Cell-Surface Fucosylation and Magnetic Resonance Spectroscopy Characterization of Human Malignant Colorectal Cells†

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Received June 2, 1992; Revised Manuscript Received August 31, 1992

ABSTRACT: Proton (^{1}H) magnetic resonance spectroscopy (MRS) has been used to distinguish lowly and highly tumorigenic human malignant colorectal cell lines based on differences in lipid, choline, and fucose resonances. The spectral patterns were comparable with those obtained for human colorectal biopsy specimens, indicating that cells grown in vitro are suitable for documenting colorectal tumor biology. For the first time, two-dimensional (2D) *co*rrelation *s*pectroscop*y* (COSY) has been used to assess the fucosylation state on the surface of intact viable cells, and differences were recorded between the highly and lowly tumorigenic cell lines. Four methyl–methine cross-peaks were assigned to covalently linked fucose on the basis of increases in volume following the addition of free fucose. Both cell lines incorporated the same amount of exogenous free fucose as determined chemically, but the COSY spectra indicated that the fucose was distributed differently by each cell line. Of the four sites containing MR-visible bound fucose, one was common to both cell lines, two characteristic of the highly tumorigenic line, and the remaining site unique to the lowly tumorigenic cells. Material released from the highly tumorigenic cells in response to increased cell density was also fucosylated (whereas shed material from lowly tumorigenic cells was not), suggesting a biological role for shed fucosylated antigens in tumor aggression.

The involvement of epigenetic factors, particularly altered cell-surface chemistry, in tumor development and progression is now becoming apparent. A better understanding and means of monitoring this phenomenon could provide a practical method for diagnosis and monitoring treatment of preinvasive and invasive cancers. Aberrant cell-surface glycosylation is integral to tumorigenesis and results in the formation of a variety of new carbohydrate antigens (Hakomori, 1989). The changes in cell-surface glycosylation reported to date include enhanced incorporation of carbohydrate into the parent oligosaccharide chains as well as elongation and increased branching of the chains themselves. As a general consequence, two of the major terminal sugars, fucose and sialic acid, are both significantly increased on the surface of malignant cells.

To date, the majority of studies investigating glycosylation of cell-surface antigens have involved isolation of these molecules and subsequent production of monoclonal antibodies. As a consequence, it has been established that the tumor-associated antigens with enhanced expression of fucose and sialic acid are predominantly the lacto-series Lewis antigens (Le^a, Le^b, Le^x, and Le^y) and, to a lesser extent, the ganglio-series and globo-series antigens (Hakomori, 1989).

Increased sialylation of the cell surface has been linked with metastatic spread (Schirrmacher et al., 1982; Bolscher et al., 1986; Dennis & Laferte, 1987; Bresalier et al., 1990), immunotolerance of malignant cells (Yogeeswawaran et al., 1983), and tumor progression (Tsuchida et al., 1987). However, the involvement of altered fucosylation in tumor development and progression is not clear. Neither is the interrelationship between the overexpression of these two sugars well understood. Yet an intricate relationship between cell-surface fucosylation and sialylation, mediated via the relative activities of fucosyltransferase and sialyltransferase enzymes, is becoming apparent (Campbell & Stanley, 1984; Hansson & Zopf, 1985; Holmes et al., 1986; Itzkowitz et al., 1988). It is the specificity of these enzymes, together with the availability of substrates, which is thought to determine the type of antigens that accumulate in a given population of tumor cells. The current knowledge of aberrant glycosylation in tumors and tumor-associated antigens is comprehensively reviewed in a recent article (Hakomori, 1989).

A second biological function thought to be involved in the development and progression of malignant cells is the shedding of membrane fragments and macromolecules from the surface of viable cells and solid tumors (Black, 1980; Schirrmacher & Barz, 1986). The material shed includes membrane proteins, glycoproteins, lipoproteins, glycolipids, and glycoaminoglycans either alone or as part of plasma membrane sections or vesicles (Black, 1980). In this paper, the term plasma membrane fragments (PMF)[1] shall be used to refer to this collective material.

A correlation has been established between the shedding properties and the metastatic potential of malignant cells.

† This project was supported by a grant from the NIH (CA 51054). C.L.L. acknowledges the receipt of an Australian postgraduate research award. E.J.D. thanks the Medical Research Council of Canada for a postdoctoral fellowship.

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[1] Abbreviations: PMF, plasma membrane fragment(s); MRS, magnetic resonance spectroscopy; 2D, two-dimensional; ^{1}H, proton; COSY, correlated spectroscopy; 1D, one-dimensional; ppm, parts per million; MR, magnetic resonance; RPMI, Roswell Park Memorial Institute; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; FBS, fetal bovine serum; PBS, phosphate-buffered saline; Hz, hertz; dB, decibels; TSP, sodium 3-(trimethylsilyl)propanesulfonate; t_1, time domain in the first dimension; t_2, time domain in the second dimension; SW1, sweep width in the t_1 time domain; SW2, sweep width in the t_2 time domain; DW, dwell time; t_1^{min}, initial delay between the two 90° pulses; FID, free induction decay; LB, Lorentzian broadening; GB, Gaussian broadening; T_2, spin–spin relaxation time; NCB, initialization of manual 2D scaling (Bruker software); $\Delta\nu_{1/2}$, line width at half-height; F_1, frequency in the first dimension; F_2, frequency in the second dimension; Thr/Fuc, threonine/fucose; Fuc, fucose; ACPS, Australian clinicopathologic staging system.

0006-2960/92/0431-11095$03.00/0

Both Barz et al. (1985) and Taylor et al. (1988) reported a greater degree of shedding from highly metastatic compared to lowly metastatic cell lines and an overall difference in the composition of shed material. The release of tumor antigens and fucosylated gangliosides from the surface of malignant cells has been extensively reported (Black, 1980; Shaposhnikova et al., 1984; Ladisch, 1987), but the possible relationship between tumorigenicity and the type or amount of material shed from cells has not been investigated. However, the strong association documented between the retention of specific gangliosides on the cell surface and tumorigenicity (Tsuchida et al., 1987) suggests a role for shed ganglioside.

Magnetic resonance spectroscopy (MRS) offers a unique method for investigating the fucosylation state of the surface of intact viable cells and material shed from these cells as it does not demand prior isolation of molecules of interest. Providing there is sufficient signal (dependent on both concentration and molecular motion), the fucosylated antigens can be studied in their native conformation in contact with the local environment.

The first indication that MRS could unravel the complexities of cell-surface glycosylation was described by Wright et al. (1988) using two-dimensional (2D) MRS. Proton (^{1}H) 2D COSY (*co*rrelation *s*pectroscop*y*) allows separation of composite one-dimensional (1D) resonances by the generation of cross-peaks in a second dimension which identify those protons which are covalently linked. Using COSY on a rat mammary adenocarcinoma metastasis model, the importance of cell-surface fucosylation, as opposed to sialylation, in tumor dissemination was established. One cross-peak (1.3–4.2 ppm) present in the COSY spectra of highly metastatic cells, tentatively assigned to the H_5–H_6 coupling of covalently linked fucose, was significantly reduced in intensity following treatment of the cells with the enzyme fucosidase. The observation that the enzyme treatment severely retarded the metastatic potential but not the tumorigenicity of the cells in vivo supported the assignment and suggested a functional role for cell-surface fucosylation in tumor dissemination.

Proton MRS has since been used to identify covalently linked fucose in the COSY spectra of plasma membrane fragments shed from human malignant colorectal cells (Lean et al., 1991). The methyl–methine cross-peak of bound fucose was assigned at a chemical shift of 1.33–4.27 ppm, almost superimposed on the methyl–methine cross-peak of threonine. This chemical shift for bound fucose is consistent with a previous assignment for fucose in a glycolipid studied in D_2O at 37 °C (Hindsgaul et al., 1982). The reported 0.07 ppm downfield shift of the H_6 protons relative to free fucose is attributed to a strong nonbonded interaction with oxygen atoms in neighboring units (e.g., sialic acid residues) of the oligosaccharide chain (Thogersen et al., 1982).

