Modulations of glycerophosphorylcholine and phosphorylcholine in Friend erythroleukemia cells upon in vitro-induced erythroid differentiation: a ^{31}P NMR study

G. Carpinelli, F. Podo*, M. Di Vito, E. Proietti+, S. Gessani+ and F. Belardelli+

Laboratorio di Biologia Cellulare and +*Laboratorio di Virologia, Istituto Superiore di Sanità, Rome, Viale Regina Elena, 299, 00161 Rome, Italy*

Received 1 August 1984

A ^{31}P NMR study has been carried out on Friend erythroleukemia cells (FLC) induced to undergo erythroid differentiation in vitro. Significant levels of glycerophosphorylcholine (GroPCho) and phosphorylcholine (P-Cho) were identified both in the untreated cells and in their PCA extracts. In FLC treated 4 days in vitro with either dimethylsulfoxide (DMSO) or hexamethylenebisacetamide (HMBA), the intracellular concentration of P-Cho was markedly increased, whereas that of GroPCho appeared to be significantly reduced. HMBA was more effective than DMSO in producing this effect. The concomitant modulations of GroPCho and P-Cho in differentiated FLC suggest the hypothesis that erythroid differentiation involves modifications of the regulatory mechanisms controlling biosynthesis and catabolism of phospholipids.

Friend erythroleukemia cell *Erythroid differentiation* *^{31}P NMR* *Glycerophosphorylcholine* *Phosphorylcholine*

1. INTRODUCTION

Friend erythroleukemia cells [1] have been widely used as a model system for the study of erythroid differentiation [2]. FLC grown in vitro show little evidence of erythroid maturation beyond the erythroblastic stage, but markedly differentiate towards normoblasts when treated with a variety of agents [3], among which DMSO and HMBA are particularly active [4]. Although the appearance of the several erythroid markers during in vitro differentiation has been investigated in great detail, only a few studies were carried out on the metabolic changes which characterize differentiated cells. NMR spectroscopy can provide significant, detailed information on intracellular metabolites and their interconversions, under non-disruptive and non-invasive conditions [5–7]. ^{31}P NMR spectra were reported for several mammalian cell lines, including FLC, in [8]. Among the various metabolites identified in these spectra, GroPCho and GroPEtn deserved particular attention. The presence of GroPCho, first detected in some muscles by authors in [9] was subsequently also identified in a variety of cell systems [9–11]. Although the biochemical bases of the modulations of GroPCho and analogous phosphodiesters have not been clarified, it has been suggested that they may play a role as 'markers' of pathological conditions, including dystrophies or malignancy [9–12]. Another potential ^{31}P NMR 'marker' has been suggested to be P-Cho, a compound generally present in high levels in tumor lines lacking GroP-

Abbreviations: Friend erythroleukemia cells, FLC; dimethylsulfoxide, DMSO; hexamethylenebisacetamide, HMBA; perchloric acid, PCA; glycerophosphorylcholine, GroPCho; glycerophosphorylethanolamine, GroPEtn; phosphorylcholine, P-Cho; phosphatidylcholine, PtdCho

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V.

Cho [12]. An increase of P-Cho in FLC during differentiation has been detected recently by ^{1}H NMR methods [13].

We aimed at studying levels and modulations of both GroPCho and P-Cho in FLC, either untreated or induced to differentiate with DMSO or HMBA.

2. MATERIALS AND METHODS

2.1. *Cells*

Friend erythroleukemia cells were grown in RPMI medium supplemented with 10% foetal calf serum (GIBCO). The cells were routinely seeded at 10^5/ml, twice a week. Clone 745A was obtained from C. Friend (New York) and clone 745-E7 was derived by cloning 745A cells serially passaged i.p. in DBA/2 mice [14]. The culture medium used for inducing erythroid differentiation was adjusted to 1.5% DMSO (v/v) or to 5 mM HMBA before the cells were added. Cells were seeded at 5×10^4/ml and grown in roller bottles (Falcon) without any further medium change. Control and DMSO- or HMBA-treated cultures reached cell densities of $2.0–2.3 \times 10^6$/ml on day 4, and had viability (as judged by trypan blue dye-exclusion test) greater than 95%. The percentage of differentiated FLC was determined by benzidine staining, according to [15].

