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Abstract We studied changes in secondary metabolites in human neutrophils undergoing constitutive or tumour necrosis factor (TNFα) stimulated apoptosis by a combination of high-performance liquid chromatography (HPLC) and NMR spectroscopy. Our results show that in contrast to freshly isolated neutrophils, neutrophil cells aged for 20 h in vitro had marked differences in the levels of a number of endogenous metabolites including lactate, amino acids and phosphocholine (PCho). There was no change in the concentration of taurine or glutamate and the ATP/ADP ratio was not affected. Levels of glutamine and lactate actually decreased. Identical changes were also observed in neutrophils stimulated to undergo apoptosis over a shorter time period (6 h) in the presence of TNFα and the phosphatidylinositol-3-kinase inhibitor wortmannin (WM). The changes in the concentration of PCho suggest possible activation of phospholipase associated with apoptosis or a selective failure of phosphatidycholine synthesis. The increased levels of apoptosis obtained with WM+TNFα, compared to TNFα by itself, suggest a synergistic effect by these compounds. The acceleration in rate of apoptosis probably arises from suppression by WM of pathway(s) that normally delay the onset of apoptosis. Changes in PCho and other endogenous metabolites, if proven to be characteristic of apoptosis in other cell systems, may permit non-invasive quantification of apoptosis.

Key words: Apoptosis; NMR; Phosphocholine; Phospholipase C; Lactate

1. Introduction

Apoptosis is vital in many normal cellular processes, involving embryonic development, clonal selection of cells in the immune system, control of viral infection and the deletion of cancer cells [1]. Once initiated, programmed cell death is rapid and results in a cell which retains membrane integrity, but has become recognisable to and can be phagocytosed by other cells by a process that does not incite an inflammatory response [2,3]. Although there are many morphological changes, such as nuclear condensation, membrane ruffling and vacuolisation of the cytoplasm [4], which are characteristic accompaniments of apoptosis, many organelles, such as the mitochondria, remain intact [5]. These factors indicate not a generalised failure of cellular metabolism, but an active programmed pathway designed to ensure that the damaged or unwanted cell is removed intact by phagocytosis. Since the functional and morphological endpoint in many cells undergoing apoptosis is the same, it is possible that many of the final effector pathways are similar, regardless of how they may be initiated. While much progress has been made on understanding the genetic controls of apoptosis, little is understood about the effector metabolic pathways involved. Although some signalling proteins have been implicated in the control of apoptosis, such as the mitogen activated protein (MAP) kinase, sphingomyelin-ceramide and protein kinase C (PKC)[1], their precise role remains uncertain. Defining the precise metabolic changes associated with apoptosis may allow important insights into these processes and permit development of methods to monitor such changes in vivo.

Neutrophils in culture undergo rapid and constitutive apoptosis (up to 80% apoptosis within 24 h). The morphological and molecular changes associated with this process have been fully characterised [2] and the endogenous rate of apoptosis of neutrophils can be modulated by various cytokines [6,7]. Neutrophil apoptosis has also been observed in vivo, where it is considered to represent a major physiological mechanism underlying the clearance of these cells from a site of inflammation [8]. This makes the neutrophil an ideal model to determine the secondary metabolic changes associated with apoptosis. In this study, we have used a combination of NMR spectroscopy and HPLC to determine changes in secondary metabolites associated with apoptosis.

2. Materials and methods

2.1. Reagents

Wortmannin and TNFα were obtained from ICN Pharmaceutical (UK). All other chemicals were of the highest purity commercially available.

2.2. Cell culture and apoptosis

Fresh neutrophils were prepared from 200 ml citrate-treated blood, taken from healthy volunteers, by dextran sedimentation and percoll gradients [2]. Naturally apoptotic cells were prepared by incubation overnight at 10×10^6 cells/ml in 10% autologous serum/Ischoves modified Dulbecco's medium until 70% apoptotic (between 20 and 26 h). Percentage apoptosis was determined by counting cells after cytospinning and two-step Difquick staining. Apoptotic cells were defined as having more than one darkly stained pyknotic nucleus. Membrane

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Abbreviations: HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; PCA, perchloric acid; PCho, phosphocholine; PI3K, phospinosotide-3-kinase; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; TNFα, tumour necrosis factor; WM, wortmannin.