While MRS does offer the advantage of allowing observation of cell-surface glycosylation on intact viable cells, the method will only report on those molecules with sufficient motion for MR detection. The elongation and increased fucosylation of tumor cell antigens make them eminently suitable for analysis by MRS. The glycosylation states of colorectal cancers and the subsequent modifications during tumor development have been extensively documented (Hakomori et al., 1984; Itzkowitz et al., 1986, 1988; Kaizu et al., 1986; Kim et al., 1986; Nudelman et al., 1986; Sogabe et al., 1991). Antigens identified for malignant colon tissue include Le^b, Le^y, extended and polyfucosylated Le^y and Le^x, sialylated Le^x, and monosialylated Le^a.

The rationale that MRS could aid in the pathological assessment of colorectal cancer has already been proposed in a study of human biopsies (Princz, 1988; Smith et al., 1990). Spectral differences were reported which broadly correlated with staging of the disease by current methods of pathological assessment. However, the lack of specificity inherent in the Dukes staging of colorectal cancer did not permit distinction between specimens histologically diagnosed as invasive.

During the last few years, human cultured cell lines with known growth characteristics, degree of differentiation, tumorigenicity, and metastatic potential have been established (Carrel et al., 1976; Leibovitz et al., 1976; Brattain et al., 1983; McBain et al., 1984; Paraskeva et al., 1984; Whitehead et al., 1985; Kirkland & Bailey, 1986; Park et al, 1987; Huschtscha & Bodmer, 1990), making it possible to simulate the biological properties characteristic of tumor development and progression in vitro and thus to study defined, reproducible samples in the laboratory. With the hypotheses that MRS provides an alternative method for mapping the structure/function and biological role of cell-surface glycosylation of intact viable cells and can aid in the diagnosis of biopsy specimens, two human malignant colorectal cell lines, LIM1215 and LIM1863, were chosen for study on the basis of their differing tumorigenicity. Both cell lines are of epithelial origin and contain two of the three major types of cells found in mature colon, columnar and goblet. In terms of their capacity to form tumors in immunodeficient mice, the LIM1215 cell line is by far the more aggressive (Whitehead et al., 1985, 1987), with the LIM1863 cell line exhibiting a high degree of organization and other features characteristic of normal colon.

Our objectives were to (a) determine if ^{1}H MRS could distinguish between the highly and lowly tumorigenic cell lines and plasma membrane fragments released from these cell lines, (b) ascertain if these cell lines have MR properties similar to colorectal biopsy specimens, (c) establish the usefulness of ^{1}H MRS for monitoring cell-surface fucosylation on intact viable cells, and, if so, (d) use ^{1}H MRS to elucidate any differences in the fucosylation state of the highly and lowly tumorigenic cell lines.

MATERIALS AND METHODS

Cell Lines. Two previously characterized malignant human colon cell lines of epithelial origin, one of high tumorigenicity, LIM1215 (Whitehead et al., 1985), and the other of low tumorigenicity, LIM1863 (Whitehead et al., 1987), were chosen for this study. The LIM1215 cells, derived from an inherited nonpolyposis invasive colorectal cancer, are capable of some differentiation and are highly tumorigenic in immunodeficient mice (6/6 1 cm^2 tumors 4 weeks after injection of 10^5 cells). In contrast, LIM1863 cells, derived from a poorly differentiated, ulcerated, and invasive colon carcinoma, are lowly tumorigenic (1/6 tumors formed in immunodeficient mice 7 weeks after injection of 10^7 cells) and unlike most cultured colorectal cells have retained a high degree of functional and morphological organization similar to that of normal colonic mucosa.

Tissue Culture Conditions. Cell lines were cultured at 37 °C in 5% CO_2 in Roswell Park Memorial Institute 1640 (RPMI-1640) culture medium (Flow Laboratories, Australia), pH 7.2, supplemented with 0.5% (w/v) *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, pK_a 7.5 (Sigma), 0.03% (w/v) L-glutamine (Cytosystems), 0.1% (w/v) sodium bicarbonate (Flow Laboratories), 0.1% (v/v) gentamycin, and 5% (v/v) fetal bovine serum (FBS) (GIBCO) from a single batch.

Under these conditions, LIM1863 cells grew in suspension as large extended clumps of organoids which could be partly separated by pipetting but not maintained as a viable single-cell suspension. Cultures were maintained in Nunclon Delta 800-mL flasks and cell numbers expanded from 1.5×10^5 to 3.0×10^5 cells/mL in 5–6 days (final pH of medium 6.4). The split ratio of cultures did not exceed 1:2. Confluent cell clumps readily adhered to the surface of the flask, and all analyses were therefore undertaken on 24-h subconfluent cells to minimize any disruption of cell-surface molecules during harvest.

LIM1863 cell organoids separated into small cell aggregates when grown in calcium-free medium. An estimation of cell number per milliliter of culture medium was therefore obtained by transferring an aliquot of cells from culture (sedimented at 1000g, 5 min) to an equal volume of Joklik's modified minimum essential medium (Ca^{2+}-free) (Flow Laboratories, catalog no. 10-323-22), supplemented with no greater than 0.02% GIBCO FBS, and incubated at 37 °C in 5% CO_2 for 24 h. Subsequent pipetting produced a viable single-cell suspension suitable for counting.

LIM1215 cells were maintained as roller suspension, rather than monolayer cultures, for comparison with the lowly tumorigenic cells, 1- or 2-L Corning roller flasks rotating at 0.75 rpm on a Bellco roller machine. The resulting suspensions of small cell clumps were gassed with 5% CO_2 in air prior to incubation at 37 °C in a closed environment. Cell numbers were expanded from 1.0×10^5 to 5.0×10^5 cells/mL in 5–6 days (final medium pH 6.5).

To separate attached cells for counting, a 10-mL aliquot of suspension culture cell clusters was exposed to a solution of 0.05% trypsin/0.02% EDTA (w/v) (Flow Laboratories) in Dulbecco's phosphate-buffered saline (PBS) containing calcium and magnesium salts (Flow Laboratories, catalog no. 17-600-22), pH 7.0, and incubated at 37 °C for 2–3 min, followed by gentle pipetting.

Preparation of Cells and Plasma Membrane Fragments. For preparation of pre-shed cell samples for ^1H MRS, 1.0×10^8 subconfluent cells were harvested, washed twice in 2–3 mL of PBS/D_2O (pH 7.2), and resuspended in PBS/D_2O to give a final volume of 400 μL.

Shed plasma membrane fragments including macromolecules and vesicles were prepared following the method originally reported by Rosi et al. (1988) and modified as described by Mackinnon (1990); 1.0×10^8 subconfluent cells were harvested, washed twice in 2–3 mL of PBS/D_2O, and resuspended in 400 μL of PBS/D_2O. The cells were then incubated at 37 °C for 40 min. Cells were subsequently removed by spinning at 800*g* for 5 min, and the resulting supernatant, containing the shed membrane fragments, was spun for a further 15 min at 12000*g* to remove any remaining cell debris.

Post-shed cell samples were prepared by harvesting 1.0×10^8 subconfluent cells and subjecting them to 2 cycles of the shedding procedure outlined above using 30- instead of 40-min incubation steps. The cells were allowed to equilibrate for an additional 30 min prior to ^1H MRS analysis.

Addition of Free Fucose to Viable Cells. Twenty-five microliters of a 1% (w/v) solution of L-fucose (Sigma) in D_2O (total of 25 μg of fucose) was added to post-shed cells immediately prior to the final 30-min, 37 °C incubation step.

Human Colorectal Biopsies. Biopsies of malignant tissue were obtained from colectomy specimens taken from the descending colon. Following excision of a small section (0.25 cm^3) for MRS analysis, histological diagnosis was performed on the remaining biopsy specimen. The material used for MRS analysis was subsequently placed in formalin and processed for histological examination. Normal specimens were samples taken from the proximal resection margin. These samples were histologically free of tumor or atypia.

Tissue was transported from the operating theatre to the pathology laboratory where a piece of the specimen was allocated for MRS study and transported at room temperature to the MRS laboratory. Prior to experimentation, the biopsy specimens had excess adipose tissue and vasculature excised, were washed with 4 × 1 mL of PBS/D_2O, and then placed on top of a glass wool plug in a 5-mm MRS tube so that the tissue would be positioned between the coils of the proton probe. Sufficient PBS/D_2O (400 μL) was used to cover the specimen. Elapsed time from surgery until MR analysis ranged from 2–5 h.

