

Ex Vivo Two-dimensional Proton Nuclear Magnetic Resonance Spectroscopy of Smooth Muscle Tumors: Advantages of Total Correlated Spectroscopy over Homonuclear J-correlated Spectroscopy¹

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Abstract

Two-dimensional total correlated proton nuclear magnetic resonance spectroscopy (TOCSY) at 600 MHz was used for an *ex vivo* biochemical analysis of a benign human smooth muscle tumor (leiomyoma) and high grade malignant smooth muscle tumor (leiomyosarcoma). While there are a number of applications of proton nuclear magnetic resonance magnitude-mode two-dimensional correlated spectroscopy (COSY) to the *ex vivo* study of tissues and cells in the literature, to our knowledge this is the first application of TOCSY for the *ex vivo* study of biological tissue. Comparison of TOCSY and purged COSY data demonstrate the potential sensitivity advantages of the TOCSY method for the study of heterogeneous biological tissues. These TOCSY spectra were used to identify and quantify a wide range of metabolites such as amino acids, peptides, triglycerides, phospholipid precursors and degradation products, bound fucose, and other saccharides. The leiomyosarcoma was found to have 5-fold higher levels of triglycerides and a 7-fold increase in the glycerophosphocholine:choline ratio compared to the leiomyoma. These metabolite changes may enhance membrane fluidity in the leiomyosarcoma compared to leiomyoma and thus may be of fundamental importance to cell motility, recognition, sarcoma tumorigenesis, and metastatic potential.

Introduction

Alterations in cell membrane glycoprotein and phospholipid and fatty acyl chain saturation are common during tumorigenesis and may play a key role in determining the metastatic behavior of tumor cells (1–3). In addition, changes in the composition of the cell may play a role in cell growth regulation, motility during invasion, and cell-cell interactions. The application of ¹H-NMR³ to cells and tissues can provide unique and important structural information about plasma membrane composition and function (3–5). The one-dimensional ¹H-NMR spectra of cells and tissues are often poorly resolved due to the heterogeneous nature of these biological samples. For sarcoma tissue, this heterogeneity results from regional variations in cell density, supporting matrix and vascularization. In addition, cells contain divalent cations such as magnesium and calcium which, due to magnetic susceptibility effects, may produce spectral line broadening. These difficulties often can make unequivocal assignment and quantitation of the resonances extremely difficult.

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³ The abbreviations used are: ¹H-NMR, proton nuclear magnetic resonance spectroscopy; COSY, homonuclear J-correlated spectroscopy; PCOSY, purged correlated spectroscopy; TOCSY, total correlated spectroscopy; PBS/D₂O, deuterated phosphate-buffered saline; GPC, glycerophosphocholine; Ch, choline; GlcNAc, N-acetylgalactosamine.

The application of two-dimensional NMR methods has alleviated these problems. Magnitude-mode COSY spectra have been used to detect and identify amino acids, phospholipids, plasma membrane triglycerides, and cell surface carbohydrates in cancer cells and tissues (6–9). From these studies, NMR-measurable changes in triglycerides and carbohydrates were found to correlate with cancer cell tumorigenesis and metastatic potential. The major limitations of these investigations have been in the sensitivity and specificity of these spectral changes for different cancer cell types. The double-quantum-filtered COSY experiment has been used to obtain high quality spectra of lymphocyte extracts (10, 11). PCOSY provides spectra of a quality comparable to the double-quantum-filtered COSY method but with twice the sensitivity (12). Homonuclear polarization transfer by isotropic mixing (also known as TOCSY; Ref. 13) has become the method of choice for two-dimensional correlation spectroscopy. Sensitivity, the capacity to produce pure in-phase absorption spectra and, most importantly, the ability to correlate resonances via multiple steps of net coherence transfer, have all contributed to its popularity.

