Early changes in glucose and phospholipid metabolism following apoptosis induction by IFN-γ/TNF-α in HT-29 cells

Norbert W. Lutz[a,*], Margaret E. Tome[b], Patrick J. Cozzone[a]

[a] *Centre de Résonance Magnétique Biologique et Médicale, Faculté de Médecine, Marseille, France*
[b] *Department of Pathology, University of Arizona, Tucson, AZ, USA*

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Abstract The effects of apoptosis induction on glucose and phospholipid metabolite levels in cancer were studied using human colon adenocarcinoma cells (HT-29). Apoptosis was induced by co-incubation with 200 U/ml tumor necrosis factor (TNF)-α for 4, 8 or 15 h, after sensitization with 500 U/ml interferon (IFN)-γ for 7 h. Perchloric acid extracts were analyzed by ^{1}H and ^{31}P nuclear magnetic resonance (NMR) spectroscopy. Significantly increased lactate and NTP (all nucleoside 5'-triphosphates) signals were detected 4 h after apoptosis-inducing IFN-γ/TNF-α treatment, but not in cells which were TNF-α-treated *without* IFN-γ preincubation. Simultaneous lactate and NTP changes, if confirmed in vivo, may serve as early, non-invasive markers of treatment response in some tumors.

Key words: Apoptosis; Cytokine treatment; HT-29 cells; Metabolism; Nuclear magnetic resonance spectroscopy

1. Introduction

^{31}P and ^{1}H nuclear magnetic resonance (NMR) spectroscopy has recently been used to monitor changes in glycolysis, cellular energetics and lipid metabolism occurring in cultured tumor cells and in solid tumors following induction of apoptosis by anticancer drugs (for recent reviews see Evelhoch et al. [1] and Hakumäki et al. [2,3]). Besides the elucidation of the biochemical mechanisms underlying, accompanying and/or modifying apoptotic cell death, the identification of 'metabolic markers' associated with the induction of apoptotic cell death has been a major goal of these studies. If sufficiently sensitive, such surrogate markers may prove to be useful for monitoring the progress of cancer treatment, potentially by in vivo NMR spectroscopy. Such a method may be particularly helpful if it allows for *early* prediction of successful therapy in patients, i.e. ideally *before* the appearance of morphological signs of cell death. In this perspective, we analyzed the temporal evolution of glucose and phospholipid metabolism in a time course study of interferon (IFN)-γ-sensitized [4–7] HT-29 cells following apoptosis induction by tumor necrosis factor (TNF)-α treatment.

The human colon adenocarcinoma cell line, HT-29, is a relevant model for human colon cancers expressing the c-*myc* proto-oncogene [8,9]. Numerous studies have demonstrated that treatment with TNF-α or TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis in HT-29 and other colon cancer cells through a ligand receptor-mediated pathway [4,5,10–16]. Furthermore, c-Myc-expressing cancer cells have been shown to exhibit high activities of lactate dehydrogenase (LDH) [17] and of other glycolytic enzymes [18]. Thus, we primarily focused on the possible glycolytic effects of apoptosis-inducing IFN-γ/TNF-α treatment in HT-29 cells. The results obtained here are intended to guide future in vivo studies of the potential of NMR-visible metabolites as diagnostic markers of apoptotic cell death in tumors.

2. Materials and methods

2.1. Cell culture

HT-29 cells were cultured and extracted as described elsewhere [19]. Briefly, cells were grown in Dulbecco's modified Eagle's medium (DMEM) (3.15 g/l glucose) supplemented with 10% fetal calf serum. All cell samples were identically subcultured from the same stock suspension; consequently, all cells were of the same passage number and were subjected to the same feeding rhythm before extraction. The treatment protocol is summarized in Fig. 1. Cells were treated with 200 U/ml TNF-α for 4, 8, 15 or 24 h prior to extraction. The cells treated for 4, 8 or 15 h had been pretreated for 7 h with 500 U/ml IFN-γ before TNF-α addition to the medium. IFN-γ alone kills cells only after 2 days of incubation [6]; in our experiments where IFN-γ was used for significantly shorter incubation periods, this cytokine essentially serves as a sensitizer by stimulating the expression of TNF-α receptors in HT-29 cells, thus enabling them to bind to the ligand [4–7].