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integrity was determined by trypan blue exclusion. Cells for experiments in which apoptosis was induced or inhibited, were counted and harvested at 6 h. Because of wortmannin's labile nature, it was added initially at 100 nM and then topped up at hourly intervals with further 50 nM aliquots. TNFα was added at 12.5 ng/ml.

For NMR spectroscopy, between 60 and 200×10^6 cells were washed three times in PBS and then extracted in 1–2 ml ice-cold 12% PCA, sonicated 2×30 s, spun at $10\,000\times g$ 2 min and the pellet kept for protein determination. The pH of the supernatant was then adjusted to 7.4 with KOH, the alkaline supernatant was respun at $10\,000\times g$ for 2 min to remove any precipitated $KClO_4$ and lyophilised for storage at −20°C. The lyophilized cell extract was redissolved in 0.6 ml D_2O and 10 μl TSP and the pH readjusted to 7.4 with DCl or NaOD.

2.3. NMR and HPLC methods

NMR spectroscopy of extracts was performed at 11.7 T (JEOL) at 21°C. Fully relaxed spectra were acquired with a 45° pulse, 16 K data points, 1024 scans. An internal standard, TSP (3-trimethylsilyl-propionic-2,2,3,3-d_4 acid, Aldrich Chemical Co, UK), was added as a chemical shift and absolute quantitation reference. Signal assignment was carried out as described by Sze and Jardetzky [9], using a combination of chemical shift information, multi-dimensional NMR and samples spiking with pure compounds. Amino acid content of cell extracts was measured by HPLC [10]. Essential amino acids were analysed by derivatisation with *o*-phthaldialdehyde (OPA) and the concentrations were calculated from external standards. A LiChroGraph HPLC system (Merck-Hitachi, Darmstadt, Germany) equipped with a LiChroGraph 100HP-18 separation column (5 mm; 4 mm i.d., 25 cm length) was used. The OPA derivatives were detected with an UV/VIS fluorometer (LiChroGraph F1050), characterised by the following wavelengths: excitation, 340 nm; emission, 450 nm. Aliquots taken from NMR samples were diluted 10–100-fold with ultrapure water, and 150–250 ml sample was mixed with 350 ml reagent containing 0.8 mg/ml OPA (Sigma). The compounds were allowed to react for 3 min. Then 150 ml mixture was injected onto the column. Elution was achieved at 40°C with a three-step linear gradient of methanol in phosphate buffer (50 mmol/l in ultrapure water, pH 6) starting at 20% methanol, increasing to 35% methanol in 5 min, then decreasing to 30% methanol in 30 min and finishing at 35 min with 20% methanol.

Protein content was determined using the BioRad reagent kit (BioRad, Watford, Herts, UK) and BSA standards as reference proteins.