Proton Magnetic Resonance Spectroscopy. All magnetic resonance experiments were carried out in triplicate on a Bruker wide-bore AM-360-MHz spectrometer equipped with an Aspect 3000 computer. A standard 5-mm probehead was used with the sample spinning at 20 Hz and the temperature maintained at 37 °C. Residual water signals were suppressed by selective gated irradiation (Jesson et al., 1973) using low power (15 and 23 dB below 0.2 W for the one- and two-dimensional experiments, respectively). Aqueous sodium 3-(trimethylsilyl)propanesulfonate (TSP) at 0.00 ppm was used as an external chemical shift reference.

One- and Two-Dimensional MR Experiments. One-dimensional spectra were acquired over a sweep width of 3597 Hz (10.0 ppm) using 8192 data points, 100 accumulations, an acquisition time of 1.139 s, and a relaxation delay of 2 s. A line broadening of 3.0 Hz was applied to whole cell data and 1.0 Hz to data generated from shed plasma membrane fragments prior to Fourier transformation.

Magnitude-mode COSY experiments were performed using standard Bruker software. A total of 2048 data points were collected in the t_2 time domain over a sweep width (SW2) of 2994 Hz (8.32 ppm). Data sampling was carried out with a dwell time (DW2) of 167 μs, an initial delay between the two 90° pulses, $t_1^{min} = 5\ \mu s + 1$ ms (to maximize small J couplings), an increment time of 334 μs (=2DW2), and a relaxation delay of 1 s, with SW1 = SW2/2 = 1497 Hz.

The number of time domain points collected in t_1 (free induction decays, FIDs) was subject to the viability limits of the sample. Cell experiments consisted of 220 FIDs, each of 32 transients over a total experiment time of 3.5 h. Cell viability over this time period was assessed by exclusion of 0.125% (v/v) trypan blue in saline (Flow Laboratories). Samples of plasma membrane fragments shed from viable cells were stable with regard to MR spectral properties for at least 48 h stored at 37 °C, 1 month stored at 4 °C, and 3 months stored at −70 °C. A total of 300 FIDs each of 96 transients were acquired over a 12-h period for each sample of plasma membrane fragments. In the case of colorectal biopsy specimens, each experiment consisted of 180 FIDs, each of 40 transients over a total experiment time of 3 h.

Processing of Two-Dimensional (2D) Data. COSY data matrices underwent zero-filling to 1K in t_1, Fourier transformation, and magnitude calculation [$\sqrt{(Real^2 + Imaginary^2)}$] to give 1024 × 1024 real data points for each 2D spectrum. Sine-bell window functions were uniformly applied in the t_1 domain, and Lorentzian–Gaussian window functions of varying width and maximum positions were applied in the t_2 domain prior to Fourier transformation as previously described by Delikatny et al. (1991).

Measurement of COSY Cross-Peak Volumes. Expansion and integration of cross-peaks using standard Bruker software gave measurement of peak volume. The reproducibility of volume measurements using this method has been assessed previously (Lean et al., 1991) with a reported maximum variation in cross-peak volumes for three identical COSY experiments of 4%. Results given are the mean ± SD of three experiments.

Hydrolysis of Shed Material. Mild acid hydrolysis of the plasma membrane fragments to release sialic acid and fucose residues was carried out on the basis of the method of Montreuil et al. (1986). Ten microliters of 2.5 M H_2SO_4 was added to 400 μL of shed membrane fragments and the sample incubated at 80 °C for 30 min. After being allowed to cool, the sample was neutralized with the addition of 10 μL of 5 M NaOH. In some cases, a small final adjustment using 0.1 M NaOD or DCl was necessary to ensure that the sample was returned to its original pH of 7.2. To a separate 400-μL sample of membrane fragments was added 20 μL of H_2O, and this sample served as a control. Results given are the mean ± SD of three experiments.

Free Fucose Determination. Free fucose levels were determined in quadruplicate by the method of Cohenford and colleagues (Cohenford et al., 1989). Fucose uptake by the cells was assessed by the measurement of free fucose remaining after exposure of the post-shed cell sample to excess additional exogenous fucose (25, 40, or 60 μg). Results given are the mean ± SD of quadruplicate samples for three experiments.

Sialic Acid Determination. The sialic acid (*N*-acetylneuraminic acid, AcNeu) content of PMF samples was determined in duplicate by an enzymatic method provided in kit form by Boehringer Mannheim (catalog no. 784192). Results given are the mean ± SD of duplicate samples for two experiments.

Lactic Acid Determination. The L-lactic acid content of PMF samples was measured in duplicate using a UV spectrophotometric method provided in kit assay by Boehringer Mannheim (L-lactic acid, UV method, catalog no. 139084). Results given are the mean ± SD of duplicate samples for two experiments.

Amino Acid Analysis. The amount of amino acids, both free and bound, in PMF samples was estimated by PICOTAG amino acid analysis. Amino acid content was determined in duplicate on a single sample before and after hydrolysis. The hydrolysis procedure involved exposure to gas-phase 6 M HCl (1% phenol), 108 °C, for 24 h.

Triglyceride Analysis. Cells [(2–3) × 10^8] were washed in saline 3 times, resuspended in a volume of 1 mL, and extracted in chloroform/methanol (14:7:1, v/v) by the method of Gottfried (1967). Triglyceride was determined colorimetrically using Sigma Diagnostic Kit 405 based on the molecular weight (885) of triolein. Results given are the mean ± SD of duplicate samples for two experiments.

RESULTS

Cells

Cell Viability. Suspension cultures of LIM1215 (highly tumorigenic) and LIM1863 (lowly tumorigenic) cell lines were easily harvested without disruption to cell integrity. Preshed and post-shed cells remained intact under the conditions of the MR experiment with a viability, as assessed by exclusion of 0.125% v/v trypan blue in saline (Flow Laboratories), greater than 80% for 2-h post preparation and greater than 70% for an additional hour.

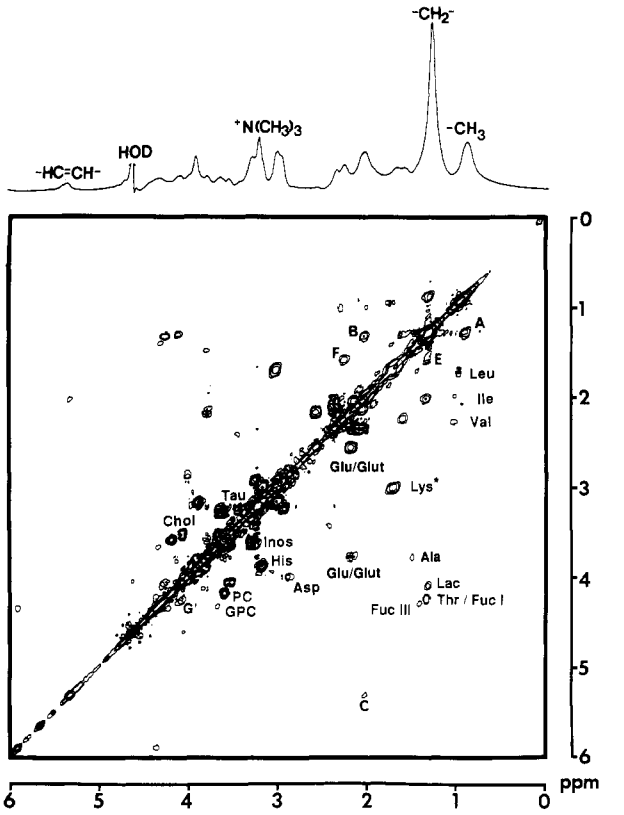

FIGURE 1: 360-MHz ^{1}H MR 1D (100 scans) and symmetrized COSY (32 scans, 220 FIDs) spectra of 10^8 subconfluent LIM1215 (highly tumorigenic) malignant colorectal cells in 400 μL of phosphate-buffered saline in D_2O. Data were obtained at 37 °C with the sample spinning. A line broadening of 3 Hz was applied to the 1D MR spectrum, and sine-bell and Lorentzian–Gaussian (LB = −30.0, GB = 0.20) window functions were used in t_1 and t_2 domains, respectively, for the COSY spectrum. Contour plots were generated with the lowest level set close to the noise level and subsequent levels increasing in powers of 2. (Asterisk) And polyamines.

