NMR studies of the lipid metabolism of T47D human breast cancer spheroids

S.M. Ronen[1], A. Stier[2] and H. Degani[1]

[1]*Isotope Department, The Weizmann Institute of Science, Rehovot, 76100 Israel* and [2]*Max-Planck-Institut für Biophysikalische Chemie, Am Fassberg, D-3400 Göttingen, FRG*

Received 18 April 1990

The in vivo ^{31}P NMR spectrum of T47D human breast cancer cells grown as spheroids shows changes in the phosphomonoester lipid precursors as a function of spheroid size. The ratio of phosphorylethanolamine (PE) to phosphorylcholine (PC) was 1.0 ± 0.3 for 3-day-old, 150 μm spheroids. This ratio increased to 2.4 ± 0.4 for spheroids 7 days and older and which were at least 300 μm in diameter. To investigate the phosphatidylethanolamine to phosphatidylcholine (PdylE/PdylC) ratio in the membranes, chloroform/methanol extracts of spheroids were performed. The ^{31}P spectrum of these extracts showed no change with spheroid size, namely the PdylE/PdylC ratio was 0.5 ± 0.06 for spheroids of all ages suggesting that membrane composition is strongly regulated at the precursor level.

Nuclear magnetic resonance; T47D; Breast cancer; Lipid metabolism; Phosphomonoester; Spheroid

1. INTRODUCTION

Increasingly, magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS) are being used for in vivo tumor detection and diagnosis in humans ([1] and references therein). Concomitantly laboratory studies of transformed cells in culture and implanted tumors in animals strive to determine the characteristics of cancer by nuclear magnetic resonance (NMR) spectroscopy ([1] and references therein). In general, the NMR spectrum of tumors during growth shows a decreasing level of energy-rich compounds such as NTP and phosphocreatine, and an increasing level of P_i and the phosphomonoesters (PME) composed of phosphorylethanolamine (PE) and phosphorylcholine (PC). It is also possible to monitor the effects of different modes of cancer treatment such as chemotherapeutic agents ([2] and references therein), cytokines ([3] and references therein) radiation ([4] and references therein) and others, on cells in culture- and animal-implanted tumors as well as human patients. The observed changes in the ^{31}P NMR spectrum as a consequence of treatment have often been shown to be a reversal of the trends observed during untreated tumor progression, namely a decrease in the PME levels and an increase in the ATP/P_i ratio.

Correspondence address: S.M. Ronen, The Weizmann Institute of Science, Rehovot, 76100 Israel

Abbreviations: MRI, magnetic resonance imagining; MRS, magnetic resonance spectroscopy; NMR, nuclear magnetic resonance; NTP, nucleotide triphosphate; PME, phosphomonoesters; PE, phosphorylethanolamine; PC, phosphorylcholine; PdylE, phosphatidylethanolamine; PdylC, phosphatidylcholine

Multicellular tumor spheroids, constitute an excellent model for tumor behavior in vivo ([5] and references therein). These mini-tumors, which can be grown under controlled laboratory conditions up to an average size of 700 μm in T47D cells, present the same characteristics as an in vivo tumor. As the spheroid size increases, only its external (ca 50 μm) layer of cells will remain in the proliferating state while the more internal layers will be in a quiescent and then necrotic state similarly to less vascularized regions of the tumor.

Our work is involved in the study of T47D clone 11 human breast cancer cells grown as spheroids. We have been able to introduce these spheroids into the NMR by encapsulating them in agarose beads and we can maintain them in a proliferating state inside the spectrometer as previously described [6]. Under these conditions we have been able to monitor variations in spheroid metabolism as a function of spheroid size. In particular, we have concentrated on variations in the PME region belonging to the water-soluble lipid precursors namely changes in phosphorylcholine and phosphorylethanolamine. The latter are precursors of phosphatidylcholine and phosphatidylethanolamine, respectively. Phosphatidylcholine is synthesized primarily through the CDP-choline route (Kennedy pathway) [7], using choline as a precursor which is phosphorylated to phosphorylcholine, converted to CDP-choline and combined with a diacylglycerol to form the membrane lipid. Phoshatidylethanolamine synthesis on the other hand is performed through either of 3 possible pathways. (i) The CDP-ethanolamine pathway, analogous to the CDP-choline route, through phosphorylethanolamine and CDP-ethanolamine [8]. (ii) The decarboxylation of phosphatidylserine [9]: this

