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Inhibition of Metastatic Potential by Fucosidase: An NMR Study Identifies a Cell Surface Metastasis Marker

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NMR spectroscopy is able to detect subtle changes to the surface chemistry of cells. We have previously shown that high-resolution ^{1}H NMR methods can identify tumor cells with the capacity to metastasize, and we now report that the long T_2 relaxation value (500–800 ms) observed in metastatic rat mammary adenocarcinoma cells is removed by treatment with fucosidase. Two-dimensional scalar-correlated NMR (COSY) spectra of fucosidase-treated cells show that a cross peak, consistent with scalar coupling between the methyl and methine groups on fucose and usually associated with malignancy and metastatic ability, is absent. Metastases were observed in only two out of ten rats injected subcutaneously with enzyme-treated cells compared to eight out of ten with untreated cells. NMR studies on isolated cellular lipids identified the long T_2 relaxation value only in the ganglioside fraction. This fraction accounts for 51% of the total ^{14}C-labelled fucose incorporated into the cells. We propose that fucogangliosides are an indicator of metastatic potential in rats. The observation that a cell surface metastasis marker has an NMR signal with a characteristically long relaxation value has important consequences for the future use of magnetic resonance imaging and spectroscopy in the cancer clinic.

Key words: NMR spectroscopy, fucoganglioside

Cancer metastasis is known to occur in several stages, some of which involve the plasma membrane [1–4]. Recently the role of cell surface glycoproteins and glycolipids in malignancy has attracted considerable interest [5–6].

High-resolution ^{1}H NMR (nuclear magnetic resonance) signals from molecules in the plasma membrane of cancer cells have been used to monitor metastatic potential [7]. Cells with metastatic potential were found to have a long T_2 (transverse) relaxation value (400–800 ms) while those not capable of generating secondary deposits had a T_2 of less than 350 ms.

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Two-dimensional (2D) NMR of whole cells and chemical analyses of highly enriched plasma membranes have identified the origin of the high-resolution signal as predominantly neutral lipids in domains in or associated with the plasma membrane [8]. These domains closely resemble proteolipid complexes produced by malignant cells [9,10], which have a core of triglyceride and cholesteryl ester surrounded by phospholipid and glycolipid.

The involvement of plasma membrane vesicles in the transfer of metastatic potential from one cell to another was first described by Poste and Nicolson (1980) [4]. We now consider the possibility that proteolipid complexes were responsible for transferring the metastatic potential in these experiments since the molecule responsible for the long T_2 is also found in the proteolipids isolated from sera of patients with malignant disease [10]. It is thus important to identify the molecule which has the long T_2 relaxation value and provides information about cancer metastasis.

Protons which resonate at 1.3 ppm generate the long T_2 relaxation value. Two-dimensional scalar-correlated spectroscopy (COSY) shows coupling of this resonance to protons resonating at 4.2 ppm. This cross peak, labelled Y, is consistent with the methyl-methine coupling of fucose, lactate, and threonine.

Trypsin/EDTA treatment decreases the long T_2, diminishes cross peak Y, and reduces metastasis in vivo [2]. Trypsinization cleaves many cell surface molecules and removes glycosaminoglycans [11] and exogenously bound gangliosides [12]. Since fucose is found in such glycolipids, the possibility that metastasis might involve surface molecules other than proteins arises.

We report here a study of the rat mammary adenocarcinoma cell line R13762 wherein the effect of fucosidase on in vivo metastatic behavior is compared with its effect in vitro. Enzyme treatment affects the metastatic but not tumorigenic properties of the cells and reduces the T_2 relaxation value from 800 to 325 ms. Fucogangliosides are identified as the origin of the long T_2 relaxation parameter. These molecules generate a distinctive NMR parameter which could be of use in future diagnosis of metastatic disease.

MATERIALS AND METHODS

Animals

Female Fischer 344 rats were bred in the Ludwig Institute for Cancer Research (Sydney Branch). Rats used in experiments were 12–16 wk old. They were fed standard rat chow and acidified water ad libidum.

Cell Cultures

The origin and properties of the rat mammary adenocarcinoma cell line have been described [13]. Cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) at 37°C and were maintained in the logarithmic phase of growth with a doubling time of 17 hr. The lightly adherent monolayers could be dislodged by tapping the flask sharply on a padded surface.