Processing and Scaling of Data Prior to 2D Fourier Transformation. The method of processing 2D MR data files was important to obtain maximum information about molecular species present at low concentration and is described in detail elsewhere (Delikatny et al., 1991). In general, a Lorentzian–Gaussian window function (LB = −30.0, GB = 0.20) was applied in the t_2 domain prior to Fourier transformation. However, it was necessary when processing 2D data containing a large contribution from lipid to use a more selective window function in the t_2 domain. This reduced the t_1 noise arising from the strong lipid signals and allowed the methyl–methine couplings from molecules with longer T_2 relaxation values and present in smaller quantities to be observed. Data from the lowly tumorigenic (LIM1863) cell line (LB = −50.0, GB = 0.25) and colorectal biopsy specimens (LB = −50.0, GB = 0.125) were filtered in this manner when the methyl–methine coupling region was to be examined in detail.

In addition, similar scaling of the data files was essential for direct comparison of the two cell lines. Data files were scaled to each other using a normalization constant for 2D data (NCB, Bruker software), and cell numbers and experimental conditions were kept constant.

Assignment of ^{1}H MR Spectra. Proton 1D and COSY MR spectra of highly (LIM1215) and lowly (LIM1863) tumorigenic human malignant colorectal cells (1.0 × 10^8) are shown in Figures 1 and 2, respectively. The 1D spectra are dominated by lipid with the methyl, methylene, and olefinic resonances at 0.90, 1.33, and 5.26 ppm, respectively. A

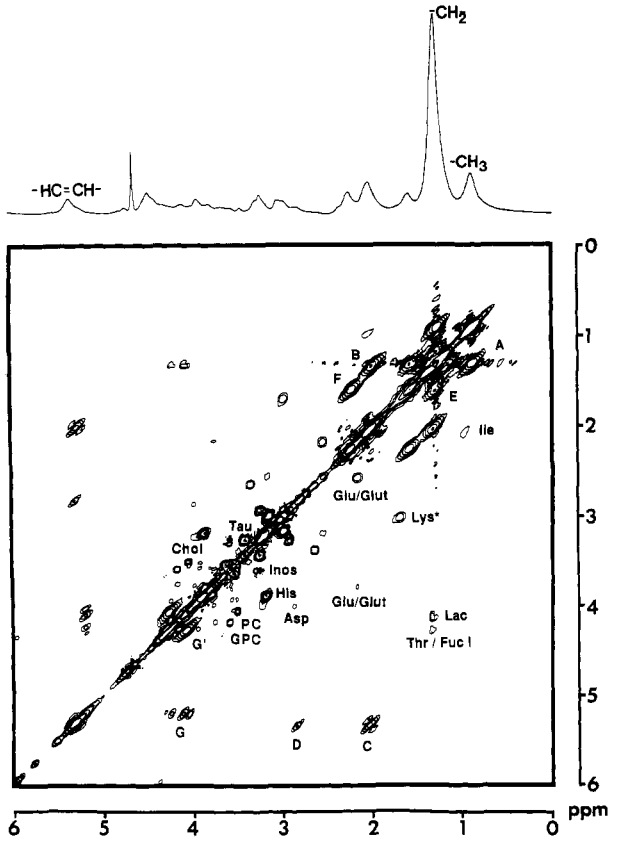

FIGURE 2: 360-MHz ^{1}H MR 1D (100 scans) and symmetrized COSY (32 scans, 220 FIDs) spectra of 10^8 subconfluent LIM1863 (lowly tumorigenic) malignant colorectal cells in 400 μL of phosphate-buffered saline in D_2O. Data were obtained, processed, and presented as for Figure 1. (Asterisk) And polyamines.

resonance at 2.18 ppm which may have a contribution from the acetyl group of sialic acid and an unassigned resonance at 4.01 ppm are also more prominent in the 1D MR spectrum of the highly tumorigenic cells.

Lipid resonances present in the COSY spectra have been previously identified as triglyceride with cross-peaks A–G′ from the lipid acyl chain and glycerol backbone connectivities (May et al., 1986). Cross-peak G, from the methylene–methine coupling of the glycerol backbone, is prominent only in the lowly tumorigenic cell spectrum (Figure 2), and its presence is indicative of a large amount of MR-visible triglyceride in this cell line (Holmes & Mountford, 1991). A component of the triglyceride is likely to be associated with the plasma membrane as previously described (May et al., 1986, 1988; Mountford & Wright, 1988; Mackinnon et al., 1992).

Chemical shifts of the cross-peaks from triglyceride, choline, phosphorylcholine, glycerophosphorylcholine, inositol, taurine, lactate, free amino acids, and peptides (Sze & Jardetzky, 1990) and other resonances yet to be assigned are summarized in Table I.

Comparison of MR Spectra of Highly and Lowly Tumorigenic Cells. The most obvious spectral differences are in the choline region from 3.0 to 3.4 ppm. The highly tumorigenic (LIM1215) cells (Figure 1) contain significantly more choline metabolites than for the lowly tumorigenic (LIM1863) cells (Figure 2). This is manifest as a strong $^+N(CH_3)_3$ resonance at 3.2 ppm in the 1D spectrum and in the COSY spectrum by an increased intensity of off-diagonal resonances from the $-CH_2-CH_2-$ coupling of choline at 3.50–4.07 ppm and phosphorylcholine at 3.61–4.25 ppm (volumes of both cross-peaks were 10-fold greater in the highly compared to the lowly tumorigenic cell spectra). When the contour levels in scaled spectra are compared, cross-peaks from amino acids (free or bound) are seen to be more intense for the highly tumorigenic cells. Triglyceride resonances are, however, more intense in the lowly tumorigenic cell spectrum with cross-peaks D and G absent for the highly tumorigenic cells. This is supported chemically with the triglyceride content of the lowly tumorigenic cells (1198 ± 100 nmol/10^8 cells, mean ± SD, n = 2) being approximately 5.5 times greater than that present in the highly tumorigenic cells (221 ± 14 nmol/10^8 cells). The line width at half-height ($\Delta\nu_{1/2}$) of the methylene resonance at 1.33 ppm is also different for the lowly (48 ± 4 Hz) and highly (35 ± 3 Hz) tumorigenic lines. Neither cell line generated a cross-peak from the methyl–methine protons of the alkyl side chains of the cholesterol ring system as previously reported for leukemic lymphocytes (May et al., 1988) or Chinese hamster ovary cells (Mackinnon et al., 1992).

Methyl–Methine Couplings. The methyl–methine coupling region (F_2, 1.00–1.70 ppm; F_1, 3.70–4.50 ppm) from the pre-shed highly tumorigenic (LIM1215) cell unsymmetrized COSY spectrum (Figure 3A) contains cross-peaks from the couplings of lactate anion (1.33–4.12 ppm) and alanine (1.49–3.79 ppm) and a cross-peak denoted Thr/Fuc I (1.33–4.27 ppm) consistent with both fucose when in a covalent linkage and the amino acid threonine (Lean et al., 1991). Cross-peaks not previously reported, denoted Fuc II (1.25–4.28 ppm) and Fuc III (1.41–4.30 ppm), are also observed.

In the COSY spectrum of the lowly tumorigenic (LIM1863) cells (Figure 3B), the cross-peak resulting from the H_5–H_6 coupling of lactate anion is less intense than in the highly tumorigenic cell spectrum, and the cross-peak from the H_5–H_6 coupling of alanine is absent. In contrast to the highly tumorigenic cell spectrum, no cross-peaks are observed at 1.25–4.28 ppm (Fuc II) or 1.41–4.30 ppm (Fuc III), but a new cross-peak is present at 1.31–4.38 ppm and denoted Fuc IV.

Perturbation by Exogenous Pools of Free Fucose. Fucosylation of the cell surface is an equilibrium process and therefore integrally dependent on substrate concentration. In order to assign cross-peaks, post-shed cells from both cell lines were exposed to an additional 25 μg of free fucose and compared with a control. In the COSY spectra of both cell lines, when exogenous pools of free fucose were increased, both α- and β-fucose anomers (1.22–4.21 and 1.26–3.81 ppm, respectively) appeared. In addition, modifications to the pattern of methyl–methine couplings were observed.

The addition of free fucose to the highly tumorigenic cells resulted in a 25 ± 11% (mean ± SD of three experiments) increase in the volume of the Thr/Fuc I cross-peak (1.33–4.27 ppm) and a 29 ± 18 and 21 ± 10% increase in the volume of cross-peaks Fuc II (1.25–4.28 ppm) and Fuc III (1.41–4.30 ppm), respectively. The spectrum of lowly tumorigenic cells with fucose added shows a significant increase in the volume of cross-peaks Thr/Fuc I (1.33–4.27 ppm) of 44 ± 3% and Fuc IV (1.31–4.38 ppm) of 40 ± 5%. These results are summarized in Table II.

