

Cell surface involvement in cancer metastasis: an NMR study

Kerry T. Holmes, Philip G. Williams, George L. May, Patricia Gregory, Lesley C. Wright, Marlen Dyne and Carolyn E. Mountford*

Ludwig Institute for Cancer Research (Sydney Branch), Blackburn Building, University of Sydney, Sydney, NSW 2006, Australia

Received 22 April 1986

NMR spectroscopy is one of the few techniques which has the sensitivity to detect subtle changes to the surface chemistry of cells. It has previously been demonstrated that high resolution ^1H NMR methods can distinguish tumour cells with the capacity to metastasise and this information appears to arise from a type of proteolipid in or attached to the plasma membrane. Here we report that the ^1H NMR signal, which we have used to identify metastatic cells in rat tumours, is significantly reduced in intensity after cultured cells are treated with trypsin/EDTA. The long T_2 relaxation value (> 350 ms) observed in metastatic cells is absent after enzyme treatment. 2D scalar correlated NMR (COSY) spectra of these treated cells show that a cross peak normally associated with malignancy and metastatic disease is markedly reduced. These findings indicate that the plasma membrane lipid particle which generates the high resolution spectrum is directly affected by trypsin/EDTA. Alterations to the cell surface properties were also demonstrated *in vivo* since reduced numbers of metastases were observed in animals injected with enzyme-treated cells. The correlation between the absence of a long T_2 relaxation value and the diminished numbers of metastases in animals suggests that the plasma membrane particle is involved in the metastatic process.

NMR Experimental metastasis Trypsin/EDTA Proteolipid

1. INTRODUCTION

The process of metastasis has many steps and some of these involve the plasma membrane. Much attention has been focussed on cell surface properties in malignancy and there is now ample evidence that variations in membrane components affect metastatic potential [1–4]. Additionally, it has been shown that tissue culture procedures alter the plasma membrane of cells and affect their behaviour when injected into animals. For example, removal of cells from flasks with standard trypsin/EDTA treatment results in diminished numbers of lung metastases in both B16 melanoma [5,6] and murine mammary tumour lines [7].

High resolution ^1H NMR spectra from lipids in the plasma membrane of cancer cells [8] are an indication of the biological status of those cells [9]. These NMR spectra have been used to monitor metastatic potential [10]. Two-dimensional (2D) NMR of whole cells [11] and chemical analysis of highly enriched plasma membranes have identified the origin of the high resolution signal as predominantly neutral lipids in a particle associated with the plasma membrane [12]. This particle closely resembles (unpublished) an mRNA proteolipid complex produced by malignant cells [13]. In the rat mammary adenocarcinoma 13762 experimental model, analysis of the NMR spectrum enables us to distinguish cells with metastatic potential [10] by the presence of a long (> 350 ms) transverse relaxation parameter, T_2 .

* To whom correspondence should be addressed

We report here a study of the N5 clone of the rat mammary adenocarcinoma line 13762, where we correlate the effect of trypsin/EDTA on in vivo metastatic behaviour with its effect in vitro on the ^1H NMR parameters diagnostic of metastatic potential.

2. MATERIALS AND METHODS

2.1. *Animals*

Female Fisher 344 rats were purchased from Charles River Laboratories (Wilmington, MA). Rats used in experiments were 8–12 weeks old. They were fed normal food and acidified water ad libidum.

2.2. *Cell cultures and cloning procedures*

The origin and properties of the rat mammary adenocarcinoma cell line have been described [14]. The N5 clone was isolated by soft agar plating [15]. Cells, grown in RPMI-1640 medium supplemented with 10% foetal calf serum (FCS) at 37°C, were maintained in the logarithmic phase of growth with a doubling time of 24–26 h. The lightly adherent monolayers could be dislodged by simply tapping the flask sharply on a padded surface.

