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Alterations of lipid composition in Friend leukemia cell tumors in mice treated with tumor necrosis factor-α

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Lipid analyses were carried out on transplantable murine Friend leukemia cell tumors, 6 h after intratumoral administration of tumor necrosis factor-α (TNF). The levels of the major phospholipid classes were uniformly decreased to about 70% of control values; free fatty acids were increased to about 170%; diacylglycerol was decreased to about 50% and triacylglycerol, the main lipid component, was not significantly altered. These results analysed in the light of concomitant alterations in the levels of phospholipid precursors and catabolites (determined in previous ^{31}P NMR studies) and histological modifications demonstrated that at early stages of TNF-induced inhibition of tumor growth (a) phospholipid catabolism was significantly enhanced; (b) morphological changes were apparently correlated with alterations in the levels of phosphatidylcholine and its catabolic products.

Tumor; Tumor necrosis factor; Friend erythroleukemia cell; Phospholipid; Glycerolipid; Free fatty acid

1. INTRODUCTION

Recent evidence demonstrates that alterations in the levels of PL metabolites may represent biochemical responses of cells to factors which regulate cell differentiation and/or proliferation in vitro and in vivo [1-7]. The interest of studying these metabolic alterations in neoplastic tissues is rapidly growing, also in the light of recent NMR investigations, showing conspicuous alterations in the levels of phospholipid precursors and/or catabolites in either experimental or clinical tumors after therapy [8-12].

Previous studies in our laboratories indicated that peritumoral injections of some cytokines, such as TNF or IL-1 β, in mice bearing established solid tumors resulted in the appearance of tumor necrosis and in marked antitumor effects [13-15]. Significant alterations in the pool sizes of five phospholipid metabolites, GroPCho, GroPEtn, PCho, GroP and Cho were observed in these cytokine-treated tumors [9,14,15]. These effects could not be simply considered as biochemical events associated with tumor necrosis, as they were detected at very short time intervals after cytokine treatment [14]. The alterations observed in the levels of these phospholipid precursors and catabolites could be consistently interpreted on the basis of alterations in the activity of at least two of the enzymes controlling the de novo biosynthesis and catabolism of phospholipids, i.e. activation of glycerophosphorylcholine phosphodiesterase (EC 3.1.4.2) and partial inhibition of choline kinase (EC 2.7.1.32) [9,14,15].

No studies have been so far reported on tumors to assess whether therapy-induced alterations in the levels of phospholipid precursors and/or catabolites are associated with modifications in the lipid composition.

This paper reports the results of analyses carried out on phospholipid classes, triglycerides, diglycerides and free fatty acids in transplantable mouse tumors, 6 h after in vivo intratumoral administration of either TNF-α or control preparations. In vivo treatment with this cytokine induced early reductions in the levels of all phospholipid classes, with no significant alteration in their relative concentration ratios. The results of lipid analyses were correlated with parallel histological examinations, as well as with previous quantitative determinations (by ^{31}P NMR) of alterations induced by TNF-α on the levels of phospholipid precursors and catabolites in the same tumor model.

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Abbreviations: FLC, Friend erythroleukemia cells; TNF-α, tumor necrosis factor-α; IL-1 β, interleukin-1 β; GroPCHo, glycerol 3-phosphorylcholine; GroPEtn, glycerol 3-phosphorylethanolamine; PCho, phosphorylcholine; PEtn, phosphorylethanolamine; GroP, *sn*-glycerol 3-phosphate; Cho, choline; PL, phospholipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SPM, sphingomyelin; FFA, free fatty acids; DG, diglycerides; TG, triglycerides; HPTLC, high performance thin layer chromatography; NMR, nuclear magnetic resonance

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2. MATERIALS AND METHODS

2.1. *Tumor preparation for lipid analyses*

Solid tumors were obtained in DBA/2 mice by s.c. injection in the dorsum of 5×10^6 FLC, clone 3Cl-8 [16]. After tumor nodules had developed, either TNF-α (4 μg in 0.2 ml) or buffered physiological saline solution (NaCl 153 mM, pH 7; 0.2 ml) were injected intratumorally. Tumors were rapidly removed 6 h after intratumoral injection of either cytokine or control preparations and cut in two pieces, one for chemical and one for histological examination.