Generally, ca. $2–3\times10^{8}$ cells were extracted with perchloric acid and prepared for NMR spectroscopy as described previously [19], except that 10–30 mM 1,2-diaminocyclohexane tetraacetic acid (CDTA) was used instead of ethylenediamine tetraacetic acid (EDTA). Following the 8-h and 15-h combination treatments, an estimated 5–10% and 25% of the cells, respectively, were found floating in the medium, or were only loosely attached to the bottom of the flask. These cells were harvested separately from the attached cells, washed twice in phosphate-free buffer and extracted. The experiments reported here were preceded by a careful quality control study (using 10 samples of untreated HT-29 cells) which had the goal of optimizing the cell culture and extraction protocol to obtain highest reproducibility for metabolite concentrations. Results indicated that, for example, a reproducibility range of ±4% relative to the mean value could be obtained for phosphocholine (PC) levels based on ^{31}P NMR spectra (data not shown).

*Corresponding author. Present address: Arizona Cancer Center, University of Arizona, P.O. Box 245024, Tucson, AZ 85724, USA. Fax: (1)-520-621 8522.
E-mail address: nwlutz@u.arizona.edu (N.W. Lutz).

Abbreviations: Fru-1,6-DP, fructose-1,6-diphosphate; GPC, *sn*-glycero-(3)-phosphocholine; Gro-3-P, glycerol-3-phosphate; IFN, interferon; LDH, lactate dehydrogenase (EC 1.1.1.27); NTP, all nucleoside 5'-triphosphates; PC, phosphocholine; TNF, tumor necrosis factor; tot. P, total ^{31}P signal integral

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2.2. ^{31}P and ^{1}H NMR spectroscopy

^{1}H NMR spectra at 400 MHz and proton-decoupled ^{31}P NMR spectra at 162 MHz were obtained at 4°C on a 9.4 T AM400-WB FT-NMR spectrometer (Bruker, Rheinstetten, Germany) using a quattro nucleus probe (QNP) for 5-mm sample tubes; acquisition and processing parameters were chosen as described elsewhere [19]. Relative metabolite signal intensities are reported here as percentages of the total integral over the entire spectrum, i.e. as percentage of total ^{1}H signal integral (%tot. H) or as percentage of total ^{31}P signal integral (%tot. P), whereby the residual water (HDO) region was not included in the integration of the total ^{1}H NMR spectrum. Percentage values rather than more conventional measures of metabolite concentration were used in view of potential in vivo applications of this NMR method, where reliable *absolute* concentrations would be rather difficult to obtain and therefore are rarely used, particularly in clinical applications. PC and *sn*-glycero-(3)-phosphocholine (GPC) values were obtained from both ^{1}H and ^{31}P NMR spectra. The results from these two methods were consistent, apart from some variation in statistical significance (P values). The numerical PC and GPC results presented below are based on ^{31}P NMR spectra unless specified otherwise.

2.3. Histological confirmation of apoptosis

Apoptosis induction in HT-29 and other colon carcinoma cells following TNF-α treatment is well documented [4,5,11,12,14–16]. To confirm the occurrence of apoptotic cell death for our treatment protocol, HT-29 cells were cultured as described above, pretreated for 7 h with 500 U/ml IFN-γ and treated for 24 h with 200 U/ml TNF-α added. Floating and loosely attached cells were separated from the attached cells for microscopic analysis. Attached cells were trypsinized, and trypsin was inactivated by adding an equal amount of serum-containing medium to the cell suspension. Cytospin slides for light microscopy were prepared, stained and scored as previously described [20]. Cells in (late) apoptosis were identified on the slides using the following characteristics: condensed chromatin, cell shrinkage, increased cytoplasmic vacuolization, and apoptotic body formation. At least 200 cells were scored for each sample and apoptotic frequency reported as a percentage of total cells scored.

