptosis (programmed cell death, PCD) in human erythroleukemia K562 cells in an additive manner. After PCD was induced in K562 cells, analysis of phospholipid composition, fatty acids and cholesterol content in their membranes showed a decrease in phosphatidylethanolamine and an increase in phosphatidylserine, cardiolipin and phosphatidic acid. Moreover, cisplatin but not TGFβ1 enhanced sphingomyeline levels in apoptotic cells, whereas TGFβ1 increased the amount of linoleic acid and, more remarkably, of cholesterol. The combination TGFβ1+cisplatin produced membrane changes similar to those provoked by each inducer individually. Furthermore, the specific activities of 5-lipoxygenase and cytosolic phospholipase A_2, both modulating the physical properties of membranes and membrane-lipid-mediated intracellular signalling, were enhanced by treatment with TGFβ1 or TGFβ1+cisplatin. These findings highlight the profound changes in cell membranes during the biochemical events of the apoptotic pathway.

Keywords: programmed cell death; biomembrane; cholesterol; lipoxygenase; phospholipase A_2.

The mechanism of apoptosis (programmed cell death, PCD) remains to be fully elucidated. Despite the large array of inducers and effectors of apoptosis, the morphological features of PCD are highly conserved, suggesting a common sequence of final events. Even though the most defining parameters of apoptosis are nuclear changes, another morphological hallmark of apoptosis is the cell shrinkage associated with membrane blebbing [1, 2]. Several PCD pathways require signal-transduction systems based on membrane receptors [2] and membrane-derived lipid precursors of second messengers [3]. While the role of membranes in cell injury has been long recognized [4], major modifications should be expected in membranes of cells undergoing apoptosis, which might allow their specific recognition and elimination by macrophages [5].

In the following study, we investigated the changes in membrane lipid composition during the induction of PCD in human erythroleukemia K562 cells. Transforming growth factor β1 (TGFβ1) and/or cisplatin were used to induce apoptosis. TGFβ1 induces PCD in several cell lines [6, 7] by interacting with specific receptors on the cell surface [8]. Cisplatin, however, forces cells into apoptosis [9] by forming complexes with nuclear DNA [10]. We also assayed the activities of membrane-related enzymes in apoptotic cells, namely lipoxygenase and phospholipase A_2. Several lipoxygenases are able to directly oxygenate biological membranes, introducing peroxides in the lipid bilayer [11–13]. In particular, 5-lipoxygenase plays a role in lipid-related signal transduction [14]. Phospholipase A_2 (PLA_2)catalyzes the hydrolysis of ester bonds at the *sn*-2 position of membrane phospholipids [15], the cytosolic form of PLA_2 being regulated by a receptor-mediated mechanism [15, 16].

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Abbreviations. PCD, programmed cell death; TGFβ1, transforming growth factor β1; $sPLA_2$, secretory phospholipase A_2; $cPLA_2$, cytosolic phospholipase A_2; Δ_4Ach, arachidonic acid; 5-HPΔ_4Ach, 5-hydroperoxyarachidonic acid; GC/MS, gas chromatography-mass spectrometry.

Enzymes. Lipoxygenase (EC 1.13.11.12); phospholipase A_2 (EC 3.1.1.4).

MATERIALS AND METHODS

Materials. Chemicals were of the purest analytical grade. Transforming growth factor β1 (TGFβ1) and cisplatin [*cis*-diamminedichloroplatinum(II)] were purchased from Sigma Chemical Co. Authentic standards used in chromatographic analysis were purchased from Fluka and Sigma Chemical Co. The cell-death detection ELISA kit was provided by Boehringer Mannheim.

Cell culture and treatment. Human erythroleukemia K562 cells were cultured in RPMI 1640 medium (Gibco BRL), supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL) and 2 mM L-glutamine. Mycoplasma-free cell cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2. PCD was induced by treating cell suspensions with either 1 ng/ml TGFβ1 [6] or 5 μM cisplatin [9] or a combination of both, for 48 h. Preliminary experiments showed that these conditions were the most effective in forcing K562 cells into apoptosis.