2.2. *Tumor necrosis factor*

Recombinant murine, TNF-α [17], a kind gift of Prof. W. Fiers, University of Ghent, Belgium, was expressed in *Escherichia coli* and purified to apparent homogeneity. Its specific activity (8×10^7 U/mg protein) and endotoxin contamination (less than 0.5 ng/mg) were determined as already described [9]. In the experiments, TNF was diluted in buffered physiological solution (NaCl 153 mM).

2.3. *Tumor extracts*

Tissues were immediately frozen at liquid nitrogen temperature and powdered by mechanical action with a freezer mill (Spex Industries Inc., Metuchen, NJ). A small quantity of this powder was used for protein assay, according to Bradford [18]. Lipids were extracted with chloroform:methanol (2:1) according to Folch et al. [19].

2.4. *Lipid analyses*

Thin layer chromatography on Silica gel 60 plates (Merck, Darmstadt, FRG) was performed with 4–5 ml of lipid extract to fractionate triglycerides, free fatty acids and diacylglycerol with the solvent system *n*-hexane/diethyl ether/acetic acid (70:30:1, by vol.). These compounds were visualized by iodine vapours and identified by comparison with a standard mixture run on the same plate. The bands were scraped off the plate, eluted with 6 ml chloroform and, after addition of the internal standard (methylheptadecanoate), transesterified with 1.5 ml of methanolic KOH solution (Supelco Inc., Bellefonte, PA) for 45 min at 40°C. The reaction was stopped by adding 1 ml of distilled water. The methylester was extracted twice with 2 ml of *n*-hexane/diethylether (1:1, by vol.). The solvent volume was reduced to about 100 μl under nitrogen. An aliquot of methylesters solution was injected into a gas-chromatographic column (2 m × 2 mm) packed with 5% diethylene glycol succinate (DEGS) supported on Supelcoport, 100–120 mesh (Supelco Inc.). Class distribution of phospholipids was performed by a HPLTLC-densitometric assay, as previously described [20]. In a selected number of cases, to assess fatty acids composition, phospholipid classes were separated on TLC with a solvent system chloroform/methanol/acetic acid/water (25:15:4:2, by vol.), visualized by iodine vapour and identified by comparison with a standard mixture run on the same plate. The bands were scraped off the gels and eluted with chloroform:methanol:H_2O (50:50:10, by vol.). The transesterification was carried out, after addition of the internal standard, with 1.5 ml of methanolic HCl solution (Supelco Inc.) for 15 h at 60°C. Extraction of methylesters and gas chromatography were performed as described above.

2.5. *Histological analyses*

Tumors were removed, cut into two pieces of approximately 0.5 cm^3 and fixed in 10% formalin for several days. Fixed tissues were subsequently processed for paraffin embedding, cut into 2–3 μm thick slices and stained with hematoxylin and eosin.

3. RESULTS AND DISCUSSION

3.1. *Lipid classes*

Table 1 shows the results of quantitative analyses on the main acylated lipid classes in tumor extracts prepared from tumors dissected 6 h after in vivo treatment with either TNF or control preparations. The levels of the major PL classes PC, PE, PS and SPM in TNF-treated tumors were uniformly decreased (by approximately 30%) and the original proportions were maintained (PC:PE:PS:SPM = 1:0.30:0.24:0.06). Lysophosphatidylcholine was present in trace amounts in either TNF-treated or control tumors (<0.1 mg/g wet weight).