2.4. Statistical evaluation

The software package StatView (version 5.0.1) from the SAS Institute, Cary, NC, USA, was used for statistical data analysis (algorithms described in StatView Reference book, SAS, 1999). Simple linear regression analysis was used to characterize linear changes in glycerol-3-phosphate (Gro-3-P) or lactate concentration as a function of the time after the onset of TNF-α treatment. The parameters obtained were (i) correlation coefficient (R^2), (ii) regression coefficient b (slope) ± S.E.M. of b, and (iii) probability level (P value for an F-test that $b = 0$). Relative metabolite levels for sample groups are reported as percentage ± S.D. One-way analysis of variance (ANOVA) with Fisher's PLSD (protected least significant difference) post hoc test for multiple comparisons was used to determine the significance ($P < 0.05$) of differences in relative metabolite concentrations between treatment groups. In this pilot study carried out with a limited number of samples, each group consisted of two samples, except for the control group ($n = 3$). The histological analysis of apoptotic cell death was performed with $n = 3$ (triplicate samples).

3. Results and discussion

3.1. Effects of IFN-γ/TNF-α treatment on the levels of glycolytic metabolites

Extracts prepared from cells that were floating in the medium at the time of harvest (up to 25% of total cells) did not give rise to substantial NMR signals. This suggests that a major proportion of the detached cells had perforated membranes so that metabolites of low molecular weight had been released into the medium before harvest or had been taken up by the buffer during the washing process. This is consistent with the observation that cell shedding is a relatively early event in apoptosis of HT-29 cells [21], and that most of the detached colon cells in the medium are apoptotic [22].

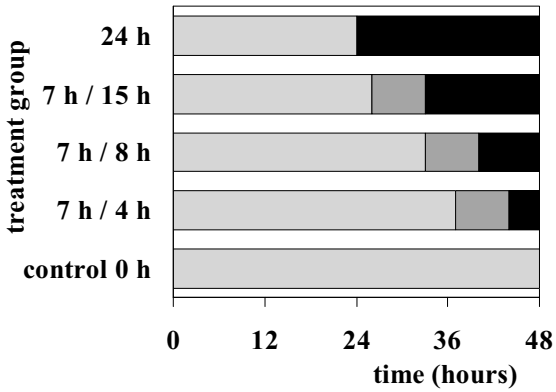

Fig. 1. Schematic representation of the treatment protocol for HT-29 cells. Cells were treated with 200 U/ml TNF-α only (for 24 h); with a combination of 500 U/ml IFN-α (for 7 h) and 200 U/ml TNF-α (for 4, 8 or 15 h); or were left untreated (controls, 0 h). Light gray: incubation without drugs; dark gray: pretreatment with IFN-γ; black: TNF-α treatment in presence of IFN-γ. For all cells, the last medium change before extraction took place at $t = 0$ of the time axis. All cells were extracted at $t = 48$ h.

The analysis by ^{31}P NMR spectroscopy of phosphorylated glycolytic intermediates from intact attached cells showed that the relative levels of Gro-3-P (Figs. 2 and 3A increased as a function of time after the onset of IFN-γ/TNF-α treatment (linear regression $R^2 = 0.650$, slope $b = 0.056 \pm 0.015$%/h, $P = 0.0087$). However, the rise in Gro-3-P was statistically significant only after 15 h of IFN-γ/TNF-α treatment, at which point Gro-3-P was twice as high as in untreated cells (0 h). In contrast, the relative level of fructose-1,6-diphosphate (Fru-1,6-DP) increased significantly already at 4 h after the beginning of IFN-γ/TNF-α treatment (by a factor of ca. 2.0 vs. controls), and remained at this elevated level (Fig. 3B). Treatment with TNF-α alone (24 h) did *not* result in significant Gro-3-P or Fru-1,6-DP changes, even after 24 h of treatment. The latter observation fits with the virtual absence of detached cells after 24 h of TNF-α treatment *without* preceding sensitization by IFN-γ pretreatment.