After 48 h culture, cell suspensions were washed in 2.7 mM KCl, 137 mM NaCl, 10 mM sodium phosphate, pH 7.4 (NaCl/P_i) and their numbers and viabilities were determined in a Thoma haemocytometer in the presence of Trypan blue. Cells were resuspended in NaCl/P_i at 10^7 cells/ml, and were then lysed by three cycles of rapid freezing (−80°C) and thawing (+25°C) and the protein content of cellular extracts was determined [17]. PCD was determined by the ELISA kit, based on the evaluation of DNA fragmentation by an immunoassay for histone-associated DNA fragments (mononucleosomes and oligonucleosomes) in the cell cytoplasm [18]. The extent of DNA fragmentation was also evaluated by cytofluorimetric analysis, showing that control K562 cells contain 2.5 ± 0.3% apoptotic bodies.

Lipid extraction and chromatographic analysis. Lipids were extracted, analyzed by two-dimensional HPTLC and quantified as described [13]. Cholesterol was scraped off the HPTLC plates, then its methylated and silylated derivatives were analyzed by gas chromatography-mass spectrometry (GC/MS). 50% of the cholesterol was hydrolyzed in methanol/5 M KOH (4:1, by vol.) for 30 min at 60°C under argon before GC/MS analysis. Thus, the amount of esterified cholesterol present in the lipid extracts could be estimated, by subtracting the amount of intact, non-hydrolyzed cholesterol from the amount of hydrolyzed cholesterol (esterified cholesterol = hydrolyzed cholesterol − intact cholesterol). The amount of total cholesterol in the K562 cells was quantified by subjecting known amounts of an authentic standard, derivatized as the lipid samples, to GC/MS.

Lipid extracts were prepared for GC/MS analysis as reported [13], then they were analyzed on an Interscience GC 800 Series gas chromatograph, equipped with a DB-1 fused-silica capillary column (30 m×0.32 mm). A temperature gradient of 140°C–280°C (at 4°C/min) was used, followed by 10 min isotemperature. The injection temperature was 250°C; the ion source temperature was 200°C. Mass spectra were recorded with a Fisons Instruments MD 800 MassLab spectrometer under electron impact with ionization energy of 70 eV. Peak identities were ascertained by comparing the diagnostic fragments of the mass spectra with those of authentic standards, methylated and silylated as the lipid extracts.

Assays of enzyme activity. A preliminary characterization of lipoxygenase activity in K562 cells showed that 5-lipoxygenase is the main isoenzyme present in these cells (unpublished work). 5-Lipoxygenase activity was assayed in K562 cell extracts in the presence of 1 mM ATP and 2 mM Ca^{2+} as described [19], using 80 μM arachidonic acid (Δ_4Ach) as a substrate. After 30 min incubation at 37°C, the 5-hydroperoxyarachidonic acid (5-HPΔ_4Ach) product was recovered by solid-phase extraction columns and quantified using the extinction coefficient at 237 nm, i.e. 29.5 $mM^{-1} \cdot cm^{-1}$. PLA_2 activity of cell homogenates was discriminated in secretory (low molecular mass) phospholipase A_2 ($sPLA_2$) and cytosolic (high molecular mass) phospholipase A_2 ($cPLA_2$). $sPLA_2$ activity of cell homogenates was assayed using 200 μM 1-acyl-2-[1-^{14}C] linoleoylphosphatidylethanolamine (specific activity 80400 dpm/nmol) in 0.2 M Tris/HCl, pH 8.5, in the presence of 10 mM Ca^{2+} as described [20]. For $cPLA_2$ activity measurements, cell homogenates were centrifuged at 200000×*g* for 30 min in a Beckman TL-100 Ultracentrifuge with a TLA 100.3 rotor. The resulting supernatant (cytosolic fraction) was assayed for $cPLA_2$ activity using 1-stearoyl-2-[1-^{14}C] arachidonoylphosphatidylcholine (specific activity 120000 dpm/nmol), according to [16]. Data reported in this paper are the mean of three independent determinations, with the indicated SD values. Reported figures are representative of triplicate experiments. Statistical analysis was performed by the Student's *t*-test.