J clone cells can partially revert to the R13762 high metastatic cell line [7] generating a low metastatic cell line RJC. These cells when injected into rats generate primary tumors which grow more slowly than the R13762 and generate secondary growths in the gut or lymph nodes.

Enzyme Treatment

Cells (1×10^8) were incubated at 37°C with fucosidase in PBS (Sigma, bovine epididymus E.C.3.2.1.51, 0.125 unit) for 30 min and then washed several times in phosphate-buffered saline (PBS) in D_2O. This enzyme was used without further purification. Less than 0.06% proteinase activity was detected, using bovine serum albumen as substrate, and β-N-acetylglucosaminidase activity was less than 0.1%; α and β-galactosidase, α-mannosidase, and β-fucosidase activities were all $< 0.2\%$ of the α-fucosidase activity. Viability of the cells was not diminished by this treatment despite a temporary clumping of the cells during incubation. Viability was determined microscopically by trypan blue exclusion.

Lactate Assay

A UV spectrophotometric method was used [Boehringer Mannheim UV-Test Kit (L-lactic acid) No. 137984].

The Distribution of ^{14}C Fucose in R13762 and RJC Cellular Components

Cells were grown for 48 hr in RPMI medium with 10% fetal calf serum containing 0.2 μCi L-[1-^{14}C] fucose (Amersham) (55 mCi/mmol) per 100 ml of cell suspension. Cells were split into two batches.

(1) The first batch was washed in saline and disrupted by sonication, and protein was precipitated with 1% tungstophosphoric acid in 0.5 M HCl. The precipitate was washed twice with tungstophosphoric acid and twice with $CHCl_3$:MeOH (2/1, v/v). The organic solvents were evaporated to dryness in a scintillation vial, and aqueous supernatant (1.5 ml) was added to it, together with 10 ml ACS scintillant. The protein pellets were dissolved in 400 μl 3 N NaOH at 60°C and neutralized after 1 hr with 600 μl 2.7 N HCl; then they were mixed with 10 ml of ACS scintillant. Samples were counted on a Rack-Beta scintillation counter.

(2) The second batch of washed cells was extracted with $CHCl_3$:MeOH by the method of Gottfried [14]. The filtered extract was evaporated to dryness and partitioned three times between $CHCl_3$:MeOH (2:1) (5 volumes) and H_2O (1 volume). The organic layer was dried over Na_2SO_4 and then evaporated to dryness in a scintillation vial, to which 1 ml H_2O and 10 ml ACS scintillant were added. The methanol-H_2O phase was dialyzed overnight against distilled H_2O at 4°C, evaporated to 1 ml, and mixed with 10 ml ACS scintillant. The samples were counted in a Rack-Beta scintillation counter.

Ganglioside Isolation

Cells (5×10^8) were washed three times in saline (0.9%) and then extracted by the method of Gottfried [14]. Partitioning against H_2O with subsequent dialysis was performed as above. The aqueous layer after dialysis was evaporated to dryness (rotary evaporator, 50°C) and transferred to a small vial in $CHCl_3$:MeOH (2:1) through an Acrodisc filter. The solvents were evaporated under nitrogen and by vacuum pump and taken up in D_2O (0.5 ml) with warming and vortexing for the NMR experiments. The organic-phase lipids were evaporated to dryness and dispersed similarly in D_2O for NMR spectroscopy.

The ganglioside fraction was spotted on HPTLC plates (Merck Silica Gel 60) and developed in $CHCl_3$:MeOH:0.02% aqueous $CaCl_2$, 55:45:10 by volume. Gangliosides were visualized with resorcinol spray and heated at 100°C for 15 min [15]. Standards

were purchased from Supelco (G_{M1}, G_{D1a}, G_{T1b} + G_{D1b}, and bovine brain mixture). These abbreviations for gangliosides follow the nomenclature system of Svennerholm, IUPAC-IUB Commission on Biochemical Nomenclature. G_{M1} = II^3 Neu Ac $GgOse_4Cer$; G_{D1a} = IV^3 Neu $AcII^3$ Neu Ac $GgOse_4Cer$; G_{D1b} = II^3 $(Neu\ Ac)_2$-$GgOse_4Cer$; G_{T1b} = IV^3 Neu Ac, II^3 $(Neu\ Ac)_2$-$GgOse_4Cer$.

