A proteolipid in cancer cells is the origin of their high-resolution NMR spectrum

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High-resolution proton nuclear magnetic resonance studies show that the spectrum of a proteolipid complex, isolated from the serum of patients with malignant diseases, is directly comparable with that obtained from intact cancer cells and solid tumours. These NMR signals have previously been shown to reveal differences between cancer cells with various biological characteristics such as metastatic capacity and drug sensitivity. The proteolipid contains cholesterol, phospholipid, triglyceride, glycolipids, ether-linked lipids, and an apoprotein of unusual electrophoretic mobility. We have yet to confirm the presence of the mRNA reported by others. NMR spectroscopy could be used as a rapid method of identifying the presence of this proteolipid complex in human serum and aiding the diagnosis of malignant disease.

NMR Proteolipid (Human serum) Malignancy

1. INTRODUCTION

High-resolution ^1H NMR signals from cancer cells correlate with their metastatic ability [1] and drug sensitivity [2] as well as being able to show malignant change prior to histological identification [3]. The origin of the NMR signal is the plasma membrane [4] and 2D NMR methods identify neutral lipid as being primarily responsible for this spectrum [5]. Lipoprotein complexes which have a core of neutral lipid [6] have been shown to have similar yet different characteristics to the plasma membrane lipid domain responsible for the high-resolution NMR spectra from malignant cells [7]. An RNA-proteolipid complex has recently been isolated from the serum of patients with malignant diseases and in the medium from cultures of malignant cells [8].

We have isolated a proteolipid and the remaining lipoproteins from the plasma of a patient with an ovarian tumour. The various lipoproteins, proteolipid, and a tumour biopsy have been studied by NMR methods. The proteolipid complex has been characterised further by lipid analysis, electron microscopy and biochemical assays.

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2. MATERIALS AND METHODS

2.1. *Isolation of lipoproteins and proteolipid*

The total lipoprotein fraction was isolated as described by Goldstein et al. [9] from the serum of a patient with an ovarian cancer. An opalescent band, visible between LDL and HDL and containing the proteolipid complex, was isolated from the total lipoprotein fraction on a KBr gradient [8].

2.2. *Chemical analysis*

Total protein, lipid, triglyceride, phospholipid, free and esterified cholesterol were measured as described in [1]. RNA was determined by the orcinol method [10] with corrections for glycolipid interference. Extraction and quantitation of gangliosides (assumed to be mainly monosialoganglioside as judged by thin-layer chromatography) were performed as described [11]. Neutral glycosphingolipid was estimated by the orcinol method [12]. Total *O*-alkyl and *O*-alkenyl glycerolipids were determined as already reported

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[13]. Isolated lipoproteins and proteolipid were examined by electrophoresis on 1% agarose with 1 M barbital buffer, pH 9.0. Gels were stained with oil red O and positions of apo A- and apo B-containing lipoproteins were ascertained from a normal plasma control.

2.3. *Electron microscopy*

Particles were examined by negative-staining electron microscopy. The preparations were stained with 1% sodium phosphotungstate (pH 7.3) and the electron micrographs obtained with a Philips 400 electron microscope, operating at 100 kV and a maximum working magnification of $\times 92000$. For the distribution of particle diameters, 400 particles were measured from electron micrographs.

2.4. *NMR spectroscopy*

^{1}H NMR spectra were recorded at 37°C using a Bruker WM-400 spectrometer equipped with an Aspect 2000 computer. 2D scalar correlated spectroscopy (COSY) experiments [14] and T_1 and T_2 measurements [15] have been described.

2.5. *Enzyme studies*

Either RNase A (0.024 IU, Sigma, EC 3.1.1.34) or lipoprotein lipase (4.2 IU, bovine pancreas, Boehringer-Mannheim, EC 3.1.27.5) was added to 1 ml of proteolipid complex which contained 367 μg protein, and incubated for 1 h at 37°C prior to NMR study.

3. RESULTS AND DISCUSSION

3.1. *Chemical analysis*

The lipid, protein, and nucleic acid content of the band containing the proteolipid complex and the remaining lipoproteins isolated from the serum are shown in table 1. The composition of the isolated proteolipid complex is similar to that reported by Wieczorek et al. [8] except for a considerably smaller RNA content as measured by the orcinol method. This method does not confirm the presence of intact mRNA, since it detects only ribose. In addition, triglyceride and ether-linked lipids as well as gangliosides and an apoprotein with an unusual electrophoretic mobility (>pre-β) are present in this complex (table 1).