In this paper, we have performed ¹H-NMR two-dimensional TOCSY (13) and PCOSY (12) at 600 MHz on *ex vivo* samples from a benign human smooth muscle tumor (leiomyoma) and high grade malignant smooth muscle tumor (leiomyosarcoma). We demonstrate that the PCOSY method can be used to detect and identify a large number of metabolites in intact leiomyoma and leiomyosarcoma tissue including free amino acids, phospholipids, triglycerides, bound fucose, and other saccharides. Furthermore, we show the sensitivity advantages of the TOCSY experiment compared to PCOSY, particularly for identifying triglycerides, phospholipids, and peptides in intact leiomyoma and leiomyosarcoma tissue. These two-dimensional TOCSY experiments of sarcoma tissue can provide new information about tissue biochemistry, cell membrane composition and function associated with metastatic potential.

Materials and Methods

Tissue Samples for NMR Measurements. Multiple 1-g tissue samples were acquired from patients with benign and malignant smooth muscle tumors of the abdomen undergoing surgical resection. All tissue samples were obtained from homogeneous and nonnecrotic portions of the resected tumors by the pathologist. Tissue samples were placed in a –80°C freezer no longer than 10 min from the time of sarcoma excision. Histological material in each sample was evaluated for tissue of origin of the dominant tumor cell type. Grade was determined by evaluating capsulation, cellularity, vascularity, pleiomorphism, necrosis, and mitotic activity along with histological cell type. The degree of malignancy was scored as low, intermediate, or high grade based on a total evaluation of the above histological features with a major emphasis placed on the mean mitotic activity (14).

Prior to the NMR measurements, a cylindrical core of tissue 3 mm in diameter and 2 cm in length was obtained using a biopsy punch and then thawed at 20°C. The piece was then incubated in PBS/D₂O for 30 min, then transferred to fresh PBS/D₂O and soaked for an additional 15 min. A glass

wool plug of 1.5 cm was placed at the bottom of the NMR tube so as to position the tissue sample in the center of the rf coil. The presoaked tissue was then placed in the NMR tube in PBS/D₂O.

NMR Measurements. NMR measurements were performed at the Francis Bitter National Magnet Laboratory on a laboratory-built 600-MHz spectrometer equipped with a Cryomagnet system 5-mm probe, with the sample maintained at 20°C. The residual water signal was suppressed by presaturation. The one-dimensional ¹H-NMR spectra were acquired with a spectral sweep width of 7000 Hz using 4 k data points with a 0.5-s relaxation delay followed by 1.5 s of low power continuous wave irradiation for solvent suppression. A 90° pulse of 14 μs was applied, and 128 transients were acquired. Peaks were referenced to aqueous 3-(trimethylsilyl) propanesulfonate. The TOCSY and PCOSY spectra were acquired and processed with software written in-house. Two thousand data points were collected in the *t*₂ dimension over a sweep width of 7000 Hz, and 256 data points were collected in the *t*₁ dimension over a sweep width of 7000 Hz. A mixing time of 72 ms was used for the TOCSY.

The data were linear predicted to 512 points in the *t*₁ dimension and were zero filled to 2K. For the TOCSY data, Gaussian line-broadening was applied along both dimensions prior to Fourier transformation. For the PCOSY data, a Gaussian window function was applied in *t*₂, and a sinebell and Gaussian window function was used in *t*₁. All of the two-dimensional spectra were symmetrized. For each tissue type, two separate samples from the same tumor were used for NMR data acquisition. Resonance assignments were based on the literature chemical shift values, spin-spin coupling patterns, and TOCSY spectra recorded by us for compounds such as phosphocholine, glycerophosphocholine, choline, phosphoethanolamine, *N*-acetylgalactosamine, galactose, and fatty acids.

Results and Discussion

The one-dimensional ¹H-NMR and two-dimensional TOCSY and PCOSY experiments were performed on *ex vivo* tissue samples confirmed by histological analyses to be a benign leiomyoma and high grade leiomyosarcoma. The leiomyoma tissue was a smooth muscle cell proliferation with a mean mitotic activity of 0.3 mitoses/10 high-powered fields. In contrast, histology of the leiomyosarcoma showed a high grade sarcoma of smooth muscle origin with a mean mitotic activity of 7.4 mitoses/10 high-powered fields.