Experimental Metastasis

R13762 cells were washed three times in PBS, incubated for 30 min at 37°C with fucosidase (0.08 unit), and then resuspended and again washed three times in PBS. Cells (1×10^7 in RPMI-1640 medium) were injected into the fat pad of the inguinal area of the mammary line of ten rats. Similarly, R13762 cells were washed as above and incubated in PBS in the absence of enzyme for 30 min at 37°C prior to injection into ten control rats. A second set of controls consisting of incubated unwashed cells was injected into a further ten animals.

Primary tumors were first evident in the inguinal area of the mammary line approximately 10 days after injection of cells. Animals were killed 4 weeks after inoculation. Tumor diameters were measured with calipers, and all animals were examined for macroscopic metastases in the lymph nodes, lungs, gut, liver, and mesentery with metastases being found only in axial lymph nodes. No examination for micrometastases was carried out on the organs.

Preparation of Samples for NMR Spectroscopy

Cell samples were prepared for NMR experiments as previously described [16] and kept at 37°C in the NMR tube for 1 hr 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 were used [16].

NMR Spectroscopy

The ^1H NMR spectra were recorded, and CPMG (Meiboom-Gill modification of the Carr-Purcell) pulse sequences were executed as previously described [8,17]. The two-dimensional scalar correlated (COSY) pulse sequence used for cells has been described elsewhere [18]. Trimethylsilylpropane-sulphonic acid, which gives a chemical shift of 0.91 ppm for the methyl resonance, was used as a reference.

RESULTS

NMR Spectroscopy

The 1D ^1H NMR spectrum of the rat mammary adenocarcinoma R13762 cell line is shown in Figure 1. The composite resonance at ~1.25 ppm is predominantly due to fatty acyl chain ($-CH_2-$) resonances of neutral lipids. This resonance can be resolved into several components [17] by Lorentzian-Gaussian deconvolution techniques (Fig. 1A), and the T_2 values of these component peaks can be measured by the CPMG pulse sequence. The long T_2 value (>350 ms) of the resonance at 1.33 ppm has previously been shown to correlate with metastatic behavior in the experimental rat model. A contour plot of the 400-MHz 2D COSY spectrum of R13762 cells is shown in Figure 2. The lipid connectivities which have been described previously [8] are illustrated in Figure 3.