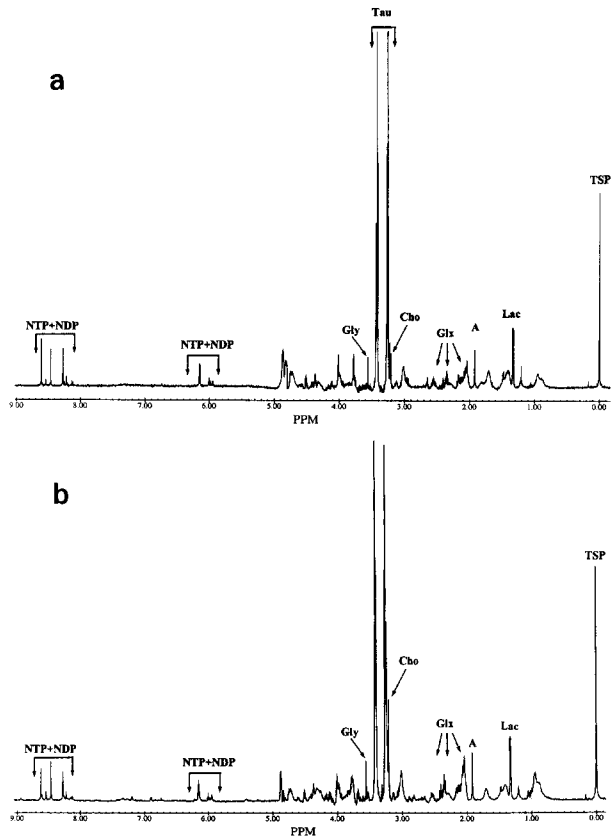

Fig. 1. High resolution ^{1}H-NMR spectra from perchloric acid extracts on (a) non-apoptotic and (b) apoptotic neutrophils. Resonances from a variety of metabolites can be readily observed including lactate (Lac), acetate (A), glutamine and glutamate (Glx), choline, phosphocholine and glycerophosphocholine (Cho), taurine (Tau), glycine (Gly), nucleotide tri/diphosphates (NTP+NDP).

3. Results and discussion

In this study we have shown, by a combination of NMR spectroscopy and HPLC, significant differences in the metabolic profile of extracts from non-apoptotic and apoptotic neutrophils. These include alterations in membrane-associated components, amino acids and intermediary metabolites. Typical ^{1}H-NMR spectra from perchloric acid extracts of non-apoptotic neutrophils are shown in Fig. 1a. Resonances from a variety of metabolites could be readily observed including lactate, acetate, amino acids, taurine, glycerophosphocholine, PCho, choline and nucleotide phosphates. Spectra from apoptotic neutrophils (Fig. 1b) showed that the concentration of some of these metabolites were significantly altered. PCho was greatly increased (Fig. 2), while the concentration of glutamine and lactate showed a marked decrease. Increased levels of tyrosine and other aromatic amino acids were also observed. The concentration of the other metabolites analysed, including taurine, glutamate and the ATP/ADP ratio, showed no significant differences between the apoptotic and non-apoptotic cells. A summary of the results is shown in Table 1.

Table 1
Metabolite concentration in fresh and apoptotic neutrophil extracts assessed by NMR and HPLC (nmol/mg protein)

	Non-Apoptotic	Apoptotic[a]
Phosphocholine	4.0 ± 2.3	11.1 ± 3.7[b]
Lactate	46.2 ± 14	20.6 ± 9.0[b]
Glutamine	58.8 ± 27	26.5 ± 12[c]
Tyrosine	0.7 ± 0.2	1.6 ± 0.3[c]
Serine	10.8 ± 3.9	18.7 ± 6.9[c]
Choline	1.5 ± 1.2	3.4 ± 1.6
Arginine	21.1 ± 13	37.3 ± 21
Glycine	28.6 ± 7.3	21.4 ± 10
Taurine	198± 25	186± 33
H-taurine	3.5 ± 1.5	1.9 ± 1.1
Alanine	17.7 ± 8.2	14.8 ± 6.4
Aspartate	39.7 ± 14	46.6 ± 27
Glutamate	62.1 ± 16	65.5 ± 25

[a]Cell underwent constitutive apoptosis in culture (see Section 2).
[b]$p < 0.01$ and [c]$p < 0.05$.

The ATP/ADP ratio of apoptotic neutrophil extracts (1.08 ± 0.47) as determined by NMR was similar to those of non-apoptotic cell extracts (1.31 ± 0.28), implying that they were still capable of energy production. This supports observations by Wallen et al. [11] that apoptosis may be energy dependent. Previously published work suggests that mitochondrial function may be compromised during apoptosis, possibly affecting oxidative phosphorylation [1,12]. If this were correct, cells undergoing apoptosis would rely on increased anaerobic respiration to maintain normal energy levels. This would in