The mechanism leading to Fru-1,6-DP accumulation following apoptosis induction is still under investigation. *Drastically* increased Fru-1,6-DP levels and a significant adenosine triphosphate (ATP) *drop* have been reported for several cell lines following treatment with apoptosis-inducing drugs [1,23–25]. In these cases, the accumulation of Fru-1,6-DP is thought to be a consequence of the inhibition of glyceraldehyde-3-phosphate dehydrogenase following NAD depletion. According to this model, NAD is consumed by the activation of the enzyme poly(ADP-ribose) polymerase (PARP), a DNA repair enzyme activated by DNA damage. However, this mechanism is less likely to occur in IFN-γ/TNF-α-treated HT-29 cells since the latter show *increased* NTP (all nucleoside 5'-triphosphates) levels (see Section 3.2) and exhibit only *modest* increases in Fru-1,6-DP levels. However, an increased production of the glycolytic intermediates, Gro-3-P and Fru-1,6-DP, in conjunction with increased lactate, alanine and NTP generation may be explained by increased aerobic glycolysis (see below).

Lac and Ala (Fig. 2) are two end products of the glycolytic pathway [26,27] whose relative concentrations were found to be changed following IFN-γ/TNF-α treatment of HT-29 cells (Fig. 3C and D). Lac increased roughly linearly with TNF-α