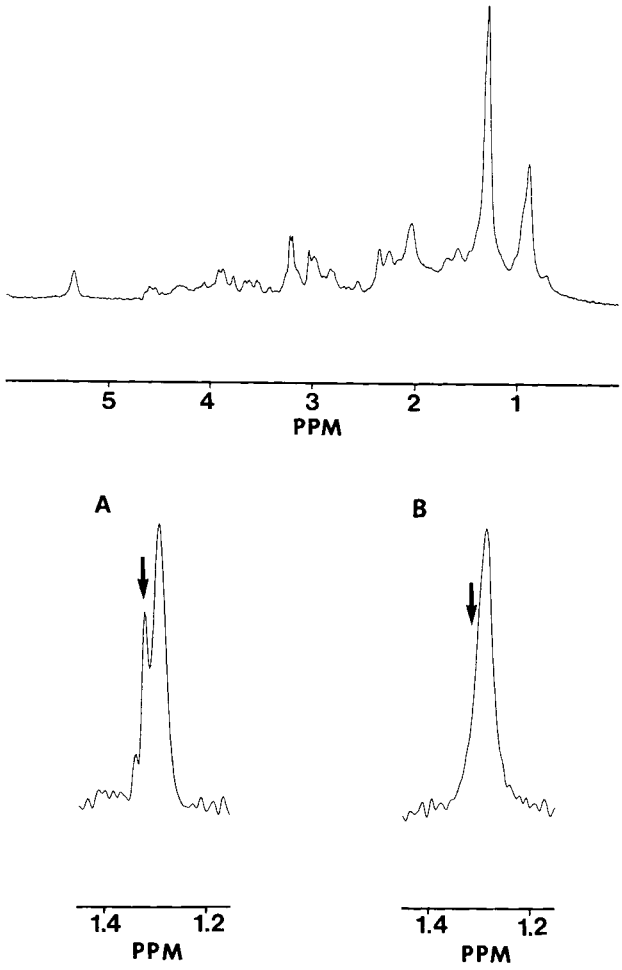

Fig. 1. 400-MHz ^1H NMR spectra of a suspension of R13762 cells (1×10^8) in phosphate-buffered saline in D_2O. Data were obtained at 37°C with the sample spinning and with suppression of the residual HOD peak by gated irradiation. The resonances under the composite (-CH_2-) peak were resolved [17] by application of the Lorentzian-Gaussian enhancement technique. The methylene region (1.2–1.4 ppm) of the resolution-enhanced spectrum is plotted on an expanded scale. **A:** Untreated cells. **B:** Cells treated with fucosidase.

Cross peak Y is of particular interest since it connects resonances at 1.3 ppm and 4.2 ppm and appears in the spectra of all metastatic cells and tumors studied so far. It coincides with the resonance at 1.33 ppm observed in the resolution-enhanced spectrum (Fig. 1A). Cross peak Y is consistent with a methyl-methine coupling on either fucose or lactate or threonine.

To distinguish between these possibilities the R13762 cells were treated with fucosidase. The treated cells were then washed to remove any free fucose. Two control samples were also studied. The first was untreated in any way, while the second was incubated for the same time as the enzyme-treated cells but in the absence of the fucosidase in order to eliminate possible loss of the relevant molecule by surface shedding.