2.2. *Chemicals*

All reagents were of analytical grade. DMSO was purchased from Merck and HMBA was kindly prepared by Dr C. Delfini (Rome). Extracts were prepared by addition of 5 ml of H_2O and 0.5 ml of 30% perchloric acid at 0°C followed by sonication. The supernatant pH was adjusted to 7.4 with KOH and the $KClO_4$ precipitate removed by centrifugation. The supernatant was evaporated to dryness with rotovapor and the residue suspended in ^2H_2O–EDTA (100 mM), reading pH 7.0, for NMR measurements.

2.3. *Nuclear magnetic resonance*

^{31}P NMR spectra were recorded at 40.5 MHz from packed cell suspensions of FLC ($2–3 \times 10^9$ cells/ml in ^2H_2O–saline) or from extracts by means of a Varian XL 100 spectrometer by following procedures previously described [11]. Chemical shifts were measured in ppm with respect to 85% phosphoric acid (external reference). Signals were identified and the concentrations of the relative compounds measured in the extracts by adding known amounts of the respective standard compounds and observing both integral variations and the pH dependence of their chemical shifts. The quantitative analysis of the extracts was carried out by using a Bruker AM 400 WB spectrometer.

3. RESULTS

The typical ^{31}P NMR spectrum obtained between +6 ppm and −3 ppm from FLC grown 4 days in vitro is shown in fig.1a. The analysis of the spectrum of the PCA extract (fig.2a) allowed the identification of the resonances II, III and IV of the cell suspension as due to inorganic phosphate (p_i, +1.81 ppm), GroPEtn (+0.35 ppm) and GroPCho (−0.20 ppm), respectively. The dif-

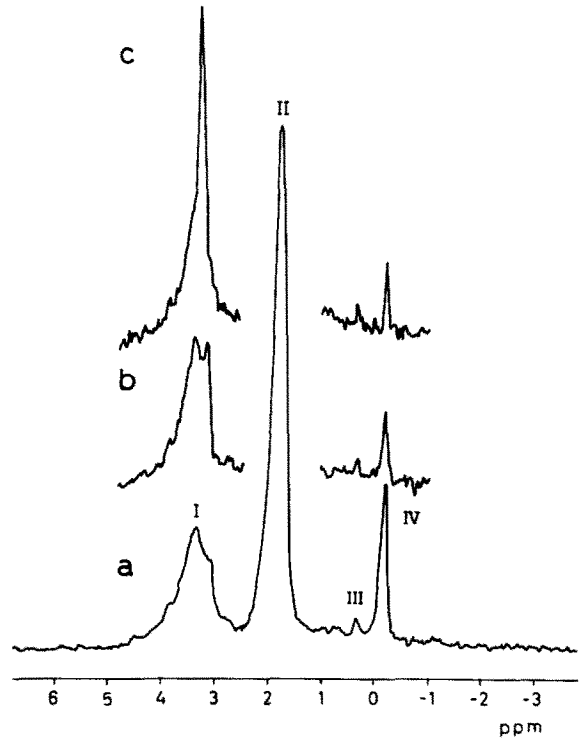

Fig. 1. ^{31}P NMR spectra (4°C, 60° r.f. pulse, acquisition time 1 s) of Friend erythroleukemia cells, clone 745A, grown for 4 days in vitro in medium containing: (a) no inducer (4412 scans); (b) 1.5% DMSO (3891 scans); (c) 5 mM HMBA (4257 scans; amplification 3 × that used for (a) and (b).