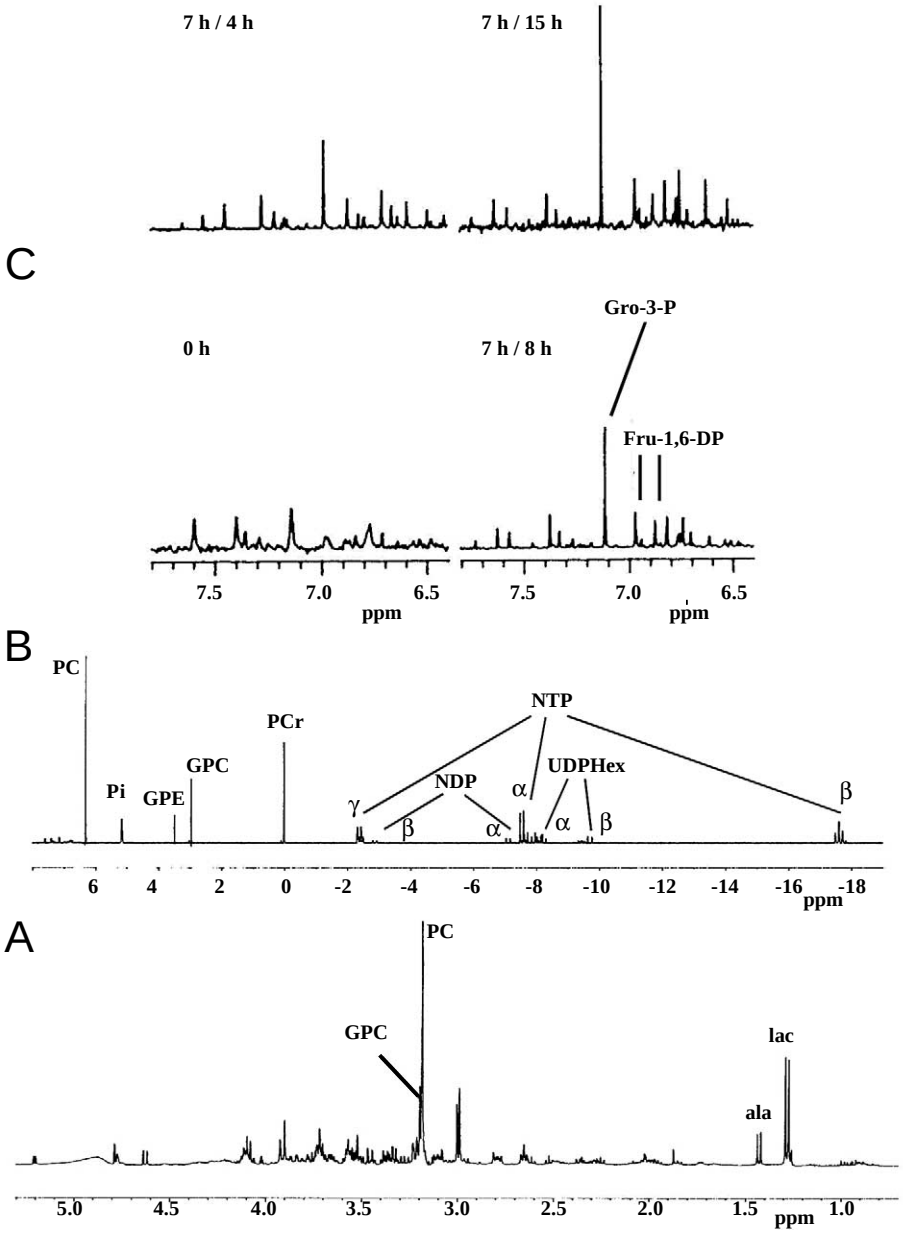

Fig. 2. A: Typical ^{1}H NMR spectrum of a perchloric acid extract of untreated HT-29 cells (control). B: Typical ^{31}P NMR spectrum of a perchloric acid extract of untreated HT-29 cells (control). C: Low-field regions of representative ^{31}P NMR spectra for untreated (0 h) HT-29 cells, and IFN-γ-sensitized HT-29 cells treated for 4, 8 or 15 h with TNF-α (see protocol shown in Fig. 1).

treatment time ($R^2 = 0.879$, slope $b = 0.221 \pm 0.031\%$/h, $P = 0.0002$) and was significantly augmented vs. control values as early as 4 h after the onset of treatment. Ala levels were somewhat increased after 4 h of treatment when compared to control values ($P = 0.052$); however, for longer treatment periods alanine levels returned to near-control values (Fig. 3D). Neither Lac nor Ala values were significantly changed for HT-29 cells treated for 24 h with TNF-α alone when compared with untreated controls, in conformity with the behavior of Gro-3-P and Fru-1,6-DP described above.

3.2. *Effects of IFN-γ/TNF-α treatment on energy metabolite levels*

The phosphocreatine signal integral ranged between 6.8 and 10.8% of total phosphate for all treatment groups; no significant differences were detected. Levels of NTP (predominantly ATP) were significantly increased ($P = 0.014$) for HT-29 cells treated with IFN-γ/TNF-α (for the *pooled* 4-h, 8-h and 15-h treatment groups designated 'average (4–15 h)' in Fig. 3E) compared to untreated controls. Moreover, nucleoside diphosphate (NDP) in the pooled 4-h, 8-h and 15-h treatment groups was significantly reduced compared to the control group (data not shown). The NTP levels of the cells treated with TNF-α alone were not significantly different from those of controls (data not shown). The relative levels of inorganic phosphate (P_i) ranged between 4.8 and 10.2% of tot. P, but no inter-group variations were observed except for a tendency towards increased P_i values for cells treated with TNF-α only. The increase in NTP levels detected in attached HT-29 cells following the onset of IFN-γ/TNF-α treatment may re-