Other cross-peaks not related to fucose showed only minor variations in volume following exposure of the cells to exogenous fucose. For example, in spectra of post-shed highly tumorigenic cells, the lactate cross-peak increased by 2.8 ± 0.9% (mean ± SD of three experiments), and cross-peaks from choline (3.50–4.07 ppm) and triglyceride (1.33–1.62 ppm) decreased by 1.6 ± 1.2 and 3.6 ± 0.6%, respectively, following addition of fucose. For spectra of the post-shed lowly tumorigenic cells, no lactate was visible before or after addition of fucose, and cross-peaks from choline and tri-

Table I: Summary of Molecules Identified in ^{1}H MR COSY Spectra of Subconfluent Human Malignant Colorectal Cells of High (LIM1215) and Low (LIM1863) Tumorigenicity and PMF from These Cells

molecules	abbreviation	coupling (H)	chemical shift, F_2–F_1 (ppm)	cells		PMF	
				215	863	215	863
triglyceride[a]	A	CH_2–CH_2–CH_3	0.90–1.33	+	+	–	–
	B	CH_2–CH_2–CH_2–CH_3	1.33–2.08	+	+	–	–
	C	CH_2–CH_2–CH=CH	2.02–5.38	+	+	–	–
	D	CH=CH–CH_2–CH=CH	2.84–5.38	–	+	–	–
	E	OC–CH_2–CH_2–CH_2	1.33–1.62	+	+	–	–
	F	OC–CH_2–CH_2–CH_2	1.60–2.30	+	+	–	–
	G	O–CH_2–CH–O	4.12–5.26	–	+	–	–
			4.26–5.26	–	+	–	–
	G′	O–CHH′	4.09–4.29	+	+	–	–
choline	Chol	N–CH_2–CH_2–OH	3.50–4.07	+	+	+	+
phosphorylcholine	PC	N–CH_2–CH_2–OP	3.61–4.25	+	+	+	+
glycero-PC	GPC	N–CH_2–CH_2–OPO–G	3.69–4.38	+	+	+	+
inositol	Inos	HOCH–HCOH (ring)	3.28–3.64	+	+	+	–
lactate anion	Lac	CH–CH_3	1.33–4.12	+	+	+	+
taurine	Tau	H_2N–CH_2–CH_2–OS	3.28–3.50	+	+	–	–
alanine	Ala	CH–CH_3	1.49–3.79	+	–	+	–
aspartic acid	Asp	CH–CH_2–COOH	2.89–4.03	+	+	+	+
glutamic acid	Glu	CH–CH_2–CH_2–COOH	2.21–2.62	+	+	–	–
glutathione	Glut	CH_2–CH(NH_2)–COOH	2.21–3.81	+	+	–	–
histidine[c]	His	CH–CH_2–ring	3.22–3.95	+	+	+	+
isoleucine	Ile	CH_2–CH_3	0.97–2.12	+	+	+	+
leucine	Leu	CH–CH_3	0.97–1.78	+	–	+	+
lysine[b]	Lys	CH_2–CH_2	1.72–3.05	+	+	+	+
threonine	Thr	CH–HOCH–CH_3	1.33–4.27	+	+	+	+
valine	Val	CH–CH_3	1.03–2.34	+	–	+	+
unassigned resonances			2.05–1.90	+	–	–	–
			2.05–2.34	+	+	+	+
			2.10–2.15	+	–	–	–
			2.64–3.44	–	+	–	–
			2.97–3.37	+	+	+	+
			3.03–3.19	+	+	+	+
			3.57–3.68	+	+	+	+
			3.58–3.97	–	+	–	–
			3.78–4.23	+	+	+	+
			3.81–4.00	+	+	+	+
			4.38–5.90	+	–	+	–
			4.38–5.98	–	+	–	–

[a] Cross-peaks A–F are from the fatty acid chains and G and G′ from the glycerol backbone. [b] And polyamines [c] This peak is substantially reduced when these cells are grown in alternative medium such as Dulbecco's modified essential medium.

glyceride increased by 4.1 ± 1.9 and 3.7 ± 2.3%, respectively, following addition of fucose.

Plasma Membrane Fragments (PMF)

The membrane macromolecules and structures described by others to be shed from the surface of malignant cells without compromise to cell viability (Black, 1980; Rosi et al., 1988) and here referred to as plasma membrane fragments (PMF) were analyzed using ^{1}H MR and chemical methods.

Scaling of COSY Data Prior to 2D Fourier Transformation. 2D COSY data from PMF were not suitable for scaling using the normalization constant (NCB, Bruker software) due to the high water content. It was therefore necessary to find an internal concentration standard. The lactate anion content was determined using chemical methods to be the same in PMF shed from the highly (4.55 ± 0.16 mM) and lowly (4.61 ± 0.05 mM) tumorigenic cell lines. Furthermore, all lactate anion was MR-visible since dialysis of the PMF samples resulted in the disappearance of the lactate anion (but not the amino acid or Fuc I cross-peaks) and no lactate was detected chemically following subsequent hydrolysis of the dialyzed samples. The lactate anion cross-peak was therefore used as a reliable, naturally occurring internal standard, and spectra of PMF shed from the lowly tumorigenic cell line underwent minor scaling (less than 2-fold) to obtain the same contour levels for this cross-peak in highly and lowly tumorigenic PMF spectra.

Assignment of ^{1}H MR Spectra. The MR spectra of PMF from LIM1215 (highly tumorigenic) and LIM1863 (lowly tumorigenic) cell lines, shown in Figures 4 and 5, respectively, are comparable in many respects. 1D spectra contain overlapping methyl (CH_3, 0.90–1.00 ppm), methylene (CH_2, 1.22–1.25 ppm), and $^+N(CH_3)_3$ (~3.2 ppm) resonances together with those (3.0–4.2 ppm) from amino acids, choline-based metabolites, and a number of unassigned resonances including those from the H_2, H_3, and H_4 protons of various carbohydrate moieties. In the COSY spectra of PMF from both cell lines, off-diagonal resonances from choline metabolites, amino acids/peptides, lactate (Table I), and a cross-peak consistent with threonine and bound fucose (Table II) are also observed.

MR spectra of PMF isolated from both cell lines differ markedly from those of the intact parental cells in the absence of resonances attributable to triglyceride (see A–G′ in Figures 1 and 2). A number of unassigned COSY cross-peaks as well as those from glutamic acid/glutathione and taurine are also absent from the PMF spectra.

Comparison of MR Spectra of PMF from Highly and Lowly Tumorigenic Cell Lines. Differences are apparent in the 1D MR spectra, particularly in the choline (3.0–3.4 ppm) and carbohydrate (3.4–4.2 ppm) spectral regions. A singlet resonance at 2.18 ppm consistent with sialic acid is only apparent in the 1D spectrum of PMF shed from the lowly tumorigenic cells (Figures 4 and 5). The absence of the alanine

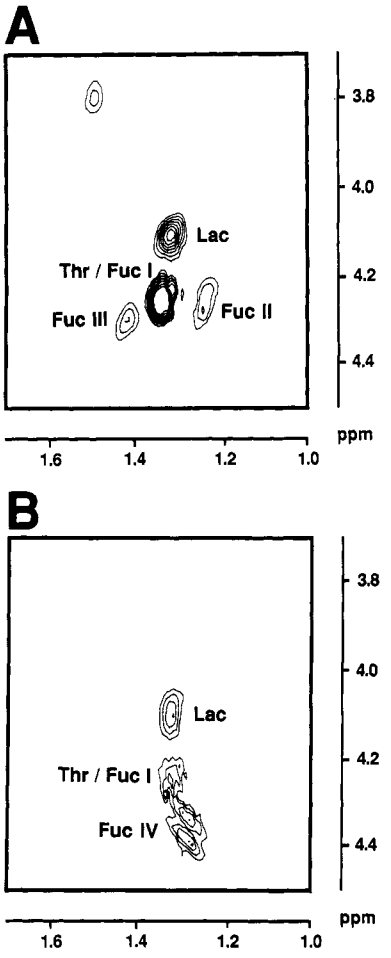

FIGURE 3: Expanded methyl–methine coupling region (F_2, 1.00–1.70 ppm; F_1, 3.70–4.50 ppm) of unsymmetrized COSY data of malignant colorectal cells. Contour plots were generated with the lowest level set close to the noise level and subsequent levels increasing linearly by increments of 1500. (A) Highly tumorigenic (LIM1215) cell line. Data processed using a Lorentzian–Gaussian in t_2 (LB = −30.0, GB = 0.20). (B) Lowly tumorigenic (LIM1863) cell line. Data processed using a Lorentzian–Gaussian in t_2 (LB = −50.0, GB = 0.25).

methyl–methine cross-peak (1.49–3.79 ppm) also distinguishes the LIM1863 from the LIM1215 PMF COSY spectrum.