Glycolipid, both neutral and acidic, constitutes 20% of the proteolipid complex. It is also present in the LDL (23%) and HDL (11.2%) fractions of the plasma. Glycolipids are only present in trace amounts in lipoproteins from healthy donors [16]. However they have been found in neoproteolipids W and S [17] which were isolated from tumours

Table 1

Lipoprotein and proteolipid complexes isolated from the plasma of a patient with an ovarian tumour

	Size (nm)	Longest T_2 (ms)	Percentage of total weight									
			RNA	Protein		Triglyceride	Ganglioside	Neutral glycosphingolipid	Phospholipid	Cholesterol		Ether-linked lipid
										Free	Esterified	
VLDL and chylomicra[a]	26–156	188	–	apo B								
LDL	19– 23	111	nil	apo B	15.7	14.2	1.1	21.9	26.8	6.6	13.2	0.4
Proteolipid	22– 25 and 8– 11	852	4.1	apo A apo B unknown	19.9	13.5	4.5	15.5	25.2	6.1	10.7	0.4
HDL	6– 9	154	nil	apo A	45.2	8.7	1.3	9.9	23.8	1.9	8.4	0.6

[a] Protein and lipid not quantitated due to a low yield

and the serum of cancer patients. The relationship between the nucleic acid containing proteolipid and the neoproteolipid complexes is unclear since no nucleic acid analysis was undertaken on the latter.

3.2. *Electron microscopy*

From electron micrographs two particle sizes, 8–11 and 22–25 nm, in approximately equal numbers were identified in the opalescent band containing the proteolipid complex. We consider these two particle types to differ from the conventional LDL and HDL, since unusual electrophoretic mobility is observed in the opalescent band but not in LDL or HDL bands which are located on either side (table 1).

3.3. *NMR spectroscopy*

The NMR method used to distinguish metastatic cells from their malignant but non-metastatic counterparts in rats is the Meiboom-Gill modification of the Carr-Purcell (CPMG) pulse sequence which provides a spin-spin (T_2) relaxation time [15]. In metastatic cells the longest T_2 is of the order of 0.4–1 s whereas in non-metastatic cells and normal serum lipoproteins [7] it is less than 0.25 s [1].

The ^{1}H NMR spectra and T_2 relaxation profiles of resonances in the methylene region are shown in fig.1 for the total plasma lipoproteins (including the proteolipid complex), the isolated proteolipid complex alone and VLDL and LDL, from the same patient. Only the proteolipid complex has a long T_2 relaxation value of 0.85 s which contrasts with the lipoproteins which are all less than 0.2 s (table 1). Resonances c and d in the spectrum of the proteolipid, both of which have a long T_2, are not present in the spectrum of any conventional lipoproteins. This long T_2 can be measured not only in the isolated complex and total lipoprotein fraction, but also in the serum and the tumour itself. Biopsy samples of tumours on both ovaries from this patient were examined and T_2 values of 0.85 and 0.65 s recorded.

Cells and tumours both gave identical values for the selective and non-selective spin-lattice (T_1) relaxation experiments [15]. The same phenomenon was observed for the proteolipid complex where the T_1 was found to be 0.45 s. These measurements rule out either diffusion or spin exchange between the molecules generating the spectrum. This was not the case for LDL or HDL from healthy donors where not only did the selective and non-selective measurements differ but a non-exponential decay was observed in the selective T_1 experiment [7]. This is an interesting comparison since the proteolipid is isolated between these two fractions on the KBr gradient. Nevertheless it is another physical measurement whereby the cells and tumours show identical behaviour to the proteolipid complex.