Table 1

Effects of TNF-α treatment on the concentration (mg/g wet tissue) of the major acyl-containing lipids in FLC tumors implanted in DBA/2 mice

Lipid class	Control		TNF	
	n	Mean ± SD	*n*	Mean ± SD
TG	18	34.41 ± 12.56	19	34.22 ± 10.71
DG	10	0.47 ± 0.19	19	0.23 ± 0.08**
FFA	19	0.20 ± 0.04	20	0.34 ± 0.11**
PC	19	5.30 ± 1.12	20	3.66 ± 0.67**
PE	19	1.56 ± 0.41	20	1.08 ± 0.25**
PS	19	1.34 ± 0.63	16	0.87 ± 0.31*
SPM	17	0.31 ± 0.15	17	0.23 ± 0.07*

n, number of mouse tumors analysed
* $P<0.05$, TNF vs control: ** $P<0.001$, TNF vs control

The levels of the other acylated lipid classes determined in this study were differently affected by TNF injection. In fact, DG was clearly decreased (by about 50%), FFA were increased (by about 70%), and TG, the main lipid component in these extracts, was not significantly changed. It is important to note that the relative order in the lipid concentrations was maintained even when the data were reffered to protein content, instead of tissue weight, since the protein content was found to be quite consistent in all experiments and not significantly altered, at least until 6 h after TNF treatment (data not shown).

The typical morphologic changes of FLC tumors, following TNF treatment, are shown in fig.1. In agreement with previous studies in our laboratories [14], morphologic examinations of these tumors 6 h after TNF injection revealed early alterations at the level of tumor blood vessels, together with some cell disaggregation, still in the absence of substantial areas of tumor necrosis. These morphologic changes were accurately evaluated as per cent arbitrary scores for disaggregation, alterations to blood vessels and tumor necrosis (table 2).

The present results support the conclusion that, at early stages of TNF-induced inhibition of tumor growth, tumor cells undergo a general reduction in their phospholipid content. This effect cannot be simply interpreted as an epiphenomenon of cell necrosis. In fact, necrosis only involves, in our conditions, less than 10% of the total histological area, a value definitely insufficient to explain a reduction of 30% in the total phospholipid content of the tumor. The latter effect