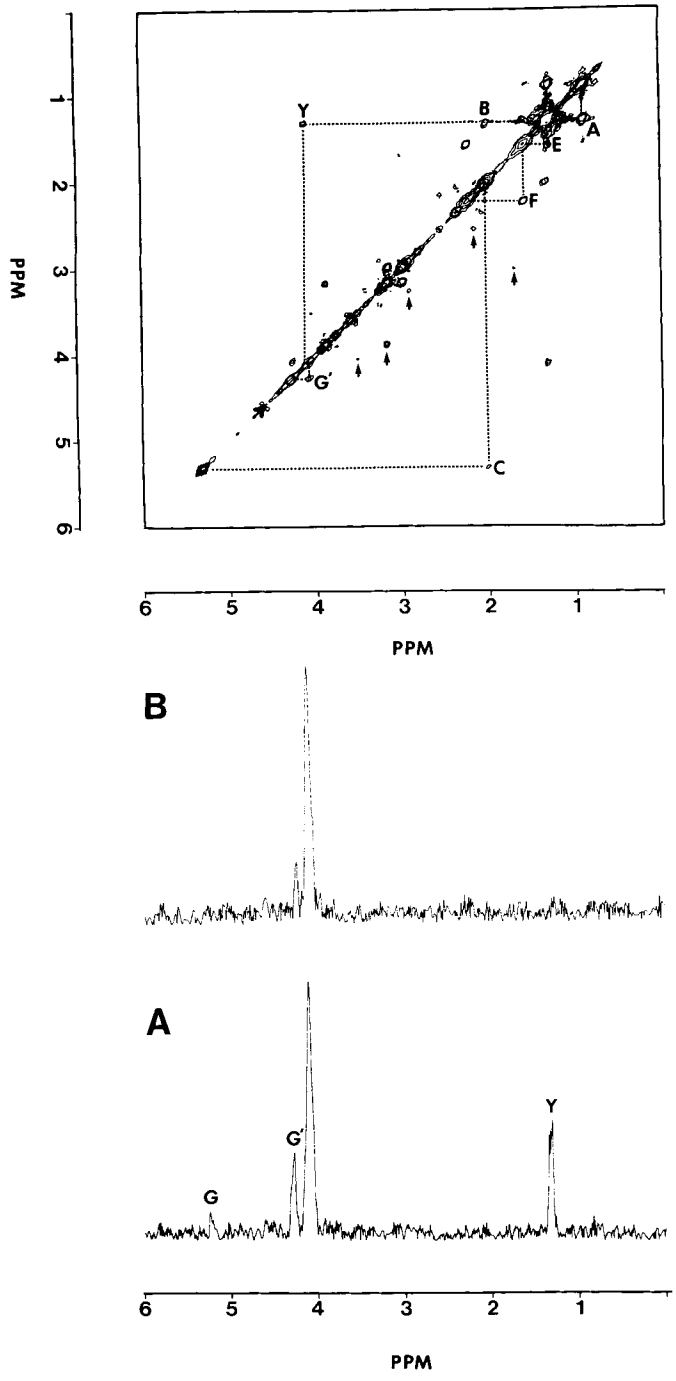

Fig. 2. 400-MHz ^{1}H NMR 2D COSY spectrum of a suspension of R13762 cells (1×10^8) in phosphate-buffered saline in D_2O. Data were obtained at 37°C with the sample spinning and with suppression of the residual HOD peak by gated irradiation. Sine-bell and Gaussian (LB = −30, GB = 0.25) window functions were applied in the t_1 and t_2 domains, respectively. Cross peaks are assigned as previously described [8], with reference to the labelled structure. A vertical slice through the COSY contour plot at 4.2 ppm yields the chemical shifts of all the protons coupled to that peak. Cross peaks G and G′ are the vicinal methylene-methine and geminal methylene couplings, respectively, in the glycerol portion of the triglyceride molecule. **A:** Untreated cells. **B:** Cells treated with fucosidase.

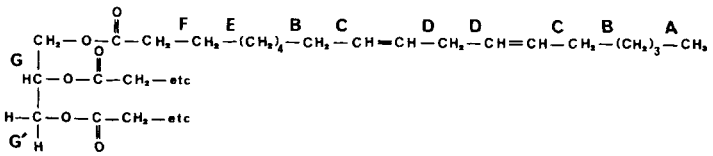

Fig. 3. Structure 1, structure of triglyceride molecule with connectivities corresponding to possible cross peaks in 2D COSY spectrum shown in Figure 2.

The resonance at 1.33 ppm remained in both control samples (Fig. 1A) but was significantly reduced in intensity after fucosidase treatment (Fig. 1B). The measured T_2 values for both control samples were > 700 ms, whereas the cells treated with fucosidase gave a T_2 of less than 350 ms (Table I).

When a vertical slice is taken through the COSY contour plot at 4.2 ppm, cross peak Y (at 1.3 ppm) is clearly observed in the untreated R13762 cells (Fig. 2A). However, this cross peak is not seen in cells treated by fucosidase (Fig. 2B). Although the enzyme contains minor contamination from other glycosidases, the other carbohydrate molecules do not have a resonance in this region of the NMR spectrum.

Threonine is eliminated as a possibility because cross peaks from the α and β CH protons at 4.1 and 4.3 ppm, respectively, are absent. Free lactate in the medium surrounding the cells in the NMR tube has been measured biochemically and found to be the same in both the metastatic R13762 and nonmetastatic J clone cells under the conditions of the NMR experiment (263 ± 1 μg/10^8 cells). Despite the same levels of free lactate, the T_2 value for R13762 is more than double (780 ms) that measured for J clone cells (325 ms), which implies that lactate is not responsible for the long T_2 of the resonance at 1.33 ppm.

We therefore conclude that the nucleus responsible for generating the long T_2 and cross peak Y has been removed by fucosidase treatment.

TABLE I. Effects of Fucosidase on the Metastasizing Capacity of R13762 Cells*

Treatment of cells	Diameter primary tumors (cm)	Metastatic deposits (cm^3)	Rats with metastases (10/group)	T_2[a] (ms)
None	4–5	64 ± 6	8	760 ± 48
Incubated in PBS at 37°C for 30 min without enzyme	4–5	62 ± 6	7	857 ± 80
Incubated in PBS at 37°C for 30 min with 0.07 units fucosidase	3–4	1 ± 0.1	2	390 ± 65
		($P < .01$)		

*1 × 10^7 cells were injected into the mammary line fat pad of Fischer rats. Rats were killed 4 wk after the initial injection and examined for macroscopic metastases. The volumes of the metastatic deposits are means ± standard deviations of the total secondary tumour volumes in the animals bearing metastases. The differences in sizes of metastatic deposits between the two control groups and the fucosidase-treated group were found to be significant ($P < .01$) by the Wilcoxon ranking test.

[a]Mean ± SD of 3 samples.