Fig. 1 shows the *ex vivo* one-dimensional ¹H-NMR spectra of a benign leiomyoma (Fig. 1A) and malignant high grade leiomyosarcoma (Fig. 1B). The one-dimensional spectra are notable for numerous overlapping resonances and are dominated by lipid resonances (Fig. 1, arrows). Although qualitative differences are found between the leiomyoma and leiomyosarcoma, due to extensive peak overlap on these one-dimensional spectra, it is clearly not possible to identify and

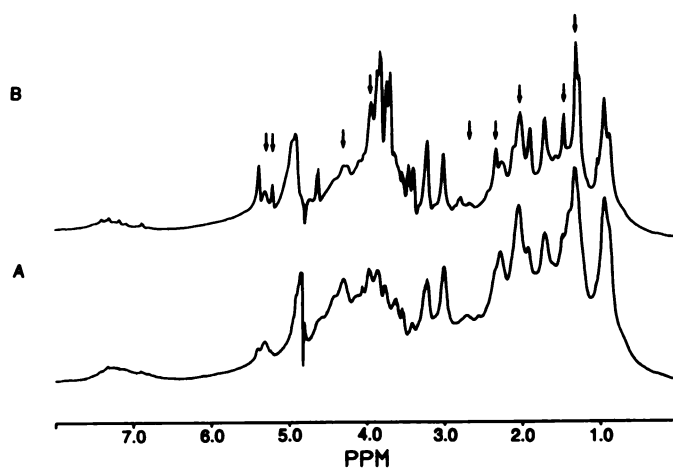


Fig. 1. *Ex vivo* one-dimensional ¹H-NMR spectra at 600 MHz and 20°C of: (A) benign leiomyoma and (B) malignant high-grade leiomyosarcoma tissues. The NMR conditions are given in "Materials and Methods." Arrows show peaks primarily characteristic of lipid resonances.

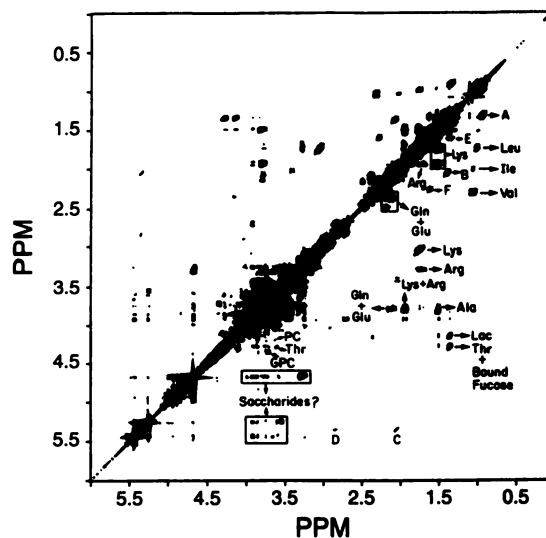


Fig. 2. *Ex vivo* symmetrized PCOSY spectrum at 600 MHz and 20°C of malignant high-grade leiomyosarcoma tissue. Total measuring time was 12 h. NMR conditions are as given in "Materials and Methods."

quantitate the various metabolites. Fig. 2 shows the PCOSY spectrum of intact tissue from the malignant high grade leiomyosarcoma with a number of cross peaks, mainly in the 0–6 ppm region. The majority of these cross peaks have been assigned (Fig. 2) and correspond to free amino acids, triglycerides (cross peaks A–F), phospholipids, and bound fucose and lactate. A particular advantage of the PCOSY experiment is the ability to distinguish multiplet structure for many of the cross peaks detected in this data obtained from intact sarcoma tissue. This is particularly helpful in making resonance assignments and illustrates that highly resolved, phase-sensitive two-dimensional spectra can be obtained from intact heterogeneous tissue specimens using the PCOSY technique.

Fig. 3 shows the TOCSY spectra of intact tissue from a benign leiomyoma (Fig. 3B) and malignant leiomyosarcoma (Fig. 3A). The two-dimensional spectra display a number of cross peaks, mainly in the 0–6 ppm region, which correspond to both small metabolites and macromolecules.

We have identified a number of amino acid spin systems (Ala, Glu, Gln, Arg, Lys, Pro, Thr, Val, Leu, and Ile) based on the spin-spin coupling pattern and the chemical shift values, and these assignments are indicated in Fig. 3B. The amino acids appear to exist as isolated molecules based on the chemical shifts, especially of the α carbon protons (15). These amino acid signals are seen in both tissue samples.