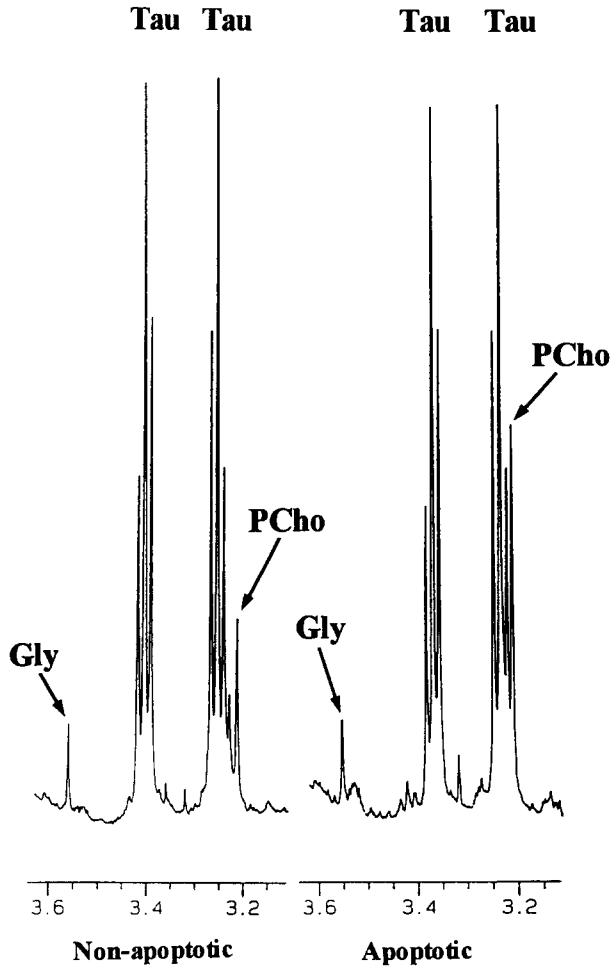

Fig. 2. High resolution ^{1}H-NMR spectra (expansion: 3.00–3.70 ppm) from perchloric acid extracts of non-apoptotic and apoptotic neutrophils. Resonance assignment: Gly, glycine; Tau, taurine; PCho, phosphocholine. There was a marked increase in the concentration of PCho in apoptotic cells compared to controls.

turn lead to increased lactate production. In this study however, we have observed a significant fall in lactate concentration in apoptotic cells (control: 46.2 ± 14 nmol/mg protein vs apoptotic: 20.6 ± 9 nmol/mg protein; $p < 0.01$), arising from either a decrease in lactate output and/or an increase in lactate metabolism. Maretzek et al. [13] have similarly shown an apoptosis-related decrease in lactate concentration, together with reduction in glucose consumption and glycogen synthesis in T47D spheroids. This suggests that decreased lactate levels may be a common factor in apoptotic cells and may further differentiate them from necrotic cells. The stage at which the lactate begins to decline during apoptosis remains to be determined, but once initiated, the energy for apoptosis may be derived from alternative fuel molecules such as lactate and glutamine. Indeed, the redox potential of the cell can affect gene transcription, as well as altering the response to certain signalling molecules possibly through NF-κB [14]. It is therefore of interest that TNFα can activate the transcriptional regulator NF-κB, possibly via the generation of reactive oxygen intermediates (ROI) [14] and that apoptosis can be induced independently by oxidative damage [15]. Agents which increase levels of glutathione can inhibit the formation of ROI and suppress apoptosis and NF-κB activity [14,15]. Hence, the redox status of the cell and apoptosis appear to be intimately linked and changes in lactate and other compounds, such as glutamine, may be indicative of energetic changes in the apoptotic process.

The increase in the aromatic amino acid tyrosine was unexpected (Table 1), but could be due to a number of reasons, including protein degradation, a selective Tyr dephosphorylation of proteins or inward leakage through the plasma membrane from the support medium. Alteration in the cell membrane associated with apoptosis could lead to an increase in porosity [16]. However, the similarity in levels of taurine and many aliphatic amino acids between apoptotic and non-apoptotic cells and the ability of apoptotic neutrophils to exclude trypan blue are consistent with an intact membrane. Therefore, the increase in aromatic amino acids might reflect the cleavage of specific aromatic rich peptides, such as membrane targeting sequences, by proteases which are known to be involved in apoptosis [17].