Cellular Uptake of ^{14}C Fucose

The high metastatic R13762 cells and the less metastatic RJC cells were incubated with ^{14}C-labelled fucose. Other workers have previously shown that there is insignificant conversion of labelled fucose into other sugars, amino acids, or glycogen in cultured cells or rat tissues [19]. The R13762 cells were found to incorporate almost twice as much label, although the same distribution among the various lipid and protein components of the cell was recorded for both cell lines (Table II). The majority of the fucose was incorporated into the nonprotein fraction (mean of 62%) and a mean of 87% of this was found in the crude ganglioside preparation.

Isolation of a Crude Gangliosides Fraction

The gangliosides were isolated from the R13762 cells and found by TLC to be a mixture, but the dominant band had the same Rf as the GM_1 standard. NMR analysis of the total ganglioside fraction showed the presence of a long T_2 (~1,000 ms), and the cross peak Y was present in the 2D COSY spectrum. No long T_2 was found in the organic phase lipids, which would include neutral lipid, neutral glycolipid, and phospholipid.

These data allow us to conclude that a crude aqueous fraction containing fucogangliosides provides key molecules in the metastatic process having a long T_2 relaxation value comparable with that of the intact cells.

Animal Experiments

Animals were divided into three groups of ten. Untreated R13762 cells injected into the mammary line of the rats resulted in the formation of lymph node metastases in eight out of ten animals, compared to seven out of ten from the cells incubated in PBS for 30 min (Table I). The sizes of the resulting primary tumors were similar in each case (4–5 cm).

Cells incubated in fucosidase prior to injection caused smaller tumors (3–4 cm), but in contrast to controls only two out of the ten rats had any metastases and these were in a single lymph node. Metastatic deposits in these two animals were 1.0 cm^3 in volume compared to a mean of 63 cm^3 in the two control groups (Table I).

Thus the treatment of R13762 cells with fucosidase prior to injection into the fat pad of the mammary line affected their metastatic but not tumorigenic property in vivo. Thus it seemed pertinent to investigate how long it took these cells to refucosylate cell surface molecules in vitro.

TABLE II. Distribution of ^{14}C Fucose in R13762 and RJC Cellular Components*

	R13762	RJC
Total uptake (dpm per 10^8 cells)	25,341 ± 1,925	14,892 ± 751
% dpm in protein	38.5 ± 0.1	37.0 ± 2.7
% dpm in lipid	61.5 ± 0.1	63.0 ± 2.7
% dpm in subfractions of lipids		
Gangliosides	86 ± 6	88 ± 2
Other lipids	14 ± 6	12 ± 2

*Cells were incubated with ^{14}C fucose for 48 hr and the uptake of label was recorded in the total protein and lipid components of the cells. The incorporation of label was further measured on the neutral and ganglioside subfractions of the lipid components. (dpm = disintegrations per minute).

Regeneration of Cell Surface Marker In Vitro

The R13762 cells which had been treated with fucosidase were cultured in enzyme-free medium for a further 24-hr period, and the NMR experiments were repeated. The long T_2 relaxation value and cross peak Y in the 2D COSY spectra were again apparent after 24 hr in tissue culture. The doubling time of these cells was approximately 17 hr.

DISCUSSION

The rat R13762 metastatic cell line has a long T_2 relaxation value not observed in the nonmetastatic phenotype J clone [7], whether grown in vitro or in vivo. The NMR data presented here indicate that the resonance associated with metastasis arises from methyl protons adjacent to the methine group in fucose. Furthermore, the removal of cell surface fucose by enzyme treatment decreased the metastatic but not tumorigenic properties of these cells.

When the cells were grown in vitro in medium supplemented with ^{14}C fucose, twice as much ^{14}C fucose was incorporated into the high metastatic R13762 cells as into the low metastatic RJC cell line. Subfractionation of the R13762 cellular components located 62% of the ^{14}C fucose in the lipids, 87% of which was located in the ganglioside fraction. We therefore conclude that the long T_2 relaxation value which has proven to be an excellent metastatic marker in the rat mammary adenocarcinoma model is generated by the methyl protons on a fucoganglioside.

Hakomori and colleagues have produced a vast literature describing fucolipids as oncofetal antigens [5,20–24]. However, most of these fucolipids are of human origin, and since the majority of human tumors are considered to be both malignant and metastatic [25], the concept of a human metastatic marker therefore has not yet been considered.