2.3. *Experimental metastasis*

Suspension cultures of N5 cells were washed twice in 0.9% NaCl, incubated for 10 min with 0.05% trypsin/0.02% EDTA and suspended for 10 min in 20 ml RPMI-1640 medium with 10% FCS at 37°C. Cells (1×10^7) were injected into the mammary line of 13 rats. Similarly suspension cultures of N5 cells were washed twice in 0.9% NaCl and incubated in the absence of enzyme for 10 min in 20 ml RPMI-1640 medium with 10% FCS at 37°C prior to injection into 13 control rats. Tumours (~4 cm diameter) were excised 4 weeks after implantation. The lungs were removed, rinsed in saline and fixed in formalin to be checked for lung colonies. Lymph nodes were examined for the presence of tumour nodules.

2.4. *Preparation of samples for NMR spectroscopy*

Cell samples were prepared for NMR experiments as described [8] and kept at 37°C in the NMR tube for 1 h prior to the commencement of the experiment. Cell viability was measured at the

end of the experiment by trypan blue exclusion and only data from cells with at least 90% viability used [9].

2.5. *NMR spectroscopy*

^1H NMR spectra were recorded and CPMG pulse sequences executed as described [12,16]. The 2D scalar correlated (COSY) pulse sequence used for cells has been described in [11]. In our previous publications resonances have been referenced to the methyl peak at 0.85 ppm. Here we reference to an external standard of trimethylsilylpropane-sulphonic acid which gives a chemical shift of 0.91 ppm for the methyl resonance.

3. RESULTS

3.1. *Animal experiments*

Animals were divided into two groups of 13. Treatment of the N5 cells with trypsin/EDTA prior to injection in the mammary line of the rats resulted in a 38% rate of lymph node metastasis, compared to 85% for the untreated control cells. No differences were seen in the size of the primary tumours in each case and no lung colonies were detected.

3.2. *T_2 relaxation measurements*

The 400 MHz ^1H NMR spectrum of the N5 cell line is shown in fig.1. The composite resonance at 1.3 ppm has been assigned to fatty acyl chain ($-\text{CH}_2-$) resonances of neutral lipids in lipoprotein/proteolipid-like particles in the plasma membrane of the cells [12,16]. This resonance can be resolved into several components by Lorentzian-Gaussian deconvolution techniques [17] (fig.1), and the T_2 values of these component peaks measured. The long T_2 value (>350 ms) of the peak at 1.33 ppm has previously been shown to correlate with metastatic behaviour in the rat mammary adenocarcinoma experimental model [10]. The measured T_2 values for all resolved peaks are decreased after enzyme treatment, but the peak at 1.33 ppm shows the most dramatic change (table 1). In the untreated N5 cells this peak has a T_2 of 794 ms which after exposure to trypsin/EDTA decreases to 176 ms. Comparison of fig.1A and B reveals that the intensity of this peak is greatly diminished by enzyme treatment, and it is this remaining signal that has the T_2 of 176 ms.