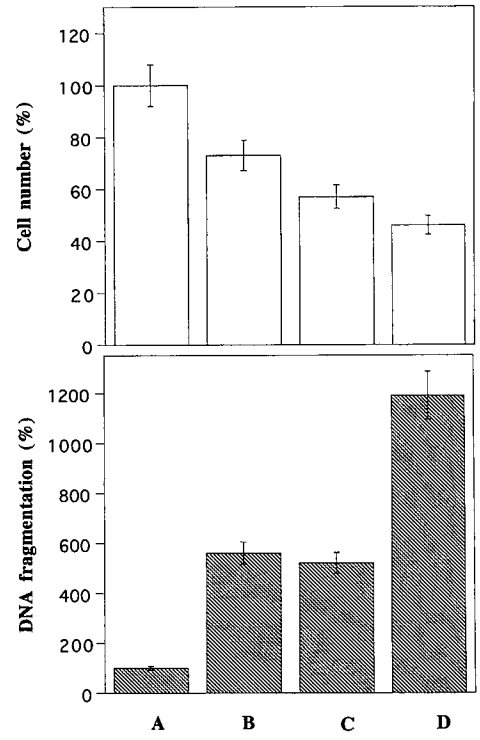

Fig. 1. Effects of PCD inducers on growth and apoptosis of K562 cells. Cell number (empty bars) and DNA fragmentation (hatched bars) of K562 cells were determined relative to the control values (arbitrarily set to 100) after treatment with TGFβ1 and/or cisplatin for 48 h. Samples: A = control cells; B = cells + TGFβ1; C = cells + cisplatin; D = cells + TGFβ1 + cisplatin.

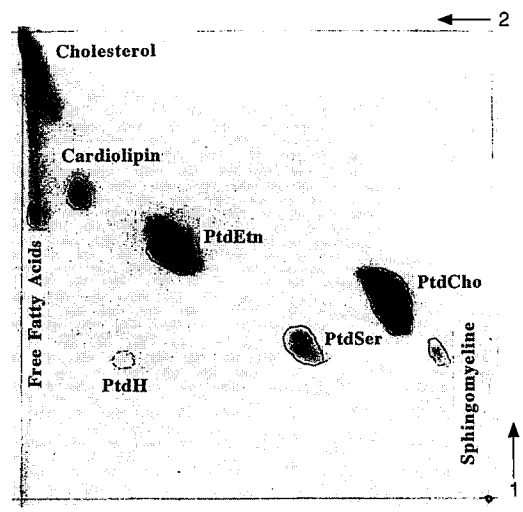

Fig. 2. Phospholipid composition of K562 cell membranes. Different lipid types of membranes of untreated K562 cells were separated by two-dimensional HPTLC as reported in [13].

RESULTS

Effects of TGFβ1 and cisplatin on K562 programmed cell death. Both TGFβ1, cisplatin and their combination induced PCD, resulting in the inhibition of cell growth (Fig. 1). The induction of apoptosis was by a 5.6-fold (TGFβ1) or 5.2-fold (cisplatin) increase of the amount of mononucleosomes and oligonucleosomes in the cell cytoplasm. The combination TGFβ1+cisplatin induced PCD in an additive manner, and yielded the highest increase in mononucleosomes and oligo-

Table 1. Quantitative analysis of K562 cell membrane lipids. Untreated cells were analyzed by two-dimensional HPTLC as shown in Fig. 2, then cholesterol was quantitated by GC/MS and phospholipids by phosphorus determination. The amount of free fatty acids was not enough for quantification. Values in parenthesis are percentages of the total lipids, 100% representing the sum of the different lipid classes (each in nmol/10^6 cells). Data are the mean of three independent experiments (± SD), each performed in duplicate.

Lipid class	Content
	nmol/10^6 cells (%)
Cholesterol	3.14 ± 0.22 (7.2)
Phosphatidylethanolamine	13.40 ± 0.67 (30.8)
Phosphatidylserine	2.90 ± 0.14 (6.7)
Phosphatidylcholine	21.80 ± 1.09 (50.2)
Sphingomyeline	1.60 ± 0.08 (3.7)
Chardiolipine	0.50 ± 0.02 (1.2)
Phosphatidic acid	0.08 ± 0.01 (0.2)

nucleosomes (11.9-fold). Cell growth was similarly arrested by TGFβ1+cisplatin to a greater degree than by either effector alone (Fig. 1).

Changes in membrane lipid composition during PCD. K562 cells were prepared for transmission electron microscopy, which clearly indicated the absence of lipid bodies or other storage lipids in this cell line (data not shown). Therefore, the lipid fraction extracted from the erythroleukemia cells represents the entire pool of cellular membranes, as confirmed by the two-dimensional HPTLC showing the typical components of eukaryotic cell membranes (Fig. 2). Quantitative analysis of the different lipid classes (Table 1) indicates that the phospholipids/cholesterol molar ratio is 13:1.