Both spectra exhibit broad cross peaks at $\omega_1 = 4.3\text{--}4.5$ ppm and $\omega_2 = 1.5\text{--}2.4$ ppm and are labeled "Peptides?" in Fig. 3B. The α -proton frequency of peptides falls in the 4.3–4.5 ppm range; based on this, we tentatively assigned these resonances to α -proton \rightarrow β protons or gamma protons of peptides. The cross-peak frequencies in this region are similar in both samples. The larger linewidths of these cross peaks are consistent with large, aggregated, or surface-bound peptides.

The cross peaks from triglycerides are labeled from A to H (Fig. 4). Fig. 4 shows a triglyceride molecule with two unsaturated sites on the acyl chain, along with a table of the chemical shifts for the fatty acid acyl proton connectivities. The triglyceride assignments are based on the TOCSY of fatty acid standards (data not shown). Cross peaks A through F are from the acyl chain and G is from the glycerol backbone. Cross peaks C and D are methylene proton couplings in the acyl chain to the unsaturated site protons; cross peak C appears only if there is at least one unsaturated site, and cross peak D appears if there

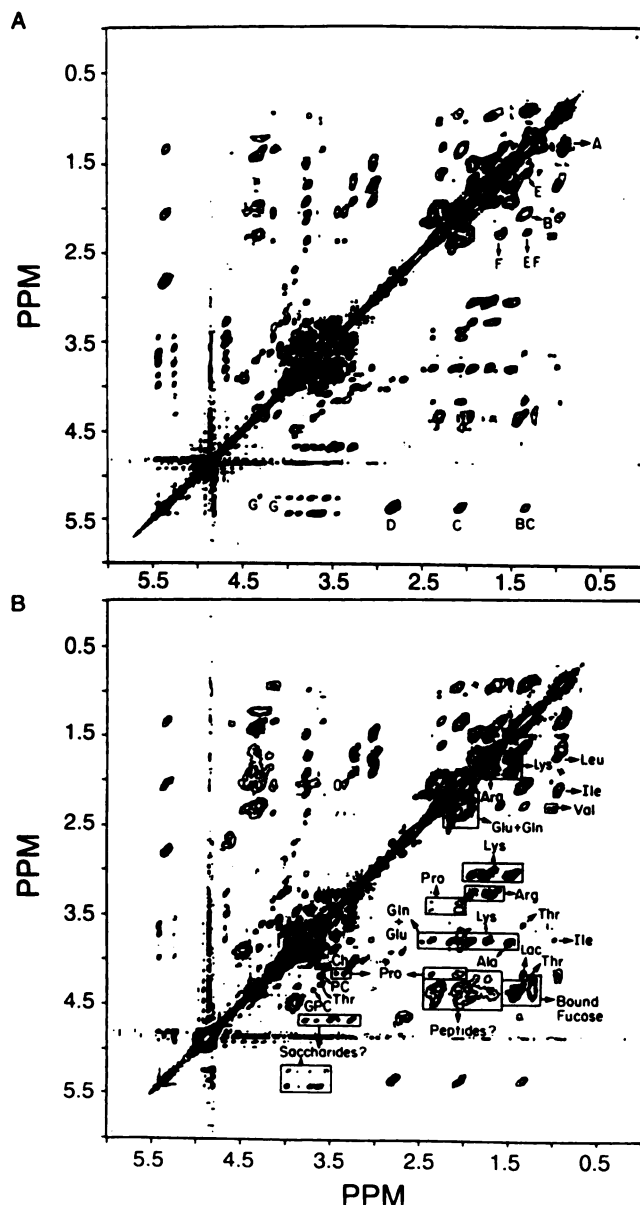


Fig. 3. *Ex vivo* symmetrized TOCSY spectra at 600 MHz and 20°C of: (A) malignant high grade leiomyosarcoma and (B) benign leiomyoma tissues. Total measuring time was 6 h for each TOCSY spectra. NMR conditions are as given in "Materials and Methods."

are at least two unsaturated sites. The cross peaks with double labels (*BC* and *EF*) are observed only in the TOCSY and are not observable in the COSY spectra, since these cross peaks connect protons more than three bonds apart. All the acyl chain cross peaks are present in both samples. The backbone cross peak *G* is seen only in the leiomyosarcoma sample and not in the leiomyoma sample, presumably because the overall triglyceride signal is weaker in the leiomyoma. The intensity of the triglycerides in the leiomyosarcoma spectra is 5 ± 3 -fold greater than in the leiomyoma spectra ($n = 3$). Based on the equal tissue volumes used for both samples, the triglyceride signals are qualitatively increased in the high-grade leiomyosarcoma compared to the benign leiomyoma.