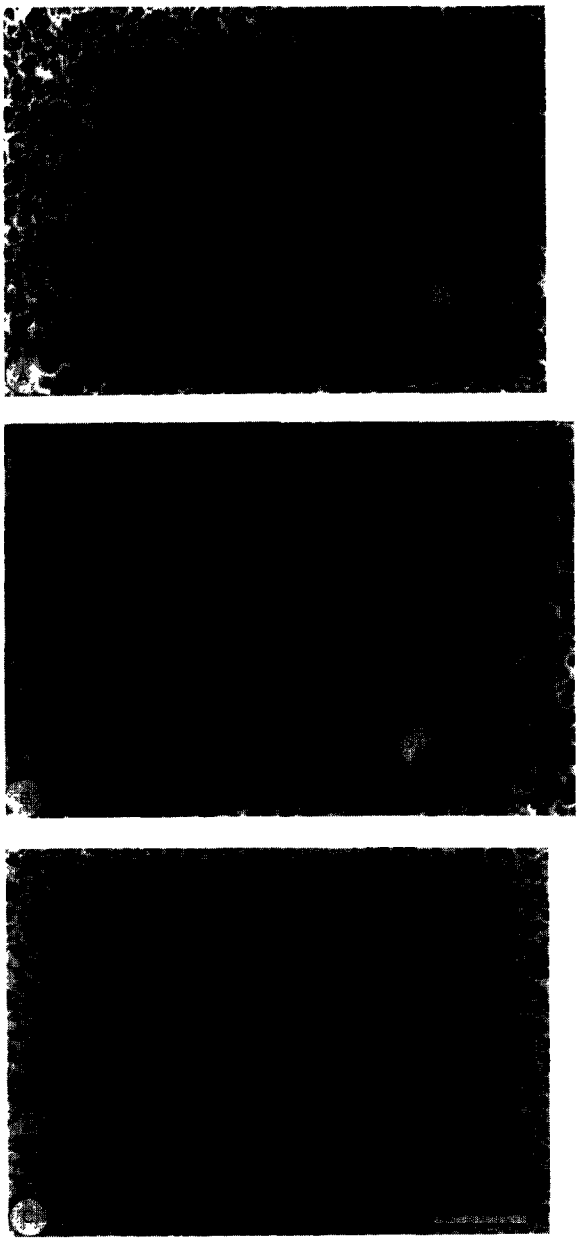

Fig.1. Histopathology of FLC tumors 6 h after injection of TNF-α. (A) Section of control treated tumor, showing closely apposed pleomorphic cells. (B) Typical section of a TNF-treated tumor, showing some blood vessels engorged with erythrocytes. (C) Typical area of tumor cell necrosis, only occasionally observed in TNF-treated tumors at 6 h after treatment. Bar = 250 nm.

seems therefore likely to derive from an altered phospholipid metabolism occurring in tumor cells in the first few hours after TNF administration.

The reduction in the levels of the major phospholipid classes might in principle be either due to a reduction in PL biosynthesis, or to an activation of PL catabolism or to both biochemical mechanisms.

Previous ^{31}P and ^{1}H NMR studies on the same tumor system allowed us to detect early changes in the concentration levels of PL precursors and catabolites [9,14]. In

Table 2

Morphologic alterations of TNF-α-treated FLC tumors, 6 h after cytokine treatment

Treatment	Mouse no.	Disaggregation	Alterations to blood vessels	Tumor necrosis
Control	1	13	25	3.0
	2	< 10	< 10	–
	3	< 10	< 10	–
	4	< 10	< 10	–
	5	13	< 10	1.5
	6	< 10	< 10	–
	7	< 10	< 10	–
	8	< 10	< 10	–
TNF	1	75	50	9.0
	2	75	50	8.0
	3	50	50	6.0
	4	100	50	10.0
	5	100	75	10.0
	6	75	50	8.0
	7	75	50	7.0
	8	50	50	5.0

Values represent the percentage of arbitrary scores over a theoretical maximum score of 100% for each group. At least 10 different histologic areas of the tumor were evaluated

particular, 6 h after TNF injection, the levels of phospholipid catabolites such as GroPCho and GroPEtn were both reduced to 50 ± 20% of their control values, with parallel conspicuous increases in the levels of GroP (7–8 ×), as well as in the [Cho]/[PCho] ratio (10–20 ×) [9]. Moreover, the level of PCho was significantly reduced (to 30–50% of control values) whereas that of PEtn remained practically unaltered.

A possible interpretation of the phospholipid analyses reported in table 1 can therefore now be attempted on the basis of the whole body of experimental evidence so far collected on this system (this paper and [9,14]). A reduced availability of the substrates destined to acylation might a priori be responsible for reductions in the cellular PL levels. However, in our case, such a mechanism could only explain the PC but not the PE reduction, since of the two precursors, PCho and PEtn, only the former appeared to be significantly decreased by the TNF treatment.

On the other hand, an activation of the PL catabolic pathway in TNF-treated tumors was clearly pointed out by our previous ^{31}P NMR studies in TNF-treated tumors [9], according to which GroPCho decreases were paralleled by increases of its two catabolic derivatives, GroP and Cho. The hydrolysis of GroPCho is known to be catalyzed by GroPCho diesterase, an enzyme which, first described by Dawson in 1956 in rat liver [21], has been successively detected in a number of animal tissues [22–25]. No detailed studies have been so far carried out, to our knowledge, on its activity in neoplastic tissues, although Victor et al. [26] recently suggested that the levels of this enzyme might be

Table 3

Effects of TNF-α treatment on the fatty acid composition of some lipid classes in FLC tumors