The effects of TGFβ1, cisplatin and their combination on the phospholipid composition of K562 cell membranes are summarized in Table 2. Major changes in the phospholipid composition of membranes of cells undergoing PCD were observed. In particular, a general decrease in phosphatidylethanolamine and increase in phosphatidylserine, cardiolipin and phosphatidic acid could be observed, whereas phosphatidylcholine was not significantly affected. Cisplatin, either alone or in combination with TGFβ1, yielded a significant increase in sphingomyelin levels.

The total phosphorus content of K562 cells exposed to either inducer of apoptosis was always lower than that of control cells. This indicates that the relative increase over untreated cells (Table 2) is an underestimation of the results in absolute value. Altogether, treatment with TGFβ1+cisplatin yielded the most pronounced effects on the phospholipid composition of K562 cell membranes. Major changes in the lipid-bilayer organization also occurred as total fatty acids and cholesterol, evaluated by GC/MS analysis. In particular, quantitative analysis of the GC profiles (Table 3) indicated that TGFβ1 alone or in combination with cisplatin caused a reduction of the palmitic (n-hexadecanoic) acid content (down to 70% of the control) and an increase of the linoleic (*cis*, *cis*-9, 12-octadecadienoic)acid content (up to 178% of the control value). Remarkably, the treatment with TGFβ1 increased the amount of cholesterol in cells up to 150% of that in the control, an effect which was enhanced by the combination with cisplatin (237% of the level of the untreated samples). Cisplatin alone was much less effective than TGFβ1 in inducing changes in fatty acids and cholesterol in the membranes, whereas the combination TGFβ1+cisplatin was the most effective (Table 2). A typical GC profile of membrane lipids extracted from K562 cells after treatment with TGFβ1+cisplatin (Fig. 3) clearly indicated the increase in cholesterol, whose identity was confirmed by the mass spectrum showing the expected fragmentation peaks at m/z = 329 and 368 (Fig. 3, inset). Treatment with PCD inducers had little effect on the free/esterified cholesterol ratio in K562 biomembranes (ratio 1:1), as demonstrated by coupled HPTLC-GC/MS analysis (data not shown).

Changes in lipid-related enzyme activities. 5-Lipoxygenase and PLA_2 activities were determined in K562 cell extracts after treatments with TGFβ1 and cisplatin. Both TGFβ1 and cisplatin led to an increase of 5-lipoxygenase specific activity (Table 4), which was higher upon combination of TGFβ1+cisplatin, levelling off at 150% of the control value.

As for PLA_2 activity, $sPLA_2$ was unaffected by the treatment with TGFβ1, cisplatin or TGFβ1+cisplatin, whereas $cPLA_2$ activity was enhanced by TGFβ1 up to 290% of the control value. Furthermore, cisplatin slightly reduced $cPLA_2$ activity, which

Table 2. Phospholipid composition of K562 cell membranes upon treatment with PCD inducers. The relative abundance of each phospholipid type, estimated by two-dimensional HPTLC analysis and phosphorus determination, is expressed relative to the total phospholipids. Total phosphorus content (Pi) is expressed as nmol/10^6cells. Values in parenthesis are percentages of the control, arbitrarily set to 100. Data are the mean of three independent experiments, each one performed in duplicate (SD < 10%). PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; PtdCho, phosphatidylcholine; PtdH, phosphatidic acid. The phospholipid composition of control K562 cells reported in this table is the same as that shown in Table 1, but here the percentage values do not include cholesterol.

Sample	PtdEtn	PtdSer	PtdCho	Sphingo-myelin	Cardiolipin	PtdH	Pi
Control	33.2 (100)	7.3 (100)	54.1 (100)	3.9 (100)	1.3 (100)	0.2 (100)	44.6 (100)
TGFβ1	25.0 (75)[a]	11.3 (155)[b]	57.1 (105)	4.2 (108)	2.0 (54)[b]	0.4 (100)[b]	24.2 (54)[b]
Cisplatin	13.8 (42)[b]	13.4 (184)[b]	61.3 (113)	8.9 (228)[b]	2.2 (169)[b]	0.4 (200)[b]	29.3 (66)[b]
TGFβ1 + cisplatin	20.9 (63)[b]	13.7 (188)b	56.0 (103)	6.8 (174)[b]	2.2 (169)[b]	0.4 (200)[b]	30.8 (69)[b]

[a] $P < 0.05$ compared to the control;
[b] $P < 0.01$ compared to the control.