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

pathway requires catalytic amounts of ethanolamine and generates more ethanolamine by formation of phosphatidylserine from phosphatidylethanolamine thus liberating ethanolamine. This is followed by phosphatidylserine decarboxylation to yield again phosphatidylethanolamine. The ethanolamine formed in this fashion is incorporated into phosphatidylethanolamine through the CDP-ethanolamine route. (iii) The calcium-dependent exchange of free ethanolamine for the head group moiety of phosphatidylserine [10].

We show here, that the PE/PC ratio in T47D spheroids increased during active spheroid growth and cell division, then leveled off when the spheroids had reached the size at which necrosis commenced. However, the cell membrane composition of these spheroids showed no modification with growth and the PdylE/PdylC ratio remained constant along the growth curve.

2. MATERIALS AND METHODS

T47D clone 11 human breast cancer cells were obtained from the pleural effusion of infiltrating duct carcinoma [11] and were a gift from Prof. I. Keydar (Tel-Aviv University).

Spheroids were prepared and introduced into the NMR following encapsulation in agarose beads as previously described [6].

Spheroid cell membrane components were obtained by a chloroform/methanol extract based on the method outlined by Bligh and Dyer [12]. The lipid containing chloroform phase was lyophilized and redissolved in a $CDCl_3$/methanol/D_2O mixture at a ratio of 8:4:1, and containing 15 mM CsEDTA as described by Meneses and Glonek [13].

The nuclear magnetic resonance experiments were performed on a Bruker AM-500 spectrometer, equipped with a quadro-nuclei (^{15}N, ^{13}C, ^{31}P, ^{1}H) software-controlled probe. ^{31}P spectra were recorded at 202.5 MHz by applying a 90° pulse, 10 s repetition delay and composite pulse proton decoupling. The in vivo spectra were recorded at (35 ± 2)°C. The cells were perfused inside the NMR probe with a sterile perfusion system previously described [14]. ^{31}P peak areas were obtained by fitting the in vivo spectra obtained during the first 2 h of the experiment and plotted at a line broadening of 25 Hz to a set of Lorentzian lines using the Bruker Glinfit program. The ^{31}P spectra of the membrane extracts were recorded at (0 ± 2)°C with the same spectral parameters as above. These peak areas were obtained using the Bruker Glinfit program and fitting to spectra plotted with no line broadening.

3. RESULTS AND DISCUSSION

The phosphomonoester region of T47D clone 11 human breast cancer cells grown as spheroids changed markedly as a function of spheroid age and size; this is demonstrated in Fig. 1. Here we show 3 typical spectra, acquired during the first 2 h subsequent to introduction into the spectrometer, for spheroids 3 days (100 μm in diameter), 6 days (200 μm in diameter), and 21 days (350 μm in diameter) after seeding. The primary changes show a marked increase in PE and a slight decrease in PC with spheroid growth, while GPC and GPE remained, within experimental error, constant. This is further demonstrated in Fig. 2. Here we show