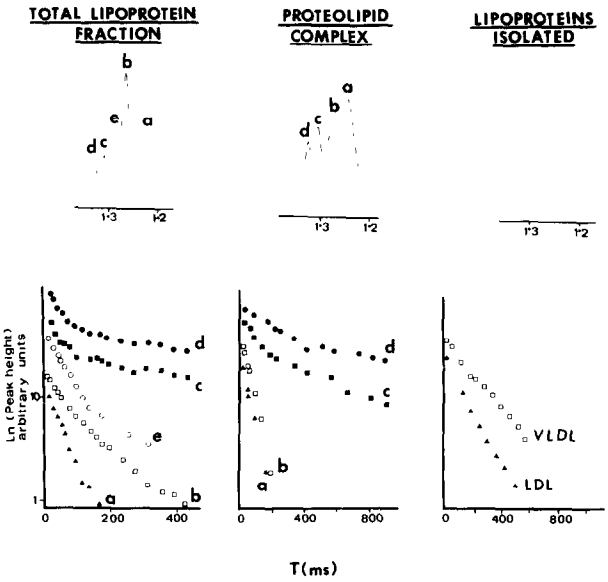


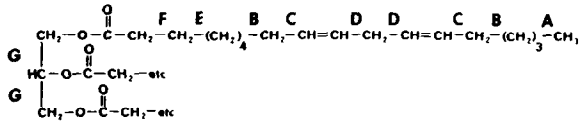
Fig.1. 400 MHz ^{1}H NMR spectra of the methylene region of: total lipoprotein fraction (including the proteolipid complex) isolated from the plasma (1); the opalescent band (containing the proteolipid complex) isolated from the total lipoprotein fraction on a KBr gradient [8] (2); lipoproteins (LDL and VLDL) which were isolated from the same plasma sample (3). The spectra are resolution enhanced by the Lorentzian-Gaussian method described in [15] using LB = −12; GB = 0.08. Below the NMR spectra the results of the CPMG T_2 relaxation experiment executed as described in [15] are shown. The natural log of each peak height is plotted against the delay between the first pulse and the nth echo. The T_2 values are calculated using a least squares method where $r^2 \geq 0.98$. The longest T_2 (ms) values of each profile are as follows: (1) a = 84, b = 181, c = 730, d = 660, e = 226; (2) a = 68, b = 63, c = 671, d = 970; (3) VLDL = 188, LDL = 111.

Attempts were made to identify the origins of the four methylene resonances which occur at 1.33,

1.31, 1.28 and 1.26 ppm in the spectrum of the proteolipid complex. RNase A had no effect on any of these resonances. This is consistent with the report by Wieczorek et al. [8] that RNase did not affect the nucleic acid content of the intact particle. Lipoprotein lipase, which degrades triglycerides caused resonances a and b at 1.26 and 1.28 ppm to decrease significantly in intensity. These two resonances have a short T_2 of less than 200 ms.

The assignment of resonances a and b to triglyceride is confirmed by 2D NMR methods (fig.2). 2D scalar correlated spectroscopy (COSY) methods allow assignment of resonances based on spin-spin (scalar) coupling. The off diagonal cross peaks denoted A–G (fig.2) indicate spin-spin coupling between protons on adjacent atoms.

The presence of cross peaks A–E (as summarised in scheme 1), and the cross peak G′ (at 4.3 ppm) which is unique to triglyceride, indicate the presence of this neutral lipid. In contrast to the cell spectrum, the assignments for which have been documented previously [5], cross peak F is not