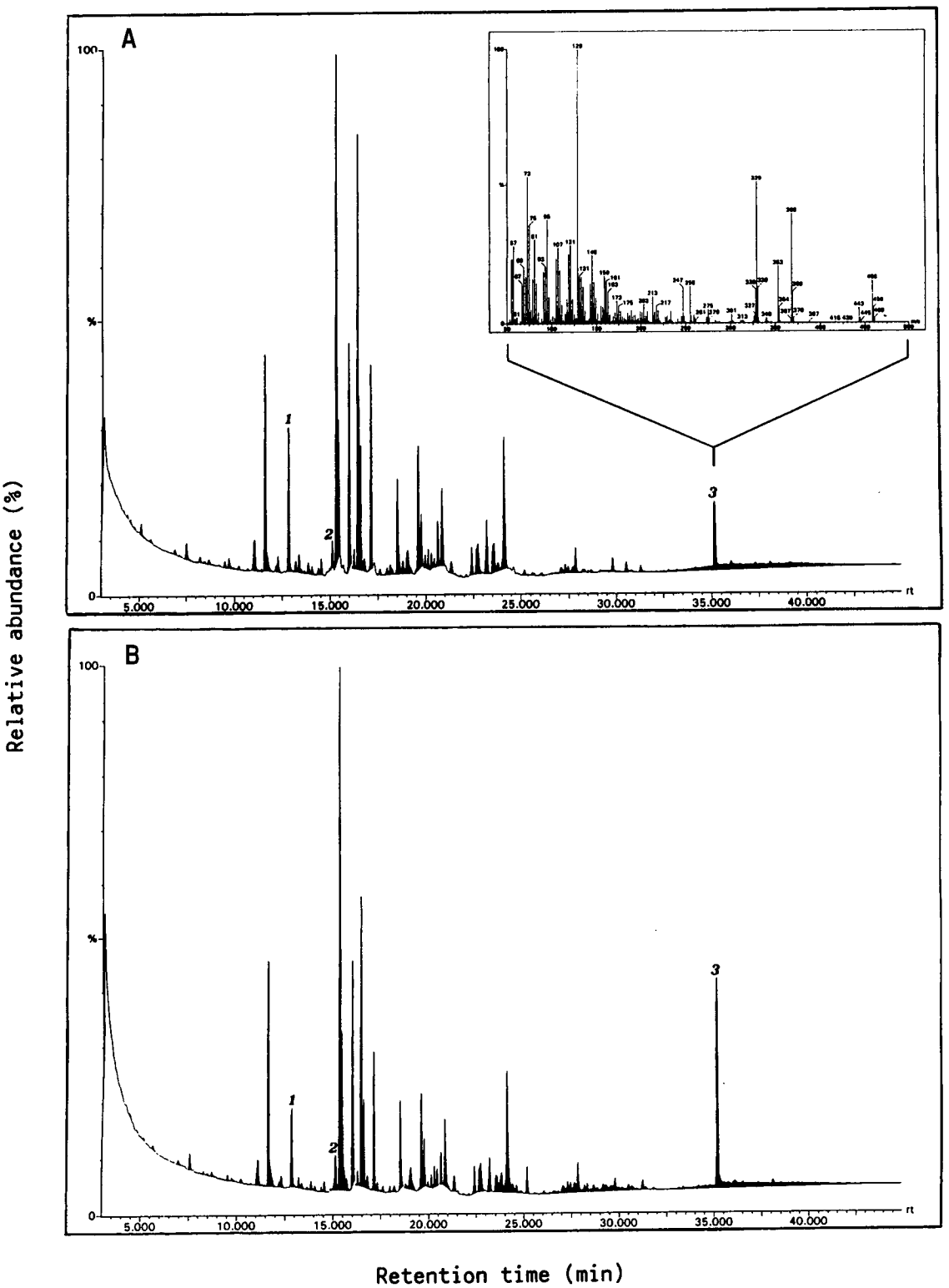

Fig. 3. Gas-chromatographic profiles of K562 cell membrane lipids. Lipids were extracted from untreated (A) or exposed to TGFβ1+cisplatin for 48 h (B) K562 cells. Extracts were methylated and converted into their trimethylsilyl ethers for GC/MS analysis. The different peaks were identified by co-injection of authentic standards, derivatized as the samples. Peak 1 = C16:0 (palmitic acid); peak 2 = C18:2 (linoleic acid); peak 3 = cholesterol. Inset. Mass spectrum of peak 3, showing the expected fragmentation pattern of cholesterol, with diagnostic peaks at *m/z* = 329 and 368.