Lipid class	n	Controls Mean ± SD	Class of saturation	n	TNF Mean ± SD
		36.6 ± 2.87	S		36.2 ± 4.48
TG	19	50.0 ± 3.14	M	13	48.8 ± 3.84
		13.3 ± 2.49	P		16.5 ± 2.69*
		71.5 ± 26.31	S		45.1 ± 6.64**
FFA	13	19.6 ± 17.96	M	11	36.7 ± 6.61**
		8.3 ± 8.03	P		19.14 ± 4.59**
		58.8 ± 7.68	S		53.6 ± 17.85
DG	11	31.6 ± 5.89	M	13	32.6 ± 11.72
		20.6 ± 12.07	P		13.7 ± 7.04*
		67.5 ± 5.60	S		63.6 ± 4.46
PC	11	22.3 ± 3.16	M	6	25.5 ± 4.03
		10.2 ± 2.41	P		10.4 ± 3.63
		54.1 ± 13.54	S		53.4 ± 4.48
PE	10	21.9 ± 3.79	M	4	25.5 ± 1.32
		21.4 ± 10.08	P		20.9 ± 3.78

For clarity, the results are reported as per cent distribution of saturated (S), monounsaturated (M) and polyunsaturated (P) fatty acids

n, number of mouse tumors analysed

* $P<0.05$; ** $P<0.001$

regulated by estrogen in breast cancer cells. GroPCho diesterase is reported to be also active on GroPEtn [21,24]. The lack of specificity of this enzyme for its substrate headgroup seems to offer a straightforward interpretation of the fact that both GroPCho and GroPEtn levels decrease to the same extent in TNF-treated tumors [9], as well as of the parallel decreases of PC and PE, observed in the present study. Moreover, studies on the properties of GroPCho diesterase in animal tissues also demonstrated that the activity of this enzyme is strongly pH dependent [21,25]. It is of interest to note that a significant alkaline shift was observed in TNF-treated tumors [9,14]. It is therefore possible to speculate that such pH shift might be involved in the activation of this enzyme.

Lastly, it has also been suggested that modulations in the levels of phosphodiesters may play a regulatory role on membrane-bound enzymes [27] and that decreased levels of phospholipids may affect the deacylation–reacylation cycle [28].

All these considerations suggest that an enhanced phospholipid catabolism is the most likely candidate to explain the observed reductions in the phospholipid contents of TNF-treated tumors at early stages of inhibition of tumor growth.

3.2. *Fatty acid distribution*

The fatty acid distribution of the main phospholipid classes is shown in table 3. These data indicate that the acyl composition of PC or PE were not significantly affected by administration of TNF. This supports the hypothesis that the metabolic rates but not the metabolic pathways are affected by TNF treatment. The highly significant increase in the proportion of polyunsaturated acids in the free fatty acid class may be justified by the activation of a phospholipase A2, although an increased uptake of polyunsaturated FFA from blood circulation cannot be ruled out.

Table 4

Correlation between the indexes of morphologic alterations (shown in table 2) and the concentration of some lipid classes (R of Pearson) in two groups of eight mice in which the FLC tumors were treated with TNF-α or normal saline

	Disaggregation		Alterations to blood vessels		Necrosis	
	Control	TNF	Control	TNF	Control	TNF
vs FFA	−0.70	0.79	−0.63	0.70	−0.71	0.74
vs PC	0.18	0.91	0.01	0.71	0.20	0.93
vs PE	0.36	0.06	0.35	−0.12	0.39	0.00

3.3. *Correlation between lipid degradation and morphological alterations*

The morphological scores reported in table 2 were correlated to the concentrations of lipids most significantly affected by TNF treatment. The results of this comparison, shown in table 4, indicated that morphological changes due to TNF treatment were well correlated to the variations in FFA and PC concentrations. No significant correlation was instead found with PE, although the latter was also significantly lowered by TNF treatment. The different degree of correlation exhibited by PC and PE levels and morphological alterations in the tissue might be due to the asymmetric distribution of the two phospholipids between the outer and inner layer of the erythroleukemic cell membrane. In conclusion, the intratumoral treatment of the FLC murine tumors with TNF induced morphological changes which were apparently correlated with alterations in the levels of PC and its catabolic products.

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