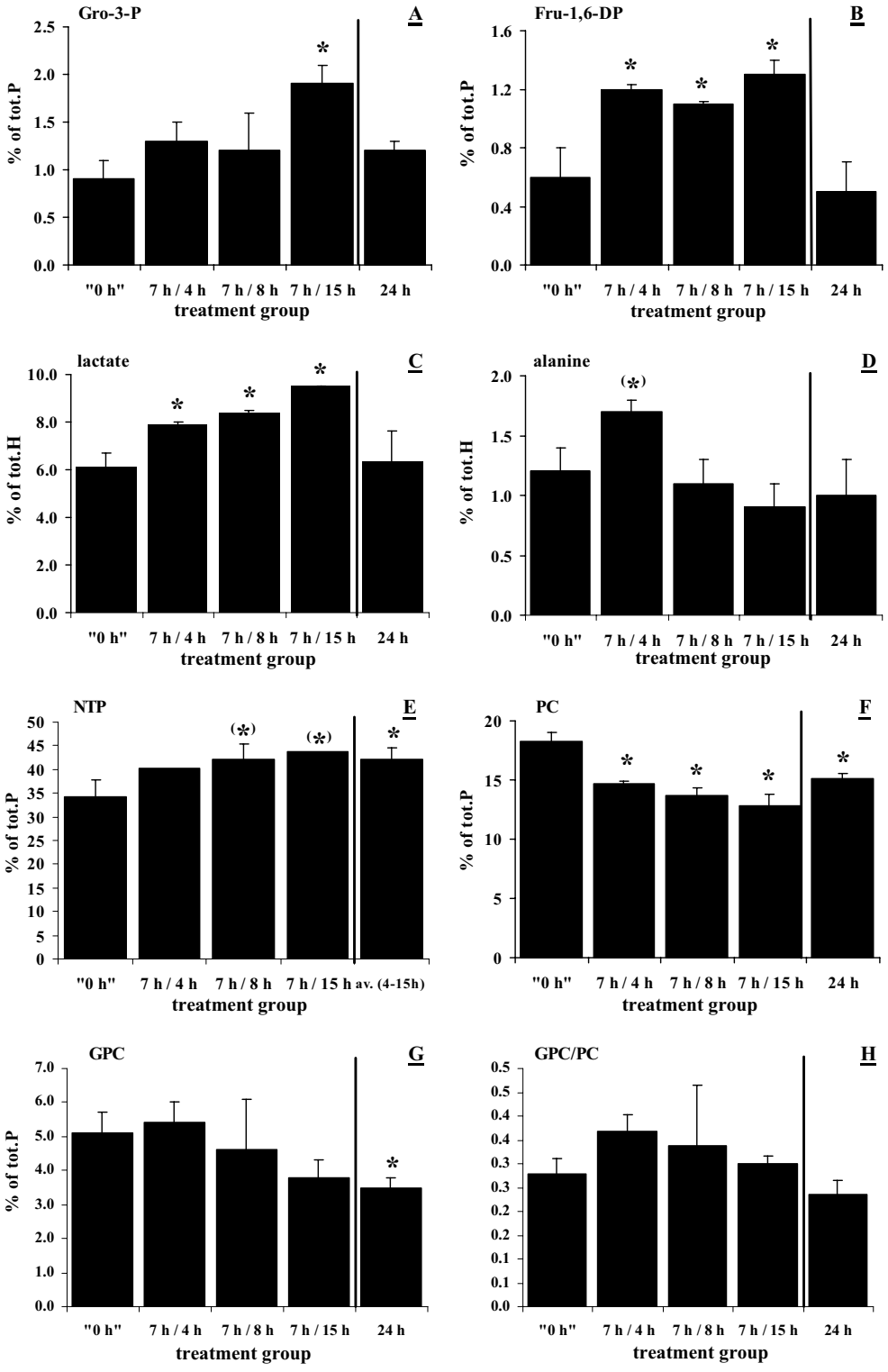

Fig. 3. Relative metabolite levels (±S.D.) for HT-29 cells treated according to the protocol shown in Fig. 1. Asterisks, *, indicate statistical significance ($P < 0.05$). Asterisks enclosed in parentheses, (*), indicate borderline significance ($0.05 \leq P < 0.07$).

flect the increased production of high-energy metabolites to support the ATP needs during early apoptosis. This phenomenon has been observed in some apoptotic cells [1,28], but not in others [24,29]. The increase in Lac levels together with enhanced NTP may be understood by assuming that a substantial part of the NTP required is generated through increased aerobic glycolysis. This hypothesis needs to be tested by more detailed experiments, but would be supported by the above-mentioned parallel increase observed for other glycolytic intermediates such as Gro-3-P and Fru-1,6-DP.