was increased up to 248% of the control by the combination TGFβ1+cisplatin. Again, the treatment with TGFβ1+cisplatin induced the most remarkable changes in lipid-related enzyme activities (Table 4), as it did with membrane lipid composition (Tables 2 and 3).

DISCUSSION

Programmed cell death is a genetically controlled process leading to the self-elimination of cells, and plays a pivotal role in the regulation of cell number by counteracting the effect of

Table 3. Changes in fatty acids and cholesterol content of K562 cells upon treatment with PCD inducers. The amount of each component, estimated by GC/MS analysis, is expressed relative to the total lipids. Values in parenthesis are percentages of the control, arbitrarily set to 100. Data are the mean of three independent experiments, each one performed in duplicate (S. D. < 10%). C14:0, myristic (n-tetradecanoic) acid; C16:0, palmitic (n-hexadecanoic) acid; C16:1, palmitoleic (*cis*-9-hexadecenoic) acid; C18:0, stearic (n-octadecanoic) acid; C18:1, oleic (*cis*-9-octadecenoic) acid; C18:2, linoleic (*cis*,*cis*-9,12-octadecadienoic) acid; C20:4, arachidonic (all-*cis*-5,8,11,14-eicosatetraenoic) acid. Not all diagnostic peaks of the authentic C20:4 were present in the sample C20:4. Therefore, the identity of the latter remains partly uncertain.

Sample	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C20:4	Cholesterol
Control	1.9 (100)	7.0 (100)	9.2 (100)	17.2 (100)	50.7 (100)	0.9 (100)	7.1 (100)	6.0 (100)
TGFβ1	1.6 (84)[b]	5.6 (80)[a]	7.7 (84)[a]	17.3 (100)	50.0 (99)	1.5 (167)[b]	7.3 (103)	9.0 (150)[b]
Cisplatin	2.1 (110)	6.1 (87)	9.7 (105)	17.4 (101)	49.0 (97)	0.9 (100)	8.8 (124)	6.0 (100)
TGFβ1 + cisplatin	2.0 (105)	4.9 (70)[b]	9.4 (102)	15.1 (88)	46.0 (91)	1.6 (178)[b]	6.8 (96)	14.2 (237)[b]

Table 4. Enzyme activities of K562 cells treated with TGFβ1 and/or cisplatin. Specific activity of 5-lipoxygenase, secretory and cytosolic phospholipases A_2 was expressed as nmol 5-HPΔ_4Ach · min^{-1} · mgP^{-1}, pmol Lin · min^{-1} · mgP^{-1} and pmol Δ_4Ach · min^{-1} · mgP^{-1}, respectively. Activity values are also expressed relative to the control, arbitrarily set to 100 (in parenthesis). Data are the mean of three independent experiments (± SD), each performed in duplicate.

Sample	5-Lipoxygenase specific activity	Secretory PLA_2 specific activity	Cytosolic PLA_2 specific activity
Control	0.397 ± 0.040 (100)	27.64 ± 0.83 (100)	19.26 ± 0.58 (100)
TGFβ1	0.496 ± 0.055 (125)[a]	27.01 ± 2.61 (98)	56.05 ± 1.68 (291)[b]
Cisplatin	0.456 ± 0.050 (115)[a]	26.55 ± 1.59 (96)	15.35 ± 0.46 (80)[a]
TGFβ1 + cisplatin	0.596 ± 0.064 (150)[b]	27.63 ± 0.88 (100)	47.67 ± 3.07 (248)[b]

[a] $P < 0.05$ relative to the control;
[b] $P < 0.01$ relative to the control.