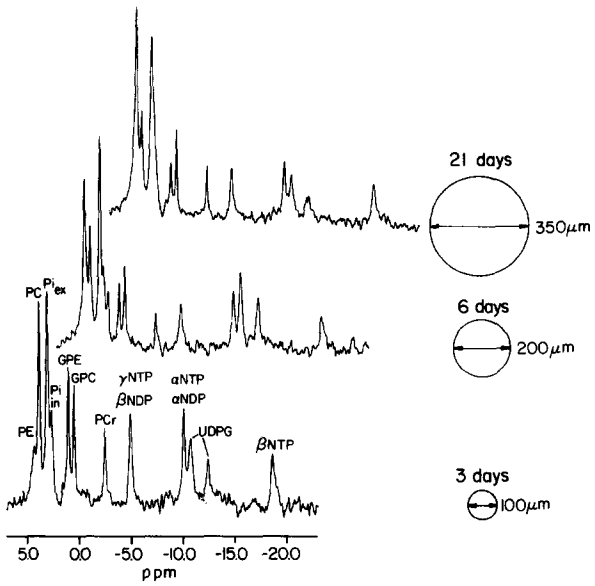


Fig. 1. ^{31}P spectra of spheroids 3, 6 and 21 days old. Each spectrum is the result of 180 fully relaxed transients (90° and 10 s relaxation delay) plotted with a line broadening of 25 Hz. Average spheroid size, determined by light microscope, is plotted schematically.

the ratio of phosphorylethanolamine to phosphorylcholine as a function of spheroid age. Starting at a value of 1.0 ± 0.3 for 3-day-old spheroids, the PE/PC ratio shows an initial increase. At this stage the spheroids are composed of a homogenous population of actively proliferating and dividing cells. The PE/PC ratio then leveled off at about 7 days at a value of 2.4 ± 0.4. Earlier studies performed in our laboratory [6] showed that 7 days is the time which correlates with the onset of necrosis in T47D spheroids and a subsequent decrease in cell division, at this point the spheroids are composed of proliferating, quiescent and necrotic cells.

Since the ^{31}P spectrum showed that the lipid precursor pool sizes change with spheroid age, the question arose as to how this affected the membrane components themselves, namely the PdylE/PdylC ratio. We in-

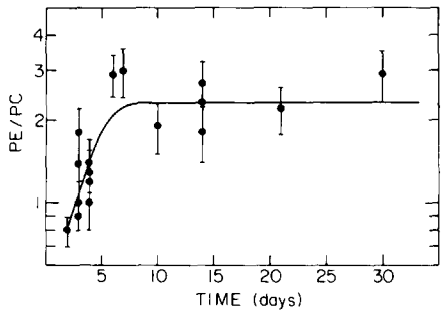
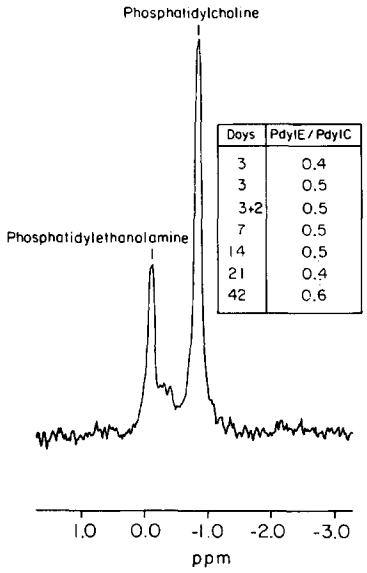


Fig. 2. Plot of phosphorylethanolamine to phosphorylcholine area ratio as a function of time from spheroid seeding. Error bars represent the error in areas calculated using the Bruker Glinfit program. Results were fit to an exponential curve.



Days	PdylE / PdylC
3	0.4
3	0.5
3+2	0.5
7	0.5
14	0.5
21	0.4
42	0.6

Fig. 3. ^{31}P spectrum of the chloroform phase obtained from a methanol/chloroform extract of 14-day-old spheroids. Spectrum was obtained as described in section 2 following 70 scans and plotted with a line broadening of 5 Hz. The two signals between the lipids are due to phosphatidylinositol and lysophosphatidylcholine [13]. The table is a summary of phosphatidylethanolamine to phosphatidylcholine area ratios (±20%) presented as a function of the extracted spheroid age. 3 + 2 represents the extraction of spheroids grown three days in the incubator and two days in the NMR spectrometer.

vestigated this question by growing spheroids to different sizes, extracting their membranes with a chloroform/methanol extract and investigating the lipid phase by ^{31}P NMR. Fig. 3 shows an example of the NMR spectrum of the chloroform phase of extracted 14-day-old spheroids. The table summarizes the different PdylE/PdylC ratios obtained from the different extracts of spheroids at different stages along the growth curve. As can be observed from these results the membrane composition is constant at a PdylE/PdylC value of 0.5 ± 0.06 and is in no way affected by the changes in the levels of the water-soluble precursors.