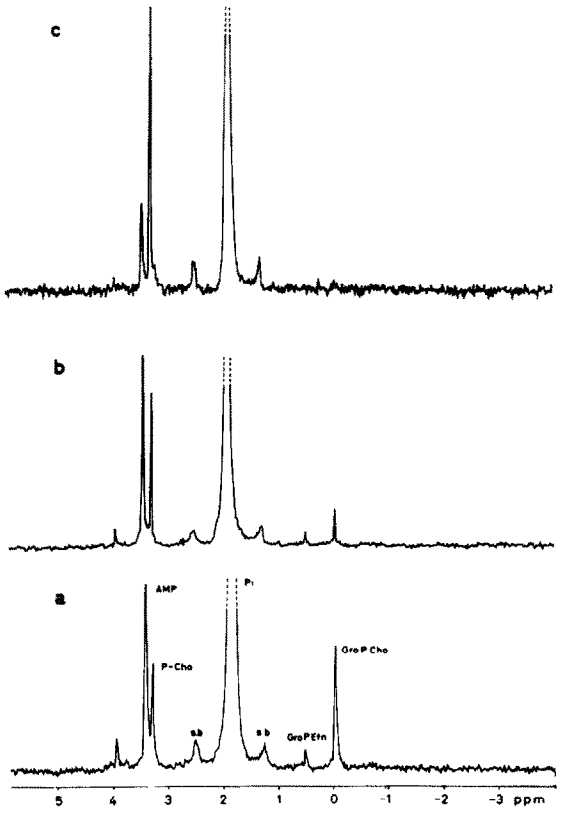

Fig. 2. ^{31}P NMR spectra (30°C, 60° r.f. pulse, acquisition time 1.5 s) of PCA extracts of the same Friend erythroleukemia cells as in fig. 1: (a) no inducer; (b) 1.5% DMSO; (c) 5 mM HMBA (amplification 1.33 × that used for (a) and (b).

ference of about 0.7 ppm between the chemical shift of GroPCho (or GroPEtn) measured here, using an electromagnet, and that reported in [8] using a superconductive magnet, is due to the different alignment of the magnetic field with respect to the sample axis in the two spectrometers (diamagnetic susceptibility effect [16]). Band I (between 3 and 4 ppm) appeared to be essentially composed of two resonances, identified as AMP and P-Cho in the PCA extract. By adjusting the pH of the latter to a value at which the chemical shift of P_i (δ = 1.88 ppm) was very close to that of intracellular P_i in the intact cells, the signals of AMP and P-Cho appeared to be separated by +0.15 ppm, whereas they tended to overlap when pH was decreased by about 0.2 units. The spectra reported in fig.1b,c were obtained from FLC treated 4 days with either DMSO or HMBA. The shift of their respective P_i resonances (not shown) indicated that the average intracellular pH decreased by about 0.1 units in cells treated with DMSO and 0.2 units in those treated with HMBA (the pH values estimated from the titration curve reported in [17] for 5 mM potassium phosphate in the presence of 0.1 M KCl, were 7.0, 6.9 and 6.8 respectively, in untreated cells and in cells differentiated with DMSO or HMBA). The slight intracellular acidification was responsible for the almost complete overlapping of the AMP and P-Cho signals in the HMBA-treated cells (fig.1c).

The spectra of intact cells showed that the GroP-Cho level decreased in both DMSO- and HMBA-differentiated FLC, whereas the P-Cho content exhibited conspicuous increments. These effects were much more evident in HMBA-treated cells. Similar results were obtained in another two experiments carried out on DMSO-induced cell cultures (clone 745A and 745-E7), while no variations in the ratio between the areas of P-Cho and GroPCho were observed in FLC (clone 745) after 2 h of treatment with DMSO (not shown).