Methyl–Methine Couplings. The methyl–methine couplings observed in the COSY spectra of the PMF are less complex than those from parental cells. The spectrum of PMF released from LIM1215 cells includes methyl–methine couplings of lactate anion (1.33–4.12 ppm) and alanine (1.49–3.79 ppm) and a composite cross-peak (1.33–4.27 ppm) assigned previously to the methyl–methine couplings of covalently linked fucose and the amino acid threonine (Lean et al., 1991). Neither cross-peak Fuc II (1.25–4.28) nor Fuc III (1.41–4.30 ppm) observed in the spectra of the parental cells is present. In the lowly tumorigenic (LIM1863) PMF spectrum, a cross-peak at the same chemical shift as Thr/Fuc I is observed, but alanine is absent as is cross-peak Fuc IV.

The assignment of bound fucose in PMF shed from the highly tumorigenic (LIM1215) cells was previously made following the release of the α and β anomers of fucose by acid hydrolysis of the sample (Lean et al., 1991). The accompanying decrease in volume of the Thr/Fuc I resonance confirmed a contribution from bound fucose to this cross-peak (no cross-peaks from free fucose were present prior to hydrolysis). We now report that, in contrast to the PMF shed from the highly tumorigenic line, no free fucose was detected by MR (Table II) or chemical methods (Table III) in PMF from the lowly tumorigenic (LIM1863) cells using the same procedures. Cross-peaks in the LIM1863 PMF spectrum are therefore assigned to lactate and threonine alone.

Chemical Analysis of PMF. Chemical analysis of PMF (Table III) confirmed the presence of all molecules assigned in the MR spectra with the exception of inositol and the choline metabolites for which no analyses were performed. The LIM1863 PMF contained 4 times the amount of sialic acid (0.054 ± 0.008 mM) present in the LIM1215 PMF (0.014 ± 0.004 mM). Significant differences were also observed in the free amino acid/peptide content of LIM1215 and LIM1863 PMF as measured chemically. The amino acid cross-peak intensities in the respective PMF spectra better reflected chemical differences recorded for free rather than total amino acid contents.

Human Colorectal Biopsies

Processing of Data Prior to 2D Fourier Transformation. The 1D and 2D COSY MR spectra of biopsies have been reported previously by others (Princz, 1988; Smith et al., 1990), but since the data acquisition and processing parameters were significantly different, they cannot be compared directly with the colorectal cell lines described here. Typical COSY spectra of colorectal biopsy specimens where the data have been processed in the same manner are described.

Assignment of ^1H MR Spectra. The MR spectrum of tissue from the resection zone contains only cross-peaks A–G′ from triglyceride and a minor contribution from the methyl–methine coupling of lactate anion (1.33–4.12 ppm). In contrast, the spectrum of a primary tumor from a patient with a low-grade malignancy staged A/A2 [Australian clinicopathologic (CP) staging system/Concord Hospital CP staging system (Chapuis et al., 1991)] displays a multitude of cross-peaks which are as assigned in the colorectal cell lines. Additional unassigned cross-peaks, not present in the cell lines, are present at 1.16–3.70, 1.33–4.60, 2.65–3.85, and 3.20–4.00 ppm.

Methyl–Methine Couplings. In order to assess the fucose present in the biopsies, the data were processed using a Lorentzian–Gaussian window function in t_2, LB = −50.0 and GB = 0.125, to reduce intense lipid resonances and enhance those molecules present in smaller quantities and with longer T_2 relaxation values. Processed in this manner, a striking difference is observed between the normal and malignant tissue in the spectral region containing the methyl–methine couplings (F_2, 1.00–1.70 ppm; F_1, 3.70–4.50 ppm) shown in Figure 6. The normal tissue spectrum (Figure 6A) contains only a weak cross-peak from lactate anion at 1.33–4.12 ppm whereas the spectrum of the malignant biopsy (Figure 6B) contains the methyl–methine couplings of lactate anion (1.33–4.12 ppm), alanine (1.49–3.79 ppm), Thr/Fuc I (1.33–4.27 ppm), Fuc II (1.25–4.28 ppm), and Fuc III (1.41–4.30 ppm).

DISCUSSION

Lipid signals are prominent in the 1D ^1H MR spectra of normal colonic mucosa and malignant colorectal tumors alike (Princz, 1988; Smith et al., 1990) with an inverse relationship existing between the line width of the lipid resonances and tumor development and progresssion. Similar differences are apparent in the spectra of the lowly and highly tumorigenic cell lines (Figures 1 and 2). Model membrane studies have shown that phospholipid bilayers can incorporate up to 3 mol % triglyceride into the bilayer (Hamilton, 1989). Once this concentration is exceeded, pools of isotropically tumbling lipid

Table II: H_5–H_6 Couplings of Free and Bound Fucose in COSY Spectra of Subconfluent Cells and PMF

		LIM1215 (highly tumorigenic)			LIM1863 (lowly tumorigenic)		
abbreviation	chemical shift (ppm) F_2–F_1	control cells	cells[a] + fucose (% increase)	PMF[b]	control cells	cells[c] + fucose (% increase)	PMF
Fuc I	1.33–4.27	yes	yes (25 ± 11)	yes	yes	yes (44 ± 3)	no
Fuc II	1.25–4.28	yes	yes (29 ± 18)	no	no	no	no
Fuc III	1.41–4.30	yes	yes (21 ± 10)	no	no	no	no
Fuc IV	1.31–4.38	no	no	no	yes	yes (40 ± 5)	no
α-Fuc	1.22–4.21	no	yes	no	no	yes	no
β-Fuc	1.26–3.81	no	yes	no	no	yes	no

[a] 11 ± 3 μg (67 ± 18 nmol) of added free fucose was incorporated by 10^8 LIM1215 (highly tumorigenic) cells. [b] Fucose was present in bound form; 7 ± 2 μg of free fucose per 400 μL of PMF shed from 10^8 cells was released by acid hydrolysis (Lean et al., 1991). [c] 10 ± 6 μg (61 ± 37 nmol) of added free fucose was incorporated by 10^8 LIM1863 (lowly tumorigenic) cells.

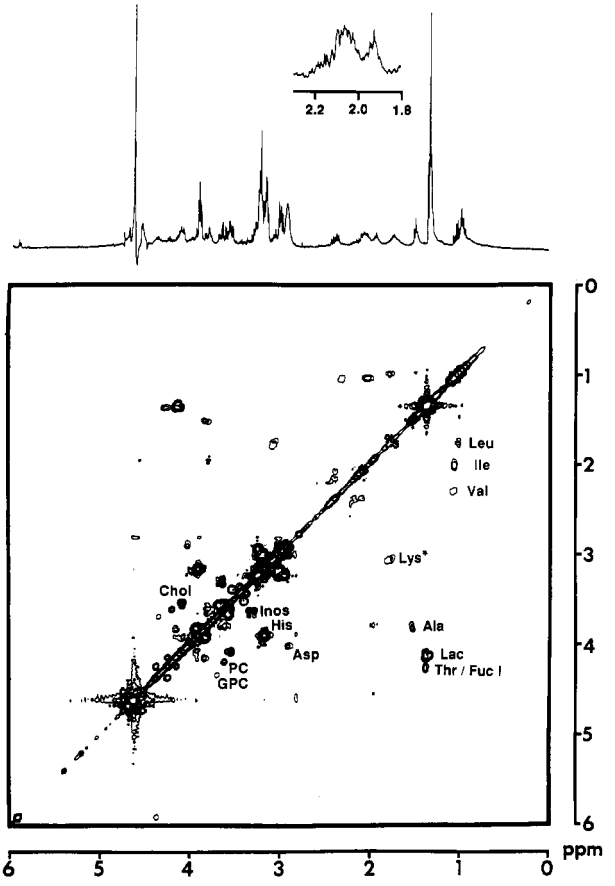

FIGURE 4: 360-MHz ^{1}H MR 1D (100 scans) and symmetrized COSY (96 scans, 300 FIDs) spectra of PMF shed in PBS/D_2O from 10^8 subconfluent LIM1215 (highly tumorigenic) malignant colorectal cells. Data were obtained at 37 °C with the sample spinning. A line broadening of 1 Hz was applied to the 1D spectrum, and sine-bell and Lorentzian–Gaussian (LB = –30.0, GB = 0.20) window functions were used in t_1 and t_2 domains, respectively, for the COSY spectrum. Contour plots were generated with the lowest level set close to the noise level and subsequent levels increasing in powers of 2. (Asterisk) And polyamines.