We conclude from this, that the membrane composition is strongly regulated. We can therefore assume that the changes that occur with spheroid age are limited to the precursor level. Possibly, there is a change in the rate-limiting stages of the lipid synthesis causing an accumulation of PE and a decrease in the PC pool size, yet in no way changing the membrane composition itself. Specifically, during synthesis of phosphatidylcholine via the Kennedy pathway, the rate-limiting phosphorylcholine cytidyltransferase step could be faster for the larger spheroids leading to a reduced PC pool size. In addition, PC can also be the product of phosphatidylcholine breakdown by phospholipase C [15], and thus the regulation of its pool size can be dependent on membrane breakdown. The synthesis rate of phosphatidylethanolamine through either of the 3 pathways earlier described (CDP-ethanolamine pathway, phosphatidylserine decarboxylation or serine-ethanolamine exchange) might be modified with growth to cause accumulation of PE. We are in the process of investigating these issues by following the incorporation of the ^{13}C-labeled lipid precursors, choline, ethanolamine and serine, into the membrane and their subsequent breakdown. In this fashion we hope to clarify the reasons for the variations in phosphorylethanolamine to phosphorylcholine ratio with spheroid age and size as well as the stage at which membrane composition is controlled. Furthermore this might explain changes observed in PME composition during tumor growth in vivo.

Acknowledgements: This research was supported by a Grant from the GIF, the German-Israeli foundation for Scientific Research and Development. S.M.R. is a recipient of the Eshkol Scholarship from the Israel Ministry of Science.

REFERENCES

[1] Daly, P.F. and Cohen, S.J. (1989) Cancer Res. 49, 770-779.
[2] Steen, R.G. (1989) Cancer Res. 49, 4075-4085.
[3] Podo, F., Carpinelli, G., Ferreti, A., Di Vito, M., Ciolli, V., Proietti, E. and Belardelli, F. (1989) in: Society of Magnetic Resonance in Medicine, 8th Annual Meeting, Book of Abstracts, vol. 1, p. 421.
[4] Steen, R.G., Tamargo, R.J., Brem, H., Glickson, J.D. and Wehrle, J.P. (1989) Magn. Reson. Med. 11, 258-266.
[5] Sutherland, R.M. (1988) Science 240, 177-184.
[6] Ronen, S. and Degani, H. (1989) Magn. Reson. Med. 12, 274-281.
[7] Pelech, S.L. and Vance, D.E. (1984) Biochim. Biophys. Acta. 779, 217-251.
[8] Kennedy, E.P. and Weiss, S.B. (1956) J. Biol. Chem. 222, 193-214.
[9] Borkenhagen, L.F., Kennedy, E.P. and Fielding, L. (1961) J. Biol. Chem. 236, PC28-PC30.
[10] Bjerve, K.S. (1973) Biochim. Biophys. Acta 296, 549-562.
[11] Keydar, I., Chen, L., Karby, S., Weiss, F.R., Delarea, J. and Radu, M. (1979) Eur. J. Cancer 15, 639-643.
[12] Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Biophys. 37, 911-917.
[13] Menses, P. and Glonek, T. (1988) J. Lipid Res. 29, 679-689.
[14] Neeman, M. and Degani, H. (1989) Cancer Res. 49, 589-594.
[15] Besterman, J.M., Duronio, V. and Cuatrecasas, P. (1986) Proc. Natl. Acad. Sci. USA 83, 6785-6789.