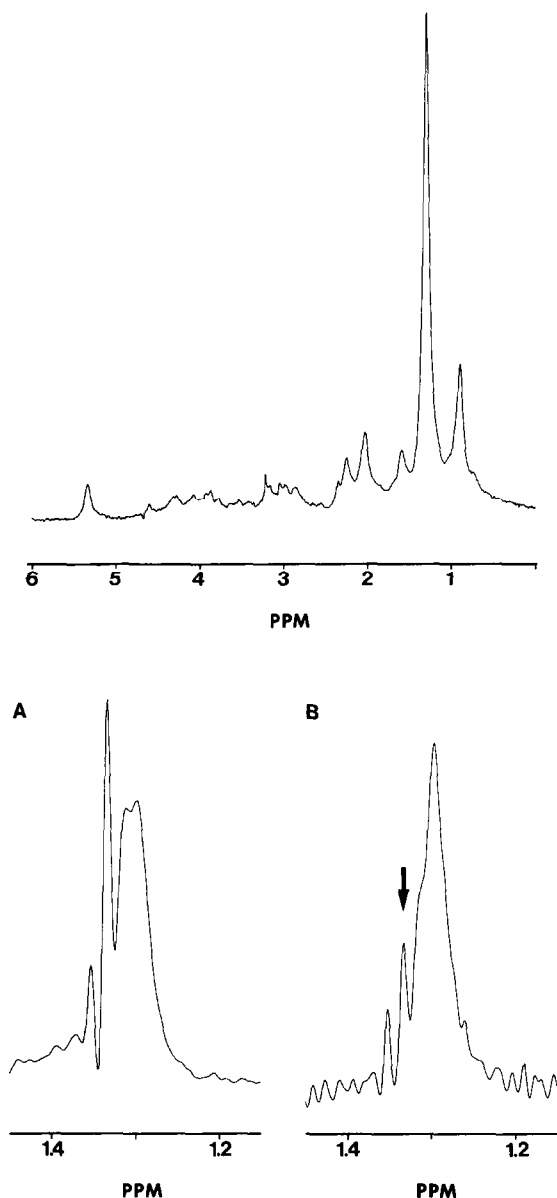


Fig.1. 400 MHz ^1H NMR spectra of a suspension of N5 cells (1×10^8) in phosphate-buffered saline in D_2O . Data were obtained at 37°C with the sample spinning and with suppression of residual HOD signals by gated irradiation. The resonances under the composite ($-\text{CH}_2-$) peak were resolved by application of the Lorentzian-Gaussian enhancement technique [17]. The resolution enhanced spectra are plotted over the methylene region (1.2–1.4 ppm), on an expanded scale. (A) Untreated cells. (B) Cells treated with 0.05% trypsin and 0.02% EDTA.

3.3. 2D COSY experiments

A contour plot of the 400 MHz 2D COSY spectrum of N5 cells is shown in fig.2. Many of the cross peaks in the 2D spectra of cancer cells arise from lipids, as shown by the lipid connectivities designated A–G in the triglyceride structure [11,12,16]. Other cross peaks arise from soluble cytoplasmic components and are indicated by arrows [12].

Cross peak Y is of particular interest. It connects resonances at 1.3 and 4.2 ppm and appears in the spectra of all malignant and metastatic cells and tumours studied thus far. The connectivity is not consistent with protons in an acyl chain and remains to be assigned. It coincides with the peaks at 1.35 and 1.33 ppm observed in the resolution enhanced spectrum. Fig.2A shows a vertical slice through the COSY contour plot at 4.2 ppm, and cross peak Y (at 1.3 ppm) is clearly observed in the untreated N5 cells. However, after treatment with trypsin or EDTA the intensity of this peak is reduced by approx. 60% (fig.2B).

4. DISCUSSION

We have been able to diminish both metastatic ability (as deduced by *in vivo* assays), and the signal which gives the long T_2 value by trypsin/EDTA treatment. These data suggest that the NMR signal giving rise to the long T_2 value arises from the outside of the cell, and that the particle we are observing is involved in the metastatic process.

Table 1

Transverse relaxation parameter (T_2) for resonances under the methylene in cell line N5 before and after treatment with trypsin/EDTA

Chemical shift (ppm)	T_2 (ms)	
	Control	Enzyme treated
1.30	188 ± 19	137 ± 18
1.31	219 ± 12	151 ± 9
1.33	794 ± 58	176 ± 12
1.35	241 ± 6	123 ± 17

Spectra were resolution enhanced by the Lorentzian-Gaussian method [17]. Results are expressed as the mean $\pm$ SE of 2 experiments