The increase in the concentration of PCho in apoptotic neutrophils is of special interest since this molecule is generated following the activation of a phosphatidylcholine-specific phospholipase D (PLD) or phospholipase C (PLC). These pathways have been linked to the induction of apoptosis in other cell models. The increased PCho concentration in apoptotic neutrophil extracts is unlikely to derive from new membrane synthesis because of the differentiated state of the neutrophil. However, in many cells, phosphatidylcholine (PtdCho) hydrolysis is elicited by a variety of growth factors, cytokines and neuropeptides hormones [18] and leads to an increase in diacylglycerol (DAG), and phosphocholine (PLC-mediated hydrolysis) or phosphatidic acid and choline (PLD-mediated hydrolysis) [19]. While neutrophils do not possess PtdCho active PLC [20], the PLD generated phosphatidic acid can be readily converted to DAG by a phosphatidate phosphohydrolase. DAG mediated activation of PKC has been implicated in growth regulation and protection from apoptosis [21]. In the neutrophil, activation of phospholipase D [22] or PKC results in upregulation of its anti-bacterial capabilities and suppression of apoptosis [6]. To ascertain whether increased PLD-mediated PtdCho hydrolysis could be implicated in the increased levels of PCho detected, neutrophil incubations were performed in the presence of Wortmannin (WM) in addition to TNFα which as well as inhibiting P13K activity also inhibits PLD activity in these cells [23,24].

Wortmannin by itself had very little effect on apoptotic rates at 6 h (WM: 2.6 ± 1.2 vs control: 1.6 ± 0.36%, $n = 3$, $p < 0.5$). TNFα alone gave a significant increase in the rate of apoptosis (TNFα: 7.4 ± 3.9 vs control: 1.7 ± 0.9%, $n = 3$, $p < 0.005$). When wortmannin was combined with TNFα, a large increase in apoptosis at 6 h was observed (TNFα+WM: 40.6 ± 3.4% vs control: 1.6 ± 0.36%, $n = 3$, $p < 0.005$). This increased rate may possibly be due to a removal of a protective effect of phosphoinositol 3,4,5-triphosphate (PIP3) on apoptosis [25]. Under these conditions, in which PLD activity is significantly attenuated [22], preliminary ^{1}H-NMR spectra still show an increased PCho signal (6.30 ± 2.1 nmol/mg protein) in TNFα+WM treated cells at 6 h, compared to control or WM treated cells alone. This would indicate that the PCho signals are unlikely to be linked to the activation of a PtdCho-directed phospholipase.

In many cells, TNFα is thought to induce apoptosis by the

release of ceramide through the activation of sphingomyelinase [21]. This reaction also releases PCho, and hence may be an alternative source for the increase in this compound. This hypothesis is supported by the observation that ceramide itself can induce apoptosis in many cells as well as inhibiting DNA synthesis [26,27]. Although ceramide does not induce apoptosis in neutrophils, its catabolite, sphingosine, is a potent apoptotic agent [21]. One of the ways in which sphingosine is thought to promote apoptosis is by inhibiting PKC activity [27]. Therefore, in the neutrophil, activation of the sphingomyelinase pathway as an initiating event, or as a consequence of apoptosis, is certainly a potential route for the generation of PCho.

In conclusion, we have shown that it is possible to study changes in secondary metabolism associated with apoptosis. In our study, the inability of wortmannin to attenuate the increase in PCho observed with both constitutive and TNFα induced apoptosis suggests a source other than stimulation of PtdCho-directed PLD activity, possibly via the breakdown of ceramide [22]. Furthermore, we have seen changes in lactate and other metabolites. When taken together, these changes may uniquely characterise cells undergoing apoptosis. If similar findings are apparent in other apoptotic cells, then it may be possible to define a metabolite fingerprint characteristic of this process, akin to the defining morphological features of apoptosis. Such observations might make it possible to use NMR for non-invasive quantification of apoptosis in cell culture and possibly in pathology, e.g. during chemotherapy of tumour masses.

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