In contrast to HT-29 cells, MCF7 cells which do *not* express c-Myc under normal growth conditions [30], do *not* exhibit a

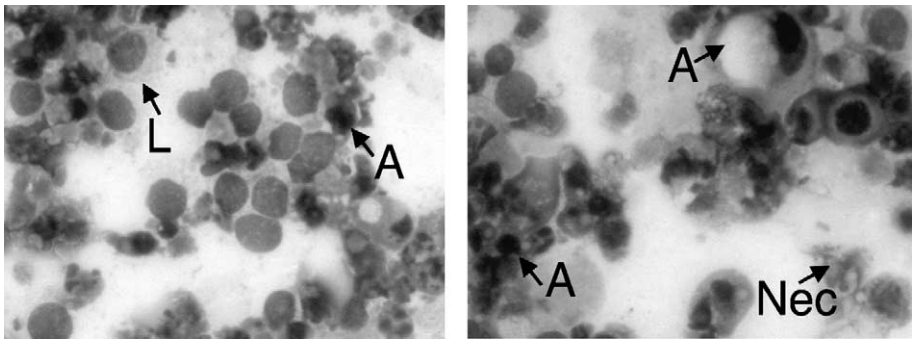

Fig. 4. Micrographs of HT-29 cells pretreated for 7 h with 500 U/ml IFN-γ, and subsequently treated for 24 h with 200 U/ml TNF-α (right), and of untreated control cells (left). L: live cells (non-apoptotic, or in early apoptosis); A: cells in late apoptosis; Nec: cells in primary or secondary necrosis. The cells shown were *detached* from the flask bottom at the time of harvest.

significant NTP increase following apoptosis induction by TNF-α treatment, and the lactate production rate appears to remain stable (Bogin et al. [29]). Further studies will reveal whether the observed Lac increase after apoptosis induction in HT-29 cells is indeed related to a high LDH expression due to c-Myc expression (see Section 1).

3.3. Effects of IFN-γ/TNF-α treatment on phospholipid metabolites

PC is an important intermediate in the synthesis and degradation of the most abundant membrane phospholipid, phosphatidylcholine. The relative PC level decreased significantly after the 4-h IFN-γ/TNF-α treatment ($P = 0.001$; Fig. 3F). Further small decreases were observed for 8- and 15-h incubations. Treatment with TNF-α alone for 24 h also reduced PC, albeit to a lesser extent than the combination treatment. In contrast to PC, the levels of GPC did not decrease after 4-h IFN-γ/TNF-α treatment, but did decrease slightly with longer incubations (statistical significance was reached only for 15-h values obtained from ^{1}H NMR spectra, where $P = 0.013$). Following a 24-h treatment with TNF-α only, GPC was reduced to the level reached by IFN-γ/TNF-α-treated HT-29 cells after 15 h. Decreased GPC levels for both IFN-γ-sensitized and non-sensitized HT-29 cells after TNF-α co-incubation may be a (somewhat delayed) consequence of decreased phosphatidylcholine turnover caused by the initial drop in cytosolic PC levels.

A decrease in the phosphatidylcholine metabolite, PC, is a frequently observed reaction of tissue to chemotherapeutic agents, and has been proposed to serve as an early indicator of successful cancer chemotherapy [31]. However, the PC decrease observed for IFN-γ/TNF-α-treated HT-29 cells is unlikely to be a specific marker of cell death since HT-29 cells treated with TNF-α *alone* also showed somewhat reduced PC levels compared to untreated controls. This observation suggests that the modest PC decrease observed for non-sensitized cells may be a metabolic effect of non-lethal TNF-α treatment. Since the GPC/PC ratio is frequently used as an indicator of changes in choline homeostasis [32–34], and often increases in cells undergoing stress or treated with cytotoxic drugs, we plotted GPC/PC vs. the duration of IFN-γ/TNF-α treatment (Fig. 3H). Although there was a trend towards a transient GPC/PC increase at 4 and 8 h after the onset of treatment, these changes did not reach statistical significance for ^{31}P nor for ^{1}H NMR values.