Scheme 1

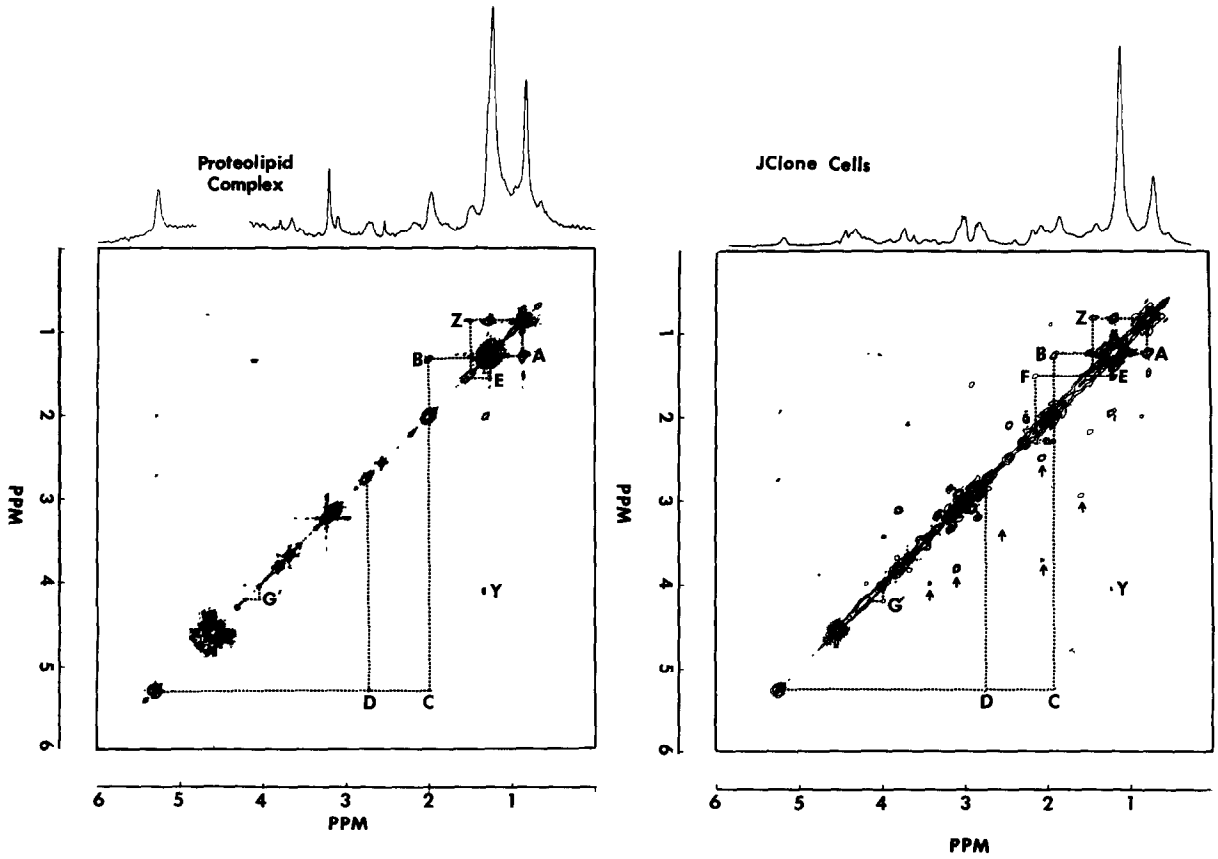


Fig.2. ^{1}H NMR spectra (400 MHz) of: proteolipid complex isolated on a KBr gradient [8] from the lipoprotein fraction of a patient with an ovarian tumour. Suspended in NaCl/D_2O (1); a suspension of the rat mammary adenocarcinoma J clone cells (1×10^8) suspended in PBS/D_2O (2). The one-dimensional spectra, transformed with a 3 Hz line broadening, are shown above the 2D scalar correlated spectroscopy (COSY) spectra executed as described in [14]. A Gaussian window function was applied in the t_2 domain (LB = −16; GB = 0.22) and a Sine-bell in the t_1 domain. Lipid acyl chain and glycerol connectivities assigned A–G are shown in scheme 1. Z denotes the cross peak between the methyl and methine protons of the alkyl side chain of the cholesterol ring system. Y is yet to be identified. The arrows in the cell spectrum point to cross peaks previously assigned to metabolites in the cell's cytoplasm [5].

observed in the spectrum of the proteolipid complex. The absence of this cross peak is likely to be due to a lack of signal since G′ is only just observable and triglyceride accounts for 18% of the total lipid in the proteolipid complex. Resonances c and d at 1.33 and 1.31 ppm have yet to be definitively assigned, but have chemical shifts consistent with the cross peak Y (fig.2).

We conclude that a proteolipid complex similar to that reported by Wieczorek et al. [8] accounts for almost all of the NMR signals from the plasma membrane of cancer cells, and for the unusually long T_2 relaxation value. Our data strongly suggest that a proteolipid is the plasma membrane lipid domain that gives high-resolution ^1H NMR signals. The presence of a proteolipid complex containing nucleic acid in the plasma membranes may provide an interesting explanation for the altered biological status of cancer cells as observed by NMR.

Furthermore, these observations may be relevant in NMR imaging which currently does not satisfactorily distinguish malignant from benign tumours [18]. Our data suggest that depending on the amount of proteolipid present in a tumour and its surrounds, the T_2 relaxation value will have contributions from this complex and may therefore be useful in tumour imaging.

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