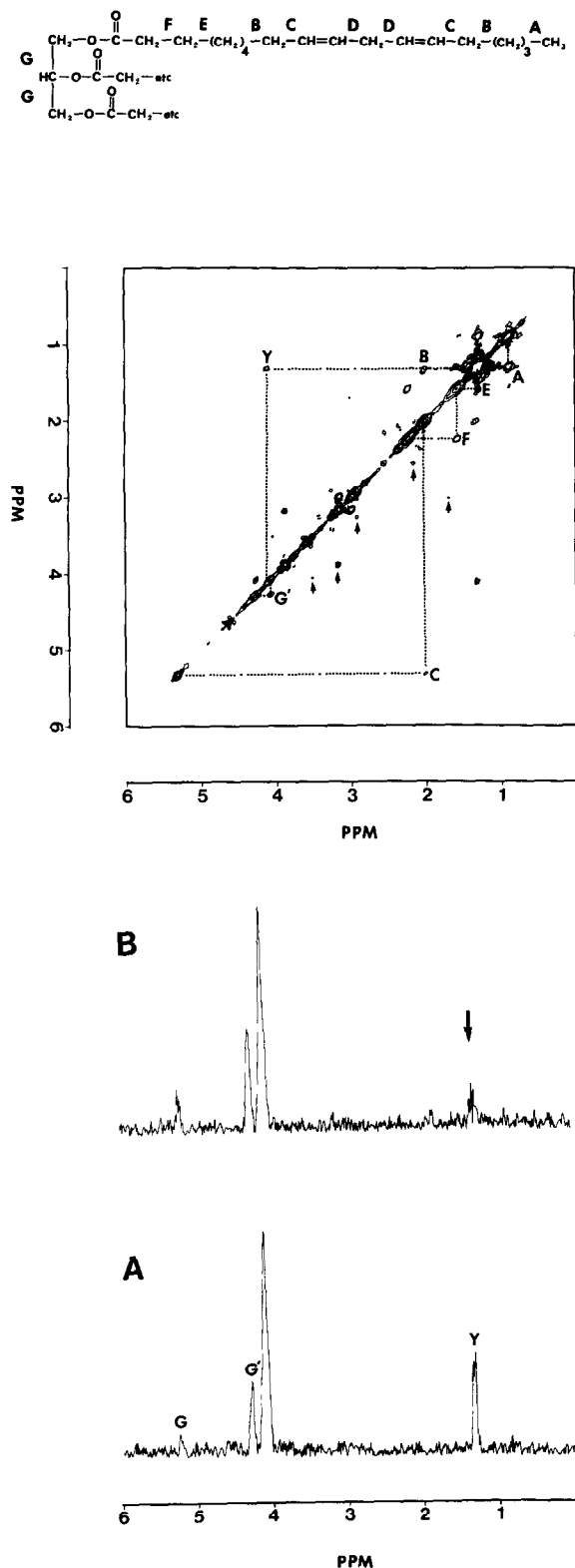


Fig.2. 400 MHz ^1H NMR 2D COSY spectrum of a suspension of N5 cells (1×10^8) in phosphate-buffered saline in D_2O . Data were obtained at 37°C with the sample spinning and with suppression of residual HOD signals by gated irradiation. Sine-bell and Gaussian ($\text{LB} = -30$, $\text{GB} = 0.25$) window functions were applied in the t_1 and t_2 domains, respectively. Cross peaks are assigned as described [11,12], with reference to the labelled structure. A vertical slice through the COSY contour plot at 4.2 ppm yields all the protons coupled to that peak. Cross peaks G and G' are the vicinal methylene-methine and geminal couplings, respectively, in the glycerol portion of triglyceride [11,12,16]. Cross peak Y is not yet unequivocally assigned. (A) Untreated cells. (B) Cells treated with 0.05% trypsin and 0.02% EDTA.

Cross peak Y in the 2D spectrum is decreased under these experimental conditions and, although the nature of the chemical species generating this NMR signal is not yet identified, these data suggest that it is an integral part of the proteolipid-like particle we detect in malignant cells using NMR spectroscopy. The significant decrease in the intensity of the resonance at 1.33 ppm after trypsin/EDTA treatment (fig.1) is consistent with the reduction of cross peak Y in the 2D spectrum (fig.2). Collectively these data suggest that an overlying resonance has been removed by the trypsin/EDTA treatment and the remaining short T_2 of 176 ms is from the underlying lipid resonance which gives rise to cross peak B (fig.2).

The involvement of plasma membrane vesicles in the transfer of metastatic potential from one cell to another was first described by Poste and Nicolson [1]. It is possible that proteolipid complexes [13] were amongst the vesicles transferred in these experiments.

Isolation of proteolipids produced by malignant cells in vitro may help to identify the functional molecules and establish their role in the metastatic process.

ACKNOWLEDGEMENT

We would like to thank Professor M.H.N. Tattersall for helpful discussions.

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