The contents of GroPCho and P-Cho determined in the PCA extracts of untreated and differentiated cells are reported in table 1. The ratio

Table 1

Concentrations of phosphorylcholine and glycerophosphorylcholine in PCA extracts of control and differentiated Friend erythroleukemia cells

Treatment	Benzidine-positive cells on day 4 (%)	[P-Cho] (μmol/10^9 cells)	[Gro-PCho] (μmol/10^9 cells	$\frac{[GroPCho]}{[P\text{-}Cho]}$
None	< 1	0.5	3.4	6.8
DMSO	59	0.8	1.1	1.4
HMBA	76	1.2	below detection	~0

between the concentrations of GroPCho and P-Cho showed a 5-fold decrease in the extract of DMSO-treated with respect to untreated FLC and practically vanished in that of HMBA-differentiated cells. The concentrations reported in table 1 represent the intracellular levels of the corresponding compounds in intact cells, within the errors brought about by the extraction procedures. The spectra of the PCA extracts actually show that the extraction yield of GroPCho is somewhat lower than that of P-Cho. In particular, although GroPCho gave rise to a signal of very small intensity in HMBA-treated cells, it was not possible to evidentiate its resonance above the noise in the corresponding PCA extract.

4. DISCUSSION

High levels of GroPCho in FLC have already been reported in [8]. The fact that the GroPCho concentration found in our control cells is about half that observed in [8] could be ascribed to several possible reasons: (i) although derived from the same original clone (745A) these cells have been passaged for many years in different culture conditions (media and percentages of foetal calf serum); (ii) the experimental conditions necessary to obtain high percentages of differentiated cells were different from those used by authors in [8]. These differences in cell growth conditions could be very relevant in modulating the levels of GroP-Cho as suggested by the modulations of this compound in other cell systems [11,12].

An increase in the ^1H NMR signal (only one) arising from both GroPCho and P-Cho was already observed [13] in FLC induced to differentiate by DMSO. However, the method used did not allow the discrimination of the individual modulations undergone by these two compounds.

To our knowledge, our results give the first example of modulations of both GroPCho and P-Cho in a cell system undergoing erythroid differentiation. A decrease in the GroPCho level occurring in differentiated FLC is in fact associated to an increase of P-Cho. Such an association is rather intriguing. According to the classical biochemical pathways of biosynthesis and degradation of phosphatidylcholine (PtdCho), GroPCho is generally assumed to be primarily the result of degradation of PtdCho via the expression of phospholipase [18] or lysophospholipase activity, whereas P-Cho would occur primarily as an intermediate in the major mechanism of PtdCho biosynthesis [19], a process known to be controlled mainly by cytidyltransferase [20]. The possibility that P-Cho might simply be a degradation product cannot be excluded a priori. However, a phospholipase C-mediated degradation of PtdCho into P-Cho appears unlikely as this enzyme has been found generally in bacteria, whereas in mammalian cells it seems to exhibit prevailing specificity for phospholipids different from PtdCho [18]. On the other hand, the existence of an enzyme with novel phosphodiesterase activity leading from GroPCho to P-Cho was reported in 1975 in rat brain homogenates [21]. Although no further studies have been carried out, to our knowledge, to investigate the presence of this enzyme in other systems, the results of these experiments indicate the interest of assessing whether such enzyme is also possibly present in FLC and in other tumor cells. In the case of a lack of such an enzyme in our system, an increase of the P-Cho level could in principle be ascribed to: (i) an increase in the pool of intracellular choline; (ii) an enhancement of phosphokinase activity, or (iii) an inhibition of the cytidyltransferase activity, resulting in the accumulation of its substrate. The observation (derived from fig. 2 of [13]) that choline content is maintained constant during DMSO-induced differentiation, seems to exclude the first hypothesis. On the other hand, a decrease in the GroPCho content might derive from (i) a reduction in the PtdCho level, or (ii) a modulation of combined phospholipase A activity [18]. The latter mechanism seems to be favored in view of the fact that PtdCho is reported to be either increased or maintained practically constant in differentiated FLC [22].

The fact that levels of GroPCho and P-Cho undergo concomitant modulations in differentiated FLC suggests the hypothesis that erythroid differentiation might result in some modifications of the regulatory mechanisms controlling biosynthesis and catabolism of phospholipids.