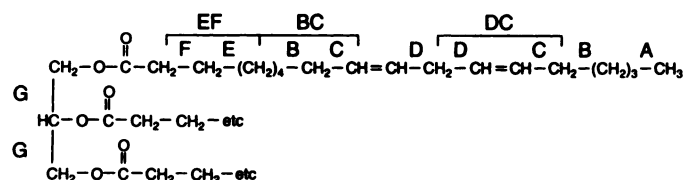
Elevated levels of these triglyceride signals have been found in studies of malignant epithelial cancer cell lines and tissues (3, 6, 16), embryonic cells (7), activated immune cells (10), and macrophages (17). For example, in colorectal cells, Lean *et al.* (8) found a 5.5-fold increase in the total triglyceride amount in high tumorigenic cells compared to low tumorigenic cells, which correlated as well with the

NMR-observable triglyceride levels from these cell lines. They propose that increased levels of these triglycerides may lead to increased cell motility and capacity of cells to migrate, which in turn may have a direct bearing on the metastatic potential of these malignant cells.

Phospholipid precursors and breakdown products such as GPC, phosphocholine, and Ch are seen in both leiomyoma and leiomyosarcoma tissues (Fig. 3B). The relative amounts of these phospholipid precursors and breakdown products, by regulating phosphatidylcholine synthesis, may play an important role in the regulation of biogenesis and function of membranes during cell growth and development (18). Measurement of cross-peak intensity for these compounds show that the phosphocholine:Ch ratio is not statistically different in the leiomyosarcoma sample (0.37 ± 0.04 ; $n = 2$) compared to the leiomyoma sample (0.32 ± 0.06 ; $n = 2$). In contrast, the GPC:Ch ratio is about 7-fold greater in the leiomyosarcoma (0.80 ± 0.20 ; $n = 2$) compared to the leiomyoma sample (0.11 ± 0.08 ; $n = 2$; $P < 0.05$). Phosphatidylcholine degradation is controlled in part by phospholipase A-forming lysolecithin. GPC is formed from lysolecithin and subsequently may be further degraded by the enzyme GPC phosphodiesterase to choline and glycerol-3-phosphate. The higher levels of GPC in the leiomyosarcoma compared to leiomyoma suggest a decreased activity of the enzyme GPC phosphodiesterase in the leiomyosarcoma. This inhibition of GPC degradation would result in increased lysolecithin levels in the leiomyosarcoma compared to leiomyoma. Lysolecithin concentrations are, in general, kept under tight control in the cell, since small increases in its concentration in membranes cause enhanced fluidity, altered activity of membrane associated enzymes, and altered cell surface recognition properties (19). The present TOCSY spectra allow for direct measurement of membrane phospholipid precursors and degradation products (Ch, phosphocholine, and GPC), which in turn are important determinants of membrane fluidity, function, and cell recognition.

Different forms of bound fucose (Fuc I, Fuc II, and Fuc III) are detected in TOCSY data from both the leiomyoma and the leiomyosarcoma (Fig. 3B). There is no qualitative difference in the fucosylation pattern seen for the smooth muscle samples examined. This is in contrast to studies of human colorectal cells where the highly tumorigenic cells were found to have Fuc I and Fuc II, while colorectal cells with low tumorigenesis displayed Fuc I and Fuc IV (8).