3.4. Histological confirmation of apoptotic death following IFN-γ/TNF-α treatment

Apoptosis induction in cytokine-treated colon carcinoma cells has been widely demonstrated [4,5,11,12,14–16]. In agreement with the published literature, we found that IFN-γ-sensitized HT-29 cells treated for 24 h with TNF-α exhibited a higher occurrence of apoptotic death than untreated controls (Fig. 4). The percentage of cells in late apoptosis was $1.0 \pm 0.3\%$ and $6.8 \pm 1.5\%$ for total control and total treated cells, respectively. Most of the cells unambiguously identified as apoptotic were found among floating cells. In these detached cells, the percentage of cells in late apoptosis was $23.7 \pm 6.6\%$ for controls and $65.7 \pm 3.8\%$ for treated cells ($P < 0.05$). The number of cells in late apoptosis was negligible in both treated and untreated *attached* cells. Since the histological method used detects apoptosis at a relatively late stage, many of the normal appearing floating cells (= live cells labeled 'L' in Fig. 4) may be at a less advanced stage of apoptosis, which would be consistent with published reports [21,22] and with the lack of significant NMR signal from intracellular metabolites due to partially leaking cell membranes. Nevertheless, the substantially increased proportion of unambiguously apoptotic cells in the treated group clearly demonstrates that the IFN-γ/TNF-α treatment protocol used in this work results in enhanced HT-29 cell death by apoptosis, in agreement with several reports based on different protocols [4,35–37]. The number of (primary and/or secondary) necrotic cells was also enhanced in the group of treated cells vs. controls, as was the amount of cell debris indicative of cell lysis.

3.5. Early metabolic events and apoptosis following IFN-γ/TNF-α treatment

In this work a variety of glycolytic and energetic changes were found to take place in IFN-γ/TNF-α-treated cells still attached to the flask bottom, *well before* the appearance of apoptotic morphology. Lac or NTP increases *as such* are not specific of apoptosis induction, although lactate increases following apoptosis induction have been reported previously [38]. However, if the *simultaneous*, *early* increases in NTP and Lac signals observed here can be reproduced in in vivo studies of treated c-Myc-expressing tumors, these changes may become useful indicators of ensuing apoptotic cell death since both metabolites can be measured by non-invasive in vivo NMR spectroscopy. In in vivo spectra, Lac signals can

overlap considerably with lipid signals which may also change following drug treatment. However, a number of lactate editing techniques are available to the NMR spectroscopist which allow him/her to separate Lac and lipid peaks unambiguously [39–41].

In vivo and in cell spheroids, the execution phase of apoptosis is completed quickly, with only a few minutes elapsing between the onset of the process and the ingestion of apoptotic bodies by nearby cells [42]. Therefore, only a small proportion of apoptotic cells can be visualized in tissue at any time by way of annexin V, TUNEL and other labeling or staining techniques [42,43], leading to small measured apoptotic indices (typically 3–4% [44,45], on rare occasions up to 15% for very late apoptosis [46]). Thus, metabolic changes occurring in these *advanced* apoptotic cells cannot be expected to result in signal alterations readily observable by in vivo NMR spectroscopy. In contrast, it is important to note that the Lac and NTP changes observed in our study occur over an *extended* period of time, and *long before* the cells are identifiable as apoptotic by the aforementioned staining techniques. Consequently, our results clearly encourage further evaluation in vivo to determine their potential as early diagnostic surrogate markers of apoptosis induction in cancer, at least for c-Myc-expressing tumors.

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