The wheat germ lectin–resistant variant of MDAY-D2 showed that greatly reduced metastatic potential correlated with lack of fucosylation [26]. This observation is consistent with our finding that the metastatic R13762 cell line has more ^{14}C fucoganglioside than the RJC cell line. Increased ganglioside levels have been found also in plasma membranes of primary and secondary tumors of rats bearing metastatic mammary carcinomas [27].

The NMR experiment on intact cells or excised tumors takes less than 2 hr. Consequently NMR is a rapid way of monitoring cell surface changes associated with the metastatic process on cells grown in vitro or in vivo. Since fucosidase treatment of R13762 cells removes the fucose resonance and the associated long T_2 for one generation of cells only, daughter cells produced after injection of the original 1×10^7 cells into the mammary line of the rat should carry the fucose-containing molecule if cellular fucosylation capacity is normal. This suggests that the state of the cells' surface at the time of injection into the animal is critical in the generation of metastases.

Fucosyltransferase A and B activity is present in the plasma membranes of malignant cells [28], and the fucosyltransferase B activity has previously been shown to be sevenfold higher in metastasizing tumors [28]. This report is consistent with the significantly higher incorporation of ^{14}C fucose in the R13762 metastasizing cells. However, in contrast to Chatterjee, a fourfold increase in the fucosyltransferase activity found in the high metastasizing R13762 compared to the low metastatic RJC cells was in the A enzyme.[1]

[1]L.C. Wright and C.E. Mountford, unpublished results.

In addition, sera from rats with the fucose-rich metastasizing R13762 primary tumor contain a molecule with a long T_2. In contrast, rats with the nonmetastasizing J clone tumor, which has a short T_2 relaxation value and therefore little apparent fucose, have no long T_2 component in their sera.[2] Others have reported that serum fucose levels in patients are a guide to recurrent malignancy [29].

The evidence that fucose-containing complexes are secreted by malignant cells, but in greater amounts by metastasizing cells, suggests that fucose might be detectable in the sera of patients with metastatic cancer. We have isolated proteolipids from the plasma of a patient with malignant disease [10] which contained a high percentage of glycolipid, a long T_2 relaxation time, and a cross peak Y when examined by 2D NMR spectroscopy, observations consistent with the involvement of secreted fucose-containing complexes.

Hakomori [5] has suggested that aberrant glycosylation in cancer cells may be a retrogenetic expression of embryogenesis and fetal development. Moreover, fetal cells and malignant cells alike have neutral lipid domains in their plasma membranes [8] and secrete proteolipid particles [30]. We have confirmed the presence of both acidic and neutral glycolipids in human serum proteolipid from patients with malignant disease [10].

The role of fucoproteins in the generation of the long T_2 and the metastatic process has yet to be evaluated. Alterations to the cell surface glycoproteins have been described in metastatic clones of the R13762 NF rat mammary adenocarcinoma line [31]. Fucosylated derivatives of the major sialoglycoprotein (ASGP1) on R13762 cell surfaces have now been sequenced [32]. Cells from DMBA8 and MAT13762 rat mammary lines showed increased binding of fucose-specific lectin to more highly metastatic variants [33].

Migratory inhibition factor (MIF) plays an important role in cellular immune systems by modulating the function of the macrophages. L-fucose has been found to inhibit the MIF present in ascites fluids of ovarian cancer patients [34]. Low levels of serum α-L-fucosidase have been associated with epithelial ovarian cancer [35]. It can be postulated that there would be a resultant increase in serum fucose and cellular surface fucose [36] which might render the surrounding macrophages unresponsive to MIF.

Surface molecules are shed from metastatic cells to a greater extent than from nonmetastatic cell surfaces [37]. It is our working hypothesis that the fucogangliosides in addition to fucoproteins are shed from malignant cells as part of the abnormal proteolipid complex and that these fucoganglioside-containing proteolipids may contribute to immunosuppression of the host [38]. Furthermore, the observation that NMR methods can detect a long T_2 from these fucolipids in intact cells and tissue could be useful in the future detection and diagnosis of cancer by magnetic resonance imaging.

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[2]L.C. Wright, M. Dyne, G.L. May, W.B. Mackinnon, K.T. Holmes and C.E. Mountford, unpublished results.

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