Cross peaks at $\omega_1 = 4.69, 5.25, \text{ and } 5.44 \text{ ppm}$ and $\omega_2 = 3.2\text{--}4 \text{ ppm}$ labeled "Saccharides?" in Fig. 3B are seen in both leiomyoma and leiomyosarcoma tissue TOCSY spectra. These peaks could arise from



Chemical shifts of scalar coupled triglyceride protons in the smooth muscle tissues^a

Cross peak symbol	Cross peak protons	chemical shift (ppm)
A	CH₂-CH₂-CH₃	1.33-0.90
B	CH-CH₂-CH₂	2.07-1.33
C	CH-CH₂-CH₂	5.38-2.07
D	CH-CH₂-CH	2.79-3.38
E	OC-CH₂-CH₂-CH₂	1.65-1.33
F	OC-CH₂-CH₂-CH₂	2.36-1.65
G	O-CH₂-CH-O	4.16-5.29
		4.37-5.29

^aThe protons in bold participate in the scalar coupling.

Fig. 4. Schematic drawing of a triglyceride molecule with the covalent linkages for the fatty acyl chain labeled A-F and for the glycerol backbone labeled G. Table of chemical shifts for the scalar connectivities.

the ring protons of saccharide molecules. The string of cross peaks at $\omega_1 = 4.69, 5.25,$ and 5.44 ppm could correspond to scalar couplings to the anomeric protons of saccharide rings. The TOCSY spectrum of GlcNAc shows cross peaks at $\omega_1 = 4.69$ and 5.25 ppm (data not shown), which corresponds to the ring 1 proton of the α and β anomers and is identical to the ω_1 cross-peak frequencies observed in the tissue samples. Some of the resonances in the $3.2\text{--}4$ ppm range of the tissue samples can be accounted for by the other ring protons of galactose or GlcNAc. These data suggest saccharides in the form of glycopeptides or as isolated monosaccharides as the chemical origin for these resonances; however, these data alone are not sufficient to assign these resonances with certainty. Galactose, glucose, GlcNAc, and *N*-acetylglutamic acid are major components of membrane glycoprotein and glycolipid and could reside on the outside surface of the cell membrane in a relatively mobile environment and thus explain part of the resonances in question.

A comparison of the PCOSY spectrum of the leiomyosarcoma tissue (Fig. 2) recorded with the same sample and shim conditions that were used for the TOCSY experiment (Fig. 3A) demonstrates that the PCOSY spectrum contains fewer cross peaks than the TOCSY spectrum. Many of the additional cross peaks observed in the TOCSY spectra relate to the potential of the TOCSY experiment to correlate resonances via multiple steps of net coherence transfer. This often has the advantage of simplifying resonance assignments (20). In addition, with the TOCSY experiment, the mixing time can be varied in order to display remote and/or related correlations. The relatively broad cross peaks seen in the TOCSY spectra, such as from the peptides, bound fucose, and from the unsaturated site protons of the triglycerides (peaks *C* and *D*) are much weaker or completely missing in the PCOSY. In the PCOSY experiment, a major source of sensitivity loss for the broad peaks is probably due to cancellation of anti-phase components of broad peaks. For this reason, less mobile metabolites such as peptides and triglycerides, which give rise to broad peaks, may be less readily detectable in the PCOSY compared to TOCSY experiment. However, for peaks that are well resolved and narrow, the multiplet structure available from PCOSY may be advantageous for metabolite identification.

In summary, PCOSY and TOCSY spectra at 600 MHz of benign and malignant smooth muscle tumors were obtained, allowing the detection of amino acids, peptides, fucose moieties, saccharides, triglycerides, and membrane phospholipid precursors and degradation products (Ch, phosphocholine, and GPC). For metabolites with poorly resolved multiplet structures as a result of tissue heterogeneity or reduced metabolite mobility, TOCSY provides enhanced sensitivity compared to PCOSY. The high-grade leiomyosarcoma contained 5-fold higher levels of triglycerides and 7-fold increase in the GPC:Ch ratio compared to benign leiomyomas. These metabolite differences may result in increased membrane fluidity in the leiomyosarcoma compared to leiomyoma and, thus, ultimately effect cell migratory

capacity and metastatic potential of these tumors. This technique would have potential applications as an adjunct to conventional histopathological analysis of sarcoma tissue, providing quantitative measures of biochemical alterations that might ultimately correlate with the metastatic potential of sarcomas.

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