Further studies are necessary in order to elucidate at which stage of in vitro-induced erythroid differentiation these modulations occur. However, although the biochemical events controlling these changes, as well as their functional correlations

with the differentiation process, are still unknown, these metabolic modulations seem to represent additional markers appearing in in vitro differentiated FLC.

ACKNOWLEDGEMENTS

We thank Professor G. D'Agnolo for advice and helpful discussions and Mr B. Santurbano and Mr M. Giannini for technical assistance. This work has been partially supported by CNR Special Programs 'Tecnologie Biomediche e Sanitarie' (no. 83.00571.57) and 'Controllo delle Malattie da Infezione' (no. 83.02916.52). G.C. is the recipient of a CNR fellowship at the Centro CNR di Biologia Molecolare, Università di Roma.

REFERENCES

[1] Friend, C., Patuleia, M.C. and De Harven, E. (1966) Natl. Canc. Inst. Monogr. 22, 505–521.
[2] Friend, C., Scher, W., Holland, J.G. and Sato, T. (1971) Proc. Natl. Acad. Sci. USA 68, 378–382.
[3] Friend, C. (1978) The Harvey Lectures 72, 253–291.
[4] Reuben, R.C., Rifkind, R.A. and Marks, P.A. (1980) in: Biochim. Biophys. Acta 605, 325–346.
[5] Radda, G.K. and Seeley, P.J. (1979) Ann. Rev. Physiol. 41, 749–769.
[6] Hollis, D.P. (1980) in: Biological Magnetic Resonance (Berliner, L.J. and Reuben, J. eds) vol. 2, Plenum Press, New York, London.
[7] Gadian, D.G. (1982) in: Nuclear Magnetic Resonance and its Applications to Living Systems, Clarendon Press, Oxford.
[8] Navon, G., Navon, R., Shulman, R.G. and Yamane, Y. (1978) Proc. Natl. Acad. Sci. USA 75, 891–895.
[9] Burt, C.T., Glonek, T. and Barany, M. (1976) Biochemistry 15, 4850–4853.
[10] Labotka, R.J., Glonek, T., Hruby, M.A. and Honig, G.R. (1976) Biochem. Med. 15, 311–329.
[11] Cassone, A., Carpinelli, G., Angiolella, L., Maddaluno, G. and Podo, F. (1983) J. Gen. Microbiol. 129, 1569–1575.
[12] Brady, T.J., Burt, C.T., Goldman, M.R., Pykett, I.L., Buonanno, F.S., Kistler, J.P., Newhouse, J.H., Hinshaw, W.S. and Pohost, G.M. (1981) in: NMR Imaging. Proceedings of an International Symposium on Nuclear Magnetic Resonance Imaging, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, NC.
[13] Agris, P.F. and Campbell, I.D. (1982) Science 216, 1325–1327.
[14] Belardelli, F., Ferrantini, M., Maury, C., Santurbano, L. and Gresser, I. (1984) Int. J. Cancer, in press.
[15] Orkin, S.H., Harosi, F.I. and Leder, P. (1975) Proc. Natl. Acad. Sci. USA 72, 98–102.
[16] Gadian, D.G., Radda, G.K., Richards, R.E. and Seeley, P.J. (1979) in: Biological Applications of Magnetic Resonance (R.G. Shulman Ed.), Academic Press, New York.
[17] Roberts, J.K.M., Wade-Jardetzki, N. and Jardetzki, O. (1981) Biochemistry 20, 5389–5394.
[18] Van den Bosch, H. (1980) Biochim. Biophys. Acta 604, 191–246.
[19] Kennedy, E.G. and Weiss, S.B. (1956) J. Biol. Chem. 222, 193–214.
[20] Vance, D.E. and Choy, P.C. (1979) Trends Biochem. Sci. 4, 145–148.
[21] Abra, R.M. and Quinn, P.J. (1975) Biochim. Biophys. Acta 380, 436–441.
[22] Zwingelstein, G., Tapiero, H., Portoukalian, J. and Fourcade, A. (1981) Biochem. Biophys. Res. Commun. 98, 349–358.