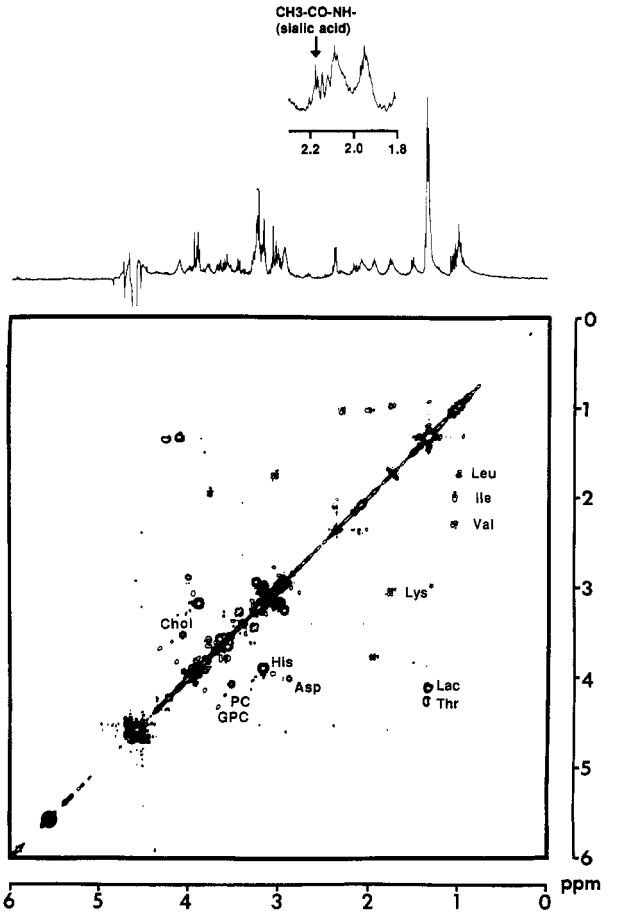

FIGURE 5: 360-MHz ^{1}H MR 1D (100 scans) and symmetrized COSY (96 scans, 300 FIDs) spectra of PMF shed in PBS/D_2O from 10^8 subconfluent LIM1863 (lowly tumorigenic) malignant colorectal cells. Data were obtained, processed, and presented as for Figure 4. (Asterisk) And polyamines.

are thought to be created which generate MR-visible signal (May et al., 1986, 1988; Mountford & Wright, 1988; Mackinnon et al., 1992).

Interestingly, COSY spectra of the PMF (Figures 4 and 5) do not contain resonances indicative of triglyceride or fatty acyl chains, suggesting that triglyceride either is not shed from the cells as part of the PMF or is not isotropically tumbling and hence not MR-visible. Others have reported a significant contribution from ganglioside, in material shed from malignant cells (Black, 1980; Shaposhnikova et al., 1984; Ladisch, 1987; Mackinnon, 1990), but the absence of cross-peaks from fatty acyl chains suggests that any ganglioside shed is in relatively immobile environments and as such not visible on the MR time-scale.

Resonances from choline and phosphorylcholine are 10-fold more intense in the COSY MR spectra of the highly tumorigenic cells compared with the lowly tumorigenic cell spectra (Figures 1 and 2). Both these metabolites are integral to phospholipid synthesis (Kuesel et al., 1990) and thus essential for increased membrane turnover associated with the increased growth rate common to tumorigenic cells. These results are in agreement with the report that phosphorylcholine is present in high concentration in malignant colorectal cells grown both in vivo and in vitro (Daley & Cohen, 1989).

The subtle but highly reproducible differences in the cell-surface fucosylation of these two cell lines as measured by MRS is perhaps the most significant observation we report here. As part of the cell-surface oligosaccharide antigens, fucose molecules are incorporated as terminal sugars, internally, or as final entities (Schirrmacher et al., 1982) on unbranched, branched, or elongated chains (Itzkowitz et al.,

Table III: Molecules Assessed by Chemical Analysis in Plasma Membrane Fragments (PMF) Shed from Human Malignant Colorectal Cells of High (LIM1215) and Low (LIM1863) Tumorigenicity

molecules	plasma membrane fragments (from 10^8 cells in 400 μL)[a] LIM1215	LIM1863
amino acids: total (free)		
alanine	3.43 ± 0.01 (1.39 ± 0.02)	0.73 ± 0.01 (0.38 ± 0.00)
aspartic acid	1.23 ± 0.06 (0.02 ± 0.00)	0.28 ± 0.07 (0.01 ± 0.00)
histidine	0.78 ± 0.04 (0.12 ± 0.00)	0.15 ± 0.01 (0.05 ± 0.00)
isoleucine	1.67 ± 0.06 (0.17 ± 0.00)	0.28 ± 0.01 (0.07 ± 0.00)
leucine	3.06 ± 0.13 (0.20 ± 0.00)	0.49 ± 0.01 (0.09 ± 0.00)
lysine	2.02 ± 0.16 (0.15 ± 0.00)	0.37 ± 0.01 (0.06 ± 0.00)
threonine	1.65 ± 0.04 (0.11 ± 0.00)	0.29 ± 0.01 (0.05 ± 0.00)
valine	2.19 ± 0.08 (0.12 ± 0.00)	0.37 ± 0.01 (0.05 ± 0.00)
other		
sialic acid	0.014 ± 0.004	0.054 ± 0.008
lactate anion	4.55 ± 0.16	4.61 ± 0.05
lactate anion after dialysis	0.00	0.00
fucose	0.00	0.00
fucose after hydrolysis	0.11 ± 0.03	0.01 ± 0.01

[a] All concentrations expressed in millimolar.

1986, 1989). ^{1}H MR has already proved to be a powerful tool in the structural analysis of isolated fucosylated molecules (Nudelman et al., 1986; Levery et al., 1986; Clausen et al., 1985). Previous studies have also shown that the H_5 and H_6 protons of fucose molecules linked at various positions on an oligosaccharide chain are deshielded to different extents according to the spatial location of neighboring carbonyl groups (Levery et al., 1986). The effect of deshielding on the H_5 and H_6 fucose protons is even larger if the fucosylated species are in their native conformation under biologically relevant conditions, i.e., in aqueous solvent at 37 °C. Lemieux and colleagues (Thogersen et al., 1982; Lemieux et al., 1980) have shown a variation in chemical shift of the fucose H_5 and H_6 protons as a function of neighboring oxygen atoms, i.e., for fucose linked to galactose (Gal) and *N*-acetylglucosamine (GlcNAc). The chemical shifts of the H_5 protons were distinctive, ranging from 4.26 to 4.46 ppm and from 4.79 to 4.89 ppm for fucose linked to Gal and GlcNAc, respectively, while the fucose H_6 protons resonated between 1.20 and 1.33 ppm irrespective of the adjacent species. The folded oligosaccharide chains were studied in D_2O at 37 °C and hence are directly comparable with the cell data presented here.

In the COSY ^{1}H MR spectrum of free α-L-fucose, the H_5–H_6 cross-peak is the most intense and resonates well clear of the diagonal. The chemical shift of the H_1–H_2 cross-peak varies depending on the environment and linkage, and those from the H_2–H_3, H_3–H_4, and H_4–H_5 couplings are situated very close to the diagonal and almost superimposed. The H_5–H_6 cross-peak is therefore considerably easier to identify than the other fucose cross-peaks. In the COSY spectra of the cells, the H_5–H_6 cross-peaks from bound fucose are well clear of most others except for the methyl–methine coupling of threonine whereas the remaining fucose cross-peaks may well be overlaid by other resonances.