mitosis. Both TGFβ1 and cisplatin were effective in reducing erythroleukemia K562 cell growth and in forcing cells into the apoptotic pathway (Fig. 1). This finding extends previous observations on the effect of these compounds on neuroblastoma, glioma, leukemia and epithelial cell lines [6–9]. The best induction of PCD was achieved by the combination TGFβ1 + cisplatin, which yielded a 12-fold increase of apoptosis over the control level. PCD is characterized by a dramatic change in plasma membranes which eventually leads to the formation of apoptotic bodies [1, 2, 9]. These cellular derivatives are characterized by extensive cross-linking of membrane proteins. The modification of membrane lipids in PCD, however, is less understood. Erythroleukemia cells proved to be a suitable model for the study of membrane lipids during PCD, as they do not contain lipid bodies which contaminate membrane lipids during the extraction procedure. Thus, in this model system, typical phospholipids of mammalian membranes were identified and quantified (Fig. 2) [21]. Interestingly, TGFβ1 and cisplatin caused similar changes in the phospholipid composition of K562 cell membranes, despite the different mechanisms of PCD induction [8, 10]. In fact, both compounds decreased phosphatidylethanolamine and increased phosphatidylserine, cardiolipin and phosphatidic acid (Table 2), thus altering the membrane surface charge, polarity and fluidity [4]. In particular, the increase in phosphatidylserine supports the hypothesis of a role for this phospholipid in the recognition of apoptotic cells by macrophages [22, 23]. Moreover, the increase of cardiolipin, a marker of mitochondrial membranes, is in keeping with the modifications of the membrane properties of these organelles, observed during PCD [24]. Cisplatin, but not TGFβ1, led to a remarkable increase in sphingomyelin that might be related to a role of its component, ceramide, in the cisplatin-induced apoptotic pathway. In fact, sphingomyelin and ceramide are able to modulate protein kinases and phosphatases [4], leading to apoptosis [25]. As far as the decrease of total phosphorus in apoptotic cells is concerned, the observed decrease might be indicative of an overall loss of membranes. Indeed, the formation of blebs on the cell surface, a typical morphological mark of PCD [1, 2], might account for this observation.

As for the fatty acids and cholesterol content, treatment with TGFβ1, alone or in combination with cisplatin, decreased the level of palmitic acid, while it increased linoleic acid and, more remarkably, cholesterol (Table 3). In contrast, cisplatin alone did not significantly affect the content of palmitic acid, linoleic acid and cholesterol. The latter is an essential structural and regulatory component of cells, mostly (about 90%) located in the plasma membrane compartment of human cells [26]. The phospholipid/cholesterol ratio modulates phase behaviour, water concentration and dynamics of phospholipid bilayers [27]. In our model of apoptosis, TGFβ1 and TGFβ1 + cisplatin reduced such phospholipid/cholesterol ratios to 9:1 and 6:1, respectively, compared to 13:1 of untreated cells. The increase of cholesterol upon apoptosis might be relevant in terms of cellular regulation. In particular, the increase of cholesterol, at the expense of the amount of prenyl precursors, might coincide with the inactivation of prenylated proteins [28], thus promoting PCD. A similar regulatory meaning might be attached to the decrease in palmitic acid (Table 3), since palmitic acid is needed for the formation of palmitoylated proteins [28]. 5-Lipoxygenase converts linoleic and arachidonic acids into hydroperoxides and these compounds have been shown to induce PCD in human-immunodeficient-virus-infected T-cells [29]. The observed increase in 5-lipoxygenase activity in the course of PCD can be associated with a more general oxidative modification of cell membranes [11–13], which might contribute to the morphological changes typical of apoptosis. Furthermore, the activity of $cPLA_2$ increased upon treatment with TGFβ1 and TGFβ1 + cisplatin, but not in cisplatin-treated cells (Table 4). However, $sPLA_2$ was unaffected, regardless of the treatment used to promote PCD. Cytosolic PLA_2, unlike $sPLA_2$, is regulated through a receptor-mediated mechanism and mediates several cell signalling pathways

[15, 16]. The activation of $cPLA_2$ by TGFβ1, which acts through a specific surface receptor [8], suggests that this enzyme might mediate receptor-triggered PCD, but not apoptotic pathways induced by DNA damage (e.g. induced by cisplatin). A role for $cPLA_2$ in a signal cascade leading to PCD might be played in synergy with 5-lipoxygenase, generating lipid second messengers in response to the activation of cell surface receptors [30]. This hypothesis would agree with other physiological circumstances where 5-lipoxygenase and PLA_2 appear to act together [14].

In conclusion, the above reported results indicate that cellular membranes are a primary target of different pathways leading to the apoptotic programme.

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