The region in the cell spectrum which includes the methyl–methine couplings of bound fucose (Lean et al., 1991) is complex and different for the two colorectal cell lines LIM1215 and LIM1863 (Figure 3). Neither cell line has the α or β anomers of free fucose apparent, suggesting that fucosyltransferase enzymes are active. As such, free fucose may by incorporated at similar or unique positions on the same or different oligosaccharide parent chains according to the types

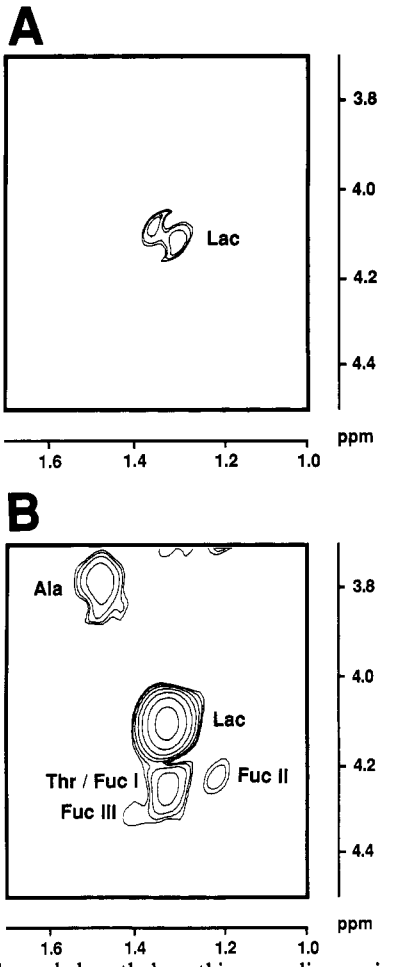

FIGURE 6: Expanded methyl–methine coupling region (F_2, 1.00–1.70 ppm; F_1, 3.70–4.50 ppm) of symmetrized COSY data from human colorectal biopsy specimens covered in PBS/D_2O. Data were obtained at 37 °C with the sample spinning and processed using sine-bell and Lorentzian–Gaussian (LB = −50.0, GB = 0.125) window functions in the t_1 and t_2 domains, respectively, prior to Fourier transformation. Contour plots were generated with the lowest level set close to the noise level and subsequent levels increasing in the first instance by an increment of 1000 and thereafter by increments advancing in powers of 2. (A) Tissue taken from the resection margin of the biopsy specimen, a zone histologically described as normal. (B) Malignant tissue from the primary tumor of a patient with a low-grade malignancy staged ACPS A, substage A2 (Chapuis et al., 1991).

of oligosaccharide chains present and the specificity of fucosyltransferase enzymes active on the cells. Elevated fucosyltransferase activity accompanying malignant transformation is a well-documented phenomenon (Chatterjee & Kim, 1978). These enzymes catalyze the transfer reactions of fucose, via GDP-fucose, to acceptor sites on the oligosaccharide chains and as a family are noted for their specificity, exhibiting preference for not only the position of covalent linkage but also the type of acceptor carbohydrate chain and in some cases very particular patterns of fucosylation and sialylation (Campbell & Stanley, 1984; Holmes et al., 1986).

Spectra from both cell lines had the methyl–methine cross-peak from Fuc I, but the highly tumorigenic cell spectrum had two other cross-peaks from bound fucose, Fuc II and Fuc III, which were not present in the lowly tumorigenic cell spectrum. The lowly tumorigenic cell spectrum had one other cross-peak from bound fucose at 1.31–4.38 ppm denoted Fuc IV (Table II).

These results therefore (1) allow assignment of the four cross-peaks to the H_5–H_6 protons of covalently linked fucose, (2) confirm that fucosyltransferases are available for the

enhanced fucosylation of vacant sites in response to an elevated concentration of surrounding free fucose in both cell lines, (3) show that a similar amount of free fucose is incorporated into both cell lines under these experimental conditions, and (4) show from the MR spectra that the incorporated fucose is distributed into one common site (Fuc I) but two sites unique to the highly tumorigenic cells (Fuc II and Fuc III) and one site unique to the lowly tumorigenic cells (Fuc IV).

It was previously shown by chemical and MR methods that the PMF from the highly tumorigenic cell line contained bound fucose resonating at the chemical shift of the Fuc I cross-peak (Lean et al., 1991). Using the same methods, we report here that the PMF from the lowly tumorigenic cell line does not contain fucose. It is therefore of interest that the fucosylated species giving rise to Fuc I which is common to both cell lines is released during the shedding of plasma membrane fragments only from the highly tumorigenic (LIM1215) cells. The release of bound fucose is in agreement with the literature describing shedding of intact tumor antigens by malignant cells and tissue (Black, 1980). The shed tumor antigens are thought to provide protection of the parent tumor by competitively interacting with the effector processes of the immune response (Alexander, 1974). Since in this case bound fucose is released from the highly but not the lowly tumorigenic cells, it suggests an integral role for the fucosylated species in the aggressive nature of the cells in vivo.

While PMF shed from the lowly tumorigenic (LIM1863) cells does not contain fucose, it has been shown by chemical methods to contain 4 times the amount of sialic acid present in the material shed from the highly tumorigenic cells. Sialic acid molecules are known to cryptically cover antigenic sites, effectively preventing recognition or access by enzymes or antibodies (Shearer et al., 1977; Altevogt et al., 1983; Urdal & Hakomori, 1983; Itzkowitz et al., 1988). The high sialic acid content of the lowly tumorigenic cells may have prevented the incorporation of additional fucose in regions of the plasma membrane subsequently shed (Beyer et al., 1979).

The differences in the cell-surface fucosylation recorded for the highly and lowly tumorigenic cell lines grown in vitro can be observed in excised human biopsies. The presence of cross-peaks Thr/Fuc I, Fuc II, and Fuc III in the spectrum of an invasive colorectal primary tumor from a patient staged ACPS A, substage A2 (Figure 6B), illustrates that with careful choice of data acquisition and processing parameters information on the degree of cell-surface fucosylation may be obtained directly from a biopsy specimen. Furthermore, we have confirmed that the fucose cross-peaks are not observed in spectra of normal colon tissue (Figure 6A), supporting a role for fucosylated species in tumor development and progression.

As mentioned previously, it is well-known that many polyfucosylated antigens exist on the surface of colorectal cells. One clue as to the origin of these fucose molecules is their chemical shift. The H_5 protons of Fuc I–Fuc IV in the colorectal cell line and biopsy spectra resonate between 4.27 and 4.38 ppm. This is consistent with the fucose moiety being linked to Gal rather than GlcNAc (Lemieux et al., 1980). Of the known antigens characteristic of colorectal cancer, the αLFuc(1→2)βDGal linkage is common to both Le^b and Le^y. However, since the extended and/or polyfucosylated Le^y antigens are most likely to have greater molecular motion, they are the likely source of the H_5–H_6 couplings, Fuc I–Fuc IV, observed in the colorectal cell and tissue spectra. Since there are many polyfucosylated antigens on the surface of the cells, this assignment remains speculative.

More specific information is available on premalignant tissues. Using monoclonal antibody techniques, Kim and colleagues (Kim et al., 1986) report that the extended Le^y antigen is a marker for the premalignant state. This may explain the cross-peak at the same chemical shift as Fuc I present in the ^{1}H COSY spectra of adenomatous colonic polyps (Princz, 1988), although this cross-peak has yet to be assigned to fucose in polyp tissue or in related cell lines. It is possible, however, that the Thr/Fuc I cross-peaks in the spectra of the highly and lowly tumorigenic colorectal cells and of plasma membrane fragments shed from the highly tumorigenic cells contain a contribution from fucose attached to the Le^y antigen.

In conclusion, ^{1}H MRS can be used to distinguish human colorectal cell lines of different tumorigenicity. The fucosylation state of the surface of intact viable cells has been assessed by 2D ^{1}H MRS, and differences have been recorded between highly and lowly tumorigenic cell lines. The MR spectra from these cell lines are comparable to those reported for human colorectal biopsies, indicating that the established cell lines grown in vitro are useful models for documenting changes to cellular chemistry and MR properties associated with tumor progression. Furthermore, the release of molecules containing MR-visible fucose from the surface of viable cells correlates with tumorigenicity, suggesting a biological role for the fucosylated species in tumor aggression.

ACKNOWLEDGMENT

We thank Dr. G. L. May, Dr. A. Kuesel, and Professor I. C. P. Smith for their continual support and constructive discourse and Rebecca Hancock for preparing the diagrams.

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